

2022

NICHD Division of Intramural Research
ANNUAL REPORT

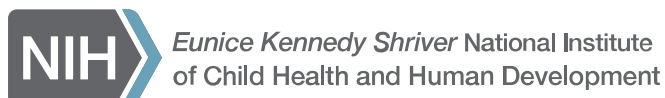


Table of Contents

6	Board of Scientific Counselors
8	Scientific Director's Preface
9	Office of the Scientific Director
11	Office of Education
15	Office of the Clinical Director
17	Clinical Trials
	Clinical Training Programs
21	Combined Maternal-Fetal Medicine/Medical Genetics Fellowship Training Program
22	NICHD-NIDDK-NIDCR Interinstitute Endocrine Training Program
24	Pediatric Endocrinology Inter-Institute Training Program
27	Pediatric and Adolescent Gynecology Training Program
31	Reproductive Endocrinology and Infertility Training Program
34	Administrative Management Branch
36	Research Animal Management Branch
	Scientific Core Facilities
38	Ryan Dale: Bioinformatics and Scientific Programming Core
41	Ryan Dale: Computer Support Services Core
44	Vincent Schram: Microscopy and Imaging Core
47	Forbes Porter: Molecular Genomics Laboratory Core
50	Ben Feldman: Zebrafish Core
53	Affinity Groups

A

60	Philip Adams: RNA-Mediated Gene Regulation in the Lyme Disease Pathogen
----	--

B

63	Tamás Balla: Phosphoinositide Messengers in Cellular Signaling and Trafficking
67	Anirban Banerjee: Structural and Chemical Biology of Membrane Proteins
74	Jeff Baron: Regulation of Childhood Growth
80	Peter Basser: Quantitative Imaging and Tissue Sciences
91	Sergey Bezrukov: Biophysics of Large Membrane Channels
96	Juan Bonifacino: Protein Sorting in the Endomembrane System
101	Andres Buonanno: Neuregulin-ErbB and NMDA Receptor Signaling in Neuronal Development and Psychiatric Disorders
109	Harold Burgess: Neuronal Circuits Controlling Behavior: Genetic Analysis in Zebrafish

C

114	Leonid Chernomordik: Cell Fusion Stages of Myogenesis and Osteoclastogenesis: Mechanisms and Physiological Role
118	Ajay Chitnis: Building the Zebrafish Lateral Line System
121	Janice Chou: Molecular Genetics of Heritable Human Disorders
126	David Clark: Chromatin Remodeling and Gene Activation

C, continued

- 130 **Bob Crouch:** Physiological, Biochemical, and Molecular-Genetic Events Governing the Recognition and Resolution of RNA/DNA Hybrids

D

- 136 **An Dang Do:** Cellular Stress in Development and Disease
140 **Mary Dasso:** Mechanisms of Nuclear Genome Organization and Maintenance
149 **Melvin DePamphilis:** Regulation of Mammalian Cell Proliferation and Differentiation
153 **Tom Dever:** Mechanism and Regulation of Eukaryotic Protein Synthesis
159 **Rena D'Souza:** Molecules and Therapies for Craniofacial and Dental Disorders
167 **Maria Dufau:** Regulation of Hormone Receptors and Gonadal Genes

F

- 172 **Jeffrey Farrell:** Transcriptional Control of Cell Specification and Differentiation
175 **Douglas Fields:** Nervous System Development and Plasticity

G

- 181 **Amir Gandjbakhche:** Translational Biophotonics in Developmental Disorders and Diseases

H

- 188 **Alan Hinnebusch:** Transcriptional and Translational Regulatory Mechanisms in Nutrient Control of Gene Expression
193 **Dax Hoffman:** Molecular Nature and Functional Role of Dendritic Voltage-Gated Ion Channels

K

- 197 **Judy Kassis:** Control of Gene Expression during Development

L

- 204 **Sergey Leikin:** Extracellular Matrix Disorders: Molecular Mechanisms and Treatment Targets
211 **Claire Le Pichon:** From Axon Damage to Disease: Common Pathways in Neurodegeneration
213 **Henry Levin:** The Biological Impact of Transposable Elements
220 **Mary Lilly:** Cell Cycle Regulation in Oogenesis
224 **Y. Peng Loh:** Neurosecretory Proteins in Neuroprotection and Neurodevelopment
230 **Jon Lorsch:** The Molecular Mechanics of Eukaryotic Translation Initiation
233 **Paul Love:** Exploring Genes and Signals Regulating Mammalian Hematopoiesis and New Approaches to Immunotherapy

M

- 241 **Todd Macfarlan:** The Arms Race between Transposable Elements and KRAB-ZFPs and its Impact on Mammals
- 247 **Matthias Machner:** Virulence Mechanisms of Microbial Pathogens
- 252 **Richard Maraia:** RNA Metabolism in Cell Biology, Growth, and Development
- 259 **Leonid Margolis:** Immune Activation and Viral Pathogenesis
- 264 **Joan Marini:** Genetic Disorders of Bone and Extracellular Matrix
- 271 **Doreen Matthies:** High-Resolution Structural Biology of Membrane Protein Complexes in Their Native Environment
- 274 **Chris McBain:** Hippocampal Interneurons and Their Role in the Control of Network Excitability
- 278 **Deborah Merke:** Pathophysiology, Genetics, and Treatment of Congenital Adrenal Hyperplasia
- 285 **Anil Mukherjee:** Childhood Neurodegenerative Lysosomal Storage Disorders

O

- 292 **Keiko Ozato:** Gene Regulation in Innate Immunity

P

- 298 **Karel Pacak:** Diagnosis, Localization, Pathophysiology, and Molecular Biology of Pheochromocytoma and Paraganglioma
- 306 **Tim Petros:** Mechanisms Regulating GABAergic Cell Development
- 311 **Karl Pfeifer:** Molecular Genetics of an Imprinted Gene Cluster on Mouse Distal Chromosome 7
- 320 **Forbes Porter:** Rare Genetic Disorders of Cholesterol Homeostasis and Lysosomal Diseases

R

- 327 **Pedro Rocha:** Three-Dimensional Organization of the Genome as a Determinant of Cell-Fate Decisions
- 330 **Katherine Rogers:** Decoding Developmental Signaling in Vertebrate Embryos
- 334 **Roberto Romero:** Mechanisms of Disease in Preterm Labor and Complications of Prematurity; Prenatal Diagnosis of Congenital Anomalies
- 341 **Tracey Rouault:** Regulation of Mammalian Iron Metabolism and Biogenesis of Iron-Sulfur Proteins

S

- 347 **Mihaela Serpe:** Mechanisms of Synapse Assembly and Homeostasis
- 355 **Sarah Sheppard:** Vascular Malformations
- 359 **Yun-Bo Shi:** Thyroid Hormone Regulation of Vertebrate Postembryonic Development
- 364 **Alexander Sodt:** Modeling the Biophysics of Cellular Membranes
- 367 **Stanko Stojilkovic:** Signaling and Secretion in Neuroendocrine Cells

S, continued

- 372 **Mark Stopfer:** Chemosensory Coding and Decoding by Neuron Ensembles
377 **Gisela Storz:** Regulatory Small RNAs and Small Proteins

T

- 382 **Christina Tatsi:** Evaluation of Hypothalamic and Pituitary Disorders
385 **Bruce Tromberg:** Point of Care and Wearable Biophotonics for Characterizing Tissue Composition and Metabolism

W

- 393 **Brant Weinstein:** Organ and Tissue Formation during Development
401 **Roger Woodgate:** Studies on DNA Replication, Repair, and Mutagenesis in Eukaryotic and Prokaryotic Cells

Y

- 404 **Jack Yanovski:** Physiology, Psychology, and Genetics of Obesity

Z

- 412 **Joshua Zimmerberg:** The Biophysics of Protein-Lipid Interactions in Influenza and Coronavirus, Malaria, and Muscular Dystrophy

- 419 Colophon

Board of Scientific Counselors

* *nominee*

Ursula Kaiser, MD, Acting Chair

7/1/19 – 6/30/24

Endocrinology, Diabetes and Hypertension

Chief, Division of Endocrinology, Diabetes, and Hypertension
George W. Thorn, MD, Distinguished Chair in Endocrinology
Director, Brigham Research Institute
Professor of Medicine, Harvard Medical School
Brigham and Women's Hospital

David R. Beier, MD, PhD*

7/1/21 – 6/30/26

Genomics

Director, Center for Developmental Biology and Regenerative
Medicine,
Seattle Children's Research Institute

Hugo J. Bellen, DVM, PhD

7/1/19 – 6/30/24

Genetics, Neuroscience, Model Organisms

Distinguished Service Professor, Departments of Molecular
and Human Genetics and Neuroscience,
Baylor College of Medicine

William T. Dauer, MD

7/1/18 – 6/30/23

Neurodevelopment and Behavior, Rare
Diseases and Genetics, Neurobiology

Professor and Director, Peter O'Donnell Jr. Brain Institute,
UT Southwestern Medical Center

P. Ellen Grant, MD

7/1/19 – 6/30/24

Newborn Medicine, Radiology

Professor of Radiology and Pediatrics, Harvard Medical
School
Founding Director, Fetal-Neonatal Neuroimaging and
Developmental Science Center,
Endowed Chair in Neonatology,
Boston Children's Hospital

Mary C. Mullins, PhD

7/1/20 – 6/30/25

Developmental Biology

Professor and Vice Chair, Department of Cell and
Developmental Biology,
Chair, Developmental, Stem Cell, and Regenerative Biology
Graduate Program,
Assistant Dean for Junior Faculty Advancement,
University of Pennsylvania

Errol Norwitz, MD, PhD, MBA

7/1/19 – 6/30/24

Obstetrics and Gynecology, Maternal Fetal
Medicine

President and CEO, Newton-Wellesley Hospital

Linda Overstreet-Wadiche, PhD

7/1/20 – 6/30/25

Neurobiology

Professor, Department of Neurobiology,

School of Medicine,

University of Alabama at Birmingham

Message from the Scientific Director

The 2022 annual report of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Division of Intramural Research (DIR) is now available at <http://annualreport.nichd.nih.gov>.

We invite you to look through the report, to review our medical and scientific discoveries of the past year, to see what work a colleague may currently be engaged in, or to identify a laboratory where you may wish to collaborate or refer a student. For potential postdoctoral fellows, graduate students, and clinical fellows, the report is fully searchable. It offers an introduction to the array of research endeavors in NICHD's DIR.

NICHD's intramural community comprises a broad array of basic, translational, and clinical researchers. We use a range of model systems in the areas of developmental biology, molecular and cellular biology, neurosciences, structural biology, imaging, and biophysics. Investigators working with a number of animal models, from fruit flies to rats and mice, are supported by a wide array of core services including bioinformatics, imaging, molecular genomics, and a large zebrafish facility. Each investigator participates in at least one, and typically more, affinity groups. These affinity groups are team-based and future-oriented—to build on thematic interests while responding to rapidly shifting scientific priorities as new knowledge is uncovered.

I encourage you to read through the [selection of our clinical trials](#) and to consider how we may collaborate through the NIH U01 grant mechanism at the NIH Clinical Research Center. This program could lead to our next great success by helping us combine expertise and patient populations, while leveraging NIH infrastructure to address topics ranging from rare disorders to the most common problems affecting human health. You can learn more about the U01 opportunities at https://ocr.od.nih.gov/new_u01/new_u01.html.

The DIR researchers whose names appear in this publication are committed to training the next generation of scientists and physician-scientists; they include tenure-track investigators, who have recently joined us, and accomplished investigators, who continue to forge new scientific paths. Visit the report on the web to learn about their work in 2022.

Our drive and purpose, on behalf of the American public and the international community, is to strive to uncover the fundamental principles that underpin reproduction and development, and to apply these findings to improve human reproductive health, healthy growth, and maturation of children and adolescents. You can reach out to me with your ideas and proposals for collaborative initiatives at mcbainc@mail.nih.gov.

Sincerely yours,
Chris J. McBain, PhD
Scientific Director, NICHD, NIH
December 2022



Office of the Scientific Director

The Office of the Scientific Director (OSD) is responsible for programmatic oversight, review, and administration of the Division of Intramural Research (DIR), NICHD. The Office has several key functions including:

- Serving our staff, investigators, administrators, trainees, and contractors to meet their needs and ensure that we are all working together to support the mission of NICHD;
- Planning, coordinating, and directing the basic and clinical scientific research programs conducted within the DIR, all with the advice of the [Board of Scientific Counselors](#) (BSC; see below) and in consultation with the Institute's Director and other offices;
- Providing oversight of scientific organization, setting of overarching research priorities, and allocation of resources;
- Coordinating the recruitment and review of Principal Investigators and other key staff;
- Providing oversight of scientific mentoring and training activities, and, as a high priority, ensuring that we provide state-of-the-art training in basic, translational, and clinical research for the next generation of scientific and clinical leaders;
- Encouraging the implementation of new technologies and the application of new techniques and treatments through clinical trials, and promoting research collaboration;
- Developing and maintaining the necessary scientific capability in the DIR to foster and guide an effective research and clinical program;
- Ensuring compliance with safety, ethics, and other legal and policy requirements;
- Providing clearance of manuscripts and other publications and ensuring the deposition of data and published manuscripts in appropriate public databases (e.g., [PubMedCentral](#), [Clinicaltrials.gov](#));
- Serving in an advisory role to the Institute Director and other senior leadership of the Institute and other institutes, offices, and centers across NIH;
- Actively participating in the governance of the NIH [Intramural Research Program \(IRP\)](#);
- Contributing to the development and support of trans-NIH initiatives;
- Working closely with other intramural research programs across NIH to support scientific and other efforts across institutes.

To maintain the highest quality of research, Principal Investigators and other key staff of the DIR are evaluated by the BSC, which meets biannually on the first Friday of December and in June of each year.



Chris J. McBain, PhD, *Scientific Director*

Sara K. King, *Chief of Staff*

Jessica Rigby, *Scientific Program Analyst*

Amaressa Abiodun, *Administrative Support Specialist*

Olga Cherkasova, *Administrative Support Specialist*

The BSC reviews site-visit reports, evaluations, and all other activities of the OSD. Each NICHD investigator is subject to a review at least once every four years, according to NIH policy ([*NIH Sourcebook Process for Reviewing NIH Intramural Science*](#)). To ensure the most effective use of public dollars toward high-quality, high-impact research, the Board is made up of accomplished senior extramural researchers. Membership of the BSC is listed at <https://annualreport.nichd.nih.gov/bsc.html>. Annually, the Scientific Director reports on the activities of the OSD, the reviews of our investigators and their accomplishments, and on all BSC recommendations to NICHD's [*National Advisory Child Health and Human Development \(NACHHD\) Council*](#).

Office of Education

The goals and objectives of the Office of Education can be summarized as follows: to meet the training needs of intramural scientists, fellows, and students at all levels, the Office of Education recruits and develops academic support programs, contributes to mentoring, evaluation, and career guidance, and creates new training initiatives. Our professional development workshops and activities typically include public-speaking workshops, job interviewing, writing and editorial services for professional school and job applications, grantsmanship workshops, academic and non-academic career presentations, one-on-one counseling, teaching opportunities, and lab-management programs. Additional areas of involvement include programming for career exploration, networking among fellows and alumni, grantsmanship, and the enhancement of fellows' competitiveness for research awards and future career opportunities, as well as support of tenure-track investigators.

Notable accomplishments of the past year

The Office of Education organizes numerous workshops, programs, and individualized opportunities for a population averaging 300 trainees, including: postdoctoral, visiting, and research fellows; clinical fellows and medical students; graduate students; and postbaccalaureate fellows and summer interns.

Our **TmT (Three-minute Talks) competition**, now in its eighth year, was held in conjunction with ten other institutes: NCATS, NIDCR, NHGRI, NEI, NIDDK, NIDCD, NIAID, NINDS, NIEHS, and NLM. **Jeremie Oliver Piña**, graduate student in the [Section on Molecules and Therapies for Craniofacial and Dental Disorders](#), was the NICHD finalist.

We continued our **Graduate Student Talks** initiative, established in 2014, which provides the Institute's graduate students with experience in presenting their thesis research to a non-specialist scientific audience.

An online **Annual Progress Review** for fellows, developed by the Office and launched in 2016, tracks scientific and career development and progress. As part of the assessments of investigators' mentoring, the reports by fellows are analyzed and provided to the site visits of the [Board of Scientific Counselors](#).

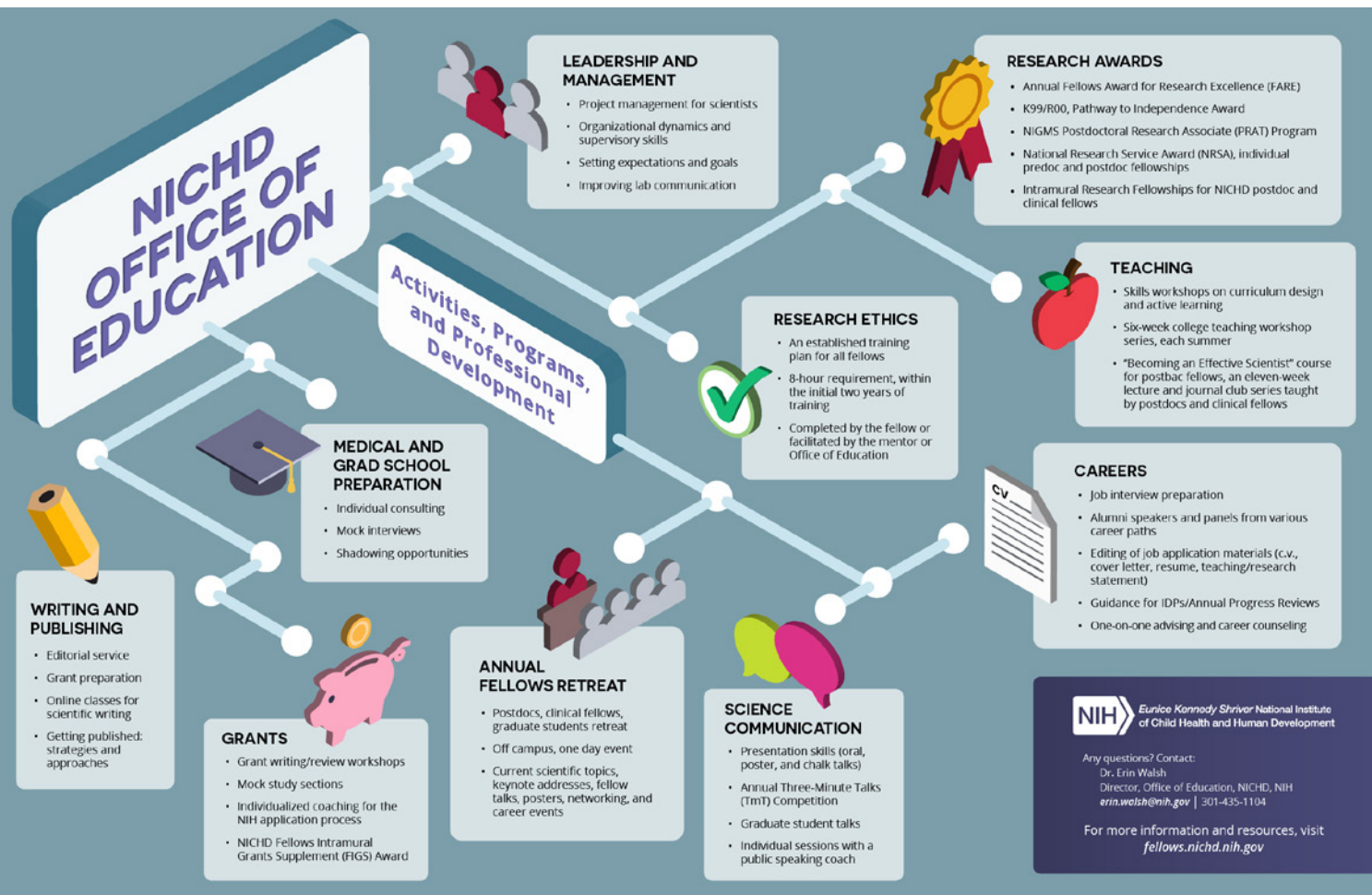
In September 2022, the Division of Intramural Research gave its Mentor of the Year awards to **Edwina Yeung**, Epidemiology Branch, in the



Erin Walsh, PhD, *Program Director, Office of Education*

Veronica Harker, Program Coordinator

Katherine Lamb, Program Specialist



LEFT TO RIGHT:
 Veronica Harker; Katherine Lamb

investigator category; and to **Laura Pillay**, [Section on Vertebrate Organogenesis](#), as fellow.

The **database of NICHD alumni** from 2008 to the present continues to be updated.

We compiled a valuable **list of organizations** that accept grant applications from NIH intramural fellows, through both NIH and non-NIH funding mechanisms. It can be found on the [NICHD fellows' wiki site](#). For NICHD, 16 Fellows Award for Research Excellence (FARE) awards were made for the 2023 competition.

The **Fellows Intramural Grants Supplement (FIGS)** continues to recognize and stimulate grant applications among fellows, and we launched the third cycle for our Intramural Research Fellowships (IRFs), a new competitive internal funding opportunity for NICHD postdoctoral and clinical fellows. Its ultimate goal is to promote fellows' grant writing and to enhance their awareness of various components for an NIH grant application.

The Institute continues its **exchange program with INSERM** (the Institut National de la Santé et de la Recherche Médicale in France), which provides a unique opportunity for US and French scientists to obtain postdoctoral training with French and US mentors, respectively. Since 2016, up to three second- and third-year medical students from **Santa Casa de São Paulo School of Medical Sciences** (Brazil) train with NICHD investigators for the **Future Researchers Program**.

The **Fellows Recruitment Incentive Award (FRIA)** continues to support investigators who recruit postdocs from populations traditionally under-represented in science.

The alumni group for our **NICHD Developing Talent Scholars program**, in its 12th year, added three new postbaccalaureate fellows in 2022. The Scholars program focuses on developing talent and supporting trainees' academic and career progression.

We are actively developing a **collaboration with Howard University** for research, training and mentoring opportunities. For the spring 2022 academic semester, we welcomed a second cohort of undergraduate student mentees from the College of Arts and Sciences into the Biology Secondary Mentors Program. Each student was paired with an NICHD Principal Investigator in the Division of Intramural Research, with whom they will work closely towards developing and executing a research project from their sophomore through their senior years of college. Our third cohort of mentor-mentee pairs will be established for spring 2023. Also in spring 2022, Office of Education Director of Communications and Outreach, Dr. Triesta Fowler, co-led a **new seminar series**, "Career Planning and Professional Development: Exploring the MD, PhD, and MD-PhD Paths," for undergraduates in the Howard University Department of Biology to provide medical and research career exploration, as well as guidance on how to proceed with medical and graduate school admissions processes.

Postdoctoral fellows were also given the opportunity to organize and teach our **annual seminar series** for postbaccalaureate trainees, which entered its 16th year. For the 2022 series, Erin Walsh and Triesta Fowler added new sessions on the medical school and graduate school search and application processes.

The **17th Annual Fellows Meeting** was held, virtually, on September 29, 2022, for about 120 people and featured keynote speaker Dr. Rosa Puertollano, NIH Senior Investigator. Each year, this retreat includes

presentations by fellows, career panel sessions, and a poster presentation by each attendee. The program is developed and run by a fellows' steering committee.

The NICHD Connection monthly newsletter continues its focus on mentoring, careers, and academic programs for young scientists, publishing its 150th issue in November 2022 and reaching all members of the intramural division and our alumni.

Dr. Triesta Fowler, who served as Office of Education Director of Communications and Outreach since 2019, left the NICHD in August 2022 to pursue a new role as a diversity officer with the National Institute of Minority Health and Health Disparities.

Contact

For further information, contact Dr. Erin Walsh (erin.walsh@nih.gov).

Office of the Clinical Director, NICHD

The NICHD Office of the Clinical Director (OCD) is responsible for research oversight, scientific review, and administration of the clinical research program within the Division of Intramural Research (DIR). The clinical research activities are coordinated with the Scientific Director and address diseases related to human health involving pediatrics and women's health.

The Institute's current clinical research portfolio includes clinical trials, natural history studies of rare diseases, and drug and device development. The clinical research program currently includes 76 protocols, conducted by 31 NICHD Principal Investigators and 170 associate investigators. Approximately half of the protocols include pediatric patients, many focusing on rare diseases. Fifteen percent



Office of the Clinical Director: Professional, Administrative, and Biorepository Staff

Top: Karim Calis, Fathy Majadly, Laverne Mensah
Center: Maryellen Rechen, Glynnis Vance, Loc Trinh
Bottom: Denise Phillips, Vincent Black, Meg Keil



Laverne Mensah, MD, Acting Clinical Director

Forbes D. Porter, MD, PhD, *Clinical Director (Former)*

Alan DeCherney, MD, *Senior Investigator*

Veronica Gomez-Lobo, MD, *Senior Staff Clinician*

Karim Calis, PharmD, MPH, *Director of Clinical Research and Compliance*

Meg Keil, PhD, CRNP, *Associate Director, Nursing and Protocol Navigation*

Maryellen Rechen, BS, RN, *Special Assistant to the Clinical Director*

Glynnis Vance, *Protocol Coordinator*

Denise Phillips, *Administrative and Credentialing Support*

Ariel Benor, MD, *Staff Clinician*

An Dang Do, MD, PhD, *Staff Clinician*

Tazim Dowlet-McElroy, MD, *Staff Clinician*

Crystal Kamilaris, MD, *Staff Clinician*

Jacqueline Maher, MD, *Staff Clinician*

Samar Rahhal, MD, *Staff Clinician*

Cristina Tatsi, MD, *Staff Clinician*

Denna Zeltser, MD, *Staff Clinician*

Sheila Brady, CRNP, MSN, *Nurse Practitioner*

Andrea Bowling, CRNP, *Nurse Practitioner*

Alberta Derkyi, FNP, CRNP, MSN, *Nurse Practitioner*

Josephine Ezemobi, CRNP, *Nurse Practitioner*

(continued)

(15%) of the protocols involve investigational drugs or devices. Current research areas include developmental disorders, healthy human development, developmental endocrinology, neurosciences, genetics, translational imaging, pediatric and reproductive endocrinology, infertility, fertility preservation, and population health. These clinical research activities are governed under the NIH Intramural Research Program (IRP) with patient participation in the NIH Clinical Center (CC). Delivery of quality and safe patient care in the NIH Clinical Center is vital in the context of NICHD's clinical research programs and services.

Partnerships and collaboration efforts, both between and within NIH institutes, and externally through research agreements with leading academic institutions nationally, and partnerships with industry, through technology transfer or cooperative research and development agreements, foster advancement of science and cutting-edge scientific research. Emphasis is placed on nurturing a clinical research environment that encourages creativity and on expanding fundamental scientific knowledge of summer interns, postbaccalaureate research trainees, as well as postdoctoral and clinical fellows in various basic science and clinical disciplines. The aim of the various components of the Office of the Clinical Director are to achieve the mission of NICHD: "NICHD leads research and training to understand human development, improve reproductive health, enhance the lives of children and adolescents, and optimize abilities for all."

Contact

For more information, email laverne.mensah@nih.gov or visit <https://www.nichd.nih.gov/about/org/dir/osd/mt/cd>.

John Perreault, CRNP, *Nurse Practitioner*
Lisa Ast, BA, RN, *Clinical Research Nurse*
Kisha Jenkins, BSN, RN, *Clinical Nurse*
Lola Saidkhodjaeva, BSN, RN, *Clinical Nurse*
Sara Talvacchio, BSN, RN, *Clinical Nurse*
Craig Abbott, PhD, *Statistician*
Samah Agabein, MD, *Protocol Coordinator*
Harveen Kaur, MPH, *Clinical Research Coordinator*
Mahlet Asrat, MHM, *Patient Care Coordinator*
Vincent Black, *Patient Care Coordinator*
Fathy Majadly, BS, *Patient Specimen Coordinator*
Loc Trinh, *Research Chemical Engineer*



New NICHD Clinical Investigators
Left to right:
Dr. Christina Tatsi, Assistant Clinical Investigator;
Dr. Sarah Sheppard, Tenure-Track Investigator;
Dr. An Dang Do, Assistant Clinical Investigator

Clinical Trials at NICHD

NICHD's Division of Intramural Research (DIR) runs numerous clinical protocols (visit <https://www.clinicaltrials.gov/ct/search?term=nichd> for a complete list of NICHD clinical trials.) The following lists and provides contact information for DIR investigators who recruit patients for clinical studies. For detailed information on all related research projects, please see the listed investigator's section of the report.

Developmental Endocrinology, Metabolism, Genetics, & Endocrine Oncology

- » Patient-oriented research into the etiology, pathophysiology, genetics, diagnosis, localization, and treatment of pheochromocytoma and paraganglioma. For more information on the study, please contact **DR. KAREL PACAK** at karel@mail.nih.gov or **MS. ALBERTA DERKYI** and **MS. SARA TALVACCHIO** at ppglgroup@mail.nih.gov.
- » Research on endocrine, genetic, and other pediatric disorders associated with endocrine and other tumors that may affect the pituitary and other related organs. For more information on the study, please contact **DR. CHRISTINA TATSI** at christina.tatsi3@nih.gov or **MS. SAMAH AGABEIN** at samah.agabein@nih.gov or 301-451-7615.
- » Investigations on the causes, complications, and treatment of primary aldosteronism. For more information on the study, please contact **DR. CRYSTAL KAMILARIS** at crystal.kamilaris@nih.gov.
- » Research investigating the long-term effects of Cushing disease in childhood. For more information on the study, please contact **DR. MEG KEIL** at keilm@mail.nih.gov or 301-435-3391.
- » Study of the safety and efficacy of pegvisomant to treat children and adolescents with growth hormone excess, including those whose disease persists after surgical and/or radiation treatment, and those who are ineligible for those treatments. For more information on the study, please contact **DR. CHRISTINA TATSI** at christina.tatsi3@nih.gov or **MS. SAMAH AGABEIN** at samah.agabein@nih.gov or 301-451-7615.
- » Studies into the role genetics plays in the development of obesity. For more information on the study, please contact **DR. JACK YANOVSKI** at yanovskj@mail.nih.gov or 301-451-3783.
- » Studies on pediatric disorders associated with the predisposition to develop obesity and diabetes, including Bardet-Biedl syndrome, Alström syndrome, Prader-Willi syndrome, leptin receptor deficiency, PCSK1 deficiency, and Pro-opiomelanocortin deficiency. For more information on the study, please contact **DR. JACK YANOVSKI** at yanovskj@mail.nih.gov or 301-451-3783.
- » Pharmacotherapy for excessive hunger and obesity in patients with Prader-Willi syndrome, Bardet-Biedl syndrome, and other rare disorders with known genetic causes. For more information on the study, please contact **DR. JACK YANOVSKI** at yanovskj@mail.nih.gov or 301-451-3783.
- » Evaluation of patients with endocrine disorders associated with excess androgen, including different forms of congenital adrenal hyperplasia. For more information on the study, please contact **DR. DEBORAH MERKE** at dmerke@nih.gov, **MS. AMY MOON** at amy.moon@nih.gov, or **MS. LEE ANN KEENER** at leeann.keener@nih.gov or 240-858-9033.
- » Clinical trial to evaluate the long-term safety and tolerability of Chronocort®, a modified release form of hydrocortisone. For more information on the trial, please contact **DR. DEBORAH MERKE** at dmerke@nih.gov or **MS. ELIZABETH JOYAL** at ejoyal@nih.gov.
- » First-in-human gene therapy trial for congenital adrenal hyperplasia. For more information on the trial, please contact **DR. DEBORAH MERKE** at dmerke@nih.gov or **MS. ELIZABETH JOYAL** at ejoyal@nih.gov.
- » Studies of patients with genetic disorders related to altered cholesterol metabolism, including those with Smith-Lemli-Opitz Syndrome (SLOS) and Niemann-Pick Disease, type C (NPC). For SLOS and related

disorders of cholesterol synthesis, please contact **DR. FORBES PORTER** at fdporter@mail.nih.gov, **DR. SAMAR RAHHAL** at samar.rahhal@nih.gov, or **MS. AISHWARYA SELVARAMAN** at aishwarya.selvaraman@nih.gov. For NPC, please contact **DR. FORBES PORTER** at fdporter@mail.nih.gov, **MS. NICOLE FARHAT** at nicole.farhat@nih.gov or 301-594-1765, or **MR. DEREK ALEXANDER** at derek.alexander@nih.gov or 301-827-0387.

- » Studies of individuals with CLN3, or juvenile neuronal ceroid-lipofuscinosis/juvenile Batten disease, and their family members. For more information on the studies, please contact **DR. AN NGOC DANG DO** at an.dangdo@nih.gov or **MS. KISHA JENKINS** at 301-594-2005.
- » Studies using exome/genome sequencing to identify novel genetic causes of idiopathic growth disorders in children and adults with either short stature or tall stature without a known cause. For more information on the study, please contact **DR. JEFFREY BARON** at baronj@cc1.nichd.nih.gov or **DR. YOUNG HEE JEE** at jeeyh@mail.nih.gov.

Lymphatic Disorders

- » Studies, awaiting Institutional Review Board approval (expected mid-2023), of patients with suspected or confirmed disorders of the lymphatic system. For more information on this study, please contact **DR. SARAH SHEPPARD** at sarah.sheppard@nih.gov or the study team at NICHD_LymphaticAnoma@mail.nih.gov.

Maternal–Fetal Medicine & Translational Imaging

- » *Studies to test and calibrate noninvasive optical imaging technology for functional brain imaging in healthy subjects.* The study is important to investigate the NIRS imaging system to explore techniques that will potentially improve the feasibility and reliability of the system according to the needs of the population whom existing imaging systems are unsuitable for. Functional near infrared spectroscopy (fNIRS) is an emerging non-invasive imaging technique to assess brain function. fNIRS measurements are based on the local changes in cerebral hemodynamic levels (oxy-hemoglobin and deoxy-hemoglobin) associated with brain activity. Due to neuro-vascular coupling, local changes in oxyhemoglobin and deoxyhemoglobin levels can serve as an indirect measure of brain activity. To probe changes in Oxy- and Deoxy-hemoglobin concentrations in the cortex that are caused by brain activity, different tasks such as the n-back, go-nogo tests will be administered to quantify spatial and temporal brain activity. For more information on the study, please contact **DR. AMIR GANDJBAKHCHE** at gandjbaa@mail.nih.gov.
- » *Studies of mirror neuron network dysfunction as an early biomarker of neurodevelopmental disorder.* In this study, functional near-infrared spectroscopy (fNIRS) combined with electroencephalography (EEG) to measure brain activity in the mirror neuron network (MNN). The MNN is associated with the development of sophisticated social behaviors that emerge in typical infants. By modeling MNN development, we hope to uncover a sensitive measure of deviations in social communication development before clinical behavioral deficits can be detected. MNN activation has been indicated through mu rhythm suppression using EEG. The first part of the study involves adult subjects to determine whether MNN activation can be elicited, using a motor observation and a simultaneous execution paradigm using EEG/fNIRS systems. The synchronicity of these signals using more advanced machine learning methods to examine how the features from both signals relate to each other and help characterize brain function in the mirror neuron network. In the next step, typically developing infants and infants at risk for developmental delays from 9–12 months of age are recruited. At-risk infants will be brought in again at 24 months of age to evaluate any deviations in their social communicative development. We will examine their developmental status

at 24 months in relation to their initial neural data to determine whether MNN activation can predict developmental outcomes. For more information on the study, please contact **DR. AMIR GANDJBAKHCHE** at gandjbaa@mail.nih.gov.

- » *Pilot study to evaluate a noninvasive multimodal biosensing device for screening and monitoring treatment response of infectious respiratory diseases.* This observational pilot study will characterize the performance of a multimodal biosensor device (portable NIRS device, PPG and temperature sensor) in measuring human vital signs, which later will be explored as a point-of-care method for screening and treatment response monitoring of individuals with an infectious respiratory illness such as COVID-19 infection. The device will measure heart, respiratory, and tissue oxygenation parameters in healthy subjects at rest and during induced hypercapnia, breath holding, and paced breathing. For more information on the study, please contact **DR. AMIR GANDJBAKHCHE** at gandjbaa@mail.nih.gov.

Pediatric and Adolescent Gynecology

- » *Data collection study on pediatric and adolescent gynecology conditions.* This study is designed to perform deep phenotyping and data collection of children and adolescents presenting with gynecologic conditions including congenital anomalies. For more information on the study, please contact **DR. VERONICA GOMEZ-LOBO** at veronica.gomez-lobo@nih.gov.
- » *Gonadal tissue freezing for fertility preservation in girls at risk for ovarian dysfunction and primary ovarian insufficiency.* This study is designed to evaluate possible mechanisms of follicle loss/dysfunction in children with Turner syndrome and classic galactosemia, and in adolescents recently diagnosed with premature ovarian insufficiency. Ovarian tissue will be cryopreserved and stored for patient's own future use. For more information on the study, please contact **DR. VERONICA GOMEZ-LOBO** at veronica.gomez-lobo@nih.gov.
- » *Biorepository for those who undergo ovarian tissue freezing before gonadotoxic therapy.* This study will create a databank of ovarian tissue obtained during ovarian tissue cryopreservation which is performed as standard care for fertility preservation in individuals who will be treated with gonadotoxic therapy. The NIH will provide ovarian tissue cryopreservation (OTC) as a clinical service and will request a portion of the tissue to use for research. For more information on the study, please contact **DR. VERONICA GOMEZ-LOBO** at veronica.gomez-lobo@nih.gov.
- » *Studies on androgen receptor sensitivity and implications for health and wellbeing: A natural history study of patients with androgen insensitivity.* Research on androgen receptor genes and receptor abnormalities to improve care for those affected and elucidate possible androgen receptor-mediated explanations for differences in physiology and health in other populations. For more information on the study, please contact **DR. VERONICA GOMEZ-LOBO** at veronica.gomez-lobo@nih.gov.
- » *Study of fertility attitudes of adolescents and young adults with Turner Syndrome and their parents/guardians (Fertility ConceptTS).* The purpose of this study is to improve our understanding of attitudes of individuals with Turner syndrome (TS) and their parents/guardians towards fertility, fertility preservation and options for building a family by developing and disseminating a fertility attitudes survey of adolescents and young adults (AYA) with TS and their parents/guardians. For more information on the study, please contact **DR. VERONICA GOMEZ-LOBO** at veronica.gomez-lobo@nih.gov.
- » *Implications of maternal 45,X mosaicism as a secondary genomic finding following cell-free DNA sequencing during pregnancy.* This natural history study will look for health risks in people with 45,X mosaicism discovered during pregnancy. Mosaicism is a condition in which cells within the same person have a different genetic

makeup. Sometimes, a type of mosaicism called 45,X may not be discovered in a woman until she undergoes routine tests during pregnancy. Little is known about how 45,X mosaicism when discovered during pregnancy may affect a person's long-term health. For more information on the study, please contact **DR. VERONICA GOMEZ-LOBO** at veronica.gomez-lobo@nih.gov.

Physical Biology and Medicine

- » Studies of genetic disorders related to fragile sarcolemma muscular dystrophy, including Limb-Girdle Muscular Dystrophy type 2B-F, I, L, Myoshi Myopathy, Becker Muscular Dystrophy, and Myoshi Muscular Dystrophy-3. For more information on the study, please contact **DR. JOSHUA ZIMMERBERG** at zimmerbj@mail.nih.gov or **MS. HANG WATERS** at watershn@mail.nih.gov.

Reproductive Endocrinology and Gynecology

- » Endometrial biopsy studies of reproductive disorders that affect the endometrium, such as recurrent implantation failure. For more information on the study, please contact **DR. ALAN DECHERNEY** at decherna@mail.nih.gov or 301-594-5494.
- » Studies of reproductive function in sickle cell disease and individuals undergoing cytotoxic gonadal therapy, including fertility preservation (oocyte freezing). For more information on the study, please contact **DR. ALAN DECHERNEY** at decherna@mail.nih.gov or 301-594-5494.

Combined Maternal–Fetal Medicine/Medical Genetics Fellowship

The goal of the Fellowship is to train individuals to provide specialized patient care in Maternal-Fetal Medicine, as well as to prepare candidates for a career in academic medicine as physician scientists. It is a three-year training program. There is the possibility of completing a combined Maternal-Fetal Medicine and Human Genetics Fellowship, and candidates can opt to complete a Ph.D. in the Department of Physiology at Wayne State University.

The 18-month clinical rotations include maternal-fetal medicine or high-risk obstetric service, obstetrical ultrasound, labor and delivery, intensive care unit, and elective rotations. The program is housed at Hutzel Women's Hospital, Detroit, and Faculty Members are Wayne State University appointees as well as Attendings of the Detroit Medical Center (DMC) in Detroit, MI. The program is approved for seven positions, two of which are funded by the Perinatology Research Branch (PRB), which is also housed at the DMC, and the remainder by the DMC itself. The Fellowship emphasizes clinical, translational, and basic science research (18 months are dedicated to research). Ideal candidates for the Program are well trained individuals from a university program who wish to pursue a career in academic medicine, thrive in a rigorous and challenging environment, and are goal-oriented and self-motivated.

The Fellowship stresses a multidisciplinary approach to the complications of pregnancy. There is a strong emphasis on prenatal diagnosis of congenital anomalies with ultrasound, and graduates are expected to be proficient in two-dimensional and three-dimensional ultrasound, fetal echocardiography, and advanced imaging techniques such as Doppler velocimetry, as well as ultrasound-guided invasive procedures such as amniocentesis. Opportunities for laboratory-based research and training are available at the PRB in the fields of parturition, reproductive immunology, placental pathology and biology, biomarker discovery, and systems biology in reproduction. Alternative opportunities are available at the C.S. Mott Center of Wayne State University.

The PRB's primary areas of interest are the mechanisms responsible for obstetrical disease, prediction and prevention of preterm birth, prenatal diagnosis of congenital anomalies, the role of infection and inflammation in perinatal disease, fetal growth and development, placental pathology, and the use of high-dimensional biology techniques to identify biomarkers for preterm labor, preterm PROM (premature rupture of membranes), preeclampsia, fetal death, and IUGR (intrauterine growth restriction).

Detailed information about the training program is available at: <http://mfmfellowship.org>. The website also contains information about the faculty and their publications and awards. The Director of the Fellowship Program is Dr. Tinnakorn Chaiworapongsa. The Program is sponsored by the PRB, the DMC, and Wayne State University. Fellows are employees of the DMC, and program oversight is with the Office of Graduate Medical Education of the DMC.

NICHD–NIDDK–NIDCR Inter–Institute Endocrine Training Program

The Inter-Institute Endocrinology Training Program (IETP) is a three-year training program that seeks to train internal medicine physicians to become first-rate endocrinologists dedicated to investigative careers. The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), and the National Institute of Dental and Craniofacial Research (NIDCR) participate in the program, with faculty from all three institutes.

Clinical and research training under the NICHD–NIDDK Inter–Institute Endocrine Training Program

Clinical training occurs largely in the first year. At any one time, fellows are responsible for five to ten patients on the inpatient service of the NIH. Under the supervision of the endocrine faculty, the trainee has complete responsibility for all aspects of a patient's care. Fellows make daily rounds, discuss patients with the attending physicians, and participate in management decisions related to both patient care and clinical investigation. Although all patients are admitted under peer-reviewed research protocols, there are many other aspects of diagnosis and patient care that fall entirely under the discretion of the endocrine fellows.

Developing an independent career as a physician-scientist is the primary focus during the second and third years of training; emphasis is placed on how to develop research questions and hypothesis-driven research protocols. To this end, the second and third years are spent primarily in the laboratory or conducting clinical research under the mentorship of a senior investigator in one of the several endocrinology branches of the NIH. During this research period, fellows continue to gain active clinical experience through bi-weekly continuity outpatient clinics (general endocrinology as well as diabetes clinics) and by participating in clinical conferences. In addition, fellows on the endocrine service serve as consultants to other services within the Clinical Center, where patients are not selected with regard to endocrine problems. Thus, fellows gain experience with the several common endocrine problems that may occur in any general medical ward. Clinical research activities include programs in all the areas of

Ranganath Muniyappa, MD, PhD,
Director, Inter-Institute Endocrine Training Program; Diabetes, Endocrinology, and Obesity Branch, NIDDK

Michael T. Collins, MD, *Director, Career Development and Research Oversight and Head, Disorders & Mineral Homeostasis Section, NIDCR*

Karel Pacak, MD, PhD, DSc,
Head, Section on Medical Neuroendocrinology, NICHD

Crystal Kamilaris, MD, *Staff Clinician*

Sanjay Jumani, MD, *Clinical Fellow*

Sonal Vaid, MD, *Clinical Fellow*

endocrine and metabolic diseases and diabetes. Study design, outcome measures, statistical analysis, and ethical and regulatory issues are stressed.

The IETP provides a comprehensive training experience that involves not only the NIH clinical branches working in endocrinology but also Georgetown University Hospital, Washington Hospital Center, and Walter Reed National Military Medical Center. The basic and clinical endocrine research facilities at the NIH are among the most extensive and highly regarded in the world. Thus, the fellowship is ideal for physicians who seek a broad education in both research and clinical endocrinology.

Publications

1. Kamilaris CDC, Stratakis CA, Hannah-Shmouni F. Molecular genetic and genomic alterations in Cushing's syndrome and primary aldosteronism. *Front Endocrinol* 2021;12:632543.
2. Gubbi S, Al-Jundi M, Del Rivero J, Jha A, Knue M, Zou J, Turkbey B, Carrasquillo JA, Lin E, Pacak K, Klubo-Gwiezdzinska J, Lin Fi. Case Report: Primary hypothyroidism associated with lutetium 177-DOTATATE therapy for metastatic paraganglioma. *Front Endocrinol* 2021;11:587065.
3. Shekhar S, Sinaii N, Irizarry-Caro JA, Gahl WA, Estrada-Veras JI, Dave R, Papadakis GZ, Tirosh A, Abel BS, Klubo-Gwiezdzinska J, Skarulis MC, Gochuico BR, O'Brien K, Hannah-Shmouni F. Prevalence of hypothyroidism in patients with Erdheim-Chester disease. *JAMA Netw Open* 2020;3(10):e2019169.
4. Al-Jundi M, Thakur S, Gubbi S, Klubo-Gwiezdzinska J. Novel targeted therapies for metastatic thyroid cancer—a comprehensive review. *Cancers (Basel)* 2020;12(8):2104.

Collaborators

- Kenneth Berman, MD, *Director, Endocrine Training Program, Washington Hospital Center, Washington, DC*
- Rebecca Brown, MD, *Diabetes, Endocrinology, and Obesity Branch, NIDDK, Bethesda, MD*
- Alan H. DeCherney, MD, *Reproductive Endocrinology and Infertility Training Program, NICHD, Bethesda, MD*
- Rachel Gafni, MD, *Craniofacial and Skeletal Diseases Branch, NIDCR, Bethesda, MD*
- Phillip Gorden, MD, *Diabetes, Endocrinology, and Obesity Branch, NIDDK, Bethesda, MD*
- Joanna Klubo-Gwiezdzinska, MD, PhD, *Thyroid Tumors and Functional Thyroid Disorders Section, NIDDK, Bethesda, MD*
- Lynnette K. Nieman, MD, *Diabetes, Endocrinology and Obesity Branch, NIDDK, Bethesda, MD*
- William F. Simonds, MD, *Metabolic Diseases Branch, NIDDK, Bethesda, MD*
- Joseph Verbalis, MD, *Director, Endocrine Training Program, Georgetown University Medical Center, Washington, DC*
- Lee S. Weinstein, MD, *Metabolic Diseases Branch, NIDDK, Bethesda, MD*

Contact

For more information, email Ranganath.Muniyappa@nih.gov or go to <https://www.niddk.nih.gov/research-funding/at-niddk/training-employment/medical-student-md/inter-institute-endocrinology-fellowship-program>.

Pediatric Endocrinology Inter-Institute Training Program

The Fellowship in Pediatric Endocrinology is a three-year program, accredited by the Accreditation Council for Graduate Medical Education (ACGME). Applicants must have completed a residency in Pediatrics or Medicine/Pediatrics and be eligible for the American Board of Pediatrics certification examination (exceptions can be made on an individual basis, according to ACGME rules). The fellowship is now closed to new trainees, although it is possible for research-oriented fellows at other institutions who are selected to participate in the Pediatric Scientist Development Program to complete their training in the program. Training takes place predominately at the National Institutes of Health Clinical Center, which is one of the largest and most sophisticated research institutions in the United States. Additional clinical training takes place at Children's National Hospital in Washington, DC, and at Walter Reed National Military Medical Center in Bethesda, MD. The fellowship is designed to provide clinical and research exposure that fosters the development of academic pediatric endocrinologists with experience in clinical, translational, and/or basic research.

The URL <https://www.nichd.nih.gov/about/org/dir/osd/tp/peitp> provides more detailed information about the program.

Program structure

The Pediatric Endocrinology Fellowship at NIH consists of one year of clinical training and two years of combined clinical and research training.

FIRST YEAR

A typical training schedule for first-year fellows includes four months at the NIH Clinical Research Center, four months at Children's National Health Systems (CNHS), two months at Walter Reed National Military Medical Center, one month on consult service, and one month elective (e.g., at The Johns Hopkins University Hospital, Baltimore, MD). Continuity clinics are held once a week and alternate between the NIH outpatient pediatric endocrine clinic and the diabetes and general endocrine outpatient clinics at CNHS. In addition, multi-disciplinary clinics in long-term follow-up for bone disorders, neuroendocrine tumors, disorders of sexual development, obesity,



- Rachel Gafni, MD, *Acting Program Director, Pediatric Endocrinology Fellowship Program and Senior Research Physician, Craniofacial and Skeletal Diseases Branch, NIDCR*
- Christina Tatsi, MD, PhD, *Assistant Clinical Investigator, NICHD*
- Marissa Lightbourne, MD, *Assistant Research Investigator/Staff Clinician, NIDDK*
- Youn Hee Jee, MD, *Staff Clinician, NICHD*
- Deena Zeltser, MD, *Staff Clinician, NICHD*
- Jeffrey Baron, MD, *Head, Section on Growth and Development, NICHD*
- Jack Yanovski, MD, PhD, *Head, Section on Growth and Obesity, NICHD*
- James Mills, MD, *Senior Investigator, Division of Epidemiology, Statistics, and Prevention Research, NICHD*
- Andrew Bremer, MD, PhD, *Program Director, Pediatric Growth and Nutrition Branch, NICHD*

(continued)

and other conditions are offered. The Clinical Center maintains clinical research protocols involving, among others, the treatment of adrenal and pituitary tumors, congenital adrenal hyperplasia, McCune-Albright syndrome, disorders of sexual development, obesity, and lipodystrophy.

SECOND AND THIRD YEARS

During the second and third years, mandatory clinical responsibilities are limited to one half-day continuity clinic per week and inpatient pediatric endocrine consultation on an on-call basis for three months per year. Fellows learn how to develop a research protocol, conduct a clinical study or basic research, evaluate the results, and generate presentations and manuscripts suitable for publication. Fellows may choose to work in a laboratory setting, clinical setting, or both, and they perform state-of-the-art basic and clinical research closely supervised by internationally known mentors. During the first year, a research mentor is chosen, and the fellow's progress is monitored by the Scholarship Oversight Committee. Many of our fellows choose academic careers following graduation.

Application information

As stated above, the program is now closed to new first-year applicants, but physician-scientists interested in completing their training at the NIH should seek admission to the Pediatric Scientist Development Program at <https://amspdc-psdp.org>.

Publications

1. Szymczuk V, Taylor J, Michel Z, Sinaii N, Boyce AM. Skeletal disease acquisition in fibrous dysplasia: natural history and indicators of lesion progression in children. *J Bone Miner Res* 2022 37:1473–1478.
2. Gladding A, Szymczuk V, Auble BA, Boyce AM. Burosumab treatment for fibrous dysplasia. *Bone* 2021 150:116004.
3. Zenno A, Brady SM, Faulkner LM, Ballenger KL, Fatima S, Yanovski JA. Circadian variation of serum leptin and adipose tissue changes in children. *Pediatr Obesity* 2022 12:e12984.

Collaborators

- Andrew Dauber, MD, MMSc, *Director, Pediatric Endocrinology, Children's National Hospital, Washington, DC*
- Karen S. Vogt, MD, *Program Director, Pediatric Endocrinology, Walter Reed National Military Medical Center, Bethesda, MD*

Miranda Broadney, MD, MPH,
*Program Director, Division of
Diabetes, Endocrinology, and
Metabolic Diseases, NIDDK*

Margaret Keil, MS, CRNP, *Director,
Pediatric Endocrine Clinical
Services, NICHD*

Deborah Merke, MD, *Adjunct
Investigator, NICHD, Chief,
Section of Congenital Disorders,
Clinical Center*

Alison M. Boyce, MD, *Lasker
Tenure-Track Investigator, Section
on Skeletal Disorders and Mineral
Homeostasis, NIDCR*

Rebecca J. Brown, MD, MHSc,
*Lasker Tenure-Track Investigator,
Diabetes, Endocrinology, and
Obesity Branch, NIDDK*

Stephanie Chung, MD, *Lasker
Tenure-Track Investigator,
Diabetes, Endocrinology, and
Obesity Branch NIDDK*

Brittney A. Corbin, BA, *Pediatric
Endocrine Fellowship Program
Coordinator, NICHD*

Zubeyir Gun, MD, *Fellow, Pediatric
Endocrinology Fellowship
Program*

Sanjay Gupta, MD, *Fellow,
Pediatric and Adult
Endocrinology Fellowship
Programs*

Vivian Szymczuk, MD, *Fellow,
Pediatric Endocrinology
Fellowship Program*

Contact

Brittney Corbin, BA, Program Coordinator

Brittney.Corbin@nih.gov

Tel: 301-496-4786

Pediatric Endocrinology Training Program

NICHD, NIH

Building 10, Room 2-5142

10 Center Drive

Bethesda, MD 20892-23330

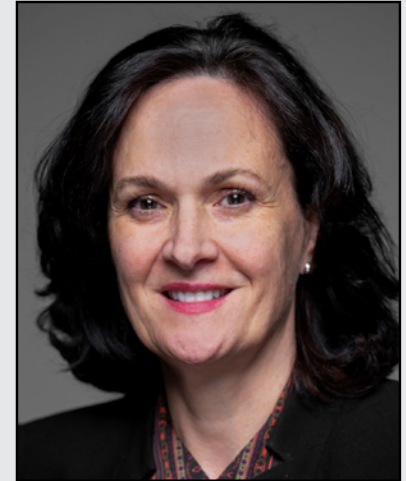
NICHD Pediatric and Adolescent Gynecology Training Program

The Pediatric and Adolescent Gynecology (PAG) program is uniquely qualified to meet the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development's mission to: "lead research and training to understand human development, improve reproductive health, enhance the lives of children and adolescents, and optimize abilities for all." PAG is a relatively new subspecialty within the field of Obstetrics and Gynecology and encompasses gynecologic care from the fetal period into adulthood. Many of the conditions managed by pediatric gynecology are rare diseases and have been poorly studied, and PAG providers have become leaders in the field of fertility preservation in children and adolescents. The NICHD PAG program comprises fellowship training as well as laboratory and clinical research programs.

Fellowship

The NICHD PAG Fellowship includes faculty from three institutions: The National Institute of Child Health and Human Development (NICHD), MedStar Washington Hospital Center (MWHC), and Children's National Hospital (CNH). The Fellowship has been training fellows since 2010; its mission is to ensure that the graduate possesses the knowledge, skills, and professional attributes essential to function as a consultant to pediatricians, family practitioners, obstetricians, and gynecologists for girls from birth to age 18–21 years with pediatric gynecologic concerns, as well as for older women born with congenital anomalies. Our fellows receive the highest quality training in clinical and surgical PAG, as well as research mentoring.

Currently, the Fellowship alternates between recruiting one or two fellows per year and trains both at NIH and Children's National Hospital (CNH). A schedule is established by which fellows rotate through general pediatric and adolescent gynecology clinics (including a heavy-menses clinic), and surgeries, specialty clinics (reproductive endocrine, Turner syndrome, PROUD [positive re-evaluation of urogenital differences clinics, or disorders of sex development], and vulvar dermatology clinics), colorectal surgery at CNH, and research and consult/clinics at the NIH Clinical Center. Fellows have thirty percent of their time protected for clinical research, which takes place throughout the two years of training. Structured training includes a



Veronica Gomez-Lobo, MD,
Program Director

Tazim Dowlut-McElroy, MD,
Associate Program Director

Jacqueline Maher, MD, *Staff Clinician*

Ariel Cohen, MD, *Clinical Fellow*

Jessica Long, MD, *Clinical Fellow*

Rama Kastury, DO, *Clinical Fellow*

Courtney Mascoe, MD, *Clinical Fellow*

Swetha Naroji, MD, *Clinical Fellow*

Taylor Badger, MD Candidate,
Medical Research Scholar Program

Victoria Huynh, MD Candidate,
Medical Research Scholar Program

Kelsey Mumford, MD Candidate,
Medical Research Scholar Program

Sreerama Balasubramanian, BS,
Intramural Research Training Award Fellow

Sarina Hanfling, BS, *Intramural Research Training Award Fellow*

(continued)

series of introductory seminars, monthly PAG lectures, which include a statistics curriculum, specialty clinic conferences (colorectal, Differences of Sexual Development, Turner's), combined REI (reproductive endocrinology and fertility)/PAG Journal Club/case conferences, and bimonthly laboratory meetings.

Research program

The research and laboratory program includes five IRB-approved clinical protocols, which are conducted in the ambulatory, in-patient, and operating rooms of the NIH Clinical Center. In addition, laboratory space within the Clinical Center allows for experiments on ovarian tissue and other samples obtained from these clinical protocols. We also work closely with the core facilities within NICHD, including the [Molecular Genomics](#), [Bioinformatic](#), and Mouse Cores.

The program includes research on fertility preservation in children and adolescents and on rare conditions. Fertility preservation has emerged as an important aspect of cancer care in pediatric and adult populations. In December 2019, the American Society of Reproductive Medicine Committee Opinion on fertility preservation in patients undergoing gonadotoxic therapies stated that that ovarian tissue cryopreservation (OTC) should no longer be considered experimental. However, there remain significant gaps in knowledge regarding OTC for fertility preservation in children and adolescents receiving gonadotoxic therapy, as the fertility effect of gonadotoxic therapy as well as the fertility benefits of OTC cannot be ascertained until 5–30 years after therapy. Recently, there has been increased interest in fertility preservation in other populations, including individuals with Turner syndrome, galactosemia, transgender youth, differences in sex development, and young women with recent premature ovarian insufficiency. Such individuals present further unknowns regarding the risks and benefits of OTC, the foremost being that they may have significantly lower populations of follicles than those planning to receive gonadotoxic therapy and may therefore not benefit from this technology. Furthermore, it is important to note that, although pregnancies have been achieved using this technology (in adults), much remains to be elucidated regarding ovarian histology, function, and mechanism of disease in the ovary, and ovarian-tissue cryopreservation has spurred new evaluation of the human ovary. The PAG program developed two protocols for ovarian-tissue cryopreservation in children and adolescents. The first is a protocol for ovarian-tissue cryopreservation for prepubertal children with Turner syndrome, classic galactosemia, and for adolescents with recent primary ovarian insufficiency (POI). The second protocol for children who will undergo gonadotoxic therapy will allow us to collect tissue and data that will inform the first protocol. In both protocols, we are performing oophorectomy for OTC and collecting a small portion of ovary for research. Through these projects we are defining the components and anatomy of the ovary, evaluating the correlation of ovarian reserve markers with histologic findings, and attempting to elucidate crucial signaling pathways regulating follicle activation and loss, through collaborations with NICHD Core laboratories, using methods including RNA-Seq and single-cell analytics.

Patients with PAG conditions (such as reproductive, endocrine, gynecologic tumors, and skin conditions, menstrual abnormalities, and congenital anomalies of the reproductive tract) may provide exceptional gain of knowledge, which may advance research in pediatric and adolescent gynecology disorders, and their evaluation may catalyze the recognition of new disease processes and new research initiatives. Through the

Bohyon Yun, MD, *Special Volunteer*
Ananya Krishnan, *Summer Intern*
Jennifer Neda John, *Summer Intern*
Rashmi Prasad, *Summer Intern*
Sajeda Shuaib, *Summer Intern*
Hong Lou, MD, *Laboratory Manager/Biologist*
Maria De La Luz Sierra, MS, *Laboratory Manager*
Harveen Kaur, MPH, *Clinical Research Coordinator*
Sofia L. Getachew, MSPM, *Fellowship Program Coordinator*

PAG conditions protocol, we are collecting deep phenotype data and specimens from PAG patients, thus creating a large database of PAG conditions, which will provide stimuli for new clinical research initiatives. One such condition, androgen-insensitivity syndrome (AIS), is a state in which the body cannot sense the male hormones in the blood or tissue. Because this is a rare condition, little is known regarding the risks and benefits of gonadectomy, optimal hormone replacement after gonadectomy, nor general health in individuals with these conditions. Furthermore, the androgen receptor is found in many tissues in the body, including skin, bone, muscle, and in the neurologic, immune, and metabolic systems. Through the PAG program Natural History study in individuals with AIS, we will provide information regarding health risks and optimal management of individuals with AIS, as well as elucidate the role of the androgen receptor.

Application information

Applications are submitted using the common application form on NASPAG. They are accepted in July before the anticipated start date, and interviews are typically held in August or September before the start date. Selection of candidates is processed through the National Resident Matching Program (NRMP). One to two positions are available in alternating years.

Please visit the URL below for detailed program information:

https://www.cc.nih.gov/training/gme/programs/pediatric_adolescent_gynecology.html

Publications

1. Tsui EL, O'Neill KE, LeDuc RD, Shikanov A, Gomez-Lobo V, Laronda MM. Creating a common language for the subanatomy of the ovary. *Biol Reprod* 2022 doi: 10.1093/biolre/ioac199.
2. O'Neill KE, Maher JY, Laronda MM, Duncan FE, LeDuc RD, Lujan ME, Oktay KH, Pouch AM, Segars JH, Tsui EL, Zelinski MB, Halvorson LM, Gomez-Lobo V. Anatomic nomenclature and 3D regional model of the human ovary: call for a new paradigm. *Am J Obstet Gynecol* 2022 S0002-9378(22)00795-5.
3. Ligon JA, Fry A, Maher JY, Foley T, Silbert S, Yates B, Gomez-Lobo V, Wiener L, Shah NN. Fertility and CAR T-cells: current practice and future directions. *Transplant Cell Ther* 2022 28(9):605.e1–605.e8.
4. Finlayson C, Johnson EK, Chen D, Fechner PY, Hirsch J, Rosoklija I, Schafer-Kalkhoff T, Shnorhavorian M, Gomez-Lobo V. Fertility in individuals with differences in sex development: provider knowledge assessment. *J Pediatr Adolesc Gynecol* 2022 35(5):558–561.
5. Maher JY, Islam MS, Yin O, Brennan J, Gough E, Driggers P, Segars J. The role of Hippo pathway signaling and A-kinase anchoring protein 13 in primordial follicle activation and inhibition. *F S Sci* 2022 3(2):118–129.
6. McElroy T, Gomez-Lobo V. Gonadectomy in individuals with Turner syndrome and Y chromosome material: fertility considerations. *J Pediatr Adolesc Gynecol* 2022 35(4):415–416.

Collaborators

- Jeffrey Baron, MD, *Division of Translational Medicine, NICHD, Bethesda, MD*
- Ryan K. Dale, MS, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*
- Alan H. DeCherney, MD, *Reproductive Endocrinology and Infertility Fellowship Training Program, NICHD, Bethesda, MD*
- Francesca Duncan, PhD, *Northwestern University Feinberg School of Medicine, Chicago, IL*
- Fabio R. Faucz, PhD, *Molecular Genetics Core, NICHD, Bethesda, MD*

- Judith L. Fridovich-Keil, PhD, *Emory University, Atlanta, GA*
- Catherine Gordon, MD, *Boston Children's Hospital, Boston, MA*
- James Graham, MS, ELD, *Shady Grove Fertility Center, Rockville, MD*
- Yasmin Jayasinghe, MBBS, FRANZCOG, PhD, *Royal Children's Hospital Melbourne, Parkville, Australia*
- Marissa Lightbourne, MD, MPH, *Section on Translational Diabetes and Metabolic Syndromes, NIDDK, Bethesda, MD*
- Laverne Mensah, MD, *Office of the Clinical Director, NICHD, Bethesda, MD*
- Kyle Orwig, PhD, *University of Pittsburgh, Pittsburgh, PA*
- Karl Pfeifer, PhD, *Section on Genomic Imprinting, NICHD, Bethesda, MD*
- James Segars, MD, *The Johns Hopkins School of Medicine, Baltimore, MD*
- Ariella Shikanov, PhD, *University of Michigan, Ann Arbor, MI*
- Ninet Sinaii, PhD, MPH, *Biostatistics and Clinical Epidemiology Service, Clinical Center, NIH, Bethesda, MD*
- Eric Widra, MD, *Shady Grove Fertility Center, Washington, DC*
- Jack Yanovski, MD, PhD, *Section on Growth & Obesity, NICHD, Bethesda, MD*
- Mary Zelinsky, PhD, *Oregon Health & Science University, Portland, OR*
- Deena Zelster, MD, *Office of the Clinical Director, NICHD, Bethesda, MD*

Contact

Veronica Gomez-Lobo, MD, Program Director

veronica.gomez-lobo@nih.gov

301-435-6926

Sofia Getachew, MSPM, Program Coordinator

sofia.getachew@nih.gov

301-435-6926

Pediatric and Adolescent Gynecology Training Program

NICHD, NIH

10 Center Drive

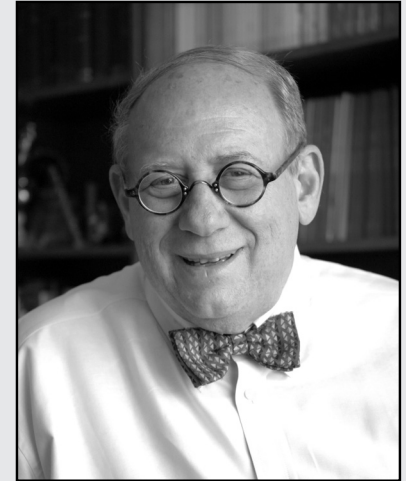
Building 10, Room 8N248, MSC 1840

Bethesda, MD 20892-1840

Reproductive Endocrinology and Infertility Training Program

The Intramural NICHD Reproductive Endocrinology and Infertility Training Program sponsors a three-year clinical fellowship in Reproductive Endocrinology and Infertility, which is accredited by the Accreditation Council for Graduate Medical Education (ACGME). The objective of the graduate medical education program is to train clinicians to serve as researchers and future leaders in the field of reproductive endocrinology with a view toward advancing basic, translational, and clinical science in reproduction. The program was started in 1978 and has since trained over 90 physicians in reproductive endocrinology. Upon completion of the rigorous scientific, clinical, and surgical curriculum, fellows may apply to the American Board of Obstetrics and Gynecology for certification in the subspecialty of Reproductive Endocrinology. The Fellowship in Reproductive Endocrinology and Infertility is served by faculty from four institutions: the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), the Uniformed Services University of the Health Sciences (USUHS), Walter Reed National Military Medical Center (WRNMMC), and the Shady Grove Fertility Center. The program accepts civilian and military graduates of U.S. residencies in Obstetrics and Gynecology and has the mission to train reproductive-endocrinology and infertility fellows who will serve as faculty in academic, government, and military institutions, and to establish and maintain high standards of training for students and residents in obstetrics and gynecology, so that they can provide evidence-based, cutting-edge treatments to couples with infertility. Program graduates have become assistant, associate, and tenured professors and departmental chairs.

Fellows in the program rotate on clinical services of NICHD-supported intramural graduate medical programs in Medical and Pediatric Endocrinology, as well as in Medical Genetics. The clinical training is robust. Thus, within the unique environment of the NIH, fellows participate in evaluation and management of rare and challenging endocrine conditions on the NIH Reproductive Endocrine Teaching Service. Clinical and surgical rotations take place at WRNMMC and the Shady Grove Fertility Center. Research is strongly emphasized. Trainees in the program must complete a thesis project and may choose among any research laboratory in the Institute's intramural research program. To complete the research project, fellows are



Alan H. DeCherney, MD,
*Deputy Director, Reproductive
Endocrinology and Infertility
Fellowship Program*

Micah J. Hill, DO, *Program
Director, Combined NICHD/
Federal Fellowship in
Reproductive Endocrinology*

Torie C. Plowden, MD, MPH,
Associate Program Director

Jasmine M. Aly, MD, *Clinical Fellow*

Marja Brolinson, MD, *Clinical
Fellow*

(continued)

given 20 months of protected research time. The curriculum includes two university-based graduate courses, one in biostatistics, the other in reproduction. In the past year, faculty and fellows published 70 peer-reviewed articles. Over the past five years, each graduate of the program published an average of five peer-reviewed manuscripts associated with the training program, and several trainees received national recognition for excellence in research.

The three-year training program is structured to capitalize on the particular strengths and resources of each participating institution. Specifically, infertility services and operative care are provided by the busy clinical services at Walter Reed Bethesda Hospital, the NIH Clinical Center, and the Shady Grove Fertility Center. Experience in Assisted Reproductive Technologies (ART) is provided by rotation in the newly renovated, state-of-the-art Walter Reed Bethesda ART facility and in the Shady Grove Fertility Center. Fellows also obtain medical endocrine, pediatric endocrine, and genetic clinical training through rotations on the active inpatient services at the NIH Clinical Center. The program staff and fellows see 1,500 patients in the NIH Clinical Center, in addition to conducting 40 surgeries and 50 oocyte retrievals, per year. Outstanding research training is available either through NIH intramural laboratories or at USUHS. The program is intended to achieve synergism between the four sponsoring institutions and to provide fellows with an experience and resources not available from a single institution.

Requirements for enrollment include graduation from a residency in Obstetrics and Gynecology in the United States that is accredited by the ACGME and an active medical license in the United States. Selection is competitive, and prospective candidates must register with the National Resident Matching Program (NRMP). Three positions per year are approved for a complement of nine fellow trainees. Trainees may meet criteria for the NIH Loan Repayment Program (LRP) for outstanding educational debt.

Didactic instruction

Structured training includes a series of introductory seminars geared to first-year fellows, which take place from July to September of the first year. The introductory seminars provide a historical perspective and basic understanding of the practice of Reproductive Endocrinology. In weekly NIH teaching-rounds conferences, fellows review and discuss challenging cases with faculty and fellows. In addition, all faculty and fellows of all years are expected to attend the weekly Preoperative and Fellows' conferences. Fellows also attend weekly research conferences sponsored by the NICHD, and they present updates on thesis work at the weekly "Research in Progress Conference." Core training objectives of the ACGME are covered in special NIH grand rounds and by courses at the NIH or the WRNMMC in Bethesda. NIH Endocrine Grand Rounds provide additional training in medical, pediatric, and reproductive endocrine conditions. Regular attendance at a monthly journal club is expected. Fellows regularly attend ART clinical meetings, during which management of patients pursuing ART is discussed and outcomes are reviewed. In addition to larger groups, mentors of individual laboratories to which the fellow is affiliated generally meet on a weekly basis to review research progress. Furthermore, fellows are encouraged to participate in didactic training offered at national meetings, such as those of the *American Society for Reproductive Medicine*, the *Society for Reproductive Investigation*, and

Jennifer Chae-Kim, MD, *Clinical Fellow*

Shelley Dolitsky, MD, *Clinical Fellow*

Ariel J. Dunn, MD, *Clinical Fellow*

Charlene Echague, DO, *Clinical Fellow*

Peter G. Lindner, MD, *Clinical Fellow*

Adina Schwartz, MD, *Clinical Fellow*

Karrie Walker, DO, *Clinical Fellow*

Grace Whiteley, DO, *Clinical Fellow*

Meghan Yamasaki, DO, *Clinical Fellow*

the *Society for the Study of Reproduction*. Moreover, fellows are encouraged to attend specialty meetings in their chosen interest areas.

Publications

1. Eubanks AA, Nobles CJ, Mumford SL, Kim K, Hill MJ, DeCherney AH, Sjaarda LA, Ye A, Radoc JG, Perkins NJ, Silver RM, Schisterman EF. The safety of low-dose aspirin on the mode of delivery: secondary analysis of the effect of aspirin in gestation and reproduction randomized controlled trial. *Am J Perinatol* 2022 39(6):658–665.
2. Glatthorn HN, DeCherney A. The efficacy of add-ons: selected IVF “add-on” procedures and future directions. *J Assist Reprod Genet* 2022 39(3):581–589.
3. Tong ZB, Otsuka N, Tu W, Wei Q, DeCherney AH. P450 side-chain cleavage enzyme (P450-SCC) is an ovarian autoantigen in a mouse model for autoimmune oophoritis. *Reprod Sci* 2022 29(8):2391–2400.
4. Wong AJ, DeCherney AH. Live birth with or without preimplantation genetic testing for aneuploidy. *N Engl J Med* 2022 386:703.
5. Zolton JR, Sjaarda LA, Mumford SL, Holland TL, Kim K, Flanagan KS, Yisihak SF, Hinkle SN, Connell MT, White MV, Perkins NJ, Silver RM, Hill MJ, DeCherney AH, Schisterman EF. Preconception hemoglobin A1c concentration in healthy women is not associated with fecundability or pregnancy loss. *F S Rep* 2022 3:39–46.

Collaborators

- William H. Catherino, MD, PhD, *Uniformed Services University of the Health Sciences, Bethesda, MD*
- Alicia Christy, MD, *Reproductive Health, Veterans Administration Central Office, Washington, DC*
- Kate M. Devine, MD, *Shady Grove Fertility, Washington, DC*
- Saioa Torrealday, MD, *Walter Reed National Military Medical Center, Bethesda, MD*
- Eric A. Widra, MD, *Shady Grove Fertility, Washington, DC*

Contact

Reproductive Endocrinology and Infertility Training Program
NICHD, NIH
Bldg. 10, Rm. 8N248
10 Center Drive
Bethesda, MD 20892-1840

Phone: 301-402-2141

Fax: 301-451-2857

Program Director: Micah J. Hill, DO; hillmicah@mail.nih.gov

Program Coordinator: Marilyn Minor; minormar@mail.nih.gov

Program Website: https://www.cc.nih.gov/training/gme/programs/reproductive_endocrinology.html

Online Application: <https://students-residents.aamc.org/training-residency-fellowship/applying-fellowships-eras>

Administrative Management Branch

The Administrative Management Branch (AMB) in the Division of Intramural Research (DIR), NICHD, assists in the planning and managing of a variety of administrative management projects. The AMB provides administrative oversight for adherence to rules and regulations, and expertise in administrative services, to ensure that the NICHD continues to move forward in its mission. The AMB staff are a key resource to NICHD's Scientific Director in the management and overall planning for the DIR.

The senior leadership within the AMB works directly with the Scientific Director and Deputy Scientific Director in strategic planning and administrative oversight, and plays a key role in maximizing the resources available to the DIR. The leadership provides guidance in all aspects of administration and represents the DIR at various NICHD programs and NIH-wide committees, as well as at focus groups concerned with administration.

The AMB administrative staff provide professional, technical, and administrative support in functional areas that further the mission of the DIR. These include budget and financial management, human resources, visas, travel, simplified acquisition, contract management and project officer support, safety and security, building and facilities management, timekeeping, program planning and evaluation, and general administrative services.

The AMB staff serve as a liaison between the laboratories and branches that they support and the many other entities at the NIH, such as the Office of the Scientific Director, NICHD; the Office of the Director, NICHD; the Office of Intramural Research, Office of the Director; the Fogarty International Center; Human Resources offices; the Office of Research Services, including the Division of International Services; the Office of Research Facilities; and the Technology Transfer Branch.

Additional Funding

- \$6.9M

Contact

For more information, email franciek@mail.nih.gov or visit <http://amb.nichd.nih.gov>.



Francie Kitzmiller, Administrative Officer, Chief, Administrative Management Branch

Valerie Leftwood, Administrative Officer, Deputy Branch Chief

Becky Preston, Administrative Officer, Deputy Branch Chief

AMB Staff

Izet Beckwith, *Administrative Officer*
Antoinette Chavez, *Administrative Officer*
Gina Elmore, *Administrative Officer, Budget*
Dena Flipping, *Administrative Officer*
Michelle Hudson, *Administrative Officer, Team Leader*
Bonnie Lancey, *Administrative Officer*
Keta Lawson-Davis, *Administrative Officer*
Wanda Logan, *Administrative Officer*
Lakeasha Mingo, *Administrative Officer, Team Leader*
Charlene Patrick, *Administrative Officer, Team Leader*
Mia Pulley, *Administrative Officer, Team Leader*
Natacha Rene, *Administrative Officer*
Nancy Richman, *Administrative Officer*
Sylvia Robinson, *Administrative Officer, Procurement Lead*
Beverley Todd, *Administrative Officer*
Marlene Taulton, *Administrative Officer*
Brittany Corum, *Administrative Technician*

Barbara Hardy, *Administrative Technician*
Ashley Mason, *Budget Analyst*
Tamika Morgan, *Facilities Assistant*
Steven Norris, *Facilities Manager*
Moona Braxton, *Procurement Technician*
Jennifer Smith, *Procurement Technician*
Anju Vergheese, *Program Analyst*
John Burton, *Purchasing Agent*
Jax Chang, *Purchasing Agent*
Towanda Daniels, *Purchasing Agent*
Roshonna Davis, *Purchasing Agent*
William Davis, *Purchasing Agent*
Sherry Jones, *Purchasing Agent*
James Law, *Purchasing Agent*
David Shen, *Purchasing Agent*
Hanumanth Vishnuvajjala, *Purchasing Agent*
Brittney Corbin, *Program Specialist*

Research Animal Management Branch

The Research Animal Management Branch (RAMB) supports all animal use and research within the NICHD Division of Intramural Research (DIR).

In addition to providing animal research support and veterinary consultation to NICHD investigators, RAMB represents the interests of the NICHD DIR on all aspects of animal research conducted within the Intramural Research Program (IRP). This includes animal facilities managed by NICHD as the lead institute, NICHD animals housed in other facilities, and all activities involving NICHD DIR-owned animals. The Branch specifically:

1. provides primary veterinary care;
2. advises the NICHD Scientific Director on animal care and use practices;
3. assures animal use within DIR is in accordance with applicable regulatory standards and guidelines;
4. coordinates intramural animal use including appropriate animal model selection, support requirements, and Animal Study Proposal review;
5. advises the scientific staff on comparative medicine, Animal Study Proposal design, disease interference, and other factors that may complicate or invalidate research results;
6. implements and coordinates animal health monitoring;
7. coordinates quarantine for incoming animals of unknown health status to prevent the introduction of agents pathogenic to humans or other animals;
8. coordinates a central ordering program for the NICHD;
9. provides administrative management of the NICHD Animal Care and Use Committee (ACUC); and
10. interfaces with organizations and institutions concerned with the ethical and humane care and use of animals in research.

RAMB operates and manages the Building 6B Shared Animal Facility (SAF), Suite 6C127 of the Ambulatory Care Research Facility (ACRF) Animal Facility, the Building 6 Shared Zebrafish facility (SZF), and the Building 49 Xenopus facility. RAMB staff and the NICHD ACUC have oversight over these facilities with regard to animal use as well as over NICHD animals in the Porter Neurosciences Research Center (PNRC) SAF, and in Central Animal Facilities (CAF). The RAMB Aquatics

Rachael Labitt, DVM, MS,
Attending Veterinarian

Daniel T. Abebe, MS, *Research Behavioral Technician*

Julie Jacobs, *Animal Program Manager*

Terrance McMeans, AA, *Rodent Facilities Manager*

Maria Publico, BS, *Animal Care and Use Committee Coordinator*

Christopher Rishell, BS, *Senior Facilities Manager*

Robin Kastenmayer, DVM, PhD,
Former Acting Animal Program Director and Senior Veterinarian

Mike Wisnieski, BS, *Former Aquatic Facilities Manager*

Program also provides aquatics animal care and husbandry to several institutes and centers (ICs) within their satellite facilities across the NIH. At the NIH, NICHD is considered a leader in the field of aquatics research animal care and support.

The Building 6B SAF houses over 11,000 cages of specific pathogen-free (SPF) rodents and provides lead institute support to the animal-research activities of three Institutes (NICHD, NEI, and NIAMS). In addition to traditional rodent research support, the program supports NICHD behavioral research studies with a dedicated technician. The NICHD ACRF Animal Facility supports the animal research activities of the NICHD with four animal rooms and two procedure rooms. The RAMB provides care and housing for rodent and aquatic species housed within this restricted-access, disease-free animal holding facility.

NICHD Aquatics are housed within the Building 6 SZF, ACRF Animal Facility, and Building 49. The Building 6 SZF supports NICHD and NHGRI with 12,500 tanks with a total capacity of over 23,000 tanks. Additional aquatics facilities provide primary animal care and research support to over 180 tanks of *Xenopus* and over 95 tanks of *Astyanax* used by NICHD DIR researchers.

As part of the NIH, RAMB staff participate in the formulation of policies and procedures that impact the care and use of laboratory animals throughout the country. RAMB leads the effort for triennial re-certification by AAALAC International. RAMB staff and many animal-user investigators have been active contributors to the NIH Animal Research Advisory Committee (ARAC) and other trans-NIH committees.

Additional Funding

- In addition to direct funding by the intramural research programs of NICHD, the RAMB is also funded by facility users from other NIH Institutes and Centers.

Contact

For more information, email rachael.labitt@nih.gov or visit <https://www.nichd.nih.gov/about/org/dir/osd/mt/ramb>.

Bioinformatics and Scientific Programming Core Facility

The goal of the Bioinformatics and Scientific Programming Core (BSPC) is to provide expert bioinformatics support to NICHD researchers, assisting at all stages, from experimental design through several iterations of analysis to final manuscript preparation. In addition, we develop software tools that can be applied to a wide range of bioinformatics, genomics, and general data analysis, both at the NICHD and in the larger international scientific community. We also coordinate training for staff and trainees in basic programming and genomic analyses to help build bioinformatics support directly within labs.

Structure

The BSPC uses a “hub and spoke” model, consisting of a central core of staff in Building 6A coordinating with embedded bioinformaticians (currently in Buildings 6, 49, and 35) working directly in laboratories. This allows us to build a centralized infrastructure that can be re-used across many research programs, while at the same time maintaining focused and custom local support in labs. Joint meetings and discussion allow everyone, central and embedded, to share lessons learned and identify new tools and methods.

Projects overview

In 2022, the BSPC worked on 90 projects, collaborating with principal investigators (PIs), fellows, staff scientists, and staff clinicians across 36 laboratories. Of these, 32 were new projects and 58 were carried over from the previous year. The projects included assays such as bulk RNA-Seq, single-cell RNA-Seq, ChIP-Seq, whole-exome sequencing, whole-genome sequencing, DNA methylation, CUT&RUN, bulk ATAC-Seq and single-cell ATAC-Seq, TRIP (Thousands of Reporters in Parallel) data, CRISPRi methods development, and long-read assembly. In addition, new projects this year included Tn-Seq, spatially-resolved RNA-Seq, proteomics, and metabolomics. Some projects involved custom algorithm development and tool development, and many projects required integration with published studies.

Projects often begin with an in-depth discussion with researchers to understand the background and goals of the project. It is important for us to understand the underlying biology and details of the experimental design (when applicable) for each project, so that we



Ryan Dale, MS, PhD, Scientific Information Officer, Head, Bioinformatics and Scientific Programming Core

Caroline Esnault, PhD, *Staff Scientist*

Apratim Mitra, PhD, *Staff Scientist*

Hongen (Henry) Zhang, PhD, *Staff Scientist*

Gennady Margolin, PhD, *Bioinformatics Scientist*

Mira Sohn, PhD, *Bioinformatics Scientist*

Kiersten Campbell, BS, *Postbaccalaureate Fellow*

Gus Fridell, BS, *Postbaccalaureate Fellow*

Eva Jason, BS, *Postbaccalaureate Fellow*

Lilly Shatford-Adams, BS, *Postbaccalaureate Fellow*

can make the most informed analysis decisions. We then provide a prioritized plan for the first round of analysis and schedule the work. There are often several iterations of analysis as a project progresses. Each iteration may add more sophisticated analyses, new data generated by the lab, or integrate results with published data. As expected for a no-cost shared resource, the time it takes for one iteration on one project is highly dependent on the existing workload across all other projects that we are handling in the Institute.

After each iteration, we meet to discuss the results in detail. The meeting includes a walk-through of the results, the computational background, discussion of how to use and interpret the tables, figures, and other output, and recommendations for next steps. Depending on the researcher's interests, this can also include a discussion of the code and help with running it or adapting it to other projects in the lab. The next iteration of analysis is then planned, prioritized, and scheduled.

The BSPC's collaboration includes writing the manuscript, producing figures and tables, consulting on interpretation, writing detailed computational methods, reviewing code, and submitting code to public repositories along with the complete software environments required to make the analyses reproducible.

Projects: computation and code

Most projects are multi-week or multi-month projects, which continue after many iterations and often require authoring substantial amounts of custom R and Python code. We work closely with NICHD's [Molecular Genomics Core](#), where much of the raw high-throughput sequencing data for NICHD are generated. We can access these data directly, avoiding the need to coordinate data transfer and/or storage space with researchers. Analysis performed by the BSPC makes extensive use of NIH's Biowulf high-performance computing cluster, and there is no direct cost to researchers for work done by the BSPC.

To ensure long-term computational reproducibility, we build a complete software environment for each project, which allows us to track all versions of software and dependencies, and any one project's environment can be updated without affecting any others. All source code is kept under version control so that the entire history of the project can be tracked. We also build reproducible workflows for each project that keep track of which results have been updated and, wherever possible, provide output as standalone, interactive HTML files, so that researchers can easily explore their results.

We also maintain R Shiny applications into which we load analysis results. After our collaborators authenticate in the system, they are able to explore their results with interactive plots and tables, which allow them to dig deeper without requiring additional computational resources or bioinformatics skills. These applications are continuously updated based on feedback from our collaborators to ensure that they remain easy to use and helpful. New this year is an interactive web application allowing our collaborators to explore their results from snRNA-Seq, scRNA-Seq, and spatially resolved RNA-Seq.

Additional software development and computational resources

The BSPC continues to develop and maintain publicly available open-source tools. One example is `lcdb-wf`, a system of workflows and pipelines to process high-throughput sequencing data, run extensive quality control, and perform differential ChIP-Seq or RNA-Seq analyses and which run on NIH's Biowulf computing cluster. We also continue to contribute to the Bioconda project, a system used by bioinformaticians worldwide to easily install biology-related software tools.

The BSPC maintains an RStudio Connect Server instance, which allows us to publish interactive applications that researchers can use to interactively explore and plot their data. We also maintain a GitLab instance in NICHD's data center, which provides source-code version control, issue tracking, and documentation for projects we work on in such a way that they can be shared with collaborators. These repositories currently store tens of thousands of lines of Python and R code and documentation written by the BSPC and used in various projects.

Contact

For more information, visit <https://www.nichd.nih.gov/about/org/dir/other-facilities/cores/bioinformatics>.

Research Informatics Support for NICHD's Division of Intramural Research

The Computer Support Services Core (CSSC) facility provides informatics and research services to intramural investigators of the Division of Intramural Research (DIR), NICHD, in the following key areas: core IT support, clinical informatics, custom software development for scientific and administrative support, and biological visualization services.

Core IT Services

During the past year, the CSSC continued supporting reliable, secure, and efficient information technology solutions. This includes acquisition, maintenance, and support for licensed software used by the DIR community, e.g., GraphPad Prism, Amira, Alamut, DNASTAR Lasergene, MathWorks MATLAB, SnapGene, PyMol, and FlowJo, as well as cross-platform desktop, server, and application hosting in the Rock Spring Data Center.

Clinical informatics

CSSC continued to support and develop applications related to clinical and translational medicine, including the Clinical Trials Database (CTDB) project. Such informatics tools allow researchers to design, collect, and report clinical observations related to natural history and interval-based studies. The total number of protocols and research projects supported by the CTDB team for 15 NIH institutes increased to 720 protocols. The Global Question Library expanded to over 274,000 research questions. Our software development group completed two CTDB releases. Features included improvements in the Quality Assurance module, Forms module, and Barcode printing. We supported the Clinical Trial Survey System (CTSS), an application for patient self-reporting, servicing 90 active protocols. The team completed one CTSS release and has rolled out 48 CTSS redesigned websites. CTDB application also supports the NICHD Office of Clinical Director central biorepository and eligibility monitoring. Through the global library in CTDB, several institutes are tracking CVs, trainings, and certificate documentation of the research teams. Since its inception, data from CTDB supported over 1,500 NICHD publications.

The database development and reporting team continued integrations with other NIH institutes. The team completed an integration with



Ryan Dale, MS, PhD, Scientific Information Officer, Head, Computer Support Services Core

Asma Idriss, PMP, MS, *Program Manager*

Patricia Pullen, MBA, *Project Manager*

Sean Ivusic, MS, *Database Support Lead*

Kesa Koresko, MS, *Senior DB/BI Specialist*

Loc Vu, BS, *Lead Software Engineer*

Matt Breymaier, BS, *Senior Bio/Application Software Engineer*

Louis Battuello, PMP, BS, *Subject Matter Expert*

Nareg Bakirci, MS, *Website Developer*

Nicki Swan, BA, *Graphic Designer*

Rana Alneaimy, MD, *Documentation Specialist*

Rolanda Bailey, BS, *Documentation Specialist*

Audrey Harrell, MS, *Documentation Specialist*

Tamara Prodanov, MD, *Documentation Specialist*

(continued)

NHBLI that receives data from the CMRCoop (Cardiac Magnetic Resonance Cooperative) system. The team continued to work with various NIMH systems to incorporate data from these systems into the CTDB data-reporting environment, specifically working towards integrating the NIH Tool box system used by investigators at NIMH. We continued supporting data marts as new reporting requirements appear and migrating data as needed. The team added extract data from CITs Biomedical Translational Research Information System (BTRIS) to support investigators research, in addition to migrating data into the data mart from various external research organizations (MedStar, Inova, John Hopkins, etc.). The team has upgraded all supporting Oracle databases to 19c and intends to complete final production upgrade to Oracle19c by the end of the calendar year. The team successfully upgraded the Cognos reporting environment to IBM Cognos 11.1.4 and is working towards upgrading the environment to IBM Cognos 11.2.2. Additionally, the team and has worked closely with various principal investigators (PIs) across the institutes to provide both management and research-related reports for clinical-related studies, publishing over 650 reports in the past year. The team applies the latest patches to all production database environments to ensure continued uninterrupted services and monitors the successful completion of backup and data mart transformation services.

Vida Bayat Mokhtari, MD,
Information Specialist
Kelly Colligan, BS, *Information Specialist*
Kami Emanuel, *Information Specialist*
Breanna Mcgriff, BS, *Information Specialist*
George Tran, *Information Specialist*

Custom software development for scientific and administrative support

The CSSC provides custom software development for the DIR's scientific and administrative community.

We continued to enhance the Manuscript Tracking System (MTrac), a web-based application that automates the clearance and approval process for manuscripts in the DIR.

The DIRweb application supports several activities: the NICHD Annual Report, PI and Fellows' retreats, Training tracking, Fellows' progress report, and [Administrative Management Branch \(AMB\)](#) personnel and travel package tracking. The DIRweb includes lab training web services for the NIH Enterprise Directory and Division of Occupational Health and Safety Training.

The team continued to release enhancements to the Fellows Annual Progress Report, a unified means for tracking and mentoring intramural trainees as well as for easing the re-appointment process. This solution provided the [Office of Education](#) with useful metrics regarding mentoring and training programs. We also updated the Exit Survey feature, a short survey allowing DIR Fellows a platform for providing feedback.

We continued developing new features and improvements for the Package Tracking module used by the DIR AMB, providing AMB staff real-time accuracy metrics for personnel and travel package compilation. The CSSC team continues to develop and support several feedback systems to support real-time customer satisfaction collection. These include surveys for the AMB, the [Office of the Clinical Director](#), laboratory administrative support staff, and NICHD's Administrative Services Branch. The system also offers more detailed feedback submissions periodically, along with comprehensive response metrics.

The team finalized a new module: the Capital Equipment/Expenditure Request Tracking System. The system will enable users to efficiently submit requests through the review process, while allowing administrative staff to

track requests through the workflow process. The project has been well received enough to allow potential offerings to the NICHD extramural community as well as the Office of the Director.

The CSSC team continued to improve Cost Tracker, an application that permits capturing, organizing, and reporting various expenses on a per-protocol basis. The work is done closely with the [Office of the Clinical Director](#) to improve protocol cost vs. effectiveness and a protocol-cost estimator module.

We helped develop and deploy a web application for visualizing single-cell RNA sequencing data from several timepoints in zebrafish embryos and larvae, enabling the research community to explore and interact with a rich dataset, so as to gain insight into early vertebrate development.

Scientific communication support

The CSSC team provided DIR laboratories with scientific communications and media services, including publication support, and website support. Those services were provided to the NICHD DIR Annual Report, the DIR Annual Fellows Retreat, the DIR Annual Scientific Retreat, the Anita B. Roberts Lecture Series, and the NICHD research labs and medical training programs. We supported the NICHD Office of Education by producing a monthly newsletter, the monthly [SD Bulletin](#) and [The NICHD Connection](#) in collaboration with Intramural Fellows. We continued maintaining websites for the NICHD DIR Annual Report.

The CSSC continued to provide a platform for conducting scientific review by the [Board of Scientific Counselors](#), administrative intranet support, and business operations.

Additional Funding

- The Clinical Trials Database (CTDB) project receives funding from other NIH Intramural Institute or Center programs, including NINR, NIMH, NIDCR, NIEHS, NIAMS, NINDS, CC, NHLBI, NIDCD, NHGRI, NCCIH, NIMHD, and NIDDK.

Collaborators

- Richard Childs, MD, *Clinical Director, NHLBI, Bethesda, MD*
- Maryland Pao, MD, *Clinical Director, NIMH, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, NICHD, Bethesda, MD*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

Contact

For more information, visit <https://www.nichd.nih.gov/about/org/dir/osd/cf/ucss>.

NICHD Microscopy and Imaging Core

The mission of the NICHD Microscopy and Imaging Core (MIC) is to provide service in four different areas: (1) histology and sample preparation for light and electron microscopy; (2) wide-field and confocal light microscopy; (3) transmission electron microscopy (TEM); and (4) image analysis and data extraction. The Facility operates as a 'one-stop shop,' where investigators can, with a minimum of effort, go from their scientific question to the final data.

Mode of operation

Located on the ground floor of the Porter Building (building 35A), the MIC is accessible 24/7, and users can reserve time on each microscope by using an [online calendar](#). The facility is available free of charge to all NICHD investigators and, resources allowing, to anyone within the Porter building. The facility is supported by the Office of the Scientific Director, NICHD. The MIC is under the joined management of Tamás Balla (Scientific and Administrative director) and Vincent Schram (acting executive director).

Schram is the point person for light microscopy and data analysis. Ling Yi is in charge of the histology/sample preparation unit. The Electron Microscopy (EM) branch of the Facility is staffed by Chip Dye. Ling Yi and Chip Dye report to Vincent Schram, who coordinates activities with Tamás Balla.

The MIC has an open-door policy with the NINDS Light Imaging Facility (LIF, Building 35). The two cores freely exchange users, share equipment, and trade support. Although not officially sanctioned, this mode of operation provides extended support hours, wider expertise, and access to more equipment than each Institute could afford on its own.

The MIC serves over 300 registered users in 68 laboratories. NICHD uses 80% of the facility's resources, NINDS 15%, and other Institutes (NIBIB, NIA, and NIMH) the remaining 5%.

Light microscopy

The MIC is equipped with six confocal microscopes, each optimized for certain applications:

1. A Zeiss LSM 710 inverted for high-resolution confocal imaging;



Vincent Schram, PhD, Acting Executive Director

Tamás Balla, MD, PhD, *Scientific & Administrative Director*

Ling Yi, PhD, *Staff Scientist*

Louis (Chip) Dye, BS, *Research Assistant*

2. the Nikon Spinning Disk/Total Internal Reflection Fluorescence (TIRF), equipped with advanced rotating TIRF capabilities;
3. a Zeiss LSM 880 2-photon confocal for thick tissues and live animals;
4. a Zeiss 800 optimized for advanced tiling experiments; and
5. a Zeiss 880 AiryScan with higher spatial resolution. The older Zeiss LSM 780 was retired in July 2022 and replaced with a modern LSM 900 equipped with an AiryScan detector.

The fully automatic Zeiss Axioscan Z1 slide scanner was heavily used in FY 2022, freeing up hundreds of personnel hours for several research units in the DIR. We also installed a high-end wide-angle fluorescence microscope to complement our equipment base for non-confocal microscopy imaging.

High-end computer workstations with imaging software (Zeiss Zen, Nikon Elements, Bitplane Imaris, SVI Hyugens and ImageJ) continue to be used for data extraction. Unfortunately, the MIC began to be handicapped by acute network connectivity issues in June 2022, a problem that shows no sign of being resolved.

The light microscopy branch of the MIC continues to rely on the same mode of operation. After an initial orientation, during which their project is researched by the staff and the best approach is decided upon, users receive hands-on training on the equipment and/or software best suited to their goals, followed by continuous support when required. Once image acquisition is complete, the staff devise solutions and train users on how to extract usable data from their images.

Electron microscopy

The electron microscopy section of the facility processes specimens from start to finish: fixation, embedding, semi-thin and ultra-thin sectioning, staining, and imaging on the JEOL 1400 transmission electron microscope. Because of the labor involved, the volume is necessarily smaller than for the light microscopy branch, in which end users perform their own processing and imaging. In the past 12 months, Chip Dye processed a total of 148 samples for morphology studies.

An automatic sample preparation device from Microscopy Solutions was acquired and installed, allowing the volume of specimens processed to be increased. John Heuser, an expert in electron microscopy, continues to use the JEOL 1400 microscope and interface with Chip Dye, who brings his extensive experience to the MIC.

Tissue preparation

The histology/sample preparation lab is the cornerstone of the MIC. Ling Yi has done a remarkable job to ensure a smooth transition after Lynne Holtzclaw's departure, and is now providing sample processing and histology services for light and electron microscopy.

Twenty-two users were trained in-person in rodent perfusion, cryopreservation, cryosectioning, immunofluorescence, and RNAscope. Perfusion and cryosectioning services were provided to eight laboratories. NICHD users include [Andres Buonanno](#), [Rena D'Souza](#), [Douglas Fields](#), [Dax Hoffman](#), [Claire Le Pichon](#), [Karl Pfeifer](#), and [Brant Weinstein](#). Users from other Institutes include Paule Joseph (NIAAA) and Mario Penzo (NIMH).

Ling Yi invested heavily in implementing RNAscope applications in the MIC, to the point where it has become

routine for many of the facility's users. She is currently working on optimizing tissue clearing methods by shortening the long incubation times these techniques require.

Image analysis

High-end computer workstations with imaging software (Zeiss Zen, Nikon Element, Bitplane Imaris, SVI Hyugens, and ImageJ) are available at the MIC.

Image processing based on neural networks (Artificial Intelligence or AI) is a remarkably powerful tool for image restoration, segmentation, and resolution improvement. The MIC is actively looking into AI-powered solutions for image restoration and segmentation. The Nikon NIS-AI suite, an advanced software for noise removal and segmentation not possible with conventional methods, is available in the Core.

Collaborators

- John Heuser, PhD, *Section on Integrative Biophysics, NICHD, Bethesda, MD*
- Carolyn L. Smith, PhD, *Light Imaging Facility, NINDS, Bethesda, MD*

Contact

For more information, email schramv@mail.nih.gov or visit <http://mic.nichd.nih.gov>.

Molecular Genomics Core Facility

With the goal of understanding the genetic changes and mechanisms underlying human diseases, the Molecular Genomics Core (MGC) Facility supports NICHD investigators by providing next-generation deep sequencing and project data analysis.

Next-Generation sequencing and bioinformatics support

The Molecular Genomics Core (MGC) provides DNA and RNA sequencing services for genomic and genetic research to investigators within the NICHD. The MGC is currently operating with four sequencing machines. Most of our work is conducted on our high-capacity, production-scale machine: an Illumina NovaSeq 6000. Two of the other sequencers, an Illumina MiSeq and an Ion Torrent Personal Genomics Machine, are smaller, faster machines, which can generate longer sequence reads of up to 400 base pairs. The fourth sequencer is a Pacific Biosciences (PacBio) Sequel IIe (recently upgraded), which can sequence long single molecules of more than 100,000 base pairs. This array of sequencers provides a suite of scales and capabilities. Our sequencing services include whole-genome, whole-exome, targeted exome, and gene-specific DNA sequencing, as well as whole-transcriptome sequencing (RNA-Seq), microRNA sequencing, microbiome sequencing, bisulfite sequencing (DNA methylome), ChIP-Seq, and ribosomal profiling. The PacBio Sequel IIe permits mutation phasing, structural variant analysis, transposon location identification, and other analyses that are not possible or practical with the other sequencers.

The MGC also operates a 10X Genomics Chromium Single Cell Controller. The Chromium converts a suspension of single cells or nuclei into cDNA libraries that are barcoded by cell or nucleus of origin. The cDNAs can then be converted into sequenceable libraries and run on our Illumina NovaSeq 6000 machine to generate thousands of cell-specific transcriptomes.

The MGC provides significant primary data-processing and downstream bioinformatic support and can assist in designing experiments or sequencing strategies (for example, optimization of targeted exome design). During FY22, MGC sequenced 3,269



Forbes D. Porter, MD, PhD,
*Director, Molecular Genomics
Core Facility*

Fabio Rueda Faucz, PhD, *Staff
Scientist*

James R. Iben, PhD, *Staff Scientist*

Tianwei Li, PhD, *Staff Scientist*

Cameron Padilla, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

samples submitted as 242 projects across the full spectrum of sequencing types, generating 16,811 gigabases of sequence; the projects involved 35 NICHD Principal Investigators from 12 Affinity Groups. In addition to sequencing and providing our standard primary analysis of the resulting data, the MGC delivered enhanced bioinformatic support to 15 NICHD investigators across seven Affinity Groups. Our mission is to offer accurate and innovative sequencing and bioinformatic tools to facilitate research into the diagnosis, counseling, and treatment of hereditary disorders, and to support basic research that promotes understanding of human health and development.

Publications

1. Kozlov G, Mattijssen S, Jiang J, Nyandwi S, Sprules T, Iben JR, Coon SL, Gaidamakov S, Noronha AM, Wilds CJ, Maraia RJ, Gehring K. Structural basis of 3'-end poly(A) RNA recognition by LARP1. *Nucleic Acids Res* 2022 50:9534–9547.
2. Zarkoob H, Allué-Guardia A, Chen YC, Garcia-Vilanova A, Jung O, Coon S, Song MJ, Park JG, Oladunni F, Miller J, Tung YT, Kosik I, Schultz D, Iben J, Li T, Fu J, Porter FD, Yewdell J, Martinez-Sobrido L, Cherry S, Torrelles JB, Ferrer M, Lee EM. Modeling SARS-CoV-2 and influenza infections and antiviral treatments in human lung epithelial tissue equivalents. *Commun Biol* 2022 5:810.
3. Rossi ÁD, Faucz FR, Melo A, de Azevedo GS, Pezzuto P, Bezerra OCL, Manta FSN, Azamor T, Schamber-Reis BLF, Tanuri A, Moraes MO, Aguiar RS, Stratakis CA, Cardoso CC. Association between maternal non-coding interferon- λ polymorphisms and congenital Zika syndrome in a cohort from Brazilian Northeast. *Viruses* 2021 13:2253.
4. Cherkasova V, Iben JR, Pridham KJ, Kessler AC, Maraia RJ. The leucine-NH $_4^+$ uptake regulator Any1 limits growth as part of a general amino acid control response to loss of La protein by fission yeast. *PLoS One* 2021 16(6):e0253494.
5. Coon SL, Li T, Iben JR, Mattijssen S, Maraia RJ. Single-molecule polyadenylated tail sequencing (SM-PAT-Seq) to measure polyA tail lengths transcriptome-wide. *Methods Enzymol* 2021 655:119–137.
6. Nguyen TH, Vicidomini R, Choudhury SD, Coon SL, Iben J, Brody T, Serpe M. Single-cell RNA sequencing analysis of the Drosophila larval ventral cord. *Curr Protoc* 2021 1(2):e38.

Collaborators

- Jeffrey Baron, MD, *Section on Growth and Development, NICHD, Bethesda, MD*
- Diana Bianchi, MD, *Prenatal Genomics & Therapy Section, NHGRI, Bethesda, MD*
- Juan Bonifacino, PhD, *Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD*
- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- Michael Cashel, MD, PhD, *Section on Molecular Regulation, NICHD, Bethesda, MD*
- Ajay Chitnis, MBBS, PhD, *Section on Neural Developmental Dynamics, NICHD, Bethesda, MD*
- David J. Clark, PhD, *Section on Chromatin & Gene Expression, NICHD, Bethesda, MD*
- Robert J. Crouch, PhD, *Section on the Formation of RNA, NICHD, Bethesda, MD*
- Mary Dasso, PhD, *Section on Cell Cycle Regulation, NICHD, Bethesda, MD*
- Rena N. D'Souza, PhD, *Section on Therapies for Craniofacial Disorders, NICHD, Bethesda, MD*
- Katie Drerup, PhD, *Unit on Neuronal Cell Biology, NICHD, Bethesda, MD*
- Maria L. Dufau, PhD, *Section on Molecular Endocrinology, NICHD, Bethesda, MD*
- Benjamin Feldman, PhD, *Zebrafish Core, NICHD, Bethesda, MD*

- Marc Ferrer, MD, *3-D Tissue Bioprinting Laboratory, NCATS, Rockville, MD*
- Judith Kassis, PhD, *Section on Gene Expression, NICHD, Bethesda, MD*
- David Klein, PhD, *Scientist Emeritus, NICHD, Bethesda, MD*
- Sergey Leikin, PhD, *Section on Physical Biochemistry, NICHD, Bethesda, MD*
- Claire E. Le Pichon, PhD, *Unit on the Development of Neurodegeneration, NICHD, Bethesda, MD*
- Henry L. Levin, PhD, *Section on Eukaryotic Transposable Elements, NICHD, Bethesda, MD*
- Mary Lilly, PhD, *Section on Gamete Development, NICHD, Bethesda, MD*
- Paul Love, MD, PhD, *Section on Cellular and Developmental Biology, NICHD, Bethesda, MD*
- Todd Macfarlan, PhD, *Unit on Mammalian Epigenome Reprogramming, NICHD, Bethesda, MD*
- Matthias Machner, PhD, *Section on Microbial Pathogenesis, NICHD, Bethesda, MD*
- Richard Maraia, MD, *Section on Molecular and Cellular Biology, NICHD, Bethesda, MD*
- Joan C. Marini, MD, PhD, *Section on Heritable Disorders of Bone & Extracellular Matrix, NICHD, Bethesda, MD*
- Deborah Merke, MD, *Section on Congenital Disorders, NICHD, Bethesda, MD*
- Keiko Ozato, PhD, *Section on Molecular Genetics of Immunity, NICHD, Bethesda, MD*
- Timothy J. Petros, PhD, *Unit on Cellular and Molecular Neurodevelopment, NICHD, Bethesda, MD*
- Karl Pfeifer, PhD, *Section on Epigenetics, NICHD, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, NICHD, Bethesda, MD*
- Pedro Rocha, PhD, *Unit on Genome Structure and Regulation, NICHD, Bethesda, MD*
- Dan Sackett, PhD, *Division of Basic and Translational Biophysics, NICHD, Bethesda, MD*
- Mihaela Serpe, PhD, *Section on Cellular Communication, NICHD, Bethesda, MD*
- Yun-Bo Shi, PhD, *Section on Molecular Morphogenesis, NICHD, Bethesda, MD*
- Stanko S. Stojilkovic, PhD, *Section on Cellular Signaling, NICHD, Bethesda, MD*
- Gisela Storz, PhD, *Section on Environmental Gene Regulation, NICHD, Bethesda, MD*
- Constantine Stratakis, MD, D(med)Sci, *Scientist Emeritus, NICHD, Bethesda, MD*
- Michael E. Ward, MD, PhD, *3-D Tissue Bioprinting Laboratory, NINDS, Bethesda, MD*
- Brant Weinstein, PhD, *Section on Vertebrate Organogenesis, NICHD, Bethesda, MD*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

Contact

For more information, email fdporter@mail.nih.gov or visit <https://www.nichd.nih.gov/about/org/dir/osd/cf/mgl>.

The NICHD Zebrafish Core

The NICHD Zebrafish Core was established in 2012 with the goal of providing its clients with consultation, access to equipment and reagents, and service in the area of zebrafish genetics. NICHD investigators as well as investigators from other NIH institutes and from outside the NIH are its clientele. The oversight committee for the Core comprises [Harold Burgess](#), [Ajay Chitnis](#), and [Brant Weinstein](#). The Core's activities consist of:

1. oversight and support of client-specific projects,
2. custom generation of genetic zebrafish models,
3. troubleshooting of new methodologies with promising application in zebrafish,
4. maintenance and improvement of equipment and infrastructure, and
5. service and educational outreach.

Oversight and support of client-specific projects

Over 2021–2022, the Core engaged in research projects with ten labs and other customers.

TRANSLATION OF DISTINCT RNA POPULATIONS BY ELONGATION INITIATION FACTORS EIF1 AND EIF5 ([DEVER LAB, NICHD](#))

Feldman advised Dever and performed several microinjection experiments to explore differences in zebrafish embryo phenotypes resulting from RNA-based over-expression or antisense morpholino-based reduction of expression of either Eif1 or Eif5. Experiments are continuing with a focus on testing the hypothesis that restoration of a balanced Eif1/Eif5 ratio can ameliorate phenotypes resulting from targeting just one of the two.

FUNCTIONAL ASSESSMENT OF TUBULIN ISOFORMS ([SACKETT LAB, NICHD](#))

The degree to which specific tubulin isotypes and/or their post-translational modification are essential for specific aspects of development in any organism remains a surprisingly open question. Feldman assisted Sackett in finding and recruiting two postbaccalaureate students with undergraduate training in reputable zebrafish labs. Since the arrival of these students in the summer of 2022, Feldman has been



Benjamin Feldman, PhD, *Staff Scientist, Director of the NICHD Zebrafish Core*

Felicia Benoit, BS,
Postbaccalaureate Intramural Research Training Award Fellow

training and mentoring them through an ambitious project to systematically knock-out each zebrafish alpha and beta tubulin isotype in F0 embryos and determine how their absence affects early development.

GENETIC DISSECTION AND CREATION OF HUMAN DISEASE MODELS OF STEROL METABOLISM (PORTER LAB, NICHD)

In previous years, the Core used CRISPR-Cas9 technology to create genetic mutant zebrafish lines for the Porter lab in five genes: *dhcr7*, *npc1*, *npc2*, *cln3*, and *ebp*, which play roles in various steps of cholesterol metabolism. Feldman is in the process of cryopreserving these lines for future use.

FUNCTION OF ZEBRAFISH ORTHOLOGS TO HUMAN GENES IMPLICATED IN DISORDERS OF THE PITUITARY-ADRENAL AXIS (STRATAKIS LAB, EX-NICHD)

In previous years, the Core used CRISPR-Cas9 technology to generate zebrafish carrying loss-of-function mutations in four zebrafish orthologs of human genes, implicated by the Stratakis lab in human growth anomalies, and eight zebrafish orthologs of human adrenal hyperplasia and Cushing's disease-associated genes. Feldman is in the process of cryopreserving these lines for future use.

FUNCTION OF ZEBRAFISH RCA2.1 (KEMPER LAB, NHLBI)

The Kemper lab is interested in zebrafish *rca2.1*s function, because it has certain similarities to human CD46 that are not found in the mouse genome. The Core previously generated two mutant *rca2.1* alleles, revealing essential roles in growth and cardiac function. Phenotypic characterization is ongoing.

FUNCTION OF ZEBRAFISH CACNA1C, WHOSE HUMAN ORTHOLOGUE IS AFFECTED IN TIMOTHY SYNDROME (GOLDEN LAB, NIDDK)

The Core previously generated a targeted AA-alteration in the *cacna1c* gene. Feldman found that recessive mutants have profound developmental anomalies, including heart edema and reduction in red blood cells. Characterization of this phenotype by trainees from the Golden lab is ongoing.

ROLE OF MINERALS IN BONE HEALTH (LAVERNE BROWN, OFFICE OF DIETARY SUPPLEMENTS)

The NICHD Zebrafish Core is working with LaVerne Brown in planning and implementing a nutritional study to explore how certain minerals influence bone health in the presence or absence of adequate vitamin D, a topic of relevance to human bone health. This year, the study parameters were refined, including acquisition of a forced-swimming exercise tunnel, to be used to stimulate bone remodeling.

Independent research by the NICHD Zebrafish Core

STRATEGIES FOR CRISPR-CAS9-BASED HOMOLOGY-DIRECTED REPAIR (HDR)

Over the past several years, Feldman and NICHD Zebrafish Core Staff explored several approaches to generating zebrafish lines with amino-acid substitutions cognate to human disease alleles of interest and generated three such alleles in house: *atp7a*, *cacna1c* and *satb1*. This past year, Feldman worked to devise a less labor-intensive pipeline that will feature outsourcing of CRISPR-based design and reagent steps to In Vivo Biosciences, followed by microinjection and allele recovery in-house via high-throughput sequencing of extruded gametes from candidate carriers.

CRYOPRESERVATION AND IN VITRO FERTILIZATION OF ZEBRAFISH SPERM

Over the last year, Feldman, assisted by Felicia Benoit, has continued to focus on improving quality control

measures to ensure viability of cryopreserved zebrafish lines and minimize variability in viability. This year, they developed an approach of pre-assessing the number and activity of sperm from individual males and only cryopreserving when yields exceeding two million active sperm are obtained.

Service

ACUC MEMBERSHIP

Feldman has served on the NICHD ACUC since 2015 and continued in this capacity this year, meeting monthly to evaluate and decide upon animal-study proposals, renewals and amendments, and ad hoc issues relevant to animal welfare.

FACULTY REPORTS

As a broader contribution to the scientific community, Feldman regularly pens recommendations for scientific articles of interest as a member of Faculty Opinions in the Developmental Biology/Pattern Formation Section. This year, he contributed two recommendations.

Additional Funding

- One-year salary support for Postbaccalaureate Fellow Felicia Benoit from the Office of Intramural Training & Education, NIH

Publications

1. Tseng WC, Johnson-Escauriza AJ, Tsai-Morris CH, Feldman B, Dale RK, Wassif CA, Porter FD. The role of Niemann-Pick type C2 in zebrafish embryonic development. *Development* 2021 148(7):dev194258.
2. Trivellin G, Tirosh A, Hernández-Ramírez LC, Gupta T, Tsai-Morris C-H, Faucz FR, Burgess HA, Feldman B, Stratakis CA. The X-linked acrogigantism-associated gene *gpr101* is a maternal regulator of early embryonic development and somatic growth in zebrafish. *Mol Cell Endocrinol* 2021 520:111091.

Collaborators

- LaVerne L. Brown, PhD, *Office of Dietary Supplements, Office of the Director, NIH, Rockville, MD*
- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- Thomas Dever, PhD, *Section on Protein Biosynthesis, NICHD, Bethesda, MD*
- Andy Golden, PhD, *Laboratory of Biochemistry and Genetics, NIDDK, Bethesda, MD*
- Claudia Kemper, PhD, *Laboratory for Complement and Inflammation Research, NHLBI, Bethesda, MD*
- Tokunbor Lawal, PhD, *Tissue Injury Branch, NINR, Bethesda, MD*
- Kenneth Olivier, MPH, MD, *Laboratory of Chronic Airway Infection, NHLBI, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, NICHD, Bethesda, MD*
- Daniel Sackett, PhD, *Division of Basic and Translational Biophysics, NICHD, Bethesda, MD*
- Constantine Stratakis, MD, D(med)Sci, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*

Contact

For more information, email bfeldman@mail.nih.gov or visit <http://zcore.nichd.nih.gov>.

Affinity Groups

Scientists and physicians in the NICHD Division of Intramural Research (DIR) are organized into 12 affinity groups (AGs). Each AG is an intellectual hub for a group of investigators, creating a forum to share ideas and collaborate around common themes in support of the DIR mission.

The AGs serve as catalysts for new initiatives. Each investigator has a primary affiliation with an AG most closely aligned with his or her scientific interests. Secondary affiliations allow for communication across specialties in support of translational research and new collaborations.

Each AG has its own mission statement, shared research goals and objectives, and resources. Collectively, the AGs contribute to recruitment, mentoring, and the annual DIR scientific retreat.

Aquatic Models of Human Development

The **Aquatic Models of Human Development Group** uses zebrafish as a model vertebrate organism to study human development. We exploit the advantages of the zebrafish model for genetic screens, genomic manipulation, microscopic imaging, and cell and behavioral biology to gain insights into mechanisms controlling human development and defects therein that can result in disease. By maintaining a high level of expertise and resources, the group lowers the barrier to entry for other researchers in the intramural program for use of zebrafish as a research tool for investigating biological processes *in vivo*. The five PIs and the Zebrafish Core Facility, along with approximately 30 postdocs, doctoral trainees and technical staff, all focusing on zebrafish as a model system and all working in the Building 6 complex, create a critical mass of talent unsurpassed at NIH.

The group specializes in the generation of induced and engineered mutations and transgenic zebrafish lines that are used to study *in vivo* the function and integration of regulatory factors and pathways that are critical to the development, health, and survival of vertebrates, including humans. Extensive formal collaborations and informal interactions between group members promote the exchange of reagents and technical information to ensure that NICHD researchers remain at the cutting edge of zebrafish research worldwide.

[Ajay Chitnis, Head](#)
[Harold Burgess](#)

[Ben Feldman \(Core\)](#)
[Katherine Rogers](#)

[Brant Weinstein](#)

Bone and Matrix Biology in Development and Disease

Matrix biology is central to the NICHD mission because it is intrinsic to the formation and reshaping of tissues before and after birth. This group aims to elucidate the mechanisms by which primary gene defects cause skeletal fragility and other matrix disorders and to apply this knowledge to the treatment of affected children. This goal is pursued with a close relationship between basic, translational, and clinical science.

The current primary focus of the group is on translational studies of skeletal dysplasias and related bone disorders. We have identified and characterized molecular mechanisms of several novel skeletal development disorders in this spectrum. In addition to building on these successes and expanding our knowledge of

mechanisms and pathology of skeletal development, we are developing novel approaches to therapeutic intervention, which will be translated to clinical trials. We are expanding our research to extracellular matrix (ECM) development and pathology in other tissues and organs. We also anticipate expanding our research program toward studies of ECM disorders in placenta, cartilage, growth plate, and other tissues and organs that are involved in fetal health, prematurity, and early child growth and development.

[Joan Marini, Head](#)

[Rena D'Souza \(NIDCR\)](#)

[Sergey Leikin](#)

Cell and Structural Biology

The **Cell and Structural Biology Group (CSBG)** conducts basic research on the molecular mechanisms that underlie fundamental cellular processes, such as organelle biogenesis and function, regulation of cell metabolism by small RNAs, small proteins, post-translational modifications and developmental programs, host-pathogen interactions, and the structure, traffic, and function of signaling proteins in the central nervous system. Knowledge gained from these studies is applied to the elucidation of the pathogenesis of various diseases, including neurodevelopmental, behavioral, and neurodegenerative disorders, metabolic disorders, and infectious diseases.

The CSBG has outstanding facilities for microscopic imaging of cell structure and function and for recombinant protein production in multiple prokaryotic and eukaryotic expression systems for X-ray crystallographic and biochemical analyses. In addition, the group has expertise in generating targeted mutations in rodents and in the anatomical, neurochemical, electrophysiological, and behavioral analyses of the mutant animals.

[Matthias Machner, Head](#)

[Philip Adams](#)

[Anirban Banerjee](#)

[Juan Bonifacino](#)

[Andres Buonanno](#)

[Mary Lilly](#)

[Gisela Storz](#)

Cell Regulation and Development

The mission and vision of the **Cell Regulation and Development Group** is to increase fundamental knowledge of basic molecular mechanisms of cell biology and animal development and enhance understanding of how dysregulation of these processes contributes to human disability and disease.

By combining expertise in the genetics of model organisms (including yeast, fruit fly, frog, zebrafish, and mouse), cell biology, biochemistry, molecular biology, electrophysiology, biophysics, genomics, and structural biology, members of the Group advance individual research objectives by regularly providing insights and advice to one another and through collaborations enabling synergy in research methods and experimental approaches. These interactions have engendered the development of novel technologies and strategies that underlie past accomplishments and will facilitate future discoveries by the Group in areas including the mechanisms and regulation of protein synthesis and transcriptional activation of gene expression, the functions and regulation of hormone receptors, signaling events and their responsive genes, mechanisms governing accurate segregation of the genetic information during cell division, mechanisms of nucleocytoplasmic trafficking, RNA processing

and RNA export, technologies based on transposable elements and deep sequencing for genome-wide profiles of gene function, the role of transposable elements in reorganizing the host genome in response to stress, technologies for analyzing gene expression at the single cell level during development, transcriptional control of the specification and differentiation of cells during vertebrate embryogenesis, the mechanisms governing adult organ formation during postembryonic vertebrate development, and molecular mechanisms of synaptic circuit assembly and function.

[Alan Hinnebusch, Head](#)
[Mary Dasso](#)
[Tom Dever](#)

[Maria Dufau](#)
[Jeffrey Farrell](#)
[Henry Levin](#)

[Jon Lorsch \(NIGMS\)](#)
[Mihaela Serpe](#)
[Yun-Bo Shi](#)

Developmental Endocrinology, Metabolism, Genetics, and Endocrine Oncology

The mission of the **Developmental Endocrinology, Metabolism, Genetics and Endocrine Oncology (DEMGE) Affinity Group** is to advance our understanding of endocrine, genetic, and metabolic disorders and endocrine tumors that impair human development, causing disease and disability. The research in this group encompasses basic, translational, and clinical science to elucidate the etiology of these diseases and to develop new diagnostic and therapeutic approaches.

[Karel Pacak, Head](#)
[Jeff Baron](#)
[Janice Chou](#)
[An Dang Do](#)
[Deborah Merke \(CC\)](#)
[Anil Mukherjee](#)

[Forbes Porter](#)
[Sarah Sheppard](#)
[Stanko Stojilkovic](#)
[Christina Tatsi](#)
[Jack Yanovski](#)

[NIH Inter-Institute
Endocrinology Training
Program
Pediatric Endocrinology Inter-
Institute Training](#)

Genetics and Epigenetics of Development

The mission of the **Genetics and Epigenetics of Development Group** is to research the genetic and epigenetic mechanisms that control reproduction, embryogenesis, and organ system development. Our vision is to apply the knowledge gained to enhance human health.

[Pedro Rocha, Head](#)
[Judy Kassis](#)

[Paul Love](#)
[Todd Macfarlan](#)

[Keiko Ozato](#)
[Karl Pfeifer](#)

Genomics and Basic Mechanisms of Growth and Development

The mission of the **Genomics and Basic Mechanisms of Growth and Development (GBMGD) group** is to do basic research into the molecular mechanisms of fundamental processes ubiquitous to all cells. Such research inevitably results in new knowledge that impacts understanding of both health and disease. The members of

the GBMGD group have a strong history of producing knowledge-changing advances in a number of fundamental processes that are essential to life; DNA replication, DNA repair, nucleotide metabolism, RNA biogenesis and metabolism, chromatin-mediated control of gene expression, and genome integrity. Simply put, the GBMGD is a unique group of world experts in processes involving DNA and RNA metabolism and the consequences of alterations of these processes to cells and organisms. Recent advances from work by GBMGD members, as well as others, has revealed that although these processes are fundamental and ubiquitous, defects in them are often manifested as specific health disorders with distinctive phenotypes affecting human development and with surprising tissue-specificity, or in cancer. Indeed, a substantial number of the most common clinically relevant genetic disorders affecting children are due to polymorphisms/mutations in factors required for the maintenance of basal cellular functions. Inherent to the GBMGD mission is to increase understanding of how natural genetic diversity in the human population contributes to these fundamental processes in ways that affect health and disease, and to apply such knowledge so that specific strategies toward improving health can be developed.

The vision of the GBMGD group is to elucidate new knowledge about fundamental processes that will promote discovery of novel strategies for treatment and prevention alternatives across a multitude of diseases that share involvement of altered nucleic acid metabolism, gene expression and genome integrity. Through basic research that incorporates modern advances in biochemistry, genetics and genomics, GBMGD group members will continue to generate new knowledge relevant to the fundamental processes essential to growth, development and health.

The GBMGD group vision includes the promotion of collaborations and communication that support its mission. This group has the ability to discover fundamental aspects of growth and development from multifaceted perspectives and disciplines, and to investigate how disturbances in one process can affect another. Because our interests are not principally focused on any particular disorder or discipline, they extend beyond classical developmental biology and the gene pattern-specific expression aspects of animal development. By focusing on basal cellular functions, the GBMGD approach provides unique and complimentary perspectives on developmental biology and growth in health and disease states. Given the NIH's tolerance to high-risk endeavors not usually fostered elsewhere, the GBMGD group, and the many collaborations among its members, fosters a greater depth and breadth of fundamental discovery than would exist in its absence.

Rich Marais, Head
David Clark

Bob Crouch
Mel DePamphilis

Roger Woodgate

Maternal-Fetal Medicine and Translational Imaging

The mission of the **Maternal-Fetal Medicine and Translational Imaging Affinity Group** is to understand the biology of normal pregnancy and its complications, as well as normal/abnormal neurodevelopment during childhood by the application of innovative, state-of-the-art cellular, molecular, and imaging methods to improve the diagnosis, treatment, and prevention of conditions responsible for maternal, perinatal, and infant morbidity and mortality.

Quantitative Imaging and Tissue Sciences (Basser) invents, develops, and translates novel *in vivo* microstructural and functional MRI methods designed to measure salient properties of the developing brain and assess and

characterize their changes in diseases and disorders. These novel quantitative imaging biomarkers are also used in neuroscience application to characterize brain network connectivity and dynamics, as well as brain tissue architectural organization.

Nervous System Development and Plasticity (Fields): Our research is concerned with understanding the molecular and cellular mechanisms by which neural activity and experience regulates development and plasticity of the nervous system, with three main areas of emphasis: (1) myelination and neuron-glia interactions, (2) cellular mechanisms of learning, and (3) gene regulation by neuronal firing.

Translational Biophotonics (Gandjbakhche) uses multi-disciplinary approaches to devise functional imaging technologies and methodologies for translating benchtop studies to the bedside. For example, near infrared spectroscopy and electroencephalogram are used to assess biomarkers for a wide range of brain development abnormalities and injuries, specifically, but not limited to, cognitive and behavioral disorders in children and traumatic brain injury. The laboratory explores endogenous (scattering and absorption) and exogenous (using fluorescence probes) optical contrast mechanisms for characterizing abnormal development and function in tissues such as the placenta. They also are involved in clinical and preclinical studies aimed at characterizing growth and development of various abnormal tissues and monitoring the efficacy of their treatment using photonics methods, such as fluorescence life time and multi spectral imaging.

Intercellular Interactions (Margolis) studies viral and non-viral pathogenesis in the context of human tissues. The laboratory developed a system of *ex vivo* human tissues that preserves their cytoarchitecture and important *in vivo* functions, and it studies lymphoid, cervico-vaginal, and placenta tissues to investigate mechanisms of cell-cell, cell-pathogen, and cell-extracellular vesicles interaction under normal as well as disease conditions.

Perinatology Research (Romero) investigates normal pregnancy and its most frequent complications such as preterm labor, preterm prelabor rupture of membranes, preeclampsia, fetal growth disorders, and fetal death—conditions which account for the excessive rate of infant mortality in the United States. The Laboratory conducts clinical and translational research and develops diagnostic, predictive, therapeutic, and preventative methods to reduce adverse pregnancy outcomes.

Biomedical Optics (Tromberg) develops models, methods, and devices for understanding and controlling light interactions with biological tissues. These methods are used to perform real-time quantitative measurements of clinically-relevant information, including: tissue blood flow, oxygen extraction, metabolic rate of oxygen consumption, and body/tissue composition (lean mass, hydration, and fat mass). Advanced capabilities include continuous dynamic monitoring of intrinsic physiological signals that can be used in feedback optimization for guiding therapies and clinical decision making. Our technology development effort includes portable, bedside, non-contact and wearable sensor platforms, as well as the design and integration of probes into instruments for minimally invasive surgical feedback and guidance.

[Roberto Romero, Head](#)
[Peter Basser](#)

[R. Douglas Fields](#)
[Amir Gandjbakhche](#)

[Leonid Margolis](#)
[Bruce Tromberg \(NIBIB\)](#)

Molecular Medicine

The **Molecular Medicine Group** brings together basic research programs that share the ultimate goal of developing treatments for human diseases through a better understanding of their pathophysiology. Main areas of research focus include the maintenance of iron homeostasis and mechanisms underlying neurodegeneration. At the intersection of our work, we hope to shed light on a group of rare diseases called neurodegeneration with brain iron accumulation or NBIA, which may also have broader implications for other types of related diseases.

[Claire Le Pichon, Head](#)

[Tracey Rouault](#)

Neurosciences

Understanding the structure and function of the nervous system is a prerequisite for predicting and treating neuropathologies. Our group uses a variety of preparations, including animal models and human tissue, and a variety of techniques to study the biology of development and function of the nervous system and underlying basic biological processes in both health and disease.

[Mark Stopfer, Head](#)

[Tamás Balla](#)

[Dax Hoffman](#)

[Y. Peng Loh](#)

[Chris McBain](#)

Jamie Morton (DIPHR)

[Tim Petros](#)

Physical Biology and Medicine

Human development, on which the future child's health depends, is a complex phenomenon within the female starting with egg-spermatozoa fusion. In each individual, a plethora of molecular recognition events mediate the development of an immune system to defend against pathogens, a musculoskeletal system to maintain the body, and flexible networks of molecular expression to manage environmental stress. Traditionally, studies of these processes are divided into biochemistry, cell biology, virology, toxicology, etc. However, nature does not know these artificial divisions, and new understandings emerge from the crucible that interfaces mathematically minded physical scientists with biomedical researchers. The **Physical Biology and Medicine Group** is a unique scientific body that approaches human development in normal life and pathology as an integral process and encompasses first-class cell biologists, physical chemists, biophysicists, virologists, and immunologists who not only successfully train postdocs and students within their own fields, but widely collaborate, building and uniquely promulgating multidisciplinary approaches to the most important biomedical problems in the framework of the NICHD mission.

By choosing carefully which biophysical projects really answer the fundamental problems that limit advancement in medicine, we as a group will suffuse these identified problems with our basic wisdom of biophysics. This wisdom includes a deep knowledge of polymer physics, membrane biology and virology, inter- and intracellular communication, the theory of transporters and diffusion of domains, the physics of channel permeation and protein conformational change, the physical chemistry of membrane hydration forces and recognition, the physiology of cell signaling and receptor activation, lipid/protein interactions,

and the physiology of secretion, viral infection, parasite invasion, fertilization, adipose transporter trafficking and insulin signaling, and developmental cell fusion. Our strategy is to sharpen our techniques and power of observation to prove our discoveries, including proteomic architecture of signaling complexes and cellular structures, TIRF microscopy, long-term live-cell time-lapse imaging of tissue and cultured cells, confocal and two-photon scanning microscopy, electrophysiology, physical theories to devise experimental tests of hypotheses, cryoelectron microscopy, and lipidomic analyses.

We are now in a position to tackle the developmental changes seen in obesity and genetic disorders, the pathophysiology of influenza, dengue, and HIV viral infection and assembly, a 3D immunohistochemical microscopy of normal and compromised placenta at super-resolution levels, the use of human brain culture in dissociated and slice culture to study traumatic brain injury and glioblastomal neoplasia, membrane domain organization, regulation of mitochondrial metabolism by cytosolic proteins, and muscle molecular ultrastructure. We currently anticipate testing if hydrogen bond theory can explain hydration repulsion of DNA and membranes, developing a deeper understanding of mechanical, electrical, and chemical interactions in complex, multicomponent cell communities, achieving the conceptual integration of cutting-edge immunological information and assays for cell-cell communication in the onset of tissue pathology and discovering the role and mechanisms of microRNA vehicles in serum.

Leonid Chernomordik, Head
Sergey Bezrukov

Doreen Matthies
Dan Sackett (SS)

Alexander Sodt
Joshua Zimmerberg

Reproductive Endocrinology & Infertility and Pediatric & Adolescent Gynecology

The **Reproductive Endocrinology & Infertility and Pediatric & Adolescent Gynecology Group** consists of the primary NICHD investigators who carry out research and clinical care in women's health. Our mission is to conduct innovative translational research and provide direct patient care in reproductive endocrinology and gynecology. Consultative services are provided to all NIH institutes.

Alan DeCherney, Head (Training Program)

Veronica Gomez-Lobo (Training Program)

RNA-Mediated Gene Regulation in the Lyme Disease Pathogen

The goal of this research is to identify and characterize gene regulation in the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease. We use genetics, RNA sequencing, and murine infection models to pinpoint genes for mechanistic study.

Lyme disease is an emerging infectious disease and the foremost vector-borne bacterial infection in the world. Given that *B. burgdorferi* inhabits tick and mammalian hosts, environments with very different temperatures, immune responses, and sources of metabolites, the bacterium must harbor robust gene-regulatory mechanisms in order to survive.

The major focus of the group has been to discover and characterize *B. burgdorferi* genes important for infectivity and stress response. We recognized the importance of mapping RNA boundaries (their 5' and 3' ends), which is critical for gene annotation, the discovery of novel transcripts, and mechanistic characterization of genes. Therefore, we performed 5'RNA-seq on total RNA isolated from *B. burgdorferi* grown in culture. This method identified RNA 5' ends and distinguished transcription starts sites (TSSs) from 5' processed ends. The data were then compared with a previous genetic screen that identified transcriptionally active sequences on the *B. burgdorferi* genome during a mouse infection, which resulted in a dataset of putative mammalian-specific *B. burgdorferi* transcriptional events and identified numerous genes for future study.

One gene identified by our approach, *bb0562*, was an annotated hypothetical protein. Targeted deletion of *bb0562* revealed that the gene encodes a protein important for disseminated infection in mice by needle inoculation and tick bite transmission. The *bb0562* gene was also found to be important for spirochete growth in low-serum media, leading to a growth defect that could be rescued by the addition of various long-chain fatty acids. We identified two canonical lipase motifs within BB0562 and demonstrated *in vitro* lipolytic activity with purified BB0562 protein. Collectively, the work established *bb0562* as a novel *B. burgdorferi* nutritional virulence determinant [Reference 1].

Ongoing work in our lab focuses on the characterization of other



Philip P. Adams, PhD, Head,
*Group on Gene Regulation in
Bacterial Pathogens*

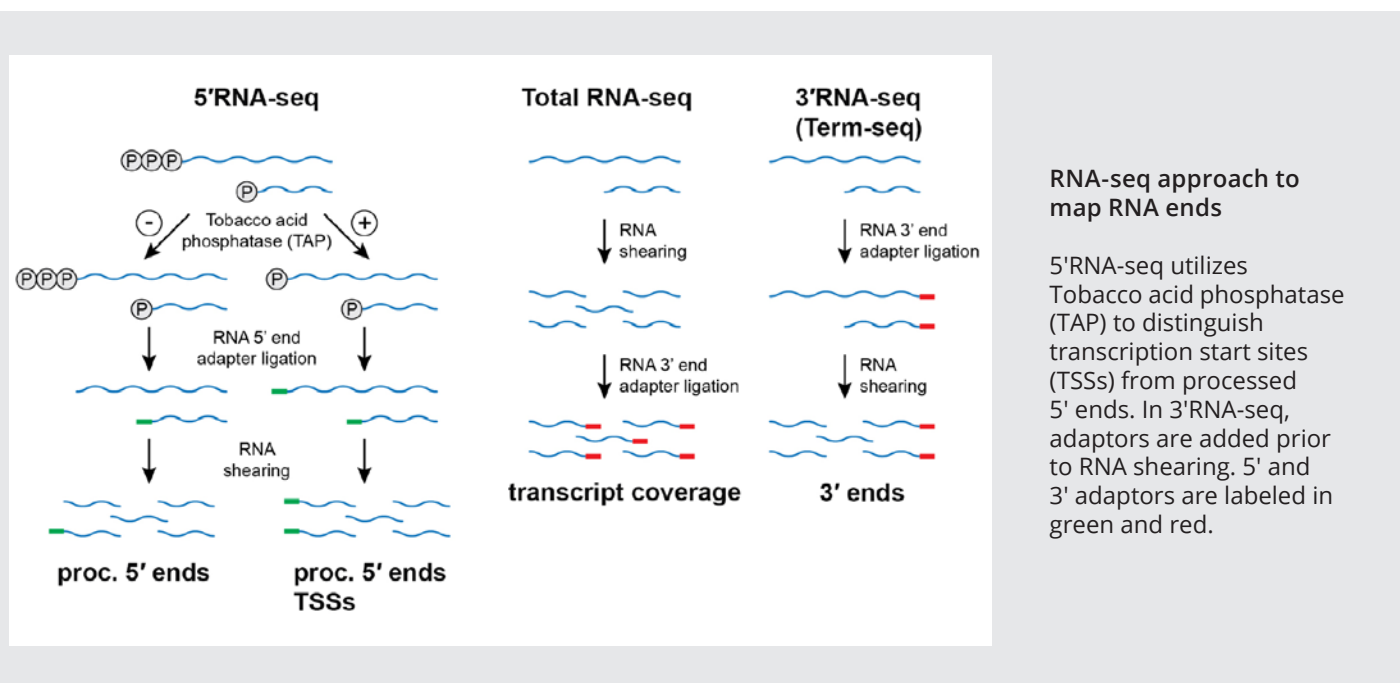
Emily Petroni, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Daniel Soliman, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Daniel Tetreault, BA,
*Postbaccalaureate Intramural
Research Training Award Fellow*

genes, particularly of regulatory RNAs, an unstudied area of *B. burgdorferi* gene regulation. To expand upon our previous study, we augmented our RNA-seq approach and now combine three techniques to further refine RNA boundaries: 5'RNA-seq, 3'RNA-seq (which captures termination events and identifies RNA 3' ends), and total RNA-seq (which sequences genes in their entirety). As proof of concept, we applied the approach to the model organism *Escherichia coli* [Reference 2]. In doing so, we identified numerous *E. coli* RNA fragments derived from 5' regions of mRNAs and internal to open reading frames (ORFs). We documented regulation for several transcripts and identified a function for a small RNA encoded internal to an essential cell-division gene. The work revealed that regulatory transcripts are derived from a wide range of locations in bacterial genomes.

Further studies to perform 3'RNA-seq in *B. burgdorferi* to better understand transcription termination and identify novel RNA regulators and their physiological roles are ongoing.



Additional Funding

- NICHD Early Career Award (2022) to Philip Adams, concluded

Publications

1. Kuhn HW, Lasseter A, Adams PP, Flores Avile C, Stone BL, Akins DR, Jewett TJ, Jewett MW. BB0562 is a nutritional virulence determinant with lipase activity important for *Borrelia burgdorferi* infection and survival in fatty acid deficient environments. *PLoS Pathog* 2021 17(8):e1009869.
2. Adams PP, Baniulyte G, Esnault C, Chegireddy K, Singh N, Monge M, Dale RK, Storz G, Wade JT. Regulatory roles of *Escherichia coli* 5' UTR and ORF-internal RNAs detected by 3' end mapping. *eLife* 2021 10:e62438.

Collaborators

- Ryan Dale, PhD, *Computer Support Services Core, NICHD, Bethesda, MD*
- Mollie Jewett, PhD, *University of Central Florida, Orlando, FL*
- Gisela Storz, PhD, *Section on Environmental Gene Regulation, NICHD, Bethesda, MD*
- Joseph Wade, PhD, *Wadsworth Center, New York State Department of Health, Albany, NY*

Contact

For more information, email philip.adams@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/adams>.

Phosphoinositide Messengers in Cellular Signaling and Trafficking

Every biochemical process in a eukaryotic cell relies upon a molecular information flow that leads from receptors that inform the cell about its environment all the way to the molecular effectors that determine the appropriate cellular response. Proper information transmission requires a high degree of organization, where the molecular players are organized into different cellular compartments so that the specificity of the cellular response can be properly maintained. Breakdown of this organization is the ultimate cause of all human diseases, even if the affected molecular pathways differ according to the kind of disease, such as cancer, diabetes, or neuro-degenerative diseases, just to name a few.

Our research focuses on the question as to how cells organize their internal membranes to provide a structural framework on which molecular signaling complexes assemble to ensure proper information processing. The lipid composition of cellular membranes is a major determinant of their biophysical properties and is unique to the different cellular organelles. How cells achieve and maintain the proper lipid composition of their membranes is poorly understood. Cellular processes that affect the membrane lipid composition of organelles are often targeted by cellular pathogens, such as viruses, to force the cells to produce the pathogen instead of performing the cells' normal functions. Better understanding of these processes can not only provide new strategies to fight various human diseases but also to intercept the life cycle of cellular pathogens, offering an alternative to antimicrobial drugs.

Lipid transport and Ca²⁺ signaling are closely interrelated in plasma membrane (PM)–endoplasmic reticulum (ER) contact sites.

Membrane contacts sites (MCS) between various organelles are emerging as key structural elements, where important communication between organelles takes place. MCS have been primarily featured in non-vesicular lipid transfer and Ca²⁺ signal propagation, but their importance is likely to reach beyond these two processes. An important class of molecules that function at MCSs are the ORPs (oxysterol binding-protein-related proteins), which are the mammalian orthologs of the yeast Osh (oxysterol-binding homolog) proteins and



Tamás Balla, MD, PhD, Head,
Section on Molecular Signal Transduction

Alejandro Alvarez-Prats, PhD,
Staff Scientist

Yeun Ju Kim, PhD, *Staff Scientist*

Gergo Gulyás, MD, PhD,
Postdoctoral Fellow

Amrita Mandal, PhD, *Postdoctoral Fellow*

Yang Niu, PhD, *Postdoctoral Fellow*

Joshua Pemberton, PhD,
Postdoctoral Fellow

Vijay Joshi, BS, *Postbaccalaureate
Intramural Research Training
Award Fellow*

Marek Korzeniowski, PhD, *Special
volunteer*

Ljubiša Vitković, PhD, *Special
Volunteer*

mediate the transport of specific lipids between cellular membranes. One of the salient features of Osh/ORP proteins is that they use a phosphatidylinositol 4-phosphate (PI4P) gradient as a driving force as they counter-transport PI4P in exchange for the specific lipids they move between membranes. Therefore, lipid transport by Osh/ORP proteins is linked to the activity of PI 4-kinases.

We investigated the impact of changing PM PI4P levels on the Ca^{2+} entry process mediated by the STIM1–Orai1 molecular complex, which underlies the refilling of the ER luminal Ca^{2+} stores during receptor stimulation. We found that changing PM PI4P levels through inhibition of the lipid kinase (PI4KA), which produces PI4P in the PM, potentially inhibits Ca^{2+} influx by interrupting the association of the calcium sensor STIM1 (stromal interaction molecule 1) with the PM. Similarly, manipulation of PM PI4P levels through the expression of ORP5 and ORP8 proteins had a major impact on Ca^{2+} influx. Our studies revealed a tight connection between Ca^{2+} entry mediated by the STIM1–Orai1 complex and the PI4P-driven lipid transport process at PM–ER contact sites.

The critical role of specific phosphoinositide lipids in the late stage of cell division

Separation of the two daughter cells at the last stage of mitosis, called cytokinetic membrane abscission, is a spatially and temporally regulated process that requires membrane remodeling at the midbody, a subcellular organelle that defines the cleavage site. The process is mediated by a multi-protein molecular complex, called ESCRT (endosomal sorting complex required for transport), and its defective function can lead to cataracts. It is not known how ESCRT defects can lead to cataracts and whether they are related to cytokinesis defects. In a collaborative study led by the Hirsch laboratory, it was found that a lens-specific cytokinetic process required the lipid kinase PI3K-C2 (phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2) and its lipid product PI(3,4)P₂ (phosphatidylinositol 3,4-bisphosphate). These studies showed that the ESCRT-II subunit VPS36 (vacuolar protein-sorting-associated protein 36) requires PI(3,4)P₂ binding, and loss of each of the ESCRT-II components led to impaired cytokinesis, triggering premature senescence in the lens of fish, mice, and humans. Importantly, the PI4P substrate for the PI3K-C2 enzyme to support this process was provided by the PI4KA enzyme. This evolutionarily conserved pathway underlies the cell type-specific control of cytokinesis, which helps to prevent early-onset cataract by protecting from senescence.

A specific calcineurin splice form targets the multi-protein complex of PI4KA.

Calcineurin, the conserved protein phosphatase and target of immunosuppressants, is a critical mediator of Ca^{2+} signaling. In a collaborative study, led by the Cyert laboratory, that focused on the understudied calcineurin isoform CNA1, it was discovered that, unlike canonical cytosolic calcineurin, CNA1 localizes to the plasma membrane and Golgi as a result of reversible palmitoylation of its divergent C-terminal tail. Palmitoylation targets CNA1 to a distinct set of membrane-associated interactors, including the multi-protein PI4KA complex containing EFR3B (palmitoylated plasma-membrane protein that controls responsiveness to G-protein-coupled receptors [GPCR]), PI4KA, TTC7B (tetratricopeptide repeat protein, a component of a complex required to localize PI4K to the plasma membrane), and FAM126A (hyacin; regulates PI4K synthesis at the plasma membrane). Hydrogen-deuterium exchange studies, performed in the Burke laboratory, found multiple contacts in the calcineurin–PI4KA complex, including a calcineurin-binding peptide motif in the disordered tail of FAM126A, which was established as a calcineurin substrate. In cellular studies, calcineurin inhibitors reduced PI4P production during Gq-coupled GPCR signaling, suggesting that calcineurin dephosphorylates and promotes PI4KA complex activity. The work thus revealed a calcineurin-regulated

signaling pathway and identified the PI4KA complex as a regulatory target. It also showed that dynamic palmitoylation provides the CNA1 enzyme with a unique localization to increase its access its substrates, lending the protein unique specificity and regulation.

PI4KA variants in human cause neurological, intestinal, and immunological disease.

The lipid kinase PI4KA generates PI4P in the PM, playing critical roles in the physiology of many cell types. PI4KA requires its assembly into a heterotetrameric complex with EFR3, TTC7 and FAM126. Sequence alterations in two of these molecular partners, TTC7 (encoded by TTC7A or TCC7B) and FAM126, in humans have been associated with a heterogeneous group of either neurological (FAM126A) or intestinal and immunological (TTC7A) conditions. In this multi-center collaborative study led by Andrew Crosby and Emma Baple, biallelic PI4KA sequence alterations in humans were shown to be associated with neurological disease, in particular hypomyelinating leukodystrophy. In addition, some affected individuals may also present with inflammatory bowel disease, multiple intestinal atresia, and combined immunodeficiency. Biochemical and structural modelling studies indicated that PI4KA-associated phenotypical outcomes probably stem from impairment of PI4KIII α -TTC7-FAM126's organ-specific functions as the result of defective catalytic activity or altered intra-complex functional interactions. Together, these data define PI4KA gene alteration as a cause of a variable phenotypical spectrum and provide fundamental new insights into the combinatorial biology of the PI4KIII α -FAM126-TTC7-EFR3 molecular complex.

Publications

1. Prinz WA, Toulmay A, Balla T. The functional universe of membrane contact sites. *Nat Rev Mol Cell Biol* 2020 1:7–24.
2. Gulyas G, Korzeniowski MK, Eugenio CEB, Vaca L, Kim YJ, Balla T. LIPID transfer proteins regulate store-operated calcium entry via control of plasma membrane phosphoinositides. *Cell Calcium* 2022 106:102631.
3. Gulluni F, Prever L, Li H, Krafcikova P, Corrado I, Lo WT, Margaria JP, Chen A, De Santis MC, Cnudde SJ, Fogerty J, Yuan A, Massarotti A, Sarijalo NT, Vadas O, Williams RL, Thelen M, Powell DR, Schueler M, Wiesener MS, Balla T, Baris HN, Tiosano D, McDermott BM Jr, Perkins BD, Ghigo A, Martini M, Haucke V, Boura E, Merlo GR, Buchner DA, Hirsch E. PI(3,4)P2-mediated cytokinetic abscission prevents early senescence and cataract formation. *Science* 2021 374:abk0410.
4. Ulengin-Talkish I, Parson MAH, Jenkins ML, Roy J, Shih AZL, St-Denis N, Gulyas G, Balla T, Gingras AC, Várnai P, Conibear E, Burke JE, Cyert MS. Palmitoylation targets the calcineurin phosphatase to the phosphatidylinositol 4-kinase complex at the plasma membrane. *Nat Commun* 2021 12:6064.
5. Salter CG, Cai Y, Lo B, Helman G, Taylor H, McCartney A, Leslie JS, Accogli A, Zara F, Traverso M, Fasham J, Lees JA, Ferla MP, Chioza BA, Wenger O, Scott E, Cross HE, Crawford J, Warshawsky I, Keisling M, Agamanolis D, Ward Melver C, Cox H, Elawad M, Marton T, Wakeling MN, Holzinger D, Tippelt S, Munteanu M, Valcheva D, Deal C, Van Meerbeke S, Walsh Vockley C, Butte MJ, Acar U, van der Knaap MS, Korenke GC, Kotzaeridou U, Balla T, Simons C, Uhlig HH, Crosby AH, De Camilli P, Wolf NI, Baple EL. Biallelic PI4KA variants cause neurological, intestinal and immunological disease. *Brain* 2021 144:3597–3610.

Collaborators

- Emma Baple, MBBS, MRCPCH, PhD, *University of Exeter Medical School, Exeter, UK*

- Evžen Boura, PhD, *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
- John Burke, PhD, *University of Victoria, Victoria, Canada*
- Andrew Crosby, PhD, *University of Exeter Medical School, Exeter, UK*
- Martha Cyert, PhD, *Stanford University, Stanford, CA*
- Emilio Hirsch, PhD, *Department of Molecular Biotechnology and Health Sciences, University of Turin, Turin, Italy*
- Juan Marugan, PhD, *Division of Pre-Clinical Innovation, NCATS, Bethesda, MD*
- Péter Várnai, MD, PhD, *Semmelweis University, Faculty of Medicine, Budapest, Hungary*

Contact

For more information, email ballat@mail.nih.gov or visit <https://ballalab.nichd.nih.gov>.

Structural and Chemical Biology of Membrane Proteins

Molecular mechanism of post-translational protein lipidation by zDHHC protein S-acyltransferases

Post-translational modifications greatly expand the structural, chemical, and functional diversity of the proteome. Of these, protein lipidation, which collectively refers to covalent modification of proteins by lipids, constitutes a centrally important class of post-translational modification. Protein S-acylation, commonly known as protein palmitoylation, is a specific form of protein lipidation whereby long-chain fatty acids, typically C16, become covalently attached to cytosol-facing cysteines through a thioester linkage. Palmitoylation is one of the most pervasive and physiologically important post-translational modifications, and the targets of palmitoylation span a very wide range of proteins, ranging from ion channels to cell-surface receptors, neuronal scaffolding proteins, and small GTPases. The repertoire of palmitoylated proteins has expanded rapidly in recent years, with thousands of proteins now known to be part of the cellular 'palmitoylome.' The physico-chemical effect of palmitoylation is to alter the local hydrophobicity of the substrate protein. The thioester bond makes S-acylation unique in that it is a labile moiety and can be cleaved, in the cellular context, by thioesterase enzymes, which makes S-acylation one of the few dynamic post-translational modifications and unique among different forms of protein lipidation. The physiological effects of S-acylation are diverse and have critical cellular importance. For example, Ras, a small GTPase that is critical for cellular growth and differentiation and is mutated in about one-third of all human cancers, is palmitoylated at the Golgi and subsequently targeted to the plasma membrane by vesicular transport. Palmitoylated Ras localizes to cholesterol-rich domains on the plasma membrane. However, it is subsequently depalmitoylated by the thioesterase APT1, dissociates from the plasma membrane, and redistributes on endomembranes, including the Golgi. Such dynamic recycling of Ras is critical for its function. On the other hand, in recent work (see below), we showed that the Spike protein of SARS-CoV-2, the causative agent of COVID-19, is S-acylated, which is important in the viral life cycle.

Protein S-acylation is catalyzed by a large group of enzymes known as zDHHC palmitoyl acyltransferases (also referred to as DHHC



Anirban Banerjee, PhD, Head,
*Unit on Structural and Chemical
Biology of Membrane Proteins*

Natalia Gomez Navarro, PhD,
Research Fellow

Elliot Murphy, PhD, *Postdoctoral
Intramural Research Training
Award Fellow*

Robbins Puthenveetil, PhD,
*Postdoctoral Intramural
Research Training Award Fellow*

Tanmay Mondal, PhD, *Visiting
Fellow*

Elvis Ongey Legala, PhD, *Visiting
Fellow*

Rahul Raina, PhD, *Visiting Fellow*

Kevin Choi, BS, *Postbaccalaureate
Fellow*

Sarah Miller, BS,
Postbaccalaureate Fellow

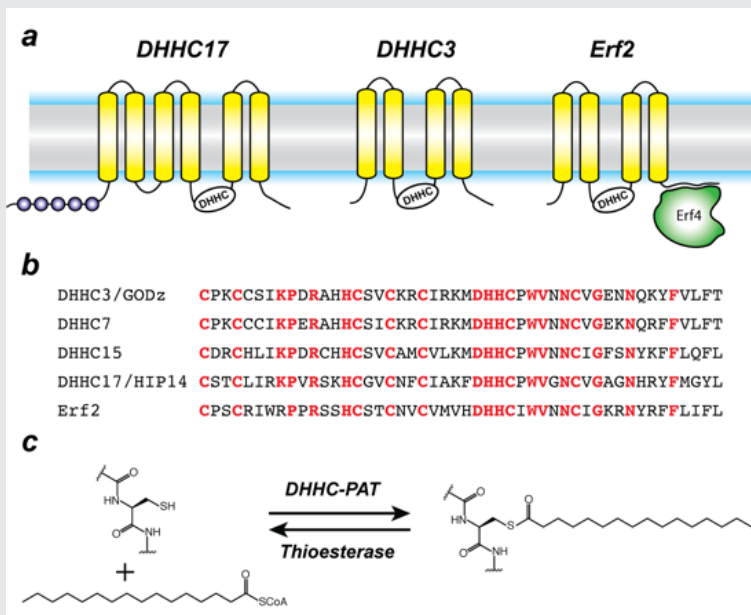


FIGURE 1. Organization and properties of DHHC palmitoyltransferases (PATs)

a. The organization of three different DHHC-PATs are shown schematically. The spheres indicate protein-protein interaction domains. Erf2 associates with a cytoplasmic subunit, Erf4, to form the active enzyme.

b. The DHHC-CRD region of a few representative DHHC-PATs are aligned. The conserved amino acids are shown in red.

c. Reaction catalyzed by DHHC-PATs; the reverse reaction is catalyzed by acylprotein thioesterases (APT).

enzymes or DHHC-PAT), so named because they contain a signature D-H-H-C motif (aspartate-histidine-histidine-cysteine) in a cysteine-rich domain (CRD) in an intracellular loop (Figure 1). These are low-abundance polytopic integral membrane proteins localized to a variety of cellular compartments. Humans have 23 DHHC-PATs encoded in their genome. Beyond the shared DHHC domain, DHHC-PATs vary considerably; some possess ankyrin repeats (structural protein motifs that mediate protein-protein interactions), a few have six transmembrane helices instead of the usual four, and at least one forms a functional heterodimer with a cytoplasmic auxiliary subunit. To date, no consensus sequences have been reported for palmitoylation. A specific DHHC-PAT can palmitoylate many substrates, and, conversely, a given substrate can be palmitoylated by many DHHC-PATs. Such redundancy has been one of the most intriguing aspects of DHHC-PATs and makes it difficult to assign substrates by overexpression/knockout strategies, given that, in the absence of one DHHC-PAT enzyme, others can take over. However, this does not necessarily reflect the true enzyme-substrate relationship. The situation is even more confounded by the lack of specific inhibitors of DHHC-PATs. Even though 2-bromopalmitate is widely used as a global inhibitor of DHHC-PATs, it has been shown that it also broadly targets other proteins involved in lipid metabolism.

Besides its broad importance in cell biology, palmitoylation has been linked to several diseases, most notably neuro-psychiatric disorders such as Huntington's disease and various forms of cancer. Recently, it was shown that zDHHC20 palmitoylates epidermal growth factor (EGFR) and is thus a potential therapeutic target for a wide range of cancers. More recently, zDHHC3 has been proposed as a target for cancer treatment owing to its activity as the palmitoyltransferase for the programmed cell-death ligand 1 (PD-L1). However, when we started working on this family, very little was known about the molecular mechanism of zDHHC palmitoyltransferases, despite their importance across a broad spectrum of biological pathways and their biomedical importance. Nothing was known about their structural organization or how they interact with substrates and the fatty acyl coenzyme A (CoA), which serves as the acyl donor.

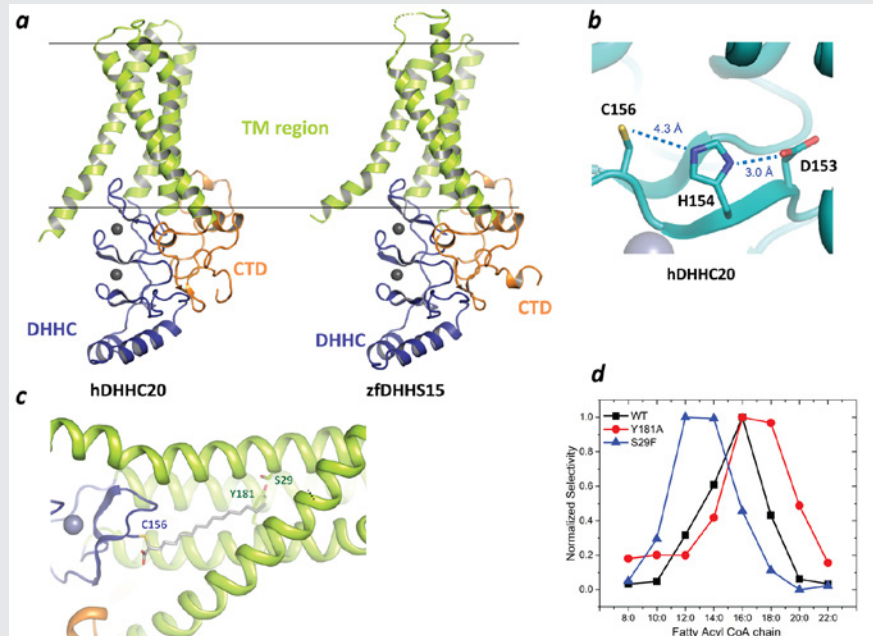
FIGURE 2. Structure, function, and membrane deformation of DHHC palmitoyltransferases

a. The structure of human DHHC20 and a catalytically inactive mutant of zebrafish DHHC15 shown in ribbon trace: the transmembrane domain (TM) is shown in green, the DHHC-containing cysteine-rich domain in blue and the C-terminal domain in orange; the grey spheres indicate Zn²⁺ ions; these are both Golgi-resident enzymes, and thus the top side faces the Golgi lumen and the active site the cytoplasm.

b. Active site of human DHHC20 showing the catalytic triad containing the active-site cysteine.

c. Structure of human DHHC20 irreversibly modified with 2-bromopalmitate, which results in the active-site cysteine linking to the alpha-carbon of palmitic acid: the acyl group of palmitic acid is shown in stick rendition; also shown are two residues towards the top of the tapering cavity to where the palmitate binds.

d. The acyl chain-length selectivity of wild-type (WT) human DHHC20: mutation of tyrosine181 to alanine (Y181A) expands the cavity and shifts the acyl selectivity to the longer side; on the other hand, mutation of serine29 to phenylalanine (S29F) contracts the cavity and thus shifts the acyl selectivity to the shorter side.

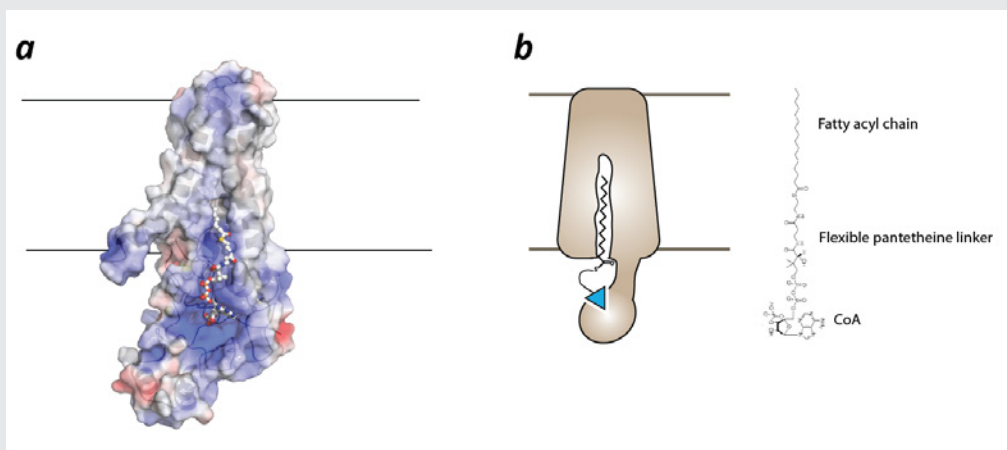


In a major breakthrough in this field, we had earlier solved the high-resolution crystal structures of two members of the zDHHC family, human zDHHC20 (hDHHC20) and zebrafish zDHHC15 (Figure 2a), the first structures of any member of this family to be characterized. They reveal a tepee-like transmembrane domain organization, which splays apart towards the cytoplasmic side and harbors the active site at the membrane-aqueous interfacial region (Figure 2b), thus readily explaining why membrane-proximal cysteines are palmitoylated. We also solved the structure of hDHHC20 irreversibly modified by a covalent inhibitor, 2-bromopalmitate. The structure mimics the auto-acylated intermediate state in the enzymatic pathway and thus reveals how the acyl group of fatty acyl-CoA binds in a cavity formed in the bilayer by the transmembrane domain (Figure 2c). Residues lining the cavity contact the acyl chain, and their mutation affects enzymatic activity. By mutating two residues at the tapering end of the cavity, we also showed that we can change the acyl chain-length selectivity of the mutant enzymes (Figure 2d). Thus, the cavity functions as a molecular ruler in determining the acyl chain-length selectivity of hDHHC20, important because, although palmitate is the most prevalent fatty acid used by DHHC palmitoyltransferases, they can use fatty acyl-CoAs of other chain lengths, a property that varies between different members of the DHHC family.

Our goal is to obtain a structural snapshot of every state in the catalytic pathway of zDHHC enzymes. To this end, we recently used a combination of x-ray crystallography, atomistic molecular dynamics simulations, and biochemistry to obtain and validate a structure of the pre-catalytic complex of human zDHHC20 with palmitoyl

FIGURE 3. Bivalent recognition of palmitoyl CoA by human zDHHC20

a. Structure of the precatalytic complex of a catalytically inactive mutant of human zDHHC20 with palmitoyl CoA: the protein is shown in surface rendition colored by electrostatic potential with positively charged surface in blue and negative charged surface in red; the palmitoyl CoA is shown in ball and stick rendition with natural atom colorings except carbon, which is shown as white spheres; the two lines indicate approximate bilayer boundaries.



b. Schematic showing bivalent recognition of long chain fatty acyl CoA by palmitoyltransferases: the chemical structure of palmitoyl CoA is also shown with the fatty acyl chain, CoA head group, and the pantetheine linker indicated.

CoA. The structure (Figure 3a) reveals that, while the hydrophobic fatty acyl chain is recognized by the transmembrane cavity of zDHHC20, the polar head group of palmitoyl CoA is recognized by the cytoplasmic charge-complementary surface. The functional and computational data show that free CoA does not compete effectively with palmitoyl CoA, leading to the hypothesis that, in this context, long chain fatty acyl CoAs act as bivalent ligands, with a hydrophobic chain and the polar head group linked by a flexible linker (Figure 3b). Coincident recognition of both modules by protein palmitoyltransferases (Figure 3b) is necessary for catalytic chemistry to take place.

S-acylation was first discovered in viral proteins and, in the wake of the COVID-19 pandemic, we turned our attention to this. It had been shown that the Spike protein of other coronaviruses or its equivalent in other pathogenic viruses are S-acylated and, in a subset of these, S-acylation is crucial to the viral life cycle. However, it was not known whether the Spike protein of SARS-CoV-2, the causative agent of COVID-19, is S-acylated and, if so, how that affects the viral life cycle. In previous work, we demonstrated that SARS-CoV-2 Spike is S-acylated and, in collaboration with Eric Freed's lab, showed that S-acylation is critically important for the viral life cycle. Remarkably, even 40 years after the discovery of S-acylation, there were still no *in vitro* reconstitution of S-acylation of any viral protein with purified components. We reconstituted the S-acylation of SARS-CoV-2 Spike protein *in vitro*, using purified zDHHC enzymes, and observed a striking correlation with our *in cellulo* experiments. The study also opened up avenues for further mechanistic dissection and laid the groundwork for identifying targeted small-molecule modulators. Recently, we expanded our focus to the S-acylation of Influenza HA protein and HIV-1 Tat protein. Both had been shown to be S-acylated, but the mechanistic underpinnings remain unclear. We are developing assays for *in vitro* reconstitution of the S-acylation of Influenza HA and HIV-1 Tat. These assays will provide the ground work for dissecting the mechanism whereby zDHHC enzymes recognize these substrates and catalyze S-acylation.

FIGURE 4. Transporters of iron and polyamine

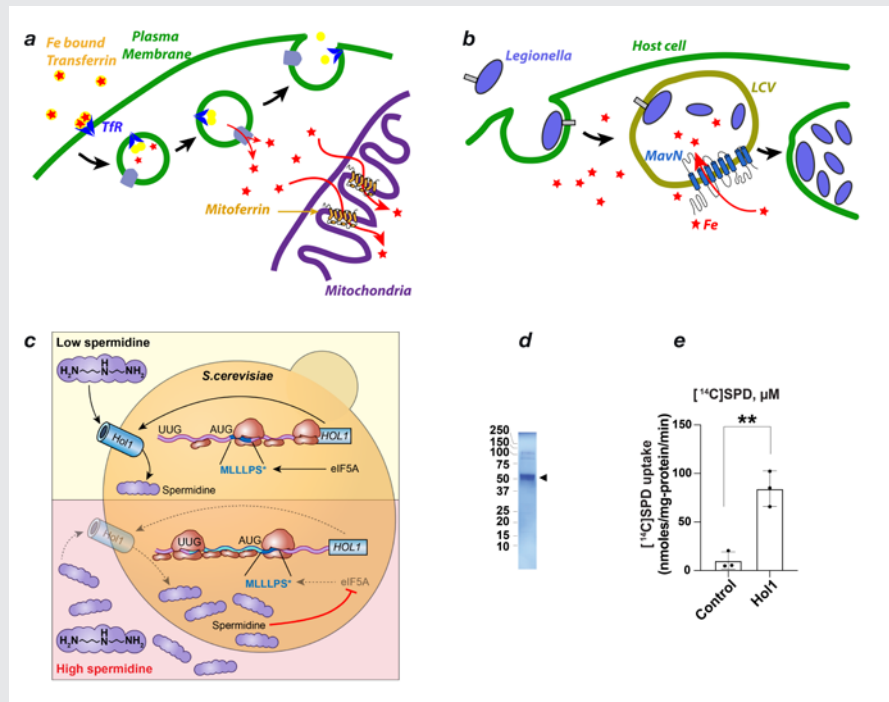
a. Iron is imported through the plasma membrane by the transferrin/transferrin receptor (blue) cycle and is transported out of endosomes by the divalent metal ion transporter (DMT) (grey); iron is delivered to mitoferrin (yellow cylinders) by unknown means; mitoferrin delivers iron to unknown partners in mitochondria, which become available for heme and Fe-S cluster biosynthesis.

b. Schematic showing *Legionella* entering a host cell and sequestering itself in a *Legionella*-containing vacuole (LCV); MavN is inserted into the membrane of the LCV and hijacks iron from the host cell.

c. Assay showing iron transport by proteoliposome-reconstituted MavN: Hol1 as the major high-affinity polyamine transporter in *S. cerevisiae*; polyamines autoregulate *HOL1* expression through feedback inhibition of *HOL1* mRNA translation, mediated by polyamine inhibition of eIF5A-dependent translation termination on a conserved upstream open reading frame in the *HOL1* mRNA leader.

d. SDS-PAGE gel of detergent-purified *K. lactis* Hol1 (arrowhead).

e. [¹⁴C]Spermidine uptake by control or *K. lactis* Hol1 proteoliposomes; error bars denote SD, ***p* < 0.01 (Student's two-tailed *t* test, *n* = 3).



Molecular mechanism of iron and polyamine transport across cellular membranes

The importance of iron in biology cannot be overstated. In higher organisms, mitochondria are the ‘hotspot’ for the cell biology of iron, because Fe-S clusters are biosynthesized and iron is inserted into heme there. Mitochondrial iron homeostasis plays a critical role in cellular iron homeostasis and in the overall physiology of the cell. In vertebrates, the only known major transporters of iron into mitochondria are mitoferrin-1 and mitoferrin-2, two homologous members of a large group of mitochondrial transporters known as the Mitochondrial Carrier family (Figure 4). Mitoferrin-1 (Mfrn1) is expressed mainly in erythroid cells, while mitoferrin-2 is expressed ubiquitously. Knockout of Mfrn1 is embryonically lethal, reflecting the importance of mitoferrins in vertebrate physiology.

Mfrn1 and Mfrn2 were discovered more than 15 years ago. However, the proposed iron-transport activity had not been demonstrated using an *in vitro* functional reconstitution assay, and nothing was known about their interaction with iron or other related metal ions, most likely because heterologous overexpression and purification of mitoferrins were not reported in the literature. There were also no reports of a reconstituted

iron transport assay starting from purified protein. In earlier work, we carried out heterologous purification, *in vitro* functional reconstitution, and mutational dissection of a vertebrate Mfrn1 (Figure 4), the first demonstration that Mfrn1 can indeed transport iron. Our studies provided the first biochemical insights into Mfrn function. In earlier work, we also used our *in vitro* proteoliposome-reconstituted iron-transport assay, the first such assay to be reported in the literature, to dissect the iron-transport activity of MavN, another highly conserved iron transporter, in the bacterial pathogen *Legionella pneumophila* (Figure 4b).

In the past two years, we shifted our interest to transporters that move polyamines across the membrane. Polyamines, small organic polycations, are essential for cell viability, and their physiological levels are homeostatically maintained by post-transcriptional regulation of key biosynthetic enzymes. In addition to *de novo* synthesis, cells can also take up polyamines; however, until recently, dedicated polyamine transporters were not known. [Tom Dever's lab](#) discovered that the *S. cerevisiae* *HOL1* mRNA is under translational control by polyamines. They also showed that Hol1 is required for yeast growth under limiting polyamine conditions. These experiments suggested that Hol1 is a high-affinity polyamine transporter (Figure 4c). We purified recombinant Hol1 and reconstituted it in proteoliposomes to demonstrate that it indeed shows robust polyamine transport activity. An extensive set of experiments from the Dever lab bolstered the conclusions that Hol1 is a high-affinity polyamine transporter and under translational autoregulation by polyamines in yeast, highlighting the extensive control cells impose on polyamine levels. We are now investigating the underlying molecular mechanisms behind Hol1 function.

Additional Funding

- Office of AIDS Research

Publications

1. Lee CJ, Stix R, Rana MS, Shikwana F, Murphy RE, Ghirlando R, Faraldo-Gómez JD, Banerjee A. Bivalent recognition of fatty acyl-CoA by a human integral membrane palmitoyltransferase. *Proc Natl Acad Sci USA* 2022 119 (7):e2022050119.
2. Puthenveetil R, Gomez-Navarro N, Banerjee A. Access and utilization of long chain fatty acyl-CoA by zDHHC protein acyltransferases. *Curr Opin Struct Biol* 2022 77:102463.
3. Murphy RE, Banerjee A. In vitro reconstitution of substrate S-acylation by the zDHHC family of protein acyltransferases. *Open Biology* 2022 12:210390.
4. Puthenveetil R, Lun CM, Murphy RE, Healy LB, Vilmen G, Christenson ET, Freed EO, Banerjee A. S-acylation of SARS-CoV-2 spike protein: mechanistic dissection, in vitro reconstitution and role in viral infectivity. *J Biol Chem* 2021 297(4):101112–101124.
5. Vindu A, Shin BS, Choi K, Christenson ET, Ivanov IP, Cao C, Banerjee A, Dever TE. Translational autoregulation of the *S. cerevisiae* high-affinity polyamine transporter Hol1. *Mol Cell* 2021 81(19):3904–3918.

Collaborators

- David Cafiso, PhD, *Department of Chemistry, University of Virginia, Charlottesville, VA*
- Thomas Dever, PhD, *Section on Mechanism and Regulation of Eukaryotic Protein Synthesis, NICHD, Bethesda, MD*
- José Faraldo-Gómez, PhD, *Theoretical Molecular Biophysics Section, NHLBI, Bethesda, MD*

- Eric Freed, PhD, *Retroviral Replication Laboratory, NCI, Frederick, MD*
- Rodolfo Ghirlando, PhD, *Laboratory of Molecular Biology, NIDDK, Bethesda, MD*
- James Inglese, PhD, *Assay Development & Screening Technology Laboratory, NCATS, Bethesda, MD*
- Ralph Isberg, PhD, *Tufts University School of Medicine, Boston, MA*
- Anant Menon, PhD, *Weill Cornell Medicine, New York, NY*

Contact

For more information, email anirban.banerjee@nih.gov or visit <http://banerjee.nichd.nih.gov>.

Regulation of Childhood Growth

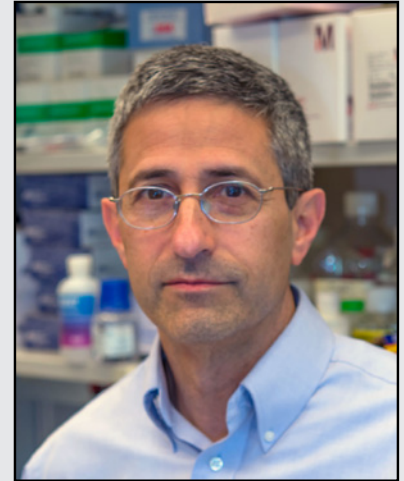
Children grow taller because their bones grow longer. Bone elongation occurs at the growth plate, a thin layer of cartilage found near the ends of juvenile bones (Figure 1). In the growth plates, new cartilage is produced through chondrocyte proliferation, hypertrophy, and cartilage matrix synthesis, and then this newly formed cartilage is remodeled into bone. The process, termed endochondral ossification, results in bone elongation, which causes children to grow in height (linear growth).

We investigate the cellular and molecular mechanisms governing childhood growth and development. We focus particularly on growth at the growth plate, which drives bone elongation and therefore determines height. One goal of this work is to gain insight into the many human genetic disorders that cause childhood growth failure or overgrowth. A second goal is to develop new treatments for children with severe growth disorders.

Novel genetic causes of childhood growth disorders

Mutations in genes that regulate growth plate chondrogenesis cause abnormal bone growth and short stature in children. Depending on the severity and nature of the genetic abnormality, the phenotype can range from chondrodysplasias with short, malformed bones, to severe, often disproportionate, short stature, to mild proportionate short stature. If the genetic defect affects tissues other than the growth-plate cartilage, the child may present with a more complex syndrome that includes other clinical abnormalities. Less commonly, mutations in these genes cause excessive growth-plate chondrogenesis and therefore abnormally tall stature. Often the increased proliferation occurs in many tissues, producing a generalized overgrowth syndrome, which can include other medical problems such as developmental delay and increased cancer risk.

For many children who are brought to medical attention for linear growth disorders, clinical, laboratory, and genetic evaluation fails to identify the underlying etiology. Genome-wide association studies and molecular studies of growth-plate biology suggest that there are hundreds of genes that control linear growth. Therefore, there are likely



Jeffrey Baron, MD, Head, Section on Growth and Development

Kevin Barnes, PhD, *Senior Research Assistant*

Julian Lui, PhD, *Staff Scientist*

Youn Hee Jee, MD, *Staff Clinician*

Joanna Curtis, BS, *Postbaccalaureate Fellow*

Benjamin Hauser, BS, *Postbaccalaureate Fellow*

Jacob Wagner, BS, *Postbaccalaureate Fellow*

Elaine Zhou, BA, *Postbaccalaureate Fellow*

Wei Wang, MSc, *Biologist*

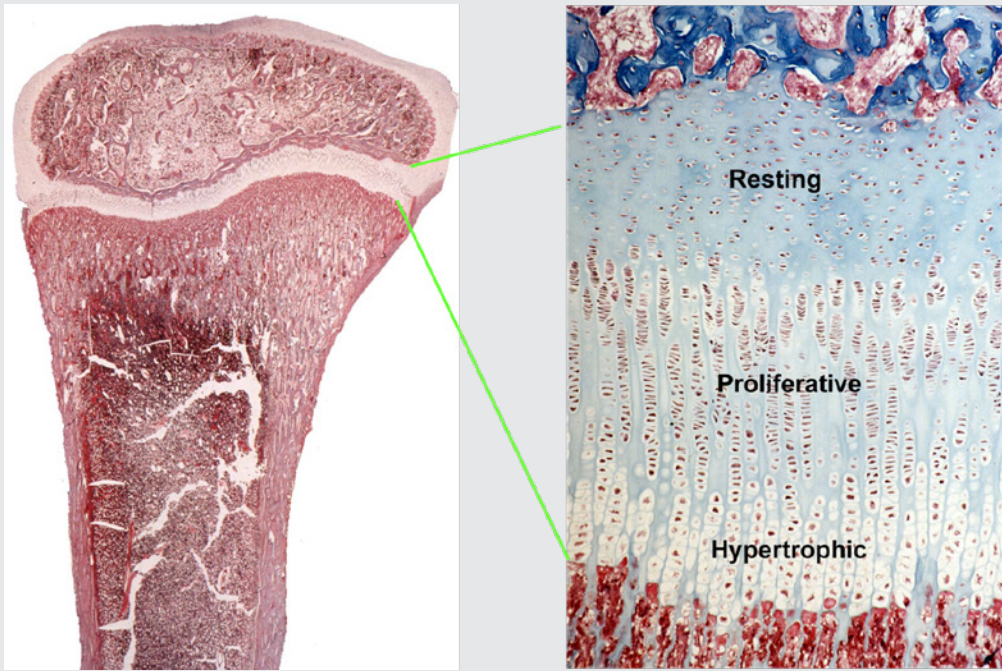


FIGURE 1.
Histological image
of a growth plate,
showing the three
principal zones

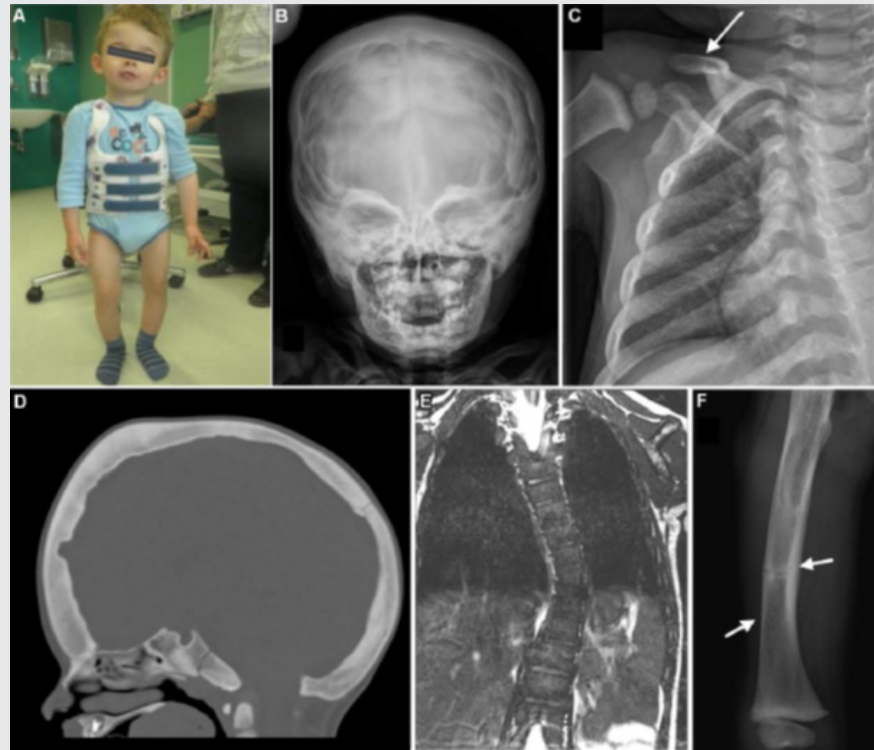
many genetic causes of linear growth disorders that remain to be discovered.

To discover new genetic causes of childhood growth disorders, we evaluated families with monogenic growth disorders using SNP (single-nucleotide polymorphism) arrays to detect deletions, duplications, mosaicism, and uniparental disomy, combined with exome sequencing to detect single-nucleotide variants and small insertions/deletions in coding regions and splice sites. When sequence variants that are likely to cause the disorder are identified, the variants and the genes in which they occur are studied in the laboratory to confirm that the variant is pathogenic, to elucidate the pathogenesis of the disorder, and to explore the role of the gene in normal growth.

Using this approach, we identified new causes of childhood growth disorders. We previously found that variants in *QRICH1*, a gene of unknown function, cause a chondrodysplasia attributable to impaired growth-plate chondrocyte hypertrophic differentiation. We also found evidence that heterozygous deletion of *CYP26A1* and *CYP26C1*, which encode enzymes that metabolize retinoic acid (RA), cause elevated RA concentrations, which accelerate bone and dental maturation in humans and cause developmental defects involving the eye and central nervous system. Our group also discovered that variants in *ACAN*, the gene encoding aggrecan, a component of cartilage extracellular matrix, cause autosomal-dominant short stature with advanced skeletal maturation, and that such patients tend to develop early-onset osteoarthritis.

We recently studied a child with a complex skeletal dysplasia who presented with severe scoliosis, thickened calvarium, craniosynostosis, osteosclerosis of the clavicles and spine, and recurring fractures in the lower extremities (Figure 2). We found that the disorder was caused by a *de novo* mutation in *SP7*. *SP7* encodes a transcription factor required for differentiation of osteoblasts, a cell type required for bone formation. We

FIGURE 2. A child with a complex skeletal dysplasia caused by a neomorphic variant in *SP7*



generated mice with a variant orthologous to that of our subject and found that the mice showed a similar complex skeletal phenotype, confirming that the variant was responsible for the disorder. The mutation shifted the DNA-binding specificity of *SP7* from AT-rich motifs to a GC-consensus sequence (typical of other *SP* family members), resulting in an aberrant gene-expression profile and abnormal osteoblast differentiation. Thus, our study identifies a novel pathogenic mechanism in which a neomorphic mutation in a transcription factor shifts DNA-binding specificity to cause disease.

We also studied disorders that causes excessive growth. We evaluated a patient with Weaver syndrome, a condition that includes overgrowth of many tissues, including the skeleton (Figure 3). The syndrome is caused by variants in *EZH2*. *EZH2* encodes a histone methyltransferase that catalyzes the trimethylation of histone H3 at lysine 27 (H3K27), which serves as an epigenetic signal for chromatin condensation and transcriptional repression. We created a mouse model that showed mild overgrowth, recapitulating the Weaver phenotype. We found that the *EZH2* variants responsible for Weaver syndrome cause a partial loss of enzymatic function. Interestingly, we also found that a more severe genetic lesion, loss of both *EZH1* and *EZH2*, impairs bone growth in mice, and we explored the molecular mechanisms involved.

Molecular and cellular mechanisms by which specific genes and pathways regulate childhood growth

Our group also studies the fundamental mechanisms governing childhood growth. Much of our work has focused on the growth plate. Growth at the growth plate is controlled by several interacting regulatory systems, involving endocrine, paracrine, extracellular matrix-related, and intracellular pathways. Previously,

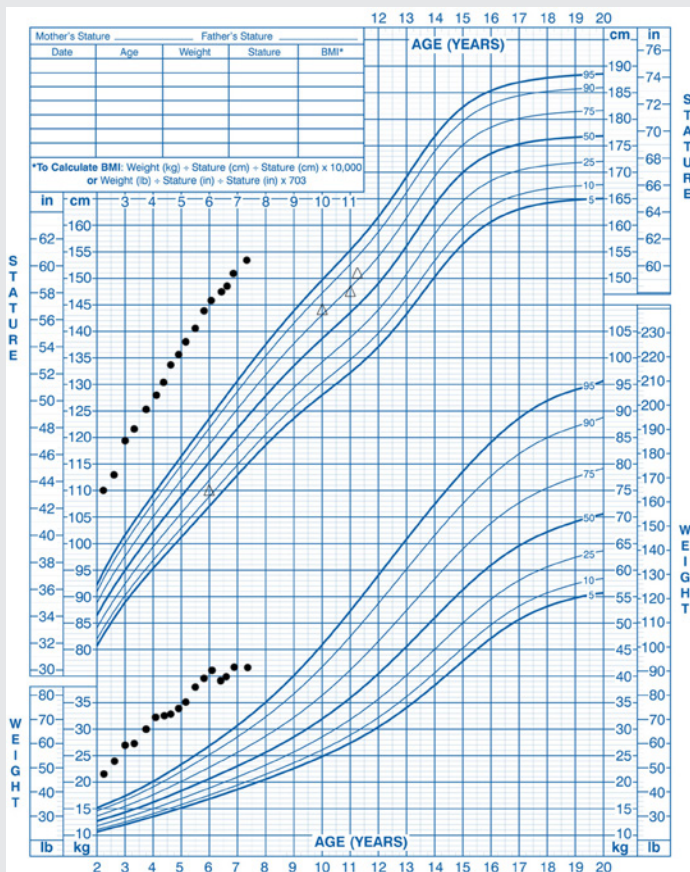


FIGURE 3. Growth chart of a child with Weaver syndrome

our group studied growth-plate regulation by FGFs, BMPs, C-type natriuretic peptide, retinoids, WNTs, PTHrP/IHH, IGFs, estrogens, glucocorticoids, and microRNAs. We also found evidence that SOX9, a transcription factor, regulates the transdifferentiation of growth-plate chondrocytes into osteoblasts.

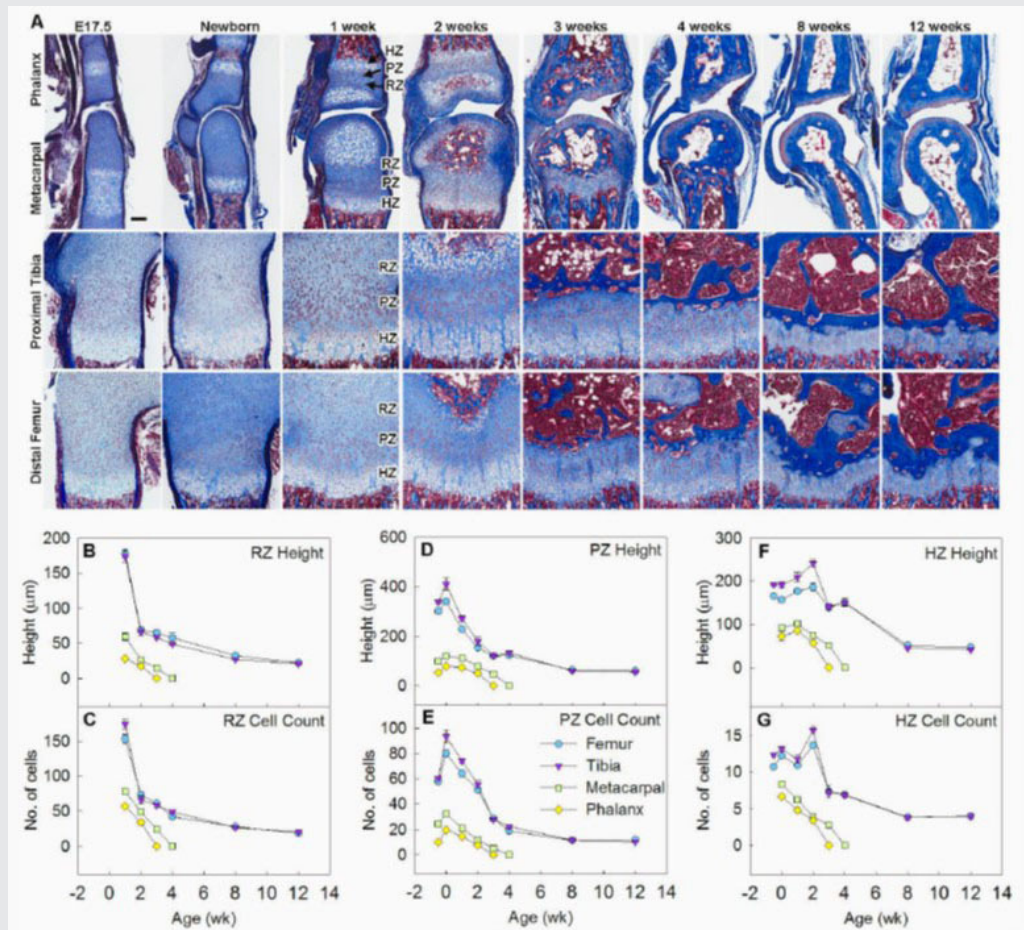
We also investigated the mechanisms that cause bone growth to occur rapidly in early life but then to slow progressively with age and eventually cease. We found evidence that the developmental program responsible for the decline in growth-plate function plays out more slowly in larger bones than in smaller bones and that such differential aging contributes to the disparities in bone length and therefore to establishing normal mammalian skeletal proportions (Figure 4).

New treatment approaches for growth-plate disorders

Recombinant human growth hormone

(GH) is commonly used to treat short stature in children. However, GH treatment has limited efficacy, particularly in severe, non-GH-deficient conditions such as chondrodysplasias, and has off-target effects. Systemic insulin-like growth factor-1 (IGF-1) treatment has similar deficiencies. There are many endocrine and paracrine factors that promote chondrogenesis at the growth plate, and which could possibly be used to treat such disorders. Targeting these growth factors specifically to the growth plate might augment the therapeutic skeletal effect while diminishing undesirable effects on non-target tissues. To develop growth plate-targeted therapy, we previously used yeast display to identify single-chain human antibody fragments that bind to cartilage with high affinity and specificity. As a first test of this approach, we created fusion proteins combining these cartilage-targeting antibody fragments with IGF-1, an endocrine/paracrine factor that positively regulates chondrogenesis. The fusion proteins retained both cartilage binding and IGF-1 biological activity, and they were able to stimulate bone growth in an organ culture system. Using a GH-deficient mouse model, we found that subcutaneous injections of the fusion proteins increased growth-plate height without increasing proliferation in kidney cortical cells, demonstrating greater on-target efficacy at the growth plate and less off-target effect on the kidney than IGF-1 alone (Figure 5). Our findings provide proof of principle that targeting therapeutics to growth-plate cartilage can potentially improve treatment for childhood growth disorders.

FIGURE 4. Growth plate senescence occurs earlier in shorter bones.



We are currently working to optimize the efficacy of targeted IGF1 therapy. In other studies, we are using cartilage-tethering antibody fragments to target other chondrogenic endocrine and paracrine factors to the growth plate. We are exploring the utility of this approach both to stimulate growth plate chondrogenesis non-specifically and also to reverse specific genetic defects in growth-plate function by modulating the abnormal molecular pathway responsible for the growth failure.

Publications

1. Lui JC, Raimann A, Hojo H, Dong L, Roschger P, Kikani B, Wintergerst U, Fratzl-Zelman N, Jee YH, Haeusler G, Baron J. A neomorphic variant in the transcription factor SP7 alters sequence specificity and causes a high-turnover bone disorder. *Nat Commun* 2022 13:700.
2. Jee YH, Gangat M, Yeliosof O, Temnycky AG, Vanapruks S, Whalen P, Gourgari E, Bleach C, Yu CH, Marshall I, Yanovski JA, Link K, Ten S, Baron J, Radovick S. Evidence that the etiology of congenital hypopituitarism has a major genetic component but is infrequently monogenic. *Front Genet* 2021 12:697549.
3. Jee YH, Won S, Lui JC, Jennings M, Whalen P, Yue S, Temnycky AG, Barnes KM, Cheetham T, Boden MG, Radovick S, Quinton R, Leschek EW, Aguilera G, Yanovski JA, Seminara SB, Crowley WF, Delaney A, Roche KW, Baron J. DLG2 variants in patients with pubertal disorders. *Genet Med* 2020 22:1329-1337.

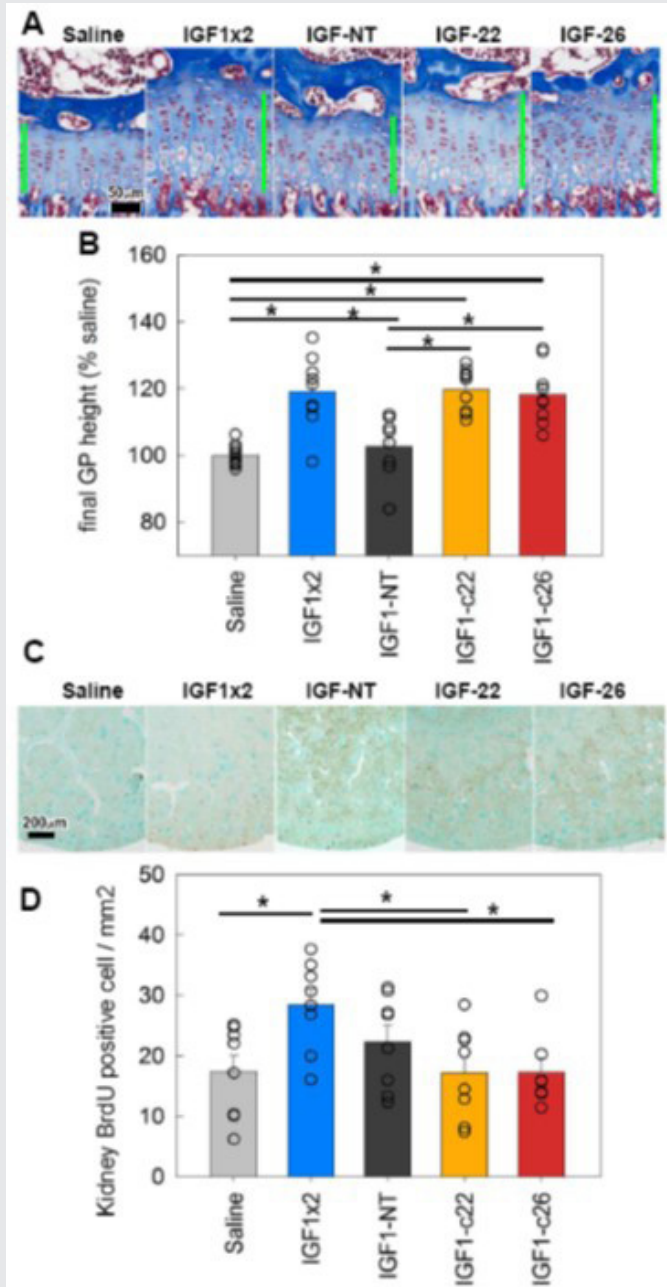


FIGURE 5. Targeted IGF-1 treatment in growth hormone-deficient mice increases growth-plate height without increasing proliferation in the kidney.

- Lui JC, Baron J. CNP-related short and tall stature: a close-knit family of growth disorders. *J Endocr Soc* 2022 6:bvac064.
- Weiss B, Eberle B, Roeth R, de Bruin C, Lui JC, Paramasivam N, Hinderhofer K, van Duyvenvoorde HA, Baron J, Wit JM, Rappold GA. Evidence that non-syndromic familial tall stature has an oligogenic origin including ciliary genes. *Front Endocrinol* 2021 12:660731.

Collaborators

- Greti Aguilera, MD, *Scientist Emeritus, NICHD, Bethesda, MD*
- Angela Delaney Freedman, MD, *St. Jude Children's Research Hospital, Memphis, TN*
- Lijin Dong, PhD, *Genetic Engineering Core, NEI, Bethesda, MD*
- Gabriele Häusler, MD, *Medizinische Universität Wien, Vienna, Austria*
- Ellen Leschek, MD, *Division of Diabetes, Endocrinology, and Metabolic Diseases, NIDDK, Bethesda, MD*
- Ola Nilsson, MD, PhD, *Karolinska Institute, Stockholm, Sweden*
- Sally Radovick, MD, *Rutgers Biomedical and Health Sciences, Robert Wood Johnson Medical School, New Brunswick, NJ*
- Gudrun Rappold, PhD, *University of Heidelberg, Heidelberg, Germany*
- Katherine W. Roche, PhD, *Receptor Biology Section, NINDS, Bethesda, MD*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

Contact

For more information, email jeffrey.baron@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/baron>.

Quantitative Imaging and Tissue Sciences

In our tissue-sciences research, we strive to understand fundamental relationships between function and structure in living tissues. Specifically, we are interested in how tissue microstructure, hierarchical organization, composition, and material properties all work together to affect biological function or dysfunction. We investigate biological and physical model systems at various length and time scales, performing biophysical measurements and developing novel physical/mathematical models (including molecular dynamics [MD] and continuum models) to explain their functional properties and behavior. Inextricably connected to these activities is our study of water and its interactions with macromolecules and ions in biological media. Experimentally, we often use water to probe tissue structure and function from nanometers to centimeters and from microseconds to lifetimes. Our armamentarium includes atomic force microscopy (AFM), small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), static light scattering (SLS), dynamic light scattering (DLS), osmometry, and multi-dimensional nuclear magnetic resonance (NMR) relaxometry and diffusometry. A goal is to develop understanding and tools that can be translated from bench-based quantitative methodologies to the bedside to aid in diagnosis and therapy.

Our activities dovetail with our basic and applied research in quantitative imaging, which is intended to generate measurements and maps of intrinsic physical quantities, including diffusivities, relaxivities, exchange rates, etc., rather than the qualitative 'weighted' MR images conventionally used in radiology. At a basic level, our work is directed toward making critical 'invisible' biological structures and processes 'visible.' Our quantitative imaging group uses knowledge of physics, engineering, applied mathematics, imaging and computer sciences, as well as key insights gleaned from our tissue-sciences research, to discover, vet, and develop novel quantitative imaging biomarkers that can detect changes in tissue composition, microstructure, and/or microdynamics with high sensitivity and specificity. The ultimate translational goal is to assess normal and abnormal developmental trajectories, diagnose childhood diseases and disorders, and characterize degeneration and trauma (such as mild traumatic brain injury). MRI is our imaging modality of choice because it is so well suited to many applications critical to the NICHD mission; it is non-invasive, non-ionizing, usually requires no exogenous contrast agents



Peter J. Basser, PhD, Head,
*Section on Quantitative Imaging
and Tissue Sciences*

Ferenc Horkay, PhD, *Staff Scientist*

Julian Rey, PhD, *Postdoctoral
Intramural Research Training
Award Fellow*

Nathan Williamson, PhD,
*Postdoctoral Research Associate
Training Award Fellow (NIGMS)*

Velencia Witherspoon, PhD,
*Postdoctoral Intramural
Research Training Award Fellow*

Teddy Cai, BS, *Predocutorial
Intramural Research Training
Award Fellow, NIH OxCam
Program*

Kristofor Pas, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Alexandru Avram, PhD,
*Collaborating Scientist funded
via the Henry Jackson Foundation
and the Center for Neuroscience
and Regenerative Medicine*

Dan Benjamini, PhD, *Collaborating
Scientist funded via the Henry
Jackson Foundation and the*

(continued)

or dyes, and is generally deemed safe and effective for use with mothers, fetuses, and children in both clinical and research settings. Critical to this enterprise is our ability to follow water as it diffuses through complex media as a probe of microstructure, and to assess its interactions with biomolecules to identify distinct water compartments in tissues.

One of our translational goals has been to transform clinical MRI scanners into scientific instruments capable of producing reproducible, accurate, and precise imaging data with which to measure and map useful imaging biomarkers for various clinical applications, including single scans, longitudinal, and multi-site studies, personalized medicine, and genotype/phenotype correlation studies, as well as for populating imaging databases with high-quality normative data. From a more basic perspective, another goal has been to apply our various MRI tools and methodologies to advance neuroscience, providing new methods to explore brain structure/function relationships, such as “imaging” the human connectome.

In vivo MRI histology

The most mature *in vivo* MRI histological technology that we invented, developed, and clinically translated is Diffusion Tensor MRI (DTI), by which we measure and map D , a diffusion tensor of water, within an imaging volume. Information derived from this quantity includes white-matter fiber-tract orientation, the orientationally averaged mean apparent diffusion constant (mADC) or mean diffusivity (MD), and other intrinsic scalar (invariant) quantities. Such imaging parameters have been used by radiologists and neuroscientists as non-invasive quantitative histological ‘stains’ that are obtained by probing endogenous tissue water *in vivo* without requiring any exogenous contrast agents or dyes. The mADC is the most widely used diffusion imaging parameter in neuroradiology to identify ischemic areas in the brain during acute stroke and to follow cancer patients’ responses to therapy. The measures of diffusion anisotropy we first proposed (e.g., the fractional anisotropy or FA) are also widely used to follow changes in normally and abnormally developing white matter and in many other clinical and neuroscience applications, such as brain white-matter visualization. Our group also pioneered the use of fiber direction–encoded color (DEC) maps to display the orientation of white matter pathways in the brain. To assess anatomical connectivity among various cortical and deep-brain gray-matter areas, we also proposed and developed DTI ‘Streamline’ Tractography, which is used to track white-matter fibers to help establish ‘anatomical connectivity’ and by neuroradiologists and neurosurgeons, so as to plan surgical procedures, and radiation dosing, so as to spare ‘eloquent’ areas of the brain. These advances also helped inspire several large federally funded research initiatives, including the NIH Human Connectome Project (HCP) and, more recently, the NIH Brain Initiative.

More recently, we invented and developed a family of advanced *in vivo* diffusion MRI methods to measure fine-scale microstructural features of axons and fascicles (also known as ‘microstructure imaging’), which otherwise could only be assessed using laborious *ex vivo* histological or pathological methods. We have been developing efficient means for performing ‘k- and q-space MRI’ in the living human brain, such as ‘Mean Apparent

Center for Neuroscience and Regenerative Medicine

Michal Komlosh, PhD,
Collaborating Scientist funded via the Henry Jackson Foundation and the Center for Neuroscience and Regenerative Medicine

Magdoom Kulam, PhD,
Collaborating Scientist funded via the Henry Jackson Foundation and the Center for Neuroscience and Regenerative Medicine

Kadharbatcha Saleem, PhD,
Collaborating Scientist funded via the Henry Jackson Foundation and the Center for Neuroscience and Regenerative Medicine

Alexandros Chremos, PhD,
Contract Scientist

Rea Ravin, PhD, *Contract Scientist*

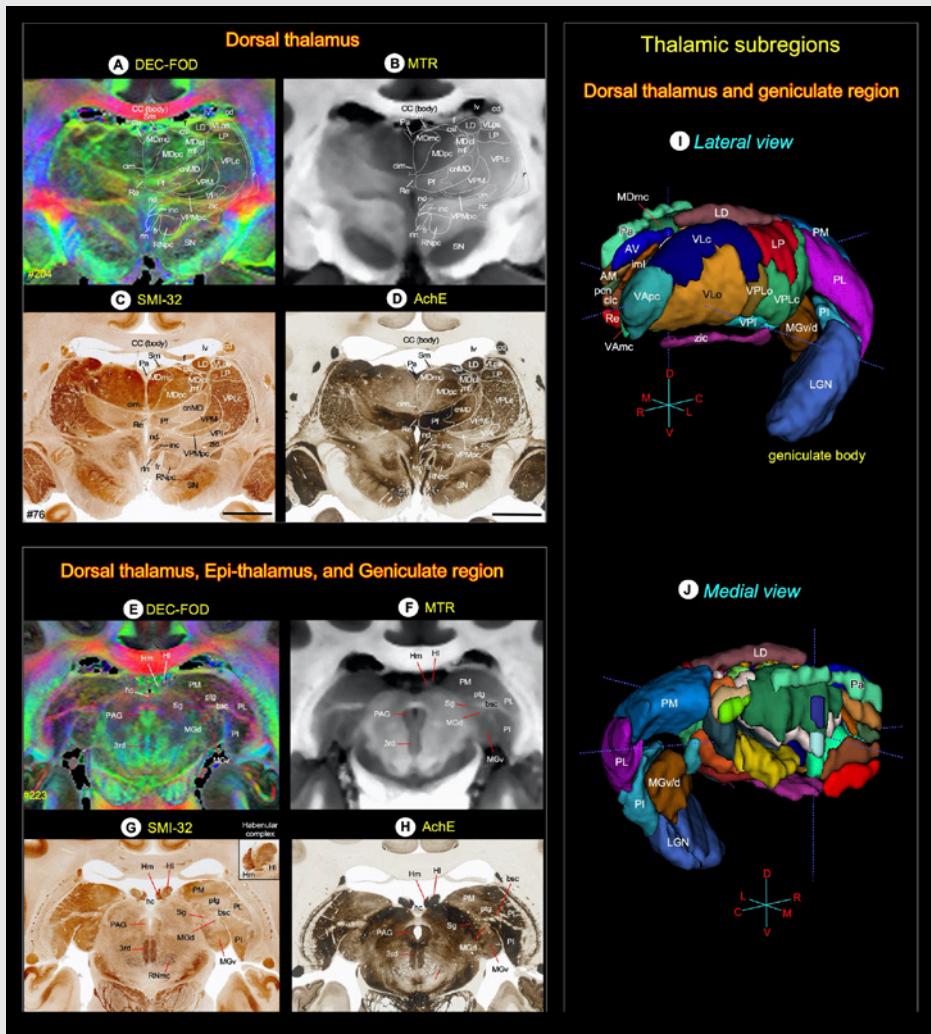


FIGURE 1. Digital “D99” Macaque Brain Atlas

Kadharbatcha Saleem created this NHP (non-human primate) brain atlas, which identifies deep gray matter areas, such as the thalamus, using both histological staining and Mean Apparent Propagator MR-derived imaging parameters. Careful correlation and integration of histology and MR images is critical to testing and vetting the sensitivity and specificity of new MR brain imaging methods. In some cases, MRI methods can be more eloquent in identifying nuclei and other structures than staining.

Propagator’ (MAP) MRI, an approach that can detect subtle microstructural and architectural features in both gray and white matter at micron-scale resolution, several orders of magnitude smaller than the typical MRI voxel. MAP MRI also subsumes DTI, as well as providing a bevy of new *in vivo* quantitative imaging biomarkers to measure and map. We recently applied this to assessing mild traumatic brain injury (TBI) and other forms of TBI. We also developed CHARMED MRI, which measures the average axon diameter (AAD), and AxCaliber MRI, which measures the axon-diameter distribution (ADD) along white-matter pathways, and we reported the first *in vivo* measurement of ADDs within the rodent corpus callosum. The ADD is functionally important, given that axon diameter is a critical determinant of conduction velocity and therefore the rate at which information is transferred along axon bundles, and helps determine the latencies or time delays between and among different brain areas. This led us to propose a novel MRI-based method to measure the ‘latency connectome,’ including a latency matrix that reports conduction times between different brain areas. We also developed a companion mathematical theory to explain the observed ADDs in different fascicles, suggesting that they represent a trade-off between maximizing information flow and minimizing metabolic demands. We developed novel multiple pulsed-field gradient (mPFG) methods and demonstrated their feasibility *in vivo*

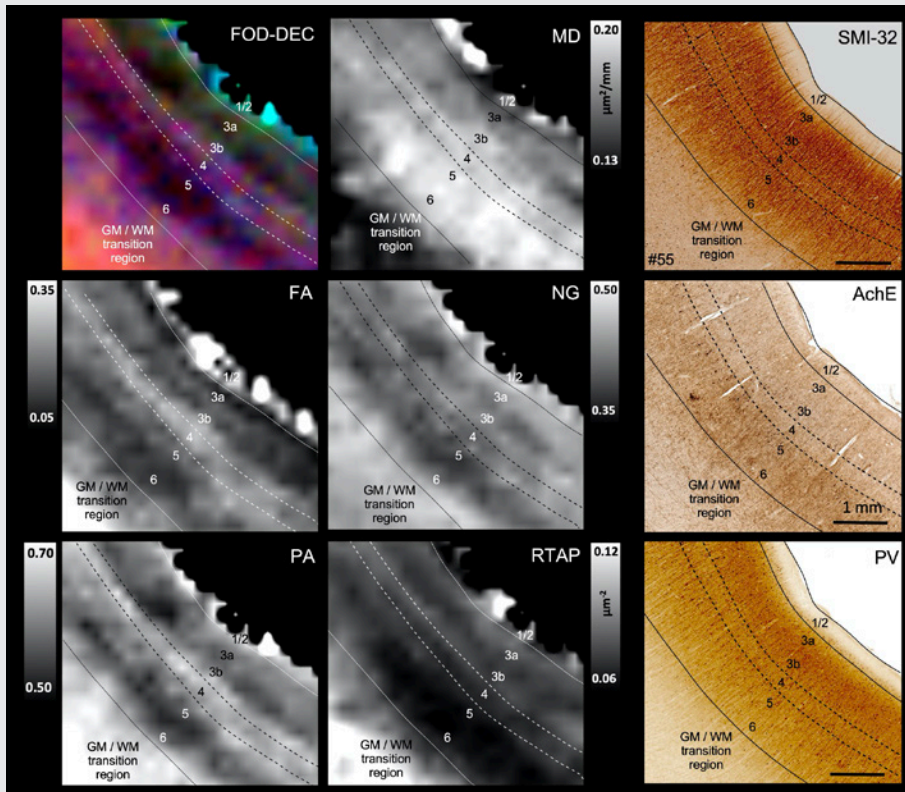


FIGURE 2. Elucidating cortical layers using MAP MRI-derived parameters

Cortical layers are generally difficult to identify using conventional MRI methods, but MAP MRI provides a family of stains that can be used to identify microstructural differences among cortical layers, allowing for non-invasive parcellation of some brain areas. The image shows maps of the diffusion-encoded color (DEC), Fractional Anisotropy (FA), Mean Diffusivity (MD), Non-gaussianity (NG), Propagator Anisotropy (PA), and Return to Axis Probability (RTAP). Shown also are the cortical layers numbered, using the convention of 1 through 6 from the cortex to the sub-cortical white matter.

on conventional clinical MRI scanners as a further means to extract quantitative features in the central nervous system (CNS), such as the AAD and other features of cell size and shape.

Although gray matter appears featureless in the brain with DTI, its microstructure and architecture are rich and varied throughout the brain, not only along the brain's cortical surface, but also within and among its various cortical layers and within deep gray-matter regions. To target this tissue, we have been developing several non-invasive, *in vivo* methods to measure unique features of cortical gray-matter microstructure and architecture that are visible in electron microscopy (EM) applications but currently invisible in conventional MRI. One example is diffusion tensor distribution (DTD) MRI, in which we use our normal tensor-variate distribution to characterize heterogeneities in these complex tissues. One of our long-term goals is to 'parcellate' or segment the cerebral cortex *in vivo* into its approximately 500 distinct cyto-architectonic areas, using non-invasive imaging methods. To this end, we are developing advanced MRI sequences and analysis pipelines to probe correlations among relaxivities and diffusivities of different water pools in the cortex. One promising avenue is to use multi-dimensional MRI relaxometry- and diffusometry-based methods to study water mobility and diffusion in gray and white matter. We continue to work to translate these and other methods to the clinic to help identify changes in normal and abnormal development, as well as in inflammation and trauma. Along these lines, we made excellent progress this past year in developing radiological-pathological correlations between MR and neuropathological images of TBI tissue specimens as a way to identify potential quantitative imaging biomarkers of injury or inflammation that may have the potential to detect TBI *in vivo*.

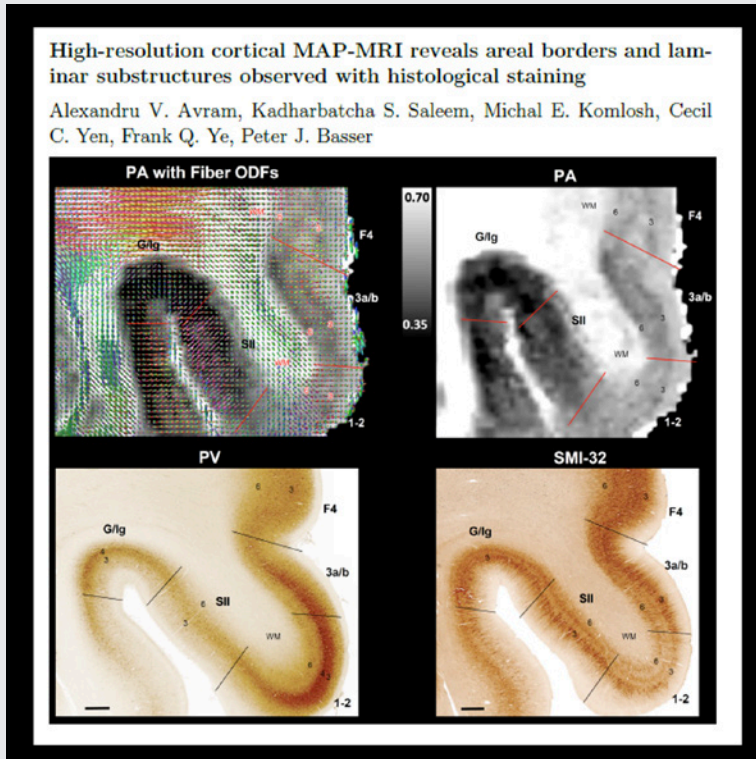


FIGURE 3. Elucidating cortical layers using MAP MRI-derived parameters

Mean Apparent Propagator (MAP) MRI-derived biomarkers are juxtaposed to images of the same specimen after being histologically stained in area F4. MR parameters are often able to detect boundaries between different cortical brain areas more reliably than by histology.

Quantitative MRI biomarker development for pediatric and fetal imaging applications

MRI is considered safer than X-ray-based methods, such as computed tomography (CT), for scanning fetuses, infants, and children. However, clinical MRI still lacks the quantitative character of CT. The scope of conventional MRI clinical applications is limited to revealing either gross morphological features or focal abnormalities. Clinical MRI also often lacks the biological specificity necessary for developing robust and reliable imaging ‘biomarkers.’ In particular, MRI assessment of normal brain development and developmental disorders has benefited greatly from the introduction of ‘quantitative’ clinical MRI techniques, with which one measures and maps meaningful intrinsic physical quantities or chemical variables that possess physical units and can be compared among different tissue regions. Quantitative MRI methods such as DTI also increase sensitivity, providing a basis for monitoring subtle changes that occur, e.g., during the progression or remission of disease, by comparing measurements in a single subject against normative values obtained from a healthy population. Quantitative MRI methods should continue to advance ‘precision imaging’ studies, in which MRI phenotypic and genotypic data can be meaningfully incorporated and used for improved diagnosis and prognosis assessments.

To advance our quantitative imaging activities, we developed numerical and statistical methods, including algorithms that generate a continuous, smooth approximation to the discrete, noisy, measured DTI field data, so as to reduce noise, and which allowed us to implement Streamline Tractography. We proposed a novel Gaussian distribution for the tensor-valued random variables that we use to design optimal DTI experiments and interpret their results. In tandem, we developed non-parametric empirical (e.g., Bootstrap) methods to

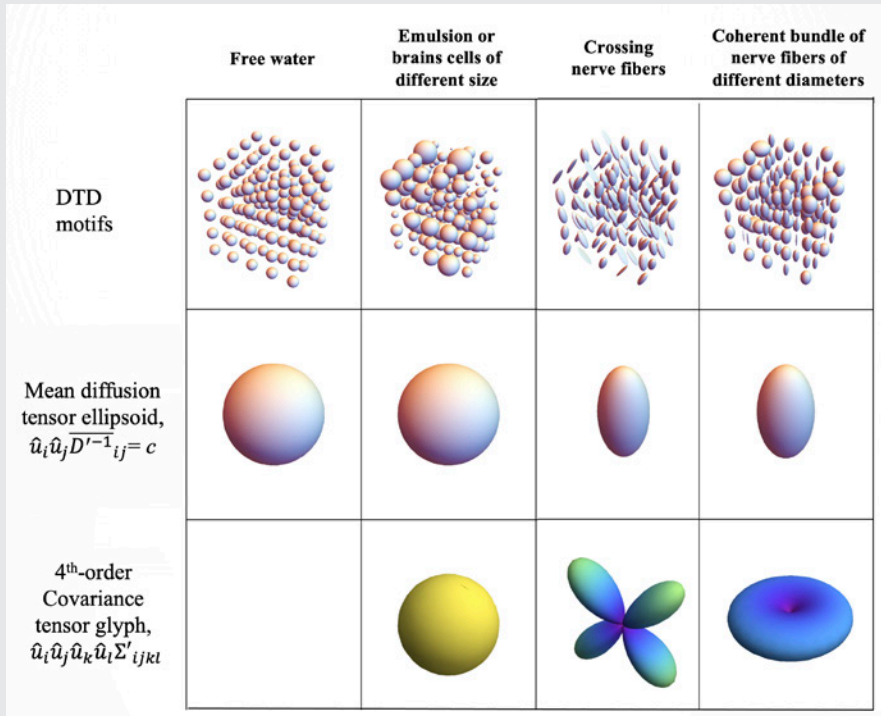


FIGURE 4. Diffusion Tensor Distribution MRI reveals microscopic heterogeneity within a voxel.

While Diffusion Tensor MRI (DTI) provides an estimate of a mean diffusion tensor within a voxel, Diffusion Tensor Distribution (DTD) MRI reveals how heterogeneous the population of microdiffusion tensors is within the same voxel, providing a wealth of new information about local microstructural variability. For instance, different microtensor motifs can lead to the same voxel averaged mean diffusion tensor. The glyph of the covariance tensor captures this variability, making it possible to distinguish among these different motifs.

determine the statistical distribution of DTI-derived quantities in order to study, e.g., the inherent variability and reliability of computed white-matter trajectories, enabling us to apply powerful hypothesis tests to assess the statistical significance of findings in a wide range of important biological and clinical applications that had been tested using *ad hoc* statistical methods. We are also developing novel methods to register different brain volumes and to generate group-average DTI data or atlases from various subject populations, based on the Kullback-Leibler divergence.

Previously, we carried out clinical studies that utilize novel quantitative MRI acquisition and analysis methods and whose aim is to improve accuracy and reproducibility of diagnosis and to detect and follow normal and abnormal development. One early example is the NIH Study of Normal Brain Development, jointly sponsored by the NICHD, NIMH, NINDS, and NIDA, which was initiated in 1998 and intended to advance our understanding of normal brain development in typical healthy children and adolescents. The [Brain Development Cooperative Group](#) is still actively publishing papers, primarily by mining the rich high-quality MRI data, many of which our lab processed, serving as the DTI Data-Processing Center (DPC). The processed DTI data collected from the project were uploaded into a database and made publicly available through the [National Database for Autism Research](#) (NDAR). Our collaborator Carlo Pierpaoli, who spearheaded this work, continues to support, update, and disseminate the processing and analysis software called "TORTOISE," which grew out of this effort and which can be downloaded from <http://www.tortoisediti.org>.

Traumatic Brain Injury (TBI) represents a significant public health challenge for our pediatric population, but also for young men and women who serve in the military. Our involvement in TBI research, particularly in detecting mild TBI (mTBI), has continued to expand through partnerships with various Department of

Defense (DoD) entities. Diffusion MRI (dMRI) provides essential information to aid in the assessment of TBI, but conventional dMRI methods have lacked sufficient specificity. To improve the accuracy and reproducibility of MAP-MRI findings, we developed a data-processing pipeline, and, in collaboration with scientists at the DoD Center for Neuroscience and Regenerative Medicine (CNRM), performed the first normative MAP-MRI studies, and applied this new and powerful method to detect tissue damage in brains of individuals who have suffered TBI, extending our NICHD TORTOISE pipeline to be able to analyze MAP-MRI data. We are now employing promising multi-dimensional MRI relaxometry-diffusometry methods to study the etiology of various types of TBI, in collaboration with the USUHS Neuropathology Research Division and under the auspices of the CNRM, and to improve the correlation and integration of neuropathology and neuroradiological imaging data, so as to speed the deployment of new MRI methods to assess TBI. We also partnered with CNRM to study ways to measure very slow flows that occur during glymphatic transport, a mechanism the brain uses to wash away harmful macromolecules, just as the lymphatic system uses in other organs. With our partners at the University of Arizona, this research is providing experimental data to enable us migrate these imaging approaches to the clinic, to be able to assess normal and pathological glymphatic transport *in vivo*.

We are also collaborating with Sara Inati, who studies focal epilepsy, a devastating disorder that is difficult to detect using conventional neuroradiological methods. We are developing and testing various new MRI-based methods that we believe may reveal pathological microstructural features and changes in architectural organization of the brain in this disorder, for example, in cortical dysplasia, to improve localization and assessment of cortical lesions.

We have been partnering with Roberto Romero and Mark Haacke to develop novel fetal brain MR imaging applications. Currently, it is challenging to measure quantitative imaging biomarkers *in utero*, particularly diffusion MRI-based ones, owing to large-scale fetal and maternal motion during the scanning session, and owing to the difficulty in acquiring image volumes with sufficient coverage, quality, and spatial resolution in a clinically feasible amount of time. Our lab has been developing novel approaches to address each of these critical issues. The suspension of clinical protocols owing to COVID-19 has reduced our data collection activities since March, 2020.

Biopolymer physics: water-ion-biopolymer interactions

Remarkably, despite their crucial role, little is known about the physical underpinnings of water-ion-biopolymer interactions, particularly in the physiological ionic strength regime. To determine the effect of ions on the structure and dynamics of key biopolymers, we developed a multi-scale experimental framework by combining macroscopic techniques (osmotic swelling-pressure measurements and mechanical measurements) with high-resolution scattering methods (e.g., SANS and SAXS), which probe the structure and interactions over a broad range of length and time scales. Macroscopic swelling-pressure measurements provide information on the overall thermodynamic response of the system, while SANS and SAXS allow us to investigate biopolymers at molecular and supramolecular length scales and to quantify the effect of changes in the environment (e.g., ion concentration, ion valance, pH, temperature) on the structure and interactions among biopolymers, water, and ions. Studies carried out on well defined model systems that mimic essential features of tissues provide important insights that cannot be obtained from experimental studies made on biological systems themselves. Mathematical models based on well established polymer-physics concepts and, more recently, molecular dynamics (MD) simulation approaches make it possible to design experiments to

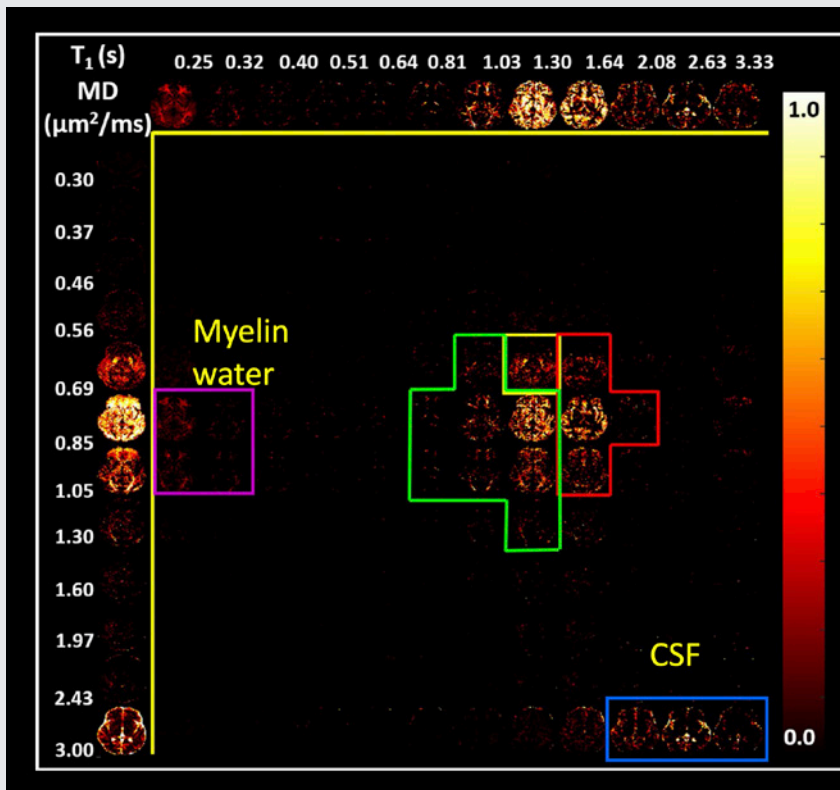


FIGURE 5. T₁-MD MRI provides a powerful probe of different water environments within each voxel.

Images showing the correlation between relaxivity T₁ and the mean diffusivity (MD) help identify distinct water domains or populations within voxels in the brain in which water may be in different chemical and physical environments. Such correlation spectra help identify white matter, gray matter, and cerebral spinal fluid compartments, as well as intra-myelin water, which is often difficult to detect.

help us quantify and explain aspects of tissue behavior and thus the underlying molecular and macroscopic mechanisms that govern key aspects of a tissue's normal functional properties.

These basic studies have led to numerous novel MRI phantom designs to support our quantitative imaging program, including diffusion MRI phantoms, which we use to calibrate scanners to assure the quality and fidelity of the imaging data in single-subject, longitudinal and multi-site studies. For instance, our U.S. Patent for a 'Phantom for diffusion MRI imaging' is now being used in the CaliberMRI phantom, enabling quantitative diffusion MRI studies to be performed at a myriad of sites. Our colleagues at NIST Boulder have incorporated our polyvinylpyrrolidone (PVP) polymer into their own diffusion MRI [NIST standard](#). We used various glass microcapillary geometries to mimic some features of white matter pathways and to interrogate our AxCaliber and dPDFG MRI models. We also developed a variety of NMR and MRI phantoms, such as a 3-D printed polymer phantom, which possess various salient features of cell or tissue systems, such as microscopic anisotropy, providing data with which to test the validity of our models and experimental designs. We are also developing novel polymer gel phantoms to calibrate exchange experiments in which to follow water moving between different microenvironments.

Measuring and mapping functional properties of extracellular matrix (ECM)

We study interactions among the main extracellular matrix (ECM) components, often using cartilage as a model system because it is aneural, avascular, and almost acellular. In cartilage ECM, collagen (type II) is

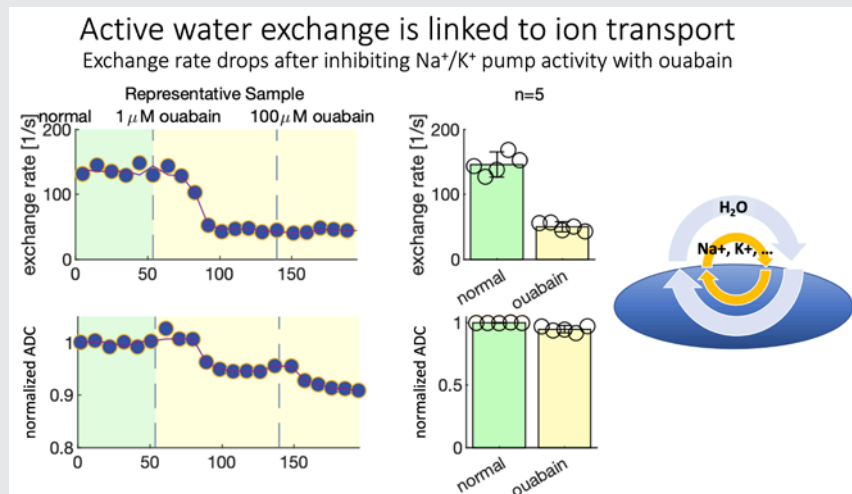
organized into fiber bundles that form a network that entraps the major proteoglycan (PG), a bottlebrush-shaped aggrecan. The biomechanical behavior of cartilage and other ECMs reflects their molecular composition and microstructure, which change during development, disease, degeneration, and aging. To determine tissue structure/function relationships, we measure various physical/chemical properties of ECM tissues and tissue analogs at different length and time scales, using a variety of complementary static and dynamic experimental techniques, e.g., osmometry, SANS, SAXS, neutron spin-echo (NSE), SLS, DLS, and AFM. Understanding the physical and chemical mechanisms affecting cartilage swelling (hydration) is essential to predicting its load-bearing ability, which is mainly governed by osmotic and electrostatic forces. To quantify the effect of hydration on cartilage properties, we previously developed a novel tissue micro-osmometer to perform precise and rapid measurements on small tissue samples (less than 1 microgram) as a function of the equilibrium water activity (vapor pressure). We also make osmotic pressure measurements to determine how the individual components of cartilage ECM (e.g., aggrecan and collagen) contribute to the total load-bearing capacity of the tissue. We also demonstrated that aggrecan-hyaluronic aggregates self-assemble into microgels, contributing to improved dimensional stability of the tissue and its lubricating ability. We further found that aggrecan is highly insensitive to changes in the ionic environment, particularly to divalent cations like calcium, which is critical for maintaining the tissue's mechanical integrity and allowing aggrecan to serve as a calcium-ion reservoir in cartilage and bone.

To model cartilage ECM, we invented and developed a new biomimetic composite material consisting of polyacrylic acid (PAA) microgel particles dispersed and embedded within a polyvinyl alcohol (PVA) gel matrix. PAA mimics the proteoglycan (i.e., hyaluronic-aggrecan complexes), while PVA mimics the fibrous collagen network entrapping them. Remarkably, the PVA/PAA biomimetic model system reproduces not only the shape of the cartilage swelling pressure curves, but also the numerical stiffness values reported for healthy and osteoarthritic human cartilage samples. Studies on these model composite hydrogels should continue to yield invaluable insights into the effects of various macromolecular factors (matrix stiffness, swelling pressure, fixed-charge density, etc.) on the tissue's macroscopic mechanical/swelling properties, and ultimately its remarkable load-bearing and lubricating abilities, and their loss in various diseases and disorders, including osteoarthritis.

We are now attempting to translate our understanding of the structure-function relationships of ECM components to develop and design novel non-invasive MRI methods, with the aim of inferring ECM composition, patency, and functional properties *in vivo*. Our goal is to use MRI for early diagnosis of diseases of cartilage and other tissue and organs to follow normal and abnormal ECM development, which entails making components of ECM (e.g., collagen and PGs) that are 'invisible' to MR 'visible' so as to predict the functional properties of the composite tissue, such as its load-bearing ability. An obstacle is that protons bound to immobile species (e.g., collagen) are largely invisible with conventional MRI methods. However, magnetization exchange (MEX) MRI (as well as other related methods) make it possible to detect the bound protons indirectly by transferring their magnetization to the abundant free water protons surrounding them. It also enables us to estimate collagen content in tissue quantitatively. In previous pilot studies with Uzi Eliav (deceased) and Ed Mertz, we applied the new MEX MRI method to determine the concentration and distribution of the main macromolecular constituents in bovine femoral-head cartilage samples. The results were qualitatively consistent with those obtained by histological techniques, such as high-definition infrared (HDIRI) spectroscopic imaging. Our novel approach has the potential to map tissue structure and functional properties *in vivo* and non-invasively. We are now developing molecular dynamics (MD)-based models of cartilage and cartilage ECM

FIGURE 6. Steady-state water exchange provides a window on cell metabolism.

We are now able to measure steady-state water exchange without using exogenous dyes or contrast agents, but simply by watching water move among distinct compartments. The exchange rate, which characterizes the intensity of water exchange, can be modified by using a number of physical and chemical perturbations. Ouabain is a channel blocker that can down-regulate the operation of the Na-K ATPase membrane pump, and surprisingly, this is accompanied by a reduction in water exchange, adding support to the notion that water accompanies ions exchanged across cell membranes and could potentially detect the rate of ion exchange as a proxy for cell activity.



analogs in order to interpret our experimental findings, develop and test novel hypotheses, and predict the behavior of our model system under different experimental conditions.

Recently, we have been employing several novel MR methodologies that use our one-sided NMR systems to study water relaxation, diffusion, and exchange processes in ECM as a means to characterize its functional properties. Most recently, Velencia Witherspoon has been using these approaches to study the organization and structure of fascia. Our specialized NMR profilers are ideally suited to these tasks, as they can probe layered media, such as cartilage and fascia, using ultra-thin slices, almost as thin as an optical microscope provides.

Patents

1. Horkay F., Bassar PJ. Composite gels and methods of use thereof, USPTO Patent number: 20200254144, August 13, 2020
2. Bassar PJ. MRI Tractography Based Transit Time Determination for Nerve Fibers. USPTO Patent number: 20210270921. Publication date: September 2, 2021

Additional Funding

- "In vivo Brain Network Latency Mapping." NIH BRAIN Initiative grant (no cost extension) 1-R24-MH-109068-01
- "Connectome 2.0: Developing the next generation human MRI scanner for bridging studies of the micro-, meso- and macro-connectome." NIH BRAIN Initiative-funded 1U01EB026996-01
- "Neuroradiology/Neuropathology Correlation/Integration Core." 309698-4.01-65310, (CNRM-89-9921)

Publications

1. Saleem KS, Avram AV, Glen D, Yen CC, Ye FQ, Komlos M, Bassar PJ. High-resolution mapping and

- digital atlas of subcortical regions in the macaque monkey based on matched MAP-MRI and histology. *Neuroimage* 2021 245:118759.
2. Avram AV, Saleem KS, Komlosh ME, Yen CC, Ye FQ, Basser PJ. High-resolution cortical MAP-MRI reveals areal borders and laminar substructures observed with histological staining. *Neuroimage* 2022 119653.
 3. Magdoom KN, Komlosh ME, Saleem K, Gasbarra D, Basser PJ. High resolution ex vivo diffusion tensor distribution MRI of neural tissue. *Front Phys* 2022 10:807000.
 4. Cai TX, Williamson NH, Ravin R, Basser PJ. Disentangling the effects of restriction and exchange with diffusion exchange spectroscopy. *Front Phys* 2022 23:805793.
 5. Benjamini D, Priemer DS, Perl DP, Brody DL, Basser PJ. Mapping astrogliosis in the individual human brain using multidimensional MRI. *Brain* 2022 awac298.
 6. Horkay F, Basser PJ. Hydrogel composite mimics biological tissues. *Soft Matter* 2022 18:4414–4426.

Collaborators

- Emiliios Dimitriadis, PhD, *Division of Bioengineering and Physical Science, NIBIB, Bethesda, MD*
- Jack Douglas, PhD, *NIST, Gaithersburg, MD*
- R. Douglas Fields, PhD, *Section on Nervous System Development and Plasticity, NICHD, Bethesda, MD*
- Raisa Freidlin, PhD, *Signal Processing and Instrumentation Section, CIT, NIH, Bethesda, MD*
- Dario Gasbarra, PhD, *University of Helsinki, Helsinki, Finland*
- Mark R. Gilbert, MD, *Neuro-Oncology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Mark Haacke, PhD, *Wayne State University School of Medicine, Detroit, MI*
- Mark Hallett, MD, PhD, *Human Motor Control Section, NINDS, Bethesda, MD*
- Iren Horkayne-Szakaly, MD, *Joint Pathology Center, Armed Forces Institute of Pathology, Washington, DC*
- Beth Hutchinson, PhD, *University of Arizona, Tucson, AZ*
- Sara Inati, MD, *Electroencephalography (EEG) Section, NINDS, Bethesda, MD*
- Edward L. Mertz, PhD, *Section on Physical Biochemistry, NICHD, Bethesda, MD*
- Michael O'Donovan, MD, PhD, *Developmental Neurobiology Section, NINDS, Bethesda, MD*
- Evren Özarlan, PhD, *Linköping University, Linköping, Sweden*
- Sinisa Pajevic, PhD, *Section on Critical Brain Dynamics, NIMH, Bethesda, MD*
- Daniel Perl, MD, *Uniformed Services University of the Health Sciences, Bethesda, MD*
- Carlo Pierpaoli, MD, PhD, *Section on Quantitative Medical Imaging, NIBIB, Bethesda, MD*
- Dietmar Plenz, PhD, *Section on Critical Brain Dynamics, NIMH, Bethesda, MD*
- Tom Pohida, MS, *Signal Processing and Instrumentation Section, CIT, NIH, Bethesda, MD*
- Randall Pursley, MS, *Signal Processing and Instrumentation Section, CIT, NIH, Bethesda, MD*
- Roberto Romero, MD, D(Med)Sci, *Perinatology Research Branch, NICHD, Detroit, MI*
- Joelle Sarlls, PhD, *In Vivo NMR Center, NINDS, Bethesda, MD*
- Brain Development Cooperative Group, *Various*

Contact

For more information, email peter.basser@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/basser>.

Biophysics of Large Membrane Channels

Our section studies the mitochondrial and bacterial membrane proteins that form “large” beta-barrel channels, which are the gateways of metabolite exchange and components of many toxins recognized as novel drug targets. Currently, we mainly focus on the metabolite-transporting channel VDAC of the outer mitochondrial membrane, but other beta-barrel channels such as MspA (major outer-membrane porin from *Mycobacterium smegmatis*), alpha-hemolysin (toxin from *Staphylococcus aureus*), OmpF (general bacterial porin from *Escherichia coli*), LamB (sugar-specific bacterial porin from *Escherichia coli*), OprF (porin from *Pseudomonas aeruginosa*), translocation pores of *B. anthracis* (PA63), and *C. botulinum* (C2IIa) and *C. perfringens* (Ib) binary toxins are also within our interest. Despite having drastically different biological functions, these channels have much in common regarding their biophysical characteristics, so that comparative analysis gives a better understanding of the underlying physical principles. We also use gramicidin A (linear pentadecapeptide from *Bacillus brevis*) as a molecular sensor to probe the surface charge and mechanical properties of membranes.

We investigate the channels under precisely controlled conditions, first isolating the corresponding channel-forming proteins from their host organisms or refolding recombinant proteins from inclusion bodies, purifying, and then reconstituting them into planar lipid membranes for electrophysiological examination. Our emphasis on single-channel experiments allows us to study transport processes at a single-molecule level. The obtained findings pave the way for the rational design of new strategies and pharmacological approaches to effectively correct the deviant molecular interactions associated with pathology in disease and development.

Voltage-activated complexation of alpha-synuclein with beta-barrel channels and its inhibition as a potential therapeutic target for Parkinson’s disease treatment

Voltage-activated protein complexation is the process by which a transmembrane potential drives complex formation between a membrane-embedded channel and a soluble or membrane-peripheral target protein (Figure 1). Metabolite and calcium flux across



Sergey M. Bezrukov, PhD, DSci,
Head, Section on Molecular Transport

Tatiana K. Rostovtseva, PhD,
Associate Scientist

Motahareh Ghahari Larimi, PhD,
Visiting Fellow

Megha Rajendran, PhD, *Visiting Fellow*

Kaitlin Abrantes, BS, *Intramural Research Training Award Fellow*

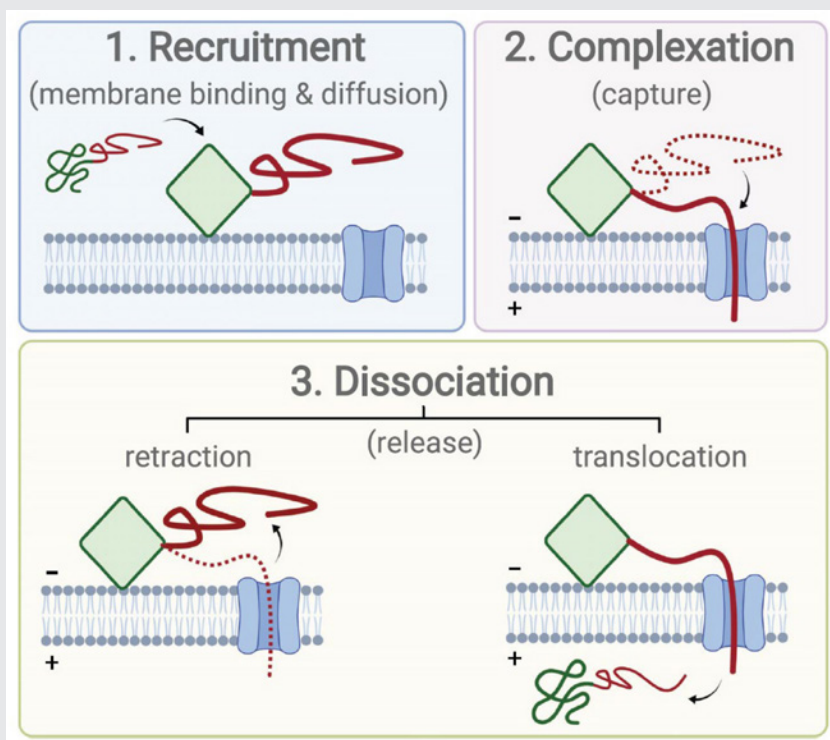


FIGURE 1. Voltage-activated complexation between a membrane-embedded channel and a peripheral protein

The peripheral protein is shown with two domains: a membrane-binding domain (*green*), which can be structured or not, and a polyanionic, disordered domain (*red*). Complexation begins with recruitment of the peripheral protein to the channel-containing membrane and diffusion along the membrane surface to the vicinity of the channel. Direct capture from solution is also possible, in which case the membrane-binding domain is not necessary, but is much less efficient because of the increased dimensionality of the diffusion space. The complex is formed when the polyanionic domain of the peripheral protein is captured into the channel by the transmembrane potential. Finally, the peripheral protein is released either by retraction from the channel or translocation of the entire (*unraveled*) polypeptide.

the mitochondrial outer membrane was shown to be regulated by voltage-activated complexation of the voltage-dependent anion channel (VDAC) and either dimeric tubulin or alpha-synuclein (α Syn). However, the roles played by the VDAC's characteristic attributes—its anion selectivity and voltage-gating behavior—have remained unclear. This past year, we conducted a comparative analysis of *in vitro* measurements of voltage-activated complexation of α Syn with three well-characterized beta-barrel channels, VDAC, MspA, and alpha-hemolysin, that differ widely in their organism of origin, structure, geometry, charge-density distribution, and voltage-gating behavior. The voltage dependences of the complexation dynamics for the different channels were observed to differ quantitatively but to have similar qualitative features. In each case, energy-landscape modeling describes the complexation dynamics in a manner consistent with the known properties of the individual channels, while voltage gating does not appear to play any role. The reaction free energy landscapes thus calculated reveal a common physical mechanism of complexation for all three channels, together with a non-trivial dependence of the complex stability on the surface density of α Syn.

It is well recognized that involvement of α Syn in Parkinson's disease (PD) is complicated and difficult to trace on cellular and molecular levels. Recently, we established that α Syn can regulate mitochondrial function by voltage-activated complexation with the VDAC, as described above. When complexed with α Syn, the VDAC pore is partially blocked, reducing the transport of ATP/ADP and other metabolites through the mitochondrial outer membrane. Further, α Syn can translocate into the mitochondria through the VDAC, where it interferes with mitochondrial respiration. Recruitment of α Syn to the VDAC-containing lipid membrane appears to be a crucial prerequisite for both the blockage and translocation processes. This year, we studied an inhibitory effect of HK2p, a small membrane-binding peptide from the mitochondria-targeting N-terminus of hexokinase

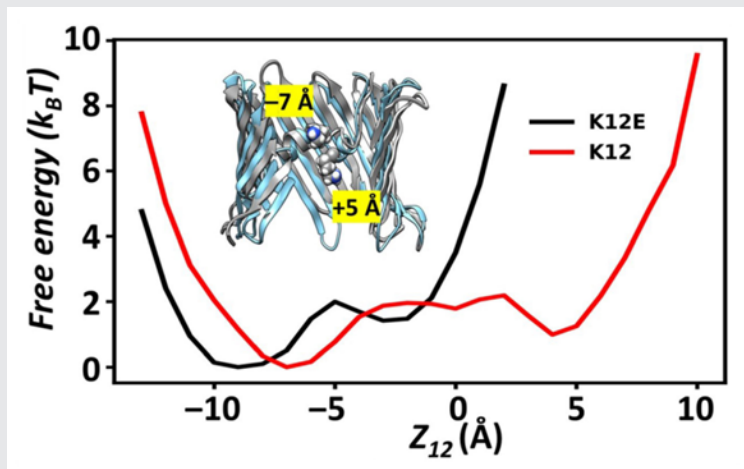


FIGURE 2. Atomistic MD simulations of the VDAC

1D Free Energy Profile of residue 12 in the wild-type (K12) or mutant (K12E) VDAC along the Z-axis of the channel. The coordinate z_{12} denotes the z-coordinates of the nitrogen atom of K12 or the carbon atom of the carboxylate group of E12, which are used to compute free-energy profiles, $F = -\ln[p(z_{12})]$, where $p(z_{12})$ is the probability density. Unlike the two stable positions (*inset graphic*) in the wild-type (WT), which occur over a broad range of z_{12} , the potential minima in the mutant are restricted to $z_{12} < 0$.

2, on α Syn membrane binding, and hence on α Syn complex formation with the VDAC and translocation through it. In electrophysiology experiments, the addition of HK2p at micromolar concentrations to the same side of the membrane as α Syn results in a dramatic reduction in the frequency of blockage events in a concentration-dependent manner, reporting on complexation inhibition. Using two complementary methods of measuring protein-membrane binding, bilayer overtone analysis and fluorescence-correlation spectroscopy, we found that HK2p induces detachment of α Syn from lipid membranes. Experiments with HeLa cells, using a proximity ligation assay, confirmed that HK2p impedes α Syn entry into mitochondria. Our results demonstrate that it is possible to regulate α Syn-VDAC complexation by a rationally designed peptide, thus suggesting new avenues in the search for peptide therapeutics to alleviate α Syn mitochondrial toxicity in PD and other synucleinopathies.

The single residue K12 governs the exceptional voltage sensitivity of VDAC gating.

The VDAC is the most abundant protein in the mitochondrial outer membrane (MOM) and is the primary conduit for ions and water-soluble metabolites, such as ATP and ADP, to cross the MOM. As such, the VDAC plays a central role in the regulation of MOM permeability and mitochondrial metabolism, and in communication between mitochondria and the rest of the cell. The VDAC responds to a transmembrane potential by “gating,” i.e., transitioning to one of a variety of low-conducting states of unknown structure. The gated state results in nearly complete suppression of multivalent mitochondrial metabolite (such as ATP and ADP) transport, while enhancing calcium transport. Voltage gating is a common property of beta-barrel channels and has been observed in bacterial outer-membrane porins as well as in anthrax, aerolysin, and alpha-hemolysin toxins, but VDAC gating is anomalously sensitive to transmembrane potential. This past year, we showed that a single residue in the pore interior, K12, is responsible for most of the VDAC’s voltage sensitivity.

Using the analysis of over 40 microseconds of atomistic molecular dynamics (MD) simulations, we explored correlations between motions of charged residues inside the VDAC pore and geometric deformations of the beta-barrel. Residue K12 is bistable (Figure 2, red curve); its motions between two widely separated positions

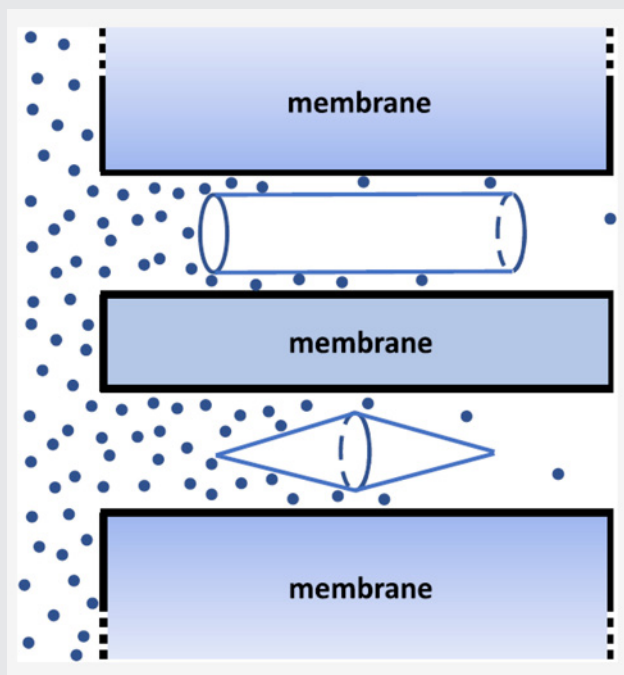


FIGURE 3. Blocker-induced increase in channel diffusion resistance

Schematic illustration of molecular flux through a cylindrical channel with a blocker of an abruptly varying radius (*cylinder, top*) and with a blocker of a smoothly varying radius (*two cones connected by their bases, bottom*).

along the pore axis enhance the fluctuations of the beta-barrel and augment the likelihood of gating. Single-channel electrophysiology of various K12 mutants reveals a dramatic reduction in voltage-induced gating transitions. The crystal structure of the K12E mutant at a resolution of 2.6 Å indicates a similar architecture of the K12E mutant to the wild type; however, 60 microseconds of atomistic MD simulations using the K12E mutant showed restricted motion of residue 12 (Figure 2, black curve), resulting from enhanced connectivity with neighboring residues, and diminished amplitude of barrel motions. We thus conclude that beta-barrel fluctuations, governed particularly by residue K12, drive VDAC gating transitions.

Intrinsic diffusion resistance of a membrane channel, mean first-passage times between its ends, and equilibrium unidirectional fluxes

Diffusion resistance is an important characteristic of channel-facilitated membrane transport and is widely used in

chemical engineering, electrochemistry, and cell biophysics. It is a diffusion analog of the electrical resistance, relating the steady-state diffusive flux of solute molecules through a membrane channel with the driving force of the transport process: the solute concentration difference in the two reservoirs separated by the membrane. We derived analytical expressions for the diffusion resistance in the case of a cylindrically symmetric blocker, whose axis coincides with the axis of a cylindrical nanopore in two limiting cases, i.e., where the blocker radius changes either smoothly or abruptly (Figure 3). Comparison of our theoretical predictions with the results obtained from Brownian dynamics simulations shows good agreement between the two. We also established a general relation between the channel diffusion resistance and the mean first-passage times of the solute molecules between the channel openings. Specifically, we showed that this direction-independent characteristic of transport is equal to the sum of the direction-dependent mean first-passage times divided by the molecule partition function in the channel. Our analysis is based on consideration of the equilibrium unidirectional fluxes flowing through the channel in opposite directions. The approach is quite general in the sense that it does not apply only to any specific model of the channel and is, therefore, universally applicable to transport in channels of arbitrary shape and tortuosity, at arbitrary interaction strength of solute molecules with the channel walls. This result promises to be of great value in computing the intrinsic diffusion resistance of the channel numerically, as it allows researchers to avoid dealing with multiple problems in analyzing transport under non-equilibrium conditions.

Publications

1. Hoogerheide DP, Rostovtseva TK, Bezrukov, SM. Voltage-activated complexation of α -synuclein with three diverse β -barrel channels: VDAC, MspA, and α -Hemolysin. *Proteomics* 2021 2100060.
2. Rajendran M, Queralt-Martín M, Gurnev PA, Rosencrans WM, Rovini A, Jacobs D, Abrantes K, Hoogerheide DP, Bezrukov SM, Rostovtseva TK. Restricting α -synuclein transport into mitochondria by inhibition of α -synuclein–VDAC complexation as a potential therapeutic target for Parkinson's disease treatment. *Cell Mol Life Sci* 2022 79:368.
3. Ngo VA, Queralt-Martín M, Khan F, Bergdoll L, Abramson J, Bezrukov SM, Rostovtseva TK, Hoogerheide DP, Noskov SY. The single residue K12 governs the exceptional voltage sensitivity of mitochondrial voltage-dependent anion channel gating. *J Amer Chem Soc* 2022 144:14564–14577.
4. Berezhkovskii AM, Bezrukov SM. Intrinsic diffusion resistance of a membrane channel, mean first-passage times between its ends, and equilibrium unidirectional fluxes. *J Chem Phys* 2022 156:071103.
5. Dagdug L, Skvortsov AT, Berezhkovskii AM, Bezrukov SM. Blocker effect on diffusion resistance of a membrane channel. Dependence on the blocker geometry. *J Phys Chem B* 2022 126:6016–6025.

Collaborators

- Vicente M. Aguilera, PhD, *Universidad Jaume I, Castellón, Spain*
- Alexandra Beilina, PhD, *Cell Biology & Gene Expression Section, NIA, Bethesda, MD*
- Alexander M. Berezhkovskii, PhD, *Division of Computational Bioscience, CIT, NIH, Bethesda, MD*
- Mark R. Cookson, PhD, *Laboratory of Neurogenetics, NIA, Bethesda, MD*
- Leonardo Dagdug, PhD, *Universidad Autónoma Metropolitana-Iztapalapa, Mexico City, Mexico*
- David Hoogerheide, PhD, *National Institute of Standards and Technology, Gaithersburg, MD*
- Jennifer C. Lee, PhD, *Biochemistry and Biophysics Center, NHLBI, Bethesda, MD*
- Ekaterina M. Nestorovich, PhD, *The Catholic University of America, Washington, DC*
- Sergei Y. Noskov, PhD, *University of Calgary, Calgary, Canada*
- Olga Protchenko, PhD, *Liver Diseases Branch, NIDDK, Bethesda, MD*
- Alexander Sodt, PhD, *Unit on Membrane Chemical Physics, NICHD, Bethesda, MD*
- Gerhard Wagner, PhD, *Harvard Medical School, Cambridge, MA*
- Michael Weinrich, MD, *Office of the Director, NICHD, Bethesda, MD*
- David L. Worcester, PhD, *National Institute of Standards and Technology, Gaithersburg, MD*
- Joshua Zimmerberg, MD, PhD, *Section on Integrative Biophysics, NICHD, Bethesda, MD*

Contact

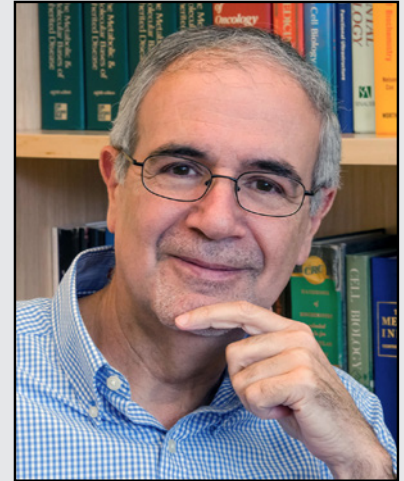
For more information, email bezrukos@mail.nih.gov or visit <http://smt.nichd.nih.gov>.

Protein Sorting in the Endomembrane System

Our laboratory investigates the molecular mechanisms by which transmembrane proteins (referred to as cargo) are sorted to different compartments of the endomembrane system in eukaryotic cells. The system comprises an array of membrane-enclosed organelles including the endoplasmic reticulum (ER), the Golgi apparatus, the trans-Golgi network (TGN), endosomes, lysosomes, lysosome-related organelles (LROs) (e.g., melanosomes, cytotoxic granules), and different domains of the plasma membrane in polarized cells such as epithelial cells and neurons (Figure 1). Transport of cargo between these compartments is mediated by vesicular or tubular carriers that bud from a donor compartment, translocate through the cytoplasm, and fuse with an acceptor compartment. Work in our laboratory focuses on the molecular machineries that mediate these processes, including (1) sorting signals and adaptor proteins that select cargo for packaging into transport carriers (Figure 1), (2) microtubule motors and organelle adaptors that drive movement of transport carriers and other organelles through the cytoplasm, and (3) tethering factors that promote fusion of transport carriers to acceptor compartments. We study these machineries in the context of different intracellular transport pathways, including endocytosis, recycling from endosomes to the plasma membrane, retrograde transport from endosomes to the TGN, biogenesis of lysosomes and LROs, autophagy, and polarized sorting in epithelial cells and neurons. Knowledge gained from this fundamental research is applied to the elucidation of disease mechanisms, including congenital disorders of protein traffic, such as the pigmentation and bleeding disorder Hermansky-Pudlak syndrome (HPS), hereditary spastic paraplegias (HSPs), and other neurodevelopmental disorders.

The adaptor protein chaperone AAGAB promotes assembly of the AP-4 complex.

Adaptor protein 4 (AP-4) is a heterotetrameric complex, composed of epsilon, beta4, mu4, and sigma4 subunits, that mediates export of transmembrane cargos, including autophagy protein 9A (ATG9A), from the TGN towards pre-autophagosomal structures (Figure 1). AP-4 has received particular attention in recent years because mutations in any of its subunits cause a complicated form of hereditary spastic paraplegia referred to as “AP-4-deficiency syndrome.” This



Juan S. Bonifacino, PhD, Head,
Section on Intracellular Protein Trafficking

Rafael Mattera, PhD, *Staff Scientist*

Xiaolin Zhu, Nurse, *Technician*

Nireekshit Addanki Tirumala, PhD, *Visiting Fellow*

Raffaella De Pace, PhD, *Visiting Fellow*

Saikat Ghosh, PhD, *Visiting Fellow*

Morié Ishida, PhD, *Visiting Fellow*

Ganesh Shelke, PhD, *Visiting Fellow*

Adriana Golding, PhD, *Intramural Research Training Award Fellow*

Jennifer M. Kunselman, PhD, *Intramural Research Training Award Fellow*

Nicole Hsiao-Sánchez, BS, *Postbaccalaureate Fellow*

Bob Hsieh, BS, *Postbaccalaureate Fellow*

Robert Kaltenbach, BS, *Postbaccalaureate Fellow*

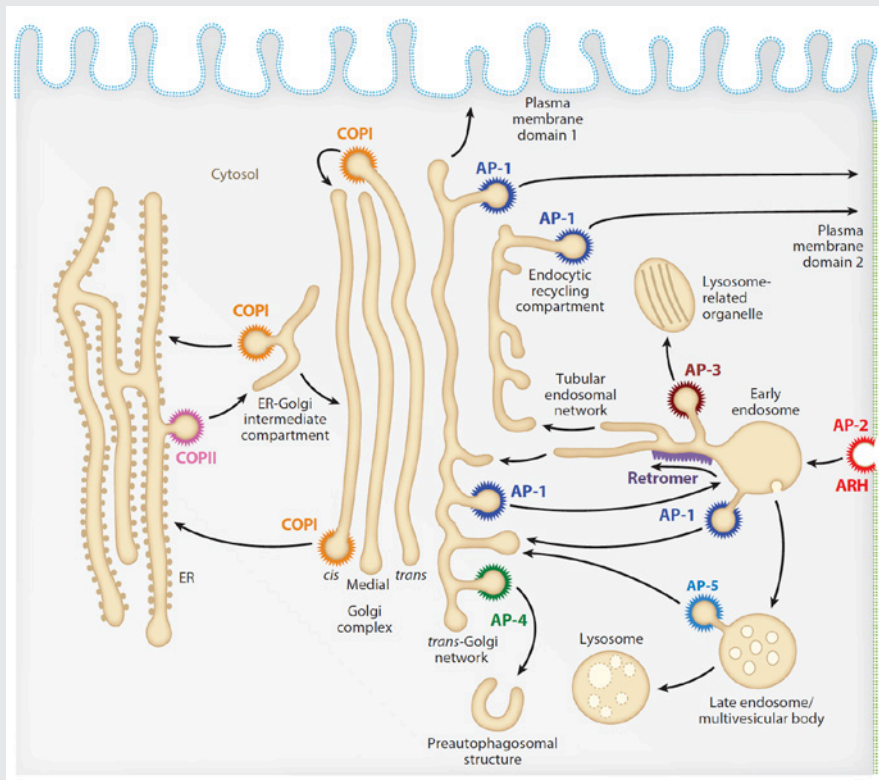


FIGURE 1. Schematic representation of the endomembrane system of eukaryotic cells showing the localization of coats involved in protein sorting

year, we reported that the alpha- and gamma-adaptin-binding protein (AAGAB, also known as p34) binds to and stabilizes AP-4 subunits, thus promoting complex assembly. The physiological importance of these interactions is underscored by the observation that AAGAB-knockout cells exhibit reduced levels of AP-4 subunits and accumulation of ATG9A at the TGN, like those in cells with mutations in AP-4-subunit genes. The findings demonstrated that AP-4 assembly is not spontaneous but AAGAB-assisted, further contributing to the understanding of an adaptor protein complex that is critically involved in the development of the central nervous system.

ATG9A transport to the cell periphery by RUSC2-mediated coupling to kinesin-1

ATG9A cycles between the TGN in the perinuclear area and pre-autophagosomal structures in the peripheral area of the cell. We recently found that the AP-4 accessory protein RUSC2 couples ATG9A-containing vesicles to the plus-end-directed microtubule motor kinesin-1 via an interaction between a disordered region of RUSC2 and the kinesin-1 light chain (KLC). The interaction is counteracted by the microtubule-associated WD40-repeat domain 47 protein (WDR47). The findings uncovered a mechanism for the peripheral distribution of ATG9A-containing vesicles that involves the function of RUSC2 as a kinesin-1 adaptor and WDR47 as a negative regulator of this function.

ATG9A enables lipid mobilization from lipid droplets.

ATG9A is a scramblase that flips phospholipids between the two membrane leaflets, thus contributing to the expansion of the autophagosome membrane. We found that depletion of ATG9A not only inhibits autophagy

but also increases the size and/or number of lipid droplets in human cell lines and in *C. elegans* (the latter work in collaboration with Andy Golden). Moreover, ATG9A depletion blocks transfer of fatty acids from lipid droplets to mitochondria and, consequently, utilization of fatty acids in mitochondrial respiration. The findings indicate that ATG9A plays a critical role in lipid mobilization from lipid droplets to autophagosomes and mitochondria, highlighting the importance of ATG9A in both autophagic and non-autophagic processes.

Autophagy-associated immune dysregulation in a patient with mutations in ATG9A

We also contributed to a study by Peter Williamson and Erwin Gelfand that concerns a patient with compound heterozygous mutations in ATG9A. The patient exhibited hyperplastic proliferations of T and B cells in lung and brain, and defects in lymphocyte memory-cell populations after developing an infection with the Epstein-Barr virus (EBV). The defects were corrected after treatment with the mTORC inhibitor rapamycin. The results point to a novel role of ATG9A and autophagy in lymphocyte biology and provide an example of how genetic studies may suggest effective specific therapeutic interventions.

SNX19 restricts endolysosome motility through contacts with the endoplasmic reticulum.

In addition to coupling to microtubule motors, interactions with other organelles also regulate the movement of endolysosomes within the cytoplasm. In this regard, we found that the sorting nexin protein SNX19 tethers endolysosomes to the endoplasmic reticulum (ER), reducing their motility and contributing to their concentration in the perinuclear area of the cell. Tethering depends on two *N*-terminal transmembrane domains that anchor SNX19 to the ER, and a PX domain that binds to phosphatidylinositol 3-phosphate on the endolysosomal membrane. The positioning and movement of endolysosomes within the cell is thus the result of a balance between movement driven by microtubule motors and immobilization by tethering to the ER.

RUFY3 is an ARL8 effector that couples endolysosomes to the microtubule motor dynein-dynactin.

The small GTPase ARL8 associates with endolysosomes, leading to the recruitment of several effectors that couple endolysosomes to kinesins for anterograde transport along microtubules, and to tethering factors for eventual fusion with other organelles. This past year, we identified RUFY3 as a novel ARL8 effector that couples endolysosomes to dynein-dynactin for retrograde transport along microtubules. This function of RUFY3 in retrograde transport contributes to the juxta-nuclear redistribution of endolysosomes upon cytosol alkalization. The findings highlight the role of ARL8 in the control of not only anterograde but also retrograde endolysosome transport.

Reduced axonal endolysosomal motility in a human iPSC-derived inducible neuronal model of NPC1 disease

Niemann-Pick disease, type C1 (NPC1) is a childhood-onset, lethal, neurodegenerative disorder caused by autosomal recessive mutations in the NPC1 gene, and is characterized by impaired cholesterol homeostasis. Mutations in NPC1 lead to deficient transport and accumulation of unesterified cholesterol in endolysosomal compartments and progressive neurodegeneration. This past year, we contributed to the characterization of a novel human iPSC (induced pluripotent stem cell)-derived, inducible neuronal model of NPC1 developed in the [laboratory of Forbes Porter](#). We found that cholesterol accumulation reduces the motility of axonal endolysosomes in such neurons, and that extraction of cholesterol with 2-hydroxypropyl-beta-cyclodextrin

remobilizes them. The findings shed light on the pathological mechanisms contributing to neuronal degeneration in NPC1.

Transcytosis and trans-synaptic retention by postsynaptic ErbB4 underlie axonal accumulation of NRG3.

This past year, we also collaborated with the [laboratory of Andrés Buonanno](#) to investigate the mechanisms by which Neuregulin 3 (NRG3) localizes to the axon. We found that pro-NRG3 undergoes proteolytic cleavage by the protease BACE1 at the TGN to generate mature NRG3. Mature NRG3 then emerges on the somatodendritic plasma membrane, from where it is re-endocytosed and anterogradely transported into axons by transcytosis. By a mechanism we call “trans-synaptic retention,” NRG3 then accumulates at presynaptic terminals by stable interaction with its receptor ErbB4 on postsynaptic GABAergic interneurons. We propose that trans-synaptic retention may account for the polarized expression of other neuronal transmembrane ligands and receptors.

PI4P and BLOC-1 remodel endosomal membranes into tubules.

In collaboration with the labs of Cédric Delevoye, Graça Raposo, Daniel Lévy, and colleagues, we found that phosphatidylinositol-4-phosphate (PI4P) and the biogenesis of lysosome-related organelles complex 1 (BLOC-1) govern the formation, stability, and functions of recycling endosomal tubules. BLOC-1 tubulates negatively charged membranes, including those containing PI4P produced by type II PI4-kinases. Reduced PI4-kinase expression impairs the recycling of endosomal cargoes and interferes with the life cycles of intracellular pathogens such as *Chlamydia* and influenza virus, which depend on endosomal recycling for replication.

Alpha-synuclein fibrils subvert lysosome structure and function for the propagation of protein misfolding between cells through tunneling nanotubes.

We collaborated with Chiara Zurzolo to demonstrate that alpha-synuclein fibrils affect the morphology of lysosomes and impair their function in neuronal cells. Furthermore, alpha-synuclein fibrils induce peripheral redistribution of lysosomes, increasing their transfer to neighboring cells through tunneling nanotubes. Lysosomal membrane permeabilization (LMP) allows the seeding of soluble alpha-synuclein for production of fibrils in lysosomes of acceptor cells. Lysosomes thus function as a Trojan horse for both seeding and propagation of disease pathology.

Publications

1. Keren-Kaplan T, Bonifacino JS. ARL8 relieves SKIP autoinhibition to enable coupling of lysosomes to kinesin-1. *Curr Biol* 2020 31:540–554.
2. Guardia CM, Jain A, Mattera R, Friefeld A, Li Y, Bonifacino JS. RUSC2 and WDR47 oppositely regulate kinesin-1-dependent distribution of ATG9A to the cell periphery. *Mol Biol Cell* 2021 32:ar25.
3. Saric A, Freeman SA, Williamson CD, Jarnik M, Guardia CM, Fernandopulle MS, Gershlick DC, Bonifacino JS. SNX19 restricts endolysosome motility through contacts with the endoplasmic reticulum. *Nat Commun* 2021 12:4552.
4. Mailler E, Guardia CM, Bai X, Jarnik M, Williamson CM, Li Y, Maio M, Golden A, Bonifacino JS. The autophagy protein ATG9A enables lipid mobilization from lipid droplets. *Nat Commun* 2021 12:6750.
5. Mattera R, De Pace R, Bonifacino JS. The adaptor protein chaperone AAGAB stabilizes AP-4 complex subunits. *Mol Biol Cell* 2022 33:ar109.

Collaborators

- Andrés L. Buonanno, PhD, *Section of Molecular Neurobiology, NICHD, Bethesda, MD*
- Cédric Delevoye, PhD, *Institut Curie, INSERM, Paris, France*
- Erwin W. Gelfand, MD, *National Jewish Medical and Research Center, Denver, CO*
- Andy Golden, PhD, *Laboratory of Biochemistry and Genetics, NIDDK, Bethesda, MD*
- Daniel Lévy, PhD, *Institut Curie, INSERM, Paris, France*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dymorphology, Clinical Director, NICHD, Bethesda, MD*
- Graça Raposo, PhD, *Institut Curie, INSERM, Paris, France*
- Peter R. Williamson, MD, PhD, *Laboratory of Clinical Immunology & Microbiology, NIAID, Bethesda, MD*
- Chiara Zurzolo, MD, PhD, *Institut Pasteur, Paris, France*

Contact

For more information, email bonifacinoj@helix.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/bonifacino>.

Neuregulin–ErbB and NMDA Receptor Signaling in Neuronal Development and Psychiatric Disorders

Failure of cortical microcircuits to properly regulate excitatory-inhibitory (E-I) balance is a key feature in the etiology of several developmental psychiatric disorders and neurological diseases, such as schizophrenia, autism, ADHD and epilepsy. E-I balance is important to synchronize the firing pattern of local neuron ensembles, and its dysregulation can degrade cognitive functions and, in extreme cases, result in epileptiform activity. Alterations in neuronal network activity, in particular oscillations in the gamma-frequency range (30–80 Hz), are associated with behavioral and cognitive deficits in psychiatric disorders. We have been investigating whether and how Neuregulins (NRG 1-3) and their major neuronal receptor ErbB4, which are genetically linked to psychiatric disorders, function in an activity-dependent fashion (i.e., experience) in the developing brain to regulate synaptic and neuronal network properties. We use genetically modified NRG and ErbB4 mouse models, in combination with optogenetic, electrophysiological, behavioral, and molecular/cellular techniques, to identify novel interactions between the NRG/ErbB4, glutamatergic, dopaminergic, and GABAergic signaling pathways associated with psychiatric disorders.

Our earlier studies demonstrated that NRG/ErbB4 signaling in GABAergic fast-spiking parvalbumin-positive (PV+) interneurons regulates E-I balance, gamma oscillation network activity, and numerous behaviors relevant to psychiatric disorders. To understand how NRGs mediate their biological functions during brain development, we investigated how different NRG ligands are proteolytically processed and trafficked in neurons, using molecular, cellular, and genetic approaches. NRGs are synthesized as unprocessed pro-proteins (proNRGs) containing either a single or two (dual) transmembrane (TM) domains. Contrary to dogma, we discovered that these two types of NRGs are processed and trafficked very differently. Single-TM NRGs are cleaved and shed from endoplasmic reticulum–plasma membrane (ER–PM) junctions on neuronal soma in an activity-dependent fashion in response to calcium entry through NMDA receptors (NMDAR), a ligand-gated channel that binds the excitatory neurotransmitter glutamate (Figure 1). By contrast, dual-TM NRGs are constitutively cleaved by the protease BACE1 in the trans-Golgi network, sorted into axons by transcytosis, and then selectively retained at presynaptic



Andrés Buonanno, PhD,
*Head, Section on Molecular
Neurobiology*

Detlef Vullhorst, PhD, *Staff
Scientist*

Eastman Lewis, PhD, *Postdoctoral
Intramural Research Training
Award Fellow*

Mara Bloom, BS,
Postbaccalaureate Fellow

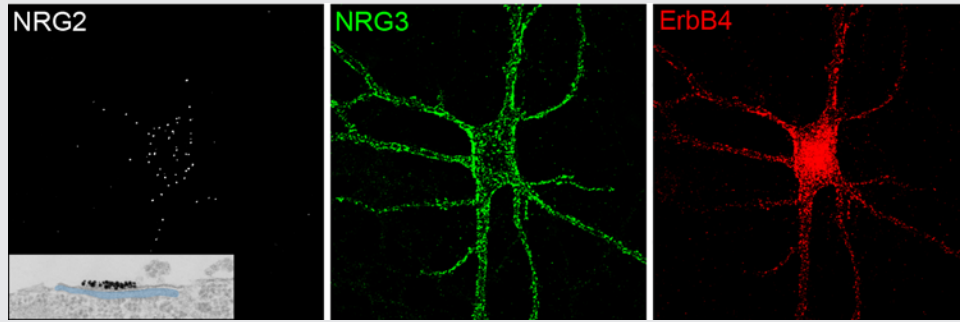
Hayli Spence, BS,
Postbaccalaureate Fellow

Jacqueline Welday, BS,
Postbaccalaureate Fellow

Ricardo Murphy, BS, *Contractor*

FIGURE 1. Distinct subcellular distribution of NRG2 and NRG3 in a cultured hippocampal ErbB4⁺ GABAergic interneuron

A dissociated hippocampal GABAergic interneuron showing pro-NRG2 surface puncta (*white*) clustered at ER-PM contacts and processed NRG3 at presynaptic terminals (*green*) that are juxtaposed to postsynaptic ErbB4 receptors (*red*). Note the restricted distribution of pro-NRG2 on the soma and proximal dendrites that correspond to clusters of NRG2 at ER-PM junctions, as confirmed by electron microscopy (*inset*). In stark contrast to NRG2, NRG3 and ErbB4 staining overlap extensively, consistent with their trans-synaptic interactions at excitatory synapses onto inhibitory interneurons (see also Figure 2).



glutamatergic terminals via juxtacrine trans-synaptic interactions with ErbB4 receptors on dendrites of GABAergic interneurons (Figure 1). The findings suggest that single-TM NRGs signal in paracrine mode at neuronal soma and proximal dendrites, whereas dual-pass TM NRGs signal in juxtacrine fashion from axons and presynaptic terminals by interacting with postsynaptic ErbB4 on GABAergic dendrites.

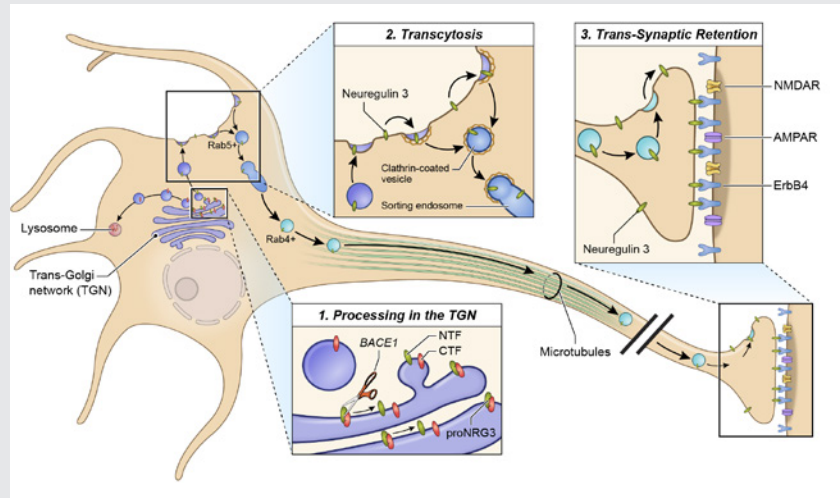
We investigated how NRG/ErbB4 signaling affects networks and behaviors associated with psychiatric disorders. The prefrontal cortex (PFC) is a site of convergence of long-range glutamatergic inputs that integrate multiple modalities of information to produce goal-directed behaviors. GABAergic PV⁺ interneurons coordinate pyramidal cell firing induced by this converging glutamatergic innervation to synchronize cortical networks that modulate goal-oriented behaviors that are disrupted in psychiatric disorders. Interestingly, drugs (i.e., ketamine) that inhibit NMDAR activity alter E-I balance, increase gamma oscillation network activity and disrupt behaviors associated with psychiatric disorders. Several lines of evidence suggest that NMDAR antagonists may disproportionately act on NMDARs expressed on PV⁺ interneurons; however, there has been significant controversy as to whether adult PV⁺ interneurons have functional NMDARs. Given the importance of PV⁺ interneurons for cortical functions, and our prior findings that NRG/ErbB4 signaling inhibits NMDAR activity in GABAergic interneurons, we embarked on an investigation into the proportion of PFC adult PV⁺ interneurons expressing functional NMDARs and their role in local network activity.

Presynaptic accumulation of NRG3 in central neurons is achieved by trans-synaptic retention, a novel mechanism for polarized axonal expression.

How stable axonal polarity is maintained remains a central question in neuroscience. We recently demonstrated that dual-TM proNRGs, comprising CRD-NRG1 type III and NRG3, are targeted to axons and accumulate at glutamatergic presynaptic terminals, where they signal in juxtacrine mode via postsynaptic ErbB4 receptors expressed at postsynaptic densities on GABAergic interneurons (Vullhorst, Ahmad *et al.*, *J Neurosci* 2017;37: 5232). In a new series of studies, we aimed to understand how and where proNRG3 is cleaved, and how its biologically active NRG3 peptide is sorted to and then retained in axons. To this end, we

FIGURE 2. Diagram summarizing the results of our NRG3 trafficking studies

The diagram depicts the different steps of pro-NGR3 processing that begin with its synthesis as a dual-TM protein in the endoplasmic reticulum, its cleavage, and sorting of the biologically active single-TM NRG3 ligand to axonal presynaptic terminals, where it is retained by virtue of its interaction with postsynaptic ErbB4 receptors. The three major steps of pro-NGR3 processing and axonal retention are:



1. Unprocessed dual-TM pro-NGR3 requires cleavage by the protease BACE1 to release the biologically active single-TM NRG3 from the *trans*-Golgi network (TGN).
2. NRG3 then traffics to axons by transcytosis, a process that involves initial transport of NRG3 to the somatodendritic plasma membrane, re-endocytosis into Rab5-positive early endosomes, sorting and anterograde axonal transport in Rab4-positive vesicles.
3. NRG3 is retained selectively at axonal terminals by virtue of its binding across the synapse to its cognate receptor ErbB4, which is expressed at glutamatergic postsynaptic densities on dendrites of GABAergic interneurons. We termed this mechanism, which is necessary for the polarized expression of NRG3 in axonal terminals, "trans-synaptic retention," and we propose that it accounts for the polarized expression of other neuronal transmembrane ligands and receptors in axons.

investigated the spatial-temporal dynamics of NRG3 processing and sorting in neurons using an optogenetic proNGR3 cleavage reporter (LA143-NGR3) that we had developed. In dark conditions, unprocessed LA143-NGR3 is retained in the trans-Golgi network (TGN) but, following blue-light photoactivation, it is cleaved by BACE1 and released from the TGN. We found that mature NRG3 initially emerges on the somatodendritic plasma membrane, from where it is re-endocytosed and anterogradely transported on GTPase Rab4-positive vesicles into axons via a process known as transcytosis. Interestingly, our work showed that NRG3 accumulation at axonal presynaptic terminals is mediated by interactions with ErbB4 receptors expressed by postsynaptic GABAergic interneurons. We then went on to demonstrate that the continuous interaction between the NRG3 EGF-like domain and its receptor ErbB4 is necessary for NRG3 retention at presynaptic sites, as either addition of a competing peptide or knock-down of ErbB4 from GABAergic neurons prevents its accumulation (Figure 2). We call this mechanism "trans-synaptic retention" and propose that it accounts for polarized expression of other neuronal transmembrane ligands and receptors [Reference 1].

ER-PM junctions on GABAergic interneurons are organized by neuregulin 2/VAP interactions and regulated by NMDA receptors.

Two major unresolved questions we recently pursued were to understand at a mechanistic level: (1) how proNGR2 clusters at ER-PM junctions; and (2) how proNGR2 dissociates from the junctions in response to NMDAR activation. We found that proNGR2 promotes the formation of ER-PM junctions in hippocampal GABAergic interneurons via interactions of its cytoplasmic tail with the ER-resident protein VAP. Interestingly, there are two stretches of amino acids in the intracellular cytoplasmic domain conserved between proNGR1

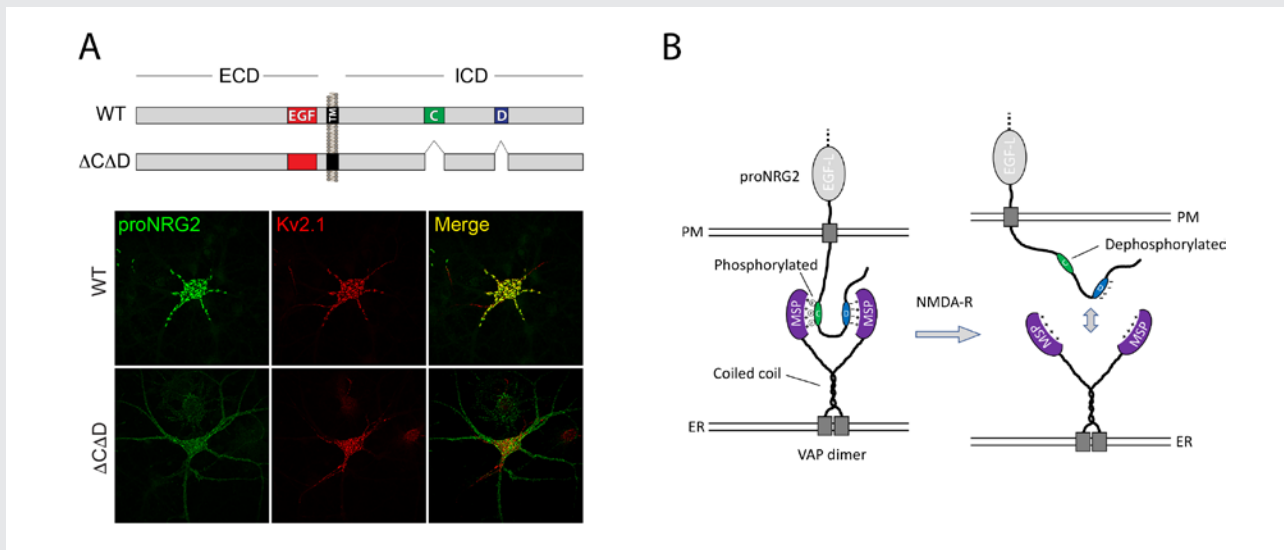


FIGURE 3. ProNGR2 clustering at ER-PM junctions is mediated by VAP binding and regulated by phosphorylation.

A. Cultured hippocampal neurons transduced with AAVs expressing wild-type proNGR2 (*top*) or proNGR2DCDD lacking the C- and D-boxes (*bottom*). WT NRG2 (*green*) forms large clusters at ER-PM junctions (revealed by Kv2.1 staining in *red*), which are located on the cell body and proximal dendrites. Removal of the VAP binding sites in proNGR2DCDD abolishes its accumulation at ER-PM junctions, causing it to broadly distribute throughout the neuronal plasma membrane.

B. Working model of proNGR2/VAP interactions. ProNGR2 cooperatively engages with a VAP dimer via its two low-affinity FFAT motifs in the C- and D-boxes. Interactions between the VAP major sperm protein (MSP) domain and the cryptic FFAT site in the C-box require Ser/Thr phosphorylation. Their dephosphorylation downstream of NMDAR activation promotes proNGR2 dissociation from VAP.

and proNGR2, denoted C- and D-boxes, that are required to stabilize proNGR2/VAP complexes during immunoprecipitation. Although the protein sequence of neither box conforms to known FFAT motifs, shown in other proteins to bind to VAP, the proNGR2 D-box contains a track of acidic residues required for VAP binding and the C-box harbors a cryptic, phosphorylation-dependent VAP binding site. Importantly, NMDAR activation stimulates dephosphorylation of Ser/Thr residues in the C-box and its dissociation from VAP, which reduces proNGR2 clustering at ER-PM junctions [Vullhorst *et al.*, under review]. These observations are interesting because, although both proNGR2 and the potassium channel Kv2.1 are colocalized at ER-PM junctions and clustering at these sites is regulated by NMDA receptor activity, their modes of interaction with VAP differ (Figure 3). Based on these findings, we hypothesize that autocrine NRG2/ErbB4 signaling and Kv2.1 function synergistically as a homeostatic protective mechanism to downregulate GABAergic interneuron excitability during periods of strong excitatory activity and/or elevated extracellular glutamate levels, which would help to protect these neurons from excitotoxicity.

A bidirectional mechanism that regulates NRG2 processing and NMDA receptor activity in GABAergic interneurons

Single-pass TM NRG1 type II and NRG2 accumulate as unprocessed proforms at ER-PM junctions on neuronal

soma and proximal dendrites. We recently demonstrated that calcium entry through NMDA receptors promotes dephosphorylation of serine/threonine residues in the proNRG2 intracellular region, which results in the dissociation of proNRG2 from ER-PM junctions, metalloproteinase ADAM10 cleavage, and the rapid release of the biologically active NRG2 ectodomain, which activates ErbB4 signaling [Vullhorst & Buonanno, *Mol Neurobiol* 2019;56:8345]. In turn, activation of ErbB4 receptors at excitatory postsynaptic densities of GABAergic interneurons selectively down-regulates NMDA receptor activity [Vullhorst *et al.*, *Nat Comm* 2015;6:7222]. We hypothesize that this bidirectional NMDAR-NRG2 (up)/ErbB4-NMDAR (down) signaling mode serves as a homeostatic mechanism that regulates the activity of GABAergic interneurons. Importantly, disruption of such a homeostatic mechanism would alter E-I balance and neuronal network activity, consequently affecting numerous psychiatric-relevant behaviors known to be altered in NRG2 and ErbB4 knockout mice [Yan *et al.* *Mol Psychiatry* 2018;23:1233; Skirzewski *et al.*, *Mol Psychiatry* 2018;23:2227; Reference 2].

NRG2 and ErbB4 knockout mice exhibit a dopamine imbalance and behavioral alterations relevant to psychiatric disorders.

NRG2 expression is more widespread than originally reported, extending to striatal and medial prefrontal cortical (mPFC) neurons. Unexpectedly, we found that, in contrast to GABAergic interneurons that express ErbB4 receptors on their soma and dendrites, mesencephalic dopamine (DA) neurons also express ErbB4 on their axons. To investigate the function of NRG2-ErbB4 signaling, we generated NRG2 and ErbB4 knockout (KO) mice. We found that NRG2 and ErbB4 KOs have higher extracellular DA levels in the dorsal striatum but lower levels in the mPFC and hippocampus, a pattern of DA imbalance that recapitulates the reported prefrontal cortical reduction and striatal increase of DA levels in schizophrenia patients. NRG2 and ErbB4 KO mice performed abnormally in a battery of behavioral tasks relevant to psychiatric disorders (Figure 4). They exhibit hyperactivity in a novelty-induced open field, deficits in pre-pulse inhibition, hypersensitivity to amphetamine, antisocial behaviors, reduced anxiety-like behavior in the elevated plus maze, and deficits in

FIGURE 4. Overlapping behavioral and neurochemical phenotypes in NRG2 and ErbB4 KO mice

Lack of either NRG2 or ErbB4 in genetically engineered mice elicits similar phenotypic alterations with relevance to psychiatric disorders, demonstrating that NRG2 is an important and non-redundant ErbB4 receptor ligand in the postnatal brain.

	NRG2^{-/-}	ErbB4^{-/-}
Open field (Hyperactivity)	increased	increased
Elevated Plus Maze (Anxiety)	reduced	reduced
Prepulse Inhibition (Sensorimotor gating)	reduced	reduced
T-Maze (Working Memory)	reduced	reduced
Amphetamine Sensitivity	increased	increased

the T-maze alteration reward test, a task dependent on hippocampal and mPFC function. In addition, ErbB4 KO mice exhibit reduced spatial learning and memory on the Barnes maze and perform markedly worse in conditioned place preference (CPP) tasks when associating cued-reward palatable food with location. However, we found that the poor performance of ErbB4 KOs in CPP likely results from deficits in spatial memory, instead of reward seeking, as ErbB4 KOs are more motivated to work for palatable food rewards [Reference 2]. Taken together, our work emphasizes the importance of NRG2–ErbB4 signaling in nigrostriatal, mesocortical, and mesolimbic DA systems, and it demonstrates that this signaling pathway regulates a wide array of behaviors relevant to psychiatric disorders, including schizophrenia.

Developmental, neurochemical, and behavioral analyses of ErbB4 Cyt-1 knockout mice

ErbB4 transcripts are alternatively spliced to generate isoforms that either include (Cyt-1) or exclude (Cyt-2) exon 26, an exon that encodes a cytoplasmic domain that imparts to ErbB4 receptors the ability to signal via the PI3K/Akt pathway rather than the MAPK pathway. To investigate the effects of germline (constitutive) and conditional (acute) deletions of the Cyt-1 exon, we generated and studied ErbB4–floxed (ErbB4-Cyt1^{fl/fl}) mice, because ErbB4 Cyt-1/2 isoforms had only been studied in cultured cells, and clinical genetic studies specifically implicated ErbB4 Cyt-1 variants as a risk factor for schizophrenia. We found that, overall, ErbB4 mRNA levels remain unchanged in germline ErbB4 Cyt-1 knockouts (Cyt-1 KOs), with all transcripts encoding Cyt-2 variants. In contrast to mice lacking all ErbB4 receptor function, GABAergic interneuron migration and number are unaltered in Cyt-1 KOs. However, basal extracellular dopamine (DA) levels in the medial prefrontal cortex are elevated in Cyt-1 heterozygotes. Despite these neurochemical changes, Cyt-1 heterozygous and homozygous mice do not manifest the behavioral abnormalities previously reported to be altered in ErbB4 null mice. To address the possibility that Cyt-2 variants compensate for the lack of Cyt-1 during development, we microinjected an adeno-associated virus expressing Cre-recombinase (AAV-Cre) into the DA-rich ventral tegmental area of adult ErbB4-Cyt1^{fl/fl} mice to acutely target exon 26. Such conditional Cyt-1 KOs were found to exhibit behavioral abnormalities in the elevated plus maze and startle response, consistent with the idea that late exon 26 ablations may circumvent compensation by Cyt-2 variants. Our findings suggest that ErbB4 Cyt-1 function *in vivo* is important for modulating DA levels and for regulating behaviors in adult mice [Reference 3].

Pathway-specific contribution of parvalbumin interneuron NMDARs to synaptic currents and thalamocortical feedforward inhibition

Despite the importance of understanding how glutamatergic inputs onto PV+ interneurons affect network activity, behavior, and disease, there continues to be controversy as to whether both AMPA and NMDA glutamate receptors or only AMPARs contribute to their excitatory drive. Using a combination of molecular, electrophysiological, and optogenetic approaches, in combination with selective gene-targeting techniques in PV+ interneurons, we resolved this long-standing controversy. We found that nearly 100% of PV+ interneurons in adult medial PFC express the NMDAR subunits GluN1 and GluN2B, and that they have functional NMDARs. With selective optogenetic stimulation of corticocortical or thalamocortical inputs onto PV+ interneurons in the PFC, we found that the relative synaptic NMDAR contribution to excitatory post-synaptic currents is pathway-specific, with NMDARs contributing more at thalamocortical synapses. The pathway-specific contribution of NMDARs to PV+ interneuron excitation is likely to explain earlier conflicting reports suggesting the absence of functional synaptic NMDARs in this GABAergic interneuron subtype in the adult PFC. We then went on to determine whether NMDAR currents in PV+ interneurons contribute significantly to PFC neuronal network activity. Indeed, we found that PV+ interneuron NMDARs contribute to thalamus-mediated feedforward

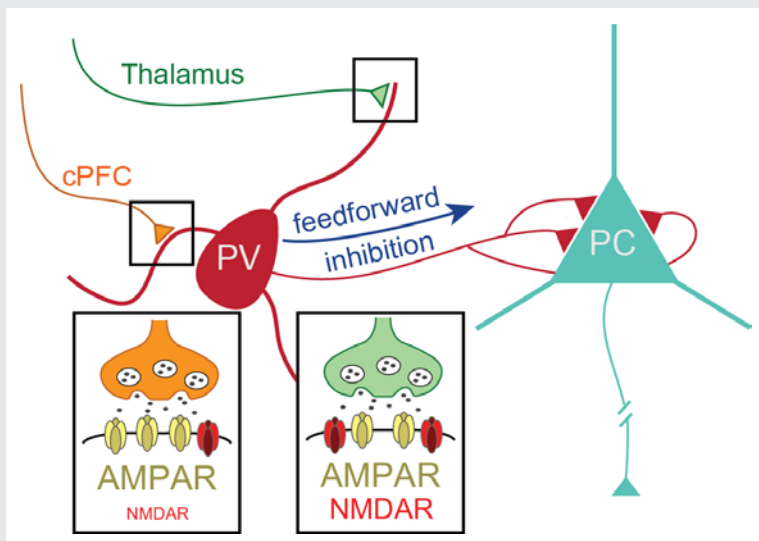


FIGURE 5. Pathway-specific contribution of PV+ interneuron NMDARs to synaptic currents and thalamocortical feedforward inhibition

Inhibitory fast-spiking GABAergic PV+ interneurons (*red*) in adult mouse PFC receive excitatory glutamatergic inputs from the contralateral PFC (cPFC; *orange*) and the ipsilateral thalamus (*green*). Optogenetic stimulation of either cPFC or thalamocortical inputs onto PV+ interneurons indicate that NMDARs contribute more to the size and shape of excitatory synaptic currents at thalamic than at cPFC synapses. By selectively knocking out NMDAR expression in adult inhibitory PV+ interneurons, we found that expression of these receptors at thalamic synapses of PFC PV+ interneurons are required for feedforward inhibition of pyramidal cells (PC).

inhibition in PFC circuits, suggesting molecular and circuit-based mechanisms for cognitive impairment under conditions of reduced NMDAR function (Figure 5). The findings represent an important conceptual advance, which has major implications for understanding the pathogenesis of psychiatric disorders [Reference 5].

Additional Funding

- NICHD DIR Director's Investigator Award (RRC# D-14-07). PI: Andres Buonanno; Co-PI: Juan Bonifacino. "Exploring the functional role of Neuregulin isoform diversity in CNS"
- Center for Compulsive Behaviors Fellowship Award

Publications

1. Ahmad T, Vullhorst D, Chaudhuri R, Guardia CM, Chaudhary N, Karavanova I, Bonifacino JS, Buonanno A. Transcytosis and trans-synaptic retention by postsynaptic ErbB4 underlie axonal accumulation of NRG3. *J Cell Biol* 2022 221:e202110167.
2. Skirzewski M, Cronin ME, Murphy R, Fobbs W, Kravitz AV, Buonanno A. ErbB4 null mice display altered mesocorticolimbic and nigrostriatal dopamine levels as well as deficits in cognitive and motivational behaviors. *eNeuro* 2020 7(3):0395–19.2020.
3. Erben L, Welday JP, Cronin ME, Murphy R, Skirzewski M, Vullhorst D, Carroll SL, Buonanno A. Developmental, neurochemical, and behavioral analyses of ErbB4 Cyt-1 knockout mice. *J Neurochem* 2022 161:435–452.
4. Erben L, Welday JP, Murphy R, Buonanno A. Toxic and phenotypic effects of AAV_Cre used to transduce mesencephalic dopaminergic neurons. *Int J Mol Sci* 2022 23:9462.
5. Lewis EM, Spence HE, Akella N, Buonanno A. Pathway-specific contribution of parvalbumin interneuron NMDARs to synaptic currents and thalamocortical feedforward inhibition. *Mol Psychiatry* 2022 27: 5124–5134.

Collaborators

- Tanveer Ahmad, PhD, *Multidisciplinary Centre for Advance Research and Studies, Jamia Millia Islamia, New Delhi, India*
- Juan Bonifacino, PhD, *Section on Intracellular Trafficking, NICHD, Bethesda, MD*
- Steve Carroll, MD, PhD, *Medical University of South Carolina, Charleston, SC*
- Carlos Guardia, PhD, *Section on Intracellular Trafficking, NICHD, Bethesda, MD*
- Lex Kravitz, PhD, *Washington University Medical School in St. Louis, St. Louis, MO*
- Miguel Skirzewski, PhD, *University of Western Ontario, London, Canada*
- Jung-Hwa Tao-Cheng, PhD, *EM Facility, NINDS, Bethesda, MD*

Contact

For more information, email buonanno@mail.nih.gov or visit <http://smn.nichd.nih.gov>.

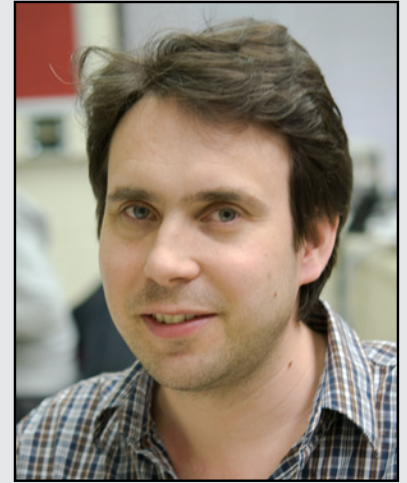
Neuronal Circuits Controlling Behavior: Genetic Analysis in Zebrafish

The Section on Behavioral Neurogenetics studies how, under diverse environmental contexts, the nervous system selects appropriate behavioral responses to sensory information in a way that best satisfies internal motivational objectives. We use the larval zebrafish as a model because its brain exhibits the basic architecture of the vertebrate brain but is much less complex than the mammalian brain. Despite the relative simplicity of their nervous system, zebrafish have a sophisticated repertoire of sensory-guided and internally driven behaviors. Furthermore, the optical clarity of the embryo facilitates visualization of individual neurons and their manipulation with genetic techniques. Behavior in larvae is innate and thus exhibits minimal variability between fish. Subtle alterations in behavior can therefore be robustly measured, making it possible to quickly assess the contribution of identified neurons to a variety of motor behaviors.

We focus on two aspects of behavioral regulation: the neuronal mechanisms by which sensory context regulates behavioral decisions and the pathways that sustain changes in behavioral state. Neuronal connections that allow the brain to integrate sensory and internal-state information are established through genetic interactions during development, and are frequently disrupted by gene mutations associated with neuro-developmental disorders. We can therefore use discoveries about sensorimotor integration pathways to understand how human disease genes disrupt brain development. To support these objectives, we develop new genetic tools and behavioral assays to probe the nexus between neuronal function and behavior at single-cell resolution.

Neuronal pathways for auditory sensory processing

Startle responses are rapid reflexes that are triggered by sudden sensory stimuli, and which help animals defend against, or escape from, potentially threatening stimuli. In both fish and mammals, startle responses are initiated by giant reticulospinal neurons in the medulla, which receive short-latency sensory input from diverse sensory modalities. Although highly stereotyped, startle responses are nevertheless modulated by sensory context and behavioral state and are therefore an excellent system in which to understand how



Harold Burgess, PhD, Head,
Section on Behavioral Neurogenetics

Tripti Gupta, PhD, *Staff Scientist*

Ashwin A. Bhandiwad, PhD,
Postdoctoral Fellow

Jennifer Panlilio, PhD, *Postdoctoral Fellow*

Svetlana Semenova, PhD, *Visiting Fellow*

Reid Doctor, BSc, *Graduate Student*

Daniel Bazan, BSc,
Postbaccalaureate Fellow

Grace Biddle, BSc,
Postbaccalaureate Fellow

George Holmes, BSc,
Postbaccalaureate Fellow

Katelyn Robertson, *Special Volunteer*

Jennifer L. Sinclair, MSc, *Zebrafish Technician*

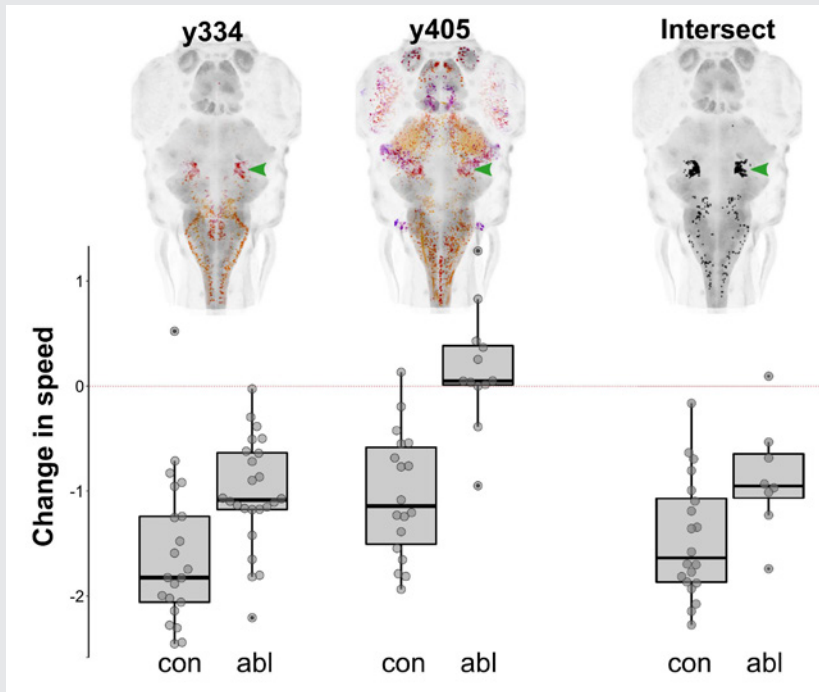


FIGURE 1. Prepontine tegmental neurons mediate tonic immobility in zebrafish.

Depth-coded projections of Gal4 expression in two lines (y334 and y405) that label neurons whose ablation interferes with the reduction in spontaneous swimming in response to an intense auditory stimulus (*bar graphs*). The computational intersectional expression of y334 and y405 is shown, revealing a common cluster of neurons in the preoptine tegmentum (*green arrowhead*). Laser ablation of the preoptine cluster diminishes immobility in response to an intensity stimulus.

such information is integrated for behavioral choice. In mammals, including humans, the startle response to a strong auditory stimulus can be inhibited by pre-exposure to a weak acoustic ‘prepulse,’ a form of startle modulation, termed prepulse inhibition, that is diminished in several neurological conditions. Previously, we showed that in zebrafish, as in mammals, several distinct cellular mechanisms mediate prepulse inhibition, depending on the time interval between the prepulse and the startle stimulus, with NMDA–receptor signaling playing a key role for intervals greater than 100 ms. Our work on resolving the core neuronal pathway that mediates prepulse inhibition provides a basis for probing how gene mutations linked to neuro-developmental disorders disrupt sensory processing. NMDA–receptor mutations have been linked to both autism-spectrum disorders and schizophrenia. Working collaboratively with the Sirotkin laboratory, we demonstrated that mutations in the NMDA–receptor subunit *grin2B* disrupt neural development [Reference 1].

Neural mechanisms for behavioral–state control

Over the course of the day, motivational goals change in response to both internal and external cues. At any given moment, an individual’s behavioral state strongly influences decisions on how to interact with the environment. A major goal in neuroscience is to identify the neural systems that maintain short-term behavioral states and to determine how they interact with central mechanisms for behavioral choice.

While studying defensive responses to auditory stimuli, we noted that cues of about an order of magnitude larger than those needed to provoke startle responses drove an unexpected freezing behavior, especially when repeatedly presented over a short period of time. In many species, overwhelming stimuli elicit a behavior known as tonic immobility, an ultimate response to inescapable threat. In humans, the experience of tonic immobility correlates with development of the post-traumatic stress disorder, yet very little is known about its underlying neural basis. Leveraging our library of Gal4 (a tool to modulate gene activity)–transgenic

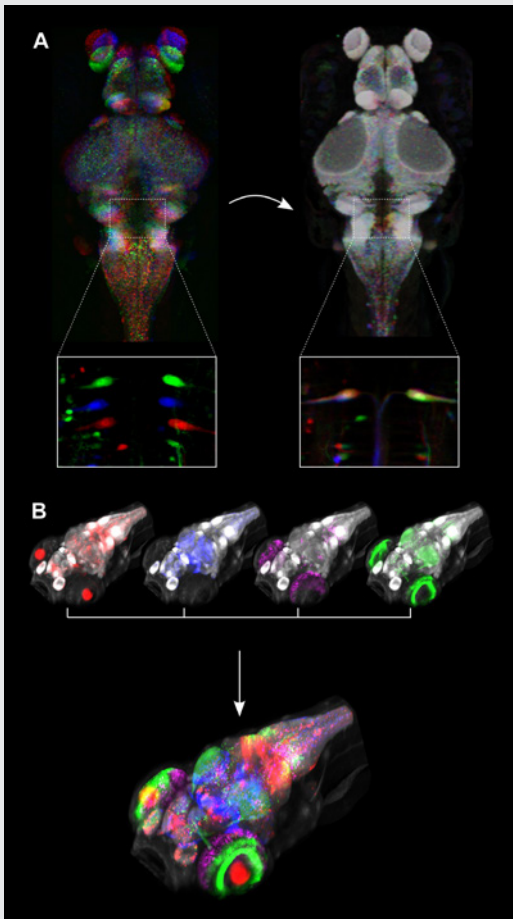


FIGURE 2. Whole brain registration in zebrafish

A. Brain registration corrects for both technical variability during imaging and biological variability between samples. Three larval zebrafish brains, pseudocolored red, green and blue, were imaged. When superimposed, it is apparent that they do not precisely align. Following registration, the same three brains are closely coordinated, even to the extent that the Mauthner cells, an identifiable bilateral cell in the hindbrain of each brain, become closely matched.

B. Brain registration allows transgene expression patterns from different individuals to be mapped onto the same reference brain coordinate system, so that they can be compared in three dimensions. In this example, four transgenic lines (*pseudocolored*) were separately imaged then, following registration, simultaneously visualized.

lines, we performed a screen to discover neurons that are required for such behavior in zebrafish. We isolated a cluster of neurons in the prepontine tegmentum that are necessary for sustained immobility after an intense auditory stimulus (Figure 1). By manually isolating and performing RNA-Seq on these neurons, we found that they express several stress-associated neuropeptides, including markers that make them likely homologs of part of the mammalian parabrachial complex, an area recently implicated in responses to noxious stimuli. Our screen also demonstrated a central role for cerebellar signaling in tonic immobility, and we found a direct projection from Purkinje neurons to the prepontine neurons, similar to recent work showing that a subset of Purkinje neurons in mammals also directly project to the parabrachial nucleus. The study identified, for the first time, a cellular pathway that mediates tonic immobility and suggests that the parabrachial has a deep evolutionary history in mediating defensive behavior [Reference 2].

Zebrafish models of neuro-developmental disorders

We collaborate widely with clinicians to generate and characterize zebrafish models for mutations discovered in humans (often through exome-sequencing) that are likely to have a neuro-developmental origin. We use the CRISPR/Cas9 system to generate lesions in zebrafish genes that are homologous to those disrupted in disorders. We then apply behavioral analysis, transcriptomics, and voxel-based morphometry as part of a broad phenotyping strategy. To promote rigorous use of zebrafish neurological disease models, we wrote a critical review outlining advantages and limitations of the zebrafish system [Reference 3 & 4].

Our work with brain morphometry arose from earlier studies in which we generated several hundred new Gal4 and Cre lines in order to provide genetic accessibility to neurons of interest. A unique feature of brain imaging in zebrafish is the ability to visualize the total architecture of the brain while

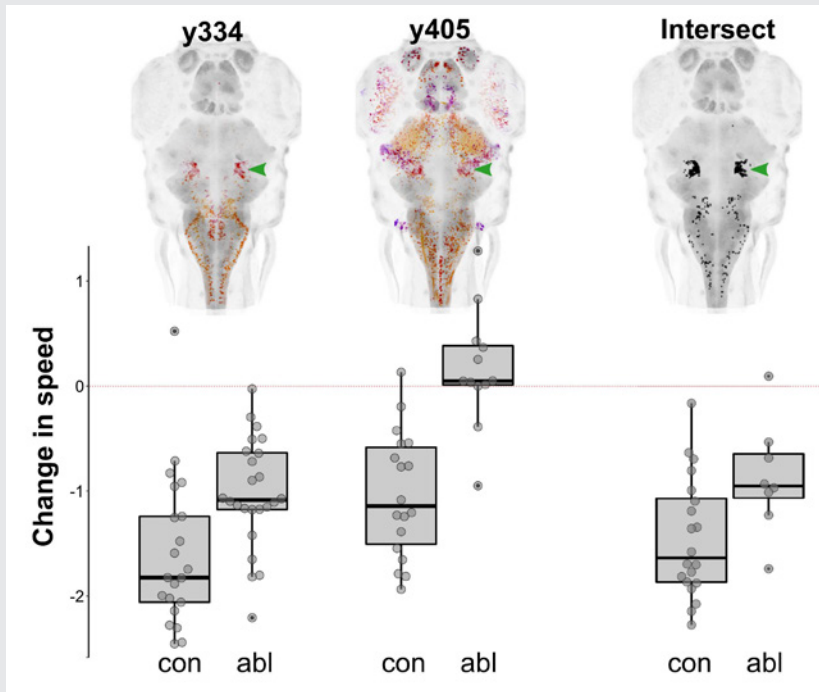


FIGURE 3. Pipeline for brain morphometry in zebrafish

A. Control and mutant brains are scanned and co-registered.

B. We calculate p-value maps of intensity and size differences at each voxel.

C. Clusters of significant voxels reveal local changes independent of anatomical boundaries.

D–E. Comparing mean values in neuroanatomically defined regions provides strong statistical power to detect subtle changes in brain composition or structure in models of neuro-developmental disorders.

simultaneously recording the position and morphology of every constituent labeled neuron. To make these transgenic lines accessible to the broader research community, we performed whole-brain imaging for each line, then registered the image of each line to the same reference brain (Figure 2). In collaboration with Nicholas Polys, we then developed an online brain atlas (<http://zbbrowser.com>) that enables researchers to quickly visualize the larval brain and locate transgenic lines to aid experiments. Such powerful visualization tools facilitate integrated analysis of reconstructed neuronal morphology in the context of the three-dimensional anatomy of the brain. Then, in order to build a brain atlas, we optimized a protocol that permits highly precise brain registration [Reference 5].

We showed that such high precision of alignment permits statistically robust whole-brain analysis of neuronal composition and morphology in zebrafish mutant models, pinpointing brain regions with changes that are difficult to detect visually (Figure 3). The technique can be applied to almost any zebrafish neuro-developmental model, thereby enabling robust and quantitative detection of subtle changes in brain structure or composition. We used this method to test brain structure and composition in zebrafish that carry mutations in genes that are homologous to human genes known to be disrupted in a variety of neuro-developmental disorders, including autism and intellectual disability. Through this work, we aim to provide insight into the fundamental molecular and cellular processes associated with each disorder.

Publications

1. Zoodsma J, Keegan E, Bhandiwad A, Napoli AJ, Burgess HA, Sirotkin H, Wollmuth L. Disruption of *grin2B*, an ASD-associated gene, produces social deficits in zebrafish. *Mol Autism* 2022 13:38.
2. Bhandiwad AA, Chu N, Semenova SA, Burgess HA. A cerebellar-prepontine circuit for tonic immobility

triggered by inescapable threat. *Sci Adv* 2022 8:eabo0549.

3. Burgess HA, Burton EA. A critical review of zebrafish neurological disease models – 1. The premise: neuroanatomical, cellular, and genetic homology, and experimental tractability. *Oxford Open Neuroscience* 2023 kvac018.
4. Burton EA and Burgess HA. A critical review of zebrafish neurological disease models – 2. Application: functional and neuroanatomical phenotyping strategies and chemical screens. *Oxford Open Neuroscience* 2022 kvac019.
5. Bhandiwad AA, Gupta T, Subedi A, Heigh V, Holmes GA, Burgess HA. Brain imaging and registration in larval zebrafish. *Zenodo* 2022 10.5281/zenodo.7312001.

Collaborators

- Edward A. Burton, MD, DPhil, FRCP, *Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh, Pittsburgh, PA*
- Andy Golden, PhD, *Laboratory of Biochemistry and Genetics, NIDDK, Bethesda, MD*
- Todd S. Macfarlan, PhD, *Section on Mammalian Epigenome Reprogramming, NICHD, Bethesda, MD*
- Reza Maroofian, PhD, *Institute of Neurology, University College, London, UK*
- Anne O'Donnell Luria, MD, PhD, *Mendelian Genomics Research Center, Broad Institute of MIT and Harvard, Cambridge, MA*
- Nicholas F. Polys, PhD, *Department of Computer Science, Virginia Tech, Blacksburg, VA*
- Howard Sirotkin, PhD, *Stony Brook University, Stony Brook, NY*
- Lonnie P. Wollmuth, PhD, *Stony Brook University, Stony Brook, NY*

Contact

For more information, email haroldburgess@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/burgess>.

Cell Fusion Stages of Myogenesis and Osteoclastogenesis: Mechanisms and Physiological Role

Diverse biological processes, in which enveloped viruses infect cells and cells from all kingdoms of life secrete, internalize, traffic and sort integral proteins, sculpt their membranes and bring together parent genomes in sexual reproduction, share a common stage: fusion of two membranes into one. Biological membrane remodeling is tightly controlled by protein machinery, but is also dependent on the lipid composition of the membranes. Whereas each kind of protein has its own individual personality, membrane lipid bilayers have rather general properties, manifested by their resistance to disruption and bending and by their charge. Our long-term goal is to understand how proteins fuse membrane lipid bilayers. We expect that better understanding of important fusion reactions will bring about new ways of controlling them and lead to new strategies for quelling diseases involving cell invasion by enveloped viruses and defects in intracellular trafficking or intercellular fusion. Our general strategy is to combine in-depth analysis of the best characterized fusion reactions with comparative analysis of diverse less explored fusion reactions that can reveal new kinds of fusion proteins and clarify the generality of emerging mechanistic insights. In our recent studies, we explored the mechanisms of myoblast fusion and osteoclast fusion in development and regeneration of muscles and bones.

Myomerger promotes fusion pore formation by elastic coupling between proximal membrane leaflets and hemifusion diaphragm.

Myoblast fusion, a key stage in the development and regeneration of skeletal muscle, is a multistep process, starting from hemifusion controlled by the muscle-specific protein Myomerger/Myomixer/Minion and followed by opening of a fusion pore controlled by another muscle-specific protein, Myomaker [reviewed in Petrány M], Millay DP. *Trends Cell Biol* 2019;29:964-973]. Molecular mechanisms by which Myomerger, a single-pass transmembrane protein containing 84 amino acids with an ectodomain that includes two alpha helices drives progression beyond early hemifusion events to complete fusion, remain to be understood.

In our earlier studies [Leikina E. *et al. Dev Cell* 2018;46:767-780; Reference 5], we demonstrated that Myomerger acts by destabilizing membranes



Leonid V. Chernomordik, PhD,
Head, Section on Membrane Biology

Eugenia Leikina, DVM, *Senior Research Assistant*

Kamram Melikov, PhD, *Staff Scientist*

Elena Zaitseva, PhD, *Staff Scientist*

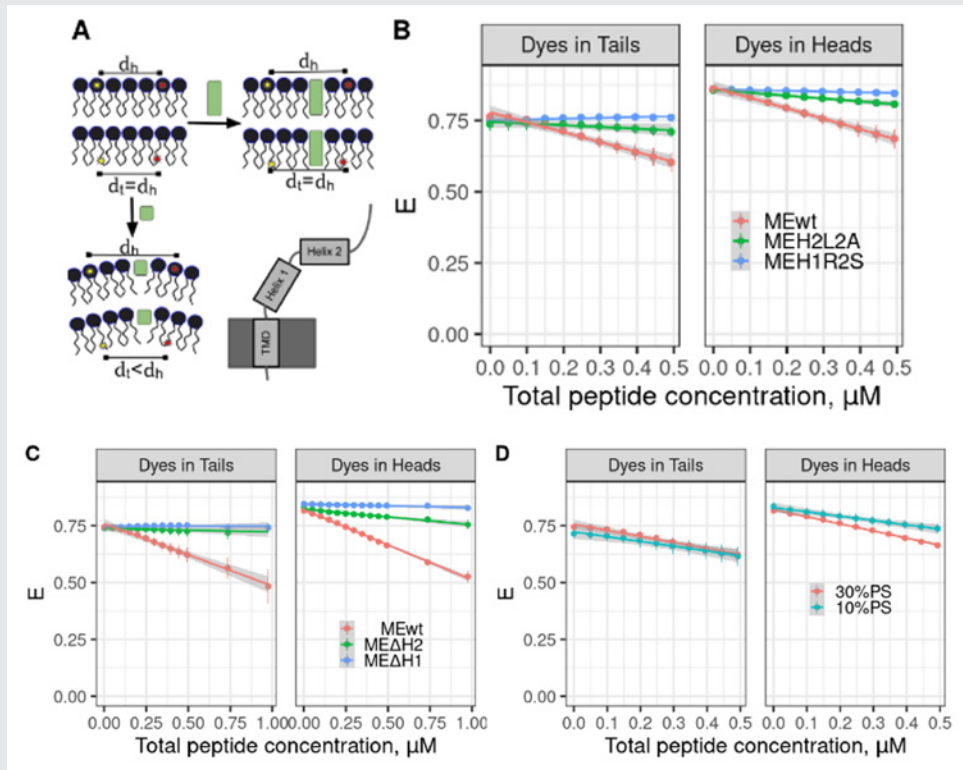
Jarred Whitlock, PhD, *Intramural Research Training Award Fellow*

Jared Cunanan, BS,
Postbaccalaureate Fellow

Wendy Zhang, BS,
Postbaccalaureate Fellow

FIGURE 1. Binding of Myomerger and generation of positive curvature require α -helix domains and depend on phosphatidylserine (PS).

A. In contrast to cylindrical molecules (green rectangles), membrane insertion of molecules generating positive spontaneous curvature (small green square) results in a larger change in the distance between donor and acceptor dye pairs located in the lipid headgroups than for the dye pairs located in the tail region. Domain diagram of Myomerger is shown in the right bottom corner.



B. Mutant Myomerger ectodomain (ME) with R to S substitution in the first α -helix (ME-H1R2S) and mutant with L to A substitution in the second α -helix (ME-H2L2A) induce smaller change in FRET for dyes both in headgroups and in tails compared with wild-type (WT) ME.

C. MEs lacking either the first α -helix (Myo Δ H1) or the second α -helix (Myo Δ H2) induce smaller change in FRET between dyes both in headgroups and in tails than in WT ME.

D. WT Myomerger induces larger changes in FRET for dyes in lipid headgroups in liposomes containing 30% PS than in those containing 10% PS.

through generation of elastic stresses in the outer leaflet of the plasma membrane. In more recent work [Reference 3], we continued our collaboration with Douglas Millay's lab to explore the specific contributions of different domains of Myomerger to functionally important protein-lipid bilayer interactions. We also examined how phosphatidylserine, a lipid previously implicated in diverse cell-cell fusion processes, including myoblast fusion [reviewed in Reference 4], regulates Myomerger activity. Under fusion conditions, phosphatidylserine, normally present only in the inner leaflet of the plasma membrane, is also transiently exposed in the outer membrane leaflet. Mechanisms that couple the phosphatidylserine signaling with the function of the fusion protein machinery are yet unclear. We focused on the membrane interactions of different domains of Myomerger and the dependence of these interactions on the lipid composition of membrane lipid bilayer. Complementing myoblast fusion assays and *in vitro* liposome assays, we found that the two helices possess unique characteristics, which are needed for full activity of the protein. We characterized membrane insertion of different regions of Myomerger by evaluating changes in the spontaneous curvature of the lipid monolayer. To characterize changes in this membrane property, which is critically important for membrane fusion,

we used our recently published assay, in which we compare the effects of inserted molecules on the FRET efficiency between dyes attached to either lipid headgroups or lipid tails [Reference 5]. We found that the membrane-proximal, amphipathic Helix-1 is normally disordered, and that its alpha-helical structure is induced by phosphatidylserine, facilitating interactions of this region with the membrane. The distal, more hydrophobic Helix-2 is intrinsically ordered, possesses an ability to insert into membranes, and promotes the membrane-stressing effects of Helix-1. The removal of either of the two alpha helices in the Myomerger ectodomain dramatically reduced its effect on Forster resonance energy transfer, suggesting that both alpha helices are necessary for efficient insertion and curvature generation. Similarly, mutations in either of the alpha helices reduced insertion and positive curvature generation. Our findings reveal that Myomerger fusogenic activity is a tightly controlled event involving its two ectodomain helices, which are regulated by changes in the lipid composition of the fusing membranes, providing an explanation as to how its membrane-stressing activity is spatially and temporally regulated during the final stage of myoblast fusion in the formation of multinucleated muscle cells. A deeper understanding of the myoblast fusion mechanism could help develop new therapeutic strategies for genetic and acquired muscle diseases. The uncovered mechanism, by which cells control the activity of proteins that merge membranes, can underlie fusion-pore formation in diverse fusion processes and in other cell-biological processes involving protein-induced membrane deformations.

Additional Funding

- Office of AIDS Research Award 2022
- Research on Women's Health (ORWH) through the Bench to Bedside Program award 2022, 2023

Publications

1. Li T, Hadigan C, Whitlock JM, Qin J, Kumar J, Kumar P, Catalfamo M. IL-27 modulates the cytokine secretion in the T cell-osteoclast crosstalk during HIV infection. *Front Immunol* 2022 13:818677.
2. Whitlock JM, Leikina E, Melikov K, de Castro LF, Mattijssen S, Maraia RJ, Collins MT, Chernomordik LV. Cell surface-bound Ia protein regulates the cell fusion stage of osteoclastogenesis. *bioRxiv* 2022.03.08.479741.
3. Gamage DG, Melikov K, Paola Munoz-Tello P, Wherley TJ, Focke LC, Leikina E, Huffman E, Diao J, Kojetin DJ, Prasad V, Chernomordik LV, Millay DP. Phosphatidylserine orchestrates Myomerger membrane insertions to drive myoblast fusion. *Proc Natl Acad Sci USA* 2022 119:e2202490119.
4. Whitlock JM, Chernomordik LV. Flagging fusion: phosphatidylserine signaling in cell-cell fusion. *J Biol Chem* 2021 296:100411.
5. Golani G, Leikina E, Melikov K, Whitlock JM, Gamage DG, Luoma-Overstreet G, Millay DP, Kozlov MM, Chernomordik LV. Myomerger promotes fusion pore by elastic coupling between proximal membrane leaflets and hemifusion diaphragm. *Nat Commun* 2021 12(1):495.

Collaborators

- Alison Boyce, MD, *Metabolic Bone Disorders Unit, NIDCR, Bethesda, MD*
- Michael Collins, MD, *Skeletal Disorders & Mineral Homeostasis Section, NIDCR, Bethesda, MD*
- Michael M. Kozlov, PhD, DHabil, *Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel*
- Richard Maraia, MD, *Section on Molecular and Cellular Biology, NICHD, Bethesda, MD*

- Leonid Margolis, PhD, *Section on Intercellular Interactions, NICHD, Bethesda, MD*
- Douglas Millay, PhD, *Cincinnati Children's Hospital Medical Center, Cincinnati, OH*

Contact

For more information, email chernoml@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/chernomordik>.

Building the Zebrafish Lateral Line System

Cells divide, move, adhere, and interact with their neighbors and their environment to determine the formation of multicellular organ systems with unique fates, morphologies, function, and behavior. Our goal is to understand how such interactions determine the self-organization of cell communities in the nervous system of the zebrafish (*Danio rerio*) embryo. The lateral line is a mechano-sensory system that helps sense the pattern of water flow over the fish and amphibian body; it consists of sensory organs called neuromasts, which are distributed in a stereotypic pattern over the body surface. Each neuromast has sensory hair cells at its center, surrounded by support cells that serve as progenitors for the production of more hair cells during growth and for the regeneration of neuromasts. The development of this superficial sensory system in zebrafish is spearheaded by the posterior Lateral Line primordia (pLLp), groups of about 150 cells formed on either side of a day-old embryo near the ear. Cells in the primordia migrate collectively under the skin to the tip of the tail, as they divide and reorganize to form nascent neuromasts, which are deposited sequentially from the lateral line's trailing end. Their journey is easily observed in live transgenic embryos with fluorescent primordium cells. Furthermore, a range of genetic and cellular manipulations can be used to investigate gene function and morphogenesis in the system. Understanding the self-organization of this relatively simple and accessible system in zebrafish will help elucidate the broader principles that determine cell-fate specification, morphogenesis, and collective cell migration in the developing vertebrate nervous system.

Signaling and mechanics influence the number and size of epithelial rosettes in the migrating zebrafish posterior Lateral Line primordium.

Protoneuromasts are formed within the migrating primordium, starting from its trailing end as clusters of cells apically constrict and form epithelial rosettes. Their formation is promoted by fibroblast growth factor (Fgf)-signaling centers that form periodically in the wake of a shrinking Wnt-active domain that inhibits epithelial rosette formation and progressively shrinks toward the leading end of the primordium (Wnt and Fgf pathways are signaling pathways).



Ajay Chitnis, MBBS, PhD, Head,
Section on Neural Developmental Dynamics

Gregory Palardy, BS, *Research Technician*

Chongmin Wang, MS, *Research Technician*

Pritesh Krishnakumar, PhD,
Postdoctoral Fellow

Sana Fatma, PhD, *Postdoctoral Visiting Fellow*

Abhishek Mukherjee, PhD,
Postdoctoral Visiting Fellow

Megan Schupp, BS,
Postbaccalaureate Intramural Research Training Award Fellow

However, the precise number and size of epithelial rosettes is not strictly dependent on a prepattern of Fgf-signaling activity, as it is broadly influenced by the balance of mechanical interactions that promote or oppose formation of epithelial rosettes. When chemokine-dependent migration of leading cells is compromised, the resulting slowing of the primordium is accompanied by the fusion of epithelial rosettes to form fewer larger rosettes. However, such fusion is not observed when Fgf signaling, responsible for migration of trailing cells, is inhibited to slow primordium migration. These observations can be accounted for by a mechanics-based model, in which local interactions associated with apical constriction and cell adhesion promote aggregation, while tension along the length of the primordium, influenced by the relative efficacy of leading and trailing cell migration, opposes such aggregation. We described the development of a computational Cellular Potts model, which allowed us to explore how the relative speed of leading versus trailing cells, as well as changes in cell adhesion and mechanical coupling, differentially regulated by Wnt and Fgf signaling, can influence the pattern of neuromast formation and deposition by the migrating primordium. Our studies illustrate how signaling and mechanics cooperate to coordinate self-organization of morphogenesis in the migrating primordium.

Sox2 stabilizes maturing epithelial rosettes in the zebrafish posterior Lateral Line primordium in part by inhibiting destabilizing Wnt-signaling activity.

Protoneuromasts are formed within the migrating primordium, starting from its trailing end, as clusters of cells sequentially reorganize to form epithelial rosettes, each around a central *Atoh1a*-expressing cell specified as a sensory hair cell progenitor. The rosettes' formation is initiated in Fgf-signaling domains that are periodically established in response to Fgfs produced by cells in an adjacent leading Wnt-active zone, where Wnt signaling also inhibits these leading cells from responding to Fgfs and forming protoneuromasts. Fgf signaling-dependent expression of the diffusible Wnt antagonist *Dkk1b* (dickkopf Wnt-signaling pathway inhibitor 1b) facilitates establishment of stable Fgf-signaling centers in nascent protoneuromasts by preventing potentially destabilizing inhibition from Wnt signaling. *Dkk1b* also contributes to progressive restriction of the initially broad Wnt-signaling domain to a smaller leading zone, as new Fgf signaling-dependent protoneuromasts form in the wake of the shrinking Wnt system. As the leading Wnt system shrinks, *Atoh1a* expression (a transcription factor that enables chromatin-binding activity) and epithelial rosette morphogenesis in maturing neuromasts formed earlier in more trailing parts of the primordium, become self-sustaining and independent of the Fgfs signals produced by leading Wnt active cells that initiated protoneuromast formation. However, *Dkk1b* is not expressed in these maturing neuromasts, raising a question about what inhibits potentially destabilizing Wnt signaling in the trailing neuromasts. We showed that *Sox2* (a member of the Sox family of transcription factors that regulate cell-fate decisions during development and is essential for maintaining self-renewal, or pluripotency, of undifferentiated embryonic stem cells) is expressed in nascent and maturing protoneuromasts in a pattern that is complementary to domains with Wnt signaling activity. Furthermore, *Sox2* functions in a partially redundant manner with *Sox1a* and *Sox3*, to inhibit Wnt signaling. This helps keep Wnt activity restricted to a leading zone, which we suggest is essential for effective stabilization of maturing protoneuromasts in the trailing zone. Together our observations show how patterning events that initiate protoneuromast formation are followed by changes in regulation requiring *SoxB1* family factors that help consolidate neuromast morphogenesis, prior to their deposition by the migrating primordium.

Collaborators

- Harshad Vishwasrao, PhD, *Advanced Imaging and Microscopy Resource, NIBIB, Bethesda, MD*

Contact

For more information, email chitnisa@mail.nih.gov or visit <http://chitnislabs.nih.gov>.

Molecular Genetics of Heritable Human Disorders

We conduct research to delineate the pathophysiology of and develop novel therapies for the glycogen storage disease type I (GSD-I) subtypes GSD-Ia and GSD-Ib. GSD-Ia is caused by a deficiency in the liver/kidney/intestine-restricted glucose-6-phosphatase- α (G6Pase- α or G6PC), and GSD-Ib is caused by a deficiency in the ubiquitously expressed glucose-6-phosphate transporter (G6PT or SLC37A4). G6Pase- α is an endoplasmic-reticulum (ER) transmembrane protein that regulates intracellular glucose production by catalyzing the hydrolysis of G6P to glucose and phosphate. The active site of G6Pase- α faces into the ER lumen and depends on G6PT, another ER transmembrane protein, to translocate G6P from the cytoplasm into the ER lumen. To function, G6Pase- α couples with G6PT to form a functional G6Pase- α /G6PT complex, which maintains interprandial glucose homeostasis. GSD-Ia and GSD-Ib patients manifest a common metabolic phenotype of impaired glucose homeostasis and long-term complications of hepatocellular adenoma/carcinoma (HCA/HCC) and renal disease. There is no cure for GSD-Ia and GSD-Ib. The current dietary therapies have enabled GSD-I patients to maintain a normalized metabolic phenotype if strictly adhered to. However, the underlying pathological processes remain uncorrected, and HCA/HCC and renal disease still occur in metabolically compensated GSD-I patients. We generated animal models of GSD-Ia and GSD-Ib, which are being exploited to both delineate the disease more precisely and develop new treatment approaches, including gene therapy. We also generated G6PC- and G6PT-expressing recombinant adeno-associated virus (rAAV) vectors and showed that rAAV vector-mediated gene therapy for GSD-Ia and GSD-Ib is safe and efficacious. Our rAAV-G6PC/rAAV-coG6PC vector (US patent #9,644,216) technology was licensed to Ultragenyx Pharmaceutical Inc. (Novato, CA), who launched a phase I/II clinical trial ([NCT03517085](#)) in 2018, followed by a phase III clinical trial ([NCT05139316](#)) in 2022, using the rAAV-GPE-coG6PC vector. To explore alternative genetic technologies for GSD-I therapies, we have established formal collaborations under the CRADA with CRISPR Therapeutics (Boston, MA) and Beam Therapeutics (Boston, MA) to evaluate the efficacy of CRISPR/Cas9-based and adenine base editor (ABE)-based gene editing systems, respectively, to correct gene-specific G6PC mutations in animal models of GSD-Ia.



Janice Y. Chou, PhD, Head, Section on Cellular Differentiation

Irina Arnaoutova, PhD, Staff Scientist

Lisa Zhang, PhD, Staff Scientist

Sudeep Gautam, PhD, Visiting Fellow

Nelson George, PhD, Visiting Fellow

Hung-Dar Chen, PhD, Contract Technician

Cheol Lee, PhD, Contract Technician

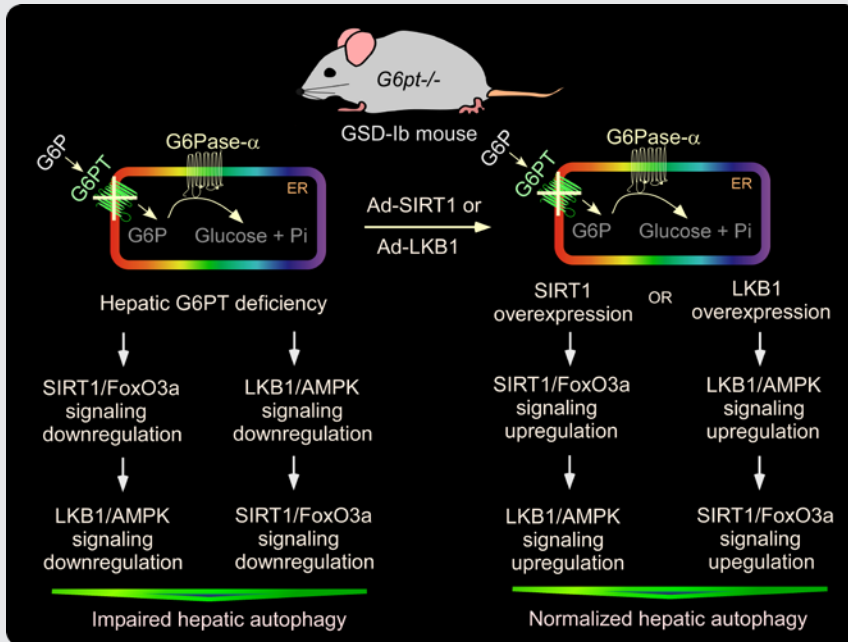


FIGURE 1. Molecular mechanism underlying hepatic autophagy impairment in GSD-Ib

Correction of metabolic abnormalities in a mouse model of glycogen storage disease type Ia by CRISPR/Cas9-based gene editing

The rAAV-coG6PC vector used in the current phase III clinical trial is episomally expressed, and the long-term durability of expression in humans is currently being established. We therefore sought to explore the use of the CRISPR/Cas9 technology to correct a pathogenic GSD-Ia variant in its native genetic locus. The most prevalent pathogenic mutation identified in Caucasian GSD-Ia patients is G6PC-p.R83C, representing 32% of diseased alleles. Using the CRISPR/Cas9-based gene editing technology, we generated a GSD-Ia mouse disease model, the *G6pc*-R83C mouse homozygous for the G6PC-p.R83C mutation and showed that the *G6pc*-R83C mice manifest impaired glucose homeostasis mimicking that of human GSD-Ia. We then used a CRISPR/Cas9-based gene-editing system to treat newborn *G6pc*-R83C mice and showed that the treated mice grew normally to age 16 weeks without hypoglycemia seizures. The treated *G6pc*-R83C mice, expressing 3% or more of normal hepatic G6Pase-α activity, maintained glucose homeostasis, displayed normalized blood metabolites, and could sustain 24 hours of fasting. Taken together, we have developed a second-generation therapy in which *in vivo* correction of a pathogenic G6PC-p.R83C variant in its native genetic locus could lead to potentially permanent, durable, long-term correction of the GSD-Ia disorder [Reference 1].

Molecular mechanism underlying hepatic autophagy impairment in GSD-Ib

Clinically, GSD-Ib patients manifest a metabolic phenotype of impaired blood glucose homeostasis and a long-term risk of hepatocellular adenoma/carcinoma (HCA/HCC). The etiology of HCA/HCC in GSD-Ib is unknown. Studies have shown that deficiency in autophagy, an evolutionarily conserved, degradative process that produces energy and building blocks through lysosomal degradation of intracellular proteins and organelles in times of nutrient deprivation and environmental stresses, contributes to hepatocarcinogenesis. Autophagy can be regulated positively by the deacetylase sirtuin 1 (SIRT1), AMP-activated protein kinase (AMPK), and

forkhead box O (FoxO) transcription factor family members. In the liver, AMPK is activated via phosphorylation of the AMPK α -subunit at residue T172 by the liver kinase B-1 (LKB1), a serine/threonine kinase.

To understand the pathways contributing to hepatocarcinogenesis in GSD-Ib, we hypothesized that impaired hepatic autophagy is a significant contributor. We showed that G6PT deficiency leads to impaired hepatic autophagy, evident from attenuated expression of many components of the autophagy network, reduced autophagosome formation, and reduced autophagy flux. The G6PT-deficient liver displayed impaired SIRT1 and AMPK signaling, along with reduced expression of SIRT1, FoxO3a, LKB1, and the active p-AMPK. Importantly, we showed that overexpression of either SIRT1 or LKB1 in G6PT-deficient liver restored autophagy and SIRT1/FoxO3a and LKB1/AMPK signaling. The hepatosteatosis in G6PT-deficient liver reduced SIRT1 expression. LKB1 overexpression reduced hepatic triglycerides levels, providing a potential link between LKB1/AMPK signaling upregulation and the increase in SIRT1 expression. In conclusion, downregulation of SIRT1/FoxO3a and LKB1/AMPK signaling underlies impaired hepatic autophagy, which may contribute to HCA/HCC development in GSD-Ib. Understanding this mechanism may guide future therapies [Reference 2].

Gene therapy for GSD-Ia

We generated four efficacious *G6PC* gene transfer rAAV vectors for GSD-Ia gene therapy: rAAV-G6PC expressing the wild-type (WT) G6PC, rAAV-coG6PC expressing a codon-optimized (co) G6PC, rAAV-G6PC-S298C expressing a G6PC-S298C variant with increased efficacy, and rAAV-coG6PC-S298C. As stated above, our rAAV-G6PC/rAAV-coG6PC vector (US patent #9,644,216) technology was licensed to Ultragenyx Pharmaceutical Inc., who launched a phase I/II clinical trial ([NCT03517085](#)) in 2018, followed by a phase III clinical trial ([NCT05139316](#)) in 2022 using the rAAV-GPE-coG6PC vector.

To examine the long-term efficacy of such rAAV vectors, we conducted a long-term (66–76 week) gene transfer study in *G6pc*^{-/-} mice using these rAAV vectors. All treated *G6pc*^{-/-} mice survived to age 66–76 weeks, and the outcomes were additive. Hepatic G6Pase- α activities in rAAV-G6PC-S298C-, rAAV-coG6PC-, and rAAV-coG6PC-S298C-treated *G6pc*^{-/-} mice were 1.7-, 1.7-, and 4.4-fold higher, respectively, than that in rAAV-G6PC-WT-treated mice. The efficacy of the rAAV-coG6PC-S298C vector is 2.6-fold higher than the rAAV-coG6PC vector currently used in phase III clinical trial ([NCT05139316](#)). Taken together, the rAAV-G6PC-S298C and rAAV-coG6PC-S298C vectors offer attractive clinical alternatives [Reference 3].

Evaluation of the adenine base editor–based gene–editing system to correct a pathogenic G6PC mutation in a humanized mouse model of GSD-Ia

We explored the adenine base editor (ABE)–based technologies that enable a programmable conversion of A•T to G•C in genomic DNA for GSD-Ia therapies. The ABE system works in both dividing and non-dividing cells, is reported to produce virtually no indels or off-target editing in the genome, and can correct a pathogenic variant in its native genetic locus, leading to permanent, therapeutically effective long-term expression. This is a collaborative study with Beam Therapeutics, Cambridge, MA under a CRADA.

The G6PC-p.R83C is the most prevalent pathogenic mutation identified in Caucasian GSD-Ia patients that contains a single G→A transition in the *G6PC* gene. We first generated a homozygous humanized R83C/R83C mouse strain, the *G6PC*-R83C mouse, by inserting the entire coding sequence of the human G6PC-p.R83C along with human *G6PC* 3' UTR into exon 1 of the mouse *G6pc* gene at the ATG start codon. The insertion places the

human transcript under the control of the native mouse *G6pc* promoter/enhancer. The mouse *G6pc* gene is disrupted by a premature STOP codon created in the mouse *G6pc* exon 1. We showed that the *G6PC-R83C* mice manifest impaired glucose homeostasis characterized by growth retardation, hypoglycemia, hyperlipidemia, hyperuricemia, hepatomegaly, and nephromegaly, mimicking the abnormal metabolic phenotype of human GSD-Ia. We then treated newborn *G6PC-R83C* mice with lipid nanoparticles encompassing the guide RNA and mRNA encoding ABE (LNP-ABE) and showed that the treated mice grew normally to age eight weeks without hypoglycemia seizures. The LNP-ABE-treated *G6PC-R83C* mice expressed significant levels of hepatic G6Pase- α activity with an editing efficiency of up to about 60% and displayed normalized blood metabolite profiles and could tolerate 24 hours of fasting. Taken together, our data demonstrate the potential of base-editing to correct the G6PC-p.R83C mutation in its native genetic locus, which could lead to potentially permanent, durable, long-term correction of the GSD-Ia disorder.

Targeting Wnt/ β -catenin signaling reduces renal fibrosis in murine GSD-Ia.

GSD-Ia patients manifest nephromegaly caused by marked glycogen accumulation and nephropathy. The current dietary therapies have significantly alleviated metabolic abnormalities and delayed chronic renal disease and renal insufficiency in GSD-Ia patients. However, the underlying pathological processes remain uncorrected: glomerular hyperfiltration, hypercalciuria, hypocitraturia and urinary albumin excretion still occur in metabolically compensated GSD-Ia patients. We showed that one mechanism underlying GSD-Ia nephropathy is fibrosis mediated by activation of the renin-angiotensin system (RAS). The Wnt/ β -catenin signaling that promotes fibrosis controls the expression of RAS genes. We hypothesized that elevated renal glycogen could elicit acute kidney injury (AKI) by activating Wnt/ β -catenin signaling and promoting fibrosis. We showed that *G6pc*^{-/-} mice displayed impaired renal glucose homeostasis and AKI. Renal levels of β -catenin increased markedly in *G6pc*^{-/-} mice during postnatal development, along with elevated renal levels of renin, angiotensinogen, and the transcription factor snail1. Renal fibrosis was evident by elevated renal levels of α -smooth muscle actin (α -SMA) and extracellular matrix (ECM) proteins. ICG-001, a β -catenin inhibitor, reduced renal levels of active β -catenin, renin, snail1, and α -SMA, indicating that targeting the Wnt/ β -catenin signaling is a promising therapeutic strategy for GSD-Ia nephropathy.

Additional Funding

- The Children's Fund for Glycogen Storage Disease Research
- CRISPR Therapeutics (Cambridge, MA), under a cooperative research and development agreement (CRADA)
- Beam Therapeutics, Inc (Cambridge, MA), under a CRADA

Publications

1. Arnaoutova I, Zhang L, Chen HD, Mansfield BC, Chou JY. Correction of metabolic abnormalities in a mouse model of glycogen storage disease type Ia by CRISPR/Cas9-based gene editing. *Mol Ther* 2021 29(4):1602–1610.
2. Gautam S, Zhang L, Lee C, Arnaoutova I, Chen HD, Resaz R, Eva A, Mansfield BC, Chou JY. Molecular mechanism underlying hepatic autophagy impairment in glycogen storage disease type Ib. *Hum Mol Genet* 2022 online ahead of print.
3. Zhang L, Lee C, Arnaoutova I, Anduaga J, Starost MF, Mansfield BC, Chou JY. Gene therapy using a novel

G6PC-S298C variant enhances the long-term efficacy for treating glycogen storage disease type Ia.
Biochem Biophys Res Commun 2020 527:824–830.

Collaborators

- Alessandra Eva, PhD, *Istituto Giannina Gaslini, Genoa, Italy*
- Brian C. Mansfield, PhD, *Foundation Fighting Blindness, Columbia, MD*
- Matthew F. Starost, PhD, *Diagnostic & Research Services Branch, Division of Veterinary Resources, NIH, Bethesda, MD*
- David A. Weinstein, MD, MSc, *University of Connecticut School of Medicine, Farmington, CT*

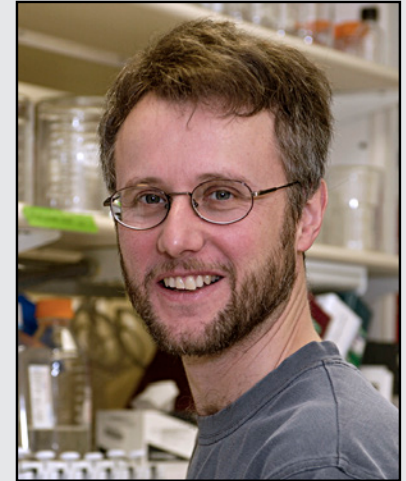
Contact

For more information, email chouja@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHDIInvestigators/chou>.

Chromatin Remodeling and Gene Activation

Aberrant gene regulation is the basis of many disease states. Our main objective is to understand how genes are activated for transcription in the context of chromatin structure. Chromatin is not merely a packaging system for DNA in eukaryotic cells; it also participates in gene regulation. The structural subunit of chromatin is the nucleosome, which contains nearly two turns of DNA coiled around a central core histone octamer. The nucleosome is a highly stable structure. Nucleosomes are generally quite regularly spaced along the DNA, like beads on a string (Figure 1). Under physiological conditions, this nucleosomal filament spontaneously coils to form a heterogeneous chromatin fiber of about 30 nm in diameter (the structure of the fiber is controversial). Chromosomes are composed of functional loops of the chromatin fiber organized by various non-histone proteins. Transcriptionally active and inactive chromatin regions are marked with different sets of post-translational histone modifications. Inactive regions are associated with highly condensed chromatin, as observed in the nucleus and referred to as heterochromatin, whereas active regions are less condensed and referred to as euchromatin. The extreme condensation of heterochromatin is thought to contribute to transcriptional repression. Thus, chromatin restricts access to DNA and is inherently repressive at several structural levels (Figure 1). Given that gene activation requires sequence-specific binding by transcription factors at their cognate sites in gene promoters and at enhancers, the question arises as to how they access their binding sites in chromatin. The answer is provided by the chromatin-remodeling enzymes.

The chromatin-remodeling enzymes can be divided into two groups: histone- or DNA-modifying enzymes, which implement the “epigenetic code”, and ATP-dependent remodeling machines, which move or displace nucleosomes. Histone modifications mark different genomic regions for activation or repression, resulting in recruitment of activating or repressing factors, respectively. Histone-modifying enzymes are typically subunits of large protein complexes containing complementary activities. Similarly, the ATP-dependent chromatin remodelers are usually large complexes containing several subunits, but they use the free energy available from ATP hydrolysis to alter chromatin structure by manipulating nucleosomes. Most of these enzymes can move nucleosomes along DNA, some can form arrays of



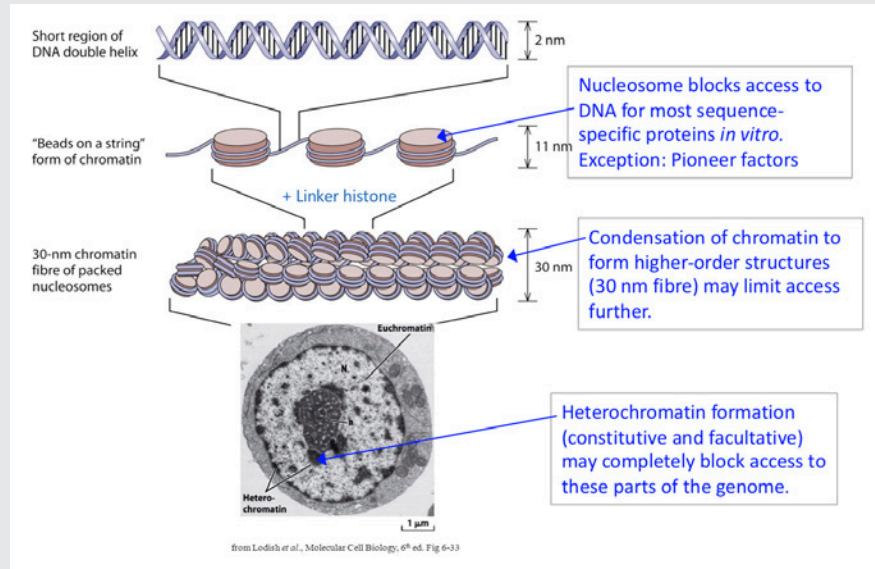
David J. Clark, PhD, Head,
Section on Chromatin and Gene Expression

Peter Eriksson, PhD, *Staff Scientist*
Christopher Coey, PhD, *Intramural Research Training Award Fellow*
Hemant K. Prajapati, PhD, *Visiting Fellow*
Zhuwei Xu, PhD, *Visiting Fellow*
Paul Elizalde, MS, *Postbaccalaureate Fellow*
Kayla Gibson, *Summer Student (Howard University)*

FIGURE 1. DNA packaging in the nucleus: to what extent does chromatin compaction limit access to the DNA?

DNA is packaged into the nucleus by histones. The basic structural subunit of chromatin is the nucleosome core, which contains about 147 bp of DNA wrapped nearly twice around a central octamer of core histones. Nucleosomes are regularly spaced along the DNA like beads on a string; the intervening DNA is called the linker DNA and is bound by linker histone (H1). The beads-on-a-string fiber spontaneously condenses into a heterogeneous fiber of about 30 nm width.

Genomic regions rich in repetitive elements form constitutive heterochromatin in all cells, in which the chromatin fiber is even more condensed. Facultative heterochromatin is formed on genes that should be permanently silent in a specific differentiated cell type. Heterochromatin is densely packed and darkly staining in the electron micrograph shown here. Euchromatin is less condensed (*light staining*) and contains active genes. We are interested in determining to what extent chromatin limits DNA accessibility. Figure adapted from Chereji *et al. Genome Res* 2019;29:1985–1995.



regularly spaced nucleosomes (e.g., CHD1, ISW1, ISW2, INO80C, SNF2h), while others can remove the histones altogether (e.g., SWI/SNF, RSC, PBAF), or replace histones with variant histones (e.g., SWR1). Human diseases have been linked to defective chromatin-remodeling enzymes. For example, mutations in the hSNF5 subunit of the human SWI/SNF complex are strongly linked to pediatric rhabdoid tumors, and the CHD remodelers have been linked to cancer and autism. Therapies and drugs aimed at epigenetic targets are being tested. Thus, a full understanding of chromatin structure and the mechanisms by which it is manipulated is vital.

Our current objectives are as follows:

1. To determine the roles of the major yeast chromatin-remodeling complexes (RSC, SWI/SNF, ISW1, ISW2, CHD1, INO80C) in chromatin organization and gene expression; why there are so many different remodelers and whether they are functionally redundant; so far, our studies indicate that each remodeling enzyme makes a different contribution to chromatin organization;
2. to test the hypothesis that nucleosomes control DNA accessibility and play a vital role in gene regulation by blocking promoters.

Sequence-specific transcription factors and DNA accessibility

Gene activation involves the recruitment of a set of factors to a promoter in response to appropriate signals, ultimately resulting in the formation of an initiation complex by RNA polymerase II and transcription, events that coincide with the removal of promoter nucleosomes to create a nucleosome-depleted region (NDR). This observation has led to the generally accepted model that promoter nucleosomes physically block

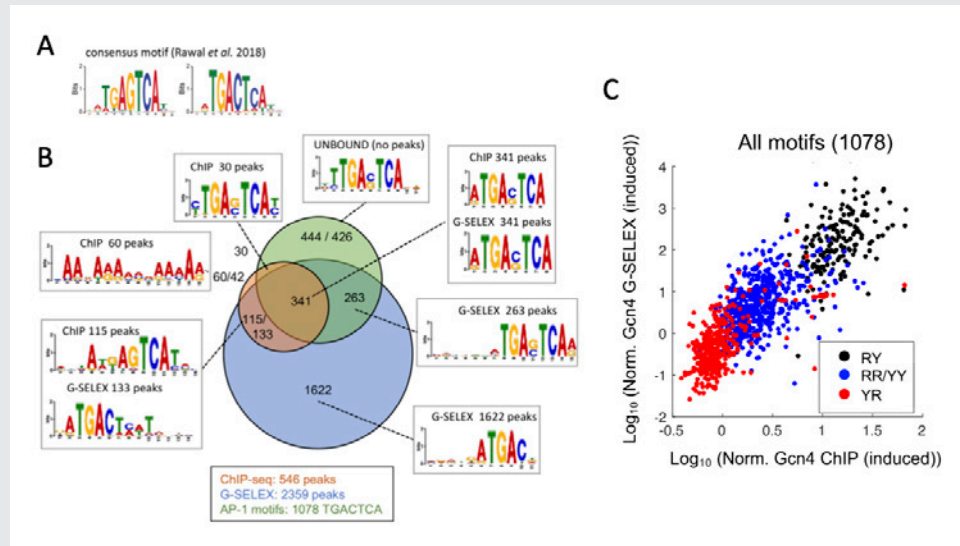
FIGURE 2. Sequence-specific binding by the transcription factor Gcn4 can be predicted by its high-affinity consensus site *in vitro* and *in vivo*.

A. Gcn4 consensus binding site derived from ChIP-Seq data.

B. Venn diagram showing the overlap of Gcn4-bound sites *in vivo* (ChIP-Seq data) and *in vitro* (G-SELEX data) and the consensus sites derived for each set of sites.

C. Comparison of Gcn4 binding to the 1078 TGACTCA motifs in the yeast genome *in vitro* and *in vivo*:

high-affinity sites (RY = RTGACTCAY), low-affinity sites (RR = RTGACTCAY and YY = YTGACTGAY), and very poorly bound sites (YTGACTCAR). Note log scale. Figure adapted from [Reference 1].



transcription initiation, acting as repressors by preventing access to specific transcription factor binding sites. The nucleosome is a highly stable structure, containing tightly wound DNA that is largely inaccessible to the majority of sequence-specific DNA-binding proteins. Activation occurs if sequence-specific 'pioneer' transcription factors are present (these proteins bind to nucleosomal sites with high affinity), and/or if 'classical' transcription factors, which are normally blocked by nucleosomes, recruit ATP-dependent chromatin remodelers to move or evict promoter nucleosomes, thus facilitating initiation complex formation.

Although the main effort of the lab focuses on the role of the ATP-dependent chromatin-remodeling enzymes in gene regulation, with particular emphasis on how they may control the accessibility of genomic DNA, we are also interested in the central role of sequence-specific transcription factors. Transcription factors usually recognize consensus DNA-binding sites, mostly containing 4 to 12 base pairs, and in which some bases may be degenerate (e.g., 'Y' for pyrimidine [C or T] or 'R' for purine [A or G]). The probability of finding such a site in the genome is often much higher than the number of actual binding sites detected empirically, using methods such as ChIP-Seq. Such consensus sites occur not only in regulatory elements, but also inside genes and elsewhere, where they may or may not be functional. The observation that consensus sites often predict far more transcription factor-binding sites than are actually bound *in vivo* has led to the proposal that consensus sites in non-regulatory regions are not bound because they are blocked by chromatin. However, our recent measurements of DNA accessibility in yeast and mouse nuclei imply that all consensus sites are likely to be accessible in some cells within a population. Such general but limited accessibility predicts detectable binding at all consensus sites, albeit lower than at sites in nucleosome-free DNA. If true, the hypothesis that chromatin prevents binding by transcription factors to consensus sites outside nucleosome-depleted regions is questionable.

We explored an alternative explanation: that consensus-site sequences derived from ChIP-Seq data may be too degenerate in some cases, such that only a subset of the predicted sites are true sites. We investigated this possibility using the well studied yeast transcription factor Gcn4 as a model [Reference 1]. Previously, we published ChIP-Seq data for Gcn4 in a collaboration with the [Hinnebusch lab](#). In that study, we derived a consensus sequence for Gcn4 binding (Figure 2A), of which there are 1,754 instances in the yeast genome, but only 546 show detectable Gcn4 binding *in vivo*. To resolve this discrepancy, it is necessary to determine which sites are bound by Gcn4 in the absence of the potential blocking effect of chromatin (i.e., using purified DNA *in vitro*). Accordingly, we developed a modified SELEX method to identify all sites bound by Gcn4 in the yeast genome, which we termed 'G-SELEX.' We used short genomic DNA fragments and purified Gcn4 attached to beads to select DNA fragments containing a Gcn4-bound site. The bound DNA was amplified and incubated with Gcn4 again in a second round of selection; three rounds of selection were performed in total. The final bound product was subjected to paired-end sequencing. We mapped the DNA fragments to the yeast genome to produce a very high-quality coverage map. We identified 2,359 Gcn4-bound sites, but most were bound at very low frequency, and corresponded to Gcn4 half-sites (Figure 2B). In contrast, the major peaks (high-affinity sites) corresponded to the 7-bp sequence TGACTCA. However, of the 1,078 instances of this sequence in the yeast genome, less than half are bound *in vitro* or *in vivo*. Further analysis revealed that the bound sites conform to a more extensive consensus: RTGACTCAY, such that RTGACTCAR or YTGACTCAY sites are bound only weakly, and YTGACTCAR sites are not bound *in vitro* or *in vivo* (Figure 2C). We conclude that the high-affinity site (RTGACTCAY) essentially accounts for Gcn4 binding *in vitro* and *in vivo*, irrespective of whether the site is located in a nucleosome-depleted promoter or inside a gene assembled into nucleosomes.

More generally, we propose that transcription-factor binding sites need to be defined more precisely using quantitative data, which should result in more accurate genome-wide prediction of real binding sites and greater insight into gene regulation. Overall, the study [Reference 1], together with our previous studies, suggests that the prevailing model that chromatin is a general block to gene expression unless specific transcription activators are present may be incorrect. Our current studies are aimed at resolving this issue.

Publications

1. Coey CT, Clark DJ. A systematic genome-wide account of binding sites for the model transcription factor Gcn4. *Genome Res* 2022 32:367–377.

Collaborators

- Jeffrey J. Hayes, PhD, *University of Rochester Medical Center, Rochester, NY*
- Alan Hinnebusch, PhD, *Section on Nutrient Control of Gene Expression, NICHD, Bethesda, MD*
- Vasily M. Studitsky, PhD, *Fox Chase Cancer Center, Philadelphia, PA*

Contact

For more information, email clarkda@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/clark>.

Physiological, Biochemical, and Molecular–Genetic Events Governing the Recognition and Resolution of RNA/DNA Hybrids

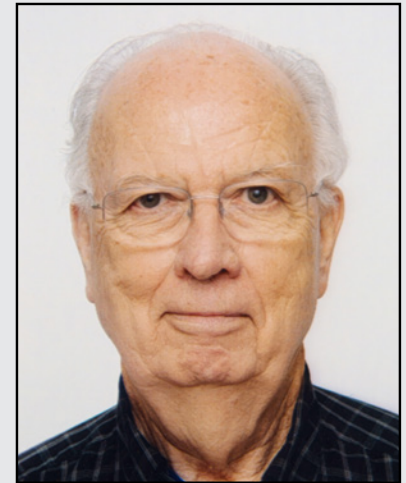
Ribonucleases H (RNases H) are considered essential enzymes in multicellular organisms, thereby placing the genes that encode the enzymes in the housekeeping category. Damaged DNA is a leading cause of many human diseases and disorders. We study the formation and resolution of RNA/DNA hybrids, which occur during DNA replication and RNA transcription. Such hybrid molecules may lead to increased DNA damage, but may also play critical roles in normal cellular processes. We are interested in how RNA/DNA hybrids are resolved and in the role that RNases H play in their elimination. Two classes of RNases H, Class I and Class II, are present in most organisms.

Patients with mutations in the *RNASEH1* gene exhibit typical mitochondrial myopathy symptoms (neuromuscular disorder). We were the first to show that RNase H1 is essential for the maintenance of mitochondrial DNA. More than 1,000 proteins are targeted to mitochondria, many of which, when mutated, are known causes of mitochondrial myopathies. Mice deleted for the *Rnaseh1* gene arrest embryonic development at day 8.5 because of failure to amplify mitochondrial DNA.

Aicardi-Goutières syndrome (AGS), a severe neurological disorder with symptoms appearing at or soon after birth, can be caused by defective human RNase H2. As many as 38 Mendelian genotypes may result in a type I interferonopathy, including mutations in each of the genes encoding the subunits of the heterotrimeric RNase H2, the hallmark of which is activation of the innate immune response.

Differences between Class I and Class II RNases H

Over the years, many of our investigations focused on RNase H1. RNase H1 recognizes the 2'-OH of four consecutive ribonucleotides (rNMPs), while the DNA strand is distorted to fit into a pocket of the enzyme. Thus, the enzyme requires more than one ribonucleotide for cleavage of RNA in RNA/DNA hybrids. In both prokaryotes and eukaryotes, RNases H1 consist of a single polypeptide. In contrast, in eukaryotes RNase H2 is a complex of three distinct polypeptides but a single polypeptide in prokaryotes. The catalytic subunit of



Robert J. Crouch, PhD, Head,
Section on Formation of RNA
Susana M. Cerritelli, PhD, *Staff*
Scientist

Kiran Sakhuja, MS, MSc, *Biologist*
Caitlin Darling, BS,
Postbaccalaureate Fellow
Andrea Mota, BS,
Postbaccalaureate Fellow
Alexander Robinson, BS,
Postbaccalaureate Fellow
Matthew Roy, BS,
Postbaccalaureate Fellow

the hetero-trimeric RNase H2 of eukaryotes is similar in its primary amino-acid sequence to the prokaryotic enzyme. RNase H2 can recognize and cleave both RNA/DNA hybrids and a single ribonucleotide embedded in DNA, making an incision at the 5' of the ribonucleotide, or the transition from the ribonucleotide in the case of RNA-primed DNA synthesis (e.g., *rrrrrD* in DNA—italics indicate transition from ribonucleotide to deoxyribonucleotide) [References 1 & 2].

Several types of RNA/DNA hybrid structures can be formed, and they are processed differently. Simple RNA/DNA hybrids consist of one strand of RNA paired with one strand of DNA. The HIV-AIDS reverse transcriptase (RT) forms such hybrids when copying its genomic RNA into DNA. The RT also has an RNase H domain that is structurally and functionally similar to the class I cellular RNase H and is necessary for several steps of viral DNA synthesis. R-loop hybrids (three-stranded nucleic acid structures) have two separated DNA strands, with one hybridized to RNA while the other is in a single-strand form. Such structures sometimes form during transcription and can lead to chromosomal breakage. However, they are also part of the normal process of switching (recombination) from one form of immunoglobulin to another, resulting in distinct isoforms of antibodies. Another form of hybrid are single or multiple ribonucleotides incorporated into DNA during replication [Reference 1]. The first two types of hybrids are substrates for class I and II RNases H. The third is uniquely recognized by type 2 RNases H, when the stretch of ribonucleotides embedded in DNA is less than four nucleotides long.

Dual activities of RNase H2; Aicardi-Goutières syndrome

Eukaryotic RNases H2 recognize and resolve RNA hybridized or covalently attached to DNA (two chemically distinct structures), using the same catalytic mechanism for hydrolysis. RNase H2 mutations that reduce catalytic activity, or fail to properly interact with *in vivo* substrates, cause Aicardi-Goutières syndrome (AGS). Mutations in seven genes are known to cause AGS, with more than 50% of AGS patients having mutations in any of the three subunits of RNase H2. We previously expressed (in *Escherichia coli*) and purified human RNases H2 with mutations corresponding to several of those seen in AGS patients; one such mutation, *RNASEH2A-G37S* (G37S), has significant loss of RNase H2 activity. Using the 3D structure of the human enzyme that we had determined, we could locate all known mutations in RNase H2 that cause AGS. The wide distribution of the mutations suggests that modest changes in stability and interaction with other unknown proteins, as well as loss of catalysis, can all cause AGS. A mutation near the catalytic center of G37S found in some AGS patients results in low RNase H2 activity for both embedded ribonucleotides in DNA and RNA/DNA hybrids [Reference 1]. We are developing mouse models of AGS to clarify which defects are associated with each RNase H2 activity.

Mice bearing the G37S mutation in homozygous form are perinatal lethal, i.e., either dead at birth or die within a few hours of birth [Reference 1]. Mutations in another gene, *TREX1* (which encodes a nuclease), also cause AGS, and it has been shown that homozygous knockout (KO) mice are viable but die after a few weeks owing to a cardiomyopathy, which can be prevented by blocking either an innate or adaptive immune response. In contrast, the G37S-mutant perinatal lethality and the fact that RNase H2 KO mice die during early embryogenesis suggest a more severe defect than that seen in *TREX1*-KO mice. Damaged DNA that finds its way into the cytoplasm can be sensed by the cGAS protein, producing the small molecule cGAMP, which interacts with the Sting protein, an important protein for the DNA-sensing in the innate immune pathway. Mice that are homozygous for G37S and deleted for the *cGAS* or *Sting* genes are mostly perinatal lethal but no longer exhibit increases in ISGs (interferon-stimulated genes). Interestingly, a small fraction of the double

G37S–*Sting* KO are viable, indicating only limited involvement of ISGs in perinatal lethality. Further studies are under way, which we expect will lead us to the cause of lethality.

To distinguish among the defects that persistent RNA/DNA hybrids and single ribonucleotides joined to DNA cause *in vivo*, Hyongi Chon, a former postdoctoral fellow, rationally designed a modified RNase H2 to make an enzyme unable to cleave single ribonucleotides embedded in DNA but that retained RNA/DNA hydrolytic activity. The mutant enzyme, which we called RED (ribonucleotide-excision deficient), resolves RNA/DNA hybrids, which are substrates of both RNase H1 and RNase H2. Unlike the mouse and human RNases H2, RNase H2 activity is not required in the yeast *Saccharomyces cerevisiae*. Employing the ease of genetic mutation studies in yeast, we demonstrated that, in yeast producing the RNase H2^{RED}, the enzyme acted *in vivo* by leaving embedded ribonucleotides (rNMPs) in DNA but was potent in removing RNA in RNA/DNA hybrids.

Embryonic lethality of mice *Rnaseh2b*–KO strains has been attributed to accumulation of rNMPs in DNA, but lethality could be the result of loss of RNA/DNA hydrolysis or a combination of both rNMP and RNA/DNA hydrolysis defects [References 1 & 2]. To distinguish among the possible causes of embryonic lethality, we generated a mouse that produces the RNase H2^{RED} enzyme. Mouse embryonic fibroblasts (MEFs) derived from *Rnaseh2*^{RED} mice have the same high level of rNMPs as seen in *Rnaseh2b*–KO MEFs [Reference 2]. Interestingly, the *Rnaseh2*^{RED} mice die around the same time as the *Rnaseh2b*–KO mice. Therefore, lethality of the KO and RED RNase H2 mouse strains may be caused by increased rNMPs in genomic DNA. *Rnaseh2a*^{G37S/RED} embryos also arrest at approximately the same stage as *Rnaseh2a*^{RED/RED} embryos because of better association of RNase H2^{RED} than RNase H2^{G37S} with DNA substrate containing embedded rNMPs. The result is important because some RNase H2–AGS patients have similar compound heterozygous mutations in which there may be a dominant mutated enzyme.

Our studies on RNase H2-RED have permitted us and others to assign specific substrates to each of the two activities and determine which functions are related to various phenotypes seen when RNase H2 is absent [References 1–3]. One of our goals is to produce an RNase H2 with robust incision at single rNMPs in DNA but with poor RNA/DNA hybrid cleavage (hybrid-defective [HD]) to complement the RNase H2-RED enzyme. We have identified amino-acid changes that appear to produce such RNase H2-HD and are in the early stages of studies in both cell cultures and mice.

Abasic substrates

The rNMPs embedded in DNA could be converted to abasic sites in which the flanking dNMPs would be connected by a ribose phosphate rather than an rNMP. In collaboration with the Storici and Tell groups, we examined the abilities of eukaryotic RNases H2 to cleave substrates containing a single ribose abasic (only sugar phosphate) site in duplex DNA. Prokaryotic RNases HII, but not eukaryotic RNases H2, can recognize and cleave at abasic rNMPs in duplex DNA. Little was known about abasic sites in RNA until, in collaboration with Vivian Cheung's group, we discovered that abasic sites are present in RNAs of yeast and human cells, and likely in all organisms. The abasic sites are located in or near R-loops. The Cheung group had previously shown that MPG (*N*-methylpurine DNA glycosylase) and APE1 (apurinic/apyrimidinic endonuclease) interact with R-loops. MPG is a glycosylase that removes the base of dNMPs or rNMPs (as reported in our papers with Cheung), which, in turn, can be cleaved by the APE1. Abasic sites in DNA are repaired by *Ape1* excision using the complementary DNA strand as template for repair. RNAs have no template to correct for the absence of a base. The association of abasic sites with R-loops is intriguing and suggests that RNAs complexed with DNA

are protected from RNase H activities. Indeed, we found that RNA transcripts, having formed an R-loop, pause and, in one case, serve as an enhancer for APOE, the most common gene associated with Alzheimer's disease. The RNA of the R-loop has an adenosine that becomes methylated and attracts MPG glycosylase, leading to an abasic site in the RNA, probably stabilizing the R-loop and protecting it from RNase H resolution [Reference 4].

Loss of RNase H1 in early B cell development activates the mitochondrial unfolded protein response without affecting the nuclear R-loops.

We made a knockout of the mouse *Rnaseh1* gene and discovered that two isoforms of RNase H1 are produced from a single mRNA by a leaky scanning method for differential translation. One protein is localized to the nucleus and a second is targeted to mitochondria. Nuclear DNA replication begins at fertilization, with mitochondrial DNA (mtDNA) beginning amplification several days later. We observed early embryonic death shortly after mtDNA replication should have begun, thereby linking the absence of mtDNA replication with lethality. We were curious to see the contribution effects in a system less complicated than embryonic development. We chose mouse B cell development because (1) B cells are not required for viability when mice are housed in a germ-free environment; (2) B cell development occurs in only a few rounds of cell duplication; (3) resting B cells are in G₀, providing a population of cells that respond together when stimulated; (4) many useful tools for analyses and manipulation are available; and (5) RNase H has potential, known substrates in B cell development.

We generated an *Rnaseh1* conditional KO mouse strain in which we can specifically knocked out the gene, using a CRE-lox method with the *Mb1* (the *Mb1* gene encodes the Ig- α signaling subunit of the B cell antigen receptor) promoter-driving CRE. Transcripts of *Mb1* are initiated from the earliest stage of B cell development and persist until plasmacytes are formed. B cells develop to the resting stage, at which point they can be stimulated to undergo isotype switching by class switch recombination (CSR), ultimately producing circulating antibodies. We found that *Mb1-CRE* KO of the *Rnaseh1* gene resulted in little or no circulating antibodies but did produce resting B cells, although yielding half as many B cells as did wild-type (WT) mice. Stimulation of these B cells initiated transitioning from G₀ to G₁ phase of the cell cycle, but essentially they never entered S-phase. The resting B cells had no RNase H1 activity, no mtDNA, and their mitochondria exhibited abnormal morphology. We performed RNA-Seq analyses of resting and 24 h-stimulated mutant and WT B cells to discover genes related to loss of mtDNA and/or a nuclear DNA-damage response. We observed reductions in the following pathways: the cell-cycle, the immune system, DNA replication, and in mitochondrion, RNA processing, and ribosomes. The 50% yield of resting B cells in the KO strain must occur during cell amplification in bone marrow. The loss of RNase H1 was initiated just prior to cell amplification and might limit the number of cell cycles. It is also possible that defects affecting the time of residence of the B cells in the bone marrow niche are affected. Loss of the *Nidogen1* gene (*NID1*) results in a 50% reduction of resting B cells compared with normal, the same reduction as in our KO mice. We noticed a significant difference between WT and mutant resting B cells for the *NID1* transcripts.

The list of genes with the highest difference between resting and stimulated KO mice are *Atf5*, *Gdf15*, *Atf3*, *Hspa9*, and *Ddit3*. *Atf5*, *Atf3*, and *Ddit3*, all hallmarks of the unfolded mitochondrial response (UPRmt). The activation of the UPRmt indicates that loss of mtDNA takes precedence over nuclear DNA-damage response, just as we observed in embryonic development when the *Rnaseh1* gene was deleted in the male and female gametes. We checked the presence of R-loops by DRIP-Seq and, surprisingly, found no alteration in R-loops, indicating the lack of a role RNase H1 in the processing of these structures.

The RNA exosome and RNases H cooperate to suppress R-loop-mediated genome instability.

In addition to RNase H processing, there are other mechanisms to resolve R-loops and/or prevent their formation. The RNA exosome is a major 3'-5' RNA degradation, multi-subunit complex in eukaryotes, which eliminates cryptic and defective transcripts, preventing their engagement with DNA and suppressing R-loop formation.

In the yeast *S. cerevisiae*, we are studying the interaction and cooperation between RNases H and the RNA exosome in preventing R-loop-mediated genome instability. We found that over-expression of RNase H1 partially suppressed the growth defects of exosome-deficient mutants, suggesting that some of the problems in these cells are caused by harmful R-loops. We observed that cells defective in both RNase H and exosome activities are hypersensitive to the drug hydroxyurea, which induces replicative stress by reducing the cellular dNTP supply, and that replicative stress in these conditions is eliminated by expressing the RNase H2-RED variant that processes R-loops.

We are using a genetic system that correlates R-loop accumulation with homologous recombination to further elucidate the interplay between RNases H and the exosome in R-loop processing. In this system, yeast strains contain a fragment of the mouse switch Mu sequence (from *IgH* locus) in an orientation that either facilitates or prevents R-loop formation. We found that RNAs with the fragment in both orientations accumulated in exosome-deficient mutants, indicating that these RNAs are targeted for degradation by the exosome. Moreover, strains with the fragment in the orientation that favors R-loop formation showed increased accumulation of RNA/DNA hybrids and recombination rates when both the exosome and RNases H were defective, compared with cells lacking only RNases H. We conclude that RNase H and the exosome pathways converge to promote genome stability by suppressing the harmful effects of R-loops.

Publications

1. Uehara R, Cerritelli SM, Hasin N, Sakhuja K, London M, Iranzo J, Chon H, Grinberg A, Crouch RJ. Two RNase H2 mutants with differential rNMP processing activity reveal a threshold of ribonucleotide tolerance in DNA for embryonic development. *Cell Rep* 2018 25:1135–1145.
2. Cerritelli SM, Iranzo J, Sharma S, Chabes A, Crouch RJ, Tollervy D, El Hage A. High density of unrepaired genomic ribonucleotides leads to Topoisomerase 1-mediated severe growth defects in absence of ribonucleotide reductase. *Nucleic Acids Res* 2020 48:4274–4297.
3. Liu Y, Rodriguez Y, Ross RL, Zhao R, Watts JA, Grunseich C, Bruzel A, Li D, Burdick JT, Prasad R, Crouch RJ, Limbach PA, Wilson SH, Cheung, VG. RNA abasic sites in yeast and human cells. *Proc Natl Acad Sci USA* 2020 117:20689–20695.
4. Watts JA, Grunseich C, Rodriguez Y, Liu Yaojuan, Li D, Burdick JT, Bruzel, Crouch RJ, Mahley RW, Wilson S, Cheung V. A common transcriptional mechanism involving R-loop and RNA abasic site regulates an enhancer RNA of APOE. *Nucleic Acids Res* 2022 50(21)12497–12514.

Collaborators

- Frederic Chedin, PhD, *University of California Davis, Davis, CA*
- Vivian G. Cheung, MD, *University of Michigan Life Sciences Institute, Ann Arbor, MI*

- Louis Dye, BS, *Microscopy and Imaging Core, NICHD, Bethesda, MD*
- Aziz El Hage, PhD, *University of Edinburgh, Edinburgh, UK*
- James Ibem, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Francesca Storici, PhD, *Georgia Institute of Technology, Atlanta, GA*
- Gianluca Tell, PhD, *Università degli Studi di Udine, Udine, Italy*
- David Tollervey, PhD, *University of Edinburgh, Edinburgh, UK*

Contact

For more information, email crouchr@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/crouch>.

Cellular Stress in Development and Disease

The overarching goal of the Unit is to build a foundation for a translational research program. The basic science component of the program will focus on investigating the regulation of the cellular integrated stress response (ISR) as (1) a mechanism for variable phenotypic expressions and (2) a potential therapeutic approach to relevant human diseases. The clinical component will focus on using lysosomal diseases, such as CLN3 (Batten disease), as models for the pursuits of investigator-initiated and sponsored interventional trials.

ISR is an evolutionarily conserved process capable of inducing pro-survival or pro-apoptotic status in cells experiencing endoplasmic-reticulum (ER) and other stresses by activating either autophagy or apoptosis. Cellular stresses, such as unfolded protein accumulation, trigger the ISR through one of the four known eIF2 α kinases. The integration occurs as Ser51-phosphorylated eIF2 α inhibits the guanine nucleotide exchange activity of eIF2B, halts the formation of the translation initiation ternary complex, and effectively attenuates global mRNA translation. The reduced ternary complex formation paradoxically allows for increased translation of selected mRNAs, many of whose protein products are involved in determining cell fate and may be specific in the response to the instigating stress (Sonenberg N, Hinnebusch AG. *Cell* 2009;136:731). As an instigator or sequelae, aberrant ISR is implicated in human disorders of metabolism (diabetes), growth (skeletal dysplasia, cancer) and neurologic processes (MEHMO, Down syndrome, Alzheimer's disease), amongst many others. Regulation of the ISR could provide therapeutic benefit.

CLN3 is a rare, fatal, pediatric, neurodegenerative, lysosomal disorder with no currently approved treatment. Syndromic CLN3 presentation includes vision loss, neurodevelopmental plateauing and decline, behavioral inflexibility and emotional lability, seizures, and motor dysfunction, as described previously (OMIM 204200) and also observed in a cohort of natural history study ([NCT03307304](#)) participants at the NIH. Accumulations of lipopigment consisting of carbohydrates, lipids, metal ions, and proteins (particularly mitochondrial ATP synthase subunit C [SCMAS]) form intracellular and lysosomal deposits that autofluorescence under UV light and present as fingerprint patterns under electron microscopy. The affected *CLN3* gene encodes a 468-amino acid, transmembrane, ubiquitously



An N. Dang Do, MD, PhD,
*Head, Unit on Cellular Stress in
Development and Diseases*

Danielle O'Mard, BS, MPH,
Laboratory Technician

Ewelina Dwojak, BS, MS,
Postbaccalaureate IRTA

expressed protein implicated in many cellular pathways, whose exact function remains to be elucidated.

Findings from cells and flies implicate perturbed ISR following stress induction in the CLN3-deficient compared with wild-type (WT) controls. Reduced expression of WT CLN3 lowered SH-SY5Y cell viability following treatment with tunicamycin (Wu D *et al. Biochem Biophys Res Commun* 2014;447:115), and reduced *Drosophila* survival following exposure to chemical inducers of oxidative stress (Tuxworth RI *et al. Hum Mol Genet* 2011;20:2037). Higher eIF2 α phosphorylation and BiP/Grp78 expression in fibroblast cell lines from individuals with CLN3 disease than in WT controls followed treatment with NH₄Cl (Wei H *et al. Hum Mol Genet.* 2008;17:469). Disturbances in the lysosome autophagic pathway may also interconnect with the ISR through the transcription factor EB (TFEB) (Martina JA *et al. EMBO J* 2016;35:479; Burton TD *et al. J Biol Chem* 2020;295:7418). TFEB is a widely studied autophagy regulator and a treatment target of interest for conditions involving lysosomes and neurodegeneration (Cortes CJ, La Spada AR. *Neurobiol Dis* 2019;122:83). Cln3 mutant mice treated with trehalose, a disaccharide activator of TFEB, had improved intracellular lipopigment accumulation, brain weight, pain sensitivity, and survival (Palmieri M *et al. Nat Commun* 2017;8:14338). Thus, further understanding of the ISR in CLN3 disease models would provide insight into the underlying pathophysiology and inform novel therapeutic approaches.

Development of a human sample biorepository and experimental models

The Unit on Cellular Stress in Development and Diseases was established in October 2021, and completed recruitment of laboratory personnel in April 2022. We are developing laboratory reagents, experimental models, and collaborations to address the overall objective of understanding the role of the integrated stress response in human diseases such as CLN3.

We continue to conduct extensive characterization of the phenotype of individuals with variants in CLN3, and establish a biorepository of corresponding biosamples (CSF, blood, urine). To date, our study contains the largest cohort of individuals with CLN3-related disorders extensively characterized and prospectively followed, with corresponding biosamples collected. This (1) permits the development and assessment of outcome measures applicable for therapeutic trials ([NCT03307304](#)); (2) builds the infrastructures for engagement in a sponsored Phase 1/2 study of miglustat therapy trial ([NCT05174039](#)), and in future investigator-initiated and sponsored interventional trials; and (3) provides the means for further CLN3 research through identification of disease-reflective biomarkers.

Using CSF samples from the natural history study, we identified the neurofilament light chain as a potential marker for disease monitoring [Reference 1], and glycerophosphodiester species as potential marker(s) for disease diagnosis [References 2&3]. Biomarker discovery efforts will continue through the use of commercial platforms (Olink® proximal extension assay), and through extramural collaborations to perform proteomic (David Sleat, NCL-Stiftung Research Award), transcriptomic (Susan Cotman), and metabolomic/lipidomic (Monther Abu-Remaileh) analyses of samples from Clinical and Basic Science projects. We also continue to work with collaborators (Jon Brudvig and Jill Weimer) on translating candidate biomarkers to screening applications

Additional Funding

- 2022 NICHD Early Career Award

Publications

1. Dang Do AN, Sinaii N, Masvekar RR, Baker EH, Thurm AE, Soldatos AG, Bianconi SE, Bielekova B, Porter FD. Neurofilament light chain levels correlate with clinical measures in CLN3 disease. *Genet Med* 2021 23(4):751–757.
2. Brudvig JJ, Swier VJ, Johnson TB, Cain JC, Pratt M, Rechtzigel M, Leppert H, Dang Do AN, Porter FD, Weimer JM. Glycerophosphoinositol is elevated in blood samples from CLN3 Δ ex7-8 pigs, CLN3 Δ ex7-8 mice, and CLN3-affected individuals. *Biomark Insights* 2022 17:11772719221107765.
3. Laqtom NN, Dong W, Medoh UN, Cangelosi AL, Dharamdasani V, Chan SH, Kunchok T, Lewis CA, Heinze I, Tang R, Grimm C, Dang Do AN, Porter FD, Ori A, Sabatini DM, Abu-Remaileh M. CLN3 is required for the clearance of glycerophosphodiesterases from lysosomes. *Nature* 2022 609:1005–1011.
4. Dang Do AN, Thurm AE, Farmer CA, Soldatos AG, Chlebowski CE, O'Reilly JK, Porter FD. Use of the Vineland-3, a measure of adaptive functioning, in CLN3. *Am J Med Genet A* 2022 188:1056–1064.

Collaborators

- Monther Abu-Remaileh, PhD, *Stanford University, Stanford, CA*
- Katharine E. Alter, MD, *Functional and Applied Biomechanics Section, NIH Clinical Center, Bethesda, MD*
- Eva H. Baker, MD, PhD, *Radiology and Imaging Sciences, NIH Clinical Center, Bethesda, MD*
- Brian P. Brooks, MD, PhD, *Ophthalmic Genetics and Visual Function Branch, NEI, Bethesda, MD*
- Carmen C. Brewer, PhD, *Audiology Unit, NIDCD, Bethesda, MD*
- Jon Brudvig, PhD, *Sanford Research, Sioux Falls, SD*
- Susan Cotman, PhD, *Massachusetts General Hospital, Boston, MA*
- Ryan K. Dale, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*
- Thomas E. Dever, PhD, *Section on Protein Biosynthesis, NICHD, Bethesda, MD*
- Alex Grinberg, DVM, *Mouse Transgenic Core, NICHD, Bethesda, MD*
- Hanna L. Hildenbrand, MS, OTR/L, *Occupational Therapy Service, NIH Clinical Center, Bethesda, MD*
- Laryssa A. Huryn, MD, *Ophthalmic Clinical Genetics Section, NEI, Bethesda, MD*
- Zou Jizhong, PhD, *Induced Pluripotent Stem Cells Core, NHLBI, Bethesda, MD*
- Mark D. Levin, MD, *Laboratory of Vascular & Matrix Genetics, NHLBI, Bethesda, MD*
- Jesse H. Matsubara, PT, DPT, *Functional and Applied Biomechanics, NIH Clinical Center, Bethesda, MD*
- Terri D. Nguyen, MS, OTR/L, *Occupational Therapy Service, NIH Clinical Center, Bethesda, MD*
- Carlo Pierpaoli, MD, PhD, *Laboratory on Quantitative Medical Imaging, NIBIB, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, NICHD, Bethesda, MD*
- David Sleat, PhD, *Center for Advanced Biotechnology and Medicine, Rutgers, The State University of New Jersey, Piscataway, NJ*
- Ariane G. Soldatos, MD, MPH, *Pediatric Neurology Consultation Service, NINDS, Bethesda, MD*
- Beth I. Solomon, MS, CCC-SLP, *Speech Language Pathology Section Rehabilitation Medicine Department, NIH Clinical Center, Bethesda, MD*
- Audrey E. Thurm, PhD, *Neurodevelopmental and Behavioral Phenotyping Service, NIMH, Bethesda, MD*
- Sara K. Young-Baird, PhD, *Uniformed Services University of the Health Sciences, Bethesda, MD*

- Qingjun Wang, PhD, *University of Kentucky College of Medicine, Lexington, KY*
- Jill Weimer, PhD, *Sanford Research, Sioux Falls, SD*
- Cristiane Zampieri-Gallagher, PhD, *Rehabilitation Medicine Department, NIH Clinical Center, Bethesda, MD*
- Wadih M. Zein, MD, *Ophthalmic Genetics and Visual Function Branch, NEI, Bethesda, MD*
- Wei Zheng, PhD, *Therapeutic Development Branch, NCATS, Bethesda, MD*

Contact

For more information, contact an.dangdo@nih.gov.

Mechanisms of Nuclear Genome Organization and Maintenance

We are interested in mechanisms of genome maintenance and organization. During interphase, chromosomes are surrounded by the nuclear envelope (NE), which separates the nuclear from cytoplasmic compartment of the cell. The sequestration of chromosomes within the nucleus has profound consequences for almost all aspects of gene expression and cell function. Communication between the nucleus and cytoplasm occurs through conduits called nuclear pore complexes (NPCs), which are embedded in the NE and consist of about 34 proteins called nucleoporins (Figure 1). Beyond nucleocytoplasmic trafficking, nucleoporins are important for chromosome organization, transcriptional control, RNA processing, cell signaling, and cell-cycle control. Both nucleoporins and soluble components of the nuclear trafficking machinery also perform transport-independent functions in mitotic chromosome segregation. The involvement of nucleoporins in such diverse events offers the intriguing possibility that they might coordinate these processes with nuclear trafficking and with each other. Moreover, nucleoporin dysfunction has important clinical implications: nucleoporin genes are frequently misregulated in cancers, and nucleoporin mutations cause congenital defects, pediatric nephrotic syndromes, and premature ovarian insufficiency. Nucleoporins are critical viral targets, and their disruption contributes to neurodegenerative conditions, including amyotrophic lateral sclerosis, frontotemporal dementia, and Huntington's disease.

Our research centers on nucleoporins, NPC-associated proteins (e.g., the SUMO pathway, spindle-checkpoint proteins), and other components of the nuclear transport machinery (e.g., the Ran pathway) throughout the cell cycle. Our goal is to define their biochemical roles and how their dysregulation causes human disease. We took a multifaceted approach toward this question, using both CRISPR-based strategies in mammalian cells and fly (*Drosophila melanogaster*) developmental genetics.

Selective degradation of nucleo-cytoplasmic transport proteins and their interphase function

Understanding the activities of individual nucleoporins has been complicated by their multifaceted nature, abundance, and unusual



Mary Dasso, PhD, Head, Section on Cell Cycle Regulation

Vasilisa Aksenova, PhD, *Contract Research Scientist*

Alexei Arnaoutov, PhD, *Staff Scientist*

Maia Ouspenskaia, DVM, *Biologist*

Abik Nandi, PhD, *Visiting Postdoctoral Fellow*

Sanjana Sundararajan, PhD, *Visiting Postdoctoral Fellow*

Reham Aljumaah, BA, *Predoctoral Intramural Research Training Award Fellow*

Sierra Ball, BA, *Postbaccalaureate Intramural Research Training Award Fellow*

Carolyn Egekeze, BA, *Postbaccalaureate Intramural Research Training Award Fellow*

Elizabeth Giordano, BA, *Postbaccalaureate Intramural Research Training Award Fellow*

Jacob Miller, BA, *Postbaccalaureate Intramural Research Training Award Fellow*

Ashley Person, BA, *Postbaccalaureate Intramural Research Training Award Fellow*

stability. To overcome these issues, we employed strategies for selective and rapid degradation of individual proteins within human tissue-culture cells (Figure 2). Specifically, we used CRISPR-Cas9 to construct cell lines in which sequences encoding auxin-induced degron (AID) domains are inserted into both alleles of targeted genes within cells that also stably express the transport inhibitor response 1 (TIR1) protein. TIR1 promotes rapid, selective degradation upon addition of the plant hormone auxin. Frequently, we also add a fluorescent tag to the targeted proteins, allowing their degradation to be monitored visually as well as biochemically. We have been successful in developing cell lines that allow conditional depletions of nucleoporins associated with different regions of the NPC (Figure 1).

Our recent findings regarding the roles of nucleoporins during interphase address three issues. First, we investigated the role of individual nucleoporins in NPC assembly and stability. Our results indicate that different regions of the NPC can persist independently after depletion of individual nucleoporins, suggesting that the NPC is a surprisingly modular structure. Second, we investigated the role of individual nucleoporins in different nuclear trafficking pathways, an assessment that includes evaluation of nuclear protein import, protein export, and RNA export. We are able to differentiate between the roles of individual nucleoporins and can specifically show that the TPR protein has a unique and important role in nuclear mRNA export via the transcription-export-2 (TREX-2) complex (see also below). Third, we investigated how organization of the nuclear transport machinery impacts development. In particular, we found that an evolutionarily controlled mechanism for association of Ran's GTPase-activating protein (RanGAP) is critical for the development of *Drosophila*. Defining the mechanism through which individual nucleoporins contribute to each of these processes will allow us to better design future experiments examining nucleoporin function in human development and disease.

Analysis of nucleoporins demonstrates that the NPC is a highly modular structure.

NPCs are built from many copies of roughly 34 distinct nucleoporins. Models of the NPC depict it as a composite of several sub-domains, which have been named the outer rings, inner ring, cytoplasmic fibrils, and nuclear basket. The outer-ring domains of the NPC are formed from the Y-complex, which contains nine core nucleoporins (SEH1, SEC13, NUP37, NUP43, NUP85, NUP96, NUP107, NUP133, and NUP160), with a tenth subunit (ELYS) required for chromatin recruitment. Other nucleoporins (NUP205, NUP188, NUP155, NUP93, and NUP35) form the inner-ring structures. The distinct roles of individual nucleoporins and their functional interactions remain poorly understood. Moreover, NPCs undergo a disassembly-reassembly cycle during mitotic division, and a lack of tools for acute manipulation of individual nucleoporins has therefore precluded the study of their roles in maintaining structures within pre-existing pores without complications from disruption of NPC assembly.

We added AID tags and fluorescent moieties by homozygously targeting gene loci encoding Y-complex and inner-ring nucleoporins. Auxin addition resulted in a rapid loss of the targeted proteins in each case, without degradation of other nucleoporins. We anticipated that loss of any Y-complex member should result in complete destabilization of the outer rings. While this was true after depletion of NUP96 or NUP107, the loss of other Y-complex members surprisingly left the outer-ring lattice in place. The findings suggest that the outer-ring structure is remarkably resistant to perturbations, once it is fully assembled, and show that its members are not of equivalent importance in sustaining its stability. Importantly, near-complete loss of the outer ring in NUP96-depleted cells did not cause collapse of the rest of the NPC, as demonstrated by immunostaining,

FIGURE 1. Auxin-induced degron (AID) tagging of nucleoporins

Left. Model of nuclear pore structure with cytoplasmic filaments (green) oriented upward and nucleoplasmic basket (reddish brown) oriented downward. Other domains of the NPC as indicated.

Right. Polypeptides associated with NPC sub-complexes, with fill colors corresponding to model domains. Text shows progress in obtaining AID-tagged cell lines for individual nucleoporins: reddish brown: successful tagging; black: in progress; yellow: tagging achieved but cell-line quality issues; grey: tagging unsuccessfully attempted.

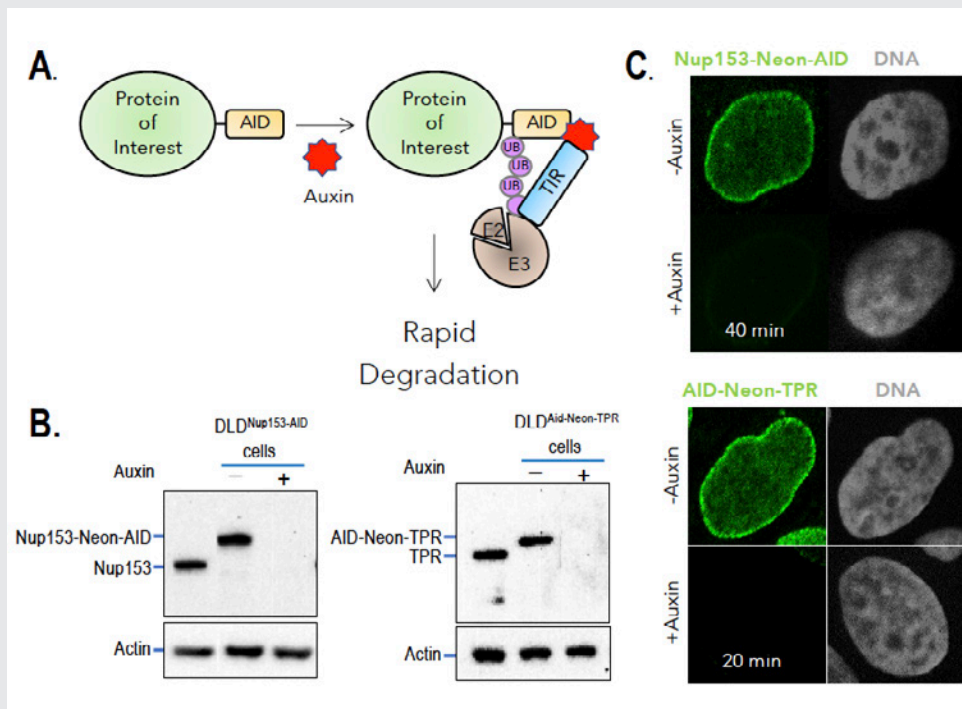
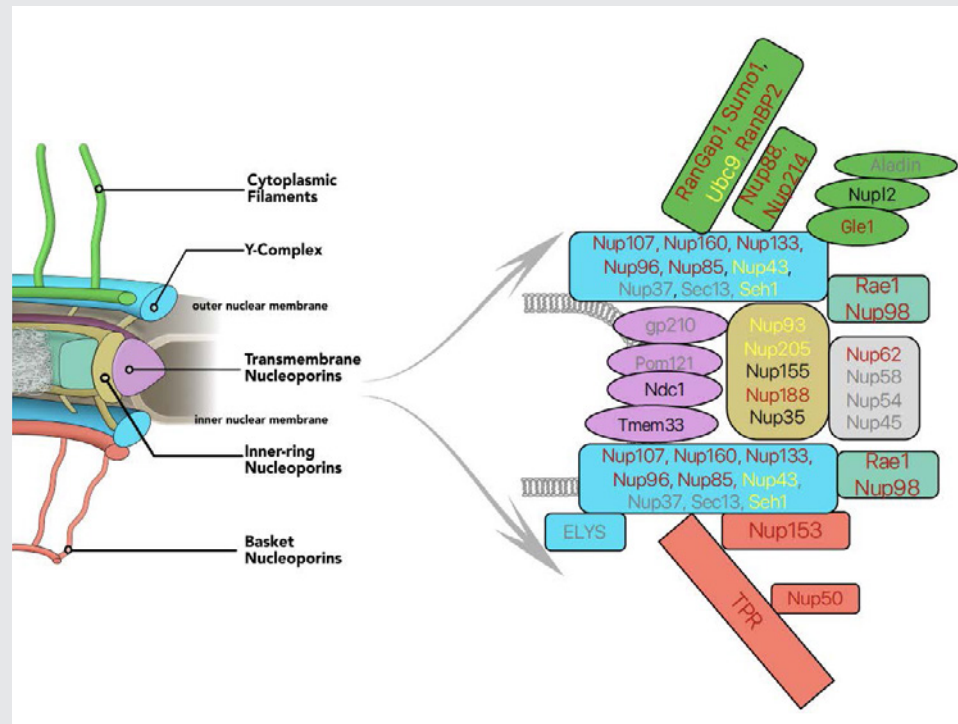


FIGURE 2. Auxin-induced degradation of AID-tagged nucleoporins

A. TIR1-expressing cells ubiquitinate and degrade proteins tagged with AID domains upon auxin addition. Nup153 and TPR were homozygously fused with the AID tag and a fluorescent marker (*neon green*) in TIR1-expressing cells. Rapid and uniform degradation is observed after auxin addition by Western blotting (B) or destruction of the fluorescent tag (C).

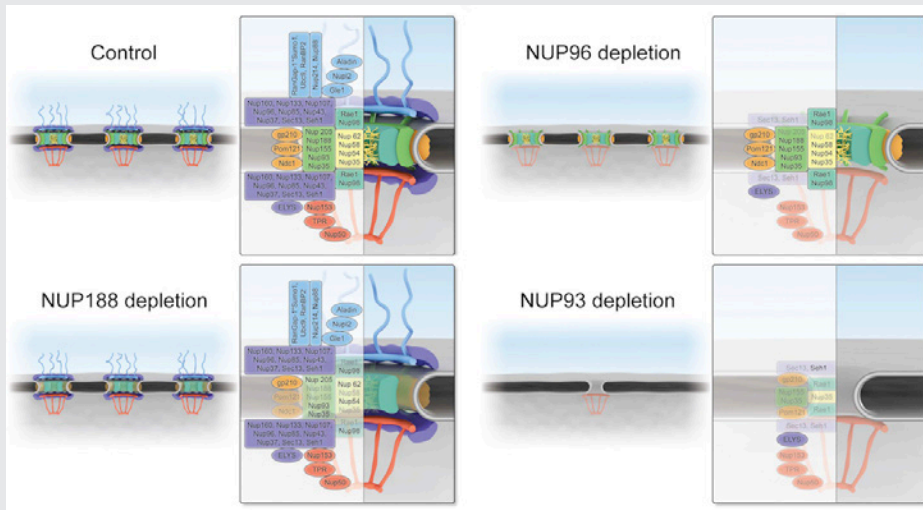


FIGURE 3. Schematic representations of NPC structure upon loss of NUP188, NUP96, or NUP93

live microscopy, and mass spectrometry. Particularly, the remarkable persistence of inner-ring nucleoporins indicated resilience of the NPC structure. Interestingly, depletion of the inner-ring nucleoporin NUP188 caused an NPC disassembly that was opposite to the profile after NUP96 depletion: inner-ring components were extensively displaced, while the components of the cytosolic fibrils, outer ring, and basket were largely unaffected. Also, there was a global reduction of almost all nucleoporins upon loss of NUP93. High-resolution scanning electron microscopy of residual NPCs after depletion of NUP96 or NUP93 further confirmed the status of these structures. Together, our results indicate that the inner and outer rings of the NPC form distinct and independent structures, and that NUP93 serves as an NPC lynchpin essential for both of them (Figure 3).

After depletion of the inner ring or outer rings, we tested whether the residual structures remained functional for the import and export of a model nuclear transport substrate. Remarkably, there were only minimal changes in both nuclear import and export rates upon loss of NUP96 or NUP188. However, NUP93 depletion caused a complete block in nuclear transport in both directions, confirming that global disruption functionally disabled NPCs. Taken together, the results indicate that persistent inner-ring or outer-ring structures could still act as conduits for vectorial nuclear trafficking and that these modules can support independent and redundant trafficking routes. Removal of both sets of structures forecloses all nuclear trafficking. Notably, the persistence of functional pores lacking a subset of canonical nucleoporins suggests that terminally differentiated cells might retain substantial nuclear trafficking even with divergent NPC composition. Differentiated cells might thus customize function through altered NPC composition, potentially modulating specific trafficking pathways or aspects of NPC activity, such as gene regulation and post-translational protein modifications.

Roles of nucleoporins in gene expression and RNA trafficking

A series of evolutionarily conserved complexes are co-transcriptionally recruited to nascent mRNAs, facilitating their processing as well as escorting them to and through the NPC, actions that are functionally linked; a failure to perform any of them during mRNA biogenesis directly impacts both upstream and downstream events. A key player in mRNA maturation is the transcription and export 2 (TREX-2) complex. Loss of the TREX-2 complex leads to defects in mRNA export, similar to the phenotype observed after loss of the major mRNA export receptor NXF1. The GANP subunit of TREX-2 localizes within the nucleus and associates with the NPC's nuclear

basket, which protrudes from the nucleoplasmic face of the NPC. In vertebrates, the nuclear basket comprises three nucleoporins (BSK-NUPs), called NUP153, TPR, and NUP50. BSK-NUPs have been implicated in numerous processes beyond protein import and export, including chromatin remodeling, control of gene expression, and protein modification, as well as mRNA processing and export.

It has been difficult to analyze discrete NPC functions in the absence of vertebrate BSK-NUPs; knockout of their genes is deleterious for organisms, and their depletion by RNAi requires extended incubations, potentially permitting the emergence of secondary phenotypes from prolonged NPC disruption or defective post-mitotic NPC re-assembly. We used AID to untangle the functions of individual BSK-NUPs in both nuclear-basket architecture and gene expression. We found that NUP153 and TPR bound to the NPC independently of each other and that loss of individual BSK-NUPs did not destabilize the NPC. We further found that TPR, but not NUP153 or NUP50, tethers the TREX-2 complex to the NPC. Loss of NUP50, NUP153, or TPR led to unique transcriptomic responses. Importantly, transcriptomic signatures after loss of TPR were more pronounced and similar to changes upon the loss of either the GANP subunit of the TREX-2 complex or of the RNA-export receptor NXF1. Moreover, similar to the case of NXF1 or GANP, loss of TPR led to retention of both upregulated and downregulated mRNA transcripts within the nucleus.

Taken together, these data support a unique role of TPR in transcription regulation and mRNA export through the TREX-2 complex. The findings both support our hypothesis that individual nucleoporins have distinct and non-redundant cellular functions, and they demonstrate the utility of the AID system to analyze their unique roles within cells.

Impact of Ran pathway organization beyond nuclear trafficking: development and mitosis

The Ran GTPase is a critical regulator of nuclear trafficking in eukaryotic cells. Ran's cytoplasmic GTPase-activating protein, RanGAP, and its chromatin-bound nucleotide exchange factor, RCC1, establish compartmental patterns for Ran's nucleotide-binding status: GTP-bound Ran (Ran-GTP) is abundant in the nucleus, while GDP-bound Ran (RanGDP) is abundant in the cytosol. The asymmetry drives nucleo-cytoplasmic trafficking through controlled cargo binding and release by karyopherins, a family of RanGTP-binding transport receptors.

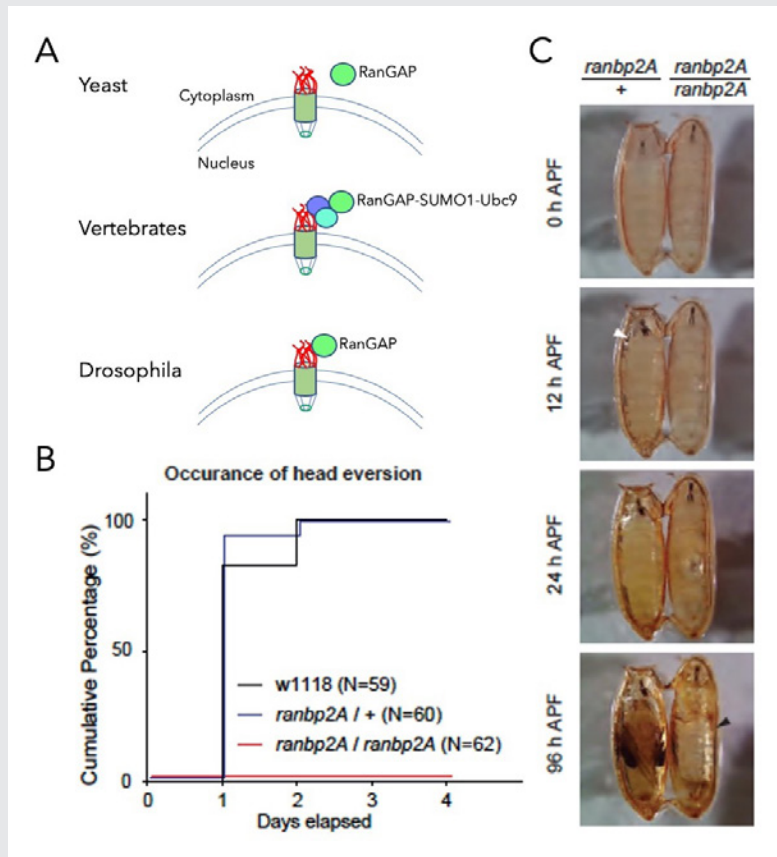
In multicellular organisms, RanGAP is tethered to the cytoplasmic side of the NE, but the functional consequences of its localization had not been clarified. To investigate the importance of RanGAP association with the NE, we used human tissue-culture cells and *Drosophila*. In mammals, RanGAP tethering is mediated by its covalent conjugation to SUMO1, a small ubiquitin-like protein. We made human tissue-culture cells in which RanGAP was neither SUMOylated nor localized to the NE. Surprisingly, the cells showed neither obvious changes in viability nor substantial defects in nucleo-cytoplasmic transport of a model substrate. In *Drosophila*, we found that SUMOylation of RanGAP did not control its association with the NE. Rather, we identified a specific region within the nucleoporin dmRanBP2 (*Drosophila melanogaster* RanBP2) that directly binds to dmRanGAP, tethering it to the NPC (Figure 4). A dmRanBP2 mutant lacking this region showed no apparent growth defects during larval stages, but arrested at the early pupal stage. The developmental arrest was rescued by a direct fusion of dmRanGAP to the dmRanBP2 mutant, indicating that recruitment of dmRanGAP to dmRanBP2 per se was necessary for the pupal ecdysis sequence during development. Collectively, the results indicate that, while the localization of dmRanGAP to the NE is widely conserved in multicellular organisms, the targeting mechanisms are not. Furthermore, the localization appears to be critical for developmental processes rather than for viability

FIGURE 4. Anchorage of dmRanGAP to dmRanBP2 is required for development.

A. Schematic representation of RanGAP association to NPCs. In yeast, RanGAP (green) is not bound to the NPC (top). In vertebrates, RanGAP binds to RanBP2 (red) in a complex that also contains Ubc9 (aqua) and SUMO1 (blue, middle). In flies, dmRanGAP binds directly to dmRanBP2 (bottom).

B. Cumulative step histogram of head eversion event during pupal stage. Late 3rd instar larvae (5-day old) were collected on day 0. While head eversion occurred robustly in controls (both *w1118* and heterozygous *ranbp2A* flies), no head eversion event was observed in homozygous *ranbp2A* flies.

C. Behavior analysis of pupal ecdysis. Heterozygous or homozygous *ranbp2A* flies were collected at the white pupal stage (APF: after puparium formation). The homozygous *ranbp2A* fly failed to undergo head eversion (white arrowhead) and air bubble translocation after 24 hours, and the air bubble occupied most of the pupa (black arrowhead) after 96 hours.



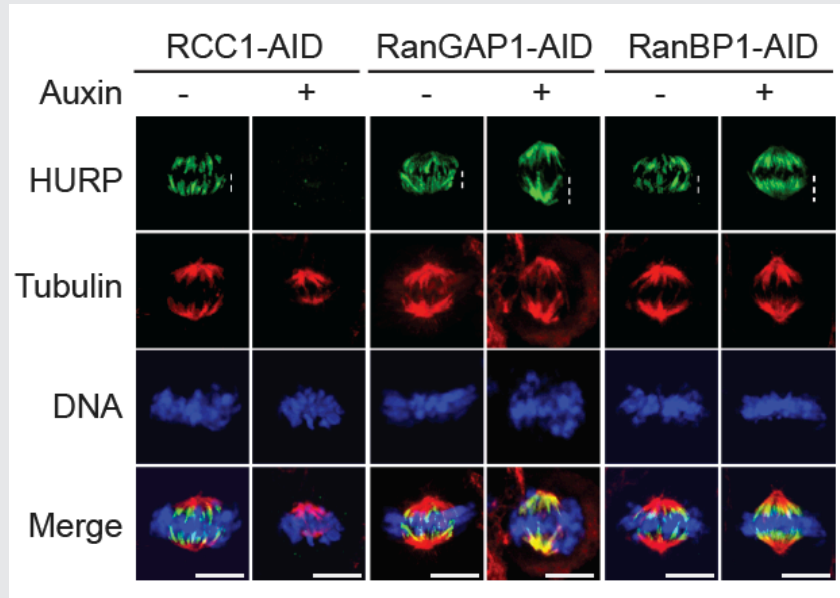
at the cellular level. We continue to explore the developmental consequences of releasing the interaction of dmRanGAP to dmRanBP2.

We have a long-standing interest in the process of chromosome segregation. The Ran GTPase pathway and nucleoporins promote chromosome segregation through their important roles in spindle assembly and cell-cycle progression. After mitotic NE breakdown, RCC1 generates RanGTP near chromosomes, while Ran distal to chromosomes is GDP-bound, directing spindle assembly through spatially regulated release of spindle assembly factors (SAFs) from karyopherins. To segregate chromosomes accurately, RanGTP distribution must be tightly regulated, both spatially and temporally. Defects in chromosome segregation lead to aneuploidy, a condition in which cells possess an abnormal number of chromosomes. The error-prone nature of Ran-dependent spindle assembly is particularly important during meiosis, potentially contributing to human pregnancy losses and genetic disorders, including Down's syndrome. Moreover, aneuploidy arising from mitotic divisions is a hallmark of many solid tumors.

RanBP1 is a RanGTP-binding protein that forms a stable heterotrimeric complex with Ran and RCC1 (RRR complex) *in vitro*, inhibiting RCC1's nucleotide exchange activity. We previously reported that RRR complex formation determines RCC1's partitioning between its chromatin-bound and soluble forms in embryonic systems, where RCC1 levels are very high, as well as specifically inhibiting the activity of soluble RCC1. Somatic

FIGURE 5. Regulation of mitotic Ran-GTP gradients by the RRR complex

Immuno-staining with HURP and tubulin antibodies of cells in HCT116^{RCC1- μ AID-3xFLAG}, HCT116^{RanGAP1-3mAID}, and HCT116^{RanBP1- μ AID-HA} cells treated with or without 1 mM Auxin for 5, 2, and 3 h, respectively. Cells also express TIR1. White dashed lines represent the length of HURP signal (*top row*). Scale bars = 10 μ m.



cells have less soluble RCC1 during mitosis, raising questions about whether RRR complex formation is important after early development. To investigate the mitotic role of the RRR complex after early development, we examined whether RanBP1 or a related protein called RanBP3 might be important for controlling mitotic RanGTP gradients within somatic cells. We systematically varied RanBP1 and RanBP3 levels in HCT116 or DLD1 cell lines through overexpression or fusion with AID tags. Consistent with earlier reports, RanBP1 was dispensable for interphase import or export of a model substrate, while RanBP3 appears to facilitate nuclear export via the Crm1 karyopherin. Within mitosis, altering RanBP1 levels substantially altered RCC1 dynamics on metaphase chromosomes, while altering RanBP3 levels did not. Moreover, we found dramatic re-localization of the SAF hepatoma up-regulated protein (HURP, a component of the spindle-assembly pathway) during metaphase, in direct correspondence with changes in RCC1 dynamics (Figure 5), showing that RanGTP levels and SAF activity near chromosomes correlate with altered RCC1 behavior. Analogous to findings in embryonic systems, the data indicate an important mitotic role in human somatic cells for RanBP1 in controlling RCC1 dynamics and determining the accurate spatial distribution and magnitude of Ran-GTP gradients, thus ensuring correct execution of Ran-dependent mitotic events.

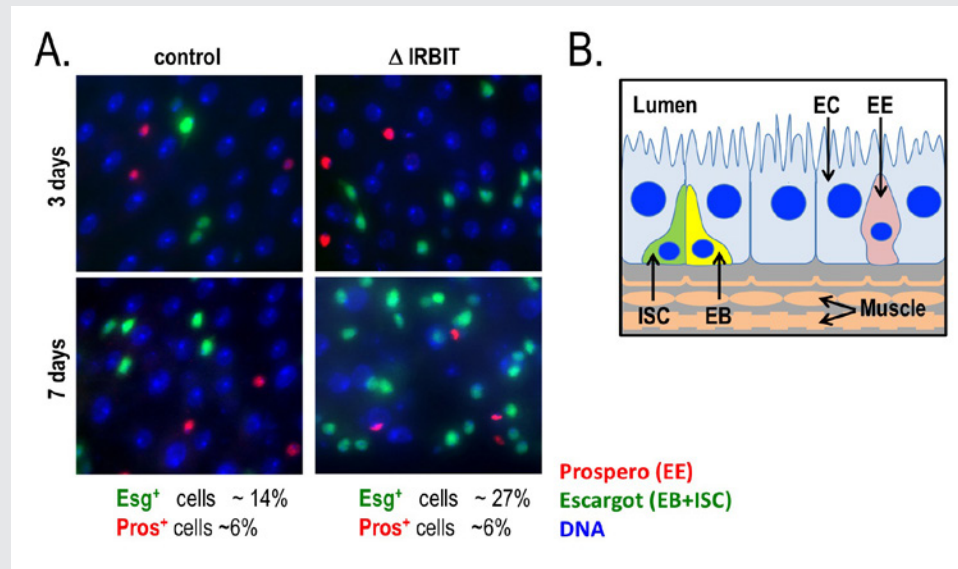
The role of the IRBIT protein in tissue homeostasis

We previously reported a conserved role for the IRBIT protein (IP₃-receptor-binding protein released with inositol 1,4,5-trisphosphate) in inhibiting ribonucleotide reductase (RNR), an enzyme that produces deoxynucleotide triphosphates (dNTPs) for DNA synthesis. We further found that mammalian tissue-culture cells show altered cell-cycle progression and genome stability in the absence of IRBIT, and that the mechanism is conserved between humans and *Drosophila*. Therefore, in collaboration with [Mihaela Serpe](#) and Brian Oliver, we used flies as a model organism to understand the role of IRBIT in development and tissue homeostasis.

In situ hybridization showed *IRBIT* expression in regions destined to become the midgut during embryogenesis, and that *IRBIT* is highly expressed in the adult midgut. The *Drosophila* midgut has a tubular structure and is

FIGURE 6. Loss of IRBIT disrupts tissue homeostasis in the *Drosophila* midgut.

A. Epithelia from control (*left*) and *IRBIT*^{-/-} (*right*) flies at three (*top*) or seven (*bottom*) days after eclosure. The guts were stained with the DNA dye Hoechst 33258 (*blue*), and antibodies against Prospero (*red*, to identify EE cells) and Escargot (*green*, to identify EB and ISC cells). We observed a rapid and progressive increase in the fraction of Escargot-positive cells in the *IRBIT*^{-/-} flies over time. In conjunction with additional experiments, the accumulation is indicative of accumulation of undifferentiated enteroblast progenitor cells.



B. Schematic of epithelium within the *Drosophila* midgut. EC: enterocyte; ISC: intestinal stem cell; EB: enteroblast; EE: enteroendocrine cell.

surrounded by visceral muscles. The adult midgut possesses a monolayered epithelium composed of four distinct cell types (Figure 6B): intestinal stem cells (ISCs), undifferentiated progenitor cells called enteroblasts (EBs), specialized absorptive enterocytes (ECs), and secretory enteroendocrine cells (EEs). The midgut is maintained through division of ISCs, giving rise to EBs, which in turn differentiate into EEs. Nutrients are absorbed from the lumen of the gut, which also contains a complex microbiota; the midgut acts both as a niche for commensal microbes and as the first line of defense against microbial pathogens. Like the intestine of vertebrates, the epithelium of the midgut has a remarkable regenerative capacity, which has been extensively exploited for the study of stem cell-driven tissue self-renewal, as well as tissue homeostasis during aging.

We examined IRBIT's role in the midgut by generating an *IRBIT* null fly (*IRBIT*^{-/-}) (Figure 6A). The midguts of one-day-old wild-type and *IRBIT*^{-/-} flies were essentially indistinguishable at the tissue-architecture level. However, we observed a rapid loss of tissue homeostasis in the *IRBIT*^{-/-} flies, with a progressive increase in relative numbers of undifferentiated enteroblast progenitor cells and tissue dysplasia. *IRBIT*^{-/-} flies also show fewer cell-cell contacts, when stained for junctional proteins in the posterior midgut epithelium, and altered gene expression patterns, reminiscent of changes associated with inflammation and aging. The phenotypes are fully rescued by expression of full-length IRBIT; further experiments suggested that altered dNTP pools likely contribute to the *IRBIT*^{-/-} phenotypes. Further analysis showed that the IRBIT-RNR pathway is essential to ensure correct differentiation of ISC progeny, and that it is a key downstream target of the GATAe transcription factor. Moreover, the GATAe-IRBIT-RNR pathway becomes dysfunctional as flies age, contributing to a characteristic accumulation of undifferentiated ISC progeny, which can be reversed by specifically inhibiting RNR within progeny cells. Collectively, our findings showed that RNR suppression by IRBIT is an important mechanism that directs differentiation of ISC progeny to maintain intestinal tissue homeostasis.

Publications

1. Li Y, Aksenova V, Tingey M, Yu J, Ma P, Arnaoutov A, Chen S, Dasso M, Yang W. Distinct roles of nuclear basket proteins in directing the passage of mRNA through the nuclear pore. *Proc Natl Acad Sci USA* 2021 118:e2015621118.
2. Chen S, Lyanguzova M, Kaufhold R, Plevock Haase KM, Lee H, Arnaoutov A, Dasso M. Association of RanGAP to nuclear pore complex component, RanBP2/Nup358, is required for pupal development in *Drosophila*. *Cell Rep* 2021 37:110151.
3. Aksenova V, Dasso M. Mapping paths through the nuclear pore complex. *Nat Cell Biol* 2022 24:6–7.
4. Aksenova V, Arnaoutov A, Dasso M. Analysis of Nucleoporin Function Using Inducible Degron Techniques. *Methods Mol Biol* 2022 2502:129–150.
5. Bley CJ, Nie S, Mobbs GW, Petrovic S, Gres AT, Liu X, Mukherjee S, Harvey S, Huber FM, Lin DH, Brown B, Tang AW, Rundlet EJ, Correia AR, Chen S, Regmi SG, Stevens TA, Jette CA, Dasso M, Patke A, Palazzo AF, Kossiakoff AA, Hoelz A. Architecture of the cytoplasmic face of the nuclear pore. *Science* 2022 376:6598.
6. Gao S, Esparza M, Dehghan I, Aksenova V, Zhang K, Batten K, Ferretti MB, Begg BE, Cagatay T, Shay JW, García-Sastre A, Goldsmith EJ, Chen ZJ, Dasso M, Lynch KW, Cobb MH, Fontoura BMA. Nuclear speckle integrity and function require TAO2 kinase. *Proc Natl Acad Sci U S A* 2022 119:e2206046119.

Collaborators

- Beatriz Fontoura, PhD, *University of Texas Southwestern Medical Center, Dallas, TX*
- Amnon Harel, PhD, *Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel*
- Lindsey Hayes, MD, *Johns Hopkins University School of Medicine, Baltimore, MD*
- André Hoelz, PhD, *Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA*
- Brian C. Oliver, PhD, *Laboratory of Biochemistry and Genetics, NIDDK, Bethesda, MD*
- Lionel Pintard, PhD, *Institut Jacques Monod, Université Paris Diderot, CNRS, Paris, France*
- Jeffrey Rothstein, MD, *Brain Science Institute, Johns Hopkins University School of Medicine, Baltimore, MD*
- Thomas U. Schwartz, PhD, *Massachusetts Institute of Technology, Cambridge, MA*
- Mihaela Serpe, PhD, *Section on Cellular Communication, NICHD, Bethesda, MD*
- Alexander Strunnikov, PhD, *Guangzhou Institutes of Biomedicine and Health, Guangzhou, China*
- Katharine Ullman, PhD, *Huntsman Cancer Institute, University of Utah, Salt Lake City, UT*
- Weidong Yang, PhD, *Temple University, Philadelphia, PA*

Contact

For more information, email dassom@mail.nih.gov or visit <http://sccr.nichd.nih.gov>.

Regulation of Mammalian Cell Proliferation and Differentiation

Our current research program focuses on one question: how cancer cells can be selectively destroyed with little or no harm to non-malignant cells. To that end, we discovered a family of small molecules (termed the 'WX8-family') that selectively target the PIKFYVE phosphoinositide kinase, an enzyme that is essential for lysosome homeostasis and autophagy in cultured mammalian cells, and the *PIKFYVE* gene, which is essential for pre-implantation mouse development. We showed that the WX8-family of PIKFYVE inhibitors can selectively terminate autophagy-dependent cancer cells and pluripotent cancer stem cells, in culture and in tumor xenografts. The NICHD/NIH now holds a patent on the application of these molecules for cancer therapy. We described the characteristics of these molecules and their therapeutic applications in two research publications [References 2 & 5] and in one manuscript that has been accepted for publication [Roy A, Chakraborty AR, Nomanbhoy T, DePamphilis ML. PIP5K1C phosphoinositide kinase deficiency distinguishes PIKFYVE-dependent cancer cells from non-malignant cells. *Autophagy* 2022, accepted with revisions; journal impact factor 17], as well as in one manuscript that is in preparation [Roy A, Chakraborty AR, DePamphilis ML. PIKFYVE inhibitors induce IL24 amplified endoplasmic reticulum stress in melanoma cells and tumors].

Developmental acquisition of p53 functions

Remarkably, the p53 transcription factor, referred to as "the guardian of the genome," is not essential for mammalian development. Moreover, efforts to identify p53-dependent developmental events have produced contradictory conclusions. Given the importance of pluripotent stem cells as models of mammalian development, and their applications in regenerative medicine and disease, resolving these conflicts is essential. We attempted to reconcile disparate data into justifiable conclusions predicated on reports that p53-dependent transcription is first detected in late mouse blastocysts, that p53 activity first becomes potentially lethal during gastrulation, and that apoptosis does not depend on p53. Furthermore, p53 does not regulate expression of genes required for pluripotency in embryonic stem cells (ESCs); it contributes to ESC genomic stability and differentiation. Depending on conditions, p53 accelerates initiation of apoptosis in ESCs in response to DNA damage, but



Melvin L. DePamphilis, PhD,
*Head, Section on Eukaryotic DNA
Replication*

Arup Chakraborty, PhD, *Research
Fellow*

Ajit Roy, PhD, *Postdoctoral Fellow*

cell-cycle arrest, as well as the rate and extent of apoptosis in ESCs, are p53-independent. In embryonic fibroblasts, p53 induces cell-cycle arrest to allow repair of DNA damage, and cell senescence to prevent proliferation of cells with extensive damage.

Cell death and the p53 enigma during mammalian embryonic development

Twelve forms of programmed cell death (PCD) have been described in mammalian cells, but which of them occurs during embryonic development and the role played by the p53 transcription factor and tumor suppressor remains enigmatic. Although p53 is not required for mouse embryonic development, some studies conclude that PCD in pluripotent embryonic stem cells from mice (mESCs) or humans (hESCs) is p53-dependent, whereas others conclude that it is not. Given the importance of pluripotent stem cells as models of embryonic development and their applications in regenerative medicine, resolving this enigma is essential. We attempted to reconcile contradictory results based on the facts that p53 cannot induce lethality in mice until gastrulation, as stated above, and that experimental conditions could account for differences in results with ESCs. Consequently, activation of the G2-checkpoint in mouse ESCs is p53-independent and generally, if not always, results in non-canonical apoptosis. Once initiated, PCD occurs at equivalent rates and to equivalent extents regardless of the presence or absence of p53. However, depending on experimental conditions, p53 can accelerate initiation of PCD in ESCs and late-stage blastocysts. In contrast, DNA damage following differentiation of ESCs *in vitro* or formation of embryonic fibroblasts *in vivo* induces p53-dependent cell-cycle arrest and senescence.

Combined inhibition of p38MAPK and PIKfyve synergistically disrupts autophagy to selectively target cancer cells.

In nutrient-poor conditions, autophagy buffers metabolic stress and counteracts the effects of chemotherapy and radiation on cancer cells, which depend on autophagy for survival. However, clinical trials targeting autophagy have failed to produce successful anticancer treatments using currently available inhibitors. Recent studies have shown that PIKfyve kinase inhibitors disrupt lysosome function in autophagy and can selectively kill certain cancer cells. Analysis of biochemical changes caused by PIKfyve inhibition revealed that resistant cells contain significantly elevated levels of cellular p38MAPK (p38 mitogen-activated protein kinase) protein and phosphorylation. Expression of the lysosomal protein LAMP2 (lysosome-associated membrane protein 2), carrying phospho-mimetic mutations of the p38MAPK phosphorylation sites, prevented all effects caused by PIKfyve inhibition-induced lysosome dysfunction. Thus, the activation of p38MAPK in response to PIKfyve inhibition revealed a novel compensatory role in maintaining lysosome function in autophagy. The functional cooperation between the cellular PIKfyve and p38MAPK pathways in regulating lysosome homeostasis was found to be especially important in cancer cells. Combined inhibition of PIKfyve and p38MAPK activities synergistically blocked autophagy-mediated protein degradation, prevented cathepsin maturation, and markedly reduced the viability of many cancer cell types without affecting the viability of normal cells. Furthermore, combined PIKfyve and p38MAPK inhibitors synergistically reduced tumor growth in mice bearing xenografts of human colorectal adenocarcinoma, suggesting a novel way to target cancer cells by prolonged inhibition of autophagy using lower drug concentrations. The study demonstrates that PIKfyve and p38MAPK cooperate to regulate lysosome homeostasis and that their combined inhibition synergistically blocks autophagy to reduce cancer cell viability *in vitro* and *in vivo*.

Selective elimination of pluripotent stem cells by PIKfyve-specific inhibitors

Inhibition of PIKfyve phosphoinositide kinase selectively kills autophagy-dependent cancer cells by disrupting lysosome homeostasis. We showed that PIKfyve inhibitors can also selectively eliminate pluripotent embryonal carcinoma cells (ECCs), embryonic stem cells, and induced pluripotent stem cells under conditions where differentiated cells remain viable. PIKfyve inhibitors prevented lysosome fission, induced autophagosome accumulation, and reduced cell proliferation in both pluripotent and differentiated cells, but they induced death only in pluripotent cells. We confirmed the ability of PIKfyve inhibitors to distinguish between pluripotent and differentiated cells with xenografts derived from ECCs. Pretreatment of ECCs with the PIKfyve-specific inhibitor WX8 suppressed their ability to form teratocarcinomas in mice, and intraperitoneal injections of WX8 into mice harboring teratocarcinoma xenografts selectively eliminated pluripotent cells. Differentiated cells continued to proliferate, but at a reduced rate. The results provide a proof of principle that PIKfyve-specific inhibitors can selectively eliminate pluripotent stem cells *in vivo* as well as *in vitro*.

PIKfyve-specific inhibitors restrict replication of many coronaviruses *in vitro* but not in a murine model of COVID-19.

The ongoing COVID-19 pandemic has claimed more than 6 million lives and continues to test the world economy and healthcare systems. To combat this pandemic, the biological research community has shifted efforts to the development of medical countermeasures, including vaccines and therapeutics. However, to date, the only small molecules approved for the treatment of COVID-19 in the United States are the nucleoside analogue Remdesivir and the protease inhibitor Paxlovid, although several compounds have received Emergency Use Authorization and many more are currently being tested in human efficacy trials. One such compound, Apilimod, is being considered as a COVID-19 therapeutic in a Phase II efficacy trial. However, at the time of writing, there were no published efficacy data in human trials or animal COVID-19 models. We showed that, while Apilimod and other PIKfyve inhibitors have potent antiviral activity in various cell lines against multiple human coronaviruses, these compounds worsen disease in a COVID-19 murine model when given prophylactically or therapeutically.

Patent

DePamphilis ML, Sharma G, Roy A, Marugan JJ, Ferrer M. Autophagy Modulators for Use in Treating Cancer: *US Patent 11,471,460*, October 18, 2022; for the WX8-family of PIKfyve inhibitors described in:

1. Sharma G, Guardia CM, Roy A, Vassilev A, Saric A, Griner LN, Marugan J, Ferrer M, Bonifacino JS, DePamphilis ML. A family of PIKfyve inhibitors with therapeutic potential against autophagy-dependent cancer cells disrupt multiple events in lysosome homeostasis. *Autophagy* 2019;15(10):1694–1718;
2. Roy A, Chakraborty AR, Nomanbhoy T, DePamphilis ML. PIP5K1C Phosphoinositide kinase deficiency distinguishes PIKfyve-dependent cancer cells from non-malignant cells. *Autophagy* 2022;under review;
3. References 2, 5, and 6 in this report.

Publications

1. Klionsky DJ, et al., DePamphilis ML, et al., Roy A, et al., Sharma G, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition). *Autophagy* 2021 17(1):1–382.
2. O'Connell CE, Vassilev A. Combined inhibition of p38MAPK and PIKfyve synergistically disrupts autophagy to selectively target cancer cells. *Cancer Res* 2021 81(11):2903–2917.

3. Jaiswal SK, Raj S, DePamphilis ML. Developmental acquisition of p53 functions. *Genes (Basel)* 2021 12(11):1675.
4. Raj S, Jaiswal SK, DePamphilis ML. Cell death and the p53 enigma during embryonic development. *Stem Cells* 2022 40(3):227–238.
5. Chakraborty AR, Vassilev A, Jaiswal SK, O’Connell CE, Ahrens JF, Mallon BS, Pera MF, DePamphilis ML. Selective elimination of pluripotent stem cells by PIKfyve specific inhibitors. *Stem Cell Rep* 2022 17(2):397–412.
6. Logue J, Chakraborty AR, Johnson R, Goyal G, Rodas M, Taylor LJ, Baracco L, McGrath ME, Haupt R, Furlong BA, Soong M, Prabhala P, Horvath V, Carlson KE, Weston S, Ingber DE, DePamphilis ML, Frieman MB. PIKfyve-specific inhibitors restrict replication of multiple coronaviruses *in vitro* but not in a murine model of COVID-19. *Commun Biol* 2022 5(1):808.

Contact

For more information, email melvin.depamphm@nih.gov or visit <http://depamphilislab.nichd.nih.gov>.

Mechanism and Regulation of Eukaryotic Protein Synthesis

We study the mechanism and regulation of protein synthesis, focusing on GTPases, protein kinases, translation factors, and mRNA features that control this fundamental cellular process. We use molecular-genetic and biochemical studies in yeast and human cells to dissect the structure-function properties of translation factors, elucidate mechanisms that control protein synthesis, and characterize how mutations in the protein-synthesis apparatus cause human disease. Of special interest are the translation initiation factors eIF2, a GTPase that binds methionyl-tRNA to the ribosome, and eIF5B, a second GTPase that catalyzes ribosomal subunit joining in the final step of translation initiation. We also investigate stress-responsive protein kinases that phosphorylate eIF2alpha, as well as viral regulators of these kinases, and how cellular phosphatases are targeted to dephosphorylate eIF2alpha. We are characterizing eIF2gamma mutations that are associated with the MEHMO syndrome, a novel X-linked intellectual disability syndrome, and we are investigating the function of the translation factor eIF5A, with a focus on its ability to stimulate the peptidyl transferase activity of the ribosome and facilitate the reactivity of poor substrates such as proline. We are also examining the role of the hypusine modification on eIF5A and the role the factor plays in polyamine-regulated gene-specific translational control mechanisms, and we are characterizing metabolite control of translation via non-canonical upstream open reading frames (uORFs) in select mRNAs.

Evolutionarily conserved inhibitory uORFs sensitize *Hox* mRNA translation to start-codon selection stringency.

Selection of the translation start site (typically an AUG codon) in eukaryotes is influenced by context nucleotides flanking the AUG codon and by levels of the factors eIF1 and eIF5. A major recent focus of the lab has been to study translational control by start-codon selection. We conducted a search of mammalian genes and identified five homeobox (*Hox*) gene paralogs initiated by AUG codons in conserved suboptimal context, as well as 13 *Hox* genes that contain evolutionarily conserved upstream open reading frames (uORFs) that initiate at AUG codons in poor sequence context [Reference 1]. Our collaborator with Rachel Green mapped the 5' end of the *Hox* mRNAs,



Thomas E. Dever, PhD, Head,
Section on Protein Biosynthesis

Ivaylo P. Ivanov, PhD, *Staff Scientist*

Byung-Sik Shin, PhD, *Staff Scientist*

Arya Vindu, PhD, *Visiting Fellow*

Sara Young-Baird, PhD,
Postdoctoral Fellow

Chune Cao, *Biological Laboratory Technician*

Joo-Ran Kim, BS, *Special Volunteer*

Thomas Saba, BS, *Predocutorial
Intramural Research Training
Award Fellow*

revealing that the mRNAs are much shorter than previously reported and lack proposed alternative translation elements. We found that the conserved uORFs inhibit *Hox* reporter expression and that altering the stringency of start-codon selection by overexpressing translation initiation factors eIF1 or eIF5 modulates the expression of *Hox* reporters. We also showed that modifying ribosome homeostasis by depleting a large ribosomal subunit protein or treating cells with sublethal concentrations of puromycin lowers the fidelity of start-codon selection. Given that the *Hox* genes encode developmental regulators of animal body plans, our findings reveal that alteration in start-codon selection stringency has the potential to regulate global gene expression programs, including *Hox* gene-directed body plan formation in animals.

Translational control by metabolite-sensing nascent peptides

In ongoing studies, we searched for additional mRNAs containing noncanonical uORFs. One such candidate was identified in plants in the mRNA encoding GDP-L-galactose phosphorylase (GGP), a control enzyme in the vitamin C biosynthetic pathway. Using reporter assays in mammalian cells and, *in vitro*, using rabbit reticulocyte lysates, we revealed that a uORF-like element in the GGP mRNA mediates translational control by vitamin C. We propose that interaction of vitamin C with the GGP uORF nascent peptide in the ribosome exit tunnel causes the ribosome to pause and that queuing of subsequent scanning ribosomes results in increased initiation on the uORF and prevents ribosome access to the GGP ORF. We hypothesize that the mechanism by which a paused elongating ribosome promotes initiation at an upstream weak start site via ribosome queuing may underlie the control of translation of other mRNAs, especially those whose translation is derepressed by conditions that impair ribosome loading.

Analysis of eIF2 γ mutations that link intellectual disability with impaired translation initiation

Protein synthesis plays a critical role in learning and memory in model systems, and our studies have linked a human X-linked intellectual disability (XLID) syndrome with altered function of eIF2. In previous studies, we showed that the MEHMO syndrome (named based on the patient phenotypes: mental [intellectual] disability, epilepsy, hypogonadism and hypogenitalism, microcephaly, and obesity) is caused by mutations in the *EIF2S3* gene, which encodes the gamma subunit of eIF2. Using genetic and biochemical techniques in yeast models of human MEHMO-syndrome mutations, we previously characterized several mutations that impair eIF2 function, disrupt eIF2 complex integrity, and alter the stringency of translation start-codon selection. Our collaborators have linked the *EIF2S3* mutations with variable levels of motor delay, microcephaly, ID, epilepsy, central obesity, and diabetes, thus revealing a broad genetic spectrum and clinical expressivity of the MEHMO syndrome [Reference 2]. Over the past year, we have been characterizing additional novel EIF2S3 mutations identified in patients with the MEHMO syndrome. The work complements our previous studies characterizing other *EIF2S3* mutations linked to the MEHMO syndrome and will further our understanding of the molecular defects in eIF2 that cause the varied symptoms of the disease.

In previous studies, we characterized induced pluripotent stem (iPS) cells derived from a patient with the MEHMO syndrome. Our studies revealed defects in general protein synthesis, constitutive induction of the integrated stress response (ISR), a cellular stress response pathway that alters protein synthesis to mount an adaptive response, and hyper-induction of the ISR under stress conditions. The *EIF2S3* mutation also impaired neuronal differentiation by the iPS cells. Interestingly, the drug ISRIB, an activator of the eIF2 guanine nucleotide exchange factor, rescued the cell growth, translation, and neuronal differentiation defects associated with the *EIF2S3* mutation, offering the possibility of therapeutic intervention for the

MEHMO syndrome [Reference 3]. Our current efforts are aimed at generating a mouse model of the MEHMO syndrome.

Molecular analysis of the hypusine-containing protein eIF5A and polyamine control of protein synthesis

The translation factor eIF5A is the sole cellular protein containing the unusual amino acid hypusine [N^{ϵ} -(4-amino-2-hydroxybutyl)lysine]. In previous studies, we showed that eIF5A promotes translation elongation and that such activity depends on its hypusine modification. Moreover, using *in vivo* reporter assays and *in vitro* translation assays, we showed that eIF5A in yeast, like its bacterial homolog EF-P, is especially critical for the synthesis of proteins containing runs of consecutive proline residues. In collaboration with Rachel Green, we reported that eIF5A functions globally to promote both translation elongation and termination. Moreover, using our *in vitro* reconstituted assay system, we showed that the structural rigidity of the amino acid proline contributes to its heightened requirement for eIF5A and that eIF5A could functionally substitute for polyamines to stimulate general protein synthesis. Together with Marat Yusupov, we found that eIF5A binds in the ribosome E site to the hypusine residue projecting toward the acceptor stem of the P-site tRNA. Based on these findings, we propose that eIF5A and its hypusine residue function to reposition the acceptor arm of the P-site tRNA to enhance reactivity towards either an aminoacyl-tRNA, for peptide bond formation, or a release factor, for translation termination.

In ongoing experiments, we are further investigating the hypusine modification on eIF5A. The modification is formed in two steps: first, an *n*-butylamine moiety from spermidine is transferred to a specific Lys side chain on eIF5A, whereupon hydroxylation of the added moiety completes the formation of hypusine. In contrast to the essential deoxyhypusine synthase, which catalyzes the first step in hypusine formation, the *LIA1* gene, encoding the hydroxylase, is non-essential in yeast. We identified mutations in eIF5A that cause synthetic growth defects in cells lacking the hydroxylase. Interestingly, the mutations map to the ribosome-binding face of eIF5A. Our results are consistent with the notion that the hydroxyl modification helps bind and position eIF5A and its hypusine residue to effectively promote the reactivity of the peptidyl-tRNA on the ribosome.

We also linked eIF5A to the regulation of polyamine metabolism in mammalian cells [Reference 4]. The enzyme ornithine decarboxylase (ODC) catalyzes the first step in polyamine synthesis. ODC is regulated by a protein called antizyme, which, in turn, is regulated by another protein called antizyme inhibitor (AZIN1). The synthesis of AZIN1 is inhibited by polyamines, and an unusual uORF in the leader of the *AZIN1* mRNA is critical for the regulation. The uORF lacks a canonical AUG start codon and initiates at a near-cognate codon instead. We found that high polyamine levels enhance translation initiation from the near-cognate start site of the uORF, and, remarkably, polyamine induction of uORF translation depends on the sequence of the encoded polypeptide, including a highly conserved Pro-Pro-Trp (PPW) motif that causes polyamine-dependent pausing of elongating ribosomes.

Extending these studies, we identified eIF5A as a sensor and effector for polyamine control of uORF translation. Using reconstituted *in vitro* translation assays, we found that synthesis of a PPW peptide, like translation of polyproline sequences, requires eIF5A. Moreover, the ability of eIF5A to stimulate PPW synthesis was inhibited by polyamines and could be rescued by increasing eIF5A levels. Taken together, our studies showed that eIF5A functions generally in protein synthesis and that modulation of eIF5A function by polyamines can be exploited to regulate specific mRNA translation [Reference 4]. We are now exploring

the possibility that polyamine regulation of eIF5A underlies translational control of mRNAs encoding other enzymes and regulators of polyamine biosynthesis.

In recent studies examining translational control by polyamines, we identified the yeast high-affinity polyamine transporter [Reference 5]. Using ribosome profiling, we identified mRNAs whose translation was sensitive to changes in polyamine levels. One of the mRNAs encoded a member of the drug-proton antiporter (DHA1) family of transporters called Hol1. We showed that *HOL1* was required for yeast growth under limiting polyamine conditions and for high-affinity polyamine uptake by yeast. Together with [Anirban Banerjee's lab](#), we showed that purified Hol1 transports polyamines. The leader of the *HOL1* mRNA contains a highly conserved uORF encoding the peptide MLLLPS. We found that polyamine inhibition of the translation factor eIF5A impairs translation termination at the Pro-Ser-stop (PS) motif of the uORF to repress Hol1 synthesis under conditions of elevated polyamines. Our findings reveal that polyamine transport, like polyamine biosynthesis, is under translational autoregulation by polyamines in yeast, highlighting the extensive control cells impose on polyamine levels. In ongoing studies, we are characterizing *HOL1* homologs in the pathogenic yeast *Candida albicans* and testing the hypothesis that combined inhibition of Hol1 and polyamine synthesis might be an effective means to block growth of this pathogenic yeast.

Analysis of the role of eEF2 and its diphthamide modification in translation elongation

We are also studying the translation elongation factor eEF2. Like its bacterial ortholog EF-G, eEF2 promotes translocation of tRNAs and mRNA from the A site to the P site on the ribosome following peptide bond formation. In most eukaryotes and archaea, a conserved histidine residue at the tip of eEF2 is post-translationally modified to diphthamide through the action of seven non-essential proteins. The function of diphthamide and the rationale for its evolutionary conservation are not well understood. The name diphthamide is derived from diphtheria, a disease of the nose and throat caused by the bacterium *Corynebacterium diphtheriae*. Infections with *C. diphtheriae* can lead to respiratory distress and even death; however, an effective vaccine is available. The bacterium expresses a toxin that ADP-ribosylates the diphthamide residue, leading to inactivation of eEF2. Several additional bacterial pathogens, including *Pseudomonas aeruginosa* and *Vibrio cholerae*, express distinct toxins that also modify the diphthamide residue and inactivate eEF2.

Based on a cryo-electron microscopy structure of eEF2 bound to the yeast 80S ribosome, obtained during our previous collaboration with Venki Ramakrishnan's lab (Cambridge, UK), we hypothesized that diphthamide has at least two functions: first, to disrupt the decoding interactions of rRNA with the codon-anticodon duplex in the ribosomal A site; and second, to help chaperone the codon-anticodon interaction as the A-site tRNA is translocated to the P site. In ongoing studies, we are further exploring the role of diphthamide in promoting the accuracy and efficiency of translation elongation. We first identified mutations in eEF2 that sensitize cell growth to the loss of diphthamide. Then, we used these mutants to characterize the translational defects associated with loss of diphthamide. Our preliminary data indicate that loss of diphthamide impairs ribosome processivity during elongation as a result of increased levels of frameshifting and translation termination at out-of-frame stop codons. Such increased frameshifting in yeast and mammalian cells lacking diphthamide occurs at both programmed frameshifting sites in the HIV and SARS-CoV-2 viruses and throughout translation elongation at non-programmed sites. We propose that diphthamide, despite its non-essential nature in yeast, has been conserved throughout evolution to maintain the fidelity of translation elongation and block spurious

frameshifting events that would impair the production of the native proteins and generate novel frameshifted proteins that might be deleterious to the cell.

Molecular analysis of eIF5B and a translational fidelity checkpoint at subunit joining

The translation factor eIF5B is a GTPase required for the last step of translation initiation: the joining of the large ribosomal subunit to the small subunit poised on the start codon of an mRNA. The eIF5B binds to the 40S subunit and collaborates in the correct positioning of the initiator Met-tRNA_i^{Met} on the ribosome in the later stages of translation initiation, gating entrance into elongation. Working with Jody Puglisi and colleagues, we helped show that eIF5B promotes subunit joining by repositioning the acceptor arm of Met-tRNA_i^{Met} and that the factor also plays an important role in translation start-site selection, ensuring high fidelity in this process, which establishes the reading frame for translation on an mRNA.

Additional Funding

- Intramural Targeted Anti-COVID-19 (ITAC) Award (2021–2023): “Control of Ribosomal Frameshifting on the SARS-CoV-2 mRNA”

Publications

1. Ivanov IP, Saba JA, Fan CM, Wang J, Firth AE, Cao C, Green R, Dever TE. Evolutionarily conserved inhibitory uORFs sensitize Hox mRNA translation to start codon selection stringency. *Proc Natl Acad Sci USA* 2022 119:e2117226119.
2. Kotzaeridou U, Young-Baird SK, Suckow V, Thornburg AG, Wagner M, Harting I, Christ S, Strom T, Dever TE, Kalscheuer VM. Novel pathogenic EIF2S3 missense variants causing clinically variable MEHMO syndrome with impaired eIF2gamma translational function, and literature review. *Clin Genet* 2020 98:507–514.
3. Young-Baird SK, Lourenço MB, Elder MK, Klann E, Liebau S, Dever TE. Suppression of MEHMO syndrome mutation in eIF2 by small molecule ISRIB. *Mol Cell* 2020 77:875–886.
4. Ivanov IP, Shin BS, Loughran G, Tzani I, Young-Baird SK, Atkins JF, Dever TE. Polyamine control of translation elongation regulates start site selection on antizyme inhibitor mRNA via ribosome queuing. *Mol Cell* 2018 70:254–265.
5. Vindu A, Shin BS, Choi K, Christenson ET, Ivanov IP, Cao C, Banerjee A, Dever TE. Translational autoregulation of the *S. cerevisiae* high-affinity polyamine transporter Hol1. *Mol Cell* 2021 81:3904–3918.

Collaborators

- John Atkins, PhD, *University College Cork, Cork, Ireland*
- Anirban Banerjee, PhD, *Unit on Structural and Chemical Biology of Membrane Proteins, NICHD, Bethesda, MD*
- Israel Fernandez, PhD, *St. Jude's Children's Research Hospital, Memphis, TN*
- Adam Geballe, MD, *The Fred Hutchinson Cancer Research Center, Seattle, WA*
- Terri Goss Kinzy, PhD, *Illinois State University, Normal, IL*
- Rachel Green, PhD, *The Johns Hopkins University School of Medicine, Baltimore, MD*
- Vera Kalscheuer, PhD, *Max Planck Institut für Molekulare Genetik, Berlin, Germany*
- Eric Klann, PhD, *New York University, New York, NY*

- Stefan Leibau, MD, *Eberhard Karls Universität Tübingen, Tübingen, Germany*
- Joseph Puglisi, PhD, *Stanford University, Palo Alto, CA*
- Matthew Sachs, PhD, *Texas A&M University, College Station, TX*
- Naomi Taylor, MD, PhD, *Pediatric Oncology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Daniel Wilson, PhD, *Institut für Biochemie und Molekularbiologie, Universität Hamburg, Hamburg, Germany*
- Marat Yusupov, PhD, *Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France*

Contact

For more information, email thomas.dever@nih.gov or visit <http://spb.nichd.nih.gov>.

Molecules and Therapies for Craniofacial and Dental Disorders

Embryonic development of the craniofacial complex requires tightly controlled molecular crosstalk between proliferating and differentiating cell networks to form the most intricate structures in the human body. During embryogenesis, perturbations in environmental and/or genetic milieu can negatively affect craniofacial development. While considerable progress has been made in studying isolated genetic mutations leading to syndromic and non-syndromic craniofacial disorders, the broad molecular-genetic mechanisms driving morphogenetic events have yet to be sufficiently explored and understood. Such gaps in our understanding have restricted clinical treatment options for patients affected by common developmental anomalies, such as cleft palate or tooth agenesis. Thus, there is a strong biologic rationale for a thorough investigation of basic molecular mechanisms driving craniofacial structure morphogenesis, which may pave the way toward translatable therapeutic developments for patients.

The overarching goal of our research program is to conduct basic and translational studies of genetic and molecular mechanisms involved in craniofacial development, with the primary aim of unveiling novel regulatory molecules and putative patient-centric therapeutic solutions for craniofacial and dental disorders. Among the molecular pathways known to control craniofacial development is Wnt/ β -catenin signaling. Wnts are also well known as upstream effectors of osteogenesis and odontogenesis. Our lab demonstrated, for the first time, the successful *in utero* correction of cleft palate defects in a *Pax9*^{-/-} mouse genetic model by small-molecule neutralizing therapy targeting Wnt-antagonizing proteins. We are now actively investigating additional drug-delivery approaches and molecular targets for the modulation of Wnt signaling *in vivo* to permit targeted correction of both cleft palate and tooth agenesis. Our research group employs basic principles of developmental biology, next-generation sequencing, regenerative medicine, tissue engineering, and drug delivery models to identify and validate novel approaches to restore molecular equilibrium in genetic models of the highly relevant human diseases cleft palate and tooth agenesis. Actively fostering collaborations with both intramural and extramural investigators, from basic scientists, engineers, to clinicians, the lab aims to pioneer innovative approaches toward the treatment of patients



Rena D'Souza, MS, DDS, PhD,
*Head, Section on Craniofacial
Genetic Disorders, Director of the
National Institute of Dental and
Craniofacial Research*

Fahad K. Kidwai, BDS, MSc, PhD,
Clinical Staff

Resmi Raju, PhD, *Postdoctoral
Fellow*

Parna Chatteraj, *Lab Manager*

Jeremie Oliver Piña, MS, MBA,
Predoctoral Fellow

affected by craniofacial disorders of development. Through these rigorous and robust preclinical, proof-of-principle studies, we hope to open up pathways toward novel clinical trials for the treatment of previously unpreventable disorders affecting the craniofacial complex.

Toward a novel *in utero* therapy for the prenatal cure of cleft palate in a mouse genetic model

Cleft palate (CP), together with cleft lip, is among the most common birth defects in humans, occurring in up to 1 in 500 live births. Such birth defects can inflict heavy physical, mental, psychosocial, and financial burdens on patients and their caregivers throughout life, often requiring many stages of complex surgical correction with varying success rates. Hence, there remains a substantial need for innovative approaches to alleviate the burden of postnatal care for these patients. While considerable progress has been made in studying isolated genetic mutations leading to syndromic and non-syndromic cleft disorders, the broad molecular genetic mechanisms driving osteogenic differentiation from palatal shelf out-growth, elevation, and fusion events have yet to be sufficiently explored and understood. Such gaps in our understanding have restricted clinical treatment options for patients affected by cleft disorders. Given that prenatal molecular diagnostics have made CP identifiable earlier in gestation, the advancement of safe and efficacious interventions *in utero* to correct CP has become feasible.

The objective of our work is to investigate the molecular genetic mechanisms driving palatal osteogenesis and to optimally manipulate key signaling environments *in utero* to promote CP correction in a mouse genetic model. If properly explored, such information can be applied to the development of novel therapeutics that can benefit individuals with isolated or syndromic CP defects who face complex surgeries and the arduous burden of life-long care. The long-term goal of this research is to investigate the spatiotemporal molecular

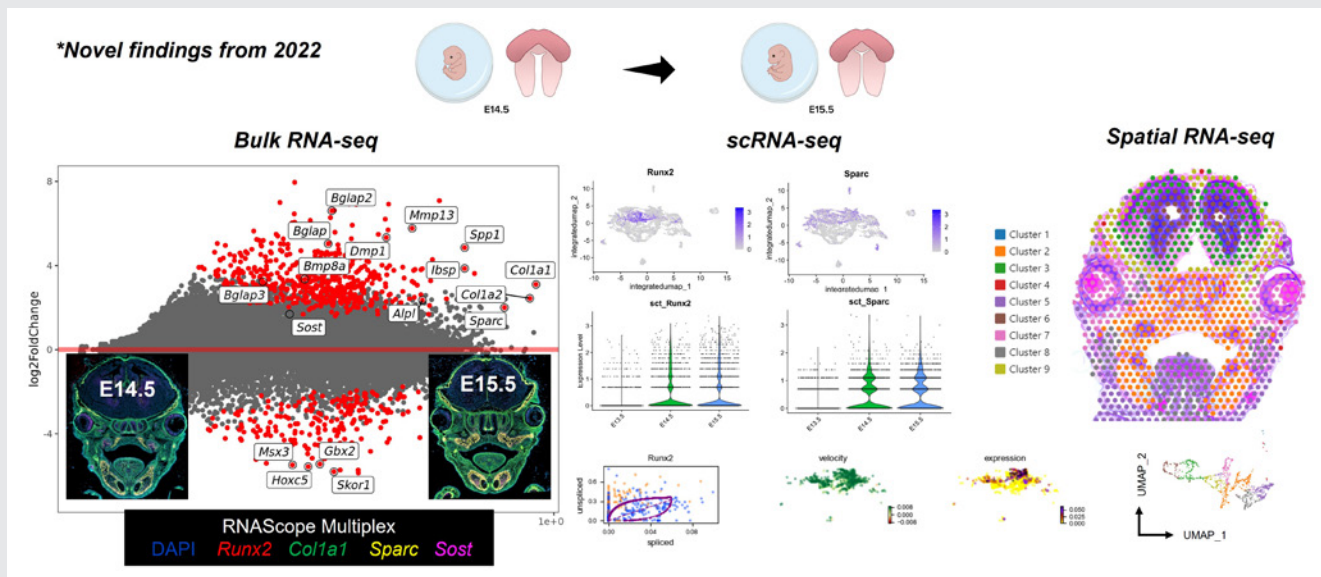


FIGURE 1. Characterization of palate osteogenesis begins with fusion.

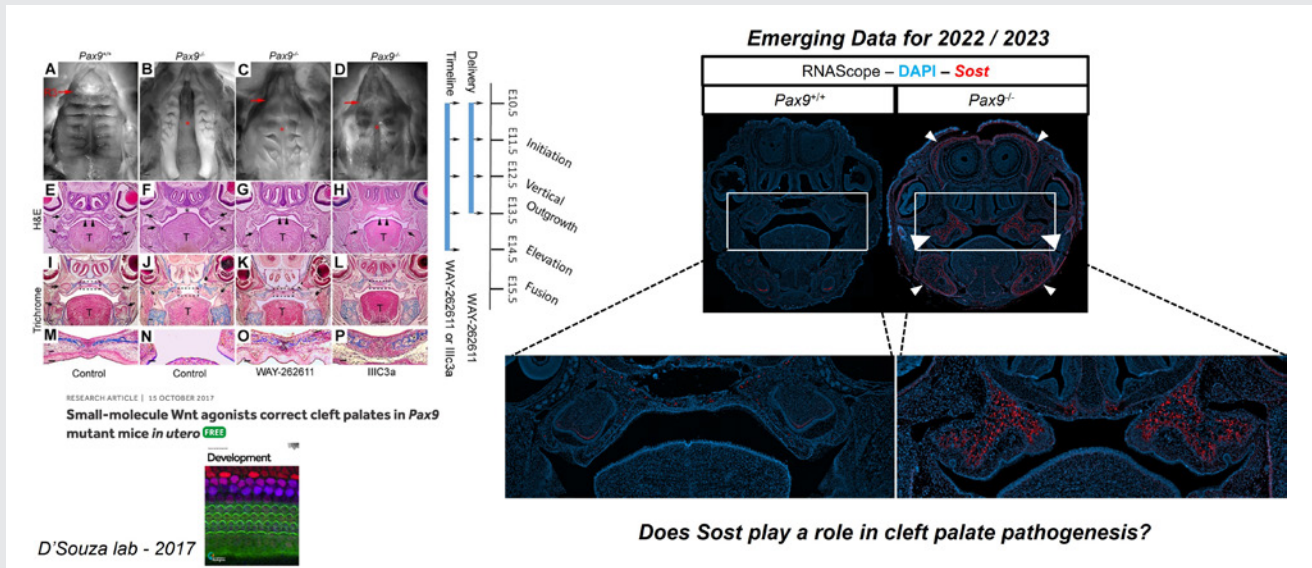


FIGURE 2. Wnt signaling drives palate osteogenesis and fusion.

mechanisms driving osteogenic differentiation in normal palate development and in CP dysmorphogenesis, using *Pax9*^{-/-} as a model. Differential multiomic profiles of expression in spatial biological context will unveil the molecular framework for the development of novel therapeutic strategies to optimize signaling environments during development. The proposed research will test the following hypotheses: (1) Wnt signaling effector function is critical for osteogenesis of the embryonic palate; and (2) loss of the up-stream master regulator of Wnt signaling homeostasis, Pax9, results in the disruption of palatal osteogenesis via up-regulation of sclerostin (*Sost*), a potent inhibitor of Wnt signaling and bone formation (Figures 1&2). The specific aims of this proposal are as follows: (1) to define the spatiotemporal transcriptomic profile of embryonic palate osteogenesis via unbiased stage-specific signature mapping of cell populations in the normal murine secondary palate; (2) to differentially compare epigenomic, proteomic and gene expression signatures of Wnt-related osteogenesis in *Pax9*^{-/-} CP; and (3) to pilot novel *in utero* drug delivery approaches and molecules, based on the molecular profiles observed *in situ*, for the prenatal cure of CP. The proposed research will add novel foundational knowledge of multiomic morphogenetic expression gradients of key Wnt signaling regulators within the embryonic palate and will propose an innovative therapeutic model whereby palatal clefts may be corrected *in utero*.

The proposed research will improve unbiased mechanistic understanding of palatal bone development and carry out the first preclinical study of intra-amniotic small-molecule and antibody-replacement drug delivery for targeted palatal osteogenesis. The validation of a translational *in utero* drug delivery system for reversal of single-gene CP disorders will lead to preventive and corrective prenatal therapeutic interventions in humans.

Role of Wnt signaling pathway genes in dentinogenesis

Dental caries is one of the most common diseases worldwide. Two mineralized tissue barriers in the tooth are composed of an outer and inner layer of enamel and dentin, respectively. Trauma or dental caries can

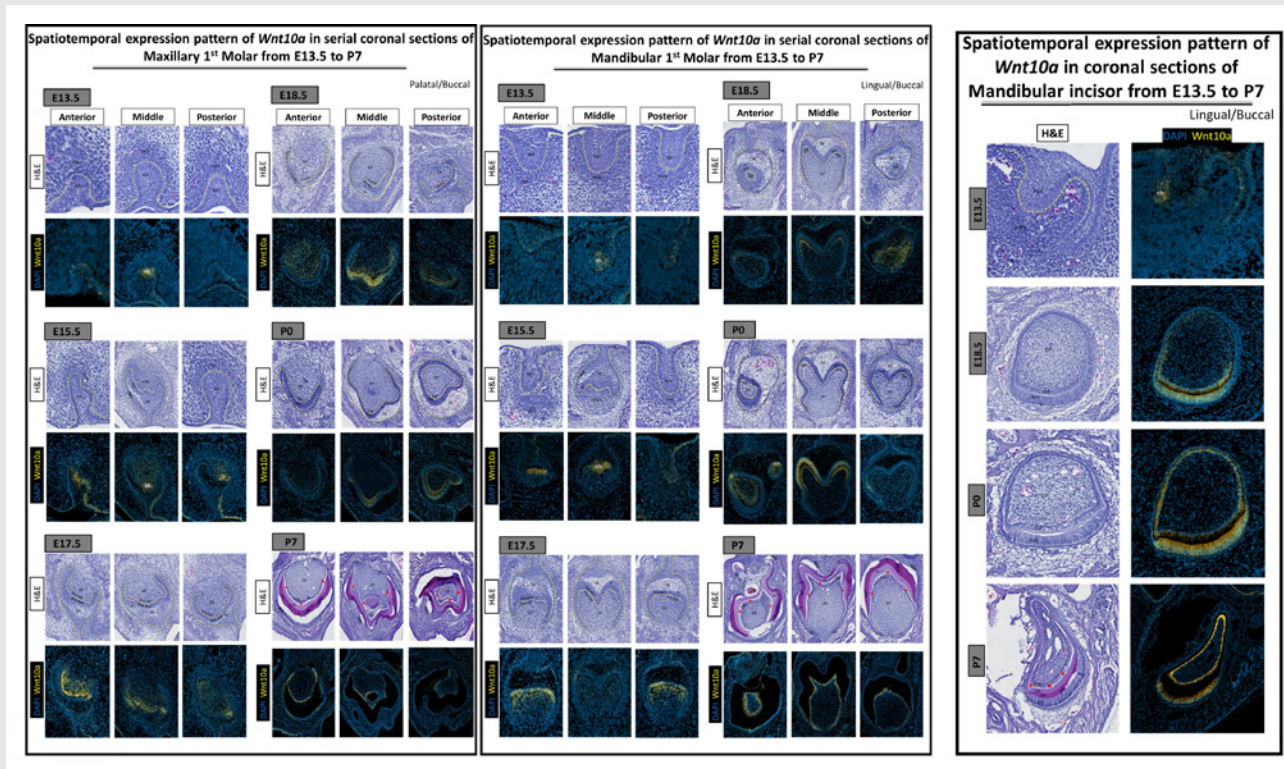


FIGURE 3. Wnt signaling drives palate osteogenesis and fusion.

lead to the destruction of this mineralized barrier as well as inner specialized cell layer (odontoblast cells) in the dental pulp. The odontoblast cell layer is responsible for forming a dentin bridge (reparative dentinogenesis) to protect the inner vital pulp tissue. However, the process of reparative dentinogenesis is limited to the critical size of the tooth caries. In a large dental decay, the inner pulp is exposed to the oral cavity and, if not treated at an early stage, can undergo necrosis. Traditional clinical treatments used to replace the affected tooth structure are based on artificial filling materials. This highlights the importance of developing a therapy that stimulates and directs the cells in dental pulp to repair injuries afflicting dentin and pulp. One of the main focuses of regenerative dental research is to preserve the natural architecture of tooth structure following deep dental cavities. Modern regenerative dentistry challenges us to apply the current scientific knowledge of developmental biology into translational dental medicine. The Wnt/ β -catenin signaling pathway plays a major role in dentinogenesis, as well as dentin repair following tooth damage. Wnt/ β -catenin signaling has thus emerged as a major target in dentin regeneration and repair by overstimulating it in a variety of ways (Figures 3&4). However, there is a gap in our understanding of how modulation of the Wnt/ β -catenin signaling genes that are involved during dentinogenesis and reparative dentinogenesis occurs in a time- and region-specific manner.

Our preliminary data confirm the spatiotemporal involvement of Wnt10a in odontoblast differentiation and dentinogenesis. A synergistic expression pattern of Wnt10a and the Wnt regulators Sost and Dkk1 was observed in early stages of normal embryonic tooth development. At the later stage of P7, following enamel and dentin development, Wnt10a expression is confined to a single layer of odontoblasts and reduced dental epithelium

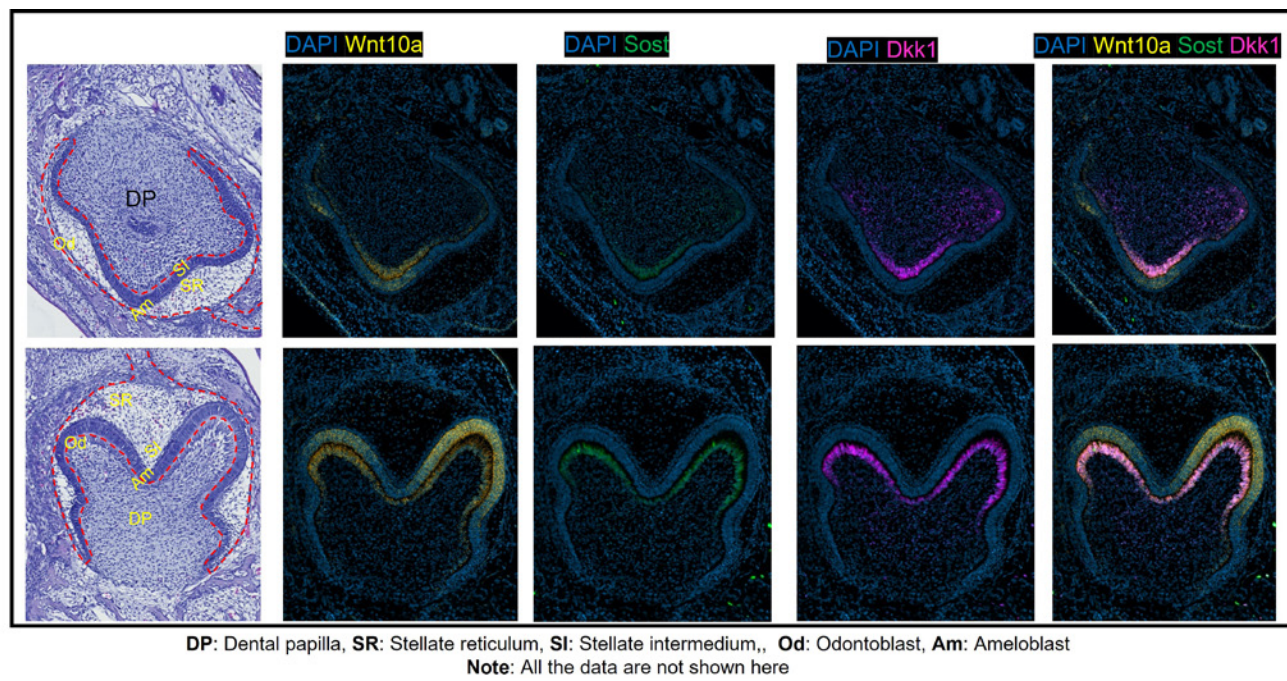


FIGURE 4. Wnt10a and Wnt modulators

with a strong expression in the cervical loop area. Expression of the Wnt modulators Sost and Dkk1 was reduced or disappeared at P7 developmental stage. Gradient expression pattern of Wnt10a expression in ameloblast and odontoblast along the cusp tips and grooves suggests the involvement in cusp morphogenesis.

Identifying and comparing the spatiotemporal expression of Wnt signaling molecules in dentin development and the dentin repair process will provide new insight into the nature of dentin regeneration and help us identify potential targets for regenerative therapy in dentistry.

Transcriptomic analysis reveals that differential signaling events control maxillary and mandibular incisor development.

Tooth development is a complex process involving the reciprocal interactions between the dental epithelium and the underlying mesenchyme. Morphogenic gradients mediate this epithelium-mesenchymal interaction. It is known that morphogenic gradients influence the distinct patterning of dentition. The broad range of patterning in dentition provides a valuable system in which to study the heterogeneity in molecular mechanisms during the process of odontogenesis. However, little is known about the distinct morphogenic gradients or signature transcriptomic factors responsible for the formation of mouse incisiform in the maxillary vs. mandibular domain. Our study therefore focuses on identifying the exclusive morphogenic gradients or signature transcriptomic factors within the maxillary vs. mandibular incisiform field during the early odontogenesis.

Our unbiased next-generation sequencing experiments revealed exclusive morphogenic gradients and signature transcriptomic patterns expression in the maxillary (Six3, Otx2 and Garta2) vs mandibular incisiform (Hand2 and

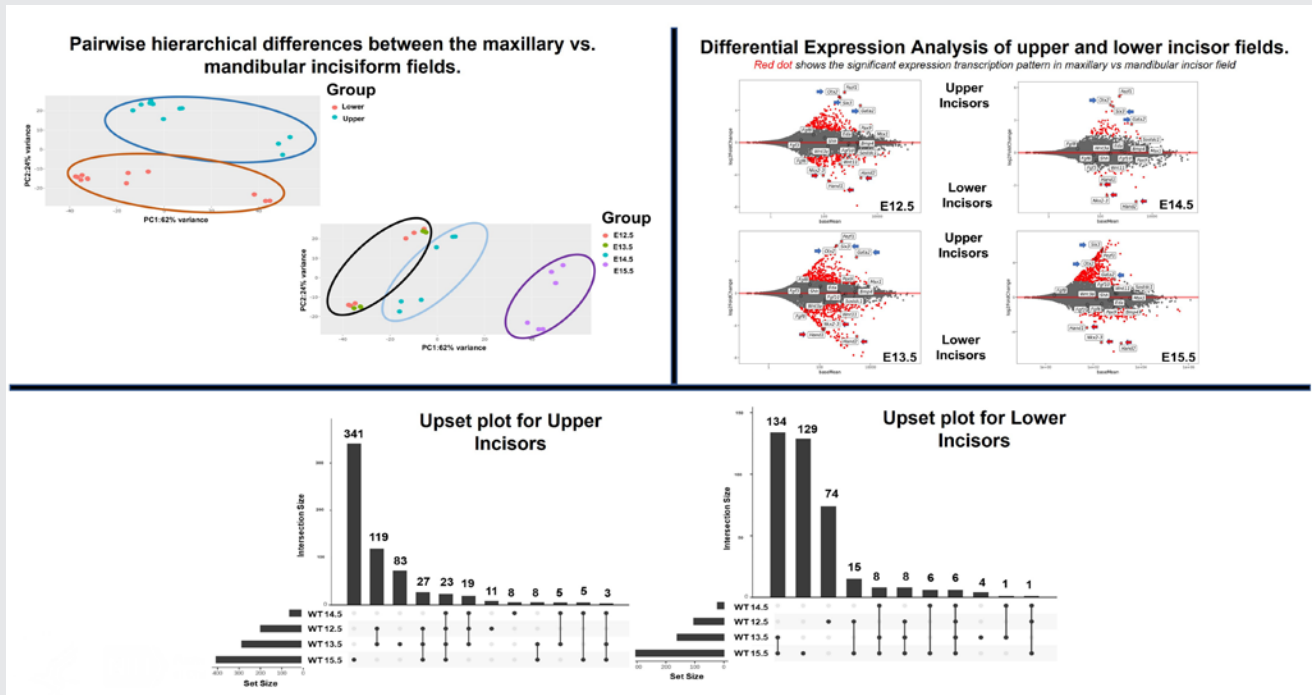


FIGURE 5. Signature transcriptomic pattern in upper and lower incisors

Nkx2-3) field. Our Enrichment Analysis revealed *Msx1* as a master regulator of signature transcriptomic patterns of maxillary and mandibular incisors (Figure 6). Our preliminary data show an association of *Msx1* with *Hand2* in lower incisiform field (Figures 5&6). *Msx1* deficiency also results in sporadic expression of *Nkx2-3* unlike in wild-type, where the expression was found to be confined to enamel knot (Figures 5&6). Experiments to identify the association of *Msx1* with *Six3*, *Otx2* and *Gata2* in maxillary incisiform development are under way.

For the first time, these studies provide a rationale for the differential effect of distinct morphogenetic gradients or signature transcription factors regulating incisiform in the maxillary vs. mandibular domain. The findings provide a framework for future studies to understand the patterns of tooth agenesis. Patterns of tooth agenesis provide valuable clues about the important roles of exclusive transcription factors and distinct morphogenic gradients in modulating during incisiform development in the maxillary vs. mandibular domain.

In vivo molecular consequences of *Wise* deficiency on *Pax9* and/or *Msx1*-dependent signaling events that control maxillary vs mandibular incisors

It is known that morphogenic gradients influence the patterning of dentition, and we and others have shown that transcription factors such as *Msx1* and *Pax9* modulate the functions of *Bmp*, *Wnt*, and *Eda* during odontogenesis (Figure 7). Recently, inhibition of a known bifunctional *BMP* and *Wnt* antagonist, *Wnt* modulator in surface ectoderm (*Wise*), was shown to selectively rescue tooth formation in mouse genetic models. Whether *Msx1* interacts with *Wise* and *Eda* in the incisor domain remains unclear. The goal of this study was to analyze the *in vivo* molecular consequences of *Wise* deficiency on *Msx1*-dependent signaling events that control

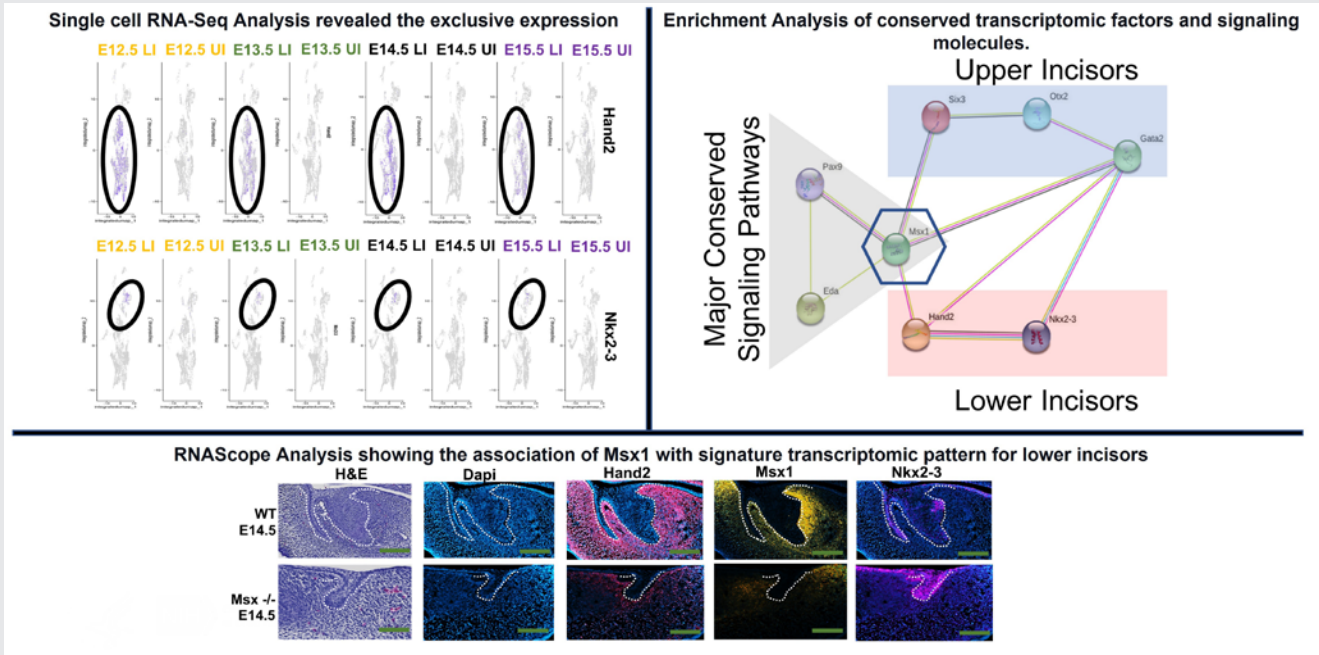


FIGURE 6. Association of Msx1 with signature transcriptomic pattern

Genotype	Maxillary or Mandibular	Phenotype Interpretation	Number of mice (n)	Numbers indicate the mice with the condition / total number of mice Percentage				
				Hypodontia (Agenesis)		Normal	Hyperdontia (Supernumerary teeth)	
				0	1	2	3	4
WT	Maxillary incisors	Normal	15			15/15 (100%)		
	Mandibular incisors	Normal				15/15 (100%)		
Eda -/-	Maxillary incisors	Normal	21			20/21 (95%)		
	Mandibular incisors	Normal				20/21 (95%)		
Msx1 +/-Eda -/-	Maxillary incisors	Normal	7			7/7 (100%)		
	Mandibular incisors	Tooth Agenesis		7/7 (100%)				
Msx1 +/-Pax9 +/-	Maxillary incisors	Normal	33			33/33 (100%)		
	Mandibular incisors	Tooth Agenesis		31/33 (93%)				
Pax9 +/-Eda -/-	Maxillary incisors	Tooth Agenesis/Normal	10		4/10 (40%)			
	Mandibular incisors	Tooth Agenesis	8	8/8 (100%)				

FIGURE 7. Disease model for tooth agenesis

maxillary vs mandibular incisors. We successfully generated the disease model with Msx1, Pax9 and Eda strain. Our preliminary data revealed a unique pattern of tooth agenesis (lower incisors).

For the first time, our studies provide a rationale for the differential effects of the Bmp and Wnt pathways in regulating Msx1-dependent events that pattern the distal maxilla and mandibular domains. The data provide a framework for the development of targeted therapeutics for human tooth agenesis.

Publications

1. Piña JO, Raju R, Chattara P, Kidwai FK, D'Souza RN. Spatiotemporal mapping of the embryonic palate transcriptome. *Commun Biol* 2022; under revision.

Collaborators

- Fabio R. Fauz, PhD, *Molecular Genomics Core, NICHD, Rockville, MD*
- Steven L. Goudy, MD, MBA, FACS, *Emory University School of Medicine, Atlanta, GA*

Contact

For more information, email rena.d'souza@nih.gov.

Regulation of Hormone Receptors and Gonadal Genes

The Section investigates the regulatory mechanism(s) involved in the progression of spermatogenesis and the control of Leydig cell function. We study novel gonadotropin-regulated genes relevant to the progression of testicular gametogenesis, Leydig cell function, and other endocrine processes. Our studies concentrate on the function and regulation of the gonadotropin-regulated testicular RNA helicase (GRTH/DDX25), a member of the DEAD-box family of RNA helicases, discovered, cloned, and characterized in our laboratory, which is essential for the progress of spermatogenesis [Dufau ML, Kavarthapu R. *Front Endocrinol (Lausanne)* 2019;10:576]. The various functions of GRTH/DDX25 provide fertile ground for the development of a non-hormonal male contraceptive.

Gonadotropin-regulated testicular RNA helicase (GRTH)

GRTH is present in Leydig cells (LCs), meiotic (pachytene spermatocytes), and haploid germ cells (round and elongated spermatids). Male null mice lacking GRTH are sterile owing to azoospermia resulting from the failure of round spermatids to elongate. We demonstrated GRTH's participation in the nuclear export/transport of specific mRNAs, the structural integrity of the Chromatoid Body (CB, an organelle prevalent in round spermatids [RS] and found to be key for the progress of spermatogenesis), storage/processing of relevant mRNAs, and their transit/association to the actively translating polyribosomes, where it may regulate translational initiation of genes. GRTH is the only DEAD-box family member regulated by hormones. *GRTH* transcription is stimulated in LCs by LH (luteinizing hormone)/cAMP through direct actions of the androgen (A)/A receptor (AR) (autocrine), and in germ cells in paracrine fashion through the AR in Sertoli cells. The upstream region of the *GRTH* gene directs its expression in germ cells and downstream in LCs. Through these regions, A/AR exerts its direct (endogenous) regulation of the *GRTH* gene in the LC, and indirectly in germ cells.

We identified functional binding sites for the germ-cell nuclear factor (GCNF), which are present in RS and spermatocytes (SP), and its regulation by A/AR in the distal region of the *GRTH* gene, operative selectively in RS. Current knowledge indicates actions of A



Maria L. Dufau, MD, PhD,
*Head, Section on Molecular
Endocrinology*

Raghuvveer Kavarthapu, PhD, *Staff
Fellow*

Muruganath Kumar Raju, PhD,
Postdoctoral Fellow

Rajakumar Anbazhagan, PhD,
Visiting Fellow

on GCNF cell-specific regulation of *GRTH* expression in germ cells (RS). Also, GRTH exerts negative autocrine regulation of GCNF, linking A actions to germ cells through GCNF as an A-regulated trans-factor that controls transcription/expression of GRTH (Kavarthapu & Dufau, *Mol Endocrinol* 2015;29:1792). These findings provide a connection between androgen action and two relevant germ-cell genes (*GRTH* and *GCNF*), which are essential for the progress of spermatogenesis, and we established their regulatory interrelationship.

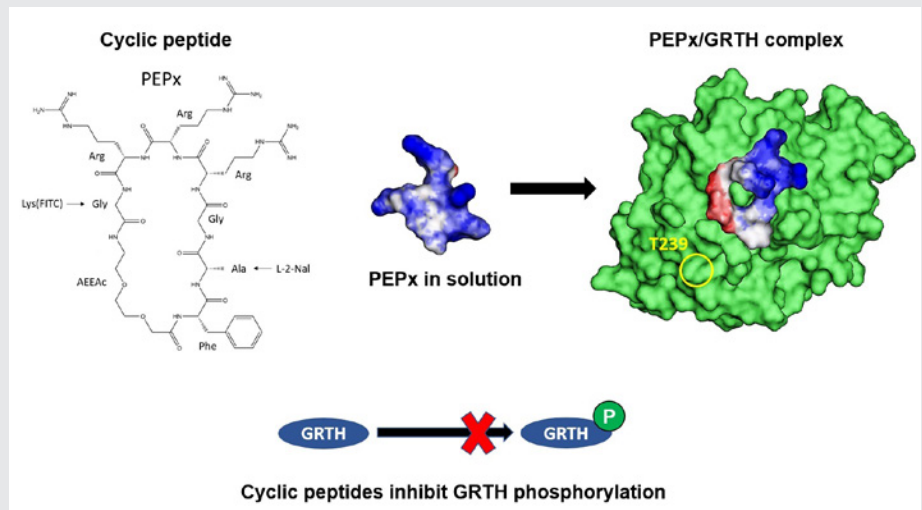
Our early studies revealed that, when the missense mutation of R to H at amino acid 242 of GRTH, found in 5.8% of 100 azoospermic patients, was transfected in COS1 cells, it caused loss of the 61 kDa phospho-species (pGRTH) with preservation of the 52 kDa non-phospho form (Tsai-Morris *et al. Mol Human Reprod* 2007;13:887). The finding provided an avenue to elucidate the function of pGRTH in spermatogenesis. We generated humanized mutant *GRTH* knock-in (KI) mice. The mutant mice are sterile, with reduction in testicular size, they lack sperm with arrest at step 8 of round spermatids (RS), and exhibit complete loss of the pGRTH species with preservation of the nuclear 52 kDa form [Kavarthapu R, Anbazhagan R, Raju M, Morris CT, Pickel J, Dufau ML. *Hum Mol Genet* 2019;28:2561]. The mouse model allows us to study the biological/biochemical functions of the cytoplasmic pGRTH. In KI mice, the nuclear export/transport and functions of GRTH are preserved (i.e., mRNA export), while the cytoplasmic functions, including shuttling of messages, storage in the CB, and translational events all requiring pGRTH, are absent. We observed a marked reduction in the size of the CB in RS and lack of pGRTH in the CBs. Germ-cell apoptosis was present in pachytene spermatocytes (PS) and in RS. In contrast to KO, KI showed no changes in miRNA biosynthesis, excluding participation of pGRTH as a transcriptional regulator of the microprocessor complex (which consists of Drosha, a miRNA-processing enzyme, and its cofactor, the RNA-binding protein DCGR), affecting pri-miRNA (primary microRNA) formation and indicative of the participation of non-phospho GRTH in such processes. In KI mice there is loss of chromatin-remodeling and related proteins, including TP2 (transition protein 2, a testis-specific DNA-condensing protein), PRM2 (a protamine that facilitates DNA condensation), and TSSK6 (a testis-specific serine/threonine-protein kinase, which is involved in the compaction of DNA during spermatogenesis). Significant reductions in their mRNA half-lives indicate that their association with pGRTH in the cytoplasm protects these mRNAs from degradation. Also, our work showed that pGRTH stimulates TP2 translation in a 3' UTR-dependent manner.

In recent studies, we elucidated the GRTH phospho-site at threonine (T239), which is structurally adjacent to the mutant site found in azoospermic patients (R242H). Molecular modelling of the phospho-site, based on the RecA domain 1 of the DDX9 crystal structure, pointed to the amino acids that formed the GRTH/protein kinase alpha (PKA) interface, solvent accessibility, and H-bonding. These include, in addition to the core residues T239 and R242, amino acids E165, K240, and D237 [Reference 1]. We demonstrated the relevance of these residues by disrupting amino acids using site-directed mutagenesis (single or double mutations), which caused reduction or abolition of the pGRTH at T239. The pGRTH form is the cytoplasmic species, which was demonstrated as essential for the progress of spermatogenesis beyond step 8 of RS and for viable sperm formation. It is important to note that the deleterious effects on GRTH phosphorylation caused by the mutations did not result from changes in PKA-catalytic binding affinity but rather in consequential structural changes that can affect PKA catalytic efficiency. Studies based on the abolition of the phospho-form provide the basis for drug design, such as for discovery of a reversible chemical inhibitor for use as male contraceptive.

During the past year, we determined that cyclic peptides that fit the shallow pocket of the GRTH/DDX5/PKA interphase are preferred compounds to block GRTH phosphorylation and amenable for use in the development of an oral male contraceptive [Reference 2] (Figure 1). In this regard, these cyclic peptides

FIGURE 1. Cyclic peptides inhibit GRTH phosphorylation.

Peptide x (*left*) ($^1\text{FAGRRRG}^7$) represents one of the simplest cyclic peptides to accommodate stringent conditions and the main elements of the pharmacophore. The molecular scaffold provided the basis for the design of cyclic peptides (CPs) that bind to GRTH. A computational multistage cyclization procedure predicted the binding modes at the interphase. The simulations indicated that, in solution, the CPs are quite flexible, an attribute that permits adaptation



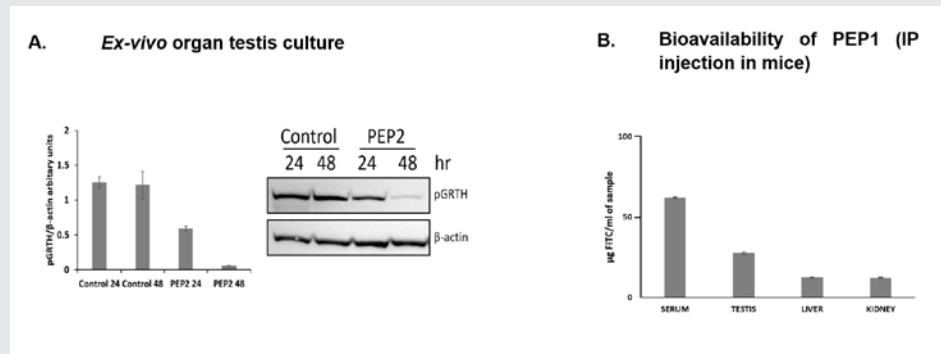
to the GRTH/PKA interphase (*center, right*) to exert its inhibitory function on the phosphorylation of GRTH at T239 (*yellow circle, right*). Binding of the CPs to GRTH was confirmed by thermal stabilization of non-phospho-GRTH and increased efficiency in FRET assay [Reference 2].

(PEP0, PEP1, and PEP2) have been designed and synthesized as promising therapeutic agents. PEP1 and PEP2, revealed by fluorescein isothiocyanate (FITC), showed effective internalization in COS-1 cells and in seminiferous tubules after a four-hour treatment. We observed a dose-dependent inhibitory effect on GRTH phosphorylation in a COS-1 stable cell line expressing GRTH, with significant reduction in pGRTH protein observed after 8–16 hour treatments. CETSA (cellular thermal shift assay) showed compound binding resulting in thermal stabilization of the soluble non-pGRTH protein when compared with control peptides. Increased efficiency in a fluorescence resonance energy transfer (FRET) assay revealed the interaction of the cyclic peptide with GRTH. Exposure of a culture of seminiferous tubules to these compounds resulted in significant inhibition of the pGRTH protein species. Similar results were obtained with the compound that lacks FITC. Taken together, effective internalization and targeted reduction in the expression of pGRTH by cyclic peptides provide a promising angle to develop effective compounds for use as a non-hormonal male contraceptive.

Also, work on transcriptome profiles of germ cells isolated from GRTH-KI mice compared with those of wild type (WT) mice, using the RNA-Seq technique, provided further insights linking phospho-GRTH with histone ubiquitination and acetylation essential for chromatin compaction and spermatid elongation during spermiogenesis [Reference 3].

We also initiated studies on the role of phosphorylated GRTH in the storage of messages in the CB. We observed absence of pGRTH from the CB of GRTH KI mice (with an insertion of the R242H human mutation that abolishes GRTH phosphorylation at T239 and spermatogenesis). RNA-Seq analysis of mRNA isolated from the CB revealed that the abundance of 947 genes was reduced and that of 474 genes increased [Reference 3]. Transcripts related to spermatid development, differentiation, and chromatin remodeling were reduced; in contrast, those encoding factors involved in RNA transport, regulation, surveillance, and transcriptional and translational regulation were increased in the CB of KI mice, which was validated by qPCR. Transcripts of

FIGURE 2. Cyclic peptides inhibit phosphorylation of GRTH in testis organ culture and bioavailability of PEP1 after IP injection in mice.



several initiation factors (eIF4e, 4ebp2, 3l, and 3m), together with mRNAs related to the 60S subunit (RpL101/RPlp0), were increased and accumulated in the CB; mRNAs that could not be transported from the CB to polyribosomes for translation remain stored in the CB in KI mice owing to loss of pGRTH [Reference 4]. Our studies demonstrated the importance of phospho-GRTH in the maintenance of the structure of the CB and its role in the storage and stability of germ cell-specific mRNAs during spermiogenesis.

In other studies, we performed single-cell transcriptomic analysis of testicular germ cells to further elucidate the role of pGRTH and non-pGRTH in germ cell development. scRNA-Seq was performed on testicular cells isolated from seminiferous tubules of GRTH KO, GRTH KI lacking pGRTH, and WT mice. Graph-based cell clustering via tSNE (t-distributed stochastic neighbor embedding) analysis identified 15 clusters. Based on known cell-type, marker genes were identified somatic cells clusters 13–15 (Sertoli, myoid macrophages). Germline markers were expressed in clusters 1–12 (spermatogonia [S]; spermatocytes [SP]; round spermatids [RS], and elongated spermatids [ES]). Pseudo-time of germ cell clusters (1–12) revealed a long, continuous trajectory of development in WT. In KO and KI, this trajectory was halted at RS stage with absence of ES. Upon differential gene expression and GO enrichment analysis on RS cluster for KI vs WT and KO vs WT, genes related to S development/differentiation, translational process, ribosome assembly, and acrosome vesicle formation and assembly were found to be significantly reduced in the RS cluster of KI and KO samples. These genes were validated by real-time qPCR. EM studies of RS of KI and KO revealed defects in the acrosome structure. These studies indicate the essential role of phospho-GRTH in the formation of the acrosome granule and in maintaining the structural integrity of acrosome. Other studies were directed at finding targets for GRTH- and pGRTH-regulated miRNAs and on the mechanism of GRTH regulation of miRNA biogenesis. The identified miRNAs regulate essential genes such as Tnp2, TSSK6, Pabpc1, Ppp1cc, and UPF2, which play a critical role in spermatid differentiation and fertility.

Previously, we demonstrated cyclic peptides with potential for use as non-hormonal male contraceptive agents through inhibition of GRTH phosphorylation at Thr 239 *in vitro* [Reference 2, and Patent USPTO No.63/250,665 “Cyclic Peptides as Non-Hormonal Male Contraceptive Agents and Methods Thereof” 2021 and revised in 2022]. Recently, we conducted *ex vivo* organ testis culture in agarose blocks soaked with or without cyclic peptide PEP2 treatment. The results showed a significant reduction in phospho-GRTH at 24 and 48 h of treatment (Figure 2A). Also, the bioavailability of cyclic peptides was tested *in vivo* by IP injection of FICT (fluorescein isothiocyanate-labeled)-PEP1 in mice after 30 mins. Significant presence of FICT-PEP1 was observed in the serum and testis but minimally in the liver and kidney (Figure 2B).

Publications

1. Raju M, Hassan SA, Kavarthapu R, Anbazhagan R, Dufau ML. Characterization of the phosphorylation site of GRTH/DDX25 and protein kinase A binding interface provides structural basis for the design of a non-hormonal male contraceptive. *Sci Rep* 2019 9:42857–42867.
2. Raju M, Kavarthapu R, Anbazhagan R, Hassan SA, Dufau ML. Blockade of GRTH/DDX25 phosphorylation by cyclic peptides provides an avenue for developing a non-hormonal contraceptive. *J Med Chem* 2021 64:14715–14727.
3. Kavarthapu R, Anbazhagan R, Sharma AK, Shiloach J, Dufau ML. Linking phospho-gonadotropin regulated testicular RNA helicase (GRTH/DDX25) to histone ubiquitination and acetylation essential for spermatid development during spermiogenesis. *Front Cell Dev Biol* 2020 8:310–321.
4. Anbazhagan R, Kavarthapu R, Coon SL, Dufau ML. Role of phosphorylated-gonadotropin regulated testicular RNA helicase (GRTH/DDX25) in the regulation of cell specific mRNAs in chromatoid bodies during spermatogenesis. *Front Cell Dev Biol* 2020 8:580019.
5. Kavarthapu R, Dufau MI. Crosstalk between PRLR and EGFR/HER signaling pathways in breast cancer. *Cancers* 2021 13:4685–4690.
6. Kavarthapu R, Dufau ML. Prolactin receptor gene transcriptional control, regulatory modalities relevant to breast cancer resistance and invasiveness. *Front Endocrinol* 2022 13:949396.

Collaborators

- Sergio A. Hassan, PhD, *Bioinformatics and Computational Biosciences Branch, NIAID, Bethesda, MD*

Contact

For more information, email dufau@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/dufau>.

Transcriptional Control of Cell Specification and Differentiation

Animals consist of a collection of cells with diverse shapes, structures, and functions, a diversity that is rebuilt from scratch by every embryo. The genetic programs that direct the process are the central mystery of developmental and regenerative biology. We are interested in how decisions about the cell type to adopt are controlled, and how the genetic programs direct the morphological and functional specialization of different cells.

The single-cell revolution in developmental biology has given us new access and new tools to address these questions. I previously developed high-temporal-resolution, single-cell RNA-sequencing approaches to identify transcriptional trajectories, i.e., the 'highways' or the most likely paths through gene expression that cells take during development. From such data, we were able to identify the sequence of genes expressed by individual cell types during early development, which provides insight into the genetic programs that regulate cells' choice of cell type and then their downstream functional transformations at a wider breadth than was previously achievable. Work in the lab focuses on more deeply exploring such processes, using the approaches we developed. Our lab combines single-cell genomics with imaging, genetic, and classical embryological approaches to investigate the genetic control of cell specification and differentiation during vertebrate embryogenesis. We focus on zebrafish embryos as a model system in which to study these questions, because, among vertebrates, they are easy to culture, image, and manipulate, both embryologically and genetically.

Genetic underpinnings of cell differentiation

Once a cell has been specified, it must acquire the particular morphology and functionality of its cell type through the process of differentiation, a process that is driven by cell type-specific expression of differentiation genes and often results in dramatic changes in basic cell-biological processes. We aim to identify those genes that drive differentiation and understand their regulation. To do this, we generated a single-cell RNA-Seq atlas spanning embryogenesis and early larval stages (62 timepoints from 3–120 hours post-fertilization), annotated over 300 cell types, and built a web portal to enable other investigators to browse these data. To better understand how



Jeffrey A. Farrell, PhD, *Stadtman Investigator, Head, Unit on Cell Specification and Differentiation*

Paulina Capar, MSc, *Technician*

Abhinav Sur, PhD, *Postdoctoral Visiting Fellow*

Sean Lee, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

Avani Modak, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

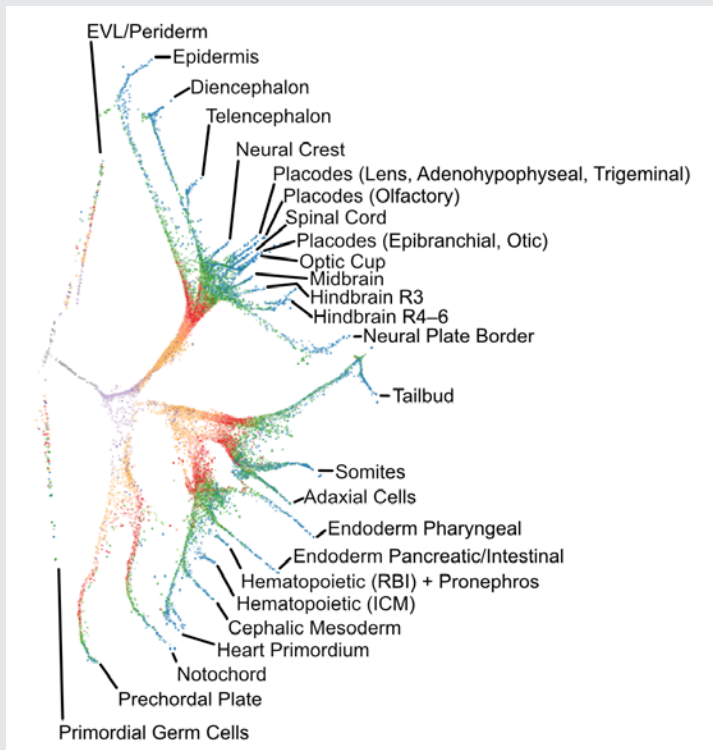


FIGURE 1. Transcriptional trajectories in early zebrafish development

Single-cell transcriptomes were isolated from zebrafish embryos at 12 developmental stages spanning 3–12 hours post-fertilization. The branching transcriptional trajectories, which represent the gene-expression events that give rise to 25 different differentiated cell types, were then reconstructed using URD, a simulated diffusion-based computational reconstruction method, which is software that we developed and published in 2018 [Reference 1].

cells acquire their cell type-specific features, we also built a catalog of shared gene-expression programs that are re-used across many different tissues during development. Additionally, our analysis uncovered uncharacterized cell types in zebrafish development that have human homologs, which will be useful for building future disease models. We therefore built developmental trajectories to describe the sequence of gene expression that likely gives rise to these cell types, and we used these trajectories to determine candidate regulatory factors that may drive their specification and which can be tested in future work.

Consequences of heterogeneous developmental trajectories

Distinct cell types can arise through many developmental trajectories or developmental histories. We and others have observed refinement at the boundaries between groups of cells specified to become different tissues; at such boundaries, some cells switch from one specification state to another. We use the axial mesoderm as a model and seek to understand (1) what drives cell-type switching; (2) the long-term consequences for a cell that switched; and (3) the mechanisms that assist in successful switching.

Effect of environmental insults on developmental choices

During early embryogenesis, a field of equipotent cells are instructed to initiate different gene expression programs by external developmental signals and cell-intrinsic cues. We recently observed that cells that experience DNA damage in early zebrafish embryos initiate an unusual transcriptional response during a very limited window in development. Moreover, most damaged cells are not eliminated but appear to be excluded from contributing to some tissues in the animal, which suggests that responding to DNA damage may affect cells' choices during development and which raises the question as to how that occurs. We are investigating (1)

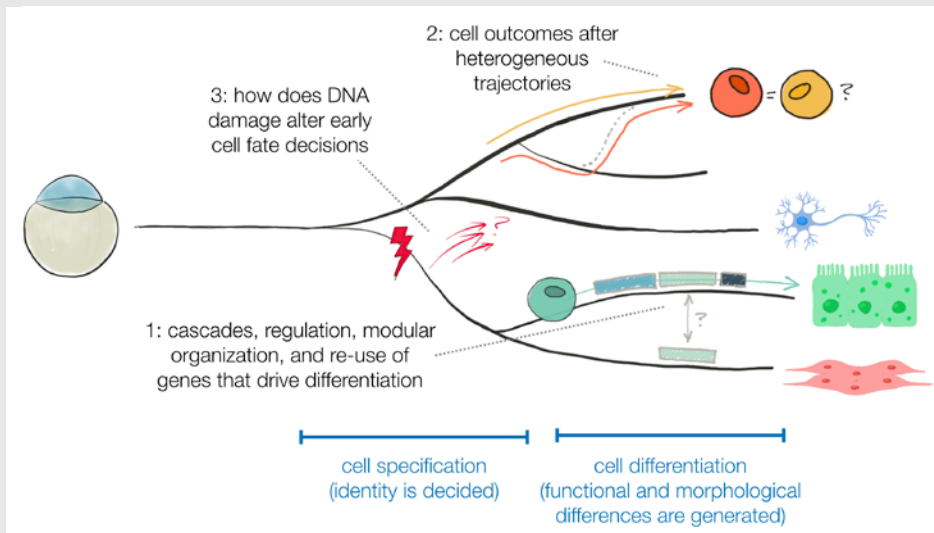


FIGURE 2. Overview of ongoing research projects

the fate of cells in early development that experience DNA damage; (2) the role this unusual transcriptional response plays; and (3) what drives the bias in damaged cells' future cell type.

Publications

1. Farrell JA, Wang Y, Riesenfeld SJ, Shekhar K, Regev A, Schier AF. Single-cell reconstruction of developmental trajectories during zebrafish embryogenesis. *Science* 2018 360:eaar3131.
2. Siebert S, Farrell JA, Cazet J, Abeykoon Y, Primack A, Schnitzler C, Juliano CE. Stem cell differentiation trajectories in Hydra resolved at single-cell resolution. *Science* 2019 365:eaav9314.
3. Raj B, Farrell JA, Liu J, El Kholtei J, Carte AN, Navajas Acedo J, Du LY, McKenna A, Relic D, Leslie JM, Schier AF. Emergence of neuronal diversity during vertebrate brain development. *Neuron* 2020 108:1058–1074.

Collaborators

- Celina Juliano, PhD, *University of California Davis, Davis, CA*
- Michal Rabani, PhD, *The Alexander Silberman Institute of Life Science, The Hebrew University of Jerusalem, Jerusalem, Israel*

Contact

For more information, email jeffrey.farrell@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/farrell>.

Nervous System Development and Plasticity

The healthy development of the brain and of cognition in children is central to the mission of the NICHD. Our research is concerned with understanding the molecular and cellular mechanisms by which functional activity in the brain regulates the development of the nervous system during late stages of fetal development and early postnatal life. In addition to synaptic plasticity, we are interested in novel mechanisms of activity-dependent nervous-system plasticity that are particularly relevant to the period of childhood, including the involvement of glia (non-neuronal brain cells). Our work has three main areas of emphasis: myelination and neuron-glia interactions; cellular mechanisms of learning; and gene regulation by neuronal firing.

Traditionally, the field of activity-dependent nervous system development has focused on synapses, and we continue to explore synaptic plasticity. However, our research is also advancing our understanding of how glia sense neural impulse activity and how the activity-dependent regulation of glia contributes to development, plasticity, and the cellular mechanisms of learning. A major emphasis of our current research is to understand how myelin (white matter in the brain) is regulated by functional activity. By changing conduction velocity, activity-dependent myelination may be a non-synaptic form of plasticity, regulating nervous system function by optimizing the speed and synchrony of information transmission through neural networks. Our studies have identified several cellular and molecular mechanisms for activity-dependent myelination, and the findings have important implications for normal brain development, learning, cognition, and psychiatric disorders. Our research shows that myelination of axons by glia (oligodendrocytes in the central nervous system [CNS] and Schwann cells in the peripheral nervous system [PNS]) is regulated by impulse activity, and we have identified several molecular mechanisms that control proliferation and differentiation of myelinating glia and myelination. Most recently, we determined that myelin thickness can be adjusted through a treadmill process that adds and removes layers of myelin from the sheath to adjust conduction velocity and improve functional performance by optimizing spike-time arrival at synapses. The findings provide evidence for a new form of nervous system plasticity and learning that would be particularly important in child development, but which also operates in adulthood, thereby improving function based on experience.



R. Douglas Fields, PhD, Head,
*Section on Nervous System
Development and Plasticity*
Philip Lee, PhD, *Staff Scientist*
William Huffman, MA, *Technician*
Maxwell Foote, *Postbaccalaureate
Fellow*
Erin Santos, BS, *Postbaccalaureate
Fellow*
Catherine Carr, PhD, *Special
Volunteer*
Javier Carvajal, PhD, *Special
Volunteer*

Learning is perhaps the most important function of childhood. Our research is determining the molecular and cellular mechanisms that convert short-term into long-term memory. If functional experiences produce lasting effects on brain development and plasticity, specific genes must be regulated by specific patterns of impulse firing. We are determining how the pattern of neural impulses, i.e., the neural information code, regulates specific genes controlling development and plasticity of neurons and glia.

Nervous system plasticity by activity-dependent myelination

The fundamental cellular mechanism of learning, memory, and neural plasticity is synaptic plasticity, in which the strength of synaptic transmission or the number of synapses is modified by experience. Our laboratory has a long-standing interest in synaptic plasticity, but our current interest is to explore new, non-synaptic mechanisms involved in these processes. In particular, we are investigating how changes in neural-impulse conduction velocity contribute to learning and plasticity, and the involvement of glial cells that form the myelin sheath in this new form of plasticity. Our research indicates that, by modifying conduction velocity to optimize the timing of neural impulse arrival at relay points in neural networks, and by influencing the phase and frequency of neural oscillations, myelin-forming glia participate in learning, neural plasticity, and nervous system development in accordance with functional activity and experience.

Myelin, the multilayered membrane of insulation wrapped around nerve fibers (axons) by glial cells, is essential for proper neural impulse transmission and nervous system function. Myelination is an essential part of brain development, but the processes controlling myelination of appropriate axons are not well understood. Myelination begins in late fetal life and continues throughout childhood and adolescence, but myelination of some brain regions is not complete until an individual's early twenties.

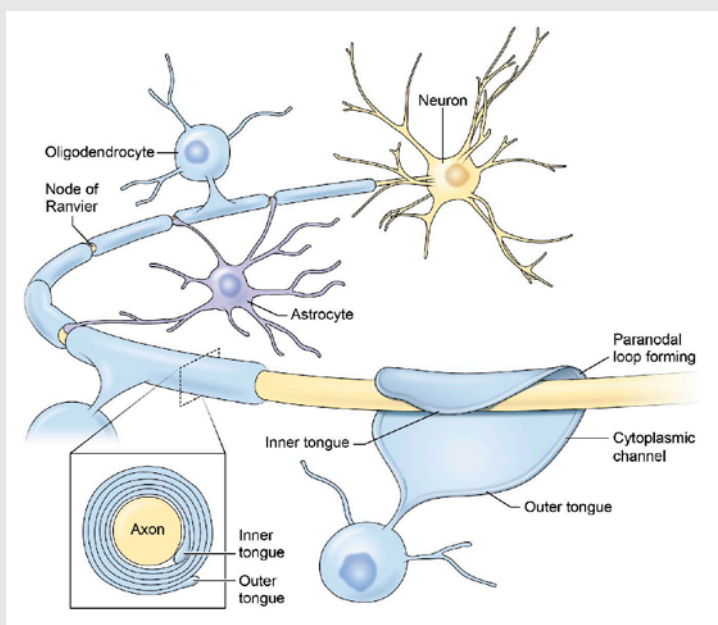


FIGURE 1. The structure of CNS myelin and nodes of Ranvier

Traditionally myelin has been viewed in terms of conduction failure after damage (for example in multiple sclerosis), but we are exploring how changes in myelin driven by functional activity affect the timing of neural-impulse arrival at synaptic relay points, which is critical for information processing and synaptic activity. In addition, the frequency, phase, and amplitude coupling of oscillations in the brain (brainwaves) requires appropriate impulse conduction velocity, which is influenced by myelination. Many neurological and psychological dysfunctions can develop when optimal neural synchrony of spike-time arrival and neural oscillations are disturbed, as, for example, in schizophrenia, epilepsy, dyslexia, and autism.

Our research shows that, to activate receptors on myelinating glia as well as on astrocytes and other cells, neurotransmitters are released not only at synapses but also along axons firing action potentials. The recipient cells in turn release growth factors, cytokines, and other molecules that regulate myelination, proliferation, and development of myelinating glia.

INDUCTION OF MYELINATION BY ACTION POTENTIALS

In addition to establishing the effects of impulse activity on proliferation and development of myelinating glia, we determined that release of the neurotransmitter glutamate from vesicles along axons triggers the initial events in myelin induction, including stimulating the formation of cholesterol-rich signaling domains between oligodendrocytes and axons and increasing the local synthesis of myelin basic protein, the major protein in the myelin sheath, through Fyn kinase-dependent signaling. We showed that, through this axon-oligodendrocyte signaling mechanism, electrically active axons become preferentially myelinated by a factor of 8 to 1 over electrically inactive axons, thus regulating myelination of axons and neural circuit function according to functional experience, which would be particularly important in the adolescent brain, for example, where environmental experience during sensitive periods can have long-lasting effects on neural circuit development and behavior. The findings are also relevant to such demyelinating disorders as multiple sclerosis and to remyelination after axon injury.

Myelin in the PNS is formed by a different type of glial cell: the Schwann cells. Our research is also investigating how neural impulse activity can influence myelination in the PNS, and the results indicate that different mechanisms are responsible from those operating on CNS myelin. These studies involve optogenetics and gene expression analysis, *in vivo* and in primary cell cultures.

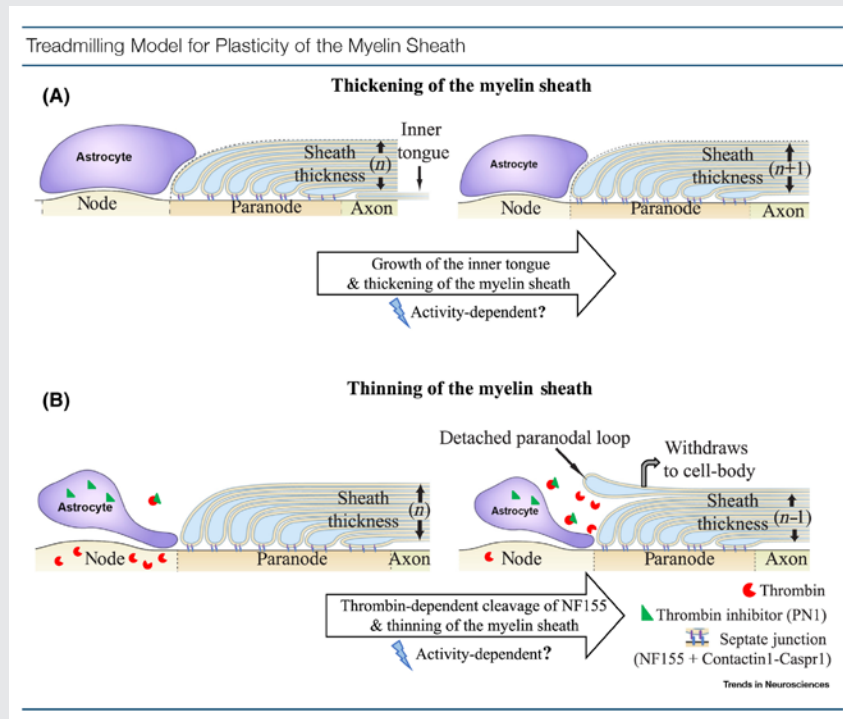
MODIFICATION OF MYELIN STRUCTURE AND CONDUCTION VELOCITY BY ASTROCYTES

Given that optimal neural-circuit function and synaptic plasticity require the proper impulse transmission speed through all axons to induce spike timing-dependent plasticity and to sustain oscillations at appropriate frequencies, mechanisms that determine and modify conduction time through axons could provide a non-synaptic mechanism of neural circuit plasticity. Conduction velocity in myelinated axons depends on the thickness of the myelin sheath and on the morphology of the electrogenic nodes of Ranvier (gaps in the myelin sheath) along axons. Our research showed that myelination of unmyelinated axons and the thickness of the myelin sheath can be increased in response to neural activity and environmental experience. Prior to our research, myelin structure was believed to be static, and there was no known mechanism that could reduce the thickness of the mature myelin sheath (except in the context of pathology). However, a mechanism would be necessary to reduce conduction velocity to achieve optimal spike-time arrival from inputs that arrive at relay points in neural networks too soon.

Our research shows that myelin thickness and nodal gap length are reversibly altered by astrocytes, glial cells that contact nodes of Ranvier, and that this alters the speed of impulse transmission and neural network function. Myelin is attached to the axon by intercellular junctions adjacent to the nodes of Ranvier. We found that one of these cell-adhesion molecules, neurofascin 155, has a binding site for the proteolytic enzyme thrombin, which is secreted by neurons and enters the brain from the vascular system. We found that thrombin-dependent cleavage of neurofascin 155 severs the tether between the axon and myelin, allowing the latter to detach and rendering the myelin sheath thinner. The process is inhibited by vesicular release of thrombin protease inhibitors from perinodal astrocytes. Previously, it was unknown how the myelin sheath

FIGURE 2. Treadmilling model for plasticity of the myelin sheath

The speed of neural impulse transmission is altered by changes in myelin structure. The thickness of the myelin sheath in CNS axons is determined by two opposing processes: one (A) that adds additional wraps of myelin to the axon, and the other (B) that removes the outer layer, thereby increasing and decreasing impulse conduction velocity, respectively. New layers of myelin are added beneath the inner tongue of myelin. Myelin is attached to the axon at the paranodal region flanking the node of Ranvier via septate junctions, consisting of neurofascin 155 on myelin interacting with the contactin1-caspr1 complex on the axon. Cleavage of neurofascin 155 by thrombin (red) can break this interaction, resulting in detachment of the outer paranodal loop from the axon, and withdrawal of the outer layer of myelin, which increases nodal gap length and reduces myelin sheath thickness; both effects slow conduction velocity. Perinodal astrocytes at the nodes of Ranvier regulate the process by secreting thrombin inhibitors (green triangle), such as protease nexin1. The treadmilling process helps achieve optimal conduction velocity in individual axons [Trends Neurosci 2019;42:443].



could be thinned, and the functions of perinodal astrocytes were not well understood. Our findings uncovered a new form of nervous system plasticity in which myelin structure and conduction velocity are adjusted by astrocytes. The thrombin-dependent cleavage of neurofascin 155 may also have relevance for myelin disruption and repair.

Regulation of gene expression by action-potential firing patterns

All information in the nervous system is encoded in the temporal pattern of neural impulse firing. Given that long-lasting changes in the nervous system require regulated gene expression, appropriate patterns of neural impulse firing driving neural plasticity must control transcription of specific genes, a fundamental question central to the processes of experience-dependent plasticity during development and learning. However, little is known about how neural firing patterns regulate gene expression. Our experiments are revealing the intracellular signaling and gene-regulatory networks that respond selectively to appropriate temporal patterns of action-potential firing to generate adaptive responses.

To determine how gene expression in neurons and glia is regulated by impulse firing, we stimulate nerve cells to fire impulses in differing patterns by optogenetics and by delivering electrical stimulation through platinum electrodes in specially designed cell-culture dishes. Live-cell calcium imaging shows that temporal aspects of intracellular calcium signaling are particularly important for regulating gene expression according

to neural-impulse firing patterns in normal and pathological conditions. After stimulation, we measured mRNA and protein expression by gene microarrays, quantitative RT-PCR (reverse transcriptase–polymerase chain reaction), RNA-Seq (RNA sequencing), Western blot, and immunocytochemistry. The results confirm our hypothesis that precise patterns of impulse activity can increase or reduce the expression of specific genes in neurons and glia. Moreover, our research shows that regulation of gene expression in neurons by specific temporal patterns of impulse activity is not a property of special genes; in general, the neuronal transcriptome is highly regulated by the pattern of membrane depolarization, with hundreds of genes differentially regulated by the temporal code of neuronal firing.

We are also pioneering new methods of transcriptional analysis in neurons. The standard approach to analyzing gene expression is by measuring the abundance of tens of thousands specific gene transcripts in cells by microarray or RNA-Seq, as described above, but this approach fails to capture the unique feature of transcriptional regulation in neurons. In contrast to other cells responding to external signals that may drive cells to a steady-state equilibrium, transcriptional networks in neurons are continually modulated dynamically by temporally varying action-potential firing frequencies and burst patterns, together with synchrony and phase relationships among populations of interconnected neurons. Such activity may not alter the abundance of a gene transcript significantly; nevertheless, the coordinated activity within transcriptional networks is being modulated dynamically to modify function.

To address this question, we applied a covariance approach using a Pearson correlation analysis, to determine how pairs of genes in mouse dorsal root ganglion (DRG) neurons are coordinately expressed in response to stimulation producing the same number of action potentials in different temporal patterns. Our analysis of 4,728 distinct gene pairs related to calcium signaling, 435,711 pairs of transcription factors, 820 pairs of voltage-gated ion channels, and 86,862 calcium-signaling genes paired with transcription factors indicated that genes become coordinately activated by distinct action-potential firing patterns. Thus, in addition to regulating the expression level of numerous genes, the temporal pattern of action-potential firing profoundly modulates how genes are networked in functional pathways.

Our findings provide a deeper understanding of how nervous system development and plasticity are regulated by information coded in the temporal pattern of impulse firing in the brain. The findings are also relevant to chronic pain, as well as to the regulation of nervous system development and myelination by functional activity.

DIFFERENCES IN CHROMATIN STRUCTURE BETWEEN NEURONS AND GLIA

In collaboration with our colleague [David Clark](#), our research is revealing fundamental differences in chromatin structure between neurons and glia. Chromatin can be visualized by electron microscopy as regularly spaced ‘beads-on-a-string,’ in which the beads represent nucleosome cores and the string is the intervening linker DNA. Using MNase (micrococcal nuclease) digestion and RNA-Seq, we compared the chromatin structure of purified mouse DRG neurons, cortical oligodendrocyte precursor cells (OPCs), and cortical astrocytes. We found that DRG neurons have shorter average nucleosome spacing (approximately 165 base pairs) than either glial cells (OPCs, with approximately 182 base pair spacing) or astrocytes (with approximately 183 base pairs). The significance of such basic differences in chromatin structure between DRG neurons and these glial cells is unknown and is currently being investigated. Interestingly, the atypical nucleosome spacing of neuronal chromatin does not extend to promoter-proximal regions.

Nanocellulose block of SARS–Cov2 and HIV infection

An important imperative is to develop methods that have broad anti-viral action that are resistant to mutations in a virus that can circumvent immunization, such as the SARS-CoV-2 virus. Thus, in response to the urgent need to develop methods for combatting Covid-19, our laboratory developed a new method to block viral infection of cells by SARS-CoV-2. We developed a compound that binds to the SARS-CoV-2 and to the HIV virus and that thus prevents cellular infection. Nanocellulose derived from the ivory nut endosperm of a South American palm yields thin nanoparticles (from 1–5 nm width), which provide a high surface area for entrapment of microbes. In research published on-line in advance of print [Reference 5], we demonstrated that the substance is a universal microbe binder that prevents viral infection of cells in culture. The material can encapsulate the S protein of SARS-CoV-2, as well as whole SARS-CoV-2 and HIV-1 virions, thereby blocking infection of cells.

Publications

1. Pajevic S, Plenz D, Basser PJ, Fields RD. Oligodendrocyte-mediated myelin plasticity. *eLIFE* 2022; in press.
2. Munyeshyaka M, Fields RD. Oligodendroglia are emerging players in several forms of learning and memory. *Commun Biol* 2022 5(1):1148.
3. Santos EN, Huffman WC, Field RD. Effects of visual deprivation on remodeling of nodes of Ranvier in optic nerve. *eNeuro* 2022 9(6):ENEURO.0194-22.2022.
4. Santos EN, Huffman WC, Fields RD. Recovery of node of Ranvier structure in optic nerve under visual deprivation. *Neurosci Lett* 2022 under revision.
5. Barriga EJC, Fitzgerald W, Dimitriadis EK, Margolis L, Fields RD. Sulfated endospermic nanocellulose crystals prevent the transmission of SARS-CoV-2 and HIV-1. *Res Sq* 2022 rs.3.rs-2163527 (preprint).

Collaborators

- Peter J. Basser, PhD, *Section on Quantitative Imaging and Tissue Sciences, NICHD, Bethesda, MD*
- David Clark, PhD, *Section on Chromatin and Gene Expression, NICHD, Bethesda, MD*
- Emilios Dimitriadis, PhD, *Trans-NIH Shared Resource on Biomedical Engineering and Physical Science, NIBIB, Bethesda, MD*
- Wendy Fitzgerald, BS, *Section on Intercellular Interactions, NICHD, Bethesda, MD*
- Dumitru Iacobas, PhD, *New York Medical College, Valhalla, NY*
- Vanja Lazarevic, PhD, *Experimental Immunology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Leonid Margolis, PhD, *Section on Intercellular Interactions, NICHD, Bethesda, MD*
- Sinisa Pajevic, PhD, *Division of Computational Bioscience, CIT, NIH, Bethesda, MD*
- Dietmar Plenz, PhD, *Laboratory of Systems Neuroscience, NINDS, Bethesda, MD*
- Shahriar Sheikhbahaei, PhD, *Cellular and Systems Neurobiology Section, NINDS, Bethesda, MD*
- Jeffrey C. Smith, PhD, *Cellular and Systems Neurobiology Section, NINDS, Bethesda, MD*
- Hiroaki Wake, PhD, *National Institute for Basic Biology, Okazaki, Japan*

Contact

For more information, email fieldsd@mail.nih.gov or visit <http://nsdps.nichd.nih.gov>.

Translational Biophotonics in Developmental Disorders and Diseases

Brain imaging and spectroscopy of developmental disorders

We used the unimodal and multimodal approaches of functional near-infrared spectroscopy (fNIRS) and electroencephalogram (EEG) to conduct two distinct lines of research: (1) to investigate the developmental trajectories of neural activity associated with social-cognitive and motor signs in children with/without elevated risk for developmental disorders; and (2) to validate the fNIRS findings with that of the gold standards of EEG and/or functional magnetic resonance imaging (fMRI) during social cognitive tasks. Our goals are to validate the use of the child-friendly neural imaging tool fNIRS in early detection of developmental disorders and for predicting later developmental trajectories. To do so, we continued our collaborations with Audrey Thurm and Nathan Fox to study the development of the mirror neuron network (MNN) in infants. The MNN is associated with the development of sophisticated social-cognitive behaviors, which emerge during infancy (e.g., complex imitation, decoding emotional states). Modeling MNN development will create a sensitive measure of deviations in social-cognitive development before clinical behavioral deficits can be detected. MNN activation has already been associated with mu desynchronization (i.e., attenuation of EEG power in the alpha frequency), using EEG. Our research group extended these findings to fNIRS-related hemodynamic changes of the MNN. Moreover, we combined the use of EEG (which has high temporal resolution) with fNIRS (which provides a more precise spatial resolution of neural activity) to provide new insights into the MNN findings.

So far, five manuscripts were published from this work [Condy EE *et al. Front Hum Neurosci* 2021;15:627983; Miguel HO *et al. PLoS One* 2021;16:e0253788; Nguyen *et al. Brain Sci* 2021;11:397; and References 1 & 2], and one manuscript is under review. First of all, we conducted a thorough review of the literature summarizing previous neural findings in the MNN using fNIRS in adults. We then conducted our own fNIRS study to examine the hemodynamic changes in the MNN during a live action-observation and execution task. Thirty adults participated in our study in which they either reached for or observed an experimenter reach for objects. Their hemodynamic changes were recorded by fNIRS probes placed on the sensorimotor and parietal regions (bilateral superior parietal lobule



Amir H. Gandjbakhche, PhD,
*Head, Section on Translational
Biophotonics*

Hadis Dashtestani, PhD,
Postdoctoral Fellow

Thien Nguyen, PhD, *Postdoctoral
Fellow*

Jinho Park, PhD, *Postdoctoral
Fellow*

Soongho Park, PhD, *Postdoctoral
Fellow*

Wan-Chun Su, PhD, *Postdoctoral
Fellow*

Wei Lun Huang, MS, *Intramural
Research Training Award Student*

Emily Blick, BS, *Postbaccalaureate
Fellow*

Aaron Buckley, BS,
Postbaccalaureate Fellow

Sara Johnson, BS,
Postbaccalaureate Fellow

William Martin, BS,
Postbaccalaureate Fellow

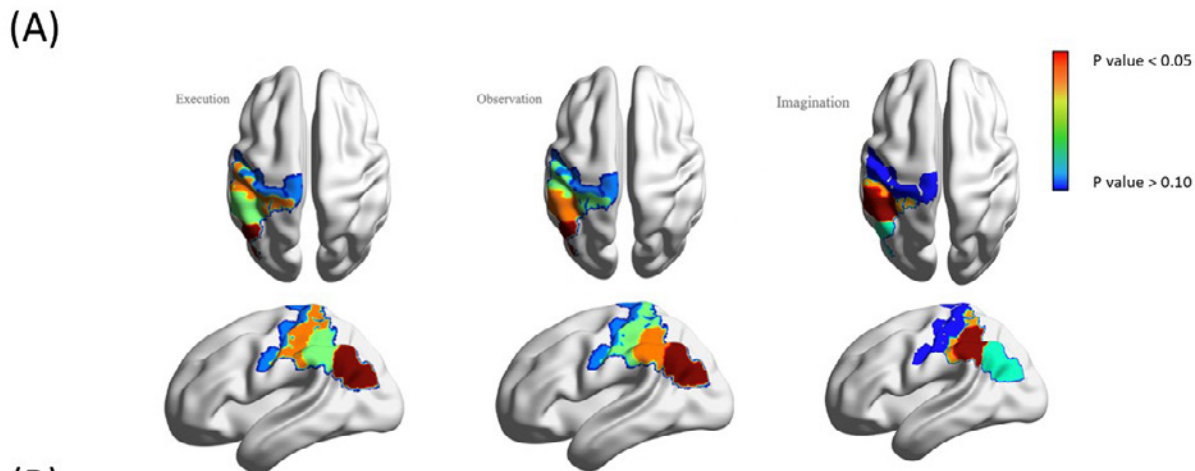
John Millerhagen, BS,
Postbaccalaureate Fellow

Vinay Veluvolu, BS,
Postbaccalaureate Fellow

(continued)

[SPL] and bilateral inferior lobule). The inferior parietal lobule (IPL), right supramarginal gyrus (SMG), and right angular gyrus (AG) were activated in both observation and execution conditions. Moreover, during execution, greater activation was found in the left precentral and postcentral regions than during observation. Besides the level of hemodynamic changes, previous neuro-imaging research suggested lateralized brain activity in the MNN during action execution, with greater activation in the contralateral than in the ipsilateral hemisphere. However, it is unknown whether the same lateralization could be found during action observation. We therefore used fNIRS to investigate the lateralization of cortical activation during a similar live action-observation and action-execution task. Our results suggested brain lateralization during action execution but not observation. In addition, we found that hand preference and dexterity between the right and left hands are related to brain lateralization patterns [Reference 1]. Our results are consistent with previous fMRI findings, supporting fNIRS as an applicable and valid method for studying the neural mechanisms of cognitive and motor signs. We also investigated the neural connectivity over MMN and

Marc Bornstein, PhD, *Special Volunteer*
 Han-Shin Hahn, PhD, *Special Volunteer*
 Kosar Khaksari, PhD, *Special Volunteer*
 Siamak Aram, PhD, *Guest Researcher*



(B)

	Execution			Observation			Imagination		
	Correlation	Corresponding region	P-value	Correlation	Corresponding region	P-value	Correlation	Corresponding region	P-value
Comp 1	0.3905	Left IPL	0.055	0.4823	Left IPL	0.0457	0.4418	Left SMG	0.0573
Comp 2	0.3821	Left POCG	0.0581	0.3753	Left SMG	0.0498	0.4080	Left POCG	0.0585
Comp 3	0.3548	Left SMG	0.0624	0.3310	Left POCG	0.053	0.3017	Left IPL	0.0638
Comp 4	0.3350	Left PRCG	0.0653	0.3351	Left PRCG	0.0617	0.300	Left PRCG	0.0664

FIGURE 1. Simultaneous recording of fNIRS and EEG

(A) Color-coded covariation in fNIRS and EEG recordings.
 (B) Regions, their cross-modality correlations, and the corresponding p-values.

its correlations with autistic traits in neurotypical adults. We defined the MNN connections with the regions that showed significantly greater connectivity in both action-execution and action-observation tasks than in a baseline, resting-state. Correlations between connectivity and autistic traits were also investigated using Pearson correlations. Strong MNN connections were found over the right precentral, right SMG, left IPL, left postcentral, and between left SMG–left AG. In addition, individuals with higher subclinical autistic traits present higher connectivity in both action execution and action-observation conditions. Positive correlations between MNN connectivity and subclinical autistic traits can be used in future studies to investigate the MNN in a developing population with ASD.

Besides the unimodal studies, we used a multimodal approach, i.e., simultaneous recordings of fNIRS and EEG, to reveal new insights in neural activity during action execution, observation, and imagery [Reference 2]. We simultaneously recorded fNIRS and EEG-based neural activity during the live action observation-execution and imagery tasks. Benefitting from concurrent recordings of brain hemodynamic and electrophysiological responses, we used a novel method, structured sparse multiset Canonical Correlation Analysis (ssmCCA), to find linear transforms of each modality and maximize the correlations between the projections of each modality. Our findings from 21 right-handed participants confirmed neural activity over the MNN system (e.g., central and parietal regions) during all three conditions. Unimodal analyses revealed differentiated activation between conditions; however, the activated regions did not fully overlap across the two modalities (fNIRS: left pre- and post-central, right angular gyrus; EEG: bilateral central, right frontal, and parietal). The discrepancies might be because fNIRS and EEG detect different signals. Using fused fNIRS–EEG data, we consistently found activation over the left inferior parietal lobe, superior marginal gyrus, and post-central gyrus during all three conditions, suggesting that our multimodal approach identifies a shared neural region associated with MNN (Figure 1). The study highlights the strengths of the multimodal fNIRS–EEG fusion technique for studying the MNN. Neural researchers should consider using the multimodal approach to validate their findings. In the future, we will use the multimodal approach to conduct longitudinal studies in children with or without elevated risks for developmental disorders. We believe that the study will facilitate early detection and intervention for children with developmental disorders.

COVID-19 point-of-care biosensor

As a result of the recent outbreak and spread of various mutants of coronavirus disease 19 (COVID-19), many people are experiencing high fever, acute respiratory diseases, and, in severe cases, death. In order to prevent the spread of COVID-19, a monitoring system that can monitor the status of the infected person real-time while minimizing contact with the infected person is needed. Patients with infectious viral diseases such as COVID-19 experience hypoxia, hypoxemia, and lung dysfunction caused by the destruction of lung cells by the virus, which changes the tissue oxygen saturation and body temperature of the infected patient. The purpose of our study is to develop a multimodal sensor device for measuring changes in acute respiratory disease-infected patients, and to develop a classification technology using data obtained from a complex sensor device. Such devices could become important tools for monitoring disease onset, progression, and recovery in the future.

At the beginning of the COVID-19 pandemic, we conducted a review study on the efficacy of infrared thermography for screening infectious diseases and found that the technology was not reliable. We therefore proposed using multiple vital signs such as blood and tissue oxygen saturation levels, to screen for an infectious disease. To prove the utility of our proposal, we started a multisite study at NIH and the

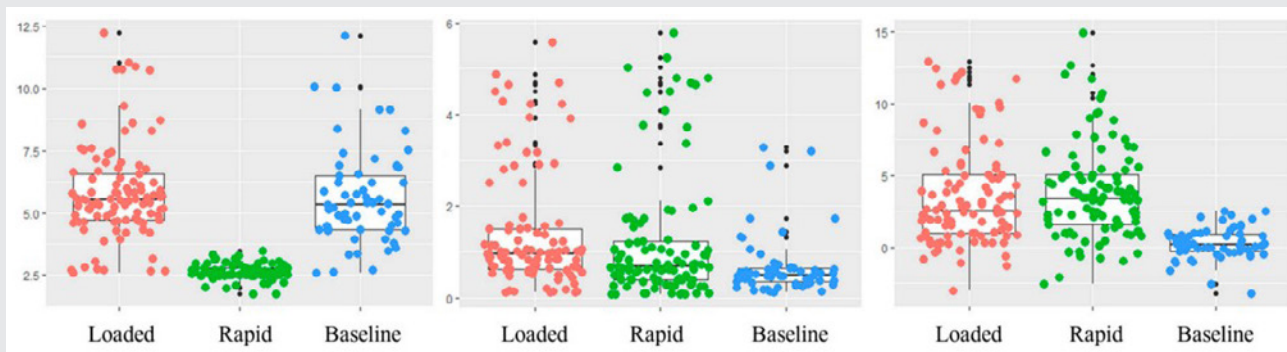


FIGURE 2. Classification of loaded, rapid, and normal breathing for monitoring COVID-19 infection

Machine learning techniques permit the classification of different breathing patterns for assessing respiratory diseases such as COVID-19.

University of British Columbia in Vancouver, Canada. In collaboration with Babak Shadgan, data were collected on 24 healthy volunteers during normal breathing, restricted breathing, and rapid breathing through a clinical protocol approved by the University of British Columbia. Tissue oxygenation levels and hemodynamics responses, including oxy-, deoxy-, and total hemoglobin, were measured using an NIRS device. The device consists of a multi-wavelength LED emitting light at 760 nm and 850 nm, and three photodiode detectors 3, 3.5, and 4 cm away from the LED. The NIRS device was placed over the participant's chest during data acquisition. In order to obtain signals similar to the breathing patterns experienced by COVID-19-infected patients, three breathing patterns, i.e., normal breathing, restricted breathing, and rapid breathing, were defined. During normal breathing, participants were instructed to breathe in a comfortable state. During the restricted breathing, nasal breathing was restricted by blocking the participant's nose with a clip, while mouth breathing was done through a breathing trainer. During the rapid breathing, participants were instructed to take about 25 shallow and rapid breaths per minute. A classification algorithm was based on machine learning for breathing patterns. We evaluated whether meaningful features can be detected from data acquired using the NIRS device and to classify various breathing patterns using machine learning technology (Figure 2). The maximum/minimum peaks of the O_2Hb data signal were detected. The interval and amplitude differences between the detected peaks were then used to generate two features: respiratory interval and breathing depth. The generated features and mean O_2Hb were used for learning and classification using random forest machine learning technology. We achieved an accuracy of 86% [Reference 3]. We are exploring the use of Deep Learning methods for real time classification.

Placenta oxygenation: from basics to point of care

The placenta plays an essential role in the health of both mother and fetus. An abnormal placenta is associated with pregnancy complications such as preeclampsia, fetal growth restriction, fetal death, preterm labor, and other complications. NIRS is an optical method for non-invasive measurement of blood oxygenated and deoxygenated hemoglobin and tissue oxygenation in deep tissue layers such as brain, muscle, and placenta. However, studies examining placental oxygenation levels have yielded conflicting results, discrepancies that may be the result of unknown placental scattering coefficients used for oxygenation calculations or differences in patient populations. A major problem in the assessment of placental oxygenation using NIRS arises from the

anatomical location of the organ; taking that into account (e.g., skin, adipose tissue, uterine wall), we designed a new wearable depth-resolving NIRS device featuring six source-detector distances in the range of 10–60 mm. Distinct source and detector distances scan different tissue depths to help distinguish between placental and maternal oxygenation. The device also uses two light sources with wavelengths of 760 nm and 840 nm so that it is sensitive to changes in blood oxyhemoglobin and deoxyhemoglobin. We evaluated the performance of the NIRS device by observing changes in the optical properties of a placental-mimicking phantom at a depth of 25 mm. In addition to evaluating the accuracy and validating the performance of the custom NIRS device, we performed *in vivo* oxygen measurements on two human subjects using a wearable NIRS device in various parts of the body, including arms, calves, and abdomen. We also compared the wearable NIRS device with a time-domain NIRS system (TRS-41 system, Hamamatsu Photonics, Japan) on different parts of the body, including the arms, calves, and abdomen, to evaluate the accuracy and validate performance. We found an average error of $2.7\% \pm 1.8\%$ between the two devices/systems, an agreement between the measurements of the wearable NIRS device and the well established TRS system that validates the high accuracy of the device in *in vivo* tissue oxygenation measurements.

We then used the NIRS device to measure placental oxygenation *in vivo* in 12 volunteers from the Advanced Obstetrics and Gynecology Research Center located at Detroit Medical Center (DMC). Measurements were performed at three locations: upper, middle, and lower parts of the placenta. Of the 12 subjects, five had maternal pregnancy complications such as short cervix, hypertension, or polyhydramnios. Eleven of the 12 participants delivered at the DMC. After delivery, the placentas of 10 of 11 participants were transferred to the DMC pathology department to examine the lesion. Chronic or acute lesions were found in five placentas, four of which were from participants with maternal pregnancy complications. The level of placental oxygenation was calculated using the intensity of backscattered light at the appropriate light source-detector separation. For each patient, three oxygenation levels at the three measurement sites were used: the upper, middle, and lower parts of the placenta. Our results suggest a possible relationship between placental oxygenation levels and pregnancy complications and placental pathology. However, the sample size used in this study is small (12 participants). We will conduct further studies to include more subjects. Results from this study have been published [Reference 4]. We are performing Monte Carlo simulations on the four-layer model to better understand the experimental results and to create theoretical indicators. Simulations are based on thickness and scattering and absorption coefficients of all maternal layers (dermis, adipose, uterus/rectus) and placenta. Based on Monte Carlo's random-walk theory and experimental results, we are attempting to develop a system that can analyze the characteristics of light scattering and propagation in the mother's maternal layers and more clearly measure the oxygenation levels of the mother's placenta. We are currently waiting for the results of the cord blood gas analysis at delivery, pregnancy outcomes, and placental pathology of 24 participants in DMC, whom we have measured. In the meantime, a two-layer model, based on Monte Carlo simulations and tissue thicknesses, is being developed to calculate the oxygenation level of the second layer. We consider maternal skin, adipose, and uterus as the first layer and the placenta as the second layer. The calculated placental oxygenation will be correlated with the oxygen gas content in the cord blood and pregnancy outcomes as well as placental pathology (Figure 3).

As a result of the COVID-19 pandemic, patient recruitment at the Center for Advanced Obstetrical Care and Research of the Perinatology Research Branch was interrupted. Although we were able to perform measurements on 24 pregnant women during our last visit to DMC in September, it will be difficult to recruit a larger population in future. Hence, we initiated a collaboration with Guoyang Luo to submit a clinical protocol

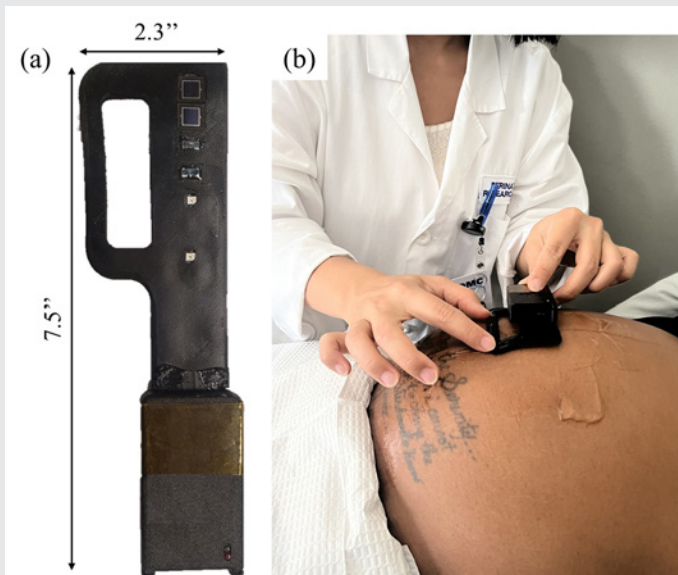


FIGURE 3. Wireless, wearable biosensors for placental oxygenation

(A) A wireless, flexible, and wearable multimodal biosensor, which consists of two multiwavelength LEDs, four PDs, and an accelerator, to monitor pregnancy health.

(B) During a measurement the biosensor was being placed on the abdomen, above the placenta, of a pregnant woman.

entitled “Continuous monitoring of the placental oxygenation during pregnancy” to the Howard University IRB. The primary objective of the protocol is to study and evaluate the oxygenation level of the placenta in order to standardize and correlate the oxygenation data with placenta pathology and pregnancy outcomes. The protocol will recruit up to 1,000 pregnant women from underserved communities over the course of five years. Also, we are developing an analysis algorithm that evaluates the behavior of placental cells in consideration of various oxygen levels, using the dynamic full-field optical coherence tomography (DFFOCT) system in parallel with measurement of placental oxygenation using the NIRS device.

We are developing an algorithm designed to analyze changes in the dynamic activity (frequency and magnitude of cells) within a cell and calculate a mean frequency representing a weighted frequency. As a preliminary experiment, we evaluated the viability status of HeLa cells, an immortal human cell line widely used in cell research, from alive to dead. With the developed algorithm, the dynamic activity of cells was quantitatively and clearly distinguishable according to changes in their viability status [Reference 5]. From these results, we believe that DFFOCT can be used to analyze changes in cellular dynamic activity depending on the nutrient and oxygen saturation contained in placental cells. We hypothesize that there is a relationship between the dynamic activity of placental cells and potential neuro-developmental disorders. For the next study, we will analyze the dynamic activity of the placenta cells, taking into account various oxygen levels in the placenta. To this end, we plan to create a special sample chamber to control and maintain the oxygen concentration in the cells while conducting the experiment.

Additional Funding

- Bench to Bedside Award 345 (2016): “Mirror neuron network dysfunction as an early biomarker of neurodevelopment” (ongoing)
- Human Placenta Project–NICHD (2016, ongoing)

Publications

1. Khaksari K, Smith EG, Miguel HO, Zeytinoglu S, Fox N, Gandjbakhche AH. An fNIRS study of brain lateralization during observation and execution of a fine motor task. *Front Hum Neurosci* 2022 15:798870.
2. Dashtestani H, Miguel HO, Condy EE, Zeytinoglu S, Millerhagen JB, Debnath R, Smith E, Adali T, Fox NA, Gandjbakhche AH. Structured sparse multiset canonical correlation analysis of simultaneous fNIRS and EEG provides new insights into the human action-observation network. *Sci Rep* 2022 12:6878.
3. Mah AJ, Nguyen T, Ghazi Zadeh L, Shadgan A, Khaksari K, Nourizadeh M, Zaidi A, Park S, Gandjbakhche AH, Shadgan B. Optical monitoring of breathing patterns and tissue oxygenation: a potential application in COVID-19 screening and monitoring. *Sensors (Basel)* 2022 22:7274.
4. Nguyen T, Khaksari K, Khare SM, Park S, Anderson AA, Bieda J, Jung E, Hsu CD, Romero R, Gandjbakhche AH. Non-invasive transabdominal measurement of placental oxygenation: a step toward continuous monitoring. *Biomed Opt Express* 2021 12(7):4119–4130.
5. Park S, Nguyen T, Benoit E, Sackett DL, Garmendia-Cedillos M, Pursley R, Boccara C, Gandjbakhche A. Quantitative evaluation of the dynamic activity of HeLa cells in different viability states using dynamic full-field optical coherence microscopy. *Biomed Opt Express* 2021 112(10):6431–6441.

Collaborators

- Franck Amyot, PhD, *Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD*
- Mehran Armand, PhD, *The Johns Hopkins University, Baltimore, MD*
- Claude Boccara, PhD, *École Supérieure de Physique et de Chimie Industrielles, Paris, France*
- Nathan Fox, PhD, *University of Maryland, College Park, MD*
- Andrea Gropman, MD, *Children's National Health System, Washington, DC*
- Jay Knutson, PhD, *Laboratory of Molecular Biophysics, NHLBI, Bethesda, MD*
- Guoyan Luo, MD, PhD, *Howard University Hospital, Washington, DC*
- Tom Pohida, MS, *Division of Computational Bioscience, CIT, NIH, Bethesda, MD*
- Randall Pursley, MSc, *Signal Processing and Instrumentation Section, CIT, NIH, Bethesda, MD*
- Jessica C. Ramella-Roman, PhD, *Florida International University, Miami, FL*
- Roberto Romero-Galve, MD, DMedSci, *Perinatology Research Branch, NICHD, Detroit, MI*
- Dan Sackett, PhD, *Division of Basic and Translational Biophysics, NICHD, Bethesda, MD*
- Babak Shadgan, MD, MSc, PhD, *University of British Columbia, Vancouver, Canada*
- Audrey Thurm, PhD, *Pediatrics & Developmental Neuropsychiatry Branch, NIMH, Bethesda, MD*
- Bruce Tromberg, PhD, *Section on Biomedical Optics, NICHD, Bethesda, MD*
- Eric Wassermann, MD, *Cognitive Neuroscience Section, NINDS, Bethesda, MD*

Contact

For more information, email gandjbaa@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/gandjbakhche>.

Transcriptional and Translational Regulatory Mechanisms in Nutrient Control of Gene Expression

We study the fundamental mechanisms involved in the assembly and function of translation initiation complexes for protein synthesis, using yeast as a model system in order to exploit its powerful combination of genetics and biochemistry. The translation initiation pathway produces an 80S ribosome bound to mRNA, with methionyl initiator tRNA (Met-tRNA_i) base-paired to the AUG start codon. The Met-tRNA_i is recruited to the small (40S) subunit in a ternary complex (TC) with the GTP-bound eukaryotic initiation factor eIF2 to produce the 43S preinitiation complex (PIC) in a reaction stimulated by eIFs 1, 1A, 3, and 5. The 43S PIC attaches to the 5' end of mRNA, facilitated by the cap-binding complex eIF4F (comprising eIF4E, eIF4G, and the RNA helicase eIF4A) and poly(A)-binding protein (PABP) bound to the poly(A) tail, and scans the 5' untranslated region (UTR) for the AUG start codon. Scanning is promoted by eIF1 and eIF1A, which induce an open conformation of the 40S and rapid TC binding in a conformation suitable for the scanning of successive triplets entering the ribosomal P site (P-out), and by eIF4F and other RNA helicases, such as Ded1 and its paralog Dbp1, that remove secondary structure in the 5'UTR. AUG recognition evokes tighter binding of the TC in the P-in state and irreversible GTP hydrolysis by eIF2, dependent on the GTPase-activating protein (GAP) eIF5, releasing eIF2-GDP from the PIC, with Met-tRNA_i remaining in the P site. Joining of the 60S subunit produces the 80S initiation complex ready for protein synthesis.

Our current aims in this research area are to: (1) elucidate the functions of eIF1, eIF5, eIF3, and 40S ribosomal proteins in TC recruitment and start-codon recognition; (2) identify distinct functions of the RNA helicases eIF4A (and its cofactors eIF4G/eIF4B), Ded1, and Dbp1, and of the poly(A)-binding protein (PABP) in mRNA activation, 48S PIC assembly, and scanning *in vivo*; (3) uncover the mechanisms of translational repression and regulation of mRNA abundance by the repressors Scd6, Pat1, the helicase Dhh1, and the mRNA-decapping enzyme Dcp2; (4) elucidate the regulation of Ded1, eIF4G, and Dhh1 functions in response to nutrient limitation or stress; and (5) elucidate the roles of the yeast orthologs of eIF2A and eIF2D in eIF2-independent initiation of translation in stress conditions.

We also analyze the regulation of amino acid-biosynthetic genes in budding yeast as a means of dissecting fundamental mechanisms



Alan G. Hinnebusch, PhD, Head,
*Section on Nutrient Control of
Gene Expression*

Hongfang Qiu, PhD, *Staff Scientist*

Fan Zhang, MS, *Senior Research
Assistant*

Swati Gaikwad, PhD, *Postdoctoral
Fellow*

Ritu Gupta, PhD, *Postdoctoral
Fellow*

Rakesh Kumar, PhD, *Postdoctoral
Fellow*

Priyanka Mittal, PhD, *Postdoctoral
Fellow*

Poonam Poonia, PhD,
Postdoctoral Fellow

Priyanka Singh, PhD, *Postdoctoral
Fellow*

Sakshi Singh, PhD, *Postdoctoral
Fellow*

Vishalini Valabhoju, PhD,
Postdoctoral Fellow

Anil Vijjamarri, PhD, *Postdoctoral
Fellow*

Qiaoyun Zheng, PhD, *Postdoctoral
Fellow*

of transcriptional control of gene expression. During amino acid limitation, transcription of such genes is coordinately induced by the activator Gcn4 as the result of its induction at the translational level. The eviction of nucleosomes that occlude promoter DNA sequences and block access by RNA polymerase is thought to be a rate-limiting step for transcriptional activation. Previous studies implicated certain histone chaperones, ATP-dependent chromatin-remodeling complexes, or histone acetyltransferase (HAT) complexes in eviction of promoter nucleosomes at certain yeast genes, but it is unclear whether these co-factors function at Gcn4 target genes. Our aim is to elucidate the full set of co-factors that participate in promoter nucleosome eviction at Gcn4 target genes, their involvement in this process genome-wide, and the transcriptional consequences of defective nucleosome eviction. Functional cooperation among the chromatin-remodeling complexes SWI/SNF, RSC, and Ino80, as well as the HAT complexes SAGA, NuA4, NuA3, and Rtt109/Asf1, in these processes are under study. We recently discovered that Gcn4 can activate transcription from binding sites within the coding sequences (CDS) of its target genes, inducing internal subgenomic sense and antisense (AS) transcripts in addition to the conventional full-length transcripts that initiate 5' of the CDS; and we are probing both the mechanism and possible regulatory functions of these internal AS transcripts, as well as the roles of co-transcriptional histone methylation, nucleosome reassembly, and mRNA decay enzymes in controlling their synthesis and abundance. We are also probing mechanisms involved in the asymmetric transcriptional induction of genes belonging to pairs of divergently oriented genes where only one gene responds to Gcn4 binding at the shared upstream activation sequences (enhancer).

Large-scale movement of eIF3 domains during translation initiation modulate start-codon selection.

In our previous cryo-electron microscopy (cryo-EM) reconstructions of yeast 48S preinitiation complexes (PIC), the eIF3 subcomplex (dubbed the *b/i/g/a* module), comprising the eIF3b subunit C-terminal domain (CTD), eIF3i, the eIF3g N-terminal domain (NTD), and an extended helical segment of eIF3a-CTD, is located near the decoding center at the 40S subunit interface, interacting with eIF1, eIF2y, eIF3c, and the 40S itself and appearing to lock the mRNA into the 40S binding cleft. The *b/i/g/a* module is found at this location in both the open and closed conformations of the PIC, which are thought to depict scanning and initiation conformations, respectively, with certain contacts restricted to either the open or closed state. Surprisingly, the *b/i/g/a* module was found at a dramatically different position on the solvent-exposed 40S surface in our more recent py48S-5N complex, where the eIF5-NTD replaces eIF1 on the 40S subunit in a later stage of the initiation pathway. We hypothesized that, following 43S PIC attachment to mRNA, the eIF3 *b/i/g/a* module relocates from the solvent side to the subunit interface of the open py48S complex to help prevent PIC drop-off from mRNA during scanning, that certain of its contacts at the interface surface are remodeled on AUG recognition, and that on dissociation of eIF1 and attendant loss of eIF3b-RRM (RNA recognition motif) interaction with eIF1, the *b/i/g/a* module relocates to the solvent side of the 40S to allow binding of the eIF5-NTD in place of eIF1 on the 40S platform. Examining eIF3b-CTD substitutions designed to disrupt interactions of its β -propeller or RRM with eIF2y, eIF1, or eIF3c, found uniquely at the interface surface, revealed that those conferring the strongest phenotypes increased discrimination against near-cognate UUG start codons (the *Ssu*⁻ phenotype). Binding assays confirmed the interaction of the eIF3b-RRM with eIF3c, found exclusively at the 40S subunit interface, in a manner perturbed by one such *Ssu*⁻ substitution at a predicted contact with eIF3c. Interestingly, strong *Ssu*⁻ phenotypes were also observed for eIF3b substitutions that perturb eIF3b interaction made exclusively at the solvent-exposed surface of the 40S subunit. The findings suggest that interactions of the *b/i/g/a* module with certain initiation factors at the subunit interface act primarily to stabilize the closed conformation of the

PIC on start-codon recognition, that relocation of the module back to the solvent interface is required to finalize start-codon selection, and that these interactions are crucial for the ability to utilize non-cognate initiation codons *in vivo*.

Amino acid residues of 40S protein uS5/Rps2 at the mRNA entry channel enhance initiation at suboptimal start codons *in vivo*.

The ribosomal protein uS3/Rps3 is positioned at the solvent side of the 40S near the mRNA entry channel. We showed previously that substituting uS3/Rps3 residues that contact mRNA preferentially destabilizes the closed conformation of the PIC, reducing initiation at both UUG codons and at AUG start codons that reside in suboptimal 'Kozak' sequence (5'-(gcc)gccRccAUGG-3') context (a sequence regarded as the optimum sequence for initiating translation in eukaryotes). Particular residues of uS5/Rps2 make distinct mRNA contacts at the 40S entry channel and also interact with rRNA elements that communicate with the 40S decoding center. We found that uS5/Rps2 substitutions V121D and I125K resemble the previously characterized uS3/Rps3 substitutions in suppressing initiation at UUG codons as well the poor-context AUG start codons in *SUI1* mRNA or an elongated form of upstream open-reading frame 1 of *GCN4* mRNA (el.-uORF1). Interestingly, the uS5/Rps2 substitutions D78A, Q89K, and K119A suppress UUG initiation but do not reduce initiation at the poor-context AUG codons, and thus appear to be specific for suppressing near-cognate initiation. Substitutions Q94D and T96K diminish initiation at the poor-context AUG codons of *SUI1* and el.-uORF1, and they efficiently suppress UUG initiation only when the UUG resides in poor sequence context. Thus, the latter two residues appear to act mainly in discriminating against poor Kozak context. The findings suggest that different uS5/Rps2 residues are involved in distinct mechanisms of discrimination against different features of poor initiation sites *in vivo*.

Reprogramming of translation in yeast cells impaired for ribosome recycling favors short, efficiently translated mRNAs.

In eukaryotes, formation of the 43S PIC, containing initiator Met-tRNA_i bound to the small ribosomal subunit, is a rate-determining step of translation initiation. Ribosome recycling after translation termination produces the free 40S subunits needed to reassemble 43S PICs for new initiation events. Yeast mutants lacking orthologs of mammalian eIF2D (Tma64), and either MCT-1 (Tma20) or DENR (Tma22), are broadly impaired for 40S recycling; however, it was unknown whether the defect alters the translational efficiencies (TEs) of mRNAs. Based on previous experiments, it was also possible that Tma64/eIF2D can substitute for eIF2 in recruitment of Met-tRNA_i during initiation. Consistent with impaired initiation, the *tma64Δtma20Δ* mutant exhibits reduced assembly of bulk polysomes. Ribosome profiling of this mutant reveals a marked reprogramming of translational efficiencies, wherein translation of the most efficiently translated ('strong') mRNAs tends to be elevated, whereas translation of 'weak' mRNAs generally declines. Profiling of the *tma64Δ* single mutant reveals none of the hallmarks of impaired 40S recycling nor changes in translation efficiencies, suggesting that the defects found in *tmaΔΔ* cells are associated with defective ribosome recycling rather than with loss of eIF2D function in Met-tRNA_i recruitment. Remarkably, we observed similar translational re-programming on reducing 43S PIC assembly by inducing phosphorylation of eIF2 or by decreasing total 40S subunit levels by depleting Rps26, without affecting ribosome recycling. Moreover, the *tmaΔΔ* mutation specifically impaired translation of mRNAs with cap-proximal secondary structures that are expected to impede PIC attachment. Our findings suggest that strong mRNAs outcompete weak mRNAs in response to 43S PIC limitation achieved in various ways at the step of 43S PIC recruitment, in accordance with mathematical modeling of how translational efficiencies of different groups of mRNAs are altered by reduced ribosome abundance. The findings also have important implications for understanding changes in translation occurring in human ribosomopathies in which 40S subunit levels are diminished.

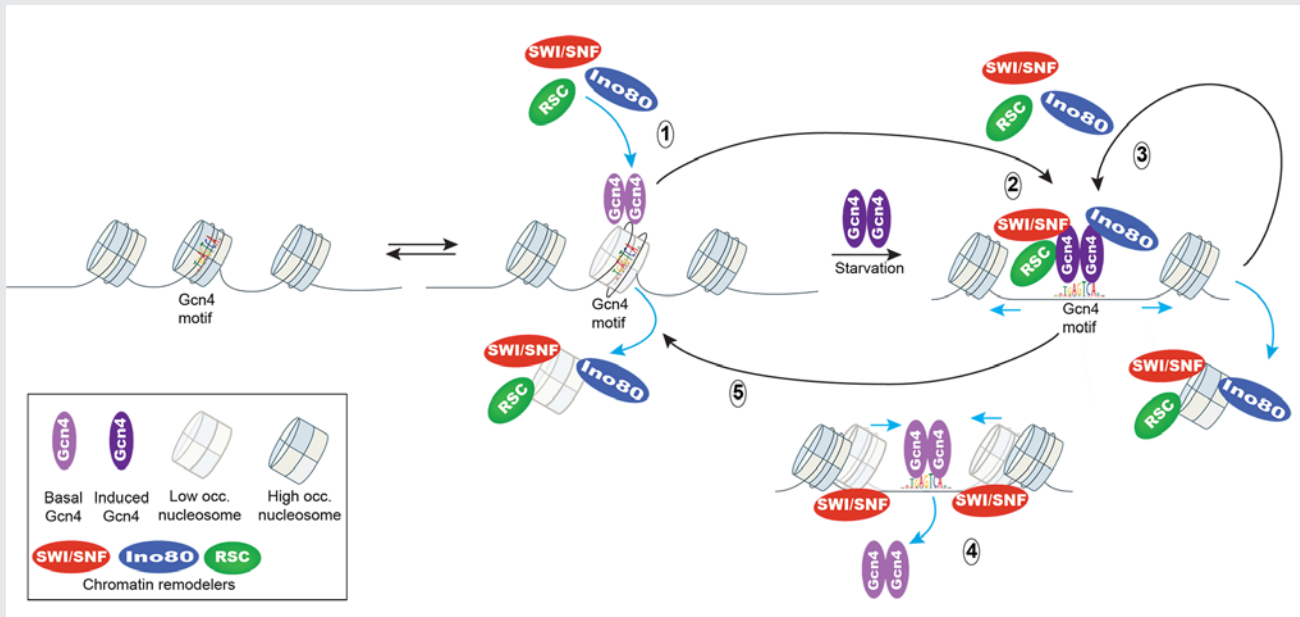


FIGURE 1. Hypothetical model depicting a positive feedback loop involving SWI/SNF, RSC, and Ino80C in stimulating Gcn4 binding to motifs in NDRs of Gcn4 target genes.

(1) Basal recruitment of the three CRs reduces nucleosome occupancy of the Gcn4-binding motif in the NDR (depicted by a semi-transparent nucleosome) to facilitate binding of Gcn4 expressed at uninduced levels (*light purple*). (2) Induction of Gcn4 expression by amino acid starvation (*dark purple*) leads to increased Gcn4 binding by mass action. The increased Gcn4 occupancy leads to additional recruitment of the CRs, which in turn increases eviction and displacement of nucleosomes from the Gcn4 motif to favor additional Gcn4 binding, thus establishing a positive feedback loop that promotes Gcn4 binding (3). At a subset of binding sites, increased SWI/SNF recruitment enhances an inhibitory effect on Gcn4 binding by an unknown mechanism, leading to dissociation of Gcn4 from the NDR (4) and reversing the stimulatory effects of other CRs on Gcn4 binding (5).

Down-regulation of yeast helicase Ded1 by glucose starvation or heat-shock differentially impairs translation of Ded1-dependent mRNAs.

Ded1 is an essential DEAD-box helicase in yeast that broadly stimulates translation initiation and is critical for mRNAs with structured 5'UTRs. Recent evidence suggests that the condensation of Ded1 in mRNA granules down-regulates Ded1 function during heat-shock or glucose starvation. We examined this hypothesis by determining the overlap between mRNAs whose relative translational efficiencies (TEs), as determined by ribosomal profiling, were diminished in either stressed wild-type (WT) cells or in *ded1* mutants examined in non-stress conditions. Only subsets of the Ded1-hyperdependent mRNAs identified in *ded1*-mutant cells exhibited strong TE reductions in glucose-starved or heat-shocked WT cells; and those down-regulated by glucose starvation also exhibited hyper-dependence on the initiation factor eIF4B, and to a lesser extent of eIF4A, for efficient translation in non-stressed cells. The findings are consistent with recent proposals that the dissociation of Ded1 from mRNA 5'UTRs and the condensation of Ded1 contribute to reduced Ded1 function during stress, and they further suggest that the down-regulation of eIF4B and eIF4A functions also contributes to the translational impairment of a select group of Ded1 mRNA targets with heightened dependence on all three factors during glucose starvation.

Distinct functions of three chromatin remodelers in activator binding and preinitiation-complex (PIC) assembly

The nucleosome-remodeling complexes (CRs) SWI/SNF, RSC, and Ino80C cooperate in evicting or repositioning nucleosomes to produce nucleosome-depleted regions (NDRs) at the promoters of many yeast genes induced by amino acid starvation. We analyzed mutants lacking the CR catalytic subunits for binding of the transcriptional activator Gcn4 and recruitment of TATA-binding protein (TBP) during PIC assembly. RSC and Ino80 enhance Gcn4 binding to UAS (upstream activation sequence) elements in NDRs upstream of many promoters as well as to unconventional binding sites within nucleosome-occupied coding sequences; and SWI/SNF contributes to UAS binding when RSC is depleted. All three CRs are actively recruited by Gcn4 to most UAS elements and appear to enhance Gcn4 binding by reducing nucleosome occupancies at the binding motifs, indicating a positive regulatory loop (Figure 1). SWI/SNF acts unexpectedly in WT cells to prevent excessive Gcn4 binding at certain UAS elements, which might involve transient nucleosome sliding that does not alter steady-state nucleosome occupancies. All three CRs also stimulate TBP recruitment, at least partly by reducing nucleosome occupancies at TBP binding sites, with SWI/SNF acting preferentially at the most highly expressed Gcn4 target genes. RSC and Ino80 function more broadly than SWI/SNF to stimulate TBP recruitment at most constitutively expressed genes, including ribosomal protein genes, whereas SWI/SNF acts preferentially at a distinct subset of highly expressed genes. Our findings point to a complex interplay among the three CRs in evicting promoter nucleosomes to regulate activator binding and stimulate PIC assembly.

Publications

1. Dong J, Hinnebusch AG. uS5/Rps2 residues at the 40S ribosome entry channel enhance initiation at suboptimal start codons *in vivo*. *Genetics* 2022 220:iyab176.
2. Llácer JL, Hussain T, Dong J, Villamayor L, Gordiyenko Y, Hinnebusch AG. Large-scale movement of eIF3 domains during translation initiation modulate start codon selection. *Nucleic Acids Res* 2021 49(20):11491–11511.
3. Gaikwad S, Ghobakhlu F, Young DJ, Visweswaraiiah J, Zhang H, Hinnebusch AG. Reprogramming of translation in yeast cells impaired for ribosome recycling favors short, efficiently translated mRNAs. *eLife* 2021 10:e64283.
4. Sen ND, Zhang H, Hinnebusch AG. Down-regulation of yeast helicase Ded1 by glucose starvation or heat-shock differentially impairs translation of Ded1-dependent mRNAs. *Microorganisms* 2021 9:2413.

Collaborators

- Jose L. Llácer, PhD, *Instituto de Biomedicina de Valencia (IBV-CSIC), Valencia, Spain*
- Jon Lorsch, PhD, *Laboratory on the Mechanism and Regulation of Protein Synthesis, NICHD, Bethesda, MD*
- Venkatraman Ramakrishnan, PhD, *MRC Laboratory of Molecular Biology, Cambridge, United Kingdom*
- Neelam Sen, PhD, *Jawaharlal Nehru University, New Delhi, India*
- Hussain Tanweer, PhD, *Indian Institute of Science, Bangalore, India*

Contact

For more information, email ahinnebusch@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/hinnebusch>.

Molecular Nature and Functional Role of Dendritic Voltage-Gated Ion Channels

The central nervous system (CNS) underlies all our experiences, actions, emotions, knowledge, and memories. With billions of neurons each firing hundreds of times per second, the complexity of the brain is stunning. To pare down the task of understanding something so complex, our research approach calls for studying the workings of a single central neuron: the pyramidal neuron from the CA1 region of the hippocampus. In humans, the hippocampus is essential for long-term memory and is among the first brain regions affected by epilepsy and Alzheimer's disease. To understand how the hippocampus stores and processes information, we focus on the CA1 pyramidal neuron, one of its principal cell types. Each of these cells receives tens of thousands of inputs onto its dendrites, and it is commonly thought that information is stored by altering the strength of individual synapses (synaptic plasticity). Recent evidence suggests that the regulation of synaptic surface expression of glutamate receptors can, in part, determine synaptic strength. However, the dendrites contain an abundance of ion channels that are involved in receiving, transforming, and relaying information in the dendrites, adding an additional layer of complexity to neuronal information processing.

We found that the A-type potassium channel subunit Kv4.2 is highly expressed in the dendritic regions of CA1 neurons in the hippocampus and, as one of the primary regulators of dendritic excitability, plays a pivotal role in information processing. Kv4.2 is targeted for modulation during the types of plasticity thought to underlie learning and memory. Moreover, we found that the functional expression level of Kv4.2 regulates the subtype expression of NMDA-type glutamate receptors, the predominant molecular devices controlling synaptic plasticity and memory. We are currently following up on these findings with more detailed investigations into the mechanisms of activity-dependent Kv4.2 regulation. In addition, we have begun to investigate the role of dendritic voltage-gated potassium and calcium channels in neuronal development and developmental disorders.

Role of voltage-gated ion channels in synaptic development and disease

ISOMERASE REGULATION OF POTASSIUM CHANNEL TRAFFICKING AND FUNCTION.

We recently identified a novel molecular cascade initiated by the



Dax Hoffman, PhD, *Head, Section on Molecular Neurophysiology and Biophysics*

Jiahua Hu, PhD, *Staff Scientist*

Lin Lin, PhD, *Microbiologist*

Ying Liu, MD, *Biologist*

Cole Malloy, PhD, *Postdoctoral Fellow*

Jon Murphy, PhD, *Postdoctoral Fellow*

Meghyn Welch, PhD, *Postdoctoral Fellow*

Maisie Ahern, BS, *Postbaccalaureate Fellow*

Ashley Pratt, BS, *Postbaccalaureate Fellow*

activation of p38 kinase and subsequent isomerase Pin1-dependent isomerization of a C-terminal motif (T607) in Kv4.2 that triggers dissociation from its auxiliary subunit DPP6, a reduction in the voltage-gated K⁺ channel I_A, and an increase in neuronal excitability. Pin1 is a prolyl isomerase that selectively binds to and isomerizes phospho-Ser/Thr-Pro (pSer/Thr-Pro) bonds. Mis-regulation of Pin1 plays an important role in a growing number of pathological conditions, including Alzheimer's disease, where it may protect against age-dependent neurodegeneration. Using biochemical and electrophysiological techniques, we showed that Pin1 activity is required for the dissociation of the Kv4.2DPP6 complex and that this action alters neuronal excitability. To investigate the consequences of this cascade on behavior and neuronal physiology, we used CRISPR-Cas9 techniques to generate a knockin mouse in which the isomerase binding site is specifically abolished (Kv4.2TA). The mice are viable and appear normal, although the activity-dependent dissociation of the Kv4.2-DPP6 complex is impaired. Kv4.2TA mice exhibit normal initial learning and memory in the Morris Water Maze; however, they exhibited better 'reversal' learning in Morris Water Maze than did wild-type (WT) mice. The data strongly support the idea that activity-dependent regulation of Kv4.2 plays an important role in cognitive flexibility. Cognitive flexibility is the ability to appropriately adjust one's behavior to a changing environment and is impaired in various neurodevelopmental disorders, such as the autism spectrum disorder.

Considering the finding that Kv4.2TA mice exhibit enhanced cognitive flexibility, ongoing experiments are investigating potential differences in synaptic properties between WT and Kv4.2TA mice. To determine the cellular/molecular correlate of this cognitive phenotype, Cole Malloy used patch clamp electrophysiology in hippocampal CA1 pyramidal cells from Kv4.2TA and WT mice to record several forms of synaptic plasticity. Kv4.2TA mice exhibit basal synaptic transmission that is similar to that of WT mice, as evidenced by comparable mini excitatory synaptic currents (mEPSCs), synaptic release probability (paired-pulse ratios), and evoked synaptic transmission magnitude. Additionally, long-term plasticity from a basal state is preserved relative to WT in stratum radiatum of the hippocampus. We performed single-cell measures of spike timing-dependent long-term potentiation (STD-LTP) and long-term depression (LTD) in CA1 pyramidal neurons in acute hippocampal slices. We observed no change in either STD-LTP, NMDA-dependent LTD, or mGluR-dependent LTD in Kv4.2TA mice compared with WT when recorded from a basal state. However, intriguingly, there was a significant enhancement in the reversal of STD-LTP (depotentiation) magnitude in Kv4.2TA mice, which appears to be driven by differences in NMDA-mediated transmission. This suggests a synapse state-dependent difference in synaptic plasticity in CA1 stratum-radiatum of the hippocampus facilitated by loss of dynamic regulation of the Kv4.2 complex. We have thus revealed a novel metaplasticity mechanism in a Kv4.2 mouse model. Further investigations into the correlation of this plasticity and the cognitive flexibility phenotype exhibited by this mouse model, as well as into more detailed molecular mechanisms, are on-going.

Seizure or neuronal activity leads to Kv4.2 protein degradation. We found that Kv4.2 degradation is dependent on the above Pin1 mechanism, given that Kv4.2 trafficking and degradation are abolished in Kv4.2TA mice. Seizure or neuronal activity triggers Kv4.2 phosphorylation and subsequent isomerization by Pin1, which results in Kv4.2 dissociation with the peptidase Dpp6 and internalization. Internalized Kv4.2 underwent ubiquitination and degradation. In order to examine whether DPP6 is required for this process, we employed DPP6 knock-out (KO) mice to test kainic acid-induced Kv4.2 protein loss. We found that seizure-induced Kv4.2 degradation is abolished in DPP6 KO mice, suggesting that seizure-induced Kv4.2 protein loss occurred in DPP6-containing Kv4.2 complex. Interestingly, we found that seizure-induced Kv4.2 phosphorylation at the Pin1 isomerization site is also abolished in DPP6 KO mice, while induction of p38 activity is normal in DPP6 KO, suggesting that seizure-induced Kv4.2 phosphorylation occurred in DPP6-containing Kv4.2 complex. These

data are consistent with and support the notion that seizure of neuronal activity induces phosphorylation of DPP6-containing Kv4.2 complex, which results in Dpp6-Kv4.2 dissociation and internalization and subsequent ubiquitination and degradation.

CA²⁺ REGULATION OF POTASSIUM CHANNEL FUNCTION

Jonathan Murphy found that Ca²⁺ entry mediated by the voltage-gated Ca²⁺ channel subunit Cav2.3 regulates Kv4.2 function both in a heterologous expression system and endogenously in CA1 pyramidal neurons through Ca²⁺-binding auxiliary subunits known as K⁺ channel-interacting proteins (KChIPs). KChIPs are calcium-sensing molecules containing four EF-hands, which are dysregulated in several diseases and disorders, including epilepsy, Huntington's disease, and Alzheimer's disease. We identified Cav2.3 as a Kv4.2-interacting protein in a proteomic screen and confirmed Cav2.3-Kv4.2 complex association using several techniques. Murphy characterized a KChIP-independent interaction between Cav2.3 and Kv4.2 using immunofluorescence colocalization, coimmunoprecipitation, electron microscopy, FRAP, and FRET. We found that Ca²⁺ entry via Cav2.3 increases Kv4.2-mediated whole-cell current in part as a result of an increase in Kv4.2 surface expression. In hippocampal neurons, pharmacological block of Cav2.3 reduced whole-cell I_A. We also found a reduction in whole-cell I_A in Cav2.3 KO mice mouse neurons, with a loss of the characteristic dendritic I_A gradient. Furthermore, the loss of Cav2.3 function leads to the enhancement of AMPA receptor-mediated synaptic currents and NMDA receptor-mediated spine Ca²⁺ influx. The findings reveal that an intermolecular Cav2.3-Kv4.2 complex impacts synaptic integration in CA1 hippocampal neurons.

DPP6 IMPACTS BRAIN DEVELOPMENT, FUNCTION AND ALZHEIMER'S DISEASE/DEMENTIA

In 2020 Lin Lin reported [Reference 6] that the novel structures in hippocampal area CA1 are significantly more prevalent in DPP6-KO aging mice than in WT mice and are also observed earlier during development in DPP6-KO mice. These novel structures apparently derived from degenerating presynaptic terminals, as clusters of large puncta that colocalize NeuN, synaptophysin, and chromogranin A, and also partially label for MAP2, β-amyloid, β-amyloid precursor protein (APP), α-synuclein, and phosphorylated tau, with synapsin-1 and VGluT1 labeling on their periphery.

Following these results, Lin Lin recently found that DPP6-KO mice show enhanced neurodegeneration associated with Alzheimer pathology. By using immunofluorescence and electron microscopy, we confirmed that both APP and β-amyloid are prevalent in these novel structures; and we showed, with immunofluorescence, that similar novel structures are found in the human hippocampal CA1 of Alzheimer's disease donors. In aged mice, we used *in vivo* MRI to show reduced size in DPP6-KO brain and hippocampus. Aging DPP6-KO hippocampi contained fewer total neurons and greater neuron death and showed diagnostic biomarkers of Alzheimer's disease, including accumulation of β-amyloid and APP and increase in expression of hyper-phosphorylated tau. The β-amyloid and phosphorylated tau pathologies were associated with neuro-inflammation characterized by increases in microglia and astrocytes. Levels of pro-inflammatory or anti-inflammatory cytokines increased in aging DPP6-KO mice. We also showed that aging DPP6-KO mice display circadian dysfunction, a common symptom of Alzheimer's disease. Together these results indicate that aging DPP6-KO mice show symptoms of enhanced neurodegeneration reminiscent of dementia associated with a novel structure resulting from synapse loss and neuronal death.

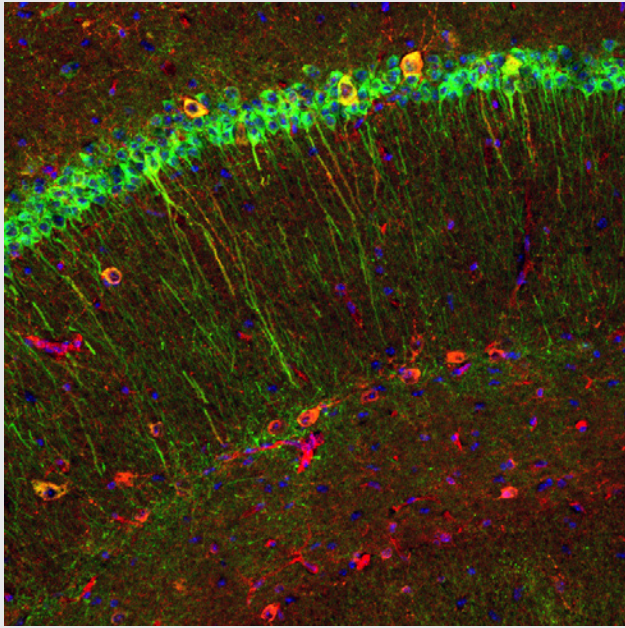


FIGURE 1. Amyloid accumulation in the CA1 hippocampus of aging DPP6-KO mice

Immunofluorescence image from an 8-week-old DPP6-KO mouse brain shows diffuse labeling for β -amyloid (*red*) in some pyramidal neuron cell bodies and apical dendrites (labeled in *green* for MAP2). In addition, labeling for β -amyloid is concentrated in some interneurons (*large, yellow*).

Publications

1. Murphy JG, Gutzmann JJ, Lin L, Hu J, Petralia RS, Wang YX, Hoffman DA. R-type voltage-gated Ca^{2+} channels mediate A-type K^{+} current regulation of synaptic input in hippocampal dendrites. *Cell Rep* 2022 38(3):110264.
2. Lin L, Petralia RS, Holtzclaw L, Wang YX, Abebe D, Hoffman DA. Alzheimer's disease/dementia-associated brain pathology in aging DPP6-KO mice. *Neurobiol Dis* 2022 174:105887.
3. Malloy C, Ahern M, Lin L, Hoffman DA. Neuronal roles of the multifunctional protein dipeptidyl peptidase-like 6 (DPP6). *Int J Mol Sci* 2022 23(16):9184.
4. Hu JH, Malloy C, Tabor GT, Gutzmann JJ, Liu Y, Abebe D, Karlsson RM, Durell S, Cameron HA, Hoffman DA. Activity-dependent isomerization of Kv4.2 by Pin1 regulates cognitive flexibility. *Nat Commun* 2020 11(1):1567.
5. Hu JH, Malloy C, Hoffman DA. P38 regulates kainic acid-induced seizure and neuronal firing via Kv4.2 phosphorylation. *Int J Mol Sci* 2020 21(16):5921.
6. Lin L, Petralia RS, Lake R, Wang YX, Hoffman DA. A novel structure associated with aging is augmented in the DPP6-KO mouse brain. *Acta Neuropathol Commun* 2020 8(1):197.

Collaborators

- Heather Cameron, PhD, *Section on Neuroplasticity, NIMH, Bethesda, MD*
- Constantine A. Stratakis, MD D(med)Sci, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*

Contact

For more information, email hoffmand@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/hoffman>.

Control of Gene Expression during Development

During development and differentiation, genes become competent to be expressed or are stably silenced in an epigenetically heritable manner. The selective activation/repression of genes leads to differentiation of tissue types. Much evidence supports the model in which modifications of histones in chromatin contribute substantially to determining whether a gene is expressed. Two groups of genes, the Polycomb group (PcG) and Trithorax group (TrxG), are important for inheritance of the silenced and active chromatin state, respectively. In *Drosophila*, regulatory elements called Polycomb group response elements (PREs) are required for the recruitment of chromatin-modifying PcG protein complexes. TrxG proteins may act through the same or overlapping *cis*-acting sequences. Our group aims to understand how PcG and TrxG proteins are recruited to DNA. Toward that end, one major project in the lab has been to determine all sequences and DNA-binding proteins required for PRE activity. In the *Drosophila* genome, there are hundreds of PREs that regulate a similar number of genes, and it was not known whether all PREs are alike. Our data showed that there is functional and architectural diversity among PREs, suggesting that PREs adapt to the environment of the gene they regulate. PREs are made up of binding sites for several DNA-binding proteins. Over the years, our lab identified Pho, Pho-like, Spps, and Combgap as DNA proteins that bind to PREs. Our recent genome-wide studies show that different PREs require distinct DNA-binding proteins. In addition, our work illustrates the combinatorial nature and redundancy of PcG recruitment in *Drosophila*.

A second major project in the lab is to determine how the PREs of the *invected-engrailed (inv-en)* gene complex control these genes in their native location. Surprisingly, we found that not all PREs are required *in vivo*, suggesting a redundancy in PRE function. To understand the interplay between PREs and enhancers (sequences important for activation of gene expression), we completed an analysis of the regulatory DNA of the *inv-en* gene complex. We found that regulatory sequences are spread throughout a region of at least 79kb in that gene complex and that the same enhancers activate both *engrailed* and *invected* expression. In addition, we showed that a 79-kb transgene (*HA-en79*), which contains the *en* gene and flanking regulatory DNA, is able to rescue a deletion for the entire *inv-en* locus. Our current studies explore the effects of the chromosomal neighborhood on gene



Judith A. Kassis, PhD, Head,
Section on Gene Expression

J. Lesley Brown, PhD, Staff
Scientist

Yuzhong Cheng, PhD, Senior
Research Technician

Taylor Crawford, BS,
Postbaccalaureate Fellow

Celine Kisimba, Postbaccalaureate
Fellow

expression. We found that there are subtle differences in the gene expression program of the 79-kb transgene and the endogenous locus. Polycomb domains are flanked by active genes or by insulators that limit the size of the domain. We hypothesized that delimiting the size of a Polycomb domain contributes to the stability of both gene activation and repression, making gene expression reproducible and robust. We recently completed experiments showing that flanking the 79-kb transgene by insulator elements strengthens the expression of the transgene, providing evidence for our hypothesis. Thus, providing “ends” to the *inv-en* domain stabilizes both its “ON” and “OFF” transcriptional states.

Polycomb group response elements (PREs)

PcG proteins act in protein complexes that repress gene expression by modifying chromatin [Reference 1]. The best studied PcG protein complexes are PRC1 and PRC2. PRC2 contains the histone methyltransferase Enhancer of Zeste, which tri-methylates lysine 27 on histone H3 (H3K27me3). The chromatin mark H3K27me3 is the signature of PRC2 function. At most well studied genes, PRC2 acts with PRC1, which binds to H3K27me3 and inhibits chromatin remodeling. In *Drosophila*, PRC1 and PRC2 are recruited to the DNA by PREs. We are interested in determining how this occurs, and, to that end, we defined all the DNA sequences and are finding all DNA-binding proteins required for the activity of a single 181-bp PRE of the *Drosophila engrailed* gene (PRE2). We found that binding sites for seven different proteins are required for the activity of the PRE2 (Figure 1). There are several binding sites for some of these proteins. Different PREs have distinct architectures (Figure 1). Our laboratory identified four PRE DNA-binding proteins: Pho, Phol, Spps, and, most recently, Combgap. The Combgap protein has 10 zinc fingers and recognizes the sequence GTGTGT.

PRE activity can be studied in transgenes, where a single PRE can recruit PcG protein complexes and silence the expression of a reporter gene. In transgenes, mutation of the Spps-, Pho-, or Combgap-binding sites within the PRE obliterates its ability to recruit PcG proteins, and the reporter gene is expressed. Thus,

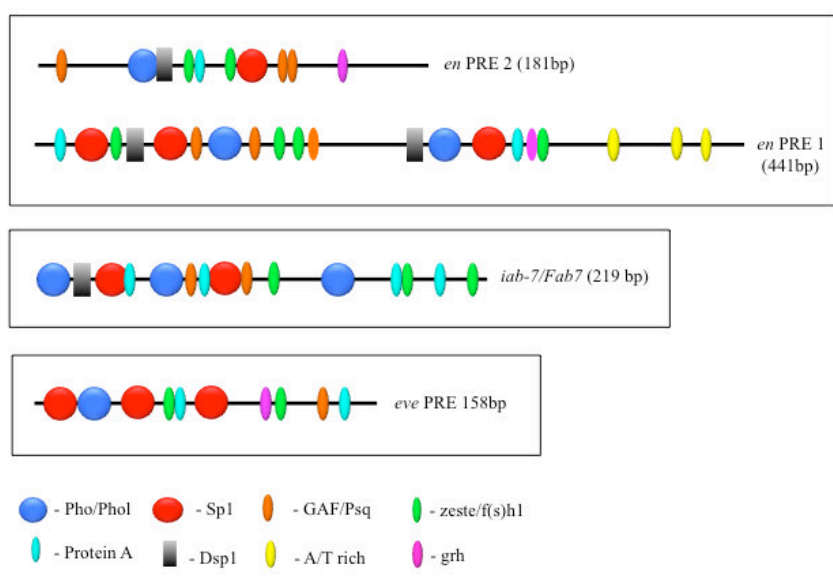


FIGURE 1. The order, number, and spatial arrangement of consensus-factor binding sites varies in different PREs.

en PRE1 and 2 are from the *engrailed* gene; *iab-7/Fab-7* PRE is from the *Abd-B* gene; *eve* PRE is from the *even-skipped* gene. The symbols represent consensus binding sites for the proteins indicated below (figure reprinted from Brown JL, Kassis JA. *Genetics* 2013;195:433).

transcriptional silencing by a single PRE in a transgene requires the combinatorial activity of many DNA-binding proteins. We were interested to determine what happens when one of the PRE DNA-binding proteins (the ‘recruiters’) from the genome is removed, and we examined the effect on PcG recruitment genome-wide. We studied PcG binding genome-wide in mutants that lack the recruiters Spps or Pho [Reference 2]. We found that PcG recruitment to some PREs was completely disrupted, whereas recruitment of PcG proteins was hardly diminished at most PREs. Most PcG domains, which are covered by the chromatin mark H3K27me3, contain several PREs. We believe that the structure of the H3K27me3 domains stabilizes genomic PREs to the loss of one recruiter. However, there are different kinds of PREs, and some are uniquely sensitive to the loss of one recruiter. Our study highlights the complexity and diversity of PcG recruitment mechanisms. Our recent experiments support the hypothesis that H3K27me3 domains stabilize PRE function.

In addition to identifying hundreds of presumptive PREs within Polycomb (H3K27me3) domains, there are thousands of presumptive PREs outside the H3K27me3 domains in larvae [Reference 2]. The question arises as to what the function of these PRE-like DNA fragments is. Our data suggest that many are tissue-specific PREs, given that H3K27me3 is detected in these locations in some cell types, while at other locations, no H3K27me3 is present in any cell type. Our on-going studies are addressing the interesting question as to whether these PRE-like DNA fragments can silence expression when included in a transgene. Our results so far argue against this, i.e., these DNA fragments do not function as PREs in a transgene silencing assay. These results show that we do not yet fully understand what constitutes a PRE.

The role of PREs at the *en* gene

The *Drosophila engrailed (en)* gene encodes a homeodomain protein that plays an important role in the development of many parts of the embryo, including formation of the segments, nervous system, head, and gut. By specifying the posterior compartment of each imaginal disc, *en* also plays a significant role in the development of the adult. Accordingly, *en* is expressed in a highly specific and complex manner in the developing organism. The *en* gene exists in a gene complex with *invected (inv)*, an adjacent gene; *inv* encodes a protein with a nearly identical homeodomain; *en* and *inv* are co-regulated and express proteins with largely redundant functions. Unlike *en*, *inv* is dispensable for *Drosophila* viability in the laboratory.

The *en* and *inv* genes exist in a 113kb domain that is covered by the H3K27me3 chromatin mark (Figure 2). Within the *en/inv* domain there are four major PREs, which are strong peaks of PcG protein binding. One popular model posits that DNA-binding proteins bound to the PREs recruit PcG protein complexes and that PRC2 tri-methylates histone H3 throughout the domain until PRC2 comes to either an insulator or an actively transcribed gene. There are two PREs upstream of the *en* transcription unit, PRE1 and PRE2 (Figure 1). Both PREs reside within a 1.5kb fragment located from -1.9kb to -400bp upstream of the major *en* transcription start site. There are also two major *inv* PREs, one located at the promoter and another about 6kb upstream of that. Our laboratory showed that all these PREs have the functional properties attributed to PREs in transgenic assays. To test their function at the intact *en-inv* domain, we set out to delete these PREs from the genome. Given that PREs work as repressive elements, the predicted phenotype of a PRE deletion is gain-of-function ectopic expression. Unexpectedly, when we made a 1.5kb deletion removing PRE1 and PRE2, flies were viable and had a partial loss-of-function phenotype in the wing. Similarly, deletion of *inv* PREs yielded viable flies with no mis-expression of *en* or *inv*. Importantly, the H3K27me3 *inv-en* domain is not disrupted in either of these mutants [Reference 3].

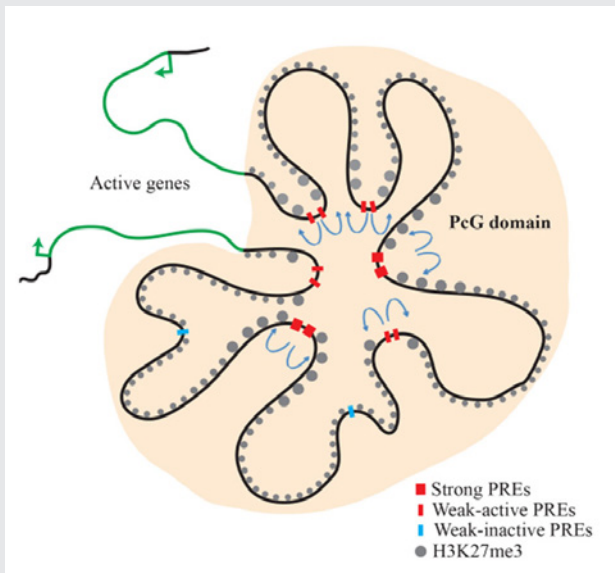


FIGURE 2. Model of the *inv-en* PcG domain in the transcriptionally silenced state

The *inv* and *en* genes are covered with H3K27me3 and are transcriptionally silent. PcG proteins are associated with this domain. There are strong, constitutive PREs, as well as ‘weak,’ tissue-specific PREs. ‘Weak’ PREs often overlap enhancers and are active in some tissues but inactive in others. Actively transcribed genes remain segregated from the PcG domain and determine the limits of the PcG domain (reprinted from Reference 3).

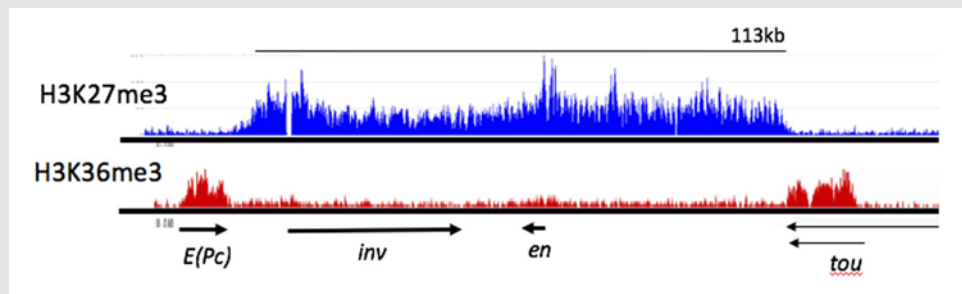
In *Drosophila*, PREs are easily recognizable in chromatin immunoprecipitation experiments as discrete peaks of PcG protein binding, but the H3K27me3 mark spreads throughout large regions. PcG proteins are conserved in mammals; however, PcG binding usually does not occur in sharp peaks, and PREs have been much harder to identify. We created a chromosome in which both the *en* and *inv* PREs are deleted [Reference 3]. Surprisingly, the flies are viable, and there is no mis-expression of *en* or *inv* in embryos or larvae. The question arises as to how PcG proteins are recruited to the *inv-en* domain in the absence of these PREs. We performed chromatin-immunoprecipitation followed by Next-Gen sequencing (ChIP-Seq) on the PcG proteins Pho and Polyhomeotic (Ph). The data showed that, in addition to the large Pho/Ph peaks at the known PREs, there are many smaller Pho/Ph peaks within the *inv-en* domain. We found that those peaks may also function as PREs. Thus, rather than a few PREs, there are many PREs controlling *inv-en* expression, and some may act in tissue-specific ways [Reference 3]. Our work shows that there are two types of PREs in *Drosophila*: strong, constitutive PREs and tissue-specific PREs that tend to overlap with enhancers (Figure 2).

The *inv-en* gene complex is flanked by *tou* and *E(Pc)*, two ubiquitously expressed genes (Figures 2 & 3). The H3K27me3 mark stops at the two genes. We believe that it is the transcription of these two genes that blocks the spreading of the H3K27me3 mark and stabilizes the repression of *inv* and *en* by PcG proteins. To test this assumption, we made a large transgene marked by HA-tagged Engrailed protein. A 79-kb *HA-en* transgene was able to correctly express En and completely rescue *inv-en* double mutants. We inserted the transgene into other places in the *Drosophila* genome [Reference 4]. Our data showed that, while the information to form the H3K27me3 domain is contained within the 79-kb *HA-en* transgene, the structure of the H3K27me3 domains differs from that at the endogenous locus. Specifically, the H3K27me3 mark spreads beyond the transgene into flanking DNA. Further, enhancers within the 79-kb *HA-en* transgene could interact with some flanking genes and drive their expression in subsets of the En pattern. Also, removal of the PREs from the transgene led to loss of PcG silencing in the abdominal segments of the flies. The data provide evidence that the endogenous *inv-en* domain imparts stability to the locus and facilitates both transcriptional activation and silencing of these two developmentally important genes. Our recent experiments show that adding insulator elements that

FIGURE 3. ChIP-Seq profile showing H3K27me3 and H3K36me3 binding to the *e(Pc)*, *inv*, *en*, and *tou* genes in *Drosophila*

H3K27me3, a mark deposited by PcG protein complex PRC2, is bound from the 3' end of the *tou* gene to the 3' end of the *E(Pc)* gene.

Arrows indicate the direction and extent of the transcription units for the genes shown. H3K36me3 is a mark of actively transcribed genes and is bound to *E(Pc)* and *tou*. Samples from *Drosophila* 3rd instar larvae, brains, and discs. In these tissues, at least 80% of the cells do not express *inv* or *en* (data from Reference 4).



block the spreading of H3K27me3 and the activity of the *inv-en* enhancers stabilizes the 79-kb *HA-en* transgene, making it behave more like the endogenous locus.

Enhancer–promoter communication

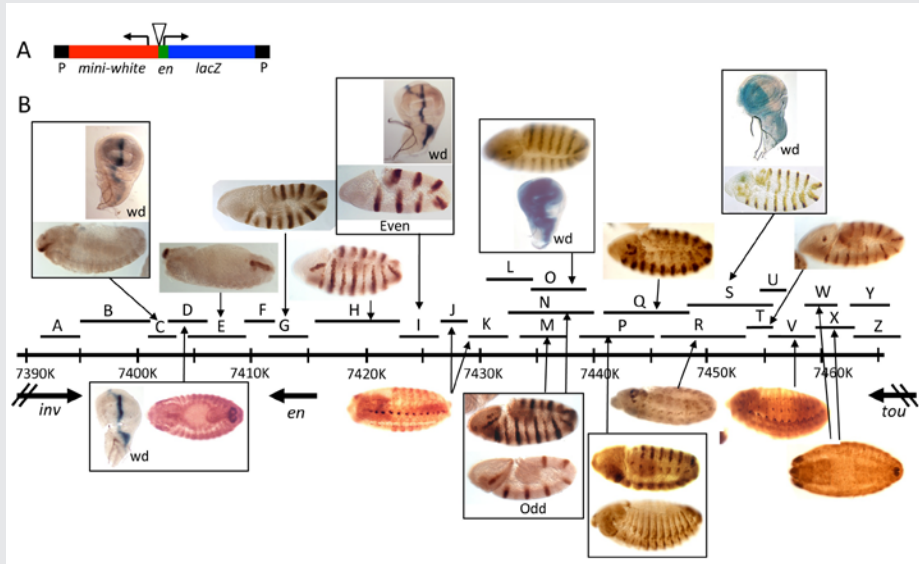
Enhancers are often located tens or even hundreds of kb away from their promoter, sometimes even closer to the promoters of genes other than the one they activate. Several years ago, we showed that *en* enhancers can act over large distances, even skipping over other transcription units, choosing the *en* promoter over promoters of neighboring genes. Such specificity is achieved in at least three ways. First, early-acting enhancers that drive *engrailed* expression in stripes exhibit promoter specificity. Second, a proximal promoter-tethering element is required for the action of the imaginal disc enhancer(s). Our data point to two partially redundant promoter-tethering elements. Third, the long-distance action of *en* enhancers requires a combination of the *en* promoter and sequences within or closely linked to the promoter-proximal PREs. The data show that several mechanisms ensure proper enhancer-promoter specificity at the *Drosophila en* locus, providing one of the first detailed views of how promoter-enhancer specificity is achieved.

As a follow-up to these studies, we located all the enhancers that regulate the transcription of *en* and the closely linked co-regulated *inv* gene (Figure 4). Our dissection of *inv-en*–regulatory DNA showed that most enhancers are spread throughout a 62kb region. We used two types of constructs to analyze the function of this DNA: P-element–based reporter constructs with small pieces of DNA fused to the *en* promoter driving *lacZ* expression (Figure 4); and large constructs with HA-tagged *en* and *inv* inserted in the genome with the phiC31 integrase. In addition, we generated deletions of *inv* and *en* DNA *in situ* and assayed their effects on *inv/en* expression. Our results support and extend our knowledge of *inv-en* regulation. First, *inv* and *en* share regulatory DNA, most of which flanks the *en* transcription unit. In support of this finding, a 79-kb *HA-en* transgene can rescue *inv en* double mutants into viable, fertile adults. In contrast, an 84-kb *HA-inv* transgene lacks most of the enhancers for *inv and en* expression. Second, there are several enhancers for *inv/en* stripes in embryos; some may be redundant, but others play discrete roles at different stages of embryonic development. Finally, no small reporter construct gave expression in the posterior compartment of imaginal discs, a hallmark of *inv/en* expression. Robust expression of *HA-en* in the posterior compartment of imaginal discs is evident from the 79-kb *HA-en* transgene, while a 45-kb *HA-en* transgene gives weaker, variable imaginal disc expression. We

FIGURE 4. Enhancers of the *invected* and *engrailed* genes

A. P-element vector (*P[en]*), used to assay the function of *en*-regulatory DNA, contains the *en* promoter, 396bp of upstream sequences, and an untranslated leader fusion between the *en* transcript and the *Adh-lacZ* reporter gene. *inv/en* DNA fragments were added to this vector at the location of the triangle.

B. The extent of each fragment cloned into *P[en]* is shown as a black line with a letter above the *inv/en* genomic DNA map (indicated by a long black line with hatch marks at 10kb intervals; numbers are coordinates on chromosome 2R, Genome Release v5). Expression pattern in embryos or the wing imaginal disc (wd) are shown above or below the genomic DNA, with arrows pointing to the fragment(s) that generate(s) the pattern (figure reprinted from Cheng Y, et al. *Dev Biol* 2014;395:131).



suggest that the activity of the imaginal disc enhancer(s) depends on the chromatin structure of the *inv-en* domain. We are currently investigating the properties of the *inv/en* imaginal disc enhancer(s) using a variety of methods, including deleting them from the endogenous *inv/en* domain using CRISPR-Cas9. Our results show there are at least two imaginal disc enhancers that behave redundantly to control *Inv* and *En* expression in the endogenous *inv-en* domain.

Defining the ends of Polycomb domains in *Drosophila*

Actively transcribed genes flank many Polycomb domains, and previous genomic studies showed that inhibition of transcription using chemical inhibitors leads to a spreading of H3K27me3 in the genome. We conducted a genome-wide analysis of Polycomb boundaries in *Drosophila* larvae [Reference 5]. We found six different types of Polycomb-domain boundaries, including those made by insulator proteins, and actively transcribed genes. The *inv-en* Polycomb domain is flanked by two actively transcribed genes, *E(Pc)* and *tou* (Figure 3). Insertion of a transcriptional stop within the *tou* gene causes an extension of the H3K27me3 mark to the point of active transcription. We also suggest that active transcription limits the range of *inv-en* enhancers and that promoter specificity is important for *inv-en* enhancer activity [Reference 5]. Currently, we are working to add actively transcribed genes, as well as, separately, to add insulators to delimit the ends of the 79-kb transgene. We hypothesize that adding boundaries will increase the accuracy and robustness of the expression of the transgene.

Reducing Wapl dosage partially corrects embryonic growth and brain transcriptome phenotypes in *Nipbl*^{-/+} embryos.

Cohesin is a multiprotein complex that encircles DNA and acts as a molecular motor that extrudes DNA. The process causes loops that fold the genome into topologically associated domains, which play a role in

regulating gene expression. Our previous work found that a mutation in Wapl, a protein that removes cohesin from DNA, caused a Polycomb phenotype. The phenotype could be corrected by reducing the dosage of Nipped-B (Nipbl in mammals), a protein that loads cohesin onto chromosomes. Heterozygous mutations in the Nipbl protein in humans cause a developmental disorder known as Cornelia de Lange syndrome. In collaboration with [Karl Pfeifer's lab](#), we conducted experiments to determine whether reducing the Wapl gene dosage in *Nipbl*^{-/+} mutant mice could correct the gene expression changes seen in this model of Cornelia de Lange syndrome. Our results showed that: (1) mice heterozygous for a loss-of-function *Wapl* allele exhibited gene expression changes in embryonic mouse brains that were similar to those seen in the *Nipbl*^{-/+} mice; this suggests that the *Wapl* gene is also haploinsufficient, and loss of one copy may cause developmental disorders in humans; (2) reducing Wapl in the *Nipbl*^{-/+} mice partially corrects the gene expression changes present in these animals. Our data support the view that the loading and unloading of cohesin is important for controlling gene expression, and balancing these two processes could help correct the developmental defects in individuals who lack a single copy of the *Nipbl* gene.

Publications

1. Kuroda MI, Kang H, De S, Kassis JA. Dynamic competition of Polycomb and Trithorax in transcriptional programming. *Ann Rev Biochem* 2020 89:235–253.
2. Brown JL, Sun M, Kassis JA. Global changes of H3K27me3 domains and Polycomb group protein distribution in the absence of recruiters Spps or Pho. *Proc Natl Acad Sci U S A* 2018 E1839–E1848.
3. De S, Mitra A, Cheng A, Pfeifer Y, Kassis JA. Formation of a Polycomb-domain in the absence of strong Polycomb Response Elements. *PLoS Genetics* 2016 12:e1006200.
4. De S, Cheng Y, Sun M, Gehred ND, Kassis JA. Structure and function of an ectopic Polycomb chromatin domain. *Sci Adv* 2019 5:eaau9739.
5. De S, Gehred ND, Fujioka M, Chan FW, Jaynes JB, Kassis JA. Defining the boundaries of Polycomb domains in *Drosophila*. *Genetics* 2020 216:689–700.
6. Kean CM, Tracy CJ, Mitra A, Rahat B, Van Winkle MT, Gebert CM, Noeker J, Calof AL, Lander AD, Kassis JA, Pfeifer K. Decreasing Wapl dosage partially corrects embryonic growth and brain transcriptome phenotypes in *Nipbl*^{-/+} embryos. *Sci Adv* 2022 8(48):eadd4136.

Collaborators

- Karl Pfeifer, PhD, *Section on Epigenetics, NICHD, Bethesda, MD*

Contact

For more information, email jkassis@mail.nih.gov or visit <https://kassislabs.nih.gov>.

Extracellular Matrix Disorders: Molecular Mechanisms and Treatment Targets

The extracellular matrix (ECM) is responsible for the structural integrity of tissues and organs as well as for maintaining an optimal environment for cellular function. ECM pathology is involved in a wide variety of disorders, ranging from rare genetic abnormalities of skeletal development (skeletal dysplasias) to such common ailments as osteoporosis, fibrosis, and cancer. Collagens are triple-helical proteins that form the structural scaffolds of the ECM. Their procollagen precursors are assembled and folded from three pro-alpha chains in the endoplasmic reticulum (ER), trafficked through the Golgi apparatus, secreted, and then converted into mature collagen by enzymatic cleavage of propeptides. The most common collagen is type I, which is a heterotrimer of two alpha1(I) chains and one alpha2(I) chain and is by far the most abundant protein in all vertebrates. Type I collagen fibers form the organic scaffold of bone, tendons, ligaments, and the matrix of skin and many other tissues. We focus on translational studies of developmental disorders of the ECM such as osteogenesis imperfecta (OI), Ehlers-Danlos syndrome (EDS), and chondrodysplasias, as well as related ECM pathologies in fibrosis, cancer, and osteoporosis. Our goal is to understand the molecular mechanisms of ECM disorders involving abnormal collagen metabolism, identify treatment targets, and bring this knowledge to clinical research and practice.

Procollagen folding and its role in ECM disorders

Osteoblasts and fibroblasts produce and secrete the massive amounts of type I procollagen needed to build the skeleton and other tissues. Type I procollagen is one of the most difficult proteins to fold. Its massive production presents a unique challenge for protein quality control and trafficking. We discovered that, above 35°C, the conformation of natively folded human procollagen is less favorable than the unfolded one. To fold procollagen at body temperature, cells use specialized ER chaperones to stabilize the native conformation. Outside the cell, the native conformation is stabilized after procollagen is converted to collagen and incorporated into collagen fibers. Unincorporated molecules denature within several hours of secretion and become susceptible to rapid proteolytic degradation. Up to 10–15% of procollagen is misfolded even under normal conditions,



Sergey Leikin, PhD, *Head, Section on Physical Biochemistry*

Elena N. Makareeva, PhD, *Staff Scientist*

Edward L. Mertz, PhD, *Staff Scientist*

Satoru Otsuru, MD, PhD, *Special Volunteer*

Muthulakshmi Sellamani, PhD, *Postdoctoral Fellow*

Megan Sousa, BA, *Postbaccalaureate Fellow*

necessitating activation of cell stress–response pathways that are responsible for degrading misfolded molecules and which force the cell to always function in a high-stress mode. Our findings indicate that one of the key pathophysiological mechanisms of OI and other hereditary type I collagen–metabolism disorders is cell stress caused by excessive accumulation of misfolded procollagen in the ER.

The most common hereditary cause of procollagen misfolding is a Gly substitution anywhere in the obligatory (Gly-X-Y)_n sequence that distinguishes all collagens. Such substitutions in type I collagen are responsible for over 80% of severe OI cases. Similar substitutions in other collagens cause EDS and a variety of other syndromes. Their pathophysiology is one of the key topics of our studies. For instance, our studies on OI patients with over 50 different Gly substitutions revealed several structural regions within the collagen where such mutations might be responsible for distinct OI phenotypes. One such region is the first 85–90 amino acids at the N-terminal end of the triple helix, mutations in which prevent normal N-propeptide cleavage. Incorporation of the uncleaved molecules into collagen fibrils leads to distinct OI/EDS with hyperextensibility and joint laxity.

While we focus mostly on hereditary disorders affecting children, excessive procollagen misfolding may also occur upon changes in the ER associated with environmental factors, inflammation, aging, etc. It is likely to contribute to fibrosis, cancer, age-related osteoporosis, and many other common ailments. Nonetheless, the pathophysiology of misfolded procollagen accumulation in the ER remains poorly understood.

Cell biology of procollagen misfolding

To understand this pathophysiology, we are investigating how misfolded procollagen is recognized by the cell, why it accumulates in the ER, and how this accumulation affects a cell's function. In one approach, we are using live-cell imaging to study the synthesis, folding, trafficking, and degradation of fluorescently tagged procollagen. To facilitate these studies, we created several plasmids enabling transient expression of fluorescent procollagen in mouse and human cells as well as in several cell lines, in which the fluorescent tag was inserted into the gene encoding endogenous pro- α 2(I) chain. These new tools for live cell imaging of procollagen have already been shared with dozens of laboratories in the USA and around the world.

Imaging of fluorescent procollagen enabled us to demonstrate that normally folded molecules are loaded into Golgi-bound transport vesicles at ER exit sites (ERESs) that are marked by the coat protein complex II (COPII). Contrary to widely held beliefs, however, such vesicles do not have a COPII coat nor do they contain HSP47, a collagen-specific ER chaperone that preferentially binds to natively folded procollagen to assist in its folding and loading into ERESs. Instead, transport-vesicle formation depends on COPI coat assembly and HSP47 release at distal regions of ERESs, potentially explaining unusual skeletal pathologies caused by mutations in HSP47, COPI, and COPII coat proteins. Misfolded procollagen is retained at ERESs, resulting in a COPII–dependent modification of ERES membranes by ubiquitin and autophagic machinery. We discovered that such ERESs are then directly engulfed by lysosomes and degraded, delineating a new ERES micro-autophagy pathway.

Rerouting of ERES–loaded cargo from secretion to micro-autophagy may have wide implications. It is likely to be a general rather than a collagen-specific phenomenon, considering the known COPII coat involvement in both protein secretion and degradation. The hypothesis is currently under investigation in our and several collaborating laboratories. From clinical and translational perspectives, our findings may explain why patients with mutations in different COPII proteins have distinct pathologies in the development of bone, cartilage, and other tissues.

In another approach, we are investigating the cell-stress response to procollagen misfolding caused by a Gly610 to Cys substitution in the triple-helical region of pro- α 2(I). We helped develop a mouse model of OI with this mutation (G610C mouse), which mimics the pathology found in a large group of patients with the same mutation. Our study of this model demonstrated that misfolding and accumulation of mutant procollagen in the ER of osteoblasts causes ER disruption, resulting in cell stress and malfunction. We are therefore investigating the mechanism of this stress and its role in pathology by altering how the cells adapt to it and by examining cell-stress response pathways activated by the mutation. For instance, we reduced autophagic degradation of ERESs containing misfolded procollagen by osteoblast-specific knockout of ATG5 (autophagy-related factor 5). Increased bone pathology caused by the resulting additional accumulation of misfolded procollagen in the ER confirmed our hypothesis that osteoblast cell stress and malfunction associated with such accumulation play a significant role in OI pathophysiology. More detailed analysis of the ATG5 knockout effects confirmed that ERES microautophagy is the primary pathway of misfolded procollagen degradation in G610C osteoblasts. Furthermore, more recent studies revealed that accumulation of misfolded G610C procollagen in osteoblast ER activates transcription of the integrated stress response genes (e.g., *Ddit3*, *Eif4ebp1*, and *Nupr1*) but not of the canonical ER stress transducers *Atf4* and *Hspa5*, suggesting non-canonical cell stress and identifying its transducers as *Atf5* and *Hspa9* paralogs of *Atf4* and *Hspa5*. We validated these findings by bulk, single-cell, and spatially resolved RNA sequencing, as well as by *in situ* RNA hybridization and Western blotting.

By combining the live-cell imaging, genetic, and biochemical analysis, we found that misfolding of procollagen in the ER of G610C osteoblasts activates the mitochondrial arm of the integrated cell stress response (ISR) rather than the canonical unfolded protein response (UPR). Misfolded G610C molecules are not recognized and retained in the ER lumen by quality-control chaperones. Instead, they are retained at ERESs, blocking the exit of all properly folded secretory proteins from the ER into the secretory pathway. The resulting ER overcrowding activates the ISR without UPR as a result of disruption of ER-mitochondria contacts. The ISR is sufficient to stabilize G610C osteoblasts in a less efficient yet functional steady state, in which procollagen synthesis is reduced and degradation is increased. However, the same ISR may not be sufficient to prevent more severe ER disruption in other cells or other mutations, which may then cause misfolding of globular proteins and concomitant secondary UPR. For instance, we found that hypertrophic chondrocytes in the growth plate of the same G610C animals undergo a secondary UPR, which blocks their transition into osteoblasts and thereby limits longitudinal bone growth. Our discovery of mitochondrial involvement not only identified a new response pathway to protein misfolding in the ER but also opened up a new research direction. We are currently investigating how the ER-mitochondria contacts are disrupted and how the resulting mitochondrial dysfunction affects the cells.

New approaches to analysis and treatment of ECM pathology

Our observations suggested that the pathology associated with procollagen misfolding may be partially reversed by improving cell adaptation to misfolded procollagen accumulation in the ER, thereby improving cellular function. Although this would not eliminate the detrimental effects of secreted mutant collagen, pharmacological treatment of cell malfunction is the most realistic short-term strategy for targeting the causes rather than the effect of bone pathology in OI. It is also likely to be a good long-term strategy for the treatment of cell malfunction caused by procollagen misfolding in cases that do not involve pathogenic mutations.

To pursue the strategy, we are examining the effects of enhancing the natural ability of cells to remove and degrade misfolded molecules via autophagy, which is the simplest way to prevent their pathogenic

accumulation in the ER. Our studies on autophagy enhancement by a low-protein diet or intermittent fasting in G610C mice revealed improved osteoblast differentiation and function, resulting in better bone quality, but we also observed stunted animal growth. We are thus evaluating other approaches that might provide the same benefits of autophagy enhancement without long-term nutrient deficiency.

In particular, we are testing drugs known to reduce ER disruption by enhancing secretion and autophagy of misfolded proteins (e.g., 4-phenylbutyrate [4PBA]) and drugs (e.g., integrated stress response inhibitor [ISRIB]) known to reduce the impact of accumulating misfolded proteins on overall protein synthesis. We found that 4PBA reduces bone pathology in a zebrafish model of OI and in G610C mice. In such mice, however, low-dose 4PBA treatment improved the function of hypertrophic chondrocytes and their conversion into osteoblasts but not the function of osteoblasts, probably because it alleviated secondary UPR more efficiently than the primary non-canonical cell stress. At the same time, a higher-dosage treatment is challenging because 4PBA is very rapidly metabolized and therefore difficult to deliver in a sustainable fashion to bone cells. We are therefore exploring other drugs, alternative delivery methods, and other approaches.

A key issue in monitoring treatment efficiency in animal models as well as in general diagnostic analysis of bone pathology is the lack of reliable methods with which to characterize the function of bone cells. Traditional histopathology relies on subjective analysis of bone-cell morphology in tissue sections, which is not a reliable indicator of cell function. Over the last several years, we developed a new approach to visualizing and quantifying mRNA expression in individual cells in bone sections. The approach enables objective and reliable cell identification as well as *in situ* characterization of cell differentiation and function. Based on the interest of bone histomorphometry experts in learning this approach, we hope that it will soon be adapted not only for research but also for clinical practice.

Translational studies of OI and other skeletal dysplasias

Over the last several years, we assisted several clinical research groups in characterizing collagen-metabolism pathology in cells from patients with newly discovered skeletal dysplasias caused by mutations in cartilage-associated protein (CRTAP), prolyl-3-hydroxylase (P3H1), cyclophilin B (CYPB), the collagen-binding molecular chaperone FKBP65, the signaling protein WNT1, the ER-membrane ion channel TRICB, the Golgi-membrane metalloprotease S2P, the transmembrane anterior-posterior transformation protein 1 (TAPT1), or collagen prolyl-4-hydroxylase 1 (P4H1). Our studies suggested that the CRTAP/P3H1/CYPB complex functions as a procollagen chaperone. A deficiency in any of the three proteins delays procollagen folding, although their exact roles remain unclear. More surprisingly, we found no detectable changes in the procollagen folding rate in cultured fibroblasts from patients with FKBP65 mutations. Our data suggested that FKBP65 may affect post-translational modification of procollagen and deposition of collagen matrix by a different mechanism. It remains unclear why some FKBP65 mutations cause severe OI with joint contractures (Bruck's disease), while others cause joint contractures without pronounced OI (Kuskokwim syndrome) or OI without pronounced joint contractures. Our study of TRICB-deficient cells revealed abnormal conformation and reduced thermal stability of type I procollagen, suggesting dysregulation of collagen chaperones in the ER or direct involvement of TRICB in procollagen folding. Our experiments indicated that the pathogenic effects of mutations in the transmembrane protein TAPT1 or in site-2 metalloprotease (S2P) might not be directly related to disruptions in synthesis, folding, or trafficking of procollagen chains. As expected, we found that patient cells with mutant P4H1 secreted abnormal procollagen, which had significantly reduced thermal stability owing to under-hydroxylation of proline residues by P4H1. Surprisingly, however, we found no abnormalities in the procollagen

folding or secretion rates, no evidence of misfolded procollagen accumulation in the cell, and no evidence of altered ER chaperone composition.

We also conducted translational studies of OI caused by missense mutations in type I collagen that are not substitutions of obligatory Gly residues. For instance, we found that substitutions of Y-position arginine (Y-Arg) residues in the Gly-X-Y triplets within the collagen triple helix cause procollagen misfolding and accumulation in the ER to almost the same extent as Gly substitutions, likely because Y-Arg enhances collagen triple-helix stability and promotes triple-helix folding through binding of HSP47.

Presently, we are focusing on understanding the causes of lung pathology in patients and mouse models of OI. Our micro-CT, histological, and transcriptomic studies revealed lung tissue pathology in G610C mice and two other mouse models of OI that could not be related to skeletal deformities. Given that cardio-pulmonary complications are the main cause of death in OI, we are investigating how disruptions of collagen metabolism in lung tissue are causing the pathology. Our preliminary experiments have already uncovered abnormal cell-ECM and cell-cell interactions affecting alveogenesis, mechanical strength of the tissue, and differentiation of lung epithelial cells. Beyond a better fundamental understanding of cell-ECM and cell-cell interactions in lungs, we hope that these studies will identify potential therapeutic targets.

Extracellular matrix pathology in tumors and fibrosis

Another aspect of our collagen metabolism pathology studies has been to characterize the pathology in fibromas and tumors, e.g., abnormal collagen composition of uterine fibroids and the potential role of type I collagen homotrimers in cancer. The normal isoform of type I collagen is a heterotrimer of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain. Homotrimers of three $\alpha 1(I)$ chains are produced in some fetal tissues, carcinomas, fibrotic tissues, as well as in rare forms of OI and EDS associated with $\alpha 2(I)$ chain deficiency. We found the homotrimers to be at least 5–10 times more resistant to cleavage by all mammalian collagenases than the heterotrimers, and we determined the molecular mechanism of this resistance. Our studies suggested that cancer cells might utilize this collagen isoform to build collagenase-resistant tracks, thus supporting invasion through stroma of lower resistance.

We also investigated bone pathology and tumors caused by defects in cAMP signaling, e.g., those associated with mutation in protein kinase A (PKA), which is a key enzyme in the cAMP signaling pathway. Initially, we studied synthesis of type I collagen homotrimers. However, over the last 3–5 years, our focus shifted to abnormal differentiation of osteoblastic cells and deposition of bone. We found that knockouts of various PKA subunits cause not only abnormal organization and mineralization of bone matrix but also novel bone structures that had not been previously reported. For instance, we observed free-standing cylindrical bone spicules with an osteon-like organization of lamellae and osteocytes but an inverted mineralization pattern, a highly mineralized central core, and diminishing mineralization away from the central core. We assisted clinical researchers in characterizing abnormal osteoblast maturation, the role of an abnormal inflammatory response, and effects of anti-inflammatory drug treatments in such animals. Improved understanding of bone tumors caused by PKA deficiencies may not only clarify the role of cAMP signaling but may also suggest new approaches to therapeutic manipulation of bone formation in skeletal dysplasias.

Multimodal imaging and mapping of tissues

Given that tissue analysis is crucial for understanding and treating collagen metabolism disorders, we are

developing methods for characterizing cellular function in relation to ECM composition and structure. The methodology builds on our advances in high-definition infrared and Raman microspectroscopy, mRNA-based histology and histomorphometry, and combining different imaging and spectroscopic modalities for tissue sections.

In particular, we developed high-definition (HD) infrared imaging and Raman micro-spectroscopic methods, improving spectral reproducibility by up to two orders of magnitude, based on thermomechanical stabilization of the light path through tissue. The technology enabled us to uncover the causes of progressive cartilage degradation in a mouse model of diastrophic dysplasia caused by mutations in the *SLC26A2* sulfate transporter gene. It was essential for the analysis of abnormal collagen matrix deposition by CRTAP- and FKBP65-deficient cells. We used it in studies of bone structure and mineralization in the mouse models of the OI and PKA deficiencies described above and for establishing new approaches to characterizing bone formation *in vitro*. Beyond nondestructive characterization of ECM composition and structure in many of our studies, the technology proved to be useful for researchers from other NICHD and NIH laboratories. For instance, we assisted NIBIB scientists in characterizing a functionalized carbon-nanotube approach to the delivery of anticancer agents into cells that overexpress hyaluronate receptors.

We are presently working on integrating the HD infrared/Raman and histological ECM imaging modalities with cellular function imaging based on *in situ* mRNA sequencing and fluorescent *in situ* mRNA hybridization. We have already combined all these modalities for proving formation of *bona fide* lamellar bone in osteoblast cultures and developing approaches to distinguishing this bone from other mineralized cell-ECM structures, which are generally more prevalent *in vitro*. We are now optimizing such structural and functional imaging for integrated characterization of the same tissue section, which would address the question of how the ECM structure and composition affect the function of adjacent cells and cell-cell interactions.

Publications

1. Omari S, Makareeva E, Leikin S. Procollagen trafficking and its implications in osteogenesis imperfecta. In: *The Collagen Superfamily and Collagenopathies*, Ruggiero F, ed. 2021 Chapter 2:23–53.
2. Gorrell L, Omari S, Makareeva E, Leikin S. Noncanonical ER–Golgi trafficking and autophagy of endogenous procollagen in osteoblasts. *Cell Mol Life Sci* 2021 78:8283.
3. Scheiber AL, Wilkinson KJ, Suzuki A, Enomoto-Iwamoto M, Kaito T, Cheah KS, Iwamoto M, Leikin S, Otsuru S. 4PBA reduces growth deficiency in osteogenesis imperfecta by enhancing transition of hypertrophic chondrocytes to osteoblasts. *JCI Insight* 2022 7:e149636.
4. Gorrell L, Makareeva E, Omari S, Otsuru S, Leikin S. Mitochondria, and ISR regulation by mt-HSP70 and ATF5 upon procollagen misfolding in osteoblasts. *Adv Sci (Weinh)* 2022 9:2201273.
5. Mertz EL, Makareeva E, Mirigian LS, Leikin S. Bone formation in 2D culture of primary cells. *JBMR Plus* 2022; <https://doi.org/10.1002/jbm4.10701>.

Collaborators

- Peter Bassler, PhD, *Section on Quantitative Imaging and Tissue Sciences, NICHD, Bethesda, MD*
- Peter H. Byers, MD, *University of Washington, Seattle, WA*
- Antonella Forlino, PhD, *Università degli Studi di Pavia, Pavia, Italy*

- Ken Kozloff, PhD, *University of Michigan, Ann Arbor, MI*
- Jennifer A. Lippincott-Schwartz, PhD, *Howard Hughes Medical Institute, Janelia Farm Research Campus, Ashburn, VA, and Adjunct Investigator, NICHD, Bethesda, MD*
- Fransiska Malfait, MD, PhD, *Center for Medical Genetics, Ghent University, Ghent, Belgium*
- Joan C. Marini, MD, PhD, *Section on Heritable Disorders of Bone and Extracellular Matrix, NICHD, Bethesda, MD*
- Roy Morello, PhD, *University of Arkansas Medical School, Little Rock, AR*
- Cathleen Raggio, MD, *Hospital for Special Surgery, New York, NY*
- Pamela G. Robey, PhD, *Craniofacial and Skeletal Diseases Branch, NIDCR, Bethesda, MD*
- Robert Sandhaus, MD, PhD, *National Jewish Health, Denver, CO*
- Sofie Symoens, PhD, *Center for Medical Genetics, Ghent, Ghent University, Belgium*

Contact

For more information, email leikins@mail.nih.gov or visit <http://physbiochem.nichd.nih.gov>.

From Axon Damage to Disease: Common Pathways in Neurodegeneration

Our work is dedicated to advancing our understanding of common molecular and cellular mechanisms of neurodegeneration, with the ultimate goal of developing treatments for neurodegenerative diseases and even preventing them. The hypothesis driving our work is that common mechanisms are responsible for neurodegeneration during development and in aging. One focus is on mechanisms of stress-response pathways in neurons, such as the evolutionarily conserved axon-damage signaling pathway under the control of DLK (dual leucine zipper kinase; MAP3K12). Another theme is to understand fundamental differences between vulnerable and resilient populations of neurons in models of acute injury and in chronic disease. The lab uses the mouse and human iPSC-derived neurons as model systems.

Elucidation of mechanisms of axon damage signaling in human neurons

DLK is an essential player in the axonal response to neuronal injury. It promotes axon degeneration, neuronal cell death, and regeneration, depending on the neuronal cell type. To elucidate regulators and substrates of DLK function, about which relatively little is known, we study DLK localization, trafficking, and interactors in a human iPSC (induced pluripotent stem cells)-derived neuron model (i3neurons; Fernandopulle MS *et al.*, *Curr Protoc Cell Biol* 2018;79: e51). Importantly, very few studies have examined DLK function in human neurons, despite DLK inhibitors being considered in clinical trials. Almost all we know comes from studies in model organisms (worm, fly, mouse).

Understanding fundamental differences between vulnerable and resilient populations of spinal motor neurons in disease

We are individually profiling transcriptomes of spinal-cord motor neurons in healthy mice and disease models to track the transcriptomic alterations that such cells undergo during disease progression. Last year, we published a single-cell transcriptomic atlas of adult mouse spinal motor neurons [Reference 1]. Previously, very few spinal motor neurons (MNs) had been resolved at the single-cell level, both because they are relatively rare among all spinal cells, and because they do not survive single-cell isolation protocols well. Our success relied on two strategies: (1) enriching for spinal MNs using a



Claire E. Le Pichon, PhD, *Head, Unit on the Development of Neurodegeneration*

Hanna Silberberg, MA, *Biologist*

Jorge Gomez Deza, PhD, *Postdoctoral Visiting Fellow*

Sangeetha Hareendran, PhD, *Postdoctoral Visiting Fellow*

Mor Alkaslasi, BS, *Graduate student*

Zoe Piccus, MA, *Graduate student*

Josette J. Wlaschin, MSc, *Graduate student*

Austin Gable, BA, *Postbaccalaureate Fellow*

Matthew Nebiyu, BS, *Postbaccalaureate Fellow*

Aditya Santoki, BA, *Postbaccalaureate Fellow*

Chat-Cre line; and (2) capturing single nuclei, which are more robust than whole cells. We were able to collect single-nucleus RNA sequencing data from 16,000 cholinergic nuclei, define the full heterogeneity of these neurons at the single-cell level, and provide a comprehensive transcriptomic description of the lower MNs that selectively degenerate in ALS and other motor neuron diseases. We observed three main classes of skeletal MNs: alpha, gamma, and a third type potentially corresponding to beta MNs. Within each skeletal MN class, we identified previously uncharacterized subtypes corresponding to anatomical and functional specializations. The data from this study can be browsed at www.spinalcordatlas.org and will soon also be available at <https://seqseek.ninds.nih.gov>.

Having laid this important groundwork, we are currently obtaining data from mouse models of motor neuron disease and will compare the transcriptomes of resilient with those of vulnerable MN types across several time points in disease.

Publications

1. Alkaslasi MR, Piccus ZE, Hareendran S, Silberberg H, Chen L, Zhang Y, Petros TJ, Le Pichon CE. Single nucleus RNA-sequencing defines unexpected diversity of cholinergic neuron types in the adult mouse spinal cord. *Nat Commun* 2021 12(1):2471.
2. Nguyen E, Smith KM, Cramer N, Holland RA, Bleimeister IH, Flores-Felix K, Silberberg H, Keller A, Le Pichon CE, Ross SE. Medullary kappa-opioid receptor neurons inhibit pain and itch through a descending circuit. *Brain* 2022 145(7):2586–2601.

Collaborators

- Ariel Levine, MD, PhD, *Spinal Circuits and Plasticity Unit, NINDS, Bethesda MD*
- Michael E. Ward, MD, PhD, *Inherited Neurodegenerative Diseases Unit, NINDS, Bethesda MD*

Contact

For more information, email claire.lepichon@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHDI/Investigators/lepichon>.

The Biological Impact of Transposable Elements

Long Terminal Repeat (LTR) retrotransposons are highly abundant and have evolved into ubiquitous families of elements that multiply through cycles of particle formation, reverse transcription, transport to the nucleus, and integration. Some families of LTR-retrotransposons acquired envelope proteins, an addition that transformed the elements into infectious retroviruses. This close relationship makes LTR retrotransposons ideal models for studying the molecular mechanisms responsible for retrovirus replication. The transposable elements (TEs) of model organisms, such as yeast, are particularly well suited to address the dynamics and global impact of their replication. We study LTR retrotransposons of the fission yeast (*Schizosaccharomyces pombe*) to determine how integration sites are selected and to understand how patterns of integration impact the physiology of the cell. In past work, we found that integration of LTR retrotransposons in *S. pombe* alters gene expression and adapts cells to environmental stress. It is through adaptation that we believe TEs form gene-regulatory networks. We also study how HIV-1 integration sites are selected.

In humans, TEs represent 50% of genomic sequences. The dominant families of TEs are Long INterspersed Element-1 (LINE-1 or L1), which constitutes 17% of the genome, and Alu Short Interspersed Elements (SINES), which are mobilized by L1 and constitute 10% of the genome. Given that TEs make up half of the human genome, it is not surprising that their regulatory features are abundant sources of tissue-specific promoter activity and are critical building blocks of gene-regulatory networks. Although the vast majority of TEs have lost mobility, each genome retains approximately 100 active copies. As a result, genome studies of human populations reveal many thousands of polymorphic TEs. Our goal is to determine the role of these genetic variants in health and disease.

Identification of an integrase-independent pathway of retrotransposition

Despite the central role of integration in the propagation of retroviruses, important questions remain about residual insertions that occur in the absence of integrase (IN) activity. Mutations in the catalytic residues of HIV-1 IN produce residual infectious titers,



Henry L. Levin, PhD, Head,
*Section on Eukaryotic
Transposable Elements*

Angela Atwood-Moore, BA, *Senior
Research Assistant*

Abhishek Anand, PhD,
Postdoctoral Fellow

Paul Atkins, PhD, *Postdoctoral
Fellow*

Hyo Won Ahn, PhD, *Visiting Fellow*

Feng Li, PhD, *Visiting Fellow*

Rakesh Pathak, PhD, *Visiting
Fellow*

Abigail Burkhart, BS,
Postbaccalaureate Fellow

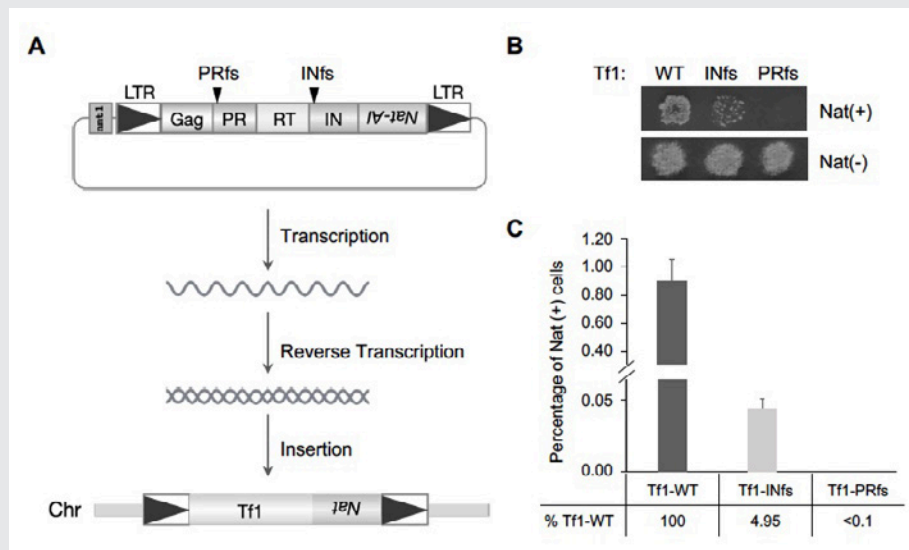
Kyla Roland, BA,
Postbaccalaureate Fellow

FIGURE 1. Tf1 insertion takes place in the absence of integrase.

A. The diagram shows the strategy of monitoring Tf1 retrotransposition. A drug-resistant gene, *nat*, with artificial intron (*nat-AI*) is introduced into Tf1, and the integration of Tf1 into host chromosomes allows cells to grow on plates containing Nat (N-acetyl transferase). The black arrows indicate the frame shift (fs) sites of PR and IN respectively. LTR: long terminal repeat; PR: protease; RT: reverse transcriptase; IN: integrase; WT: wild-type.

B. Growth phenotypes of Tf1-WT, Tf1-INfs, and Tf1-PRfs on medium containing Nat after inducing Tf1 expression.

C. Quantitative transposition analysis Tf1-WT, Tf1- INfs, and Tf1-PRfs.



typically with a 3 to 4-log reduction. However, in continuous cultures of HIV-1 lacking IN activity, insertion efficiency can be as high as 0.2–0.8% of a wild-type (WT) virus. These findings indicate that retroviruses possess a secondary, IN-independent pathway, which incorporates viral DNA into the host genome. Given that IN-independent infections could compromise the treatment of HIV-1 patients with IN inhibitors, it is important to identify the nature of this pathway.

LTR retrotransposons are important models of retroviruses because of their structural and mechanistic similarities. Tf1 and Tf2 are extensively characterized LTR retrotransposons with high integration activity in *S. pombe*. Studies of Tf1 expressed with genetic markers demonstrate that the Gag protein, protease (PR), reverse transcriptase (RT), and IN all contribute to transposition. Importantly, the resulting integration is directed to specific RNA pol II promoters by the DNA-binding factor Sap1. To identify a model system that can be used to study the mechanisms of IN-independent insertion, we measured the insertion of Tf1 lacking IN activity. We performed an insertion assay with Tf1 encoding a frameshift mutation at the start of IN (Tf1-INfs) that blocks expression of IN without altering RT expression or cDNA synthesis. We found Tf1-INfs retained 4.95% of the insertion activity of Tf1-WT [Reference 1]. These results indicate that, in the absence of IN activity, Tf1 cDNA inserted into the host genome with surprising efficiency. Genome-wide insertion profiles of Tf1 lacking IN (Tf1-INfs) were significantly different from those of Tf1 expressing active IN. DNA logo analysis showed that the sequences downstream of the Tf1-INfs insertion sites had a prominent bias for ATAAC, and upstream flanks showed a preference of CAA. Interestingly, the downstream logo matches that of the primer binding site (PBS), an 11 bp sequence retained after reverse transcription on the 3' end of the plus-strand cDNA. The CAA matches the last three base pairs of the poly purine tract (PPT), which is retained on the 3' end of the minus-strand cDNA. The PBS and PPT preferences indicated that these single-stranded sequences contributed to insertion through homologous recombination (HR). If IN-independent insertions

are directed to sites with homology to the PBS and PPT, we would expect that large numbers of insertions would occur at the 13 pre-existing copies of Tf2 that have PBS and PPT sequences identical to those of Tf1. By analyzing the raw downstream sequences, we found that approximately 70% of the IN-independent insertions occurred at homologous sequences within the pre-existing 5' LTRs of Tf2s. Whole genome sequencing of these events revealed that the most common outcome of these insertions resulted in tandem copies of Tf1 and Tf2 elements.

Our data suggest that IN-independent insertion of Tf1 is likely mediated by a form of homologous recombination. To determine whether homologous recombination factors contribute to IN-independent insertion, we measured insertion frequencies of strains lacking *mre11*, *rad50*, *nbs1*, *rad51*, or *rad52*. The results revealed that the insertions occurred through Rad52-dependent single-strand annealing (SSA), as Rad51 was dispensable. The *rad52*-R45A mutation, which specifically abolishes the SSA activity of Rad52, significantly reduced the frequency of Tf1-INfs insertions and resulted in dissociation of Rad52 from Tf1 cDNA. These data indicate that Rad52 plays a critical role in IN-independent insertions by binding to the ends of the cDNA, causing recombination with sequences similar to PBS and PPT.

The efficiency of HR-mediated IN-independent insertion of Tf1 raised questions about whether this pathway has a biological function. Our efforts to determine whether IN-independent events occur naturally showed that cultures with continuing expression of WT Tf1 produced insertions that were predominantly IN-independent [Reference 1]. These data demonstrate that Tf1 possesses two efficient insertion pathways, one relying on IN and the other being IN-independent but requiring Rad52. Significantly, we found in previously published data of HIV-1 IN-independent insertions that five of 69 sites had strong similarity to the HIV-1 PBS. Together, these results indicate that homology-dependent SSA provides a significant pathway of IN-independent insertion.

Retrotransposon insertions associated with risk of neurologic and psychiatric diseases

Mental disorders affected about 970 million people worldwide in 2017. In 2020, 21% of adults in the United States suffered from some form of mental illness. Consequently, these diseases cause great social and economic burden. Studies of identical twins show that the heritability of diseases such as attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), bipolar disorder (BIP), and schizophrenia is extremely high, ranging from 74% to 81%. Because of the complexity of the mammalian nervous system, the genetic and cellular etiology of such diseases remains largely unclear. Progress in genetic methodology has provided the potential to identify mechanisms that underlie the diseases. One approach that has successfully identified important disease loci is genome-wide association studies (GWAS). However, in the cases of neurologic and major psychiatric disorders, GWAS have identified large numbers of loci, each associated with small increases in risk. Importantly, there is extensive overlap of the loci that contribute to major psychiatric disorders, indicating that related molecular mechanisms may underlie distinct clinical phenotypes.

TASs (trait-associated single-nucleotide polymorphisms [SNPs]) of GWAS are genetic tags identifying a genomic region that contains the causal mutation(s), which lead to increased disease risk. Limits on the design of GWAS typically prevent such studies from identifying causal gene alleles. Thus, determining causal variants remains the most challenging and rate-limiting, but also the most important, step in defining the genetic architecture of diseases. The vast majority of GWAS TASs lie in intergenic or intronic regions and therefore do not alter coding

sequence. For such SNPs to be causal they would likely have regulatory effects on transcription. Structural variants such as rearrangements, copy number variants, and transposable element (TE) insertions constitute a substantial and disproportionately large fraction of the genetic variants found to alter gene expression.

In humans, the dominant families of TEs are long interspersed element-1 (LINE-1 or L1) and Alu elements, which are short interspersed elements (SINEs) and are mobilized by L1. TEs readily alter gene expression because they have evolved various sequences that act on enhancers. Given that TEs make up approximately 45% of the human genome, it is not surprising that their regulatory features are abundant sources of tissue-specific promoter activity.

Relatively recent TE insertions can proliferate in the population and become common alleles. The 1000 Genomes Project described genetic variation of diverse human populations by sequencing whole genomes of 2,504 individuals. The extensive survey of genetic variation detected 17,000 polymorphic insertions of TEs, which have the potential to alter gene expression and affect common disease risk. There may be functional consequences of common TE insertion variants that affect common disease risk. Some common polymorphic TEs have been implicated at disease loci detected by GWAS. Common polymorphic *Alu* (short transposable elements) insertions occur disproportionately near disease loci of GWAS, underscoring the fact that *Alu* insertions are potential causative variants.

Given the difficulty in identifying genetic variants responsible for neurologic and psychiatric disorders and the regulatory capacity of TEs, we tested whether polymorphic TEs are potential causative variants of such diseases [Reference 2]. We analyzed 593 GWAS of neurologic and psychiatric diseases, which in total reported 753 TASS. From the 17,000 polymorphic TEs, we found that 76 were in linkage disequilibrium (LD) with TASS, indicating that the TEs were among the variants with the potential to be causative. We extended our analysis by evaluating each candidate TE for a role in altering expression of proximal genes. In one approach, we determined whether polymorphic TEs could disrupt regulatory sequences, as annotated with the epigenomic data of the NIH Roadmap Epigenomics Consortium. In all, we identified 10 polymorphic TEs to examine further as causal candidates because they were positioned in enhancer, promoter, heterochromatin, or transcribed sequences present in neurologic tissues.

We hypothesized that the polymorphic TEs have a causal relationship with risk of psychiatric and neurologic disorders by altering expression of genes in *cis*. For evidence of altered gene expression, we queried the Genotype-Tissue Expression (GTEx) database, which contains expression data for 948 donors across 54 tissues. GTEx readily identifies changes in tissue-specific gene expression associated with loci-specific genetic variation. SNPs in LD are identified as eQTL (expression quantitative trait loci) if the genetic loci with the variants are significantly associated with altered expression of a gene in a specific tissue. We found that 31 of the TASS linked to TEs were variants that are associated with changes in expression of one or more adjacent genes within regions of the brain.

Having identified a number of polymorphic *Alu* elements that are significantly associated with disease risk detected by GWAS and that are correlated with altered gene expression in neurologic tissues by eQTL analysis, we developed a luciferase reporter assay to test whether the insert sequences in the context of flanking sequence can influence transcription activity. We measured the impact of candidate *Alu* and flanking sequences on the function of a minimal promoter in NCRM-1 (human neural stem cells). Of six

candidate *Alu* insertions evaluated for their impact on promoter activity, we found that five significantly altered the expression of luciferase. Taken together, we identified 10 polymorphic TE insertions that are potential candidates on par with other variants for having a causal role in neurologic and psychiatric disorders.

Dense transposon integration reveals that essential cleavage and polyadenylation factors promote heterochromatin formation.

In eukaryotes, the assembly of DNA into highly condensed heterochromatin is critical for a broad range of functions related to genome integrity. The methylation of histone H3 on lysine 9 (H3K9me) is central to the formation of heterochromatin by creating binding sites for a range of chromatin proteins important for silencing transposable elements, chromosome segregation, and epigenetic inheritance. Used extensively for this purpose, *S. pombe* is an excellent model in which to study the molecular mechanisms that generate and regulate heterochromatin. Centromeres, subtelomeres, and the mating-type region are packaged into constitutive heterochromatin, while meiosis genes are silenced by facultative heterochromatin until cells are starved of nitrogen. Importantly, Clr4, the H3K9-specific histone methyltransferase, is recruited to heterochromatin regions by several mechanisms. Constitutive heterochromatin results from RNAi factors that include the Ago1 (a major component of RNA silencing complexes)-containing, RNA-induced transcriptional silencing complex (RITS). Facultative heterochromatin at meiosis genes is independent of RNAi and relies on the RNA elimination (i.e., degradation) of factors Red1 and Mmi1 and on the nuclear exosome. However, gaps exist in our understanding of how RNA elimination generates heterochromatin. A new approach to identifying gene function is the high-throughput sequencing of integration profiles, also known as Tn-Seq, which identifies genes important for growth under selective conditions. Genes necessary to sustain growth under a specific condition do not tolerate insertions in that condition. Tn-Seq has been applied to identify pathogenic genes in bacteria. However, we were the first to develop the method for a eukaryote [Reference 3].

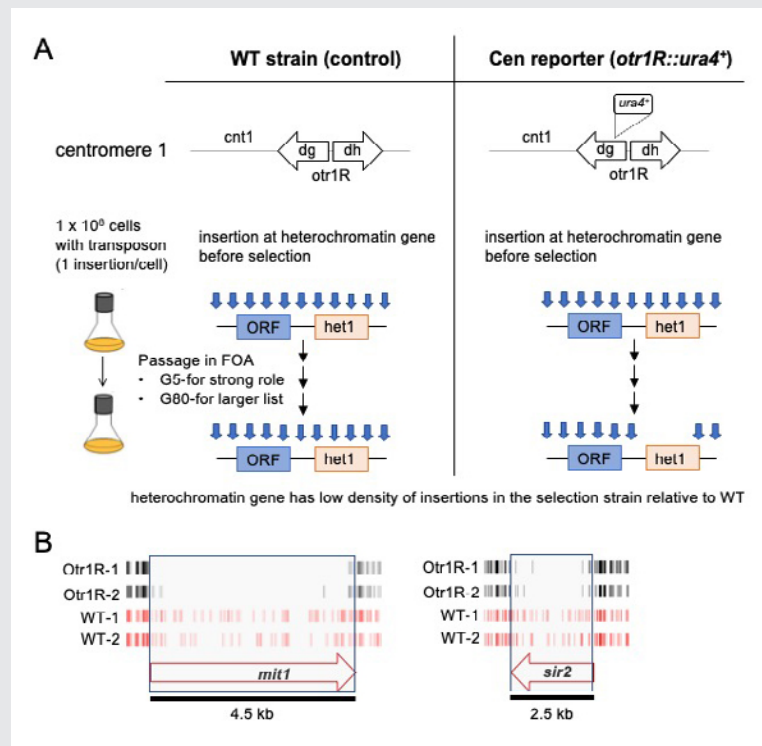
With the goal of identifying novel factors important for heterochromatin, we produced dense profiles of integrations using the Hermes transposable element and a silencing reporter (*ura4*) positioned in the outer repeats of centromere 1. Inserts that disrupted genes important for heterochromatin activated *ura4*, and thus the cells were unable to grow when passaged in 5-fluoroorotic acid (FOA) (Figure 2A). Genes with established roles in heterochromatin assembly had significantly fewer insertions in cells with the centromere reporter *otr1R::ura4* than in cells lacking the reporter (Figure 2B). The list of candidates consisted of a total of 199 genes and, importantly, 65 are known to be essential for viability. These essential genes were candidates because they tolerated many insertions in their 3' sequences that reduced heterochromatin but not viability. The high number of essential genes is significant in that most proteins found to be important for heterochromatin are identified in screens of deletion strains that cannot include essential genes. The 199 candidates showed highly significant enrichments for functions in silencing at centromere outer repeats and included all four factors that produce siRNA.

We identified other RNA-processing factors that were not previously linked to heterochromatin structure. Strikingly, four of the RNA-processing candidates form an interaction module of the canonical mRNA polyadenylation and cleavage factor CPF, as predicted from highly homologous proteins in *S. cerevisiae*. To determine whether polyadenylation and cleavage contribute to heterochromatin structure at the centromere repeats, we focused on the function of Iss1, a subunit of CPF. We generated a C-terminal truncation of Iss1 (Iss1-deltaC) by removing 38 amino acids that, based on the Hermes insertions, were not important for viability. Iss1-deltaC showed no growth restriction on nonselective medium but exhibited a heterochromatin

FIGURE 2. Dense maps of transposable element integration identify genes important for heterochromatin at centromere repeats.

A. Single insertions of the transposable element Hermes were generated in cells with wild-type *cen1* and *cen1 otr1R::ura4*. Cultures were passaged in 5-fluoroorotic acid (FOA) for 5 or 80 generations. Cells with insertions in heterochromatin genes (*het1*) express *ura4* and cannot grow in FOA. After growth on FOA, fewer insertions were detected in *het* genes in cells with *cen1 otr1R::ura4*.

B. Genes involved in forming centromere heterochromatin such as *mit1* and *sir2* had fewer inserts in cells with the *cen1 otr1R::ura4* (black, dupl. libraries) than in cells with WT *cen1* (red, dupl. libraries).



defect, as demonstrated by growth in the absence of uracil and reduced levels of H3K9 dimethylation (H3K9me2) at *otr1R::ura4*. The results demonstrated that the Hermes screen correctly identified *Iss1* as important for heterochromatin structure at the *otr1R::ura4* reporter. Interestingly, we found that *Iss1* contributes to the heterochromatin of centromere repeats in cells that lack the *otr1R::ura4* reporter but, in this case, the contribution to H3K9me2 was only observed when the RNAi pathway was disabled by deletion of *ago1*. This role at the outer centromere repeats is therefore independent or redundant with RNAi.

We expanded our study of the *Iss1*-deltaC mutation to evaluate changes in expression and transcription termination genome-wide. RNA-Seq data revealed that *Iss1*-deltaC did not significantly impact canonical transcription termination, but 73 genes were found to have higher expression. Importantly, the genes overlapped significantly with genes upregulated in cells lacking Rrp6, the 3'-5' exonuclease subunit of the nuclear exosome. As a key subunit of the nuclear exosome, Rrp6 plays an important role in RNA surveillance in the degradation of meiotic transcripts expressed during vegetative growth and the resulting formation of heterochromatin at these genes. The elimination of meiotic mRNAs depends on the RNA-binding protein Mmi1 to bind to the determinant of selective removal (DSR) sequence in order to recruit the exosome. Our co-immunoprecipitation experiments revealed that *Iss1* interacted with Rrp6, Mmi1, and the polyA polymerase Pla1, indicating that *Iss1* is associated with this network of elimination factors. Significantly, the interaction with Mmi1 was disrupted by the *Iss1*-deltaC mutation, a mutation that greatly reduced H3K9me2 at meiotic genes. We tested whether *Iss1* plays a direct role in the heterochromatin of meiotic genes by performing ChIP-Seq of *Iss1*-FLAG. While a subset of *Iss1*-bound genes were highly expressed and associated with the canonical function of *Iss1* in mRNA termination, most *Iss1*-bound peaks showed a strong correlation with genes

regulated by RNA elimination and heterochromatin. Importantly, the *iss1-deltaC* mutation caused significant increases in the RNA levels of these genes. Taken together, our studies of RNA levels, *Iss1* association with chromatin, and H3K9me2 indicate that *Iss1* plays a direct role in the formation of heterochromatin at meiotic genes. Our application of Hermes profiles to identify genes important for heterochromatin formation demonstrates the significance of the approach, especially given that we were able to identify large numbers of essential genes, a result not obtainable with other screens.

After these results were published, we received significant interest in the protocols we used for Tn-Seq with *S. pombe*. We compiled detailed instructions, provided specific timing, and described reagents [Reference 3]. The step-by-step method includes inducing transposition, selecting insertions that reduce heterochromatin, and generating libraries of insertions. Of particular note is an extensive discussion of troubleshooting together with suggested solutions.

Additional Funding

- FY2022 Office of AIDS Research Innovative Funds Program

Publications

1. Li F, Lee M, Esnault C, Wendover K, Guo Y, Atkins P, Zaratiegui M, Levin HL. Identification of an integrase-independent pathway of retrotransposition. *Sci Adv* 2022 8:1–17.
2. Ahn H, Worman Z, Lechsinska A, Payer L, Wang T, Malik N, Li W, Burns K, Nath A, Levin HL. Retrotransposon insertions associated with risk of neurologic and psychiatric diseases. *EMBO Reports* 2022 e55197:1–17.
3. Li F, Hung S, Esnault C, Levin HL. A protocol for transposon insertion sequencing in *Schizosaccharomyces pombe* to identify factors that maintain heterochromatin. *STAR Protoc* 2021 2:100392.

Collaborators

- Kathleen Burns, MD, PhD, *Dana-Farber Cancer Institute, Boston, MA*
- Avindra Nath, MD, PhD, *Division of Neuroimmunology & Neurovirology, NINDS, Bethesda, MD*
- Mikel Zaratiegui, PhD, *Rutgers, The State University of New Jersey, Piscataway, NJ*

Contact

For more information, email henry_levin@nih.gov or visit <http://sete.nichd.nih.gov>.

Cell Cycle Regulation in Oogenesis

Our long-term goal is to obtain a comprehensive understanding of how metabolic signaling pathways influence oocyte growth, development, and quality. Chromosome mis-segregation during female meiosis is the leading cause of miscarriages and birth defects in humans. Recent evidence suggests that many meiotic errors occur downstream of defects in oocyte growth and/or in the hormonal signaling pathways that drive differentiation of the oocyte. Thus, understanding how oocyte development and growth impact meiotic progression is essential to studies in both reproductive biology and medicine. We use the genetically tractable model organism *Drosophila melanogaster* to examine how meiotic progression is instructed by the developmental and metabolic program of the egg.

In mammals, studies on the early stages of oogenesis face serious technical challenges in that entry into the meiotic cycle, meiotic recombination, and the initiation of the highly conserved prophase I arrest all occur during embryogenesis. By contrast, in *Drosophila* these critical events of early oogenesis all take place continuously within the adult female. Easy access to the early stages of oogenesis, coupled with available genetic and molecular-genetic tools, makes *Drosophila* an excellent model for studies on the role of metabolism in oocyte development and maintenance.

The GATOR complex: integrating developmental and metabolic signals in oogenesis

The Target of Rapamycin Complex 1 (TORC1) regulates cell growth and metabolism in response to many inputs, including amino-acid availability and intracellular energy status. In the presence of sufficient nutrients and appropriate growth signals, the Ragulator and the Rag GTPases (a complex that regulates lysosomal signaling and trafficking) target TORC1 to lysosomal membranes, where TORC1 associates with its activator, the small GTPase Rheb. Once activated, TORC1 is competent to phosphorylate its downstream targets. The Gap activity towards Rags (GATOR) complex is an upstream regulator of TORC1 activity.

The GATOR complex consists of two subcomplexes (Figure 1). The GATOR1 complex inhibits TORC1 activity in response to amino-acid



Mary Lilly, PhD, Head, Section on Gamete Development

Chun-Yuan Ting, PhD, Staff Scientist

Kuikwon Kim, MS, Biologist

Shu Yang, PhD, Postdoctoral Fellow

Lucia Bettedi, PhD, Visiting Fellow

Yingbiao Zhang Zhang, PhD, Visiting Fellow

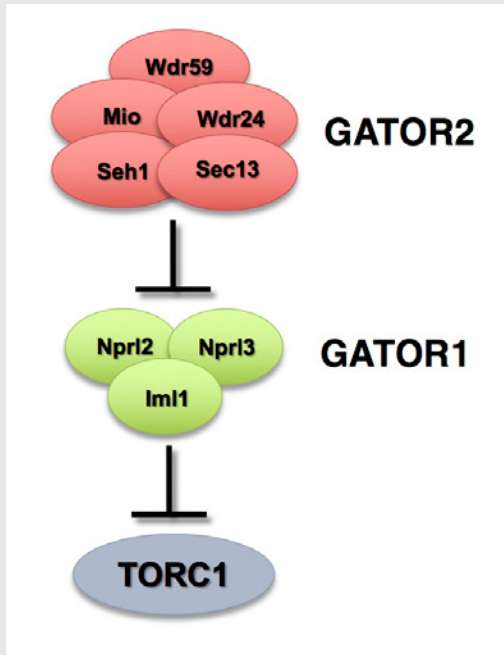


FIGURE 1. The GATOR complex regulates TORC1 activity.

The GATOR2 complex opposes the activity of the TORC1 inhibitor GATOR1.

starvation. GATOR1 is a trimeric protein complex consisting of the proteins Npr12, Npr13, and Iml1. Evidence from yeast and mammals indicates that the components of the GATOR1 complex function as GTPase-activating proteins (GAP) that inhibit TORC1 activity by inactivating the Rag GTPases. Notably, *Npr12* and *Iml1* are tumor-suppressor genes, while mutations in *Iml1*, known as *DEPDC5* in mammals, are a leading cause of hereditary epilepsy.

Our work, as well as that of others, found that the GATOR2 complex activates TORC1 by opposing the TORC1-inhibitory activity of GATOR1. Intriguingly, computational analysis indicates that Mio and Seh1, as well as several other members of the GATOR2 complex, have structural features consistent with coatomer proteins and membrane-tethering complexes. In line with the structural similarity to proteins that influence membrane curvature, we showed that three components of the GATOR2 complex, Mio, Seh1, and Wdr24, localize to the outer surface of lysosomes, the site of TORC1 regulation. However, how GATOR2 inhibits GATOR1 activity, thus allowing for the robust activation of TORC1, remains unknown. Additionally, the role of the GATOR1

and GATOR2 complexes in both the development and physiology of multicellular animals remains poorly defined. Over the past year, we used molecular, genetic, and cell-biological approaches to define the *in vivo* functions of the GATOR1 and GATOR2 complexes in the model organism *Drosophila*.

The GATOR2 component Wdr59 promotes or inhibits TORC1 activity depending on cellular context.

As mentioned above, the GATOR2 complex inhibits GATOR1 and thus serves to activate TORC1. However, the mechanism by which GATOR2 inhibits GATOR1 remains unknown. In its initial functional characterization in mammalian and *Drosophila* cultured cells, the GATOR2 complex was reported to contain five protein Mios/Mio, Seh1, Sec13, Wdr24, and Wdr59 (Figure 1A). In these earlier studies, knockdowns of GATOR2 components resulted in constitutive activation of GATOR1 and reduced TORC1 activity. Similarly, *Drosophila* mutants of the GATOR2 components *mio*, *seh1*, and *wdr24* exhibit reduced TORC1 activity and growth in the female germline. However, a recent study from *Schizosaccharomyces pombe* reported that SEA3/WDR59 inhibits TORC1 activity as a component of the GATOR1 complex. Notably, this is the opposite of the role assigned to Wdr59 based on studies in both human and *Drosophila* cultured cells. Additionally, deletions of Wdr59 in HEK293 (human embryonic kidney) cells and mouse embryonic fibroblasts result in a partial resistance to nutrient deprivation. Thus, the exact function of Wdr59 within the GATOR-TORC1 signaling pathway remains unclear.

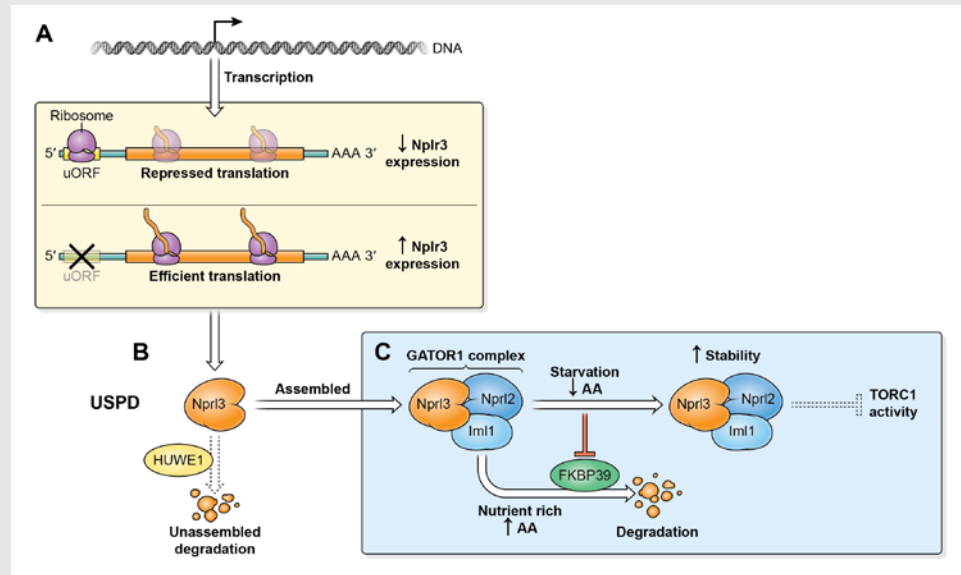
Over the last year, we showed that Wdr59, originally assigned to the GATOR2 complex based on studies

FIGURE 2. Many pathways regulate the levels of the TORC1 inhibitor Nprl3.

A. The *nprl3* mRNA contains a functional uORF that reduces Nprl3 translation.

B. Nprl3 forms the trimeric GATOR1 complex with the proteins Nprl2 and Iml1. When not assembled into the GATOR1 complex, Nprl3 is degraded via a pathway dependent on the ubiquitin ligase HUWE1.

C. In nutrient-replete conditions, FKBP39 associates with Nprl3 and promotes its degradation. Upon amino acid starvation, the FKBP39-dependent destruction of Nprl3 is blocked, and the increased levels of GATOR1 result in reduced TORC1 activity.



performed in tissue culture cells, unexpectedly has a dual function in TORC1 regulation in *Drosophila*. We demonstrated that, in the ovary and the eye imaginal disc brain complex, Wdr59 inhibits TORC1 activity by opposing the GATOR2-dependent inhibition of GATOR1. Conversely, in the *Drosophila* fat body, Wdr59 promotes the accumulation of the GATOR2 component Mio and is required for TORC1 activation. Similarly, in mammalian HeLa cells, Wdr59 prevents the proteolytic destruction of GATOR2 proteins Mio and Wdr24. Consistent with the reduced levels of the TORC1-activating GATOR2 complex, Wdr59 knockout (KO) HeLa cells have reduced TORC1 activity, which is restored along with GATOR2 protein levels upon proteasome inhibition. Taken together, our data support a model in which the Wdr59 component of the GATOR2 complex functions to promote or inhibit TORC1 activity depending on cellular context. These studies broaden our understanding of the GATOR-TORC1 signaling axis in metazoans and highlight the complexity of metabolic regulation in metazoans.

Many independent pathways converge on Nprl3 to regulate TORC1 activity in *Drosophila*.

In collaboration with the laboratory of Youheng Wei, we characterized several pathways that regulate the expression of the GATOR1 component Nprl3 in *Drosophila* (Figure 2). We determined that the stability of Nprl3 is impacted by the Unassembled Soluble Complex Proteins Degradation (USPD) pathway. In addition, we found that FK506 binding protein 39 (FKBP39)-dependent proteolytic destruction maintains Nprl3 at low levels in nutrient-replete conditions. Nutrient starvation abrogates the degradation of the Nprl3 protein and rapidly promotes Nprl3 accumulation. Consistent with a role in promoting the stability of a TORC1 inhibitor, mutations in *fkbp39* reduced TORC1 activity and increased autophagy. We also demonstrated that the 5' UTR of *nprl3* transcripts contain a functional upstream open-reading frame (uORF) that inhibits main ORF translation. In summary, our work uncovered novel mechanisms of Nprl3 regulation and identified an important role for *Drosophila* FKBP39 in the control of cellular metabolism and growth.

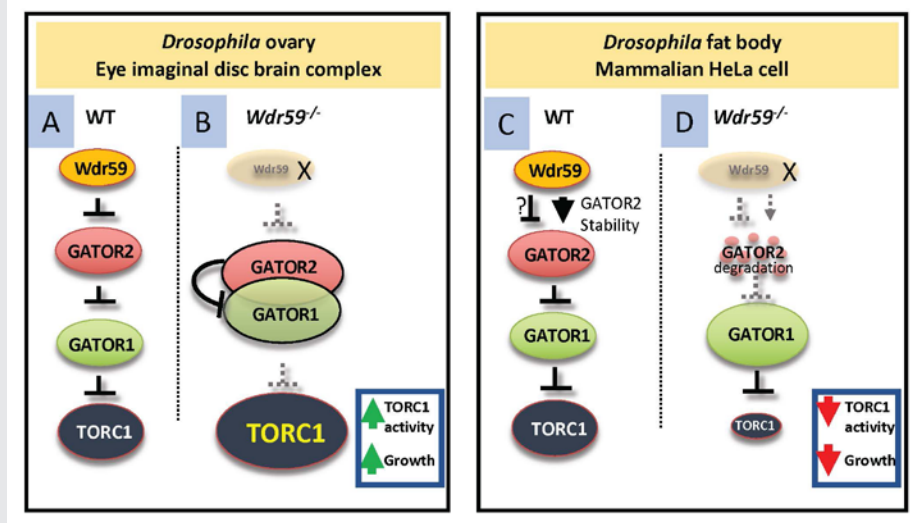
FIGURE 3. Model for the dual role of Wdr59 in TORC1 regulation

A. In wild-type animals, Wdr59 inhibits TORC1 activity upstream of the GATOR2 complex in the ovary and eye imaginal disc brain complex.

B. In *wdr59* mutants, GATOR2 increases its association with GATOR1, further inhibiting GATOR1 activity, and allowing for the increased activation of TORC1.

C. In the fat body of *Drosophila* and mammalian HeLa cells, Wdr59 protects GATOR2 components from degradation by the proteasome.

D. In the absence of Wdr59 from the *Drosophila* fat body or from HeLa cells, GATOR2 components are destroyed by the proteasome, resulting in derepression of GATOR1, increased interaction of GATOR1 with RagA, and concomitant reduction in TORC1 activity. Note that the GATOR1 complex regulates TORC1 activity by acting as a GAP for the RagA component of the GTPase, which functions to activate TORC1.



Publications

1. Zhou Y, Guo J, Wang X, Cheng Y, Guan J, Barman P, Sun MA, Fu Y, Wei W, Feng C, Lilly MA, Wei Y. FKBP39 controls nutrient dependent Npr13 expression and TORC1 activity in *Drosophila*. *Cell Death Dis* 2021 12(6):571.

Collaborators

- Brian Calvi, PhD, *Indiana University, Bloomington, IN*
- Ryan Dale, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*
- Thomas Gonatopoulos-Pournatzis, PhD, *Functional Transcriptomics Section, NCI, Frederick, MD*
- Mark Nellist, PhD, *Erasmus University Medical Center, Rotterdam, Netherlands*
- Youheng Wei, PhD, *Yangzhou University, Yangzhou, Jiangsu, China*

Contact

For more information, email mlilly@helix.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/lilly>.

Neurosecretory Proteins in Neuroprotection and Neurodevelopment

Mechanism of sorting, transport, and regulated secretion of neuroproteins

The intracellular sorting of pro-neuropeptides and neurotrophins to the regulated secretory pathway (RSP) is essential for processing, storage, and release of active proteins and peptides in the neuroendocrine cell. We investigated the sorting of proopiomelanocortin (POMC, also known as pro-ACTH/endorphin), proinsulin, and brain-derived neurotrophic factor (BDNF) to the RSP. Our studies showed that these pro-proteins undergo homotypic oligomerization as they traverse the cell from the site of synthesis in the endoplasmic reticulum (ER) to the *trans*-Golgi network (TGN). In the TGN, the pro-proteins are sorted into the dense-core granules of the RSP for processing by prohormone convertases and carboxypeptidase E (CPE) and then secreted. We showed that the sorting of prohormones to the RSP occurs by a receptor-mediated mechanism. Site-directed mutagenesis studies identified a 3-D consensus sorting motif consisting of two acidic residues found in POMC, proinsulin, and BDNF. We identified the transmembrane form of CPE as an RSP-sorting receptor that is specific for the sorting signal of these pro-proteins.

We also investigated the role of secretogranin III (SgIII) as a surrogate sorting receptor for membrane CPE in targeting POMC to the RSP. Using RNA interference (siRNA) to knock down SgIII or CPE expression in pituitary AtT20 cells, we demonstrated in both cases that POMC secretion via the constitutive secretory pathway was elevated. In double CPE-SgIII knock-down cells, elevated constitutive secretion of POMC and stimulated secretion of ACTH were perturbed. Thus, CPE mediates trafficking of POMC to the RSP; SgIII may play a compensatory role for CPE in POMC sorting to the RSP.

Transport of vesicles containing hormone or BDNF to the plasma membrane for activity-dependent secretion is critical for endocrine function and synaptic plasticity. We showed that the cytoplasmic tail of a transmembrane form of CPE in hormone- or BDNF-containing dense-core secretory vesicles plays an important role in their transport to the vesicles' release site. Overexpression of the CPE tail inhibited the movement of BDNF- and POMC/CPE-containing vesicles to the processes in hippocampal neurons and pituitary cells, respectively. The transmembrane CPE tails on the POMC/ACTH and



Y. Peng Loh, PhD, Head, Section on Cellular Neurobiology
Xuyu Yang, PhD, *Staff Scientist*
Vinay Sharma, PhD, *Contractor*
Ashley Xiao, MD, PhD, *Contractor*

BDNF vesicles interact with dynactin and the microtubule-based motors KIF1A/KIF3A to effect anterograde vesicle movement to the plasma membrane for activity-dependent secretion. Additionally, in collaboration with Joshua Park, we showed that another player, snapin, binds directly to the cytoplasmic tail of CPE and connects with the microtubule motor complex, which consists of kinesin-2 and kinesin-3, to mediate post-Golgi anterograde transport of POMC/ACTH vesicles to the process terminals of AtT20 cells for secretion. Knockdown of snapin reduced stimulated ACTH secretion, while protein kinase A (PKA) activation by forskolin significantly increased the interactions of kinesin-2 and kinesin-3 with CPE and levels of ACTH vesicles at the terminus, and enhanced secretion of ACTH in AtT20 cells. Thus, our study uncovered a novel complex consisting of the CPE cytoplasmic tail snapin–kinesin-2 and -3, which mediates anterograde transport of ACTH/POMC vesicles to the process terminals for secretion in a PKA-dependent manner in neuroendocrine cells.

Serpinin, a chromogranin A–derived peptide, regulates secretory granule biogenesis, cell survival, cardiac function, and angiogenesis.

Our previous studies in pituitary AtT-20 cells provided evidence that an autocrine mechanism up-regulates large dense-core vesicle (LDCV) biogenesis to replenish LDCVs following stimulated exocytosis of the vesicles. We identified the autocrine signal as serpinin, a novel 26 amino-acid, chromogranin A (CgA)–derived peptide cleaved from the C-terminus of CgA. Serpinin is released in an activity-dependent manner from LDCVs and activates adenylyl cyclase to raise cAMP levels and protein kinase A in the cell. This leads to translocation of the transcription factor Sp1 from the cytoplasm into the nucleus and enhanced transcription of a protease inhibitor, protease nexin 1 (PN-1), which then inhibits granule protein degradation in the Golgi complex, stabilizing and raising granule protein levels in the Golgi and enhancing LDCV formation. We also identified modified forms of serpinin, pyroglutamyl-serpinin (pGlu-serpinin), and serpinin-RRG, a C-terminally extended form, in the secretion medium of AtT20 cells and in rat heart tissue. pGlu-serpinin is synthesized and stored in secretory granules and secreted in an activity-dependent manner from AtT20 cells. We observed pGlu-serpinin immunostaining in nerve terminals of neurites in mouse brain, olfactory bulb, and retina, suggesting a role as a neurotransmitter or neuromodulator. Additionally, pGlu-serpinin exhibited neuroprotective activity against oxidative stress in AtT20 cells and against low K^+ -induced apoptosis in rat cortical neurons. In collaboration with Bruno Tota, we found that pGlu-serpinin has positive inotropic activity in cardiac function, with no change in blood pressure and heart rate. pGlu-serpinin acts through a β 1-adrenergic receptor/adenylyl cyclase/cAMP/PKA pathway in the heart. pGlu-serpinin and other CgA–derived cardio-active peptides thus emerge as novel β -adrenergic inotropic and lusitropic modulators. Together, they can play a key role in the myocardium's orchestration of its complex response to sympatho-chromaffin stimulation. Additionally, pGlu serpinin is a powerful cardio-protectant after ischemia. The mechanism involves the activation of the reperfusion-injury salvage kinase (RISK) pathway. In collaboration with Angelo Corti, we showed that serpinin-RRG had anti-angiogenic activity.

Role of CPE/NF- α 1 in neuroprotection and anti-depression during stress

Several null and homozygous nonsense mutations in the CPE (also known as neurotrophic factor- α 1 [NF- α 1]) gene were identified in seven children and a young adult woman from five different families. They display clinical features that include childhood onset obesity, type 2 diabetes, intellectual disabilities, hypogonadotropic hypogonadism, and infantile hypotonia, indicating the importance of CPE in human disease. To study the physiological functions of CPE/NF- α 1 *in vivo*, we generated a *Cpe* knock-out (KO) mouse. The KO mouse exhibited obesity, infertility, and diabetes. Further analysis of *Cpe*-KO mice in the

Morris water maze and by object-preference tests revealed defects in learning and memory and depressive-like behavior in the forced swim test. Electrophysiological measurements showed a defect in the generation of long-term potentiation in hippocampal slices. We discovered that a major cause of the neurological defects in such mice was the loss of CA3 neurons in the hippocampus after weaning stress. Hippocampal neurons in the CA3 region are enriched in CPE. *Cpe*-KO mice showed normal hippocampal cytoarchitecture at three weeks of age just before weaning, indicating that the defect was not a developmental problem. Rather, we hypothesized that the degeneration of the CA3 neurons was likely caused by glucocorticoid-induced epileptic-like neuronal firing of the granule cells in the dentate gyrus, releasing large amounts of glutamate during the weaning-stress paradigm, which includes emotional stress from maternal separation, and physical stress from ear-tagging and tail clipping for genotyping. The hypothesis was supported by the finding that treatment with carbamazepine, an anti-epileptic drug, prior to weaning prevented the stress-induced degeneration of the CA3 neurons in the *Cpe*-KO mice. Hence, CPE/NF- α 1 is important for the survival of CA3 neurons during severe stress. To determine whether the neuroprotective effect of CPE/NF- α 1 depends on the BDNF-TrkB pathway (Trk: tropomyosin receptor kinase), we treated mice with ANA12, a trkB inhibitor. Interestingly, downregulation of the BDNF-TrkB pathway had no detrimental effect on the survival of the CA3 neurons after the weaning-stress paradigm, unlike the *Cpe*-KO mice, which showed complete degeneration, suggesting that CPE/NF- α 1 is more critical than BDNF in protecting CA3 neurons from severe stress-induced cell death [Reference 1].

Furthermore, we showed that a mutant mouse expressing an enzymatically inactive form of CPE/NF- α 1 (E342Q) had a normal hippocampus and learning and memory after the weaning-stress paradigm, indicating that the neuroprotective action of CPE/NF- α 1 is independent of its enzymatic activity [Reference 1]. We showed that CPE/NF- α 1 (E342Q), either overexpressed or applied externally to cultured hippocampal or cortical neurons, protected the neurons from apoptosis induced by oxidative stress with hydrogen peroxide or glutamate treatment. Likewise, the enzymatically inactive form of CPE/NF- α 1, applied extracellularly, had the same neuroprotective effect. We thus demonstrated that CPE/NF- α 1 acts extracellularly as a signaling molecule to mediate neuroprotection. To this end, we showed that ^{125}I -CPE/NF- α 1 bound specifically to the cell surface of HT22 cells, an immortalized hippocampal neuronal cell line, in a saturable manner, suggesting the existence of a receptor. K235a, a Trk family inhibitor, and PD16285, a fibroblast growth factor receptor (FGFR1-3) inhibitor, did not prevent the neuroprotective action of CPE/NF- α 1 in hippocampal neurons treated with H_2O_2 , suggesting that CPE/NF- α 1 likely uses a different class of receptors than those of the Trk family or FGFRs. The mechanism of action of CPE/NF- α 1 in neuroprotection in mouse and rat hippocampal neurons involves the activation of the ERK1/2 signaling pathway during stress, which then leads to enhanced expression of a pro-survival mitochondrial protein, BCL2, inhibition of caspase 3 activation, and promotion of neuronal survival [Reference 2]. We then screened a human GPCR (G protein-coupled receptor) library using CPE/NF- α 1 as a ligand, and identified the serotonin receptor 5-HTR1E as a binding partner. This interaction was confirmed by co-immunoprecipitation and pulldown assays. Binding studies revealed a K_d of 13.82 nM. Molecular dynamics studies indicated that CPE/NF- α 1 interacts with 5-HTR1E via three salt bridges stabilized by several hydrogen bonds, and is independent of the serotonin binding pocket. Immunohistochemistry revealed co-localization of 5-HTR1E and CPE/NF- α 1 on the surface of hippocampal neurons. Signal transduction studies showed that HTR1E-CPE/NF- α 1 interaction activated the ERK1/2 (extracellular signal-regulated kinases)-CREB pathway via recruitment of beta-arrestin. This in turn activated the BCL2 pro-survival pathway. We showed that the 5-HTR1E-CPE/NF- α 1 interaction mediated neuroprotection of human primary neurons against H_2O_2 -induced cytotoxicity and glutamate-induced neurotoxicity. The findings indicate that CPE/NF- α 1 interacts with 5-HTR1E to promote neuronal survival

[Reference 3]. 5-HTR1E is only found in humans, primates, and guinea pig but not in mice or rats. Work is ongoing to identify a mouse receptor for CPE/NF-alpha1.

Examination of the pathway during stress *in vivo* revealed that, after mild chronic restraint stress (CRS) for 1 hour per day for seven days, mice showed significantly elevated levels of CPE/NF-alpha1 mRNA and protein, as well as the anti-apoptotic protein Bcl2, in the hippocampus. *In situ* hybridization studies indicated particularly elevated CPE/NF-alpha1 mRNA levels in the CA3 region and no gross neuronal cell death after mild CRS. Furthermore, primary hippocampal neurons in culture showed elevated CPE/NF-alpha1 and Bcl2 expression and a decline in Bax, a pro-apoptotic protein, after dexamethasone (a synthetic glucocorticoid) treatment. The up-regulation was mediated by glucocorticoid binding to glucocorticoid-regulatory element (GRE) sites on the promoter of the *Cpe* gene. Thus, during mild CRS, when glucocorticoid is released, CPE/NF-alpha1 and Bcl2 expression are coordinately up-regulated to mediate neuroprotection of hippocampal neurons. The importance of CPE as a neuroprotective agent was demonstrated by the absence of an increase in Bcl2 in the hippocampus of *Cpe*-KO mice after CRS, and degeneration of the CA3 neurons. Furthermore, CRS also elevated expression of the signaling protein FGF2. We demonstrated that mouse primary hippocampal neurons treated with CPE/NF-alpha1 increased FGF2 expression. Thus, another action of CPE/NF-alpha1 may be by increasing FGF2, which is known to have neuroprotective effects. In summary, CPE/NF-alpha1 is a critical neurotrophin for protecting CA3 neurons against stress-induced cell death via the Erk-Bcl2 signaling pathway.

The relevance of CPE/NF-alpha1 in neuroprotection in humans was underscored by our studies on a mutation of the *CPE* gene found in an Alzheimer's disease (AD) patient [Reference 4]. Our search of the GenBank EST database identified a sequence entry from the cortex of an AD patient that had three adenosine inserts in the *CPE* gene, thereby introducing nine amino acids, including two glutamines, into the mutant protein, herein called CPE-QQ. Overexpression of CPE-QQ into neuroblastoma cells indicated that the mutated protein aggregates with the wild-type (WT) protein in the ER, causing ER stress, reduced Bcl2 levels, and neuronal cell death. We generated transgenic mice overexpressing CPE-QQ and showed that, at 50 weeks but not at 11 weeks of age, the animals exhibited memory deficits and depressive-like behavior compared with WT mice, but that their spatial learning ability was unimpaired. The CPE-QQ mice were neither obese nor diabetic, as there is some CPE activity in these mice, given that the endogenous WT gene was not deleted. However, they had significantly fewer neurites in the CA3 region, the dentate gyrus of the hippocampus, and in the medial prefrontal cortex, indicative of neurodegeneration. Moreover, they exhibited reduced neurogenesis in the subgranular zone and hyperphosphorylation of the microtubule-associated protein tau at ser³⁹⁵, a hallmark of AD. The studies thus identified a human mutation in the *CPE* gene resulting in expression of a CPE-QQ protein, which caused neurodegeneration and impairment of memory function, as well as depressive-like behavior in a mouse model, linking the gene for the first time to neurodegenerative disease and depression [Reference 4]. We therefore explored whether CPE could rescue cognitive dysfunction in AD mice. We injected AAV-CPE into the hippocampus of an AD mouse model, which was able to rescue cognitive dysfunction in these mice.

Stress also induces depression. Huda Akil's group (University of Michigan) reported that FGF2 is an anti-depressant. We found that prolonged (6 hours per day for 21 days) restraint stress reduced CPE/NF-alpha1 and FGF2 in the hippocampus of mice and induced depressive-like behavior. However, after short-term restraint stress (1 hour per day for 7 days), mice did not show depressive-like behavior despite elevated corticosterone levels indicative of stress. Moreover, hippocampal CPE/NF-alpha1, FGF2, and doublecortin, a marker for neurogenesis, were elevated in these mice, suggesting that the anti-depressive effects of CPE/NF-alpha1 are

mediated, at least in part, through increased neurogenesis. Indeed, we found that exogenously applied CPE/NF- α 1 could up-regulate *FGF2* mRNA and protein expression in cultured hippocampal neurons, indicating that CPE/NF- α 1 regulates FGF2 expression. CPE/NF- α 1-KO mice exhibited severely reduced hippocampal FGF2 levels and immature neuron numbers in the subgranular zone. The mice displayed depressive-like behavior, which was rescued by FGF2 administration. Thus, we propose that CPE/NF- α 1 prevents stress-induced depressive-like behavior by up-regulating hippocampal FGF2 expression, which leads to enhanced neurogenesis and anti-depressive activity [Reference 5]. Furthermore, we found that rosiglitazone, an anti-diabetic drug, can trigger this pathway [Reference 5]. Interestingly, rosiglitazone has previously been shown to be effective in treating diabetic patients with bi-polar disorders.

Role of CPE/NF- α 1 and CPE- δ in embryonic brain development

Embryonic mouse brains express three forms of CPE/NF- α 1 mRNA (2.1kb, 1.9kb, and 1.73kb in size), which encode a 53kD WT CPE/NF- α 1, and two terminal-truncated isoforms of CPE/NF- α 1-DN (47kD and 40kD). The three mRNAs are expressed as early as E8.5 and increase significantly in two waves at E10.5 and postnatal day 1 [Reference 5]. Interestingly, CPE/NF- α 1-DNs are not expressed in adult mouse brain. *In situ* hybridization studies indicate that CPE/NF- α 1 is expressed primarily in the forebrain in mouse embryos, suggesting that CPE/NF- α 1 plays a role in neurodevelopment. We examined the effect of CPE/NF- α 1 on E13.5 neocortex-derived neurospheres, which contain stem cells and neuro-progenitors. Application of recombinant CPE/NF- α 1 reduced the number and size of the neuro-spheres formed, suggesting inhibition of proliferation and maintenance of the 'stemness' of the stem cells in the neuro-spheres. CPE/NF- α 1 down-regulated the wnt pathway in the neuro-spheres, leading to reduced levels of beta-catenin, a protein known to enhance proliferation, suggesting that CPE/NF- α 1's inhibitory effect on proliferation is brought about by negatively regulating the wnt pathway.

We also carried out differentiation studies using neuro-spheres from seven-day cultures that were dissociated into single cells and cultured for an additional five days. We observed an increase in astrocytes after CPE/NF- α 1 treatment, without alteration in the percentage of neuronal and oligodendrocyte populations. We also observed this phenomenon when the cultured embryonic stem cells were treated with a non-enzymatic form of CPE, indicating that the effect was independent of enzymatic activity. Interestingly, dissociated cells from neuro-spheres derived from *Cpe/NFalpha1*-KO mouse embryos showed fewer astrocytes but more neurons, which was reversed by CPE/NF- α 1 application. *In vivo*, *Cpe/NF-alpha1*-KO mouse cortex (at P1, the time of astrocytogenesis) showed about half the astrocyte numbers of those in WT animals, confirming the *ex vivo* data. Our results suggest a novel role for CPE/NF- α 1 as an extracellular signal to inhibit proliferation and induce differentiation of neural stem cells into astrocytes, thus playing an important role in neurodevelopment [Reference 6].

Neurite outgrowth is key to the formation of synapses and the neural network during development. We found that CPE/NF- α 1 prevented Wnt-3a inhibition of nerve growth factor (NGF)-stimulated neurite outgrowth in PC12 cells, a neuro-endocrine cell line, and in cortical neurons. Moreover, CPE/NF- α 1 augmented Wnt-5a-mediated neurite outgrowth. Thus, the interplay between NGF preventing neurite outgrowth, which is inhibited by Wnt-3a, and augmenting neurite outgrowth, which is mediated by Wnt-5a and CPE/NF- α 1, could play an important role in regulating these positive and negative cues, which are critical for neurodevelopment. Analysis of the brain of 6- to 14-week-old *Cpe*-KO mice revealed poor dendritic pruning in cortical and hippocampal neurons, which could affect synaptogenesis.

We also studied the function of 40kD CPE/NF- α 1-DN and showed that it is translocated from the cytoplasm into the nucleus of rat embryonic neurons. Overexpression of 40kD CPE/NF α 1-DN in HT-22 cells, a hippocampal cell line, resulted in an increase in the expression of IGF binding protein2 (IGFBP2), death-associated protein (DAP1), and Ephrin 1A mRNAs and proteins (receptor protein tyrosine kinases), which are involved in neuronal proliferation, programmed cell death, and neuronal migration, respectively. We demonstrated that IGFBP2 is involved in proliferation in a CPE/NF- α 1-DN-dependent manner in HT22 and mouse cortical neurons [Xiao L *et al.*, *FASEB J* 2019;33:808]. Thus, 40kD CPE/NF- α 1-DN functions to regulate expression of genes important in neurodevelopment. Further studies aimed at determining the role of CPE/NF- α 1-DN *in vivo* are in progress.

Publications

1. Xiao L, Sharma VK, Toulabi L, Yang X, Lee C, Abebe D, Peltekian A, Arnaoutova I, Lou H, Loh YP. Neurotrophic factor- α 1, a novel tropin is critical for the prevention of stress-induced hippocampal CA3 cell death and cognitive dysfunction in mice: comparison to BDNF. *Translat Psychiatry* 2021 11:24.
2. Xiao L, Loh YP. Neurotrophic factor- α 1/carboxypeptidase E functions in neuroprotection and alleviates depression. *Front Mol Neurosci* 2022 15:918852.
3. Sharma VK, Yang X, Kim K-S, Mafi A, Saiz-Sanchez D, Villanueva-Anguita P, Xiao L, Toulabi L, Inoue A, Goddard III W, Loh YP. Novel interaction between neurotrophic factor- α 1/carboxypeptidase E and serotonin receptor, 5-HTR1E, protect human neurons against oxidative/neuroexcitotoxic stress via β -arrestin/ERK signaling. *Cell Mol Life Sci* 2021 79:24.
4. Cheng Y, Cawley NX, Yanik T, Murthy SRK, Liu C, Kasicki F, Abebe D, Loh YP. A human carboxypeptidase E gene mutation in an Alzheimer's disease patient causes neurodegeneration, memory deficits and depression. *Translat Psychiatry* 2016 6(12):e973.
5. Cheng Y, Rodriguiz RM, Murthy SRK, Senatorov V, Thouennon E, Cawley NX, Aryal D, Ahn S, Lecka-Czernik B, Wetsel WC, Loh YP. Neurotrophic factor- α 1 prevents stress-induced depression through enhancement of neurogenesis and is activated by rosiglitazone. *Mol Psychiatry* 2015 20:744–754.
6. Selvaraj P, Xiao L, Lee C, Murthy SRK, Cawley NX, Lane M, Merchenthaler I, Ahn S, Loh YP. Neurotrophic factor- α 1: a key Wnt-beta-catenin dependent anti-proliferation factor and ERK-Sox9 activated inducer of embryonic neural stem cell differentiation to astrocytes in neurodevelopment. *Stem Cells* 2017 35:557–571.

Collaborators

- Angelo Corti, MD, *San Raffaele Scientific Institute, Milan, Italy*
- William A. Goddard, III, PhD, *California Institute of Technology, Pasadena, CA*
- Beata Lecka-Czernik, PhD, *University of Toledo, Toledo, OH*
- Joshua J. Park, PhD, *University of Toledo, Toledo, OH*
- Daniel Saiz-Sanchez, MD, *Universidad de Castilla-La Mancha, Ciudad Real, Spain*
- Bruno Tota, MD, *Università della Calabria, Cosenza, Italy*

Contact

For more information, email lohp@mail.nih.gov or visit <http://scn.nichd.nih.gov>.

The Molecular Mechanics of Eukaryotic Translation Initiation

The goal of our research is to elucidate the molecular mechanisms underlying the initiation phase of protein synthesis in eukaryotic organisms. We use the yeast *Saccharomyces cerevisiae* as a model system and employ a range of approaches, from genetics to biochemistry to structural biology, in collaboration with [Alan Hinnebusch's](#) and [Tom Dever's labs](#) and several other research groups around the world.

Eukaryotic translation initiation is a key control point in the regulation of gene expression. It begins when an initiator methionyl tRNA (Met-tRNA_i) is loaded onto the small (40S) ribosomal subunit. Met-tRNA_i binds to the 40S subunit as a ternary complex (TC) with the GTP-bound form of the initiation factor eIF2. Three other factors, eIF1, eIF1A, and eIF3, also bind to the 40S subunit and promote the loading of the TC. The resulting 43S preinitiation complex (PIC) is then loaded onto the 5' end of an mRNA with the aid of eIF3 and the eIF4 group of factors: the RNA helicase eIF4A; the 5' 7-methylguanosine cap-binding protein eIF4E; the scaffolding protein eIF4G; and the 40S subunit- and RNA-binding protein eIF4B. Both eIF4A and eIF4E bind to eIF4G and form the eIF4F complex. Once loaded onto the mRNA, the 43S PIC is thought to scan the mRNA in search of an AUG start codon. The process is ATP-dependent and likely requires several RNA helicases, including the DEAD-box protein Ded1p. Recognition of the start site begins with base pairing between the anticodon of tRNA_i and the AUG codon. Base pairing then triggers downstream events that commit the PIC to continuing initiation from that point on the mRNA, events that include ejection of eIF1 from its binding site on the 40S subunit, movement of the C-terminal tail (CTT) of eIF1A, and release of phosphate from eIF2, which converts eIF2 to its GDP-bound state. In addition, the initiator tRNA moves from a position that is not fully engaged in the ribosomal P site [termed P_(OUT)] to one that is [P_(IN)], and the PIC as a whole converts from an open conformation, which is conducive to scanning, to a closed one, which is not. At this stage, eIF2•GDP dissociates from the PIC, and eIF1A and a second GTPase factor, eIF5B, coordinate joining of the large ribosomal subunit to form the 80S initiation complex. In a process that appears to result in conformational reorganization of the complex, eIF5B hydrolyzes GTP and then dissociates along with eIF1A.



Jon Lorsch, PhD, *Chief, Section on the Mechanism and Regulation of Protein Synthesis*

Fujun Zhou, PhD, *Research Fellow*

Meizhen Hou, MS, *Biologist*

Julie Bocetti, BA,

Postbaccalaureate Intramural Research Training Award Fellow

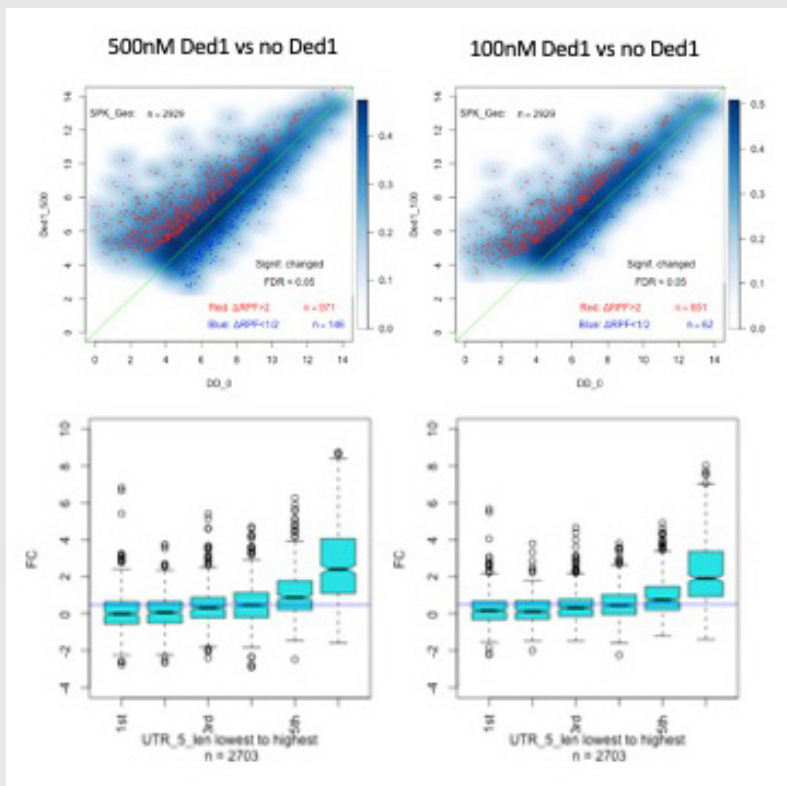


FIGURE 1. Dependency of mRNA recruitment on Ded1

Ded1 specifically enhances recruitment of mRNAs with long 5'-untranslated regions (5'-UTRs).

We continued our studies of the mechanism of translation initiation using our recently developed Rec-Seq transcriptome-wide approach. Rec-Seq utilizes our fully reconstituted yeast translation initiation system and is able to simultaneously monitor the recruitment of each mRNA in the yeast transcriptome to the 43S ribosomal PIC. We spent considerable effort this year honing the method to provide reproducible, quantitative results. A central goal was the development of a reliable approach to normalize the sequence read data to an absolute standard, so that direct comparisons can be made across experiments and replicates. After trying a number of different strategies, we believe we have finally found a way to perform this normalization step using a “spike in,” consisting of PICs assembled on luciferase mRNAs, which is added in a standard concentration to each experimental sample before processing. We also repeated our previous experiments to examine the function of the RNA helicase Ded1, using additional replicates to test reproducibility. This replication experiment yielded results similar to those of the original experiments and confirmed that Ded1 preferentially enhances mRNA recruitment to the 43S PIC for mRNAs with long, structured 5'-untranslated regions. We also performed an experiment to assess the effects of varying 40S ribosomal subunit and mRNA concentrations relative to each other. Initial results appeared to support a previously proposed model that limiting 40S subunit concentrations would specifically disfavor translation of “weak” mRNAs that compete poorly for binding to the PIC compared with “strong,” more competitive mRNAs. However, we will reanalyze these data using our new normalization approaches to determine how this affects the results and conclusions.

Additional Funding

- Funding is provided via a Memorandum of Understanding (MOU) between NIGMS and NICHD.

Publications

1. Gulay S, Gupta N, Lorsch JR, Hinnebusch AG. Distinct interactions of eIF4A and eIF4E with RNA helicase Ded1 stimulate translation in vivo. *eLife* 2020 9:e58243.

Collaborators

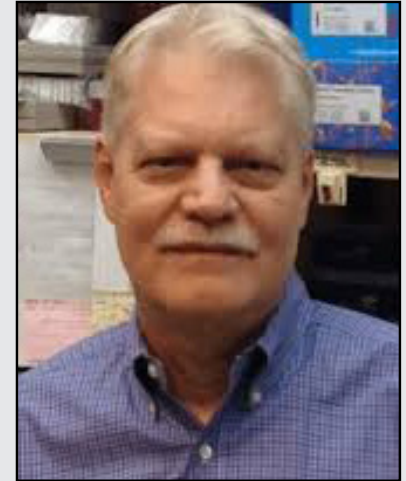
- Thomas Dever, PhD, *Section on Protein Biosynthesis, NICHD, Bethesda, MD*
- Alan Hinnebusch, PhD, *Section on Nutrient Control of Gene Expression, NICHD, Bethesda, MD*
- Venki Ramakrishnan, PhD, *MRC Laboratory of Molecular Biology, Cambridge, United Kingdom*

Contact

For more information, email jon.lorsch@nih.gov or visit <https://irp.nih.gov/pi/jon-lorsch>.

Exploring Genes and Signals Regulating Mammalian Hematopoiesis and New Approaches to Immunotherapy

Our research primarily focuses on the development of the mammalian hematopoietic system. A long-term area of interest for our laboratory is the study of signal-transduction molecules and pathways that regulate T lymphocyte maturation in the thymus. Currently, we are analyzing transgenic and conditional non-signaling mutants of T cell antigen receptor (TCR)-signaling subunits, made in our lab, to evaluate the importance of individual TCR-signaling chains and motifs at specific stages of T cell development and in mature T cells. In newer studies, we are investigating whether modification of TCR-signaling subunits can be used to enhance the tumoricidal activity of T cells for cancer treatment. Using gene profiling, we seek to identify proteins that are important for fine-tuning the T cell-signaling response in developing and mature T lymphocytes. In conjunction with checkpoint inhibitors for immunotherapy in humans, such molecules may also be targets to enhance anti-tumor activity. We also investigate the function of new T cell-specific proteins that we identified by subtraction-library screening. Our studies revealed a critical role for one of these proteins, called Themis, in T cell development by enhancing the TCR-signaling response in thymocytes. We found that another newly identified protein, Fbx12, is important for regulating proliferation during early T cell development. Another area of investigation focuses on hematopoietic stem cells (HSCs) and early stages of T cell, B cell, and erythrocyte development. We initiated characterization of a protein (Ldb1) that is important for the generation and maintenance/self-renewal of HSCs, which revealed a critical function for Ldb1 as a key subunit of multimeric DNA-binding complexes in controlling the self-renewal/differentiation cell-fate decision in HSCs. Current work, which involves genome-wide screening by RNA-Seq and CHIP-Seq, seeks to extend our knowledge of the role of Ldb1 complexes in regulating gene transcription and to explore the function of such complexes in other hematopoietic lineages. For example, we have begun to investigate the importance of Ldb1 complexes for regulating self-renewal in immature thymocytes and in the induction of T cell acute lymphoblastic leukemia (T-ALL). Our results suggest that Ldb1 complexes represent potential therapeutic targets for the treatment of an aggressive form of childhood leukemia called early T progenitor T cell acute lymphoblastic leukemia (ETP-T-ALL).



Paul E. Love, MD, PhD, Head,
Section on Hematopoiesis and Lymphocyte Biology

LiQi Li, MD, PhD, *Staff Scientist*

Guillaume Gaud, PhD, *Research Fellow*

Avik Dutta, PhD, *Visiting Fellow*

Teri Hatzihristidis, PhD, *Visiting Fellow*

Dalal El-Khoury, BS, *Technician*

Jan Lee, BS, *Technician*

Seeyoung Choi, PhD, *Contract employee*

Layla Ahmadi, BS,
Postbaccalaureate Fellow

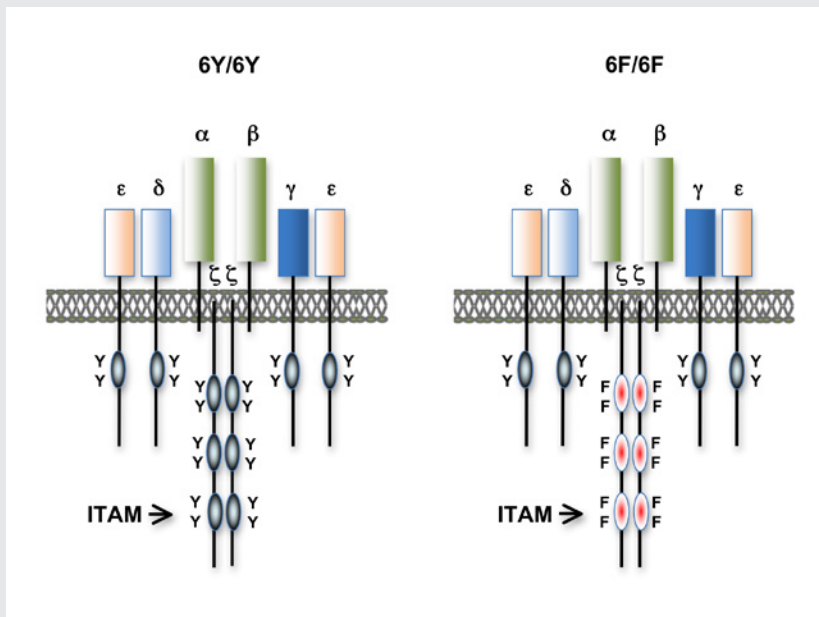


FIGURE 1. T cell antigen receptors expressed in 6Y/6Y and 6F/6F knock-in mice

Subunit composition of the T cell antigen receptors in 6Y/6Y and 6F/6F mice. 6Y/6Y mice express wild-type zeta chain dimers with functional ITAM signaling motifs that contain two tyrosine (Y) residues. 6F/6F mice express mutant zeta chain dimers in which the ITAM tyrosines have been changed to phenylalanine (F).

T cell antigen receptor (TCR) signaling in thymocyte development

Much of our research in the past has focused on the role of TCR signal transduction in thymocyte development. Signal transduction sequences, termed immuno-receptor tyrosine-based activation motifs (ITAMs), are contained within four distinct subunits of the multimeric TCR complex (CD3-zeta, CD3-gamma, CD3-delta, and CD3-epsilon). Di-tyrosine residues within ITAMs are phosphorylated upon TCR engagement; their function is to recruit signaling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell-activation cascade. Though conserved, ITAM sequences are non-identical, raising the possibility that the diverse developmental and functional responses controlled by the TCR may be partly regulated by distinct ITAMs through the recruitment of different effector molecules. We previously generated CD3-zeta-deficient and CD3-epsilon-deficient mice by gene targeting. We genetically reconstituted the mice with transgenes encoding wild-type or signaling-deficient (ITAM-mutant) forms of CD3-zeta and CD3-epsilon and characterized the developmental and functional consequences of the alterations for TCR signaling. We found that TCR-ITAMs are functionally equivalent but act in concert to amplify TCR signals and that TCR signal amplification is critical for thymocyte selection, the process by which potentially useful immature T cells are instructed to survive and differentiate further (positive selection) and by which potentially auto-reactive cells, which may cause autoimmune disease, are deleted in the thymus (negative selection).

We also found that a complete complement of TCR-ITAMs is not required for most mature T cell effector functions. However, recent work demonstrated a requirement for ITAM multiplicity for the generation of T follicular helper cells, which are necessary for optimal B cell antibody responses. One possible explanation for the relatively mild phenotype observed in the TCR ITAM-reduced mice is that ITAM-mediated signal amplification is not required for most mature T cell activation responses; another is that some ITAMs perform more subtle, regulatory functions. To investigate this question further, we recently generated a TCR-zeta chain conditional knock-in mouse in which T cell development and selection can occur without attenuation of TCR signaling (i.e., in the presence of a wild-type 3-ITAM '6Y' zeta chain), but in which mature, post-selection

T cells may be induced to express TCRs containing signaling-defective (0-ITAM '6F') zeta chains in lieu of wild-type zeta chains (Figure 1). Thus, mature T cell signaling should not be influenced by potential compensatory mechanisms that operate during T cell maturation, and T cells in such mice should be faithful indicators of the role of multiple TCR ITAMs in mediating specific, mature T cell responses. We confirmed that the knock-in zeta locus functions as predicted. We next evaluated the effect of late 'switching' from 6Y zeta to 6F zeta in mature T cells generated with wild-type 6Y zeta-containing TCRs and found that the phenotype was identical to germline inactivation of zeta ITAMs, demonstrating that compensation does not explain the mild phenotype of zeta 6F mice. Unexpectedly, we also discovered an inhibitory role for zeta ITAM signaling in response to weak (low-affinity) antigens. Strikingly, inactivation of the zeta ITAMs resulted in enhanced TCR signaling and enhanced T cell effector functions when the TCR is engaged by low-affinity ligands, but zeta ITAMs contributed positively to signaling by high-affinity ligands. This revealed a dual (activating and inhibitory) function for zeta ITAMs in TCR signaling depending on the affinity of the TCR–ligand interaction. Given that most tumor-specific antigens are low affinity and that this property limits current TCR-based approaches to tumor immunotherapy, we explored the effect of zeta ITAM inactivation on T cell–tumorcidal activity. Notably, we found that inactivation of zeta ITAMs markedly enhanced T cell–tumorcidal activity against low-affinity tumor antigens. Such experiments should provide information relevant to the design of engineered tumor antigen-specific TCRs and possibly chimeric antigen receptor T cells (CAR T cells), which are currently configured to express zeta ITAM–signaling module(s).

Identification and characterization of TCR–tuning proteins that may serve as targets for immunotherapy

We extended our analysis of TCR–signaling subunits to other molecules that participate in or influence the TCR–signaling response. The cell-surface protein CD5 negatively regulates TCR signaling and functions in thymocyte selection. Examination of CD5 expression during T cell development revealed that surface levels of CD5 are regulated by TCR–signal intensity and by the affinity of the TCR for self-peptide ligands in the thymus that mediate selection. To determine whether the ability to regulate CD5 expression is important for thymocyte selection, we generated transgenic mice that constitutively express high levels of CD5 throughout development. Over-expression of CD5 significantly impaired positive selection of some thymocytes (those that would normally express low levels of CD5) but not of others (those that would normally express high levels of CD5). The findings support a role for CD5 in modulating TCR signal transduction, thereby influencing the outcome of thymocyte selection. Current studies center on identifying the mechanism by which CD5 inhibits TCR signaling and on determining whether the protein's regulated expression during development is important for preventing autoimmunity. For that purpose, we generated a conditional CD5–deletion mouse in which CD5 expression can be removed before, during, or after T cell development. The ability of individual thymocytes to regulate CD5 expression represents a mechanism for 'fine tuning' the TCR–signaling response during development so that the integrated signaling response can be adjusted to permit T cell functional competency without causing autoimmunity. Reasoning that, in addition to CD5, other molecules participate in TCR tuning, we initiated microarray-based screening for genes differentially expressed in developing T cells under conditions of high- or low-affinity TCR interactions. We identified several genes from this screen for further study, including CD6, a surface receptor that is structurally similar to CD5, and we are validating their function as tuning molecules. Given that the molecules regulate TCR signaling, they represent potential autoimmune-disease susceptibility markers and potential targets for treatment of patients with cancer or autoimmune disease, similar to current 'checkpoint inhibitor' therapies that are based on blocking the function of the induced inhibitory molecules PD-1 and CTLA-4. Experiments are under way to investigate this translational potential.

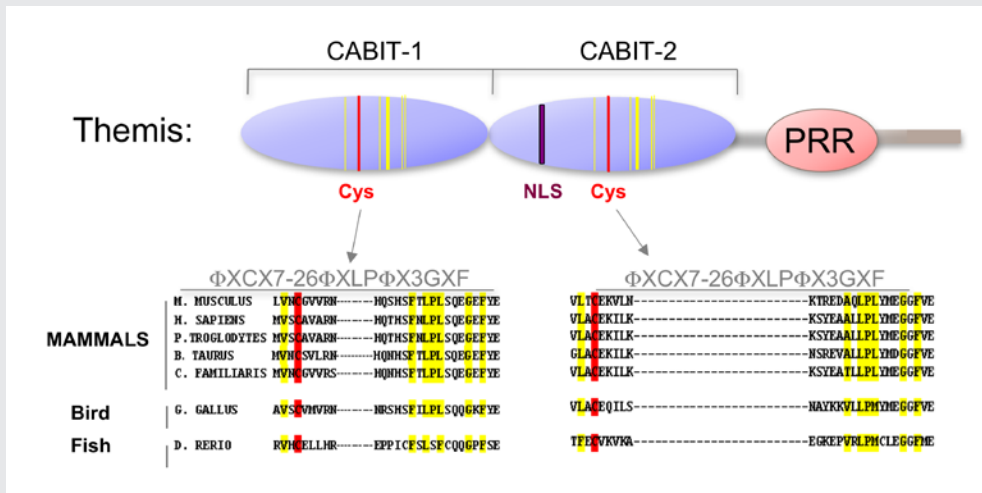


FIGURE 2. Themis is highly conserved in vertebrates.

Themis contains two novel CABIT domains, each with a conserved cysteine (red) and conserved flanking residues (yellow), a nuclear localization signal (NLS), and a proline-rich region (PRR).

Identification and characterization of Themis, a novel protein required for T cell development

Using a subtractive cDNA library–screening approach, we identified Themis, now known as Themis1, a novel T cell–specific adapter protein (Figure 2). To investigate the function of Themis1 in T cell signaling and development, we generated Themis1 knock-down cell lines, Themis1 knock-out mice (conventional and conditional), and Themis1 transgenic mice. Analysis of the effects of modulating Themis1 expression revealed a critical role for the protein in late T cell development. In a collaboration with Richard Cornall, we also investigated the phenotype of Themis2 knockout mice generated in our lab. Our results identified an important role for Themis2 in facilitating B cell activation by low-avidity, but not high-avidity, B cell receptor (BCR)–antigen interactions.

In the past few years, we focused on determining the molecular function of Themis1. Themis1, Themis2, and a large family of related metazoan proteins contain a novel globular domain of unknown function called the CABIT (cysteine-containing, all beta in Themis) module (Figure 2). Using cell transfection and biochemical and protein-assay techniques, we determined that CABIT modules bind to the catalytic domain of SHP-1, a key hematopoietic protein tyrosine phosphatase. In the presence of reactive oxygen species (ROS), which are generated in activated T cells, Themis1, via its CABIT module, promoted oxidation of the SHP-1 catalytic cysteine and therefore inactivated SHP-1. The CABIT modules from all five mammalian Themis-family proteins also inhibited SHP-1, indicating that this activity was common to the CABIT module. Given that SHP-1 is an inhibitory phosphatase that functions to dampen TCR signaling by de-phosphorylating several targets, including protein tyrosine kinases, the finding established an activating function for Themis1 in cell signaling through its ability to bind to and inhibit SHP-1. Interestingly, Themis1 is highly expressed in developing thymocytes at the stage at which they undergo positive selection. It had been known for years that thymocytes are more sensitive to TCR stimulation than are mature T cells, but the reason for this sensitivity was unknown. The function of Themis1, together with its high expression in thymocytes, provides an explanation for the sensitivity of thymocytes to TCR signaling. By showing that deletion of the gene encoding SHP-1 rescues T cell development in *Themis1*^{-/-} mice, we confirmed that the primary role of Themis1 is to inhibit SHP-1. In addition to identifying the function of CABIT modules, our results provide insight into the role of

other metazoan CABIT-containing proteins (which number in the hundreds). Our ongoing studies are focusing on further characterization of CABIT proteins, including the intestine-specific mouse protein Themis3, and determining their role in development and their possible involvement in human disease.

Role of the F-box protein Fbx12 in thymocyte development

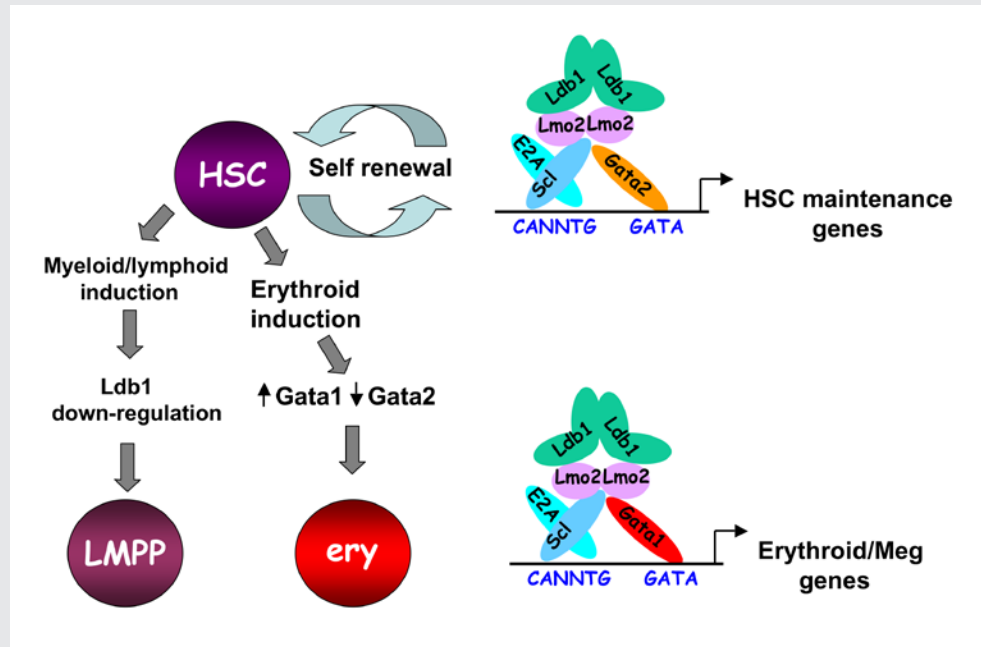
A major aspect of the T cell maturation process is the precise regulation of cell proliferation. Rather than being a shared property among all or most developing thymocytes, proliferation is strictly limited to two stages during early development. The initial proliferative phase is driven by thymus-expressed cytokines, the second coincides with 'beta selection' (i.e., is initiated in cells that have productively rearranged the TCRbeta chain and express a signaling complex called the pre-TCR). The proliferative burst that accompanies beta selection is estimated to result in a 100–200 fold expansion and is essential for further differentiation and for maximizing TCR diversity. Previous work showed that beta selection-associated proliferation requires concurrent signals by the pre-TCR and Notch receptors, but how these signals induce cell-cycle progression and why they need to be coordinated has remained unclear. Initiation of proliferation in beta-selected thymocytes requires the ubiquitin-mediated degradation of the cyclin-dependent kinase inhibitor Cdkn1b, which acts to prevent cell-cycle progression. In a recent study, we examined the molecular control of beta selection-associated proliferation. We confirmed prior findings that Cdkn1b degradation is induced by an SCF E3 ubiquitin ligase that contains the ligand-recognition subunit Fbx1. Deletion of Fbx1 partially blocked beta selection-associated proliferation, a defect that was rescued by co-deletion of Cdkn1b. We identified a new F-box protein, Fbx12, that is highly expressed in thymocytes. We found that Fbx12 also functions as an SCF E3 ligase subunit, which, like Fbx1, directs Cdkn1b degradation. The phenotype of Fbx12-deficient mice generated in our lab was strikingly similar to that of Fbx1-deficient mice, and deletion of both Fbx1 and Fbx12 resulted in a severe block in beta selection-associated proliferation, indicating that Fbx1 and Fbx12 act in concert to regulate thymocyte proliferation. Interestingly, we found that Fbx1 expression is induced by Notch signaling, whereas Fbx12 expression is induced by pre-TCR signaling. Both Fbx1 and Fbx12 are required for thymocyte proliferation; thus, their selective regulation by Notch and the pre-TCR, respectively, provides an explanation for why concurrent Notch and pre-TCR signaling are necessary for cell-cycle progression and proliferation at the beta-selection checkpoint.

Role of Ldb1 transcription complexes in hematopoiesis and in T cell acute lymphoblastic leukemia (T-ALL)

Lim domain binding protein-1 (Ldb1) is a ubiquitously expressed nuclear protein that contains a LIM-zinc finger protein-interaction motif and a dimerization domain. In hematopoietic cells, Ldb1 functions by interacting with and/or recruiting specific partners (including the LIM-only protein Lmo2 and the transcription factors Lyl1 or Tal1 and Gata1 or Gata2) to form multi-molecular transcription complexes (Figure 3). Within the hematopoietic lineage, expression of *Ldb1* is highest in progenitor cells, which include hematopoietic stem cells (HSCs). We initially investigated the role of Ldb1 in hematopoiesis by following the fate of *Ldb1*^{-/-} embryonic stem cells (ESCs) in mouse blastocyst chimeras and by conditional, stage-specific deletion of *Ldb1* in HSCs. We found that Ldb1 is not required for ESC maintenance but is required for HSC maintenance. More recent data indicate that the loss of *Ldb1*^{-/-} HSCs results from differentiation rather than cell death. We performed a genome-wide screen for Ldb1-binding sites using ChIP-Seq. Analysis of the ChIP-Seq data revealed that Ldb1 complexes bind at the promoter or at regulatory sequences near a large number of genes known to be required for HSC maintenance. Examination of the function of Ldb1 in cell lineages downstream of HSCs identified an essential function in the erythroid lineage but not in myeloid cells or lymphoid cells.

FIGURE 3. Model of Ldb1 function in the hematopoietic lineage

Ldb1 forms a multimeric DNA-binding complex in hematopoietic cells with the adapter Lmo2 and the transcription factors Scl and Gata1 or Gata2. In hematopoietic stem cells (HSCs), in which Gata2 is highly expressed, Ldb1-Lmo2-Scl-Gata2 complexes positively regulate expression of HSC maintenance genes. Differentiation of HSCs to the myeloid or lymphoid lineage (LMPP) is triggered by downregulation of Ldb1 complexes, whereas commitment to the erythroid lineage (ery) is triggered by induction of Gata1 and downregulation of Gata2, resulting in the formation of an Ldb1-Lmo2-Scl-Gata1 complex, which positively regulates expression of erythroid-specific genes.



Interestingly, ChIP-Seq analysis of Ldb1 DNA-binding complexes demonstrated that Ldb1 complexes in HSCs contain the transcription factor Gata2, whereas Ldb1 complexes in erythroid progenitors contain Gata1 (which is highly expressed in the erythroid lineage). The results indicate that multimeric Ldb1 transcription complexes have distinct functions in the hematopoietic system depending on their subunit composition, with Gata2-containing complexes regulating expression of HSC-maintenance genes and Gata1 complexes regulating expression of erythroid-specific genes (Figure 3). Current studies aim to determine how Ldb1 complexes regulate gene expression and the role of Ldb1 dimerization in mediating long-range promoter-enhancer interactions in hematopoietic cells. In addition, we are investigating a potential role for Ldb1 in regulating self-renewal of T cell progenitors in the thymus.

Acute lymphoblastic leukemias are the most common type of cancer in children. T cell acute lymphoblastic leukemia (T-ALL) results from oncogenic transformation of immature T cell progenitors (thymocytes). Mouse models of T-ALL have been generated, and one of the most informative is the Lmo2-transgenic (*Lmo2*-tg) mouse, which expresses high levels of the nuclear adapter Lmo2 in thymocytes. The model closely mimics a prevalent type of human T-ALL, which is associated with chromosomal mutations that result in increased expression of LMO2. We recently reported that overexpression of Lmo2 in mouse thymocytes induces T-ALL at two distinct stages of development (an early 'ETP' stage and a later 'DN3' stage). Notably, human T-ALLs can also occur at two similar stages of thymocyte maturation. The most immature forms of T-ALL in *Lmo2*-tg mice and in humans express high levels of the transcription factor Hhex and are designated early T progenitor (ETP) T-ALL, whereas later-stage tumors are low in Hhex but express high levels of more mature markers of T cell development,

including Notch1, Dtx1, Ptcra, and Hes1. Lmo2 functions as a subunit of the multimeric Ldb1–nucleated DNA–binding complexes described above. We found that normal ETP thymocyte progenitor cells express the same Ldb1 complex subunits as are present in HSCs and that ETPs exhibit HSC characteristics, including the self-renewal potential. ETPs in *Lmo2*-tg mice appear to be ‘locked’ into a pattern of perpetual self-renewal and are refractory to normal inductive signals that promote further differentiation. *Hhex* is a target of Ldb1 complexes in HSCs and ETPs, a result that strongly suggests that Ldb1 complexes are responsible for the aberrant self-renewal in *Lmo2*-tg mice that predisposes to oncogenesis. We hypothesized that Ldb1 complexes regulate self-renewal in ETPs as well as in HSCs. *Lmo2* is normally down-regulated when thymocytes undergo T lineage commitment, suggesting that extinguishing expression of *Lmo2* (and by extension, Ldb1 complexes) is important for T cell differentiation and that failure to do so predisposes to oncogenesis via ‘second-hit’ transforming events.

In RNA-Seq gene expression experiments, we found that the RNA–expression signatures of *Lmo2*-tg immature thymocytes and HSCs are very similar, consistent with the notion that *Lmo2* overexpression ‘freezes’ cells in a stem-cell self-renewal state. To determine whether Ldb1 complexes are in fact required for ETP self-renewal and to explore the genes regulated by these complexes, we conditionally deleted *Ldb1* in *Lmo2*-tg mice. We found that Ldb1 is required for *Lmo2*-tg–induced thymocyte self-renewal and T-ALL induction, indicating that *Lmo2* overexpression promotes T-ALL by functioning as a subunit of Ldb1 complexes. Currently, we are addressing several key questions, including whether, as predicted, Ldb1, and by extension Ldb1 complexes, regulate expression of genes that control a self-renewal genetic program in ETPs and whether Ldb1 complexes are necessary for the transcriptional/developmental effects of *Lmo2*. We are also determining the subunit structure and binding sites of Ldb1 complexes expressed in *Lmo2*-tg thymocytes. We anticipate that our results will provide insights into the mechanisms controlling T-ALL oncogenesis in humans and may thus provide new therapeutic avenues for the treatment of this devastating pediatric disease.

Additional Funding

- NICHD Director's Award
- NIH IRF Award to Avik Dutta

Publications

1. Dutta A, Zhao B, Love PE. New insights into beta-selection. *Trends Immunol* 2021 42:735–750.
2. Stamos DB, Clubb LM, Mitra A, Chopp L, Nie J, Ding Y, Das A, Venkataganesh H, Lee J, El-Khoury D, Li L, Bhandoola A, Bosselut R, Love PE. The histone demethylase Lsd1 regulates multiple repressive gene programs during T cell development. *J Exp Med* 2021 218(12):e20202012.
3. Yang C, Blaize G, Marrocco R, Rouquié N, Bories C, Gador M, Mélique S, Joulia E, Benamar M, Dejean AS, Daniels-Treffandier H, Love PE, Fazilleau N, Saoudi A, Lesourne R. THEMIS enhances the magnitude of normal and neuroinflammatory type 1 immune responses by promoting TCR-independent signals. *Sci Signal* 2022 15(742):eab15343.
4. Schuster C, Kiaf B, Hatzihristidis T, Ruckdeschel A, Nieves-Bonilla J, Ishikawa Y, Zhao B, Zheng P, Love PE, Kissler S. CD5 controls gut immunity by shaping the cytokine profile of intestinal T cells. *Front Immunol* 2022 13:906499.
5. Gao Y, Zamisch M, Vacchio M, Chopp L, Ciucci T, Paine EL, Lyons GC, Nie J, Xiao Q, Zvezdova E, Love PE,

- Vinson CR, Jenkins LM, Bosselut R. NuRD complex recruitment to Thpok mediates CD4+ T cell lineage differentiation. *Sci Immunol* 2022 7(72):eabn5917.
6. Zhang M, Lin X, Yang Z, Li X, Zhou Z, Love PE, Huang J, Zhao B. Metabolic regulation of T cell development. *Front Immunol* 2022 13:946119.

Collaborators

- Gregoire Altan-Bonnet, PhD, *Center for Cancer Research, NCI, Bethesda, MD*
- Cheryl Arrowsmith, PhD, *University of Toronto, Toronto, Canada*
- Avinash Bhandoola, MB, PhD, *Laboratory of Genome Integrity, Center for Cancer Research, NCI, Bethesda, MD*
- Remy Bosselut, MD, PhD, *Laboratory of Immune Cell Biology, NCI, Bethesda, MD*
- Utpal P. Davé, MD, *Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN*
- Richard J. Cornall, FRCP, FMedSci, *Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom*
- Lauren Ehrlich, PhD, *The University of Texas at Austin, Austin, TX*
- James Gulley, MD, PhD, *Center for Cancer Research, NCI, Bethesda, MD*
- Christian Hinrichs, MD, *Rutgers Cancer Institute, New Brunswick, NJ*
- Aravind Iyer, PhD, *Protein and Genome Evolution Research Group, NLM/NCBI, Bethesda, MD*
- Park Jung-Hyun, PhD, *Experimental Immunology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Stephan Kissler, PhD, *Joslin Diabetes Center, Harvard Medical School, Boston, MA*
- Renaud Lesourne, PhD, *INSERM, Toulouse, France*
- Dorian McGavern, PhD, *Viral Immunology and Intravital Imaging Section, NINDS, Bethesda, MD*
- Keiko Ozato, PhD, *Section on Molecular Genetics of Immunity, NICHD, Bethesda, MD*
- Rita Perlingeiro, PhD, *University of Minnesota Medical School, Minneapolis, MN*
- Karl Pfeifer, PhD, *Section on Epigenetics, NICHD, Bethesda, MD*
- Alfred Singer, MD, *Experimental Immunology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Nevil Singh, PhD, *University of Maryland School of Medicine, Baltimore, MD*
- Naomi Taylor, MD, PhD, *Pediatric Oncology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Juan Carlos Zúñiga-Pflücker, PhD, *University of Toronto, Toronto, Canada*

Contact

For more information, email lovep@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/love>.

The Arms Race between Transposable Elements and KRAB-ZFPs and its Impact on Mammals

The central mission of the NICHD is to ensure that every human is born healthy. Despite much progress in understanding the many ways the mother interacts with the fetus during development, we still know little about the molecular changes that promoted the emergence of placental mammals from our egg-laying relatives over 100 million years ago, nor about those mechanisms that continue to drive phenotypic differences amongst mammals. One attractive hypothesis is that retroviruses and their endogenization into the genomes of our ancestors played an important role in eutherian evolution, by providing protein-coding genes such as syncytins (derived from retroviral *env* genes that cause cell fusions in placental trophoblasts) and novel gene-regulatory sequences that contributed to mammalian-specific traits, including the evolution of the placenta. Our primary interest is to explore the impact of such endogenous retroviruses (ERVs), which account for about 10% of our genomic DNA, on embryonic development and on the evolution of new traits in mammals. This has led us to examine the rapidly evolving Kruppel-associated box zinc-finger protein (KZFP) family, the single largest family of transcription factors (TFs) in most, if not all, mammalian genomes. Our hypothesis is that KZFP gene expansion and diversification was driven primarily by the constant onslaught of ERVs and other transposable elements (TEs) on the genomes of our ancestors, as a means to transcriptionally repress them. The hypothesis is supported by recent evidence demonstrating that the majority of KZFPs bind to TEs and that TEs and nearby genes are activated in KZFP-knockout mice. We will continue to explore the impacts of the TE/KZFP “arms race” on the evolution of mammals. We will also begin a new phase of our research to explore whether KZFPs play broader roles in genome regulation, beyond gene silencing, and how such functions impact mammalian development and evolution.

Kruppel-associated box zinc-finger proteins (KRAB-ZFPs)

Kruppel-associated box zinc-finger (ZF) proteins (KRAB-ZFPs) are rapidly evolving transcriptional repressors that emerged in a common ancestor of coelacanths, birds, and tetrapods, and they constitute the largest family of transcription factors in mammals (estimated to be several hundred in mice and humans). Each species has its own



Todd S. Macfarlan, PhD,
*Head, Section on Mammalian
Development and Evolution*

Sherry Ralls, BA, *Biologist*

Melania Bruno, PhD, *Visiting
Fellow*

Jinpu Jin, PhD, *Visiting Fellow*

Mohamed Mahgoub, PhD, *Visiting
Fellow*

Anna Dorothea Senft, PhD,
Visiting Fellow

Rachel Cosby, PhD, *Postdoctoral
Intramural Research Training
Award Fellow*

Marja Brolinson, *Clinical Fellow*

Karrie Walker, MD, *Clinical Fellow*

Grace Whiteley, *Clinical Fellow*

Mary Elmore DeMott, BS,
Postbaccalaureate Fellow

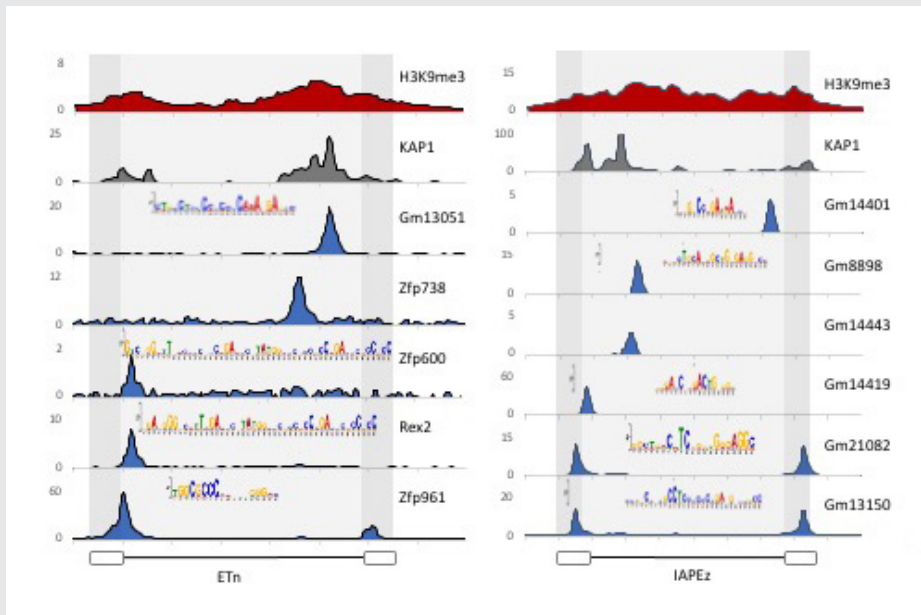


FIGURE 1. KRAB-ZFPs bind redundantly to the active retrotransposons ETn and IAP.

ChIP-Seq track for indicated KRAB-ZFPs displayed across a consensus sequence of ETn and IAP retrotransposons. ChIP-Seq was performed by expressing epitope-tagged KRAB-ZFPs in F9 ECs or ESCs and ChIP'ing with antibodies that recognize the epitope.

unique repertoire of KRAB-ZFPs, with some shared by closely related species and others specific to each species. Remarkably, there was an explosion of KRAB-ZFP genes in the earliest mammals, many of which have been retained by purifying selection, but the function of these (as well as the hundreds of species-restricted KRAB-ZFPs) have been largely unexplored. KRAB-ZFPs consist of an N-terminal KRAB domain that binds to the co-repressor KAP1 and a variable number of C-terminal C2H2 ZF domains that mediate sequence-specific DNA binding. KAP1 directly interacts with the KRAB domain, which recruits the histone methyltransferase (HMT) SETDB1 and heterochromatin protein 1 (HP1) to initiate heterochromatic silencing. Several lines of evidence point to a role for the KRAB-ZFP family in ERV silencing. First, the number of C2H2 ZF genes in mammals correlates with the number of ERVs. Second, the KRAB-ZFP protein ZFP809 was isolated based on its ability to bind to the primer-binding site for proline tRNA (PBSpro) of murine leukemia virus (MuLV). Third, deletion of the KRAB-ZFP co-repressors *Trim28* or *Setdb1* leads to activation of many ERVs. We therefore began a systematic interrogation of KRAB-ZFP function as a potential adaptive repression system against ERVs.

We began a systematic analysis of KRAB-ZFPs using a medium-throughput ChIP-Seq screen and functional genomics of KRAB-ZFP clusters and individual KRAB-ZFP genes. Our ChIP-Seq data demonstrate that the majority of recently evolved KRAB-ZFP genes interact with and repress distinct and partially overlapping ERVs and other retrotransposon targets. The hypothesis is strongly supported by the distinct ERV reactivation phenotypes we observed in mouse ESC lines lacking one of five of the largest KRAB-ZFP gene clusters. Furthermore, KRAB-ZFP cluster knockout (KO) mice are viable, but have elevated rates of somatic retrotransposition of specific retrotransposon families, providing the first direct genetic link between KRAB-ZFP gene diversification and retrotransposon mobility.

KRAB zinc-finger protein ZFP809

We initially focused on ZFP809 as a likely ERV-suppressing KRAB-ZFP, given that it was originally identified as

part of a repression complex that recognizes infectious MuLV (murine leukemia virus) by binding directly to the 18 nt primer binding site for proline (PBSpro) sequence. We hypothesized that ZFP809 functions *in vivo* to repress other ERVs that utilize the PBSpro. Using CHIP-Seq of epitope-tagged ZFP809 in embryonic stem cells (ESCs) and embryonic carcinoma (EC) cells, we determined that ZFP809 binds to several sub-classes of ERV elements via the PBSpro. We generated *Zfp809* knockout mice to determine whether ZFP809 was required for silencing the ERV element VL30pro. We found that *Zfp809* knockout tissues displayed high levels of VL30pro elements and that the targeted elements display an epigenetic shift from repressive epigenetic marks (H3K9me3 and CpG methylation) to active marks (H3K9Ac and CpG hypo-methylation). ZFP809-mediated repression extended to a handful of genes that contained adjacent VL30pro integrations. Furthermore, using a combination of conditional alleles and rescue experiments, we determined that ZFP809 activity was required in development to initiate silencing, but not in somatic cells to maintain silencing. The studies provided the first demonstration of the *in vivo* requirement of a KRAB-ZFP in the recognition and silencing of ERVs.

KRAB zinc-finger proteins ZFP568, ZFP110, and ZFP661

Although our data show that many KRAB-ZFPs repress ERVs, we also found that more ancient KRAB-ZFPs, which emerged in a human/mouse common ancestor, do not bind to or repress ERVs. One such KRAB-ZFP, ZFP568, plays an important role in silencing a key developmental gene that may have played a critical role in the onset of viviparity in mammals. Using CHIP-Seq and biochemical assays, we determined that ZFP568 is a direct repressor of a placental-specific isoform of the *Igf2* gene called *Igf2-P0*. Insulin-like growth factor 2 (*Igf2*) is the major fetal growth hormone in mammals. We demonstrated that loss of *Zfp568*, which causes gastrulation failure, or mutation of the ZFP568 binding site at the *Igf2-P0* promoter, cause inappropriate *Igf2-P0* activation. We also showed that the lethality could be rescued by deletion of *Igf2*. The data highlight the exquisite selectivity by which members of the KRAB-ZFP family repress their targets, and they identify an additional layer of transcriptional control of a key growth factor that regulates fetal and placental development. In a follow-up to these studies, we determined that ZFP568 is highly conserved and under purifying selection in eutheria with the exception of human. Human ZNF568 allele variants have lost the ability to bind to and repress *Igf2-P0*, which may have been driven by the loss of the *Igf2-p0* transcript in human placenta. We solved the crystal structure of mouse ZFP568 ZFs bound to the *Igf2-P0* binding site, which reveals several non-canonical ZF-DNA contacts, highlighting the ability of individual ZFs to change conformation depending upon ZF context and DNA structure. The structures also explain how mutations in human ZNF568 alleles disrupt *Igf2-P0* interactions, which contain either deleted ZFs or mutations of key ZF-DNA contact residues. Taken together, our studies provide important insights into the evolutionary and structural dynamics of ZF-DNA interactions, which play a key role in regulating mammalian development and evolution.

In the past year, we continued our exploration of two conserved KRAB-ZFPs with important functions in mammals: ZFP110, which binds very specifically to a motif contained within the mmSAT4 repeat that encodes the 3' exon of zinc finger genes; and ZFP661, which antagonistically binds at loop boundaries of the clustered protocadherin gene loci, adjacent to the site of CTCF transcription-factor binding. We showed that *Zfp110* is essential for regulating KZFP genes during embryonic development, whereas ZFP661 plays an important role in balancing the expression of clustered protocadherin genes in the cortex.

KRAB zinc-finger protein PRDM9

We also began a new exploration of the function of PRDM9, the most ancient KRAB-ZFP, which emerged in jawless fish and plays a highly specialized role in meiotic recombination (MR). MR generates genetic diversity

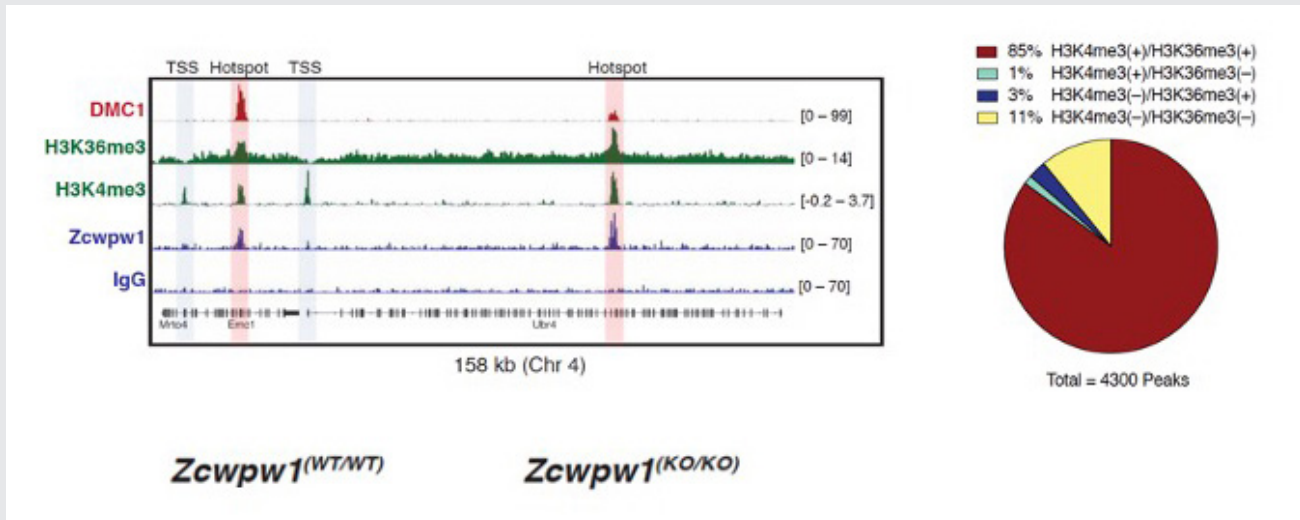


FIGURE 2. ZCWPW1 binds to meiotic recombination hotspots in spermatocytes harboring dual H3K4me3 and H3K36me3 marks.

ChIP-Seq or Cut & Run was performed with indicated antibodies in mouse spermatocytes; read pileup is displayed across a region on Chr 4. The pie chart at the right displays percentage of ZCWPW1 peaks that overlap with peaks of either H3K4me3, H3K36me3, or both marks.

in sexually reproducing organisms and ensures proper synapsis and segregation of homologous chromosomes in gametes. Errors in MR that lead to mis-segregation of chromosomes are a leading cause of miscarriage and childhood disease. MR is initiated by programmed double-strand breaks (DSBs) in DNA that are distributed non-randomly at thousands of specific 1–2 kb regions called hotspots. In most mammals, hotspots are defined by PRDM9, a protein that contains a rapidly evolving DNA-binding ZF array and a specialized HMT activity that catalyzes dual trimethylation marks on histone H3 at lysine 4 and 36 (H3K4me3 and H3K36me3), both of whose activities are required for hotspot specification. *Prdm9* loss-of-function causes sterility in mice, and PRDM9 mutations have been associated with male infertility in humans. In species lacking *Prdm9*, including yeast, plants, and birds, hotspots are located in H3K4me3-rich regions at gene promoters. Thus, the emergence of PRDM9 during evolution reshaped the MR landscape by relocating DSBs away from promoters to chromatin sites bound by the rapidly evolving PRDM9, which allowed for rapid interspecies hotspot diversification.

Histone readers ZCWPW1 and ZCWPW2

We set out to address whether other factors, in addition to PRDM9, are required to ‘re-engineer’ hotspot selection and how the DNA break and repair machinery is recruited to sites marked by PRDM9. We identified the dual histone methylation readers *Zcwpw1*, which co-evolved with and is tightly co-expressed with *Prdm9*. Using a mouse model, we found that ZCWPW1 is an essential meiotic recombination factor required for efficient repair of PRDM9-dependent DSBs and pairing homologous chromosomes in male mice. However, ZCWPW1 is not required for the initiation of DSBs at PRDM9 binding sites. Our results indicate that the evolution of a dual histone methylation writer (PRDM9) and reader (ZCWPW1) system in vertebrates

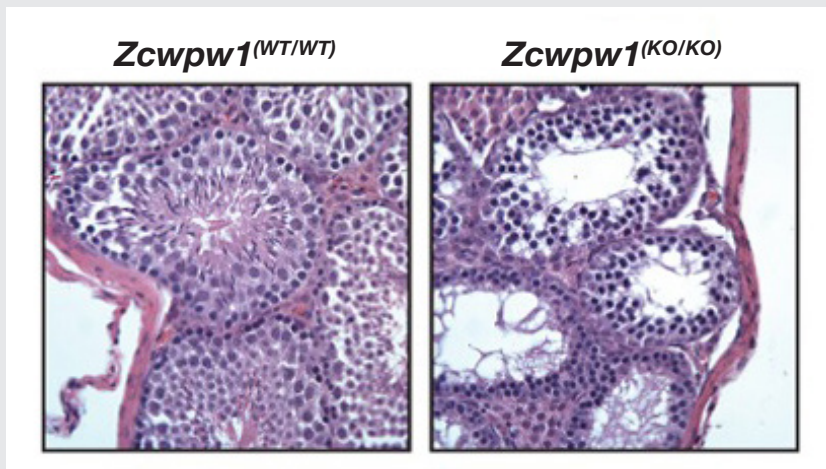


FIGURE 3. *Zcwpw1* KO mice are azoospermic.

H&E staining of adult testes from wild-type (WT) or *Zcwpw1* knockout (KO) mice. Sperm are completely lacking in *Zcwpw1* mutants as the result of defective meiotic double-strand-break repair.

remodeled genetic recombination hotspot selection from an ancestral static pattern near genes towards a flexible pattern controlled by the rapidly evolving DNA-binding activity of PRDM9. Since publishing these findings, we identified a *Zcwpw1* paralog, which was initially mis-annotated in the mouse genome, called *Zcwpw2*. Importantly, in the past year we found that *Zcwpw2* is essential for both mouse meiosis and fertility in males and females, and that it is important for the efficient generation of double-strand breaks at hotspots relative to promoters. The studies have thus revealed a three-component system, comprising a rapidly evolving DNA-binding histone methyltransferase (PRDM9), and two dual histone methylation readers (ZCWPW2 and ZCWPW1), which play at least partially separable roles in mediating the PRDM9-dependent generation of DNA DSBs and their repair at meiotic recombination hotspots.

Additional Funding

- NIGMS PRAT (Rachel Cosby)
- NICHD Individual Research Fellowship (Anna Senft)
- NICHD Fellows Recruitment Incentive Award (Mohamed Mahgoub)
- NICHD Young Investigator Award (Rachel Cosby)
- NICHD Young Investigator Award (Melania Bruno)
- NICHD Young Investigator Award (Mohamed Mahgoub)

Publications

1. Senft AD, Macfarlan TS. Transposable elements shape the evolution of mammalian development. *Nat Rev Genet* 2021 22(11):691–711.
2. Sun MA, Wolf G, Wang Y, Senft AD, Ralls S, Jin J, Dunn-Fletcher CE, Muglia LJ, Macfarlan TS. Endogenous retroviruses drive lineage-specific regulatory evolution across primate and rodent placentae. *Mol Biol Evol* 2021 38(11):4992–5004.
3. Bertozzi TM, Elmer JL, Macfarlan TS, Ferguson-Smith AC. KRAB zinc finger protein diversification drives

mammalian interindividual methylation variability. *Proc Natl Acad Sci U S A* 2021 117:31290–31300.

4. Agarwal S, Bonefas KM, Garay PM, Brookes E, Murata-Nakamura Y, Porter RS, Macfarlan TS, Ren B, Iwase S. KDM1A maintains genome-wide homeostasis of transcriptional enhancers. *Genome Res* 2021 31(2):186–197.

Collaborators

- Anne Ferguson-Smith, FRS, FMedSci, *University of Cambridge, Cambridge, United Kingdom*

Contact

For more information, email todd.macfarlan@nih.gov or visit <https://macfarlan.nichd.nih.gov>.

Virulence Mechanisms of Microbial Pathogens

Our main research goal is to define mechanisms by which pathogenic bacteria subvert the human host defense and cause disease. In parallel, we investigate whether these mechanisms may be manipulated for preventative and/or therapeutic purposes. As a model organism, we use the bacterium *Legionella pneumophila*, the causative agent of a potentially fatal respiratory infection known as Legionnaires' disease. According to the CDC (Centers for Disease Control), the number of Legionnaires' disease cases in the U.S. has risen more than four-fold over the past 15 years, making *L. pneumophila* an emerging pathogen of increasing relevance. Contrary to what its name may imply, Legionnaires' disease occurs in individuals of all ages, including children who receive respiratory therapy, newborns who had recently undergone surgery or under-water birth, and children who are immune-compromised. We are committed to an in-depth analysis of the mechanisms that allow *L. pneumophila* to exploit the human host and cause disease. Insights gained from our studies will ultimately improve our ability to diagnose, prevent, and fight Legionnaires' disease and related illnesses, thereby contributing to the success of NICHD's mission.

Within freshwater environments, *L. pneumophila* exists as an intracellular parasite of single-cell organisms known as amoeba. Upon inhalation of contaminated water droplets, *L. pneumophila* enters the lung and is phagocytosed (taken up) by specialized immune cells known as alveolar macrophages (Figure 1). Instead of being degraded by these cells, the pathogen establishes a protective membrane compartment, the *Legionella*-containing vacuole (LCV). Within this intravacuolar niche, *L. pneumophila* can replicate to high numbers before killing the host cell and infecting neighboring cells.

The virulence of *L. pneumophila* relies on the activity of close to 300 proteins, or effectors, that are delivered into the host cytosol by a specialized translocation apparatus called the Dot/Icm type IV secretion system (T4SS) (Figure 1). *L. pneumophila* mutants with a non-functional T4SS are degraded by macrophages, underscoring the importance of the translocated effectors for host-cell manipulation and bacterial virulence.

Our main objective is to obtain detailed mechanistic insight into *L. pneumophila* effectors by investigating their biological role at molecular, cellular, and structural levels. Knowledge obtained from



Matthias Machner, PhD,
*Chief, Section on Microbial
Pathogenesis*

Devanand Bondage, PhD, *Visiting
Fellow*

Katherine Bonnington, PhD,
Visiting Fellow

Candice Chang, PhD, *Visiting
Fellow*

Nicole Ellis, PhD, *Visiting Fellow*

Stephanie Lehman, PhD, *Visiting
Fellow*

Xiao Li, PhD, *Visiting Fellow*

Sabrina Bouchard, BS,
Postbaccalaureate Student

Jacob Olondo-Kuba, BS,
Postbaccalaureate Student

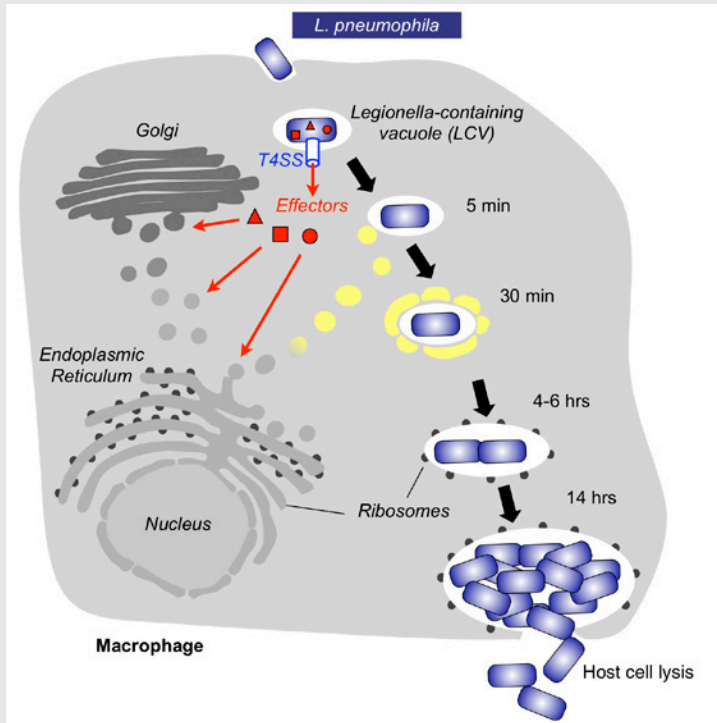


FIGURE 1. Intracellular replication cycle of *Legionella pneumophila*

Upon uptake by a macrophage, *L. pneumophila* delivers a large number of effector proteins (red) through the Dot/Icm type IV secretion system (T4SS) into the host cytosol. The effectors manipulate signaling and trafficking pathways in order to establish conditions favorable for *L. pneumophila* growth. Eventually, the host cell is lysed, and *L. pneumophila* bacteria infect neighboring cells.

these studies can help in the development of novel therapeutics aimed at treating or preventing Legionnaires' disease and related illnesses.

Comprehensive phenotypic screening strategy to identify modulators of cargo translocation by the bacterial type IVB secretion system

Multi-drug-resistant pathogens are an emerging threat to human health. Because conventional antibiotics target not only the pathogen but also eradicate beneficial microbiota, they often cause additional clinical complications. Thus, there is an urgent need for the development of "smarter" therapeutics that selectively target pathogens without affecting beneficial commensals. The bacterial type IV secretion system (T4SS) is essential for the virulence of a variety of pathogens but dispensable for bacterial viability in general and can, however, be considered a pathogen's Achilles heel.

Like many other pathogens, *L. pneumophila* relies on a T4SS (called Dot/Icm) for colonization and proliferation within a wide range of host cells, including freshwater amoeba in the environment and alveolar macrophages during Legionnaires' pneumonia in humans. *L. pneumophila* mutants with a non-functional T4SS fail to control trafficking of their LCV and are quickly delivered to lysosomes for degradation, thus underscoring the importance of the Dot/Icm system for *Legionella* pathogenesis.

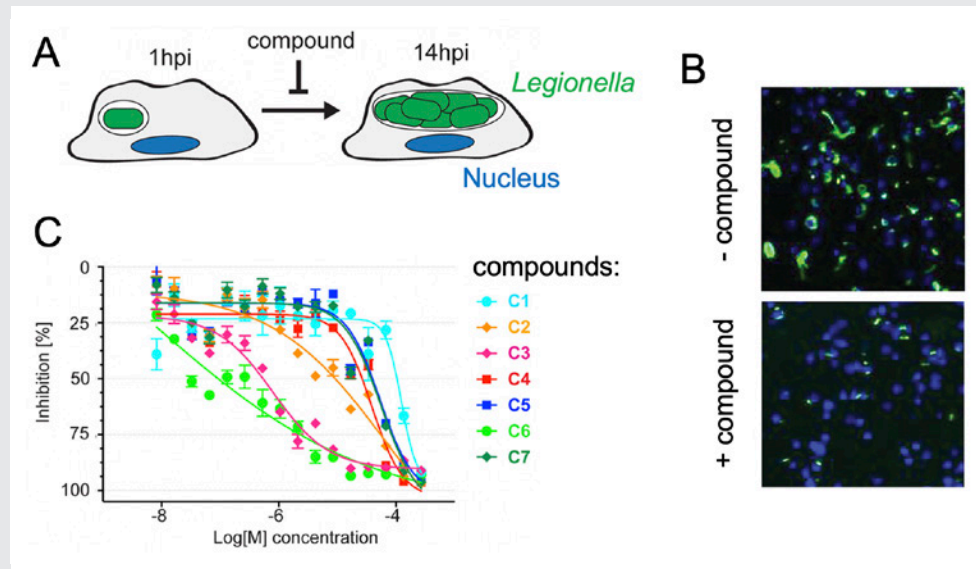
We designed an automated high-throughput screening approach for the identification of compounds that interfere with the delivery of a reporter-effector fusion protein from *L. pneumophila* into RAW264.7 mouse macrophages. Using a fluorescence resonance energy transfer (FRET)-based detection assay in a

FIGURE 2. Compounds that target the T4SS attenuate *L. pneumophila* intracellular growth

(A) Schematic overview of the intracellular growth assay with bacterial in green and host cell nuclei in blue.

(B) Growth of *Legionella* in macrophages is attenuated in the presence of hit compounds but not in untreated control cells.

(C) The inhibition of *L. pneumophila* growth by select compounds (C1 to C7) is dose-dependent.



bacteria/macrophage coculture format, we screened a library of over 18,000 compounds and, upon vetting compound candidates in a variety of *in vitro* and cell-based secondary screens, isolated several hits that efficiently interfered with biological processes that depend on a functional T4SS, such as intracellular bacterial proliferation or lysosomal avoidance, but had no detectable effect on *L. pneumophila* growth in culture medium, conditions under which the T4SS is dispensable. Notably, the same hit compounds also attenuated, to varying degrees, effector delivery by the closely related T4SS from *Coxiella burnetii*, notably without impacting growth of this organism within synthetic media. Together, the results support the idea that interference with T4SS function is a possible therapeutic intervention strategy, and the emerging compounds provide tools to interrogate, at a molecular level, the regulation and dynamics of these virulence-critical translocation machines. Our study represents the first step in our pursuit toward precision medicine by developing pathogen-selective therapeutics capable of treating the infections without causing harm to commensal bacteria.

VpdC is a ubiquitin-activated phospholipase effector that regulates *Legionella* vacuole expansion during infection.

Phospholipids are a major component of membranes. They are composed of two lipophilic fatty acids, a glycerol backbone, and a hydrophilic head with a phosphate group that can be esterified to other biomolecules. As a result of their amphipathic character, phospholipids have the tendency to form bilayers that allow lateral fluidity while providing a diffusion barrier with mechanical strength against rupture. *L. pneumophila* exploits those features by hiding inside a membrane-enclosed phagosomal compartment that provides protection from immune detection by the host. At the same time, the phagosomal membrane confines *L. pneumophila* to a tight space that needs to be gradually expanded during intracellular replication to accommodate the growing number of offspring. How vacuole expansion is controlled by pathogens has remained unclear.

We discovered that the phospholipase effector VpdC plays a major role in this process. Phospholipases are enzymes that hydrolyze phospholipids to generate free fatty acids and lysophospholipids. Owing to their

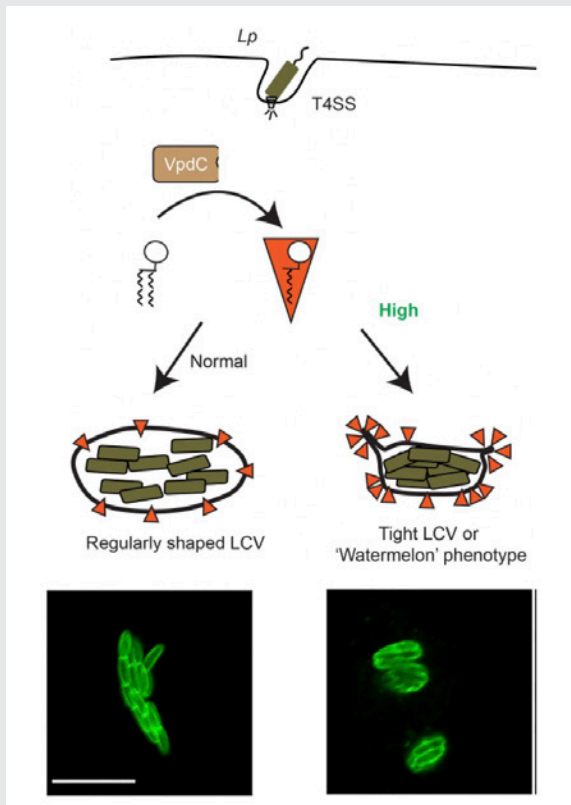


FIGURE 3. Lysophospholipids play an important role in regulating vacuole expansion during *Legionella* growth.

Upon infection of human macrophages, the effector protein VpdC from *Legionella* (*Lp*) generates moderate levels of lysophospholipids (orange triangle) within the membrane of its vacuole (LCV) to promote expansion. Overproduction of lysophospholipids prevents membrane acquisition and expansion of the LCV, trapping the bacteria (labeled green in the micrograph) within spatially confined vacuoles and reducing virulence. Scale bar, 5 μm .

inverted-cone shape, lysophospholipids can affect membrane curvature and thus profoundly influence membrane fusion. We discovered that the phospholipase effector VpdC generates lysophospholipids within the phagosomal membrane surrounding *L. pneumophila*. While moderate levels of lysophospholipids can promote membrane fusion, elevated levels obstruct fusion. Consequently, we found that VpdC overproduction blocked membrane expansion of *Legionella*-containing vacuoles, trapping the bacteria inside a spatially confined compartment and attenuating their virulence. Together, our findings not only demonstrate an important role for bacterial phospholipases in vacuolar expansion, but also suggest that exploiting lysophospholipids for their membrane-bending capability might be a common strategy among pathogens to control membrane dynamics.

Additional Funding

- NICHD Early Career Awards FY22 (to Yuen Yan Chang)
- NICHD Early Career Awards FY22 (to Stephanie Lehman)

Publications

1. Li X, Anderson DE, Chang Y-Y, Jarnik M, Machner MP. VpdC is a ubiquitin-activated phospholipase effector that regulates Legionella vacuole expansion during infection. *Proc Natl Acad Sci U S A* 2022 119(48):e2209149119.
2. Cheng E, Dorjsuren D, Lehman S, Larson CL, Titus SA, Sun H, Zakharov A, Rai G, Heinzen RA, Simeonov A,

Machner MP. A comprehensive phenotypic screening strategy to identify modulators of cargo translocation by the bacterial Type IVB secretion system. *mBio* 2022 13:e0024022.

Collaborators

- Eric D. Anderson, PhD, *Advanced Mass Spectrometry Core, NIDDK, Bethesda, MD*
- Robert Heinzen, PhD, *Coxiella Pathogenesis Section, Rocky Mountain Laboratories, NIAID, Hamilton, MT*
- Michal Jarnik, PhD, *Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD*
- Anton Simeonov, PhD, *Division of Pre-Clinical Innovation, NCATS, Bethesda, MD*
- Chad Williamson, PhD, *Advanced Microscope Facility, NICHD, Bethesda, MD*

Contact

For more information, email machnerm@nih.gov or visit <https://machnerlab.nichd.nih.gov>.

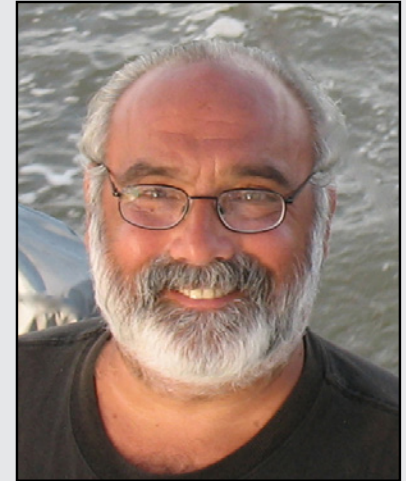
RNA Metabolism in Cell Biology, Growth, and Development

We study the biogenesis, processing, modification, and decay of tRNAs and mRNAs, as well as some of their specific interacting proteins that contribute to cell proliferation, growth, and development during health and disease. Some of our studies focus on the La protein, which interacts with nuclear precursor tRNAs and other nascent transcripts synthesized by RNA polymerase III (Pol III) that are matured to small noncoding (nc)RNAs with a diversity of critical functions. Other studies focus on the La-related proteins (LARPs) 1 and 4, which interact mostly with cytoplasmic mRNAs, and the tRNA anticodon-loop modification enzyme known as tRNA-isopentenyltransferase-1 (TRIT1).

tRNAs are produced at over 10-fold higher molar levels than are ribosomes during cellular proliferation. La protein serves as a chaperone for pre-tRNAs, which must undergo proper folding, multiple processing, and enzymatic modifications prior to nuclear export. Many diseases are attributable to defective tRNA biogenesis and the consequent failure to support mRNA translation.

Pol III synthesizes high levels of tRNA by a conserved process of transcription termination-associated reinitiation, relevant to development and cancer. Termination occurs within a short tract of T residues in the non-template (NT) DNA strand at the ends of Pol III-transcribed genes. The nascent transcripts bear a copy of this terminator, U(n)U-3'OH, a recognition motif for the nuclear La protein, which binds in a sequence- and length-dependent manner. Notably, the 3'U(n) length-dependence of La binding is usually shorter than the minimal T-length required for efficient Pol III termination, the latter of which is 6, 5, and 4 Ts for *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and human, respectively. This suggests a La link with tRNA expression, a link that fits with human Pol III, which evolved a minimal 4T termination mechanism; the data suggest that this may direct some post-transcriptional events. Studies on development, structure, and its gene variants indicate that human Pol III evolved termination mechanisms to control gene-regulatory programs with greater intricacy.

The human La protein is a target of auto-antibodies in patients with chronic inflammatory diseases such as systemic lupus erythematosus, Sjögren's syndrome (SS) (La is also known as SS antigen-B, SSB), and



Richard J. Maraia, MD, Head,
*Section on Molecular and Cellular
Biology*

Sergei Gaidamakov, PhD, *Biologist*

Sandy Mattijssen, PhD, *Staff
Scientist*

Alex Vassilev, PhD, *Staff Scientist*

Alan Kessler, PhD, *Postdoctoral
Fellow*

Abdul Khaliq, PhD, *Visiting
Fellow*

Amitabh Ranjan, PhD, *Visiting
Fellow*

Leah Pitman, BS,
Postbaccalaureate Fellow



FIGURE 1. The fission yeast *Schizosaccharomyces pombe* as a model organism

Red-white colony differentiation by tRNA-mediated suppression

neonatal lupus. Although La is active in other immune pathways, evidence for its primary involvement in autoimmunity is lacking. As noted above, La binds to POL III transcripts in all cells and serves as a chaperone during their nuclear maturation. La not only protects pre-tRNAs from 3' exonucleases, but also directs and temporally orders the first step in the 5' processing by the RNase P pathway. We propose a role for La in a novel pathway, different from tRNA maturation. Our data suggest that La is a determinant for a subset of pre-tRNAs (genes) to enter an alternative pathway to specific activation of the type-I interferon (IFN) response. We are studying a dual-activity tRNA gene(s) for which La is a key determinant of whether the nascent 4T-terminated transcripts are directed to tRNA maturation or to an alternate pathway of innate immune activation. Accordingly, some dual activity tRNA genes may serve as endogenous immune adjuvants or may contribute to autoimmunity. We are currently exploring these possibilities.

The rate of RNA production by Pol III was shown to affect the efficiency by which pre-tRNAs acquire the common $m^{2,2}G26$ modification, which increases tRNA activity. Disrupted tRNA biogenesis, attributable to gene variants, leads to developmental and other diseases, as well as to neurodegeneration and intellectual impairment. About 120 modifications occur on tRNAs, of which about 40 have been documented for human cytoplasmic (cy-) tRNAs and several others for human mitochondrial (mt-) tRNAs. Many tRNA-modification enzymes (TME) are multisubunit, and some composite modifications require several gene products. Thus, mutations in numerous TMEs cause disease.

Critical modifications to tRNA anticodon loops fine-tune base pairing for optimal decoding and wobble decoding of synonymous codons, which is an important component of a tunable system of high-fidelity cy-translation. It is important to note that the 61 sense codons for 20 amino acids (aa) are decoded by cy-tRNAs, which collectively carry only 45 anticodons, a feature of tRNA genes, named anticodon-sparing, that is widely conserved, although the number and identity of the 'missing' anticodons differ among kingdoms, anti-correlated with wobble-base modifications. In humans, about 15 standard codons must be wobble-decoded by cy-tRNAs whose activities are controlled by anticodon modifications. Thus, Pol III transcription of tRNA genes is only one level on which the translation of a range of cy-mRNAs with biased codon content can be regulated. While this constitutes a nuclear-cytoplasmic translation system with much potential for intricate regulation, the complexities of overlapping/redundant decoding activities also create potential for translation infidelity in cases of faulty modification or tRNA pool imbalance.

All TMEs are nuclear-encoded, and a minority subset modify both cy- and mt-tRNAs, with potential to synchronize translation in both compartments. Distinct decoding rules apply in mitochondria, in part because each mt-DNA encodes only one tRNA for each of 18 amino acids. Synonymous codons occupy mt-mRNAs, but

are not distinguished by different tRNAs, as is the case for cy-mRNAs. For example, while four codons each for Ala, Gly, Pro, Thr, and Val are decoded by three cy-tRNAs, a single mt-tRNA for each must wobble-decode its four cognate codons. Furthermore, these mt-tRNAs use unmodified U34 as their wobble base, whereas cy-tRNA wobble bases are part of an elaborate modification system, especially for U34. Each mt-tRNA is critical; point mutations impair mt-translation with unique association with oxidative phosphorylation diseases, whereas most nuclear tRNA genes are buffered by multiple copies; a known exception is a unique, single-copy, brain-specific tRNA. Yet, some diseases caused by a mutation in a mt-tRNA exhibit a phenotype like that resulting from a nuclear gene mutation to a TME whose substrate is the mt-tRNA.

Numerous pathogenic alleles that encode defective subunits of Pol III cause hypomyelinating leukodystrophy (HLD), whose pathobiology is consistent with poor formation of axonal myelin sheaths rather than demyelination. Pol III produces several ncRNAs in addition to tRNAs. We suggest that another disruption mechanism of a tRNA biogenesis–CNS network is also possible. As TME defects often manifest as neurological, reflecting tissue reliance on mitochondria, and because cy- and mt-translation are synchronized, including by tRNA modifications and other factors, disruption of general tRNA homeostasis may impair mt-function, with pathophysiological contribution to disease, including in developing oligodendrocytes.

mRNA levels are determined in significant part by levels of cognate tRNAs, from yeast to human. In addition, certain mRNAs are particularly sensitive to tRNA levels. The LARP4 mRNA has a tract of about 70 codons with a very poor match to cellular tRNA levels. Thus, unprogrammed changes in tRNA output (e.g., genetic mutation in POL III) could shift mRNA stabilities and translation efficiencies in unpredictable ways with uncertain outcomes.

Translation of LARP4 mRNA produces LARP4, which binds to poly(A) and to poly(A)-binding protein (PABP/PABPC1). LARP4 stabilizes mRNAs by opposing deadenylation of their poly(A) tails, substrates of Ccr4-Not deadenylase (a multiprotein complex that functions in gene expression in the nucleus, where it regulates transcription, and in the cytoplasm, where it associates with translating ribosomes and RNA processing bodies). Potential for regulation is that Ccr4-Not monitors mRNA–ribosomes for codon–tRNA match. For ribosomal protein–encoding mRNAs stabilized by LARP4, this supports a working model in which LARP4 mRNA senses tRNA levels and relays this by producing LARP4 to regulate ribosome biogenesis, perhaps with LARP1.

It has been known for some time that human Pol III and associated factors are dysregulated in cancer. More recently, functionally related mRNAs favoring proliferative or differentiation states were shown to be biased in synonymous codons and in the cognate tRNAs differentially expressed in those cells. Strikingly, the first Pol III structures provide insight into activities specific to the higher eukaryotic 17-subunit complex. First, the cancer-associated RPC7a subunit paralog (encoded by *POLR3G*) appeared to interfere with binding of the Pol III–negative regulator and tumor suppressor MAF1, whereas the RPC7b (*POLR3GL*) paralog subunit is enriched in cells programmed for differentiation (limited proliferation). Yeast Pol III has only one homolog. The second example involves the most striking feature of higher eukaryote–specific Pol III, the multi-domain expansion of RPC5 and hypothesized associated higher eukaryote promoter–type specificity, and its link to termination–reinitiation recycling.

Mechanistic control of ncRNA genes appears to have evolved activities that extend hPol III beyond the housekeeping activities of its yeast counterpart. This fits with a view of hPol III in self vs. non-self surveillance functions. Notable are Pol III Vault (Vt) ncRNAs with involvement in two activity types, in innate immune

surveillance, and differentiation vs. maintenance of undifferentiated states. Both Pol III Vt and snaR (small nuclear factor 90-associated RNA) ncRNAs are processed to miRNAs that exert downstream effects on mRNA profiles and/or differentiation/cancer.

Activities of RNA polymerase III (RNAP III) and associated factors

The Pol III multisubunit enzyme complex consists of 17 integral subunits, whereas Pols I and II consist of 14 and 12 respectively. However, during early vertebrate evolution, one of the Pol III subunits, POLR3G/RPC7, was duplicated, and both gene paralogs evolved as essential, such that present-day organisms use both. Thus, each molecule of cellular Pol III contains one or the other paralog, i.e., POLR3G or POLRGL, and this subunit can control Pol III-intrinsic activity and to direct cellular pathways toward differentiation-quiescence or proliferation-cancer type phenotypes.

The transcription factor TFIIIC, composed of six subunits, binds to A- and B-box promoters (promoter elements of tRNA genes) and recruits TFIIIB to direct Pol III to the correct start site. TFIIIB–Pol III complexes appear highly stable and demonstrate great productivity in supporting the many cycles of initiation, termination, and re-initiation necessary to produce the more than tenfold molar excess of tRNAs relative to ribosomes that is required to drive translation during growth and development. In contrast to all other multisubunit RNA polymerases, termination and re-initiation by Pol III are functionally, if not physically, linked. POLR3G and POLRGL can control the extent to which Pol III may be sensitive to inhibition by the Pol III–negative regulator Maf1 and to recycling. Our laboratory developed *in vivo* and biochemical methods to examine the unique mechanisms used by Pol III. Hereditary mutations in Pol III cause hypomyelinating leukodystrophy (HLD), as well as defects in innate immunity. In addition to its being essential for cell proliferation, Pol III is also linked to aging.

Transcription termination delineates 3' ends of gene transcripts, prevents otherwise runaway Pol from intruding into downstream genes and regulatory elements, and enables release of the Pol for recycling. While other Pols require complex *cis* signals and/or accessory factors to accomplish these activities, eukaryotic Pol III does so autonomously with high efficiency and precision at a simple oligo(dT) stretch of 5–6 bp. A basis for this high-density *cis* information is that both the template and non-template strands of the Pol III terminator carry distinct signals for different stages of termination. High-density *cis* information is a feature of the Pol III system that is also reflected in the dual functionalities of the tRNA promoters as both DNA and RNA elements. Furthermore, the TFIIIF-like Pol III subunit C37 is required for this function of the non-template strand signal. Our results reveal the Pol III terminator to be an information-rich control element. While the template strand promotes destabilization via a weak oligo(rU:dA) hybrid, the non-template strand provides distinct sequence-specific destabilizing information through interactions with the C37 subunit.

Control of the differential abundance or activity of tRNAs can be an important determinant of gene regulation. Pol III synthesizes all tRNAs in eukaryotes, and its derepression is associated with cancer. MAF1 is a conserved general repressor of Pol III under the control of TOR (target of rapamycin), which acts to integrate transcriptional output and protein-synthetic demand toward metabolic economy. We used tRNA–Hydro-Seq (hydrolysis-based tRNA sequencing) to document that little change occurred in the relative levels of different tRNAs in *maf1* mutated cells. By contrast, the efficiency of *N*²,*N*²-dimethyl G26 (*m*^{2,2}G26) modification on certain tRNAs was reduced in response to *maf1* deletion and associated with anti-suppression, which we

validated by other methods. Overexpression of Trm1 (tRNA dimethyl transferase), which produces m^{2,2}G26, reversed *maf1* anti-suppression. The model that emerges is that competition by elevated tRNA levels in *maf1-delta* cells leads to m^{2,2}G26 hypo-modification resulting from limiting Trm1, thus reducing the activity of suppressor tRNA^{Ser}UCA (UCA is the anticodon for serine) and accounting for anti-suppression. Consistent with this, Pol III mutations associated with HLD reduce tRNA transcription, increase m^{2,2}G26 efficiency, and reverse anti-suppression. Extending this more broadly, a reduction in tRNA synthesis by treatment with rapamycin leads to increased m^{2,2}G26 modification, a response that is conserved among highly divergent yeasts and human cells [Arimbasseri AG *et al.*, *PLoS Genetics* 2015;11:e1005671].

The ability of Pol III to efficiently recycle from termination to re-initiation is critical for abundant tRNA production during cellular proliferation, development, and cancer. We used two tRNA-mediated suppression systems to screen for Rpc1 (subunit of Pol III) mutants with gain- and loss-of-termination phenotypes in *Schizosaccharomyces pombe*. We mapped 122 point mutation mutants to a recently solved 3.9 Å structure of the yeast Pol III elongation complex (EC); they cluster in the active-center bridge helix and trigger loop, as well as in the pore and funnel formation, the latter indicating involvement in termination of the RNA-cleavage domain of the C11 subunit of Pol III. Biochemical, kinetic, and genetic data indicate that mutants with the RT (readthrough) phenotype synthesize more RNA than wild-type cells and, surprisingly, more than can be accounted for by the mutants' increased elongation rate. Importantly, similar mutations in spontaneous cancer suggest this is an unforeseen mechanism of Pol III activation in disease.

Role of La-related protein-4 (LARP4) in poly(A)-mediated mRNA stabilization

Ubiquitous in eukaryotes, La proteins are involved in two broad functions: first, metabolism of a wide variety of precursor tRNAs and other small nuclear RNAs by association with these RNAs' common UUU-3'OH-transcription termination elements; and second, translation of specific subsets of mRNAs, such as those containing 5' IRES (internal ribosome entry site) motifs. LARP4 emerged later in evolution, and we found it to be an mRNA-associated cytoplasmic factor associated with poly(A)-binding protein C1 (PABPC1, PABP). LARP4 uses two regions to bind to PABPC1. We showed that the N-terminal domain (NTD, amino acids 1–286) of LARP4, consisting of an N-terminal region (NTR, amino acids 1–111) followed by two tandem RNA-binding motifs known as an 'La module' (111–285), exhibits preferential binding to poly(A). The NTR contains a unique PAM2w motif that binds to the MLLE (a peptide-binding domain) of PABP. The group of our collaborator Maria Conte showed that the NTR itself is responsible for most of the poly(A) binding and that, moreover, this involves conserved residues unique to the PAM2w of LARP4. The La module is flanked by a different motif on each independently interacting with PABP. LARP4 is controlled at the level of mRNA stability: one level of control is by an A+U-rich element (ARE) in its 3' UTR via interactions with the protein tristetraproline (TTP), the latter of which is regulated in mammals by tumor necrosis factor alpha (TNF α); a second level of control was found for the LARP4 mRNA-coding sequence in an unusual group of synonymous codons with poor match to cellular tRNA levels [Reference 1]. The LARP4 protein controls the metabolism/homeostasis and translation of heterologous mRNAs by affecting their poly(A) tail length. Working with researchers in the NICHD [Molecular Genomics Core](#) facility, we developed a single-molecule, high-throughput nucleotide-resolution poly(A)-tail sequencing method, referred to as SM-PAT-Seq, which yielded insights into LARP4 function and mechanism. LARP4 is a global factor involved in mRNA poly(A) length homeostasis and appears to effect mRNA stabilization by opposing the action of deadenylases when poly(A) tails are short.

Fission yeast as a model system for the study of tRNA metabolism and function in translation

More than 20 years ago, we began developing, refining, and advancing a tRNA-mediated suppression (TMS) system in the fission yeast *Schizosaccharomyces pombe*, which provides a 'red-white' phenotypic real-time assay that can be used to investigate various aspects of tRNA biogenesis, maturation, and metabolism of tRNAs *in vivo*. In *S. pombe*, the human La protein can replace the tRNA-processing/maturation function of Sla1p, the *S. pombe* equivalent of the La protein. Moreover, in *S. pombe*, human La is faithfully phosphorylated on Ser-366 by protein kinase CKII, the same enzyme that phosphorylates Ser-366 in human cells, and this phosphorylation event promotes pre-tRNA processing. We use the system to study transcription by RNAP III, post-transcriptional processing, and tRNA modifications by conserved enzymes that produce tRNA isopentenyl-adenosine-37 and dimethyl-guanosine-26 (N²,N²-dimethyl G26, m^{2,2}G26).

tRNAs, codon use, and mRNA metabolism in growth and development

A major interest of ours is to decipher what we refer to as 'secondary information' in the genetic code, information that is derived from mRNAs' biased use of synonymous codons. This can produce a layer of information beyond the amino acid sequence of a protein; i.e., in addition to providing the template for the sequence of a protein, the use of certain synonymous codons can also produce additional biochemical effects, which we refer to as 'secondary information.' The effects can be related to ribosome pausing, which can affect protein folding, or to alterations in the stability of the mRNA. Other types of secondary information can also be encoded in synonymous codons; for example, sets of mRNAs that share similar patterns of synonymous codon bias are similarly sensitive to tRNAs with the same anticodon modification and exhibit similar patterns of efficiency of translation elongation. The components of the secondary information system are the tRNA pool, the tRNA-modification enzymes, and the codon-bias distribution among the mRNAs. We recently found that synonymous codon use by the human LARP4 mRNA is a key determinant in the control of the expression levels of its mRNA and protein, and that increases in otherwise limiting tRNAs, that are cognate to these codons, increase LARP4 production, which in turn activates LARP4, to promote a net increase in the poly(A) tail length of heterologous mRNAs, including those that encode ribosomal protein subunits [Reference 1], which may be important because ribosome production is regulated during growth and development, and because the potential circuit involving LARP4 control by tRNA could be an important point of control.

Publications

1. Kozlov G, Mattijssen S, Jiang J, Nyandwi S, Sprules T, Iben JR, Coon SL, Gaidamakov SA, Noronha AM, Wilds CJ, Maraia RJ, Gehring K. Structural basis of 3'-end poly(A) RNA recognition by LARP1. *Nucleic Acids Res* 2022 50:9534–9547.
2. Khaliq A., Mattijssen S, Maraia RJ. A versatile tRNA modification-sensitive northern blot method with enhanced performance. *RNA* 2022 28:418–432.
3. Mishra S, Hasan SH, Sakhawala RM, Chaudhry S, Maraia RJ. Mechanism of RNA polymerase III termination-associated reinitiation-recycling conferred by the essential function of the N terminal-and-linker domain of the C11 subunit. *Nat Commun* 2021 12:5900.
4. Mattijssen S, Kozlov G, Gaidamakov S, Ranjan A, Fonseca BD, Gehring K, Maraia RJ. The isolated La-module of LARP1 mediates 3' poly(A) protection and mRNA stabilization, dependent on its intrinsic PAM2 binding to PABPC1. *RNA Biol* 2021 18:275–289.
5. Mattijssen S, Iben JR, Li T, Coon SL, Maraia RJ. Single molecule poly(A) tail-seq shows LARP4 opposes

- deadenylation throughout mRNA lifespan with most impact on short tails. *eLife* 2020 9:e59186.
6. Khaliq A, Mattijssen S, Haddad AF, Chaudhry S, Maraia RJ. Targeting mitochondrial and cytosolic substrates of TRIT1 isopentenyltransferase: specificity determinants and tRNA-i6A37 profiles. *PLoS Genet* 2020 16:e1008330.

Collaborators

- Maria R. Conte, PhD, *King's College, University of London, London, United Kingdom*
- Kalle Gehring, PhD, *McGill University, Montreal, Canada*
- James R. Iben, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*

Contact

For more information, email maraiar@mail.nih.gov or visit <http://maraialab.nichd.nih.gov>.

Immune Activation and Viral Pathogenesis

The general goal of the Section is to understand the mechanisms of pathogenesis of human pathogens in the context of complex tissue microenvironments. In particular, we focused on the role of cytokines in viral pathogenesis. Our studies include extracellular vesicles (EVs) that mediate cell-cell communications and can carry cytokines. We found that EVs, including ones that carry cytokines, are altered in women experiencing pregnancy complications, and can be used as biomarkers of such pathologies. Also, EVs in people with psychoneurological symptom clusters are different from control ones. Both free cytokines and EV-associated cytokines are associated with the pattern of HIV infection and may determine the distinct course of HIV disease in women living with HIV undergoing anti-retroviral therapy. Our current studies are designed according to NICHD Strategic goals on reproductive health, healthy pregnancies, life-long wellness, and gender-based health disparities.

Cytokines in HIV disease

Among the determinants of an effective pathogen immune responses, and to viruses in particular, the complex network of cytokines plays an important role. Cytokines are protein messengers of intercellular communication between multiple immune and nonimmune cells. They are part of the language used by the innate and adaptive immune system to orchestrate an effective immune response to infectious pathogens. Although antiretroviral therapy (ART) efficiently suppresses HIV replication to undetectable levels, it does not fully restore immune functions. We investigated whether ART differentially affects the cytokine network in different body compartments. In addition to cytokines in blood plasma, our study focused on cytokines in semen, given that the majority of HIV transmission occurs through sexual intercourse. We characterized the cytokine network in blood and semen in individuals longitudinally sampled before they began ART, and after achieving suppression of HIV RNA, by measuring the concentrations of 34 cytokine/chemokines using a multiplex bead-based assay. We used Partial Least Squares Discriminant Analysis (PLS-DA) to visualize the differences in cytokine patterns between the time points. Cytokines with VIP scores (which reflect the difference in cytokines before and after antiretroviral therapy) exceeding 1 were deemed important for predicting suppression status and were



Leonid Margolis, PhD, Head,
*Section on Intercellular
Interactions*

**Christophe Vanpouille, PhD, Staff
Scientist**

Wendy Fitzgerald, BS, Biologist

subsequently tested using Wilcoxon Signed Rank Tests. While PLS-DA projections in blood were fairly similar before and after viral suppression, they were very different in semen before and after ART. When tested individually, four cytokines were significantly different across time points in semen (MIG, IL-15, IL-7, I-TAC), and two in blood (MIG and IP-10). Our results identified specific changes in the cytokine networks in semen and blood as the immune system acclimates to chronic, suppressed HIV infection.

Distinct biological manifestations of sex-based differences have been described in people living with HIV. Women with HIV have lower plasma viral loads, especially during the early phases of infection, and a 1.6-fold higher risk of developing AIDS when accounting for viral load levels in chronic infection. Substantial differences in immune activation between men and women with HIV have been described, but the reasons for these differences have not been fully elucidated. One important factor may be the cytokine response evoked during infection. We investigated the sex differences in blood plasma of men and women with chronic HIV on suppressive ART, using Luminex, and investigated whether cytokines contribute to maintaining higher immune activation in women despite suppressive therapy with ART. We identified five cytokines that were significantly higher in women than in men, namely the pro-inflammatory chemokines CXCL1 (Gro- α), CCL5 (RANTES), CCL3 (MIP-1 α), CCL4 (MIP-1 β), as well as the T cell homeostatic factor IL-7. Thus, inflammatory cytokines remained higher in virologically suppressed women than in men living with HIV, suggesting that cytokines contribute to maintaining higher immune activation in women despite suppressive therapy with ART. Moreover, CCL3, CCL4, and CCL5, which are β chemokines that share the same receptor, CCR5, have been identified as strong HIV-suppressive factors produced by CD8 T cells. This observation could provide a possible explanation for the differences of viral loads previously reported between women and men. Overall, our results are consistent with the higher cellular activation observed in women. More studies are needed to better understand the underlying mechanisms that contribute to sex-based immune cell regulation in people with HIV. Our study, together with others, reveals the importance of sex/gender-specific studies, which too often remain a neglected area of viral pathogenesis.

Although ART dramatically reduces HIV viral load and improves the life span and quality of life for most patients, people living with HIV are more likely to develop serious non-AIDS comorbidities, in part because of recurrent immune activation. The drivers of recurrent immune activation in both men and women living with HIV under ART remain to be fully understood. Cytomegalovirus (CMV) is considered to be one of the driving forces of persistent immune activation. Although the associations between CMV replication and systemic inflammation in people living with HIV during suppressive ART have been well documented, it is not clear whether CMV replication is associated with systemic immune activation already during the earliest phase of untreated HIV infection, and thus a potential target for early CMV intervention. We investigated whether genital CMV shedding contributed to systemic immune activation, as evaluated by the concentration of 34 blood cytokines in people living with HIV in the acute/early phase of HIV infection. Independent of CMV, we found that the concentrations of IP-10, MIG, MCP-1, I-TAC 10, IL-16, and MIP-1 β were modulated in the earliest phase of HIV infection compared with control individuals without HIV. In people with HIV, there was no difference in blood cytokines among CMV shedders vs non-shedders. Our results suggest that CMV shedding in the male genital tract is not the main driver of systemic immune activation in the early phase of HIV infection, in contrast with the later phase of HIV infection. Early ART initiation should remain the priority. Similar studies in women should reveal whether there is a sex-related difference in the role of CMV in early infection. Whatever the role of CMV is in HIV acquisition, our results on cytokine distribution may already explain why women progress faster to AIDS than men at a given viral load.

Extracellular vesicles as markers of complicated pregnancies

Extracellular vesicles (EVs) are released by all cells of the human body and report on the physiology of their cells of origin. Therefore, EVs can, in principle, report on the pathologic development in the organism. In particular, EVs can serve as biomarkers in complicated pregnancies. We found that the composition of EVs in maternal blood is significantly changed in case of fetal death. Preliminary results found that 16 soluble cytokines, growth factors, and angiogenic factors are significantly different between maternal blood plasma of mothers experiencing fetal death and age-matched mothers without complications. Eight proteins were significantly different in the EV fractions, many of which were the same as the significant soluble proteins, but one protein CD163 was significantly downregulated only in EV-associated form. Ongoing analysis will determine whether the EV-associated cytokines improve the prediction accuracy of poor outcomes, and future functional studies will focus on determining whether these EV-associated cytokines play distinct roles in pathology.

The cause of fetal death is not always known, but viral infection is a major cause of fetal mortality, in particular CMV infection. Congenital CMV (cCMV) infection is the most common congenital infection and is often associated with severe neurological disabilities or perinatal death. The prediction of neonatal status in the case of CMV infection is limited and therefore the identification of new prognostic markers in amniotic fluid that is sampled by amniocentesis for the diagnosis of fetal infection could improve timely prenatal assessment of infected fetuses. Among biological processes involved in the innate immunity, many cytokines are involved in the immune control of cCMV infection in fetuses. Earlier, we reported that EVs carry cytokines and that these cytokine-carrying EVs constitute a system of cytokine delivery to particular cells. EVs are present in many biological fluids, including amniotic fluid.

We studied whether cytokines measured in the amniotic fluid, in particular cytokines associated with EVs, can report on CMV infection in pregnancy. The study enrolled 80 pairs of women and fetuses/newborns, including 40 infected fetuses and 40 negative controls. Our data suggest that cCMV infection and related symptoms at birth are associated with changes in the immunological signature of the amniotic fluid. Four soluble pro-inflammatory mediators (IP-10, IL-18, ITAC, and TRAIL) and one mediated by EV (IP-10) were elevated in the case of cCMV infection. Among these proteins, five were related to symptoms at birth (IP-10 internal, IP-10 surface, IP-10 soluble, IL-18 soluble, and TRAIL soluble). Seven other cytokines, not related to cCMV infection, were significantly associated with symptomatic status at birth; therefore, a pattern for severe infection can be related with a specific increase in the presence and concentration of six mediators (IL-18 soluble, TRAIL soluble, CRP soluble, TRAIL surface, MIG internal, and RANTES internal). Thus, our data suggest that cCMV infection and its severity are associated with differential expression of cytokines, in particular EV-associated ones, in amniotic fluid at mid-gestation and may serve as candidate biomarkers of severity in case of fetal infection diagnosed by CMV-PCR. Considering trafficking of EVs, EV-associated cytokines may also prove to be promising biomarkers in maternal blood, allowing less invasive tests than amniocentesis.

Extracellular vesicles as markers of psychoneurological symptom clusters

We investigated the associations between EV-associated and soluble cytokines with immune markers and symptom clusters in men with non-metastatic prostate cancer and women with breast cancer. Because extracellular vesicle EV-associated cytokines, both encapsulated and surface bound, have been associated with symptom severity, and may vary over the lifespan, they may be potential biomarkers to uncover underlying mechanisms of various conditions.

Psycho-neurological symptom clusters are co-occurring and interrelated physiological symptoms that may include cancer-related fatigue, pain, depressive symptoms, cognitive disturbances, and sleep disturbances. It is hypothesized that these symptoms share a common systemic pro-inflammatory etiology. We investigated the associations between extracellular vesicle EV-associated and soluble cytokines with immune markers and symptom clusters in men with non-metastatic prostate cancer. Our observational study included 40 men with non-metastatic prostate cancer at the start (T1) of external beam radiation therapy (EBRT) and three months post treatment (T2), as well as 20 men with non-metastatic prostate cancer on active surveillance (AS) seen at one time point.

Both EV-associated and soluble forms of RANTES significantly correlated with the symptom cluster for EBRT at T1, whereas, at T2, soluble IFN α 2, IL-9, and IL-17 correlated with the corresponding symptom cluster. For the AS group, soluble survivin (an inhibitor of apoptosis highly expressed in most cancers) correlated with psycho-neurological symptoms. Linking specific inflammatory cytokines with psycho-neurological symptom clusters in men receiving prostate cancer treatment can enhance our understanding of the underlying mechanisms of this phenomenon and aid in developing targeted interventions.

We also investigated the associations of soluble and EV-associated cytokine concentrations with distinct symptom profiles reported by 290 women with breast cancer. Patients were classified into older (60 years or more, $n = 93$) and younger (less than 60 years, $n = 197$) cohorts within two previously identified distinct symptom-severity profiles, which included pain, depressive symptoms, sleep disturbance, and fatigue (i.e., High Fatigue Low Pain and All Low). Results of this study suggest that levels of cytokine concentrations differ between EV and soluble fractions. Several EV and soluble pro-inflammatory cytokines had positive associations with depressive symptoms and fatigue within both age cohorts and symptom profiles. In addition, in the older cohort with the High Fatigue Low Pain symptom profile, EV GM-CSF (a white cell growth factor) concentrations were significantly higher than for the All Low symptom profile. These exploratory analyses provide new information on the association between cytokines and symptom profiles of older and younger cohorts. We found unique EV-associated cytokines in older patients and in specific symptom classes. These results suggest that EVs are potential biomarker discovery tools, as well as underlying distinct symptom class profiles, and may thus inform intervention trials and offer precision-medicine approaches.

Additional Funding

- NICHD Director Award

Publications

1. Ñahui-Palomino RA, Vanpouille C, Costantini PE, Margolis L. Microbiota–host communications: bacterial extracellular vesicles as a common language. *PLoS Pathog* 2021 17(5):e1009508.
2. Mercurio V, Fitzgerald W, Vanpouille C, Molodtsov I, Margolis L. Mechanisms of residual immune activation in HIV-1-infected human lymphoid tissue ex vivo. *AIDS* 2021 35:1179.
3. Sass D, Saligan L, Fitzgerald W, Berger AM, Torres I, Barb JJ, Kupzyk K, Margolis L. Extracellular vesicle associated and soluble immune marker profiles of psychoneurological symptom clusters in men with prostate cancer: an exploratory study. *Translat Psychiatry* 2021 11:440.
4. Vanpouille C, Wells A, Wilkin T, Mathad JS, Morris S, Margolis L, Gianella S. Sex differences in cytokine

profiles during suppressive antiretroviral therapy. *AIDS* 2022 36:1215.

5. Rawlings SA, Torres F, Wells A, Lisco A, Fitzgerald W, Margolis L, Gianella S, Vanpouille C. Effect of HIV suppression on the cytokine network in blood and seminal plasma. *AIDS* 2022 36:621.

Collaborators

- Michael Bukrinsky, MD, PhD, *George Washington University, Washington, DC*
- Leonid Chernomordik, PhD, *Section on Membrane Biology, NICHD, Bethesda, MD*
- Sara Gianella Weibel, MD, *University of California San Diego, La Jolla, CA*
- Michael Lederman, MD, *Case Western University, Cleveland, OH*
- Eva Povedra, PhD, *Galicía Sur Health Research Institute, Vigo, Spain*
- Roberto Romero-Galve, MD, DMedSci, *Perinatology Research Branch, NICHD, Detroit, MI*
- Leorey Saligan, PhD, RN, CRNP, FAAN, *Symptom Biology Unit, NINR, Bethesda, MD*
- Yves Ville, MD, *Paris Descartes University, Paris, France*
- Beatrice Vitali, PhD, *Università di Bologna, Bologna, Italy*

Contact

For more information, email margolis@helix.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/margolis>.

Genetic Disorders of Bone and Extracellular Matrix

In an integrated program of laboratory and clinical investigation, we study the molecular biology of the heritable connective tissue disorders collectively known as osteogenesis imperfecta (OI). Our objective is to elucidate the mechanisms by which the primary gene defect causes skeletal fragility and other connective-tissue symptoms and to apply this knowledge to patient treatment. We identified several key genes causing recessive and X-linked OI. Discoveries of defects in collagen modification generated a new paradigm for OI as a collagen-related disorder of matrix. We established that structural defects in collagen cause dominant OI, while deficiency of proteins that interact with collagen for folding, post-translational modification, or processing cause recessive OI. Our challenge now is to understand the cellular and biochemical mechanisms of recessive OI. We generated a knock-in murine model for OI with a classical collagen mutation as well as a murine model for recessive type IX OI and one for X-linked type XVIII OI, and we are using these models to study disease pathogenesis, the skeletal matrix of OI, and the effects of pharmacological therapies. Our clinical studies involve both children with the more prevalent types III and IV OI, as well as those with the rare recessive forms, who form a longitudinal study group enrolled in age-appropriate clinical protocols for the treatment of their condition.

We are also investigating melorheostosis, a very rare bone dysostosis, which is characterized by radiographic patterns of either 'dripping candle wax' or endosteal bone overgrowth. We recently identified mosaic mutations in the oncogene *MAP2K1* as the cause of 'dripping candle wax' melorheostosis and somatic mutations in *SMAD3* as the cause of endosteal melorheostosis. In each gene, the causative mutations occur at a hot spot and result in gain of function. We are now developing animal models for studies of melorheostosis pathophysiology and treatment.

Mechanism of rare forms of osteogenesis imperfecta

Recessive null mutations in *SERPINF1*, which encodes pigment epithelium-derived factor (PEDF), cause OI type VI. PEDF is well known as a potent anti-angiogenic factor. Type VI OI patients lack serum PEDF and have elevated alkaline phosphatase (ALPL)



Joan C. Marini, MD, PhD, Head,
*Section on Heritable Disorders of
Bone and Extracellular Matrix*

Aileen M. Barnes, MS, *Biologist*

Ying Liu, PhD, *Biologist*

Gali Guterman-Ram, PhD,
Postdoctoral Fellow

Milena Jovanovic, PhD,
Postdoctoral Fellow

Elena F. Evans, BS,
Postbaccalaureate Fellow

as children and bone histology with broad unmineralized osteoid and a fish-scale pattern. However, we identified a patient with severe atypical type VI OI, whose osteoblasts displayed minimal secretion of PEDF, but whose *SERPINF1* sequences were normal, despite typical type VI OI bone histology [Reference 1]. Surprisingly, exome sequencing on this proband and family members yielded a *de novo* mutation in *IFITM5* (the gene encoding interferon-induced transmembrane protein 5, which is mutated in type V OI) in one proband allele, causing a p.S40L substitution in the intracellular domain of the encoded protein BRIL (an osteoblast-specific, mineralization-modifying, IFITM-like membrane protein). The *IFITM5* transcript and BRIL were normal in proband fibroblasts and osteoblasts. *SERPINF1* expression and PEDF secretion were reduced in proband osteoblasts. In contrast, osteoblasts from a typical case of type V OI have elevated *SERPINF1* expression and PEDF secretion during osteoblast differentiation. Together, the data suggest that BRIL and PEDF occur in connected cellular pathways that affect bone mineralization. We generated a murine model for atypical type VI OI. Investigations published to date have focused on bone material properties and on the lacunar canicular network [Reference 2]. When *Ifitm5*/BRIL p.S42L murine bone is embedded with rhodamine, it is apparent that canicular density is lower than in wild-type (WT) mice at all ages studied. This feature is also seen in normal bone with aging, where it is associated with impaired signal propagation in response to loading. Second harmonic generation was used to examine the organization of collagen in bone. Although the *Ifitm5*/BRIL p.S42L mouse does not have a defect in collagen structure, collagen is massively disorganized in their bone.

Type VI OI itself has been investigated in a knock-out murine model. We demonstrate that loss of PEDF delays osteoblast maturation as well as extracellular matrix (ECM) mineralization [Reference 4]. Barium sulfate perfusion reveals significantly higher vessel density in the tibial periosteum of *Serpinf1*^(-/-) mice than in WT littermates. The increased bone vascularization in such mice correlated with elevated numbers of CD31(+) / Endomucin(+) endothelial cells, which are involved in the coupling angiogenesis with osteogenesis. Global transcriptome analysis by RNA-Seq of *Serpinf1*^(-/-) mouse osteoblasts reveals that osteogenesis and angiogenesis are the biological processes most impacted by loss of PEDF. Intriguingly, TGF- β signaling is activated in type VI OI cells, and *Serpinf1*^(-/-) osteoblasts are more sensitive to TGF- β stimulation than WT osteoblasts. TGF- β stimulation and PEDF deficiency showed additive effects on transcription suppression of osteogenic markers and stimulation of pro-angiogenic factors. Furthermore, PEDF attenuated TGF- β -induced expression of pro-angiogenic factors. These data suggest that the functional antagonism between PEDF and TGF- β pathways controls osteogenesis and bone vascularization and is implicated in type VI OI pathogenesis. The antagonism may be exploited in developing therapeutics for type VI OI utilizing PEDF and TGF- β antibody.

The endoplasmic reticulum (ER)-resident procollagen 3-hydroxylation complex is responsible for the 3-hydroxylation of type I collagen α 1(I) chains. Deficiency in components of the collagen P3H (prolyl 3-hydroxylase) complex causes recessive OI Types VII, VIII, and IX [Reference 1]. The third member of the complex, cyclophilin B (CyPB), encoded by *PPIB*, is an ER-resident peptidyl-prolyl *cis-trans* isomerase (PPIase). CyPB is the major PPIase catalyzing collagen folding. Our group generated a *Ppib* knock-out (KO) mouse. Intracellular collagen folding occurs more slowly in CyPB null cells, supporting the enzyme's role as the rate-limiting step in folding. However, treatment of KO cells with the cyclophilin inhibitor cyclosporin A caused further delay in folding, providing support for the existence of a further collagen PPIase. We found that CyPB supports collagen lysyl hydroxylase 1 (LH1) activity, demonstrating significantly reduced hydroxylation of the helical crosslinking residue K87, which directly affects both the extent and type of collagen intermolecular crosslinks in bone.

In collaboration with Vorasuk Shotelersuk and Cecilia Giunta, we identified a new OI-causative gene on the X-chromosome. This is the first type of OI with X-linked inheritance, and it causes moderate to severe bone dysplasia with pre- and postnatal fractures of ribs and long bone, bowing of long bones, low bone density, kyphoscoliosis and pectal deformities, and short stature. Affected individuals have missense mutations in *MBTPS2*, which encodes the protein S2P [Reference 1]. S2P is a transmembrane protein in the Golgi and is a critical component of regulated intramembrane proteolysis (RIP). In RIP, regulatory proteins are transported from the ER membrane to the Golgi in times of cell stress or sterol depletion, where they are sequentially cleaved by S1P/S2P to release activated N-terminal fragments, which enter the nucleus and activate gene transcription. Mutant S2P protein has impaired RIP cleavage of the transcription factors OASIS, ATF6, and SREBP. The mutations in *MBTPS2* demonstrate that RIP plays a fundamental role in bone development.

C-propeptide cleavage-site mutations increase bone mineralization.

Type I procollagen is processed to mature collagen by the removal of both N- and C-terminal propeptides. The C-propeptide is cleaved at the Ala-Asp peptide bond between the telopeptide and the C-propeptide of each chain by procollagen C-proteinase (also known BMP-1 or bone-morphometric protein). Proband with substitutions at any of the four cleavage-site residues have a high-bone-mass form of OI, first reported by our lab in a former collaboration with Katarina Lindahl [Lindahl et al., *Hum Mutat* 2011;32:598]. The patients have elevated bone-density DEXA Z-scores and, in bone histology, patchy unmineralized osteoid. The processing of the C-propeptide from collagen secreted by proband cells is delayed. Using bone mineralization density distribution (BMDD), we investigated mineralization to show that, in the alpha2(I) cleavage site mutation, the bone had a uniformly higher mineral density, while in the alpha1(I) mutation, the average mineral density was markedly heterogeneous, with areas of either very high or low bone density.

To investigate the role of the C-propeptide in bone mineralization and development, we developed a knock-in murine model with a *COL1A1* (the gene encoding pro-alpha1 type I collagen chain) cleavage site mutation. Bone collagen fibrils showed a 'barbed-wire' appearance consistent with the presence of the processing intermediate pC-collagen, which was detected in extracts of bone from mutant mice, and with impaired collagen processing *in vitro*. Impaired C-propeptide processing affects skeletal size and biomechanics. The mice are small, and their femora exhibit extreme brittleness on mechanical testing, as well as reduced fracture load. BMDD measurements on their femora show significantly higher mineralization than in WT mice, which continues to increase in the high bone-mass mice (HBM), even after in the WT mice mineralization plateaus at six months. PINP and TRAP, serum markers of bone remodeling, are significantly elevated in such HBM. Osteocyte density is reduced, but the lacunar area is increased.

Mutations in the COL1A1 C-propeptide

The C-propeptide of type I collagen (COL1A1 C-propeptide) is processed before collagen is incorporated into matrix. Mutations in the C-propeptide occur in about 6% of OI patients. Our investigation into the biochemical consequences of C-propeptide mutations revealed both intra- and extracellular differences. Procollagen with C-propeptide defects was mis-localized to the ER lumen, in contrast to the ER-membrane localization of normal procollagen. Furthermore, cleavage of the C-propeptide was defective, contributing to abnormal osteoblast differentiation and matrix function.

Insights from the Brtl mouse model for OI

The Brtl mouse model for OI, generated by our lab, is a knock-in mouse that contains a Gly349Cys substitution

in the alpha1(I) collagen chain. Brtl was modeled on a type IV OI child and accurately reproduces type IV OI features. Brtl has provided important insights into the mechanism of OI and its treatment.

We collaborated with Kenneth Kozloff's group to investigate a potential anabolic therapy, sclerostin antibody (Scl-AB), which stimulates osteoblasts via the canonical Wnt pathway. Scl-AB stimulated bone formation in young Brtl mice and increased bone mass and load-to-fracture. Treatment with Scl-AB caused no detrimental change in Brtl bone material properties. Nano-indentation studies indicated unchanged mineralization, unlike the hyper-mineralization induced by bisphosphonate treatment. In addition, Scl-AB was successfully anabolic in adult Brtl mice, and may thus be a therapy for adult patients who have fewer treatment options. Because Scl-AB is a short-acting drug, we recently investigated sequential Scl-AB/bisphosphonate treatment. The study showed that administration of a single dose of bisphosphonate after cessation of Scl-AB treatment preserved the anabolic gains from Scl-AB. Alternatively, a single low dose of bisphosphonate concurrent with Scl-AB treatment facilitated the anabolic action of Scl-AB by increasing the availability of trabecular surfaces for new bone formation. Because a lifelong deficiency of sclerostin leads to patterns of excessive cranial bone growth and nerve compression, we undertook dimensional and volumetric measurements of the skulls of Brtl mice treated with Scl-AB. Treated mice showed calvarial thickening but minimal effects on cranial morphology and anatomic landmarks. Narrowing of vascular but not of neural foramina was seen. The anti-sclerostin antibody is now entering clinical trials for pediatric OI from two pharmaceutical companies.

Brtl mice provided important information on the cytoskeletal organization in OI osteoblasts and their potential role in phenotypic variability. We observed abnormal cytoskeletal organization involving vimentin, stathmin, and cofilin-1 in lethal pups. Reduced vimentin (an intermediate filament) can lead to cytoskeletal collapse, and increased stathmin (a regulatory factor that promotes microtubular disassembly) and cofilin-1 (an inducer of actin depolymerization) work in concert to disrupt cytoskeletal cellular functions. The alterations affected osteoblast proliferation, collagen deposition, integrin, and TGF-beta signaling. The data suggest that cytoskeletal elements present novel OI treatment targets. Another potential novel treatment may be 4-PBA, a chemical chaperone. When the drug is used to treat OI cells, it enhances autophagy, as opposed to apoptosis, of the cells and stimulates protein secretion. Interestingly, the enhanced protein secretion reflects a broad range of cellular proteins rather than simply the retained mutant collagen and relieves the ER stress along the PERK pathway.

Natural history and bisphosphonate treatment of children with types III and IV OI

Pulmonary issues are the most prevalent cause of morbidity and mortality in OI. We previously published the cardiopulmonary aspects of our natural history study on types III and IV OI. Longitudinal evaluations were completed in 23 children with type III OI and 23 children with type IV OI, who had pulmonary function tests every 1–2 years. Compared with size-matched children, our patients showed a significant decline in pulmonary function over time, including in lung volumes and flow rates. The decline was worse in the 36 children with scoliosis but also occurred in 20 participants without scoliosis, who had declining function with restrictive disease, suggesting that pulmonary dysfunction of OI is attributable to a primary defect related to abnormal collagen in the lung. We are currently reporting comprehensive pulmonary phenotyping results from a cohort of 37 individuals with OI evaluated at the NIH Clinical Center. Lung function measurements, CT scans, and radiographic images from children and young adults with five different types of OI, predominantly the classical types III and IV OI, but also including the rare recessive types VI, VII, and XIV OI, were analyzed. We showed,

for the first time, that arm span or ulnar lengths are comparable height surrogates for calculating pulmonary function testing (PFT) results in patients with OI. Most patients had restrictive lung disease even at this young age, accompanied by reduced gas exchange, pointing to parenchymal issues. In depth analyses of CT scan images demonstrate a high prevalence of bronchial thickening at the level of small airways, which may be directly related to abnormal collagen or a secondary inflammatory response in OI. The functional impact of thickening of the walls of small bronchi is supported by reduced FEV_{25-75%} air flow, which also measures small airways, in all patients with type III OI. In general, severity of pulmonary manifestations was more pronounced in patients with type III OI, which is consistent with overall severity of disease in patients with this disorder. We also found that decline in pulmonary function correlates with severity of scoliosis, supporting a role for extrinsic as well as intrinsic factors in OI lung disease.

Although short stature is a cardinal feature of OI, OI-specific growth curves were not previously available. We assembled longitudinal length, weight, head circumference, and body mass index (BMI) data on 100 children with types III and IV OI with known mutations in type I collagen, to generate sex- and type-specific growth curves for OI. The data show that gender and OI type, but not the specific mutant collagen chain, have significant effects on height in OI. A pubertal growth spurt is generally absent or blunted in types III/IV OI. The BMI 50th and 95th centile curves are distinctly shifted above respective CDC curves in both genders. Interestingly, head circumference does not differ by gender, OI type, or collagen mutation.

We examined the effect of OI genotype and clinical phenotype on adiposity and resting energy expenditure in children and young adults with OI [Reference 3], comparing them with healthy controls of matched age and BMI. The fat mass percent differed only for those patients with non-collagenous mutations, in whom it was higher than in matched controls. The same subgroup of OI patients had lower resting energy expenditure, which may contribute intrinsically to their adiposity.

Our trial of bisphosphonate in children with types III and IV OI was the first randomized controlled bisphosphonate trial for OI in the United States. It examined direct skeletal and secondary gains reported in uncontrolled trials. We found increased BMD (bone mineral density) Z-scores and improved vertebral geometry. Vertebral BMD improvement tapered off after two years' treatment. Our treatment group did not experience fewer long-bone fractures, coinciding with equivocal improvement in fractures in other controlled trials. Our trial did not support claims for improved ambulation level, lower-extremity strength, or pain alleviation, suggesting these were placebo effects. Our current recommendation is for treatment for two to three years, with subsequent follow-up of bone status. We are now engaged in a dose-comparison trial, comparing the dose from our first trial with a lower dose, achieved by increasing the cycle interval at the same dose/kg/cycle. Our preliminary analysis indicates that OI children obtain comparable benefits from lower and higher doses of pamidronate.

Melorheostosis: genetic and clinical delineation

Melorheostosis is a very rare sporadic bone dysostosis that is characterized by metabolically active bone in the appendicular skeleton, which leads to asymmetric bone overgrowth, seen radiographically as 'dripping candle wax,' functional impairment, and pain. Skin overlying the bone lesion sometimes has a hyperpigmented, vascular lesion. Because attempts to identify germline mutations causing melorheostosis were unsuccessful, we proposed that somatic mutations were causative. Our collaborative team (with Tim Bhattacharyya and Nadja Fratzi-Zelman) was the first to look directly at bone samples.

Fifteen patients with melorheostosis had paired biopsies of both affected and contralateral unaffected bone. DNA from each patient's two bone samples was subjected to whole-exome sequencing (WES), and sequences from each individual patient were compared. We identified two genes causing somatic mutations in melorheostotic lesions [Reference 5]. Each gene was associated with one radiographic form of melorheostosis, and the bone lesions had distinct histology and mechanism along the TGF β pathway. Eight of the 15 patients had somatic mutations for *MAP2K1* (dual-specificity mitogen-activated protein kinase 1), located in two adjacent residues of the negative regulatory domain and that would be expected to increase MEK1 (meiotic chromosome axis-associated kinase) activity. Erythematous skin lesions overlying the affected bone are often mosaic for the *MAP2K1* mutations and have increased vascularity [Reference 5]. Our data show that the *MAP2K1* oncogene is important in human bone formation, and they implicate *MAP2K1* inhibition as a potential treatment avenue for melorheostosis.

Four patients were determined to have causative somatic mutations in *SMAD3*, a component of the canonical TGF β pathway. *SMAD3* phosphorylation was increased in affected bone, and downstream target genes of TGF β signaling had elevated expression. The mutations were associated with an endosteal radiographic pattern. Cultured osteoblasts from affected bone exhibited reduced proliferation *in vitro*, increased expression of osteoblast differentiation markers, and increased mineralization. However, the constitutive activation of the *SMAD3* dampened the activity of *BMP2*, because addition of *BMP2* to culture media reduced osteoblast differentiation and mineralization *in vitro*. Bone lesions with *SMAD3* mosaicism did not show increased cellularity or osteoid accumulation and were more highly mineralized.

Melorheostotic bone from both *MAP2K1*-positive and *SMAD3*-positive patients showed two zones of distinct morphology. In *MAP2K1*-positive melorheostosis [Reference 5], the inner osteonal zone is intensely remodeled and has increased osteoid. The zone is covered by an outer zone containing compact multi-layered lamellae. The remodeling zone has low bone mineralization and high porosity, reflecting high vascularity. The lamellar portion is less mineralized than the remodeling zone, indicating a younger tissue age. Nano-indentation was not increased in the lamellar zone, indicating that the surgical hardness of this bone reflects its lamellar structure. We propose that the genetically induced deterioration of bone micro-architecture in the remodeling zone triggers a periosteal reaction. Our current interests are to investigate communication between mutant and non-mutant cells in the affected bone, and to understand the mechanism of the *SMAD3* mutation, using a recently generated murine model.

Additional Funding

- NICHD DIR Director's Award

Publications

1. Jovanovic M, Guterman-Ram G, Marini JC. Osteogenesis Imperfecta: Mechanisms and signaling pathways connecting classical and rare OI types. *Endocr Rev* 2022 43(1):61–90.
2. Hedjazi G, Guterman-Ram G, Blouin S, Schemenz V, Wagermaier W, Fratzl P, Hartmann M, Zwerina J, Fratzl-Zelman N, Marini JC. Alterations of bone material properties in growing *Ifitm5/BRIL* p.S42 knock-in mice, a new model for atypical type VI osteogenesis imperfecta. *Bone* 2022 162:116451.
3. Ballenger KL, Tugarinov N, Talvacchio SK, Knue MM, Dang Do AN, Ahlman MA, Reynolds JC, Yanovski JA,

- Marini JC. Osteogenesis Imperfecta: the impact of genotype and clinical phenotype on adiposity and resting energy expenditure. *J Clin Endocrinol Metab* 2022 107(1):67–76.
4. Kang H, Aryal Ac S, Barnes AM, Martin A, David V, Crawford S, Marini JC. Antagonism between PEDF and TGF- β contributes to type VI Osteogenesis Imperfecta bone and vascular pathogenesis. *J Bone Miner Res* 2022 37:925–937.
 5. Jha S, Ivovic A, Kang H, Meylan F, Hanson EP, Rimland C, Lange E, Katz J, McBride A, Warner AC, Edmondson EF, Cowen EW, Marini JC, Siegel RM, Bhattacharyya T. Distribution and functional consequences of somatic MAP2K1 variants in affected skin associated with bone lesions in melorheostosis. *J Invest Dermatol* 2021 141:688-692.e11.

Collaborators

- Timothy Bhattacharyya, MD, *Clinical and Investigative Orthopedics Surgery Unit, NIAMS, Bethesda, MD*
- Susan E. Crawford, DO, *North Shore University Research Institute, University of Chicago Pritzker School of Medicine, Chicago, IL*
- David Eyre, PhD, *University of Washington, Seattle, WA*
- Antonella Forlino, PhD, *Università degli Studi di Pavia, Pavia, Italy*
- Nadja Fratzi-Zelman, PhD, *Ludwig Boltzmann-Institut für Osteologie, Hanusch Krankenhaus der WGKK und Unfallkrankenhaus Meidling, Vienna, Austria*
- Cecilia Giunta, PhD, *Kinderspital Zürich, Zürich, Switzerland*
- Wolfgang Högler, MD, DSc, FRCPC, *Birmingham Children's Hospital NHS Foundation Trust, Birmingham, United Kingdom*
- Kenneth Kozloff, PhD, *University of Michigan, Ann Arbor, MI*
- Sergey Leikin, PhD, *Section on Physical Biochemistry, NICHD, Bethesda, MD*
- Scott Paul, MD, *Rehabilitation Medicine, NIH Clinical Center, Bethesda, MD*
- Cathleen L. Raggio, MD, *Weill Medical College of Cornell University, New York, NY*
- Vorasuk Shotelersuk, MD, FABMG, *King Chulalongkorn Memorial Hospital, Bangkok, Thailand*

Contact

For more information, email marinij@mail.nih.gov.

High-Resolution Structural Biology of Membrane Protein Complexes in Their Native Environment

We are interested in the structure and function of membrane protein complexes in their native lipid-membrane environment to understand their mechanism and the influence of their immediate surrounding and how these affect human health and disease. A cell contains many different lipid membranes with various lipid contents and distributions, which are very important for a membrane's morphology and function. However, very little is understood about how the various micro-environments are formed and maintained and how they influence the structure and function of membrane proteins. Studying membrane protein complexes in their native biological membrane is therefore required.

We use a combination of molecular biology, biochemistry, and biophysical methods to study molecular transport across membranes, with a focus on how the immediate native environment influences the structure and function of membrane proteins, but also how proteins and lipids shape and functionalize a lipid membrane.

Cryo-electron microscopy (Cryo-EM) is one of the main structural-biology methods of the lab. Using single-particle Cryo-EM, we solve high-resolution structures of membrane proteins in artificial environments such as in detergent micelles and lipid nano-discs. However, we are extending this approach to studying membrane proteins in their native environment, using native lipid nano-discs, membrane fractions in forms of vesicles, and intact cells and tissues, using a combination of correlative light and electron-microscopy techniques, including cryo-fluorescent microscopy, cryo-focused ion beam-scanning electron microscopy (Cryo-FIBSEM), Cryo-EM, and cryo-electron tomography (Cryo-ET).

Structure and function of magnesium channels

Magnesium (Mg^{2+}) is the most abundant divalent cation inside cells, with an average Mg^{2+} concentration of about 20 mM, most of it bound to proteins and ATP. Magnesium plays an essential role in cellular physiology, acting as a cofactor for more than 600 enzymes, including protein kinases, ATPase, exonucleases, and other nucleotide-related enzymes. Deficiency in Mg^{2+} is associated with diseases such



Doreen Matthies, PhD, Head, Unit on Structural Biology
Fei Zhou, PhD, *Scientist*
So-Young Kim, *Visiting Postdoctoral Fellow*
Louis Tung Faat Lai, PhD, *Visiting Postdoctoral Fellow*
Elissa J. Moller, BS, *Graduate Student*



Group picture in front of NIH building 29B in April 2022

From left to right: Fei Zhou, Rick K. Huang, Allison Zeher, Louis Tung Faat Lai, Elissa Moller, Doreen Matthies

as muscular dysfunction, bone wasting, immunodeficiency, cardiac syndromes, and neuronal disorders. The bacterial magnesium channel CorA is a homo-pentameric channel, which forms a symmetric closed state at normal to high concentrations of magnesium, with magnesium-binding sites between protomers as well as near the membrane pore. At low magnesium concentrations, the channel undergoes an asymmetric opening, which is likely to be caused by the destabilization of protomer interactions when magnesium ions dissociate from their binding site. Louis Lai is expanding the research on magnesium channels, including looking at eukaryotic magnesium channels. To investigate the structure and mechanism of these channels, structural studies on synthetic and native nano-discs, as well as in liposomes are planned.

Structural determination of the full-length SARS-CoV-2 spike protein and drug development

COVID-19, caused by the SARS-CoV-2 virus, has posed a global threat since it was first identified at the end of 2019. The rapid development of vaccines has helped counteract the severeness of the disease. However, vaccines for children under the age of 12 have only just been approved. More children have been infected by more contagious variants of the virus, and the recent surge in COVID-19 cases has put an unprecedented pressure on the pediatric health care system. The SARS-CoV-2 spike protein is responsible for the initial binding of the virus to the ACE2 receptor on human cells. Better understanding of the function and structure of the spike protein is critical for primary prevention and for the development of a vaccine and therapeutic treatments to combat the COVID-19 pandemic. Structures of the spike protein's soluble ectodomain have been determined, but the full-length spike, including its membrane domain, have not been well studied. We are working towards determining the structures of full-length spike protein complexes and identifying the key vaccine- and drug-binding interfaces in order to develop treatments that block viral entry into human cells with high efficiency and specificity, and which are also safe for children. Fei Zhou has successfully cloned and expressed the full-length spike protein, and we are working towards high-resolution structural determination of different variants and complexes.

Collaborations

Our collaborations involve structural and computational studies on a variety of membrane-protein complexes, including transporters, channels, and receptors, virus-like particles (VLP), SARS-CoV-2 accessory membrane proteins, extracellular vesicles, and lipid transport across cells, as well as novel detergents and polymers to gently extract membrane-protein complexes from their native lipid environment for high-resolution structural studies.

Publications

1. He S, Chou H-T, Matthies D, Wunder T, Meyer MT, Atkinson N, Martinez-Sanchez A, Jeffrey PD, Port SA, Patena W, He G, Chen VK, Hughson FM, McCormick AJ, Mueller-Cajar O, Engel BD, Yu Z, Jonikas MC. The structural basis of Rubisco phase separation in the pyrenoid. *Nat Plants* 2020 6(12):1480–1490.
2. Qiu B, Matthies D, Fortea E, Yu Z, Boudker O. Cryo-EM structures of excitatory amino acid transporter 3 visualize coupled substrate, sodium, and proton binding and transport. *Sci Adv* 2021 7:1–9.
3. Wasmuth EV, Broeck AV, LaClair JR, Hoover EA, Lawrence KE, Paknejad N, Pappas K, Matthies D, Wang B, Feng W, Watson PA, Zinder JC, Karthaus WR, de la Cruz MJ, Hite RK, Manova-Todorova K, Yu Z, Weintraub ST, Klinge S, Sawyers CL. Allosteric interactions prime androgen receptor dimerization and activation. *Mol Cell* 2022 82:2021–2031.
4. Petersen J, Lu J, Fitzgerald W, Zhou F, Blank PS, Matthies D, Zimmerberg J. Unique aggregation of retroviral particles pseudotyped with the delta variant SARS-CoV-2 spike protein. *Viruses* 2022 14:1024.

Collaborators

- Tamir Gonen, PhD, *Laboratory of Molecular Electron Microscopy, UCLA, Los Angeles, CA*
- Rick K. Huang, PhD, *NIH Intramural Research CryoEM Consortium, NCI, Bethesda, MD*
- Maria S. Ioannou, PhD, *Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Canada*
- Marzena E. Pazgier, PhD, *Uniformed Services University of the Health Sciences, Bethesda, MD*
- Alexander J. Sodt, PhD, *Unit on Membrane Chemical Physics, NICHD, Bethesda, MD*
- Gisela Storz, PhD, *Section on Environmental Gene Regulation, NICHD, Bethesda, MD*
- Sergei I. Sukharev, PhD, *University of Maryland, College Park, MD*
- Joshua J. Zimmerberg, MD, PhD, *Section on Integrative Biophysics, NICHD, Bethesda, MD*
- Manuela Zoonens, PhD, *Institut de Biologie Physico-Chimique, Centre National de la Recherche Scientifique, Paris, France*

Contact

For more information, email doreen.matthies@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/matthies>.

Hippocampal Interneurons and Their Role in the Control of Network Excitability

Cortical and hippocampal GABAergic inhibitory interneurons (INs) are 'tailor-made' to control cellular and network excitability by providing synaptic and extrasynaptic input to their downstream targets via GABA_A and GABA_B receptors. The axons of this diverse cell population make local, short-range projections (although some subpopulations project their axons over considerable distances) and release the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) onto a variety of targets. Distinct cohorts of INs regulate sub- and supra-threshold intrinsic conductances, regulate Na⁺- and Ca²⁺-dependent action-potential generation, modulate synaptic transmission and plasticity, and pace both local and long-range large-scale synchronous oscillatory activity. An increasing appreciation of the roles played by INs in several neural-circuit disorders, such as epilepsy, stroke, Alzheimer's disease, and schizophrenia, has seen this important cell type take center stage in cortical circuit research. With almost 30 years of interest in this cell type, the main objectives of the lab have been to understand: (1) the developmental trajectories taken by specific cohorts of INs as they populate the nascent hippocampus and cortex; (2) how ionic and synaptic mechanisms regulate the activity of both local-circuit GABAergic INs and principal neurons (PN) at the level of small, well defined networks; and (3) how perturbations in their function alter the cortical network in several neural-circuit disorders. To this end, we use a variety of electrophysiological, imaging, optogenetic, immunohistochemical, biochemical, molecular, and genetic approaches with both wild-type and transgenic animals.

NMDARs drive the expression of neuropsychiatric-disorder risk genes within GABAergic interneuron subtypes in the juvenile brain.

Medial ganglionic eminence (MGE)-derived parvalbumin (PV)+, somatostatin (SST)+ and neurogliaform (NGFC)-type cortical and hippocampal interneurons have distinct molecular, anatomical, and physiological properties. However, the molecular mechanisms regulating their maturation remain poorly understood. Via single-cell transcriptomics, we showed that the obligate NMDA-type glutamate receptor (NMDAR) subunit gene *Grin1* mediates transcriptional regulation of gene expression in specific subtypes of MGE-derived



Chris J. McBain, PhD, Head,
Section on Cellular and Synaptic Physiology

Ramesh Chittajullu, PhD, *Staff Scientist*

Kenneth Pelkey, PhD, *Staff Scientist*

Adam Caccavano, PhD,
Postdoctoral Fellow

June Hoan Kim, PhD, *Postdoctoral Fellow*

Vivek Mahadevan, PhD,
Postdoctoral Fellow

Geoffrey Vargish, PhD,
Postdoctoral Fellow

Steven Hunt, BS, *Biologist*

Xiaoqing Yuan, MSc, *Biologist*

Connie Mackenzie-Gray Scott,
BSc, *Predoctoral Fellow*

Anna Vlachos, BSc,
Postbaccalaureate Fellow

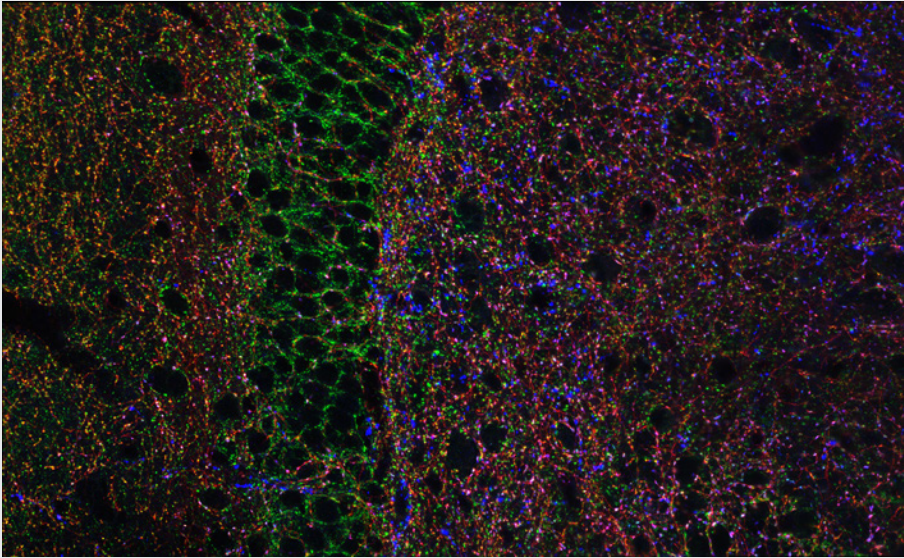


FIGURE 1. Hippocampal GABAergic terminals

Three major types of GABAergic terminal are shown in the hippocampal dentate gyrus (*red, blue, and green punch*). The black negative space shows the cell bodies of principal cells innervated by the GABAergic terminals.

interneurons, leading to altered subtype abundances. Notably, MGE-specific early developmental *Grin1* loss results in a broad downregulation of diverse transcriptional, synaptogenic, and membrane-excitability regulatory programs in the juvenile brain. These widespread gene-expression abnormalities mirror aberrations that are typically associated with neuro-developmental disorders. Our study hence provided a road map for the systematic examination of NMDAR signaling in interneuron subtypes, revealing potential MGE-specific genetic targets that could instruct future therapies of psychiatric disorders.

NPTX2 loss-of-function and schizophrenia

Memory loss in Alzheimer's disease (AD) is attributed to pervasive weakening and loss of synapses. We presented findings supporting a special role for excitatory synapses connecting pyramidal neurons of the hippocampus and cortex with fast-spiking parvalbumin (PV) interneurons, which control network excitability and rhythmicity. Excitatory synapses on PV interneurons are dependent on the AMPA receptor subunit *GluA4*, which is regulated by presynaptic expression of the synaptogenic immediate early gene *NPTX2* by pyramidal neurons. In a mouse model of AD amyloidosis, *Nptx2*^{-/-} results in reduced *GluA4* expression, disrupted rhythmicity, and increased pyramidal neuron excitability. Postmortem human AD cortex shows profound reductions in *NPTX2* and coordinate reductions in *GluA4*. *NPTX2* in human CSF is reduced in subjects with AD and shows robust correlations with cognitive performance and hippocampal volume. The findings implicate failure of adaptive control of pyramidal neuron-PV circuits as a pathophysiological mechanism contributing to cognitive failure in AD.

Resilient hippocampal gamma rhythmogenesis and parvalbumin-expressing interneuron function before and after plaque burden in 5xFAD Alzheimer's disease (AD) model

Recent studies have implicated impaired parvalbumin fast-spiking interneuron (PVIN) function as a precipitating factor underlying abnormalities in network synchrony, oscillatory rhythms, and cognition associated with AD. However, a complete developmental investigation into potential gamma deficits, induced

by commonly used carbachol or kainate in *ex vivo* slice preparations, within AD model mice is lacking. We examined gamma oscillations, using field recordings in acute hippocampal slices from *5xFAD* (which carry five familial AD mutations) and in control mice, through the period of developing pathology, starting at three months of age, when there is minimal plaque presence in the hippocampus, through to 12 or more months of age, when plaque burden is high. In addition, we examined PVIN participation in gamma rhythms, using targeted cell-attached recordings of genetically reported PVINs, in both wild-type and mutant mice. In parallel, we compared a developmental immunohistochemical characterization, probing the PVIN-associated expression of PV and perineuronal nets (PNNs), between control and *5xFAD* mice. Remarkably, this comprehensive longitudinal evaluation failed to reveal any obvious correlations between PVIN deficits (electrical and molecular), circuit rhythmogenesis (gamma frequency and power), and A β deposits/plaque formation. By six to 12 months, *5xFAD* animals exhibit extensive plaque formation throughout the hippocampus. However, a deficit in gamma oscillatory power was only evident in the oldest *5xFAD* animals (over 12 months), and only when using kainate, and not carbachol, to induce the oscillations. We found no difference in PV firing or phase preference during kainate-induced oscillations in younger or older *5xFAD* mice compared with control, and a reduction of PV and PNNs only in the oldest *5xFAD* mice. The lack of a clear relationship between PVIN function, network rhythmicity, and plaque formation in our study highlights an unexpected resilience in PVIN function in the face of extensive plaque pathology associated with this model, calling into question the presumptive link between PVIN pathology and AD progression.

A versatile viral toolkit for functional discovery in the nervous system

The ability to precisely control transgene expression is essential for basic research and clinical applications. Adeno-associated viruses (AAVs) are non-pathogenic and can be used to drive stable expression in virtually any tissue, cell type, or species, but their limited genomic payload results in a trade-off between the transgenes that can be incorporated and the complexity of the regulatory elements controlling their expression. Resolving these competing imperatives in complex experiments inevitably results in compromises. We assembled an optimized viral toolkit (VTK) that addresses these limitations and allows for efficient combinatorial targeting of cell types. Moreover, their modular design explicitly enables further refinements. We achieved this in compact vectors by integrating structural improvements of AAV vectors with innovative molecular tools. We illustrated the potential of this approach through a systematic demonstration of their utility for targeting cell types and querying their biology, using a wide array of genetically encoded tools.

Additional Funding

- Connie Mackenzie Gray-Scott was funded by an NIH-Wellcome Trust Graduate Student award.

Publications

1. Mahadevan V, Mitra A, Zhang Y, Yuan X, Peltekian A, Chittajallu R, Esnault C, Maric D, Rhodes C, Pelkey KA, Dale R, Petros T, McBain CJ. NMDARs drive the expression of neuropsychiatric disorder risk genes within GABAergic interneuron subtypes in the juvenile brain. *Front Mol Neurosci* 2021 14:712609.
2. Xiao MF, Roh SE, Zhou J, Lucey B, Craig MT, Hays L, Jia M, Chien CC, Xu D, Zhou W, Talbot C, Weinberg D, Sawa A, Pelkey KA, McBain CJ, Savonenko A, Worley PF. A biomarker-authenticated model of schizophrenia implicating NPTX2 loss of function. *Sci Adv* 2021 7(48):eabf6935.
3. Mackenzie-Gray Scott CA, Pelkey KA, Caccavano A, Abebe D, Lai M, Black KN, Brown N, Trevelyan AJ,

McBain CJ. Resilient hippocampal gamma rhythmogenesis and parvalbumin-expressing interneuron function before and after plaque burden in 5xFAD Alzheimer's disease model. *Front Synaptic Neurosci* 2022 14:857608.

4. Pouchelon G, Vergara J, McMahon J, Gorissen B, Lin J, Vormstein-Schneider D, Niehaus JL, Burbridge T, Wester JC, Sherer M, Fernandez-Otero M, Allaway K, Pelkey K, Chittajallu R, McBain CJ, Fan M, Nasse J, Wildenberg J, Fishell G, Dimidschstein J. A versatile viral toolkit for functional discovery in the nervous system. *Cell Rep Methods* 2022 2:100225.

Collaborators

- Jordan Dimidschstein, PhD, *The Broad Institute, Cambridge, MA*
- Gordon Fishell, PhD, *The Broad Institute, Cambridge, MA*
- Timothy J. Petros, PhD, *Unit on Cellular and Molecular Neurodevelopment, NICHD, Bethesda, MD*
- Andrew Trevelyan, DPhil, MBBCh, MA, *Newcastle University, Newcastle, United Kingdom*
- Paul F. Worley, MD, *Johns Hopkins University School of Medicine, Baltimore, MD*
- Kareem Zaghloul, MD, PhD, *Functional and Restorative Neurosurgery Unit, NINDS, Bethesda, MD*

Contact

For more information, email mcbainc@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/mcbain>.

Pathophysiology, Genetics, and Treatment of Congenital Adrenal Hyperplasia

In its most severe classic form, congenital adrenal hyperplasia (CAH) is a life-threatening, rare orphan disease that is part of the neonatal screen performed in all 50 U.S. states. In its mildest nonclassic form, CAH is one of the most common autosomal recessive diseases and may be a common cause of female infertility. Our research program strives to elucidate the pathophysiology and genetics of CAH, thus facilitating the development of new approaches to the diagnosis, evaluation, and treatment of the disease. We are conducting the largest ever Natural History Study of CAH, with over 450 patients enrolled. We were the first to identify adrenaline deficiency as a new hormonal imbalance in CAH and the first to report smaller-than-normal amygdala, the emotion regulator of the brain, in CAH, providing insight into hormonal effects on the brain. We found that approximately 15 percent of patients with CAH owing to 21-hydroxylase deficiency have a contiguous gene-deletion syndrome resulting in connective tissue dysplasia and a hypermobility-type Ehlers-Danlos syndrome, which represents a novel phenotype named CAH-X. Central to our work is the study of new treatments, including a long-term trial testing sex hormone blockade in children, and novel ways of replacing cortisol, aimed at mimicking the normal circadian rhythm of cortisol secretion. The NIH Clinical Center is the ideal venue in which to carry out such studies and is one of the few places in the world that facilitates the conduct of long-term studies of rare diseases.

Genotype-phenotype studies of CAH-X

CAH is most commonly caused by 21-hydroxylase deficiency. The gene encoding 21-hydroxylase, *CYP21A2*, and a highly homologous pseudogene, *CYP21A1P*, map to the short arm of chromosome 6 within the human leukocyte antigen histocompatibility complex. The deleterious sequence in the *CYP21A1P* pseudogene can be transferred to the *CYP21A2* functional gene by homologous recombination, and such events produce common mutations that account for approximately 95% of all *CYP21A2* disease-causing mutations. Of the common mutations, approximately 30% are large deletions. The *TNXB* gene, encoding tenascin-X, an extracellular matrix protein that is highly expressed in connective tissue, and a highly homologous pseudogene, *TNXA*, flank *CYP21A2* and *CYP21A1P*, respectively. Autosomal recessive tenascin-X deficiency was described as a cause of Ehlers-Danlos syndrome in 2001.



Deborah P. Merke, MD, MS,
*Adjunct Investigator, NICHD,
and Chief, Section of Congenital
Disorders, Clinical Center*

Deepika D. Burkardt, DO, *Staff
Clinician*

Rida Javaid, MD, *Staff Clinician*

Vipula Kolli, PhD, *Staff Scientist*

Qizong Lao, PhD, *Staff Scientist*

Elizabeth Joyal, MSN, *Nurse
Practitioner*

Lee Ann Keener, MSN, *Nurse
Practitioner*

Sarah Kollender, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Annie Schulman, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Charles Sukin, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

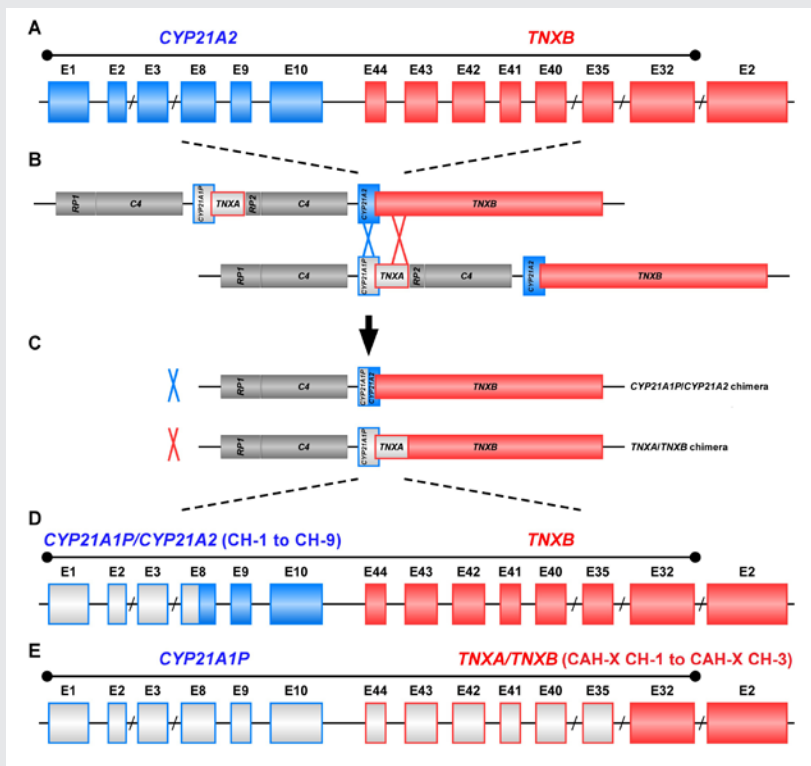


FIGURE 1. Schematic of *CYP21A1P/CYP21A2* and *TNXA/TNXB* chimeric genes

Formation of chimeric genes occurs as a result of misalignment of homologous genes during meiosis. Active genes are in solid colors; pseudogenes are in grey and are framed with the color of the corresponding functional gene. Representative chimeric genes are shown. In total, there are nine known *CYP21A1P/CYP21A2* chimeras (CH-1 to CH-9), and we identified three different types of *TNXA/TNXB* chimeras (CAH-X CH-1 to CAH-X CH-3) with different junction sites. Approximately 10 percent of patients with CAH owing to 21-hydroxylase deficiency carry at least one *TNXA/TNXB* chimera, resulting in hypermobility-type Ehlers-Danlos syndrome or CAH-X syndrome.

We hypothesized that deletions of *CYP21A2* might commonly extend into the *TNXB* gene, and we have been studying this phenomenon in our Natural History Study.

The first evaluation of the potential clinical implications of *TNXB* heterozygosity in CAH patients was performed in our Natural History Study of CAH (www.ClinicalTrials.gov Identifier No. [NCT00250159](https://clinicaltrials.gov/ct2/show/study/NCT00250159)) at the NIH Clinical Center. In 2013, we prospectively studied 193 consecutive unrelated patients with CAH with clinical evaluations for manifestations of Ehlers-Danlos syndrome and genetic evaluations for *TNXB* mutations. Heterozygosity for a *TNXB* deletion was present in 7% of CAH patients; such CAH patients were more likely than age- and sex-matched CAH patients with normal *TNXB* to have joint hypermobility, chronic joint pain, multiple joint dislocations, and a structural cardiac valve abnormality detected by echocardiography. Six of 13 probands had a cardiac abnormality, including the rare quadricuspid aortic valve, a left ventricular diverticulum, and an elongated anterior mitral valve leaflet. As a result of the study, the term CAH-X was coined to describe the subset of CAH patients who display an Ehlers-Danlos syndrome phenotype resulting from the monoallelic presence of a *CYP21A2* deletion extending into the *TNXB* gene.

The study of CAH-X has provided insight into the recombination events that occur in the class III region of the major histocompatibility complex (MHC) locus, a region in the genome that is predisposed to genetic recombination and misalignment during meiosis. The majority of deletions generate chimeric *CYP21A1P/CYP21A2* genes. Chimeric recombination between *TNXB* and *TNXA* also occurs (Figure 1). The recombination event deletes *CYP21A2* and therefore represents a CAH disease-causing allele. We described three unique types of *TNXA/TNXB* chimera (CH): CAH-X CH-1 renders the gene nonfunctional, resulting in reduced dermal and serum TNX

expression; CAH-X CH-2 alters protein structure; and CAH-X CH-3 is predicted to reduce protein folding energy. Our laboratory continues to investigate how *TNXB* contributes to the phenotype of CAH patients.

To date, we have described 24 patients (19 families) with monoallelic CAH-X and three patients with biallelic CAH-X. It is now estimated that approximately 15% of patients with CAH resulting from 21-hydroxylase deficiency are affected by CAH-X. Overall, CAH-X patients have generalized joint hypermobility, subluxations, and chronic arthralgia, and about 25% have cardiac structural abnormalities. Patients with biallelic CAH-X show severe skin hyperextensibility with delayed wound healing and significant joint hypermobility. Other connective-tissue disease manifestations in CAH-X patients include chronic tendonitis and/or bursitis, rectal prolapse, severe gastroesophageal reflux, and cardiac abnormalities. Genetic testing for CAH-X is complex and complicated by pseudogene interference and the large (70kb) size of the *TNXB* gene. In 2019, we developed a PCR-based, high-throughput, cost-effective assay that accurately identifies CAH-X. The assay had 100% sensitivity and 99.2% specificity.

The study of the CAH-X syndrome provides insight into the complex clinical and genetic characteristics associated with CAH and promises to improve patient outcome through the development of focused medical management aimed at preventing long-term consequences.

Pathophysiology of tumor formation

Patients with CAH are at risk for tumor formation, with the common development of adrenal tumors and adrenal rest tumors (ARTs). ARTs are extra-adrenal-like masses with similarities to adrenocortical tissue. Testicular ARTs (TARTs) are commonly observed in men with CAH, often cause infertility, and are easily detected by ultrasound. The etiology of TARTs and factors contributing to their origin and progression are not completely clear. Some studies support the concept that these benign tumors arise from pluripotent progenitor cells or from cells that are adrenal in origin and which descend with the testis during embryogenesis.

Understanding the pathogenesis and functional features of tumor formation is essential for developing treatment strategies. We performed the first study describing the structural morphology of the cells residing in adrenals from patients with CAH in comparison with ARTs, and we also performed gene-expression studies. We found that CAH-affected adrenal glands and ARTs have similar expression profiles and morphology, demonstrating mostly zona-reticularis characteristics and lymphocytic infiltration, suggesting a common origin that is similarly affected by the abnormal hormonal milieu. The study provided a comprehensive characterization of CAH-affected adrenals and ARTs in relation to control tissues, thus providing insight into disease-specific tissue transformation. In addition, immune system modulators may play a role in tumor formation in CAH.

New and improved biomarkers of CAH

The diagnosis and management of CAH has been limited by inadequate biomarkers. Several pitfalls were identified in the use of 17 α -hydroxyprogesterone (17-OHP) and androstenedione, the traditional biomarkers used for disease management. The development of liquid chromatography-tandem mass spectrometry (LC-MS/MS) panels of adrenal steroids has expanded the repertoire of potential new and improved steroid biomarkers. Adrenal-derived 11-oxygenated androgens have emerged as potential new biomarkers, given that traditional biomarkers are subject to variability and are not adrenal-specific, contributing to management challenges. We found that steroids synthesized with the participation of 11 β -hydroxylase (11-oxygenated C19 steroids) are abundant in patients with classic CAH resulting from 21-hydroxylase deficiency, and correlate

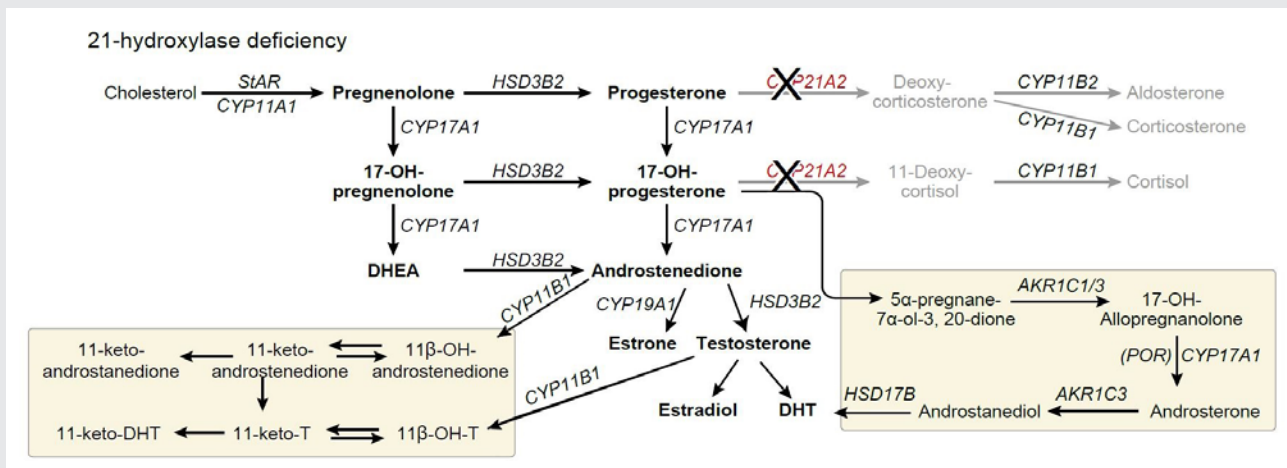


FIGURE 2. Classic and alternative steroidogenesis pathways leading to adrenal androgen production

In 21-hydroxylase deficiency, elevations of 17 α -hydroxyprogesterone and androstenedione can activate alternative steroidogenic pathways (yellow boxes).

well with long-term disease control and disease-specific comorbidities (e.g., increased adrenal volume, TARTs, menstrual irregularity, hirsutism) (Figure 2).

With our collaborators Richard Auchus and Adina Turcu, we evaluated 24-hour 11-oxygenated androgens and found diurnal variability in these promising novel biomarkers. We also compared traditional and 11-oxygenated androgens in patients with nonclassic (mild) CAH resulting from 21-hydroxylase deficiency and patients with symptoms of hyperandrogenism from other causes. Patients with nonclassic CAH present with clinical manifestations of hyperandrogenism, features that are shared with other disorders of androgen excess. In particular, the clinical phenotype of women with nonclassic CAH is similar to the more common polycystic ovary syndrome. The diagnosis of nonclassic CAH is based on serum 17-OHP and usually requires dynamic testing with synthetic ACTH (cosyntropin). We found that 11-oxygenated C19 steroids are disproportionately elevated compared with conventional androgens in nonclassic CAH, and that steroid panels can accurately diagnose nonclassic CAH in unstimulated blood tests.

In a retrospective analysis of approximately 2,800 laboratory assessments obtained as part of the Natural History Study of CAH at the NIH Clinical Center, we found discrepant 17-OHP and androstenedione in 469 (17%) of laboratory assessments. Of these, 403 (86%) had elevated 17-OHP with androstenedione in the reference range. Using frozen serum, we evaluated the utility of 11-oxygenated C19 steroids in the setting of inconclusive conventional biomarkers. We found that 11-hydroxytestosterone provided the best discrimination between poor and good clinical control. We continue to explore the utility of these newly described steroids in the diagnosis and management of CAH.

Novel treatment approaches: circadian cortisol replacement

Humans have biological clocks with characteristic patterns of hormone secretion. Cortisol has a circadian

rhythm, with levels low at sleep onset, rising between 0200hr and 0400hr, peaking in the early morning, and then declining throughout the day. Existing glucocorticoid replacement is non-physiologic, and the lack of diurnal rhythm may contribute to the many adverse outcomes observed in patients with adrenal insufficiency. In CAH, physiologic cortisol replacement might improve control of adrenal androgens at lower glucocorticoid doses, thus improving patient outcome. A promising treatment approach we have thus been studying is circadian cortisol replacement in patients with CAH.

In 2016, we successfully replaced cortisol in a physiologic manner through the use of a pump usually used to deliver insulin. A programmed 24-hour infusion of hydrocortisone was delivered subcutaneously for six months to eight patients with adrenal insufficiency resulting from 21-hydroxylase deficiency and with multiple comorbidities. Following six months of pump therapy, patients experienced significant improvement in disease control at similar or lower daily doses of glucocorticoid, and significant improvement in their quality of life and fatigue compared with oral conventional therapy. The improvements achieved in androgen control, lean body mass, and health-related quality of life after six months of pump therapy were maintained at eighteen months.

Our group was the first to study circadian cortisol replacement in CAH patients with the use of a modified-release formulation of hydrocortisone, (MR-HC, Chronocort®, CRADA #02800). We successfully completed a phase 2, open-label trial of 16 adults with classic CAH. Compared with various forms of conventional therapy prior to entry, six months of twice daily MR-HC yielded improved disease control throughout the day, using a lower hydrocortisone dose equivalent. Successful completion of this phase 2 study, carried out at the NIH Clinical Center, resulted in a multicenter international phase 3, parallel arm, randomized, open-label study to determine whether this new modified-release preparation of hydrocortisone improves short-term clinical outcome. 122 Adults with classic CAH completed the phase 3 study in 2022. The primary endpoint, 17 α -hydroxyprogesterone 24-hour area under the curve standard deviation, did not differ between the two groups; however, improved biochemical control of CAH was observed in the morning and early afternoon in those receiving the MR-HC compared with standard treatments. Sustained benefits with decreased dosage were observed in 18 months extension. Based on these data, MR-HC is now licensed in the UK and Europe. Patients who were enrolled in the phase 3 study are continuing MR-HC therapy in a long-term follow-up study, and future US studies are being planned.

Studies of circadian cortisol replacement provide insight into the role that circadian rhythm plays in the development of the comorbidities associated with adrenal insufficiency. Physiologic cortisol replacement represents a novel treatment approach that promises to improve treatment outcome for patients with CAH, as well as other forms of adrenal insufficiency.

Novel treatment approaches: sex steroid blockade and inhibition

As an alternative approach to the treatment of CAH, the effects of elevated androgen and estrogen could be prevented through the use of sex steroid blockade. Short-term (two-year) administration of an antiandrogen and aromatase inhibitor and reduced hydrocortisone was shown to normalize linear growth rate and bone maturation. A prospective long-term randomized parallel study of the effect of an antiandrogen (flutamide) and an aromatase inhibitor (letrozole), as well as reduced hydrocortisone dose vs. conventional treatment, on adult height is near completion; we will compare data between the treatment groups. The goal of this novel treatment approach is to normalize the growth and development of children with CAH and, ultimately, to determine whether the treatment regimen is effective in improving the growth of children with CAH.

Since the inception of our study of peripheral blockade of sex hormones using an antiandrogen and aromatase inhibitor, new and improved drugs that block sex steroids have been developed. In collaboration with the group of Perrin White, we are studying abiraterone, an irreversible inhibitor of 17 α -hydroxylase, a key enzyme required for testosterone synthesis, in a multicenter Phase 1/2 study in prepubescent children ([NCT02574910](#)).

Novel treatment approaches: hypothalamic–pituitary–adrenal axis suppression

Potential strategies to address the drivers of excess androgen production in CAH include suppression of the hypothalamic-pituitary-adrenal axis. Newly developed small-molecule antagonists of the corticotropin-releasing factor type 1 (CRF1) receptor antagonist are being studied. In two multicenter, open-label phase II studies of adults with classic CAH, Tildacerfont (Spruce Biosciences, USA) was given at various doses once or twice daily over two weeks and, as a 400 mg dose, for three months. Efficacy analysis was performed based on baseline androstenedione criteria (poor disease control was defined as androstenedione levels more than two times the upper limit of normal and good disease control as less than the upper limit of normal). In comparison with baseline in patients with poorly controlled disease, Tildacerfont treatment induced reductions of disease biomarkers including an average reduction of 74% for circulating levels of ACTH, 82% for levels of 17-hydroxyprogesterone, and 55% for levels of androstenedione. By contrast, ACTH and androgen biomarkers remained stable for patients with good disease control. Overall, Tildacerfont was well tolerated. Multicenter, randomized, double-blind, placebo-controlled, long-term (52 weeks) clinical trials in adults with CAH ([NCT04490915](#), [NCT04457336](#), [NCT04544410](#)) are under way to study the effect of CRF-1 antagonists on outcomes such as improved adrenal androgen control, as well as the potential of reducing glucocorticoid dose as an add-on therapy.

Additional Funding

- Cooperative Research and Development Agreement (CRADA) #02800 for Age-Appropriate Hydrocortisone Formulations for the Treatment of Adrenal Insufficiency including Congenital Adrenal Hyperplasia
- NIH U Grant: Abiraterone Acetate in Children with Classic 21-Hydroxylase Deficiency

Publications

1. Merke DP, Mallappa A, Arlt W, Brac de la Perriere A, Hirschberg AL, Juul A, Newell-Price J, Perry CG, Prete A, Rees DA, Reisch N, Stikkelbroeck N, Tourraine P, Maltby K, Treasure FP, Porter J, Ross RJ. Modified-release hydrocortisone in congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 2021 106(5):e2063–e2077.
2. Kolli V, Werneck da Cunha I, Kim SA, Iben JR, Mallappa A, Li T, Gaynor A, Coon SI, Quezado MM, Merke DP. Morphologic and molecular characterization of adrenals and adrenal rest affected by congenital adrenal hyperplasia. *Front Endocrinol* 2021 12:730947.
3. Torky A, Sinai N, Jha S, El-Maouche, Desai J, El-Maouche D, Mallappa A, Merke DP. Cardiovascular disease risk factors and metabolic morbidity in a longitudinal study of congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 2021 106(12):e5247–5257.
4. Mallappa A, Merke DP. Management challenges and therapeutic advances in congenital adrenal hyperplasia. *Nat Rev Endocrinol* 2022 18(6):337–352.
5. Sarafoglou K, Barnes CN, Huang M, Imel EA, Madu IJ, Merke DP, Moriarty D, Nakhle S, Newfield RS, Vogiatzi MG, Auchus RJ. Tildacerfont in adults with classic congenital adrenal hyperplasia: results from two phase 2 studies. *J Clin Endocrinol Metab* 2021 106(11):e4666–4679.

Collaborators

- Richard J. Auchus, MD, PhD, *University of Michigan, Ann Arbor, MI*
- Veronica Gomez-Lobo, MD, *Pediatric and Adolescent Gynecology, NICHD, Bethesda, MD*
- James Marko, MD, *Radiology and Imaging Sciences, NIH Clinical Center, Bethesda, MD*
- Martha Quezado, MD, *Laboratory of Pathology, Center for Cancer Research, NCI, Bethesda, MD*
- Richard J. Ross, MD, *University of Sheffield, Sheffield, United Kingdom*
- Ninet Sinaii, PhD, MPH, *Biostatistics and Clinical Epidemiology Service, NIH Clinical Center, Bethesda, MD*
- Adina Turcu, MD, *University of Michigan, Ann Arbor, MI*
- Perrin White, MD, *University of Texas Southwestern Medical Center, Dallas, TX*

Contact

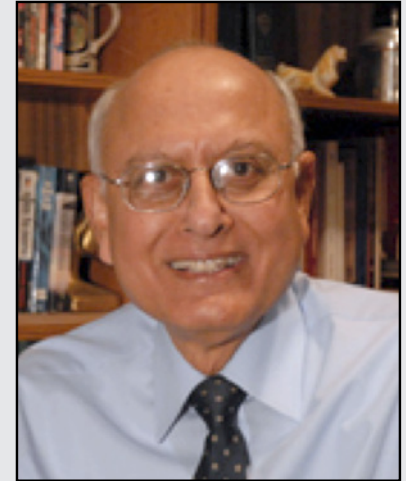
For more information, email dmerke@nih.gov or visit <https://irp.nih.gov/pi/deborah-merke>.

Childhood Neurodegenerative Lysosomal Storage Disorders

We conduct both basic and translational research into a group of the most common childhood neurodegenerative lysosomal storage disorders (LSDs), called neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease, diseases that mostly affect children, and there is no curative treatment for any of the NCLs. Mutations in at least 13 different genes (called *CLNs*) underlie various forms of NCLs. The infantile NCL (INCL), a fatal neuro-degenerative LSD, is caused by inactivating mutations in the *CLN1* gene. Our investigations focus on understanding the molecular mechanism(s) of pathogenesis underlying INCL (CLN1 disease), juvenile NCL (JNCL: CLN3 disease), and congenital NCL (CNCL: CLN10 disease). Interestingly, all NCL types share some common clinical features such as epileptic seizures, progressive psychomotor decline, and visual impairment resulting from retinal degeneration. The pathologic features include intracellular accumulation of autofluorescent material, neuroinflammation, cortical atrophy, and shortened lifespan.

Several years ago, we initiated investigations on INCL. Numerous proteins, especially in the brain, require *S*-palmitoylation (also called *S*-acylation). While *S*-palmitoylation plays important roles in membrane anchorage of soluble proteins, protein-protein interactions, protein trafficking, and protein stability, such lipid-modified proteins must also be depalmitoylated for recycling or degradation and clearance by lysosomal hydrolases. Dynamic *S*-palmitoylation requires coordinated actions of two types of enzyme with opposing functions. The enzymes that catalyze *S*-palmitoylation are palmitoyl acyltransferases (PATs), which are zinc-finger proteins with a common DHHC (Asp-His-His-Cys) motif, and they are called ZDHHC PATs or simply ZDHHCs. The mammalian genome encodes a family of 23 ZDHHC PATs. Similarly, the palmitoyl thioesterases, which depalmitoylate *S*-acylated proteins, are localized either in the lysosomes like PPT1 or in the cytoplasm like acyl-protein thioesterase-1 (APT1). Recently, several protein depalmitoylases, called ABHD17, were identified that catalyze the turnover of N-Ras (a GTP-ase signal-transduction protein).

The aim of our translational research is to apply the knowledge gained from our basic laboratory investigations to develop novel therapeutic strategies for Batten disease. The results of our earlier investigations on INCL led to a bench-to-bedside clinical trial. Using *Cln1*-knockout



Anil B. Mukherjee, MD, PhD,
*Head, Section on Developmental
Genetics*

Maria B. Bagh, PhD, *Staff Scientist*

Abhilash Appu, PhD, *Visiting
Fellow*

Avisek Mondal, PhD, *Visiting
Fellow*

Nisha Plavelil, PhD, *Visiting Fellow*

Tamal Sadhukhan, PhD, *Visiting
Fellow*

Koyel Roy, MSc, *Contract Biologist*

Sriparna B. Sadhukhan, MSc,
Contract Biologist

(*Cln1*^{-/-}) mice, which recapitulate virtually all clinical and pathological features of INCL, we discovered that PPT1 deficiency causes endoplasmic-reticulum (ER) and oxidative stress, which at least in part causes neuronal death by apoptosis. During the past several years, we also delineated a mechanism by which PPT1 deficiency disrupts the recycling of synaptic-vesicle (SV) proteins, which are essential for generating fresh SVs to replenish the SV pool size at the nerve terminals so as to maintain uninterrupted neurotransmission. We also discovered that ER and oxidative stress contribute to neuronal apoptosis and neuro-inflammation in INCL. Further, we found that PPT1 deficiency causes mis-routing of the V0a1 subunit of v-ATPase (the proton pump on the lysosomal membrane), which dysregulates lysosomal acidification, causing elevated pH and thus adversely affecting lysosomal degradative function.

We also developed a non-invasive method, using MRI and MRS (magnetic resonance spectroscopy) to evaluate the progression of neurodegeneration in *Cln1*^{-/-} mice. The methods permit repeated evaluation of potential therapeutic agents in treated animals. Application of such methods in our clinical trial with INCL also allowed us to evaluate the progressive decline in brain volume and neurodegeneration. In collaboration with Wadih Zein, we are also conducting studies to determine whether electro-retinography can be used to assess the progressive retinal deterioration in *Cln1*^{-/-} as well as in *Cln1*-knockin (KI) mice, which carry the nonsense mutation in the *CLN1* gene commonly found in the INCL patient population in the US. Moreover, we discovered that the blood-brain barrier is disrupted in *Cln1*^{-/-} mice and that this pathology is ameliorated by treatment with resveratrol, which has antioxidant properties. More recently, we discovered that a nucleophilic small molecule with antioxidant properties, *N*-(*tert*-butyl) hydroxylamine (NtBuHA), ameliorates the neurological abnormalities in *Cln1*^{-/-} mice and extends their lifespan. The compound is currently undergoing preclinical evaluation for the approval of an IND by the FDA. Intriguingly, we discovered that in *Cln1*^{-/-} mice the lysosomes contain insufficient amounts of PPT1 protein and PPT1-enzymatic activity, contributing to neuro-pathology in this disease. These and related studies provide insight into the complex mechanisms of heritable disorders of neurodegeneration such as CLN1 disease (INCL), as well as CLN3 disease (JNCL), and identify several potential therapeutic targets. Our results suggest that thioesterase-mimetic small molecules such as NtBuHA are potential therapeutics for INCL and may even be for JNCL. More recently, we discovered that cathepsin D (CD) deficiency in lysosomes is a common pathogenic link between CLN1 disease and CLN10 disease (CNCL). Our ongoing laboratory and translational investigations are attempting to advance our knowledge of INCL, JNCL, and congenital NCL (CNCL) diseases.

Mistargeted NPC1 protein to the plasma membrane promotes cholesterol-mediated mTORC1 activation, contributing to INCL pathogenesis.

As stated above, inactivating mutations in the *CLN1* gene cause INCL. *CLN1* encodes palmitoyl-protein thioesterase-1 (PPT1), a lysosomal depalmitoylating enzyme. Numerous proteins, especially in the brain, undergo *S*-palmitoylation, which confers hydrophobicity, increases membrane-affinity, and promotes protein-protein interactions. Moreover, dynamic *S*-palmitoylation (palmitoylation-depalmitoylation) facilitates intracellular protein trafficking. Despite the discovery that inactivating mutations in the *CLN1* gene encoding PPT1 cause INCL, a clear picture of the pathogenic mechanism of this devastating lysosomal storage disease (LSD) has not emerged for more than two decades. In addition to its degradative function, the lysosome plays a pivotal role in cholesterol homeostasis; it is the major cellular sorting station for dietary cholesterol. Cholesterol is transported to the late endosome/lysosome and is exported to diverse cellular compartments,

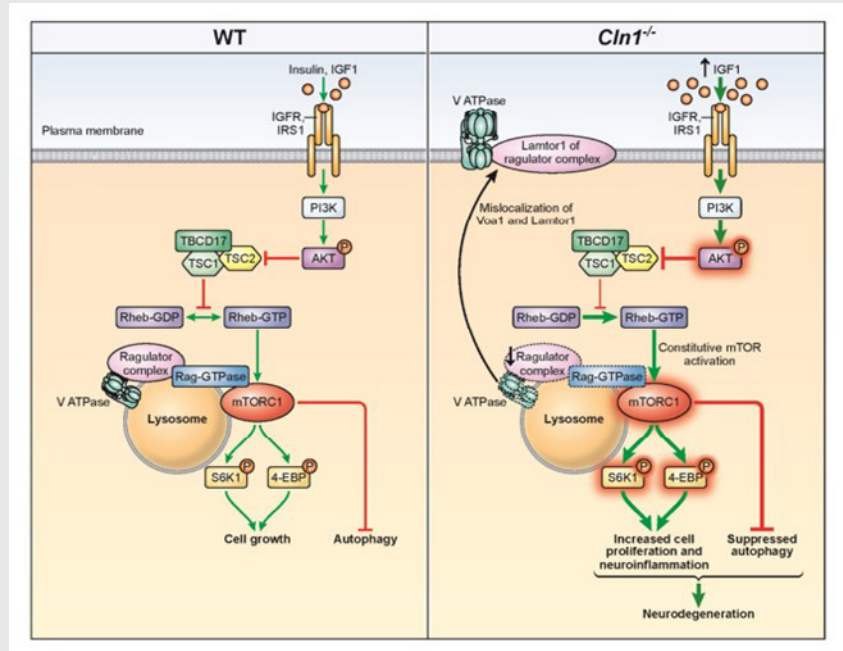
including to the plasma membrane and the endoplasmic reticulum (ER). The Niemann Pick C1 (NPC1) protein, localized to the lysosomal limiting membrane, plays a critical role in sterol trafficking, and its inactivation causes the hereditary neurodegenerative lipid storage disorder Niemann-Pick type C (NPC). Recently, it was reported that cells from patients with NPC had increased cholesterol on the lysosomal limiting membrane, which mediates the activation of the mechanistic (mammalian) target of rapamycin complex 1 (mTORC1) protein kinase. The mTORC1 kinase integrates intracellular as well as environmental cues to regulate cell growth and metabolism. Aberrant activation of mTORC1 signaling negatively regulates autophagy, which is the principal pathway for lysosomal degradation and clearance of abnormal protein aggregates and damaged organelles. Remarkably, in all three types of autophagy, the lysosome plays pivotal roles in the degradation of cargo contained in the autophagosomes. Most notably, the dysregulation of autophagy has been implicated not only in the pathogenesis of common neurodegenerative diseases such as Alzheimer's and Parkinson's, but also in most of the LSDs, in which neurodegeneration is a frequent and devastating manifestation.

To understand the mechanism of INCL pathogenesis, we used the *Cln1*^{-/-} mouse, a reliable animal model of INCL. In the brain of these mice, total cholesterol levels have been reported to be significantly higher than those in their WT littermates. However, in that study the levels cholesterol in lysosomes were not evaluated. We thus first determined the cholesterol levels in total lysates of cortical tissues from 2-, 4- and 6-month-old wild-type (WT) and *Cln1*^{-/-} mice as well as in lysosomal fractions from those tissues. We found that cholesterol levels in total lysates as well as in lysosomal fractions from *Cln1*^{-/-} mice of all three age groups were significantly higher than those in their WT littermates. These results were further confirmed by confocal imaging in neurons from *Cln1*^{-/-} mouse brain, which showed a substantially higher level of colocalization of Filipin III-stained cholesterol with lysotracker red. Taken together, these results raised the possibility that in the brain of *Cln1*^{-/-} mice lysosomal cholesterol homeostasis is dysregulated. Cholesterol enters the cell in its esterified form packaged with lipoproteins. The low-density lipoprotein (LDL) receptor (LDLR)-bound cholesterol enters the cell via a receptor-mediated pathway. Once within the cell, cholesterol esters are hydrolyzed by lysosomal acid lipase, liberating cholesterol, which is then transported to various cellular structures, including the ER and the plasma membrane. The Niemann-Pick C1 (NPC1) and NPC2 proteins mediate lysosomal cholesterol egress and import, respectively. Moreover, a lysosomal integral membrane protein (LIMP) 2/SCARB2 has also been reported to bind cholesterol and, like NPC1, it transports cholesterol through a transglycolyx tunnel, a membrane domain rich in glycoproteins and glycolipids. Under normal circumstances, the balance between the export and import maintains lysosomal cholesterol homeostasis. Thus, inactivating mutations in either the *NPC1* or *NPC2* gene dysregulates cellular cholesterol homeostasis, causing a profound neurological dysfunction, leading to a fatal neurodegenerative LSD, Niemann-Pick type C disease.

We found that Niemann-Pick C1 (NPC1) protein, which mediates lysosomal cholesterol egress, requires dynamic S-palmitoylation for trafficking to the lysosomal membrane. Intriguingly, in *Cln1*^{-/-} mice, NPC1 mistargeting to the plasma membrane caused increased oxysterol-binding protein (OSBP) on lysosomal membrane, activating cholesterol-mediated mTORC1 signaling. Activated mTORC1 signaling suppressed autophagy, contributing to neurodegeneration. Importantly, treatment of *Cln1*^{-/-} mice with a pharmacological inhibitor of OSBP suppressed mTORC1 activation, rescued autophagy, and ameliorated neuropathology. Our findings reveal a previously unrecognized pathway to INCL pathogenesis and suggest that suppression of cholesterol-mediated activation of mTORC1 signaling may have therapeutic implications.

FIGURE 1. Palmitoyl-protein thioesterase-1 deficiency disrupts the lysosomal nutrient-sensing scaffold, promoting IGF1-mediated aberrant mTORC1 activation, thus contributing to INCL pathogenesis.

Schematic representation of mTORC1 activation in WT (left panel) and *Cln1*^{-/-} (right panel) mice. mTORC1 integrates signals from growth factors, nutritional status, energy status and stress to regulate cell growth and proliferation through the phosphorylation of substrates that activate various anabolic processes like protein, lipid and nucleotide synthesis and inhibit catabolic processes like autophagy. The LNSS complex (consisting of V-ATPase and Rag-Regulator) plays a critical role in amino acid sensing and mTORC1 activation. The growth factor pathway consisting of the PI3K and AKT also regulates mTORC1 activation through the TSC complex (TSC1, TSC2, and TBCD17) and RheB. On activation, AKT phosphorylates and inhibits TSC2 and promotes the conversion of RheB-GDP (inactive) to RheB-GTP (active), which can then activate mTORC1 on the lysosomal surface. Despite mis-localization of the components of the LNSS complex in *Cln1*^{-/-} cells, the mTORC1 is activated via the growth factor pathway. Constitutive activation of mTORC1 in *Cln1*^{-/-} cells contributes to suppression of autophagy and increased cell proliferation leading to neurodegeneration.



Abbreviations used: mTORC1, mechanistic target of rapamycin complex 1; AKT, Protein kinase B; TSC, Tuberous sclerosis complex; RheB, Ras homolog enriched in brain; IGF1R, Insulin-like growth factor receptor; IGF1, Insulin-like growth factor 1; PI3K, Phosphoinositide 3-kinases

Disruption of the lysosomal nutrient-sensing scaffold promotes aberrant activation of mTORC1 signaling via IGF1, contributing to neurodegeneration in a mouse model of INCL.

Emerging evidence has remarkably transformed our understanding of the lysosome as a terminal degradative organelle into a critical signaling hub for fundamental metabolic processes. Sensing of essential nutrients by lysosomes has emerged as an important function in coordinating cellular metabolism and growth. Signals from nutrients such as glucose, amino acids, fatty acids, and cholesterol are integrated by the lysosome, turning the cellular events from anabolic to catabolic processes such as autophagy. Whereas materials from extracellular sources are transported to the lysosome by endocytosis, those originating from intracellular sources are delivered by autophagy. Notably, activation of the mTORC1 pathway, situated at the crossroads of nutrient signaling, suppresses autophagy. The loss of autophagy in the central nervous system has been reported to cause neurodegeneration. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy, in all of which the lysosome plays a pivotal role in degrading the cargo contained in the autophagosomes. Indeed, dysregulation of autophagy has been implicated not only in the pathogenesis of common neurodegenerative diseases such as Alzheimer's and Parkinson's but also underlies pathogenesis in many members of a family of over 60 LSDs, for which neurodegeneration is a devastating

manifestation. Neuronal ceroid lipofuscinoses (NCLs, Batten disease) constitute a group of the most common neurodegenerative LSDs, which mostly affect children. Mutations in at least 13 different genes (*CLN1–CLN13*) underlie various forms of NCL. Despite the discovery more than two decades ago that the LSD INCL is caused by inactivating mutations in the *CLN1* gene, the mechanism of *CLN1* disease pathogenesis has remained elusive. Children afflicted with *CLN1* disease are phenotypically normal at birth, but by 6–18 months of age they manifest psychomotor retardation. Around two years of age, these children develop complete retinal degeneration, causing blindness. At around four years of age, an isoelectric electroencephalogram (EEG) attests to a vegetative state. They remain in this condition for several more years before eventual death. These grim facts underscore an urgent need for understanding the mechanism underlying pathogenesis of *CLN1* disease, which may facilitate the development of an effective treatment.

The *CLN1* gene encodes palmitoyl-protein thioesterase-1 (PPT1), a lysosomal depalmitoylating enzyme. Many proteins in the brain undergo S-palmitoylation (also called S-acylation), a post-translational modification in which a 16-carbon, saturated fatty acid (generally palmitate) is attached to specific cysteine residues in polypeptides via thioester linkage. S-palmitoylation confers hydrophobicity to soluble proteins, increases membrane affinity, promotes protein-protein interactions, and facilitates protein trafficking. Dynamic S-palmitoylation (palmitoylation-depalmitoylation) facilitates endosomal trafficking and the localization of many proteins, especially in the brain. While S-palmitoylation is catalyzed by palmitoyl acyltransferases (called ZDHHC-PATs or simply ZDHHCs), depalmitoylation is mediated by thioesterases. Inactivating mutations in the *CLN1* gene causing PPT1 deficiency result in lysosomal accumulation of autofluorescent ceroid lipofuscin. When the ceroid lipofuscins are organized within lysosomes, they are called GRODS (granular osmiophilic deposits), a characteristic finding in neurons and other cell types from patients with *CLN1* disease. The lysosomes are the dynamic regulators of the function of many proteins, especially in the brain, and their importance is underscored by the fact that impaired lysosomal function contributes to the pathogenesis of the LSDs. These inherited diseases are characterized by metabolic dysfunction, neurodegeneration, and shortened lifespan. Moreover, it has been suggested that the lysosomal pathway plays critical roles in many cellular functions, including signaling in response to environmental cues. We reasoned that impaired dynamic S-palmitoylation of important proteins that are likely substrates of Ppt1 may impair their ability to traffic to their destination. This abnormality may result in varying impairment of functions of these proteins, cumulatively contributing to neurodegeneration.

In the present study, we used cortical tissues from *Cln1*^{-/-} mice, a reliable animal model of *CLN1* disease, because in a pilot study (www.clinicaltrials.gov; [NCT00028262](https://clinicaltrials.gov/ct2/show/study/NCT00028262)), magnetic resonance imaging (MRI) of the brain of children with *CLN1* disease showed rapid degeneration of cortical tissues. We also used cultured cells from INCL patients to determine whether the loss of *Cln1*/Ppt1 causes aberrant activation mTORC1 signaling, suppressing autophagy and contributing to neurodegeneration. We found that in the brain of *Cln1*^{-/-} mice, which mimic *CLN1* disease, Ppt1 deficiency caused aberrant activation of mechanistic target of rapamycin complex 1 (mTORC1), which suppressed autophagy, contributing to neurodegeneration. Emerging evidence indicates that sensing essential nutrients is an important function of the lysosome. Intriguingly, Ppt1 deficiency disrupted the lysosomal nutrient-sensing scaffold (LNSS), upon which mTORC1 must attach to activate. Despite this defect, mTORC1 was activated by IGF1 via PI3K/Akt-mediated pathway. Importantly, treatment of *Cln1*^{-/-} mice with pharmacological inhibitors of PI3K/Akt suppressed mTORC1 activation, restored autophagy and improved motor function. Our findings reveal a previously unrecognized mechanism by which *Cln1*/Ppt1 deficiency contributes to pathogenesis of *CLN1* disease.

Impaired lysosomal Ca²⁺ homeostasis contributes to pathogenesis in INCL mice.

The lysosome is an organelle long known for mediating degradation and clearance of cellular waste. In recent years, it has become evident that it is a highly dynamic structure that also plays important roles in cell metabolism in response to environmental cues. Impaired lysosomal degradative function leads to a family of about 60 inherited LSDs. Dysregulation of cellular Ca²⁺ homeostasis is reported to play important roles in the pathogenesis of several human diseases, including the LSDs. Defective lysosomal Ca²⁺ homeostasis has also been reported to impair autophagy. In most of the LSDs, defective autophagy leads to neurodegeneration.

The ER is the major Ca²⁺ repository in the cell, and Ca²⁺ plays a key regulatory role in autophagy. It is an intracellular degradative process that requires Ca²⁺-dependent lysosomal hydrolases for the degradation and clearance of the cargo contained in the autophagosomes. Lysosomal Ca²⁺ homeostasis is mediated by inositol 3-phosphate receptor 1 (IP3R1)-mediated transport of Ca²⁺ from the ER to the lysosome. It has also been reported that selective interaction of IP3Rs with the ER-lysosome contact sites is required for the delivery of Ca²⁺ to the lysosome. Moreover, antagonists of IP3Rs rapidly and completely block lysosomal Ca²⁺ refilling. Interestingly, IP3R1 has been reported to undergo S-palmitoylation for regulating Ca²⁺ flux in immune cells. Furthermore, disruption of Ca²⁺ homeostasis may dysregulate neurotransmitter release, contributing to neurodegeneration. Autophagy is impaired by dysregulation of Ca²⁺ homeostasis in many LSDs including in *Cln1*^{-/-} mice. We sought to test the hypothesis that *CLN1* mutations dysregulate lysosomal Ca²⁺ homeostasis and suppress the catalytic activities of Ca²⁺-dependent lysosomal hydrolases, which impair the degradation of undigested cargo in autophagosomes, causing neuro-pathology in INCL.

We sought to determine the mechanism by which PPT1 deficiency impairs lysosomal degradative function and contributes to INCL pathogenesis. We found that in *Cln1*^{-/-} mice low levels of IP3R1 dysregulate lysosomal Ca²⁺ homeostasis. Intriguingly, the transcription factor NFATC4, which regulates *IP3R1* expression, required S-palmitoylation for trafficking from the cytoplasm to the nucleus. We identified two palmitoyl acyltransferases, ZDHHC4 and ZDHHC8, which catalyzed S-palmitoylation of NFATC4. Notably, in *Cln1*^{-/-} mice, reduced ZDHHC4 and ZDHHC8 levels markedly lowered S-palmitoylated NFATC4 (active) in the nucleus, which inhibited *IP3R1* expression, thereby dysregulating lysosomal Ca²⁺ homeostasis. Consequently, Ca²⁺-dependent lysosomal enzyme activities were markedly suppressed. Impaired lysosomal degradative function impaired autophagy, which caused lysosomal storage of undigested cargo. Importantly, IP3R1 overexpression in *Cln1*^{-/-} mouse fibroblasts ameliorated this defect. Our results reveal a previously unrecognized role of *Cln1/Ppt1* in regulating lysosomal Ca²⁺ homeostasis and suggest that the defect contributes to INCL pathogenesis.

Publications

1. Mukherjee AB, Appu AP, Sadhukhan T, Casey S, Mondal A, Zhang Z, Bagh MB. Emerging new roles of the lysosome and neuronal ceroid lipofuscinoses. *Mol Neurodegener* 2019 14(1):4.
2. Sarkar C, Sadhukhan T, Bagh MB, Appu AP, Chandra G, Mondal A, Saha A, Mukherjee AB. *Cln1*-mutations suppress Rab7-RILP interaction and impair autophagy contributing to neuropathology in a mouse model of INCL. *J Inherit Metab Dis* 2020 43:1082–1101.
3. Sadhukhan T, Bagh MB, Appu AP, Mondal A, Zhang W, Liu A, Mukherjee AB. In a mouse model of INCL reduced S-palmitoylation of cytosolic thioesterase APT1 contributes to microglia proliferation and neuroinflammation. *J Inherit Metab Dis* 2021 44:1051–1069.

4. Sadhukhan T, Bagh MB, Sadhukhan S, Appu AP, Mondal A, Iben JR, Li T, Coon SL, Mukherjee AB. Ablation of microRNA-155 and neuroinflammation in a mouse model of CLN1-disease. *Biochem Biophys Res Commun* 2021 571:137-144.

Collaborators

- Aiyi Liu, PhD, *Biostatistics & Bioinformatics Branch, NICHD, Bethesda, MD*
- Rafael M. Previde, PhD, *Section on Cellular Signaling, NICHD, Bethesda, MD*
- Stanko Stojilkovic, PhD, *Section on Cellular Signaling, NICHD, Bethesda, MD*
- Wadih Zein, MD, *Ophthalmic Genetics and Visual Function Branch, NEI, Bethesda, MD*

Contact

For more information, email mukherja@exchange.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/mukherjee>.

Gene Regulation in Innate Immunity

The laboratory is interested in chromatin and gene regulation in innate immunity. We study the role of three nuclear factors, histone H3.3, BRD4, and IRF8. Histone H3.3 is a variant histone that is incorporated into nucleosomes along with transcriptional elongation, an unusual but defining feature of the variant. Most other histones are deposited into nucleosomes during replication. For this reason, H3.3 is thought to be involved in epigenetic memory created by transcription, although experimental evidence for memory formation/maintenance is scant. BRD4 is a bromodomain protein of the BET family, expressed broadly in many cells, from early embryos to adults. Through the bromodomain, BRD4 binds to acetylated histones, not unacetylated histones. BRD4 is thus called a 'chromatin reader,' a type of regulatory factor capable of conveying epigenome information to gene expression. Furthermore, BRD4, binds to the elongation factor complex P-TEFb through the C-terminal domain, and drives transcription of many genes by causing RNA polymerase II to move through the gene body, generating nascent mRNA. Many recent reports point out that BRD4 promotes growth of cancer cells, including various blood cancers, by mediating the formation of super-enhancers involved in cell-cycle progression. As we reported in 1990, IRF8 is a DNA-binding transcription factor that plays an essential role in innate resistance to a wide array of pathogens (IRF8's structure is shown in Figure 1A). IRF8 is expressed mostly in cells of the myeloid lineage, including monocytes/macrophages, dendritic cells, and microglia. IRF8 is strongly induced when stimulated by interferons (IFN). In addition, it is upregulated when myeloid cells encounter pathogen-derived molecules and agents produced by stress. In turn, IRF8 activates many genes important for host resistance. IRF8-induced genes include those involved in autophagy and lysosome-mediated pathogen clearance. IRF8 does so by binding to small DNA motifs present in promoter and enhancer regions of the target genes.

IRF8 sets microglia-specific epigenome structure and defines the transcriptome program.

Microglia are the only cell type in the brain that protect from pathogen infection and are also important for shaping neuronal development and cellular connections. Recent genome-wide single nucleotide



Keiko Ozato, PhD, Head, Section on Molecular Genetics of Immunity

Anup Dey, PhD, *Biologist*

Tiyun Wu, PhD, *Staff Scientist*

Sakshi Chauhan, PhD, *Visiting Fellow*

Fuki Kudoh, PhD, *Visiting Fellow*

Eunju Lee, PhD, *Visiting Fellow*

Keita Saeki, MD, PhD, *Visiting Fellow*

Vishal Nehru, PhD, *Contractor*

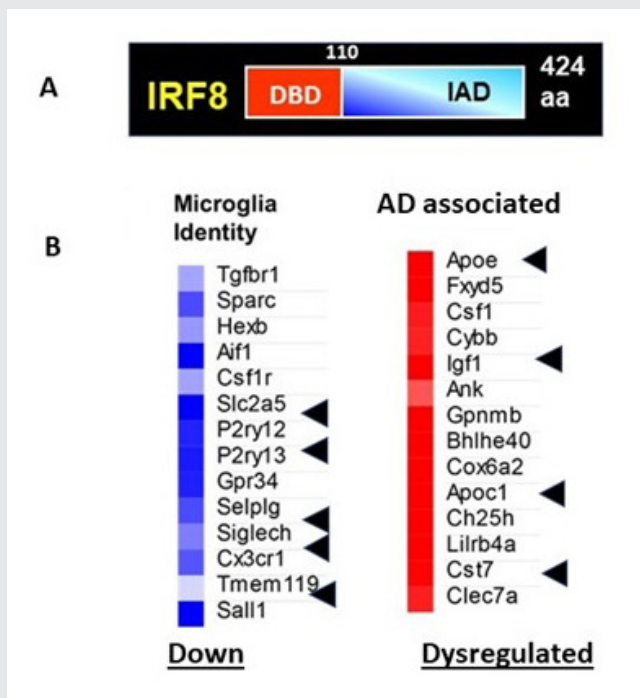


FIGURE 1. IRF8 regulates microglia transcription

A. IRF8 domain structure

B. IRF8 directs microglia identity and DAM genes (*right*). Many genes that define microglia are down regulated in *Irf8*KO cells (*left*), while AD associated genes are aberrantly expressed (*right*).

endow microglia with specific properties were missing or downregulated, including cell-surface markers such as P2ry12, Iba1, Cx3cr1, or Ccr5. On the other hand, some of IFN-stimulated genes (ISGs) and disease-associated microglia (DAM) genes expressed in AD microglia were expressed in *Irf8* KO microglia (Figure 1B). In addition, we found that IRF8 is required for the expression of two transcription factors critical for adult microglia function, i.e., *Sall1* and *Batf3*. Our results show that IRF8 directs a transcriptional cascade that defines microglia transcriptome program.

It was important to determine the DNA sites in the microglia genome to which IRF8 binds, information that is missing from the literature. This was technically difficult, because the number of harvestable microglia is low. A modified Cut&Run assay provided reproducible IRF8-binding profiles. Our data showed that IRF8 binds mostly over distant enhancer regions, located upstream and downstream of its target genes (Figure 2). IRF8-binding sites were strongly enriched with DNA motifs containing GAAA. In some enhancers, IRF8 binding was closely clustered, and some sites were within the large stretched enhancers enriched with H3K27ac histone marks. Large enhancers are known to support transcription of genes essential for cell type-specific properties. IRF8 containing large enhancers neighbored genes essential for microglia, including *Sall1* and *Batf3*, transcription factors that direct microglia activity. Deletion of *Irf8* led to loss of large enhancers associated with

polymorphism (SNP) analyses showed that genetic risk factors for Alzheimer's disease (AD) are either exclusively or most highly expressed in microglia, not in neurons. It is thought that microglia play a central role in AD onset and progression. Microglia originate from embryonic yolk sac as progenitors, which then migrate into the embryonic brain, where they differentiate into functional microglia in the postnatal stage. In the adult, microglia are distributed throughout the brain, including cortex and hippocampus. Previous reports demonstrated that the transcription factor Spi1 (PU.1) and interferon regulatory factor 8 (IRF8) take part in early progenitor differentiation in the embryonic brain. However, IRF8's function in adult microglia has not been fully understood. We examined microglia from adult brain in *Irf8* knockout (KO) mice. We found that *Irf8*KO microglia have an abnormal morphology and do not express a number of microglia-specific surface markers. We then FACS-sorted microglia from adult wild-type (WT) and *Irf8*KO mice and performed bulk and single cell RNA-Seq. Our results revealed that, without IRF8, many genes that

IRF8 binds to large enhancers in microglia

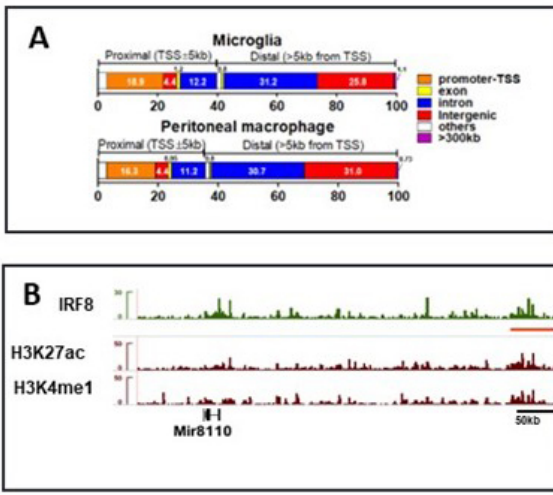


FIGURE 2. IRF8 binds to large enhancers in microglia.

A. IRF8 distribution in microglia and peritoneal macrophage genome

B. IRF8 binds as clusters colocalizing with H3k27ac and H3K4me1, markers for enhancers.

microglia identity genes. Furthermore, an ATAC-Seq assay found that IRF8 is important for setting open chromatin necessary for microglia's large enhancers. Our results clearly show that IRF8 directs the formation of the microglia-specific epigenome landscape. Consistent with this view, DNA methylome profiles revealed extensive changes in CpG methylation in IRF8KO microglia, indicating that IRF8 regulates overall CpG-island methylation patterns.

Microglia respond to external immune stimuli such as bacterial lipopolysaccharides (LPS), despite the blood brain barrier, and induce inflammatory genes. We found that IRF8 is required for the expression of many inflammatory genes induced by LPS in microglia. We next sought to determine whether IRF8 impacts the brain's health. We therefore investigated whether IRF8 affects AD progression, using a mouse model of AD (5xFAD). 5xFAD mice accumulate plaques containing polymerized β -amyloid peptides in the brain near microglia. β -Amyloid

plaques are thought to be the main cause of AD pathology, as they lead to neuronal death. We found that 5xFAD mice lacking *Irf8* gene have smaller amyloid plaques in the cortex, indicating that IRF8 facilitates plaque formation in this model (Figure 3A). Given that IRF8 shapes microglia identity, our observations are in line with previous reports that microglia worsen AD pathogenesis in the 5xFAD model. NeuN immunostaining found evidence of substantial neuronal loss in the one-year-old 5xFAD brain. However, NeuN immunoreactivity was more intense in *Irf8*KO 5xFAD brain, indicating reduced neuronal damage in the absence of *Irf8* (Figure 3B). These results are consistent with the view that microglia can negatively impact AD pathogenesis

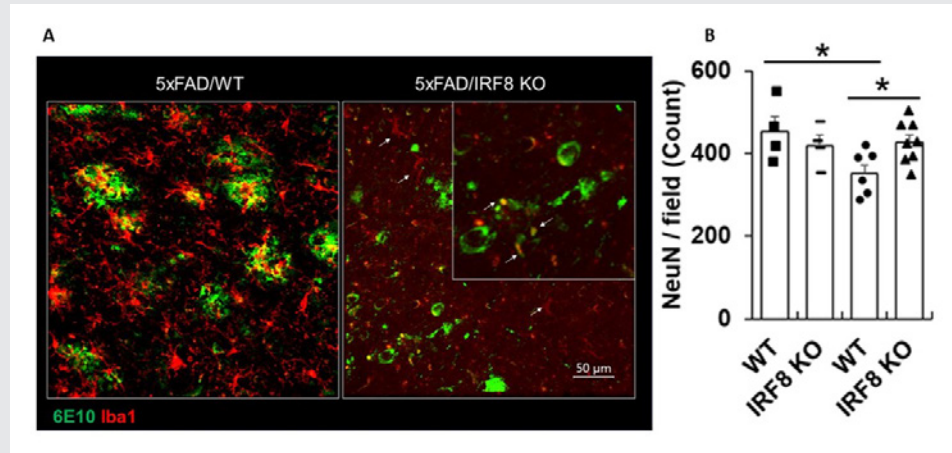
BRD4 promotes cell-cycle progression by preventing DNA damage.

Cell proliferation depends on continuous rounds of cell-cycle progression, which is driven by sequential activation of transcription factors, and other post-translational effectors. Chromatin-binding factor BRD4 is known to promote proliferation of many cancers. BRD4 inhibitors (BETi) can arrest cancer growth (See Figure 4A for BRD4 structure). BETi, and BRD4 Protac inhibitors are thought to represent new therapeutic possibilities against cancer. However, the role of BRD4 in the proliferation of normal cells has remained elusive. We examined proliferation of embryonic fibroblasts from *Brd4* conditional knockout (KO) mice. Cell-cycle analysis of WT and *Brd4* KO cells showed that BRD4 is required for transition from G0-G1, G1-S, and G2-M (Figure 4B). At G2 to M stage, many *Brd4* KO cells underwent catastrophic mitotic failure, as chromosomes failed to align and segregate properly. Transcriptome analysis found that many cell cycle-regulated genes were markedly downregulated in *Brd4* KO cells, including several histone genes at S phase, as well as the G2/M master regulators FOXM1 and

FIGURE 3. IRF8 modulates Alzheimer's disease progression in mice.

A. Large amyloid β plaques accumulate near microglia in 5xFAD brain, but amyloid plaques are smaller in Irf8KO 5xFAD brain (cortex). 6E10: polymerized amyloid plaques; Iba1: surface marker for microglia.

B. NeuN immunostaining quantification.



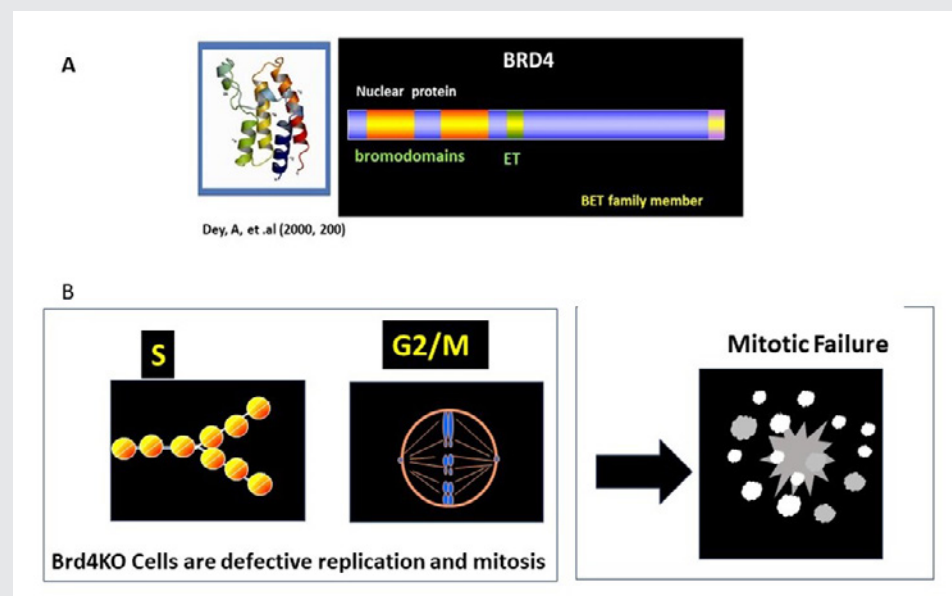
ATM/ATR. FOXM1 is a transcription factor of the forkhead family and promotes transcription of many G2/M genes. ATM/ATR are kinases previously known to be involved in DNA-damage repair. ATR has been recently shown to be activated at S and necessary for G2-M passage. Our results indicate that BRD4 drives transcription of numerous cell cycle-regulated genes. Consistent with these results, BRD4 occupied numerous cell-cycle genes throughout all stages, as revealed by ChIP-Seq analysis. BRD4 bound to these genes at all stages of cell cycle, seen at the transcription start site (TSS) and gene body.

Remarkably, Brd4KO cells suffered from DNA damage at all stages of cell cycle, which we found by extensive deposition of phospho(γ)-H2AX foci in the nucleus (Figure 4). H2AX is a variant histone H2, phosphorylated upon DNA damage through activation of ATM. Comet assay, another method to detect DNA damage corroborated

FIGURE 4. Structure and function of BRD4

A. BRD4 has two bromodomains and an ET domain.

B. BRD4 controls two fundamental events required for cell growth, replication and mitosis. Brd4KO cells were defective in both and result in mitotic failure.



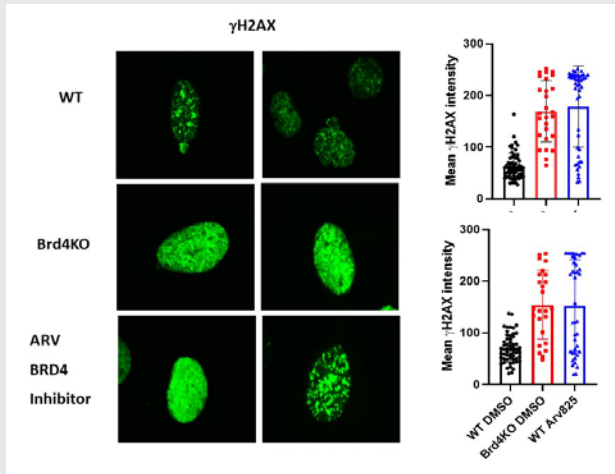


FIGURE 5. BRD4 limits endogenous DNA damage.

BRD4 depletion leads to DNA damage. WT cells, Brd4KO cells, and BRD4 inhibitor-treated WT cells were stained with antibody against γ -H2AX. Increased DNA damage is verified by quantification on the right.

gH2AX results. DNA damage prompts DNA repair response by a series of enzymes and associated factors, and activates p53. p53 is a central factor, which determines the downstream pathway that causes either cell-cycle arrest or apoptosis. We found that the expression of many DNA-damage control factors, including ATM/ATR, H2AX, and p53, is controlled by BRD4. These results indicate that BRD4 is critical for suppressing endogenous DNA damage, thus playing a major role in the maintenance of genome integrity.

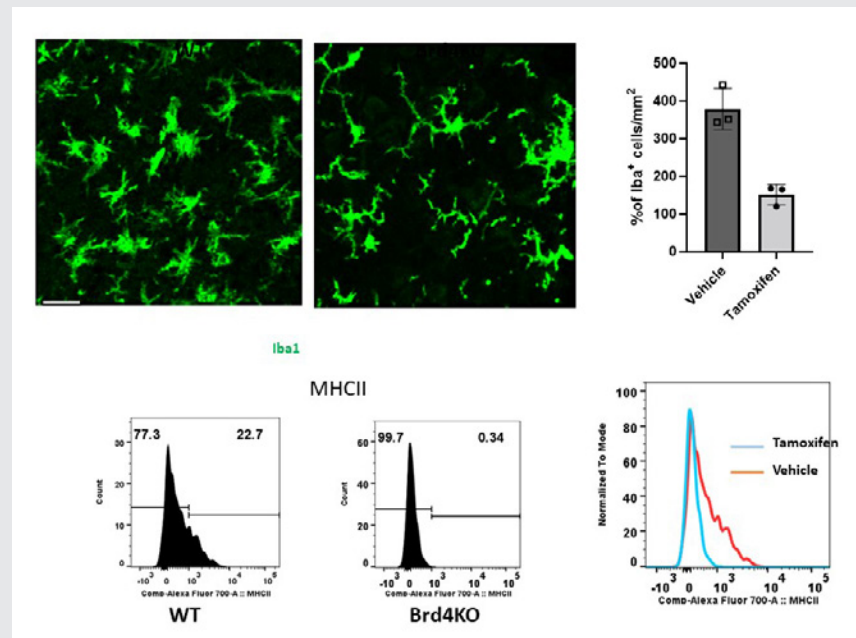
BRD4 in microglia and neuroinflammation

To further study transcriptome and epigenome regulation of microglia, we began investigating the function of BRD4. We constructed mice in which Brd4 is knocked out in microglia in adult brain (Brd4^{fl/fl} Cx3cr1Cre), where Brd4 is deleted after Tamoxifen injection. To study how BRD4 regulates neuroinflammation,

we analyzed experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis. EAE causes microglia to proliferate from the resident progenitor cells. These microglia, along with T cells and macrophages infiltrating from periphery, promote demyelination and neuronal damage, which results in paralysis. Brain

FIGURE 6. BRD4 promotes neuroinflammation in the mouse model of multiple sclerosis.

Brd4KO microglia in EAE. Microglia were visualized by Iba1 immunohistology. The numbers of microglia are reduced in Brd4KO brain (quantification on the right). MHCII expression is absent from Brd4KO microglia. Brd4 deletion in microglia reduces EAE disease phenotypes.



histology revealed that the number of microglia is considerably reduced in Brd4KO brain in EAE (Figure 5A). In addition, Brd4KO microglia exhibited aberrant morphology with stunted extensions and failed to express MHCII, a representative marker for active microglia (Figure 5B). Remarkably, mice with a microglia Brd4 deletion had less demyelination, resulting in reduced tissue damage, and reduced paralysis. Neuro-inflammatory cytokines and chemokines such as interleukin-1 β (Il1b) and Ccl were also lower in Brd4KO EAE microglia. These results indicate that BRD4 is required for microglia to detect incoming antigenic and inflammatory signals, interacting with and further stimulating infiltrated T cells. Our study revealed that BRD4 plays a central role in the pathogenesis of neuro-inflammation. It will be of interest to clarify the role of BRD4 in microglia development and AD pathogenesis.

Publications

1. Chen L, Ozato K. Innate immune memory in hematopoietic stem/progenitor cells: myeloid-biased differentiation and the role of interferon. *Front Immunol* 2021 12:621333–621434.
2. Murakami K, Sasaki H, Nishiyama A, Kurotaki D, Kawase W, Ban T, Nakabayashi J, Kanzaki S, Ozato K, Tamura T. A RUNX-CBFbeta-driven enhancer directs the Irf8 dose-dependent lineage choice between DCs and monocytes. *Nat Immunol* 2021 22:301–313.
3. Das A, Wang X, Kang J, Coulter A, Shetty AC, Bachu M, Brooks SR, Dell'Orso S, Foster BL, Fan X, Ozato K, Somerman MJ, Thumbigere-Math V. Monocyte subsets with high osteoclastogenic potential and their epigenetic regulation orchestrated by IRF8. *J Bone Miner Res* 2021 36:199–214.
4. Milner JJ, Toma C, Quon S, Omilusik K, Scharping NE, Dey A, Reina-Campos M, Nguyen H, Getzler AJ, Diao H, Yu B, Delpoux A, Yoshida TM, Li D, Qi J, Vincek A, Hedrick SM, Egawa T, Zhou MM, Crotty S, Ozato K, Pipkin ME, Goldrath AW. Bromodomain protein BRD4 directs and sustains CD8 T cell differentiation during infection. *J Exp Med* 2021 218:e20202512.
5. Nakasuji-Togi M, Togi S, Saeki K, Kojima Y, Ozato K. Herbal extracts that induce type I interferons through Toll-like receptor 4 signaling. *Food Nutr Res* 2022 66:5524. eCollection.
6. Kurotaki D, Kikuchi K, Cui K, Kawase W, Saeki K, Fukumoto J, Nishiyama A, Nagamune K, Zhao K, Ozato K, Rocha PP, Tamura T. Chromatin structure undergoes global and local reorganization during murine dendritic cell development and activation. *Proc Natl Acad Sci U S A* 2022 119(34):e2207009119.

Collaborators

- Steven L. Coon, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Robert J. Crouch, PhD, *Section on Formation of RNA, NICHD, Bethesda, MD*
- Daisuke Kurotaki, PhD, *Kumamoto University, Kumamoto City, Japan*
- Justin Milner, PhD, *University of North Carolina, Chapel Hill, NC*
- Tomohiko Tamura, MD, PhD, *Tokyo University, Tokyo, Japan*
- Vivek Thumbigere Math, DDS, PhD, *University of Maryland, School of Dentistry, Baltimore, MD*
- Jinfang Zhu, PhD, *Laboratory of Immunology, NIAID, Bethesda, MD*

Contact

For more information, email ozatok@mail.nih.gov or visit <http://ozatolab.nichd.nih.gov>.

Diagnosis, Localization, Pathophysiology, and Molecular Biology of Pheochromocytoma and Paraganglioma

Pheochromocytomas (PHEOs) and paragangliomas (PGLs) are rare but clinically important chromaffin-cell tumors that typically arise, respectively, from the adrenal gland and from extra-adrenal paraganglia. The clinical features and consequences of PHEO/PGL, collectively known as PPGLs, result from the overproduction and release of catecholamines (norepinephrine and epinephrine). An undetected PHEO/PGL poses a hazard to patients undergoing surgery, childbirth, or general anesthesia because of the potential for excess catecholamine secretion, which can result in significant, often catastrophic outcomes. Diagnosing and localizing a PHEO/PGL can be challenging. Plasma and urinary catecholamines, as well as their metabolites, and radio-iodinated metaiodobenzylguanidine (MIBG) scanning can yield false-positive or false-negative results in patients harboring the tumor, and computed tomography (CT) and magnetic resonance imaging (MRI) lack sufficient specificity. The molecular mechanisms by which genotypic changes predispose to the development of PHEO/PGL remain unknown, even in patients with identified mutations. Moreover, in patients with hereditary predispositions, PPGLs differ in terms of their growth, malignant potential, catecholamine phenotype, responses to standard screening tests, various imaging modalities, and therefore to different therapeutic options. We focus on developmental, molecular, genetic, epigenetic, proteomic, metabolomic, immunologic, and other types of studies to investigate the bases for a predisposition to develop PPGLs and the expression of various neurochemical phenotypes and malignant potentials, including therapeutic responses and appropriate follow-up.

Clinical and genetic aspects of pheochromocytoma and paraganglioma

We used single-nuclei RNA-Seq and bulk-tissue gene-expression data to characterize the cellular composition of pheochromocytomas (PC), paragangliomas (PGs), and normal adrenal tissues, to refine tumor gene-expression subtypes, and to make clinical and genotypic associations. We confirmed seven PCPG gene-expression subtypes with significant genotype and clinical associations. Tumors with mutations in *VHL* (Von-Hippel-Lindau gene), *SDH*-encoding genes (*SDHx*), or *MAML3* (mastermind like transcriptional coactivator 3)-fusions



Karel Pacak, MD, PhD, DSc,
*Head, Section on Medical
Neuroendocrinology*

Sara Talvacchio, BSN, *Research
Nurse*

Alberta Derkyi, CRNP, *Nurse
Practitioner*

Josephine Ezemobi, CRNP, *Nurse
Practitioner*

Tamara Prodanov, MD, *Clinical
Trial Database (CTDB)
Coordinator*

Thanh-Truc Huynh, MS, *Biologist*

Matthew Arman Nazari, MD,
Visiting Fellow

Suman Goshal, PhD, *Postdoctoral
Visiting Fellow*

Katerina Hadrava Vanova, PhD,
Postdoctoral Visiting Fellow

Abishek Jha, MBBS, *Postdoctoral
Visiting Fellow*

Ondrej Uher, MSc, *Predoctoral
Visiting Fellow*

Kailah Charles, BS, *Predoctoral
Fellow*

Mayank Patel, MD, *Volunteer*

were characterized by hypoxia-inducible factor signaling and neoangiogenesis. PCPGs had few infiltrating lymphocytes but abundant macrophages. While neoplastic cells transcriptionally resembled mature chromaffin cells, early chromaffin and neuroblast markers were also features of some PCPG subtypes. The gene-expression profile of metastatic SDHx-related PCPG indicated that these tumors have elevated cellular proliferation and a lower number of non-neoplastic Schwann cell-like cells, while GPR139 (G-protein coupled receptor 139) is a potential theranostic target. Our findings therefore clarified the diverse transcriptional programs and cellular composition of PCPGs and identified biomarkers of potential clinical significance.

Metabolic dysfunction mutations can impair energy sensing and cause cancer. Loss of function of the mitochondrial tricarboxylic acid (TCA) cycle enzyme subunit succinate dehydrogenase B (SDHB) results in various forms of cancer typified by PC. We delineated a signaling cascade in which the loss of SDHB induces the Warburg effect, triggers dysregulation of $[Ca^{2+}]_i$, and aberrantly activates calpain and protein kinase Cdk5, through conversion of its cofactor from p35 to p25. Consequently, aberrant Cdk5 initiates a phospho-signaling cascade in which GSK3 inhibition inactivates energy sensing by AMP kinase through dephosphorylation of the AMP kinase γ subunit PRKAG2. Overexpression of p25-GFP in mouse adrenal chromaffin cells also elicits this phosphorylation signaling and causes PC. A potent Cdk5 inhibitor, MRT3-007, reversed this phospho-cascade, invoking a senescence-like phenotype. This therapeutic approach halted tumor progression *in vivo*. Thus, we revealed an important mechanistic feature of metabolic sensing and demonstrated that its dysregulation underlies tumor progression in PC and likely other cancers.

Another retrospective study included 582 patients with PCPGs and 57 with HNGPLs (Head and neck paragangliomas). Disease-specific survival (DSS) was assessed according to age, location and size of tumors, recurrent/metastatic disease, genetics, plasma metanephrines, and methoxytyramine. Among all patients with PCPGs, multivariable analysis indicated that, apart from older age and presence of metastases, shorter DSS was also significantly associated with extra-adrenal tumor location and higher plasma methoxytyramine and normetanephrine. Among patients with HNPGLs, those with metastases presented with significantly longer DSS than patients with metastatic PCPGs, and only plasma methoxytyramine was an independent predictor of DSS. For patients with metastatic PCPGs, multivariable analysis revealed that, apart from older age, shorter DSS was significantly associated with the presence of synchronous metastases and higher plasma methoxytyramine burden. We concluded that DSS among patients with PCPGs/HNPGLs relates to several presentations of the disease that may provide prognostic markers. In particular, the independent associations of higher methoxytyramine with shorter DSS in patients with HNPGLs and metastatic PCPGs suggest the utility of this biomarker to guide individualized management and follow-up strategies in affected patients.

In the hope of discovering new markers for metastatic or aggressive phenotypes of PCPGs, we also analyzed the noncoding transcriptome from patient gene-expression data in The Cancer Genome Atlas. Differential expression of miRNAs was observed between PCPG molecular subtypes. We specifically characterized candidate miRNAs that are upregulated in pseudohypoxic PCPGs with mutations in SDHB and/or SDHD (succinate dehydrogenase complex subunit D), which are mutations associated with unfavorable clinical outcomes. Our computational analysis identified four candidate miRNAs that showed higher expression in metastatic than in non-metastatic PCPGs: miR-182, miR-183, miR-96, and miR-383. We also found six candidate lncRNAs harboring opposite expression patterns from the miRNAs when we analyzed the expression profiles of their predicted target lncRNAs. Three of these lncRNA candidates, USP3-AS1, LINC00877, and AC009312.1, were found to have lower expression in metastatic than in non-metastatic PCPGs. Using univariate and

multivariate analysis, we found miRNA miR-182 to be an independent predictor of metastasis-free survival in PCPGs. In summary, we identified candidate miRNA and lncRNAs associated with metastasis-free survival in PCPGs.

Imaging of pheochromocytomas and paragangliomas

The first study identified the importance of positron emission tomographic (PET) and anatomic imaging modalities and their individual performances in detecting succinate dehydrogenase A (*SDHA*)-related metastatic PCPGs. The detection rates of PET modalities ^{68}Ga -DOTATATE, ^{18}F -FDG, and ^{18}F -FDOPA, along with the combination of computed tomography (CT) and magnetic resonance imaging (MRI), were compared in a cohort of 11 patients with metastatic PCPGs in the setting of a germline *SDHA* mutation. We evaluated the imaging detection performances at three levels: overall lesions, anatomic regions, and on a patient-by-patient basis. ^{68}Ga -DOTATATE PET demonstrated an average lesion-based detection rate of 88.6%, while ^{18}F -FDG, ^{18}F -FDOPA, and CT/MRI showed detection rates of 82.9%, 39.8%, and 58.2%, respectively. The study found that ^{68}Ga -DOTATATE best detects lesions in a subset of patients with *SDHA*-related metastatic PCPGs. However, ^{18}F -FDG did detect more lesions in the liver, mediastinum, and abdomen/pelvis anatomic regions, showing the importance of a combined approach using both these PET modalities in evaluating *SDHA*-related PCPGs.

Recent professional society guidelines for radionuclide imaging of sporadic PC recommend ^{18}F -fluorodihydroxyphenylalanine (^{18}F -FDOPA) as the radiotracer of choice, deeming ^{68}Ga -DOTATATE and FDG to be second- and third-line agents, respectively. An additional agent, ^{18}F -fluorodopamine (^{18}F -FDA), remains experimental for PC detection. A paucity of research has performed head-to-head comparison among these agents. Thus, the purpose of the second study was to perform an intra-individual comparison of ^{68}Ga -DOTATATE PET/CT, FDG PET/CT, ^{18}F -FDOPA PET/CT, ^{18}F -FDA PET/CT, CT, and MRI in visualization of sporadic primary PC. This prospective study enrolled patients referred with clinical suspicion for sporadic PC. Patients were scheduled for ^{68}Ga -DOTATATE PET/CT, FDG PET/CT, ^{18}F -FDOPA PET/CT, ^{18}F -FDA PET/CT, whole-body staging CT (portal venous phase), and MRI within a three-month period. PET/CT examinations were reviewed by two nuclear medicine physicians, and CT and MRI were reviewed by two radiologists; differences were resolved by consensus. Readers scored lesions in terms of confidence in diagnosis of PC (1–5 scale; 4–5 considered positive for PC). Lesion-to-liver SUV_{max} (maximum standardized uptake value) was computed using both readers' measurements. Inter-reader agreement was assessed using intra-class correlation coefficients (ICCs) for SUV_{max} . Analysis included only patients with histologically confirmed PC on resection, i.e., 14 patients (eight women, six men; mean age, 52.4 ± 16.8 years). Both ^{68}Ga -DOTATATE PET/CT and FDG PET/CT were completed in all 14 patients, ^{18}F -FDOPA PET/CT in 11, ^{18}F -FDA PET/CT in 7, CT in 12, and MRI in 12. Given that ^{18}F -FDOPA PET/CT yielded the maximum positivity rate, the findings from this small intraindividual comparative study support ^{18}F -FDOPA PET/CT as a preferred first-line imaging modality in evaluation of sporadic PC.

Immune, proteomic, and metabolic aspects of pheochromocytoma and paraganglioma

PCPGs are rare neuroendocrine tumors derived from neural crest cells. Germline variants in approximately 20 PCPGs susceptibility genes are found in about 40% of patients, half of which are found in the genes that encode SDH. Patients with *SDHB*-mutated PCPGs exhibit a higher likelihood of developing metastatic disease, which can be partially explained by the metabolic cell reprogramming and redox imbalance caused by the mutation. Reactive oxygen species (ROS) are highly reactive molecules involved in a many important signaling

pathways. A moderate level of ROS production can help regulate cellular physiology; however, an excessive level of oxidative stress can lead to tumorigenic processes, including stimulation of growth factor-dependent pathways and the induction of genetic instability. Tumor cells effectively exploit antioxidant enzymes in order to protect themselves against harmful intracellular ROS accumulation, which highlights the essential balance between ROS production and scavenging. Exploiting ROS accumulation can be used as a possible therapeutic strategy in ROS-scavenging tumor cells. We focused on the role of ROS production in PCPGs, predominantly in SDHB-mutated cases, and on potential strategies and approaches to anticancer therapies by enhancing ROS production in these difficult-to-treat tumors.

To identify new therapeutic targets, we performed a detailed membrane-focused proteomic analysis of five human PG samples. Using the Pitchfork strategy, which combines specific enrichments of glycopeptides, hydrophobic transmembrane segments, and non-glycosylated extra-membrane peptides, we identified over 1,800 integral membrane proteins (IMPs). We found 45 “tumor enriched” proteins, i.e., proteins identified in all five PGs but not found in control chromaffin tissue. Among them, 18 IMPs were predicted to be localized on the cell surface, a preferred drug targeting site, including prostate-specific membrane antigen (PSMA), a well established target for nuclear imaging and therapy of advanced prostate cancer. Using specific antibodies, we verified PSMA expression in 22 well characterized human PCPG samples. Compared with control chromaffin tissue, PSMA was markedly overexpressed in high-risk PCPGs belonging to the established Cluster 1, which is characterized by worse clinical outcomes, pseudohypoxia, multiplicity, recurrence, and metastasis, specifically including *SDHB*, *VHL*, and *EPAS1* mutations. Using immunohistochemistry, we localized PSMA expression to tumor vasculature. Our study provides the first direct evidence of PSMA over-expression in PPGLs, which could translate to therapeutic and diagnostic applications of anti-PSMA radio-conjugates in high-risk PCPGs.

Immunotherapy has become an essential part of cancer treatment. Discovery of tumor-specific epitopes through tumor sequencing has revolutionized patient outcomes in many types of cancers that were previously untreatable. However, the majority of solid metastatic cancers, such as PHEO, are resistant to this approach. Therefore, understanding immune cell composition in primary and distant metastatic tumors is important for therapeutic intervention and diagnostics. Combined mannan-BAM (biocompatible anchor for cell membrane), TLR ligand (Toll-like receptor), and anti-CD40 (CH40 is a costimulatory protein found on antigen-presenting cells) antibody-based intra-tumoral immunotherapy (MBTA therapy) previously resulted in the complete eradication of murine subcutaneous PHEO and demonstrated a systemic antitumor immune response in a metastatic model. We further evaluated this systemic effect using a bilateral PHEO model, performing MBTA therapy through injection into the primary tumor and using distant (non-injected) tumors to monitor size changes and detailed immune cell infiltration. MBTA therapy suppressed the growth not only of injected but also of distal tumors and prolonged the survival of MBTA-treated mice. Our flow-cytometry analysis showed that MBTA therapy led to increased recruitment of innate and adaptive immune cells in both tumors and the spleen. Moreover, adoptive CD4⁺ T cell transfer from successfully MBTA-treated mice (i.e., subcutaneous PHEO) demonstrates the importance of such cells in long-term immunological memory. In summary, the study unravels further details on the systemic effect of MBTA therapy and its use for tumor and metastasis reduction or even elimination

We further extended the MBTA therapy to other tumors and applications in close collaboration with NCI investigators. For example, emerging evidence is demonstrating the extent of T cell infiltration within the tumor micro-environment and thus has favorable prognostic and therapeutic implications. Hence, immuno-

therapeutic strategies that augment the T cell signature of tumors hold promising therapeutic potential. Recently, immuno-therapy based on intra-tumoral injection of MBTA demonstrated promising potential to modulate the immune phenotype of injected tumors, including PHEO. The strategy promotes the phagocytosis of tumor cells to facilitate the recognition of tumor antigens and induce a tumor-specific adaptive immune response. Using a syngeneic colon carcinoma model, we demonstrated MBTA's potential to augment CD8⁺ T cell tumor infiltrate when administered intra-tumorally or subcutaneously as part of a whole tumor cell vaccine. Both immuno-therapeutic strategies proved effective in controlling tumor growth, prolonged survival, and induced immunological memory against the parental cell line. Collectively, our investigation demonstrates MBTA's potential to trigger a potent anti-tumor immune response.

We also reviewed the most promising glioblastoma vaccination strategies to contextualize the MBTA vaccine. By reviewing current evidence using translational tumor models supporting MBTA vaccination, we evaluated the underlying principles that validate its clinical applicability. We also showed the translational potential of MBTA vaccination as a possible immunotherapy in glioblastoma, along with established surgical and immunologic cancer treatment paradigms.

Therapeutic aspects of pheochromocytoma and paraganglioma

Aggressive PCPGs are difficult to treat, and molecular targeting is being increasingly considered, but with variable results. We investigated established and novel molecular-targeted drugs and chemotherapeutic agents for the treatment of PCPGs in human primary cultures and murine cell line spheroids. In PCPGs from 33 patients, including seven metastatic PCPGs, we identified germline or somatic driver mutations in 79% of cases, allowing us to assess potential differences in drug responsiveness between pseudohypoxia-associated cluster 1-related (n = 10) and kinase signaling-associated cluster 2-related (n = 14) PCPG primary cultures. Single anti-cancer drugs were either more effective in cluster 1 (cabozantinib, selpercatinib, and 5-FU) or similarly effective in both clusters (everolimus, sunitinib, alpelisib, trametinib, niraparib, entinostat, gemcitabine, AR-A014418, and high-dose zoledronic acid). High-dose estrogen and low-dose zoledronic acid were the only single substances more effective in cluster 2. Neither cluster 1- nor cluster 2-related patient primary cultures responded to temozolomide, dabrafenib, octreotide, or HIF-2a inhibitors. We showed particular efficacy of targeted combination treatments (cabozantinib/everolimus, alpelisib/everolimus, alpelisib/trametinib) in both clusters, with higher efficacy of some targeted combinations in cluster 2 and overall synergistic effects (cabozantinib/everolimus, alpelisib/trametinib) or synergistic effects in cluster 2 (alpelisib/everolimus). Cabozantinib/everolimus combination therapy, gemcitabine, and high-dose zoledronic acid appear to be promising treatment options with particularly high efficacy in SDHB-mutant and metastatic tumors. In conclusion, only minor differences regarding drug responsiveness were found between cluster 1 and cluster 2: some single anti-cancer drugs were more effective in cluster 1 and some targeted combination treatments were more effective in cluster 2.

SDH tumors, including PCPGs, hereditary leiomyomatosis and renal cell cancer-associated renal cell carcinoma (HLRCC-RCC), and gastrointestinal stromal tumors (GISTs) without KIT or platelet-derived growth factor receptor alpha mutations are often resistant to cytotoxic chemotherapy, radiotherapy, and many targeted therapies. We evaluated guadecitabine, a dinucleotide containing the DNA methyltransferase inhibitor decitabine, in these patient populations. Phase II study of guadecitabine (subcutaneously, 45mg/m²/day for five consecutive days, planned 28-day cycle) assessed clinical activity (according to Response Evaluation Criteria in Solid Tumors v.1.1) across three strata of patients with dSDH GIST, PCPG, or HLRCC-RCC. A Simon

optimal two-stage design (target response rate 30% rule out 5%) was used. Biologic correlates (methylation and metabolites) from peripheral blood mononuclear cells (PBMCs), serum, and urine were analyzed. Nine patients (seven with dSDH GIST, one each with PGL and HLRCC-RCC, six females and three males, age range 18–57 years-old) were enrolled. Two patients developed treatment-limiting neutropenia. No partial or complete responses were observed (range 1–17 cycles of therapy). Biologic activity assessed as global demethylation in PBMCs was observed. No clear changes in metabolite concentrations were observed. We concluded that guadecitabine was tolerated in patients with SDH tumors with manageable toxicity. While 4/9 patients had prolonged stable disease there were no objective responses. Thus, guadecitabine did not meet the target of 30% response rate across SDH tumors at this dose, though signs of biologic activity were noted.

Animal model of pheochromocytoma and cell culture studies

We previously identified the syndrome of multiple paragangliomas and pheochromocytomas, duodenal somatostatinoma, and polycythemia resulting from post-zygotic *EPAS1* (*HIF2A*)-gain-of-function mutations (also called Pacak-Zhuang syndrome). The mutations, located in the oxygen-degradation domain (ODD) of hypoxia-inducible factor-2alpha (HIF-2alpha), have been shown to impair hydroxylation by prolyl hydroxylase domain-containing protein 2 (PHD2) and subsequent association with the von Hippel-Lindau (VHL) protein. In that situation, degradation of HIF-2alpha is impaired, resulting in its stabilization, prolonged activation, lack of response to normal or increasing oxygen tension, and activation of the transcription of many genes participating in tumorigenesis. Recently, in collaboration with NCI investigators, we developed transgenic mice with a gain-of-function *Epas1*^{A529V} mutation (corresponding to human *EPAS1*^{A530V}), which demonstrated elevated levels of erythropoietin and polycythemia, a reduced urinary metanephrine-to-normetanephrine ratio, and increased expression of somatostatin in the ampullary region of the duodenum. The findings demonstrate the vital roles of *EPAS1* mutations in development of the syndrome and the great potential of the *Epas1*^{A529V} animal model for further pathogenesis and therapeutics studies. The model is also being used to study other malformations in animals as well as to match them with those seen in our patients (neurological, vascular, and ocular malformations) as described below.

Patients referred to the NIH for new, recurrent, and/or metastatic PHEO/PGL were confirmed for the *EPAS1* gain-of-function mutation; imaging was conducted for vascular malformations. We evaluated the *Epas1*^{A529V} transgenic syndrome mouse model, corresponding to the mutation initially detected in the patients (*EPAS1*^{A530V}), for vascular malformations by intravital 2-photon microscopy of meningeal vessels, terminal vascular perfusion with Microfil silicate polymer and subsequent intact *ex vivo* 14T MRI and micro-CT, and histologic sectioning and staining of the brain and identified pathologies. Further, we evaluated retinas from corresponding developmental time points (P7, P14, and P21) and the adult dura by immunofluorescent labeling of vessels and confocal imaging. We identified a spectrum of vascular malformations in all nine syndromic patients and in all our tested mutant mice. Patient vessels had higher variant allele frequency than adjacent normal tissue. Veins of the murine retina and intracranial dura failed to regress normally at the expected developmental time points. The findings add vascular malformation as a new clinical feature of the *EPAS1* gain-of-function syndrome.

Publications

1. Zethoven M, Martelotto L, Pattison A, Bowen B, Balachander S, Flynn A, Rossello FJ, Hogg A, Miller JA, Frysak Z, Grimmond S, Fishbein L, Tischler AS, Gill AJ, Hicks RJ, Dahia PLM, Clifton-Bligh R, Pacak K,

- Tothill RW. Single-nuclei and bulk-tissue gene-expression analysis of pheochromocytoma and paraganglioma links disease subtypes with tumor microenvironment. *Nat Commun* 2022 13:1–18.
2. Ligon JA, Sundby RT, Wedekind Malone MF, Arnaldez FI, Del Rivero J, Wiener L, Srinivasan R, Spencer M, Carbonell A, Lei H, Shern J, Steinberg SM, Figg WD, Peer CJ, Zimmerman S, Moraly J, Xu X, Fox S, Chan K, Barbato MI, Andresson T, Taylor N, Pacak K, Killian JK, Dombi E, Linehan WM, Miettinen M, Piekarz R, Helman LJ, Meltzer P, Widemann B, Glod J. A phase II trial of guadecitabine in children and adults with SDH-deficient GIST, pheochromocytoma, paraganglioma, and HLRCC-associated renal cell carcinoma. *Clin Cancer Res* 2023 29(2):341–348.
 3. Pamporaki C, Prodanov T, Meuter L, Berends AMA, Bechmann N, Constantinescu G, Beuschlein F, Remde H, Januszewicz A, Kerstens MN, Timmers HJLM, Taieb D, Robledo M, Lenders JWM, Pacak K, Eisenhofer G. Determinants of disease-specific survival in patients with and without metastatic pheochromocytoma and paraganglioma. *Eur J Cancer* 2022 169:32–41.
 4. Jha A, Patel M, Carrasquillo JA, Ling A, Millo C, Saboury B, Chen CC, Wakim P, Gonzales MK, Meuter L, Knue M, Talvacchio S, Herscovitch P, Rivero JD, Chen AP, Nilubol N, Taieb D, Lin FI, Civelek AC, Pacak K. Sporadic primary pheochromocytoma: a prospective intraindividual comparison of six imaging tests (CT, MRI, and PET/CT using 68Ga-DOTATATE, FDG, 18F-FDOPA, and 18F-FDA). *Am J Roentgenol* 2022 218:342–350.
 5. Nölting S, Bechmann N, Taieb D, Beuschlein F, Fassnacht M, Kroiss M, Eisenhofer G, Grossman A, Pacak K. Personalized management of pheochromocytoma and paraganglioma. *Endocr Rev* 2022 43:199–239.

Collaborators

- James Bibb, PhD, *University of Alabama Comprehensive Cancer Center, University of Alabama at Birmingham Medical Center, Birmingham, AL*
- Clara C. Chen, MD, *Nuclear Medicine Department, Clinical Center, NIH, Bethesda, MD*
- Jaydira Del Rivero, MD, *Pediatric Oncology Branch, NCI, Bethesda, MD*
- Graeme Eisenhofer, PhD, *Universität Dresden, Dresden, Germany*
- Hans Ghayee, DO, *Department of Internal Medicine, University of Florida, Gainesville, FL*
- Peter Herscovitch, MD, *PET Department, Clinical Center, NIH, Bethesda, MD*
- Frank I. Lin, MD, *Molecular Imaging Program, NCI, Bethesda, MD*
- W. Marston Linehan, MD, *Urologic Oncology Branch, NCI, Bethesda, MD*
- Corina Millo, MD, *PET Department, Clinical Center, NIH, Bethesda, MD*
- Jirí Neuzil, PhD, *Institute of Biotechnology, Czech Academy of Sciences, Prague, Czech Republic*
- Seyedehmoozhan Nikpanah, MD, *Nuclear Medicine Department, Clinical Center, NIH, Bethesda, MD*
- Naris Nilubol, MD, FACS, *Surgical Oncology Program, NCI, Bethesda, MD*
- Ondrej Petrak, MD, PhD, *Third Department of Medicine, General University Hospital, Prague, Czech Republic*
- Margarita Raygada, PhD, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*
- Mercedes Robledo, PhD, *Human Cancer Genetics Programme, Spanish National Cancer Centre (CNIO), Madrid, Spain*
- Jared Rosenblum, MD, *Neuro-Oncology Branch, NCI, Bethesda, MD*
- Douglas Rosing, MD, *Translational Medicine Branch, NHLBI, Bethesda, MD*
- Kelly Roszko, MD, PhD, *Skeletal Disorders and Mineral Homeostasis Section, NIDCR, Bethesda, MD*
- Babak Saboury, MD, *Nuclear Medicine Department, CC, NIH, Bethesda, MD*
- Arthur S. Tischler, MD, PhD, *New England Medical Center, Boston, MA*
- Richard Tothill, PhD, *University of Melbourne Centre for Cancer Research, Melbourne, Australia*

- Brad Wood, MD, PhD, *Radiology Department, Clinical Center, NIH, Bethesda, MD*
- Chunzhang Yang, PhD, *Neuro-Oncology Branch, NCI, Bethesda, MD*
- Deena Zeltser, MD, *Office of the Clinical Director, NICHD, Bethesda, MD*
- Zhengping Zhuang, MD, PhD, *Neuro-Oncology Branch, NCI, Bethesda, MD*

Contact

For more information, email karel@mail.nih.gov or visit <https://irp.nih.gov/pi/karel-pacak>.

Mechanisms Regulating GABAergic Cell Development

The incredible diversity and heterogeneity of interneurons was observed over a century ago, with Ramon y Cajal hypothesizing in “Recollections of My Life” that “The functional superiority of the human brain is intimately linked up with the prodigious abundance and unaccustomed wealth of the so-called neurons with short axons.” Although interneurons constitute the minority (20%) of neurons in the brain, they are the primary source of inhibition and are critical components in the modulation and refinement of the flow of information throughout the nervous system. Abnormal development and function of interneurons has been linked to the pathobiology of numerous brain diseases, such as epilepsy, schizophrenia, and autism. Interneurons are an extremely heterogeneous cell population, with distinct morphologies, connectivities, neurochemical markers, and electrophysiological properties. With the advent of new technologies such as single-cell sequencing to dissect gene expression and connectivity patterns, the classification of interneurons into specific subtypes is ever evolving.

Interneurons such as GABAergic projection neurons are born in the ventral forebrain during embryogenesis and undergo a prolonged migratory period to populate nearly every brain region. However, our general understanding of the developmental mechanisms that generate such GABAergic cell diversity remains poorly understood. The goal of our lab is to dissect the genetic and molecular programs that underlie initial fate decisions during embryogenesis and to explore how the environment and genetic cascades interact to give rise to such a stunning diversity of GABAergic cell subtypes. We take a multifaceted approach, utilizing both *in vitro* and *in vivo* strategies to identify candidate mechanisms that regulate interneuron fate decisions. We strive to develop cutting-edge techniques that will overcome the many challenges faced when studying interneuron development. We believe that our pursuits will act as a springboard for future research and provide new insights into both normal development and various neurodevelopmental diseases.

Transcriptional heterogeneity of cycling VZ and SVZ cells throughout the embryonic mouse forebrain

The medial ganglionic eminence (MGE) gives rise to the majority of



Timothy J. Petros, PhD, Head,
*Unit on Cellular and Molecular
Neurodevelopment*

Yajun Zhang, BM, *Biologist*

Dongjin Lee, PhD, *Postdoctoral
Fellow*

Jianing Li, PhD, *Postdoctoral Fellow*

Matthew Manion, PhD,
Postdoctoral Fellow

Chris Rhodes, PhD, *Postdoctoral
Fellow*

Dhanya Asokumar, BS,
Postbaccalaureate Fellow

Samra Beyene, MS,
Postbaccalaureate Fellow

Lielle Elisha, BS, *Postbaccalaureate
Fellow*

Anna Kim, *Student Internship
Program*

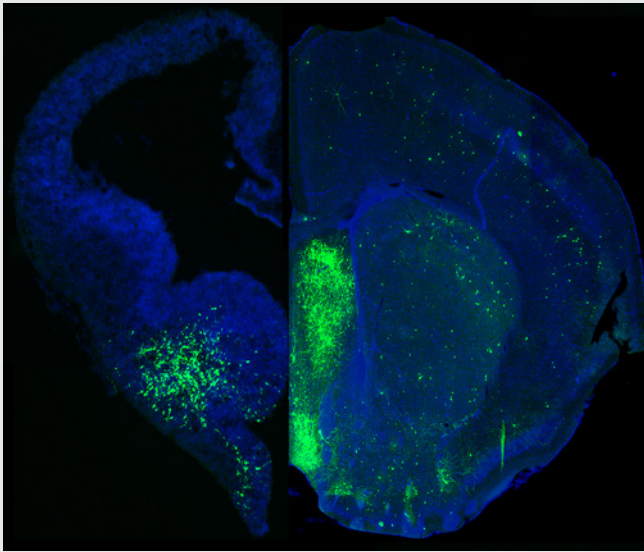


FIGURE 1. MGE-derived GABAergic cells populate many different brain regions.

The image depicts a section of an embryonic brain (*left*) that has been electroporated to label cells derived from the medial ganglionic eminence (MGE), merged with a section of an adult brain (*right*), displaying the incredible spatial and morphological diversity of MGE-derived cells in the mature brain. Understanding how this heterogeneous population is generated from one embryonic brain structure is the focus of this laboratory.

forebrain interneurons, most notably the somatostatin- and parvalbumin-expressing (SST⁺ and PV⁺) subtypes, and nNOS (neuronal nitric oxide synthetase)-expressing neurogliaform and ivy cells in the hippocampus. The MGE is a transient, dynamic structure, which arises around E10 and bulges into the lateral ventricle over the next several days before dissipating towards the end of embryogenesis. Given that initial fate decisions are generated within the MGE, there has been much focus on identifying a logic for interneuron generation from this region. Previous experiments characterized both a spatial and temporal gradient within the MGE, which regulates the initial fate decision to become either PV⁺ or SST⁺ interneurons. SST⁺ interneurons are preferentially born early in embryogenesis from the dorsoposterior MGE, whereas PV⁺ interneurons are born throughout embryogenesis with a bias of originating from the ventroanterior MGE. Earlier, I had discovered an additional mechanism regulating this fate decision: the mode of neurogenesis. Using *in utero* electroporations, I found that PV⁺ interneurons are preferentially born from basal progenitors (also known as intermediate progenitors), whereas SST⁺ interneurons arise more commonly from apical progenitors.

Recently, we built on this observation to characterize heterogeneous gene expression in neural progenitors throughout the embryonic forebrain. We performed a comprehensive single-cell RNA sequencing (scRNA-Seq) analysis of ventricular zone (VZ) and subventricular zone (SVZ) cells in four brain regions that give rise to distinct cell types: the MGE, LGE (lateral ganglionic eminence), CGE (caudal ganglionic eminence), and cortex. This allows us to compare gene expression in neural progenitors both between distinct brain regions (MGE vs. CGE) and within specific subdomains of these regions (dorsal vs. ventral LGE). We verified many of these gene expression profiles via *in situ* hybridization and revealed previously unknown transcriptional heterogeneity in VZ cells throughout the forebrain. The findings were published earlier this year [Reference 2]. We are currently following up on several intriguing candidates to better understand their role in cell fate.

Characterization of the epigenetic landscape during embryonic neurogenesis

In multicellular organisms, cells are genetically homogenous but structurally and functionally heterogeneous

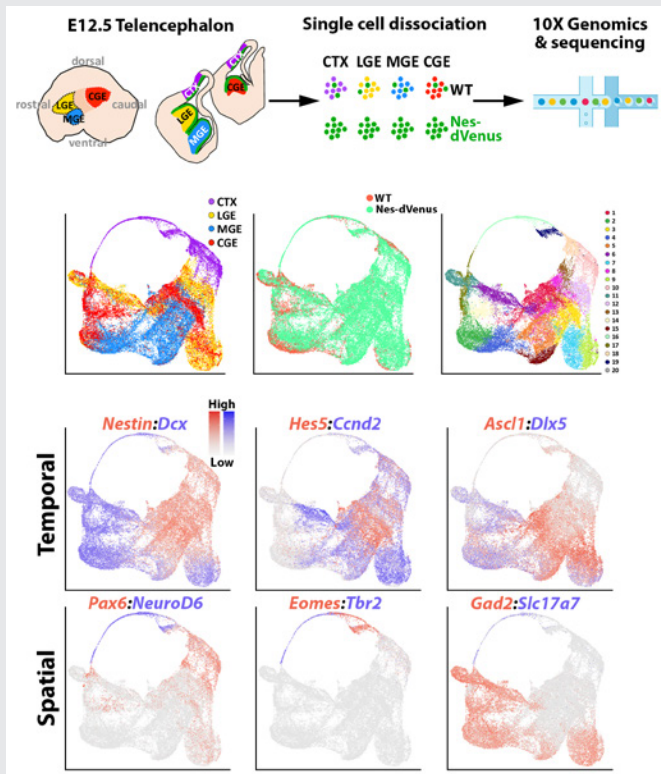


FIGURE 2. Single-cell sequencing in the embryonic mouse forebrain

Top row. Experimental paradigm to harvest cells from four distinct brain regions (MGE, LGE, CGE, cortex) from wild-type and Nestin-dVenus embryonic mouse brains for single-cell RNA sequencing.

Middle row. UMAP (uniform manifold approximation and projection) plots of single cells categorized by brain region (*left*), mouse line (*middle*), or putative cell cluster (*right*).

Bottom rows. UMAP plots depicting gene expression profiles enriched in different stages of neural progenitors (*top*) or spatial domains (*bottom*).

as a result of differential gene expression, which is often mitotically heritable. The mechanisms regulating such expression are ‘epigenetic,’ as they do not involve altering the DNA sequence itself; they include DNA methylation (DNAm), histone modifications, and higher-order chromatin structure. In particular, DNA and histone modifications often follow specific rules termed the ‘epigenetic code,’ similar to the genetic code. Collectively, DNAm and histone modification have been reported to regulate transcription and chromatin structure in many stem-cell and developmentally critical processes. Previous scRNA-Seq experiments on the ganglionic eminences (GEs) identified surprisingly few region-specific genes in cycling progenitors (immature cells that are still cycling and have not exited the cell cycle), despite the fact that these regions produce distinct GABAergic cell populations. Because there are dynamic changes in the chromatin landscape during development, a prevailing hypothesis is that epigenomic signatures may be a better predictor of cell fate during development, revealing both potential distal enhancers and/or genetic loci that may be ‘poised’ but not yet expressed. However, direct support for this hypothesis is lacking. The idea is particularly relevant, given that epigenetic changes are observed in many neurological and psychiatric diseases and that most single-nucleotide variants (SNVs) identified in diseases-specific genome-wide association studies (GWAS) map to non-coding regions, implying that epigenetic regulation of gene expression may underlie some disease etiologies.

We performed single-cell assay for transposase-accessible chromatin with sequencing (scATAC-Seq), in combination with Cut&Tag (to characterize histone modifications) and Hi-C/Capture-C (to analyze higher order chromatin interactions), in order to generate an ‘Epigenome Atlas’ of four different regions in the embryonic mouse brain. This comprehensive, unbiased analysis reveals striking differences in gene expression and chromatin organization between adjacent brain regions and revealed new candidates for region-specific

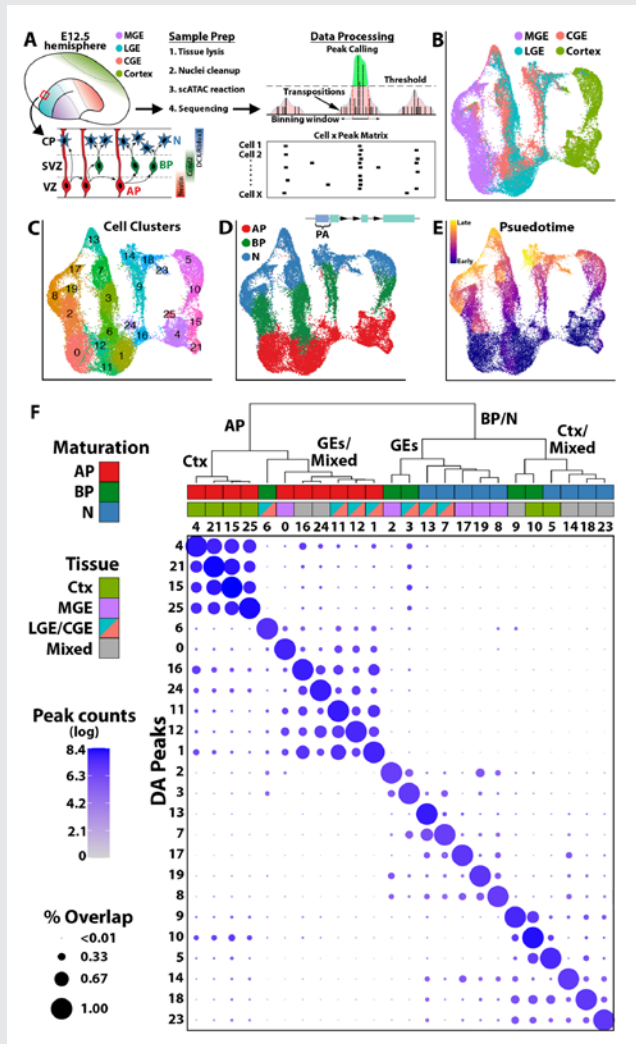


FIGURE 3. snATAC-Seq in distinct regions of the mouse embryonic forebrain

A. Schematic of snATAC-Seq (single-nucleus analysis) of transposase-accessible chromatin using sequencing) workflow and neurogenic cell types: apical progenitors (APs), basal progenitors (BPs) and neurons (Ns). B. UMAP (uniform manifold approximation and projection) visualization of single nuclei clustered by brain region; C. SLM (Smart Local Moving algorithm); D. neurogenic cell type. PA (promoter accessibility) representing reads mapping within 2 kb upstream of TSSs (transcription start sites); E. pseudotime. F. Embryonic snATAC-Seq dot plot of differentially accessible peaks (DA peaks) for each cluster. Dot diameter indicates the percent of DA peaks from one cluster (column cluster labels) that are detectable in any other cluster (row cluster labels). Color intensity represents the total DA peak count per cluster. Hierarchical clustering was performed using correlation distance and average linkage.

promoter-enhancer interactions that may be critical for neurons' fate and maturation. We are currently following up on several intriguing avenues based on these observations. The work was published earlier this year [Reference 1], and [the entire dataset is publicly available](#) in a searchable format on the UCSC Genome Browser platform. Additionally, based on this study and the one above, we recently prepared a manuscript detailing our procedures to obtain single-cell and single-nuclei dissociations from embryonic and adult mouse brains for numerous downstream applications [Reference 3].

Mechanisms regulating fate determination of CGE-derived interneurons

While significant progress has been made characterizing mechanisms regulating initial fate decisions of MGE-derived interneurons, our understanding of CGE-derived interneurons lags significantly. This is in part because we lack genetic tools to specifically target and manipulate CGE-derived cells. There is an expansion of CGE-derived interneuron subtypes in humans and primates compared with mice, so a better understanding of the developmental trajectory of these cells is warranted.

To this end, we are currently performing two types of experiments to better understand the developmental logic of CGE-derived cells. First, we are performing cell transplantation studies to determine whether specific CGE-derived interneuron subtypes are derived from distinct regions of the CGE. These types of transplantation experiments generated initial insights into the spatial logic of the MGE, but surprisingly similar types of experiments have not been performed in the CGE. By combining this spatial logic

with the scRNA-Seq and scATAC studies detailed above, we hope to link early transcription and chromatin accessibility profiles in CGE progenitors with mature interneuron fates. Second, we are developing a Perturb-Seq approach using mESCs to identify activation (or repression) of genes that promote a CGE fate. We hope to make significant progress in both these approaches in the upcoming year.

Publications

1. Rhodes CT, Thompson JJ, Mitra A, Asokumar D, Lee DR, Lee DJ, Zhang Y, Jason E, Dale RK, Rocha PP, Petros TJ. An epigenome atlas of neural progenitors within the embryonic mouse forebrain. *Nat Commun* 2022 13(1):4196.
2. Lee DR, Rhodes CT, Mitra A, Zhang Y, Maric D, Dale RK, Petros TJ. Transcriptional heterogeneity of ventricular zone cells in the ganglionic eminences of the mouse forebrain. *eLife* 2022 <https://doi.org/10.7554/eLife.71864>.
3. Lee DR, Zhang Y, Rhodes CT, Petros TJ. Generation of single cell and single nuclei suspensions from embryonic and adult mouse brains. *STAR Protocols* 2022 4(1):101944.
4. Chakraborty S, Kopitchinski N, Zuo Z, Eraso A, Awasthi P, Chari P, Mitra A, Tobias IC, Moorthy SD, Dale RK, Mitchell JA, Petros TJ, Rocha PP. Enhancers that activate target genes across CTCF boundaries increase phenotypic robustness. *Nat Genet* 2022 in press.

Collaborators

- Susan Amara, PhD, *Laboratory of Molecular and Cellular Neurobiology, NIMH, Bethesda, MD*
- Ryan Dale, PhD, *Bioinformatics Core, NICHD, Bethesda, MD*
- Claire Le Pichon, PhD, *Unit on the Development of Neurodegeneration, NICHD, Bethesda, MD*
- Chris McBain, PhD, *Section on Cellular and Synaptic Physiology, NICHD, Bethesda, MD*
- Pedro Rocha, PhD, *Unit on Genome Structure and Regulation, NICHD, Bethesda, MD*

Contact

For more information, email tim.petros@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/petros>.

Molecular Genetics of an Imprinted Gene Cluster on Mouse Distal Chromosome 7

Our lab is interested in epigenetic mechanisms that regulate gene expression in order to understand how the epigenome is established and then modified during development and how disruptions in the epigenome can lead to developmental disorders and disease. A simple definition of the epigenome is that it is the total array of heritable changes in chromosome structures that influence gene function. Known epigenetic mechanisms include DNA modifications (such as cytosine methylation), altering histone localization and chemical structures, and DNA looping. Our approach is to build and then characterize mouse models for human disorders in which the fundamental problem is disruption of the normal epigenome. By understanding disease progression in these models, we hope to learn about mechanisms for regulating the epigenome. But we also hope that clarifying the exact molecular defects in these specific diseases will identify new therapeutic targets. We are currently investigating two disease models. First, we are continuing our long-term and comprehensive analysis of maternal loss of imprinting (LOI) at the *H19/Igf2* locus. As detailed in the next paragraph, maternal LOI is a model for Beckwith Wiedeman syndrome. Second, we are generating and characterizing new mouse models that disrupt cohesin accumulation on the chromosomes. Altered cohesin patterns are the molecular cause for the Cornelia de Lange syndrome.

Genomic imprinting is an unusual form of gene regulation by which an allele's parental origin restricts allele expression. For example, almost all expression of the noncoding RNA tumor-suppressor gene *H19* is from the maternal chromosome. In contrast, expression of the neighboring Insulin-like Growth Factor 2 gene (*Igf2*) is from the paternal chromosome. Imprinting represents the cleanest possible example of epigenetic regulation of gene expression, and therefore understanding imprinting is an excellent means for understanding epigenetics. Imprinted genes are not randomly scattered throughout the chromosome but rather are localized in discrete clusters, where monoallelic expression is regulated by a common *cis*-acting DNA-regulatory element called the Imprinting Control Region (ICR). We study a cluster of imprinted genes on the distal end of mouse chromosome 7 (Figure 1). The syntenic region in humans (11p15.5) is highly conserved in gene organization and expression patterns. Imprinting of *H19* and of *Igf2* is regulated by the *H19* ICR, which is located just upstream of the



Karl Pfeifer, PhD, Head, Section on Epigenetics

Claudia Gebert, PhD, *Biologist*

Beenish Rahat, PhD, *Postdoctoral Fellow*

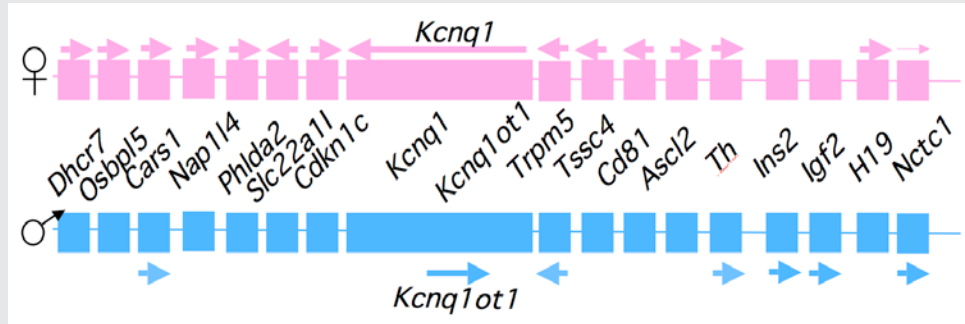
Hyungchul Lee, PhD, *Visiting Fellow*

Colter Brainard, BS, *Postbaccalaureate Fellow*

Rachel Muti, BS, *Postbaccalaureate Fellow*

FIGURE 1. An imprinted domain on mouse distal chromosome 7

Maternal (*pink*) and paternal (*blue*) chromosomes are indicated. Horizontal arrows denote RNA transcription.



H19 promoter. We showed that the molecular function of the *H19* ICR is to organize the region into alternative 3D structures. In humans, epigenetic mutations that disrupt *H19* ICR function result in loss of monoallelic expression. Mutations in the paternal *H19* ICR lead to loss of *Igf2* expression and biallelic (2X) *H19* expression, and are associated with the Russell-Silver syndrome. Mutations in the maternal *H19* ICR lead to loss of *H19* but biallelic (2x) *Igf2* expression, and are associated with the Beckwith-Wiedemann syndrome and several pediatric cancers. Our lab generated mouse models that phenocopy the human diseases, and our goal is to characterize the molecular defects associated with mis-expression of *Igf2/H19* and to understand how these molecular defects lead to disease and cancer.

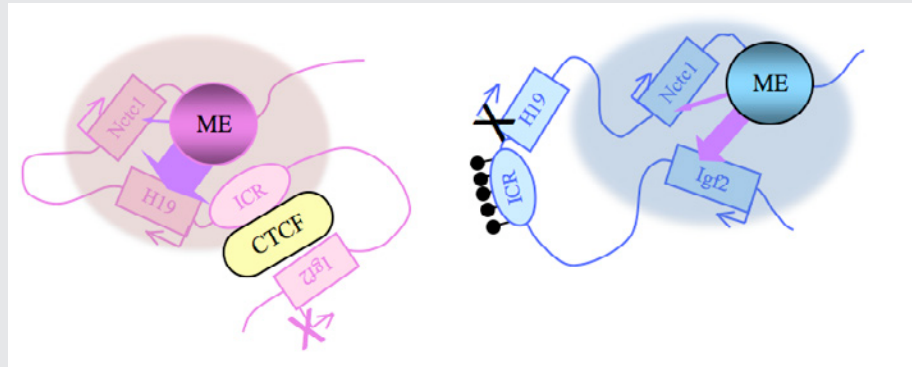
Alternative long-range interactions between distal regulatory elements establish allele-specific expression at the *Igf2/H19* locus.

Paternally expressed *Igf2* lies about 80 kb upstream of the maternal-specific *H19* gene. Using cell-culture systems as well as transgene and knockout experiments *in vivo*, we identified the enhancer elements responsible for activation of the two genes. The elements are shared and are all located downstream of the *H19* gene (Figure 2). Imprinting at the *Igf2/H19* locus depends on the 2.4 kb *H19* ICR, which lies between the two genes, just upstream of the *H19* promoter (Figure 2). On the maternal chromosome, binding of the CTCF protein, a transcriptional repressor, to the *H19* ICR establishes a transcriptional insulator that organizes the chromosome into loop structures that bring the *H19* promoter into contact with downstream enhancers but exclude the *Igf2* promoter from these enhancer interactions. The loops favor *H19* expression but block interactions between the maternal *Igf2* promoters and the downstream shared enhancers, thus preventing maternal *Igf2* expression. Upon paternal inheritance, the cytosine residues within the ICR DNA sequences are methylated, which prevents binding of the CTCF protein, so that a transcriptional insulator is not established. Thus, paternal *Igf2* promoters and the shared enhancers interact via DNA loops, and expression of paternal *Igf2* is facilitated. Taken together, we find that the fundamental role of the ICR is to organize the chromosomes into alternative 3-D configurations that promote or prevent expression of the *Igf2* and *H19* genes.

The *H19* ICR is not only necessary but is also sufficient for genomic imprinting. To demonstrate this, we used knock-in experiments to insert the 2.4 kb element at heterologous loci and demonstrated its ability to imprint these regions. Furthermore, analyses of the loci confirmed and extended the transcriptional model described above. Upon maternal inheritance, even ectopic ICR elements remain unmethylated, bind to the CTCF protein, and form transcriptional insulators. Paternally inherited ectopic ICRs become methylated,

FIGURE 2. Distinct maternal and paternal chromosomal conformations at the distal 7 locus

Epigenetic modifications on the 2.4 kb ICR generate alternative 3D organizations across a large domain on paternal (*blue*) and maternal (*pink*) chromosomes and thereby regulate gene expression. ICR, imprinting control region; ME, muscle enhancer; filled lollipops, CpG methylation covering the paternal ICR.



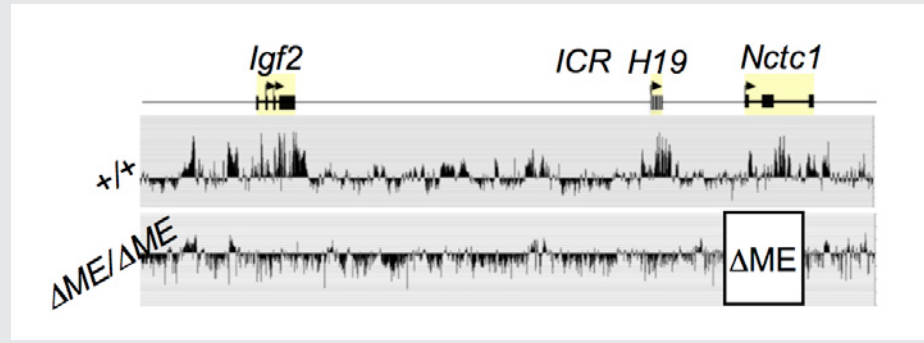
cannot bind to the CTCF, and therefore promote alternative loop domains distinct from those organized on maternal chromosomes. Most curious was the finding that DNA methylation of ectopic ICRs is not acquired until relatively late in development, after the embryo implants in the uterus. In contrast, at the endogenous locus, ICR methylation occurs during spermatogenesis. The findings thus imply that DNA methylation is not the primary imprinting mark that distinguishes maternally from paternally inherited ICRs.

The *Nctc1* gene lies downstream of *H19* and encodes a spliced, polyadenylated long noncoding RNA (lncRNA), which is transcribed across the muscle enhancer element (ME in Figure 2), which is shared by *Igf2* and *H19*. *Nctc1* expression depends on this enhancer element. Concordantly, the shared enhancer interacts with the *Nctc1* promoter, just as it interacts with the maternal *H19* and paternal *Igf2* promoters. We showed that all three co-regulated promoters (*Igf2*, *H19*, and *Nctc1*) also physically interact with each other in a manner that depends on their interactions with the shared enhancer. Thus, enhancer interactions with one promoter do not preclude interactions with another promoter. Moreover, we demonstrated that such promoter-promoter interactions are regulatory; they explain the developmentally regulated imprinting of *Nctc1* transcription. Taken together, our results demonstrate the importance of long-range enhancer-promoter and promoter-promoter interactions in physically organizing the genome and establishing the gene expression patterns that are crucial for normal mammalian development.

Molecular mechanisms for tissue-specific promoter activation by distal enhancers

Normal mammalian development is absolutely dependent on establishing the appropriate patterns of expression of thousands of developmentally regulated genes. Most often, development-specific expression depends on promoter activation by distal enhancer elements. The *Igf2/H19* locus is a highly useful model system for investigating mechanisms of enhancer activation. First, the biological significance of the model is clear, given that expression of these genes is so strictly regulated. Even twofold changes in RNA levels are associated with cancer and developmental disorders. Second, we already know much about the enhancers in this region and have established powerful genetic tools to investigate their function. *Igf2* and *H19* are co-expressed throughout embryonic development and depend on a series of tissue-specific enhancers that lie between 8 and more than 150 kb downstream of the *H19* promoter (or between 88 and more than 130 kb

FIGURE 3. The shared muscle enhancer (ME) directs RNAP binding and RNA transcription across the entire 150 kb locus.



downstream of the *Igf2* promoters). The endodermal and muscle enhancers have been precisely defined, and we generated mouse strains carrying deletions that completely abrogate enhancer function. We also generated insulator insertion mutations that specifically block muscle enhancer activity. We used these strains to generate primary myoblast cell lines so that we can combine genetic, molecular, biochemical, and genomic analyses to understand the molecular bases for enhancer functions.

The lncRNA, encoded by *Nctc1*, is an essential element of the muscle enhancer, as demonstrated by transient transfection analyses that define a 300-bp element that is both necessary and sufficient for maximal enhancer activity. However, stable transfection and mouse mutations indicate that this core element is not sufficient for enhancer function in a chromosomal context. Instead, the *Nctc1* promoter element is also essential; the *Nctc1* RNA itself is not required (at least in *trans*). Mutational analysis demonstrates that it is *Nctc1* transcription through the core enhancer that is necessary for enhancer function. Curiously, the *Nctc1* promoter has chromatin features typical of both a classic enhancer and a classic peptide-encoding promoter. Several recent genomic studies also suggested a role for noncoding RNAs in gene regulation and enhancer function. We will use our model system to characterize the role of *Nctc1* transcription in establishing enhancer orientation, enhancer promoter specificity, and enhancer tissue specificity.

The muscle enhancer (ME) directs RNA polymerase (RNAP) II not only to its cognate promoters (i.e., to the *H19* and *Igf2* promoters) but also across the entire intergenic region. To demonstrate this, we used ChIP-on-chip to analyze RNAP localization on chromatin prepared from wild-type and enhancer-deletion (DME) cell lines (Figure 3). As expected, RNAP binding to the *H19* and *Igf2* promoters is entirely enhancer-dependent. Curiously, we also noted enhancer-dependent RNAP localization across the entire locus, including the large intergenic domain between the two genes. Furthermore, RNAP binding is associated with RNA transcription. Thus, the enhancer regulates accessibility and RNAP binding not only at specific localized sites but across the entire domain. The results support a facilitated tracking model for enhancer activity.

RNAP binding at 'real' genes and across the intergenic regions is qualitatively different. To demonstrate this, we used naturally occurring single-nucleotide polymorphisms (SNPs) to investigate allelic differences in binding of RNAP and activation of gene expression in wild-type cells and in cells carrying enhancer deletions or insulator insertion mutations. RNAP binding across the *Igf2* and *H19* genes is both enhancer-dependent and insulator-sensitive; that is, a functional insulator located between an enhancer and its regulated gene prevents RNAP binding and likewise prevents RNA transcription. Across the intergenic regions, RNAP binding

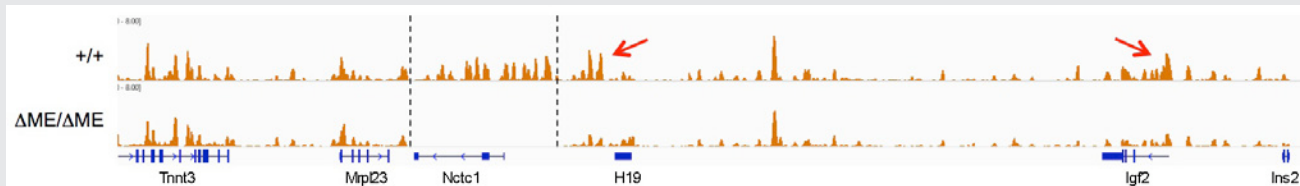


FIGURE 4. Chromatin patterns at the *Igf2/H19* locus are independent of enhancer activity.

Chromatin was isolated from wild-type and enhancer-deletion muscle cells, using antibodies against H3K4me1, and analyzed by DNA sequencing.

and RNA transcription are similarly enhancer-dependent (see above). However, intergenic RNAP binding and transcription are not insulator-sensitive. The results indicate that insulators do not serve solely as a physical block for RNAP progression, but rather they specifically interfere with certain RNAP states or activities.

The muscle enhancer regulates RNAP binding and RNA transcription, but does not establish chromatin structures, because both RNA transcription and RNAP binding across the *Igf2/H19* domain are entirely dependent upon the muscle enhancer. For example, levels of *H19* RNA are reduced more than 10,000-fold in muscle cells in which the enhancer has been deleted. To test the dependence of chromatin structure on enhancer activity, we performed ChIP-Seq on wild-type and on enhancer-deletion cell lines using antibodies against the histones H3K4me1, H3K43me3, and H3K36me3. Surprisingly, we saw no changes in the patterns of chromatin modification (Figure 4). Thus, a functional enhancer and active RNA transcription are not important for establishing chromatin structures at the *Igf2/H19* domain.

Functions of *H19* lncRNA in regulating cell-cycle progression and senescence

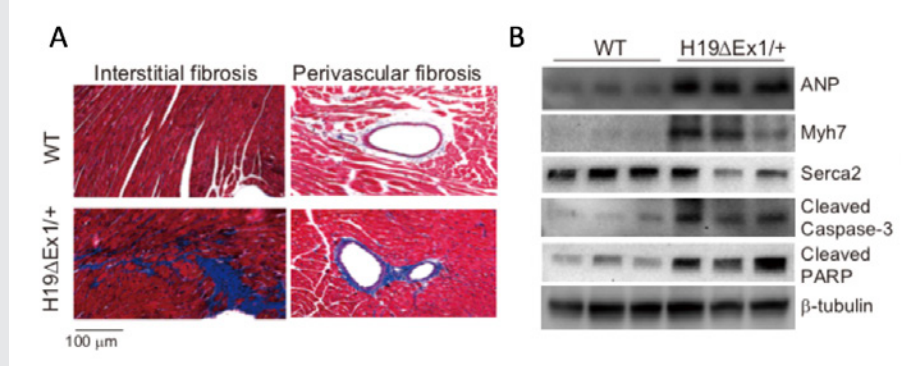
To determine the biochemical functions of *H19* lncRNA, we use *in vitro* models, including primary myoblasts, C2C12 myoblasts, and NIH3T3 cells. Abrupt depletion of *H19* by either siRNA or cre-induced recombination of *H19*-floxed alleles results in increased p21 RNA (p21 is a cyclin-dependent kinase inhibitor involved in cell-cycle arrest) and peptide, and such increased p21 activity in turn prevents cell-cycle progression and induces cellular senescence. *H19* lncRNA regulation of p21 is at the level p21 mRNA stability and translation efficiency and occurs via the p21 3' UTR. Genetic and biochemical analyses suggest that *H19* lncRNA facilitates interactions between p21 mRNA and Wig1. Current experiments focus on identifying the molecular mechanisms for these regulatory actions.

Functions of *H19* lncRNA in regulating cardiac development

The Beckwith-Wiedemann syndrome (BWS) is a developmental disorder characterized by generalized overgrowth of the fetus and a high risk for several neonatal cancers. Many BWS patients also display cardiac problems. BWS can be explained by one of two different genetic lesions: loss of function of the *CDKN1C* gene or maternal loss of imprinting at the *H19/Igf2* locus. Maternal loss of imprinting has the effect of doubling *Igf2* expression while concomitantly reducing *H19* RNA levels. Curiously, children born via artificial reproductive technology (ART) show increased incidence of BWS, which can be explained by increasingly frequent loss of *H19/Igf2* imprinting in such children. Moreover, the children show high frequency of cardiac dysfunction. Taken

FIGURE 5. Cardiac disease in *H19*-deficient mice

Mice lacking *H19* are hypertrophic, fibrotic (*panel A*), display protein expression profiles typical of cardiac failure (*panel B*), and show aberrant function on echocardiograms.



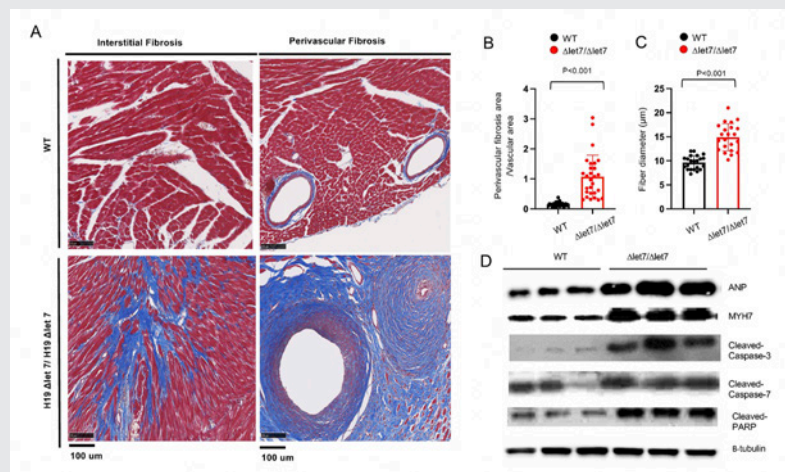
together, such findings suggest that abnormal expression of the *H19/Igf2* locus can lead to cardiac problems.

We observed that our BWS mouse model also results in cardiac dysfunction, as measured by echocardiography and ECG analyses. Molecular and molecular-genetic analyses demonstrate that biallelic *Igf2* expression and loss of *H19* play independent and distinct roles in generating the BWS phenotype. Biallelic expression of *Igf2* results in elevated levels of circulating IGF2 peptide, which super-activates insulin and insulin-like receptor kinases in cardiomyocytes, resulting in hyper-activation of AKT/mTOR signaling pathways, which in turn causes cardiomyocyte hypertrophy and hyperplasia. Such effects result in a cardiac hypertrophy that is non-pathologic and transient, i.e., the hearts function normally and, as long as *H19* levels are normal, the heart size normalizes after birth once *Igf2* expression is repressed. Thus, there are no significant health effects associated with loss of imprinting of *Igf2* only.

Loss of expression of *H19* is pathologic (Figure 5). Hearts show progressive heart disease, as manifested by hypertrophy, increased fibrosis, expression of cardiac failure markers, and reduced and abnormal heart function, as measured by echocardiography. *H19* expression in hearts is restricted to endothelial cells. *In vivo*

FIGURE 6. Let7 binding sites on *H19* lncRNA are essential for normal heart function.

Mice carrying *H19* alleles that do not include let7 binding sites are fibrotic (*panels A and B*), hypertrophic (*panel B*), and display protein expression profiles typical of cardiac failure.



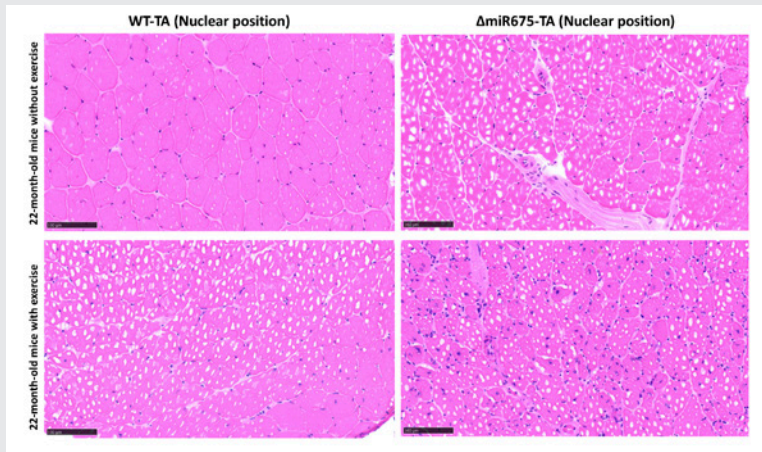


FIGURE 7. *H19*-encoded microRNA mir675 is essential for muscle injury recovery.

functional gene product. The *H19* precursor RNA is processed into two final products: a 2.3 kb lncRNA and microRNA 675. To determine which *H19* RNA is essential to prevent cardiomyopathy, we developed two new mouse strains that selectively disrupt accumulation of the lncRNA or the miRNA. Disruption of the lncRNA is necessary and sufficient to induce cardiac disease. To understand the molecular bases for *H19* lncRNA function, we generated alleles that disrupt specific domains. We observed that disruption of *let7* miRNA binding sites on the H19 RNA results in the same myopathies as complete gene ablation (Figure 6).

Function of H19 RNAs in muscle development

Studies in mice and humans indicate that *H19* gene function is critical for normal development and function of skeletal muscle. As described in the previous section, H19 precursor RNA is processed into two distinct products: 2.3 kb lncRNA and a small microRNA (mi675). We generated mouse models that specifically ablate one or the other. The *H19*^{DeltaMIR} allele generates an H19 lncRNA with a 40 bp deletion that is expressed at normal levels and maintains all H19 lncRNA functions that we have tested. For example, the *H19*^{DeltaMIR} lncRNA is sufficient to prevent cardiomyopathy and to prevent cellular senescence in myoblasts. However, *H19*^{DeltaMIR}*H19*^{DeltaMIR} mice present defects in muscle development and muscle regeneration. Specifically, muscle and muscle fibers are significantly smaller in mutant mice, and mutant mice cannot recover efficiently from muscle injury (Figure 7). The latter phenotype is exacerbated by age, indicating that *H19* mutant mice are a model for sarcopenia. Current research focuses on identifying the molecular bases for these phenotypes

Reducing *Wapl* dosage partially corrects embryonic growth and brain transcriptome phenotypes in *Nipbl*^{+/-} embryos.

Cohesin rings interact with DNA and modulate expression of thousands of genes. Cohesin is loaded onto chromosomes by NIPBL (a protein that is required for the association of cohesin with DNA) and unloaded by WAPL. Haploinsufficiency for *NIPBL* leads to a developmental disorder, the Cornelia de Lange syndrome (CdLS), that is modeled in *Nipbl*^{+/-} mice. Key phenotypes in this mouse model include slow embryonic growth, dysregulation of thousands of genes, and perinatal lethality. In *Drosophila*, reduced *Nipbl* function also causes developmental defects. Importantly, reducing *Wapl* and *Nipbl* function together alleviates many of the

analyses of whole hearts and *in vitro* analyses of isolated endothelial cells show that reduction in *H19* results in increased endothelial-to-mesenchymal transition (EMT). EMT is an essential feature of normal cardiac development; for example, formation of cardiac valves requires EMT. However, elevated frequency of EMT is associated with heart disease. Our data support the notion that *H19* regulates the cell fate of endothelial cells, and future experiments aim to identify the underlying molecular mechanisms.

The *H19* gene does not encode a protein. Rather H19 RNA is the

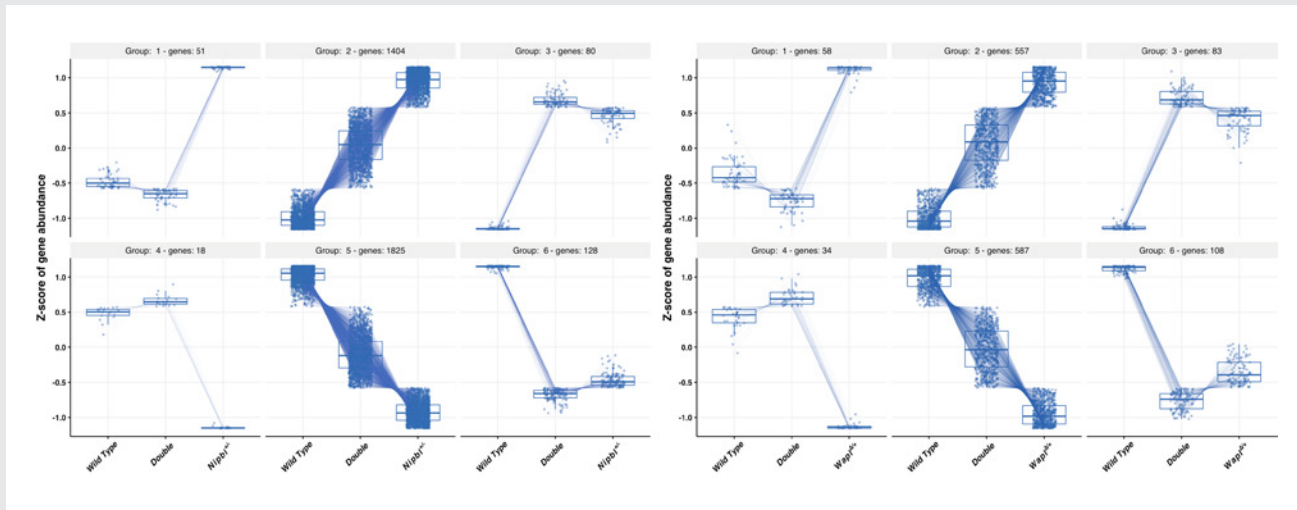


FIGURE 8. Rescue of transcriptome phenotypes in *Wapl*^{+/-} *Nipbl*^{+/-} double heterozygotes

A. 94% of 3,506 genes dysregulated in *Nipbl*^{+/-} embryonic brains are partially or fully rescued by reducing *Wapl* gene function.

B. Similarly, 87% of 1,427 genes dysregulated in *Wapl*^{+/-} embryonic brains are rescued by reducing *Nipbl* function.

developmental defects. To determine whether this is true in mammals, we generated mice with novel *Wapl* hypomorph alleles and then compared the phenotypes of *Wapl*^{+/+} *Nipbl*^{+/+} (wild type), *Wapl*^{+/-} *Nipbl*^{+/+} (*Wapl* hets), *Wapl*^{+/+} *Nipbl*^{+/-} (*Nipbl* hets), and *Wapl*^{+/-} *Nipbl*^{+/-} (double hets) littermates. Disruption of *Wapl* and *Nipbl* each lead to reduced growth and dysregulation of a (mostly) overlapping set of genes. Interestingly, these phenotypes are not exacerbated but instead are partially rescued in the double mutant (Figure 8). The results suggest that *Nipbl/Wapl* balance (i.e., loading/unloading balance) is the critical factor in regulating cohesin function, and also suggest novel therapeutic approaches for CdLS patients.

Publications

1. Park K-S, Rahat B, Lee HC, Noeker J, Mitra A, Kean CM, Knutsen RH, Springer D, Gebert CM, Kozel BA, Pfeifer K. Cardiac pathologies in mouse loss of imprinting models are due to misexpression of H19 long noncoding RNA. *eLife* 2021 10:e67250.
2. Flores DJ, Duong T, Brandenberger LO, Mitra A, Shirali A, Johnson JC, Springer D, Noguchi A, Yu ZX, Ebert SN, Ludwig A, Knollmann BC, Levin MD, Pfeifer K. Conditional ablation and conditional rescue models for *Casq2* elucidate the role of development and of cell-type specific expression of *Casq2* in the CPVT2 phenotype. *Hum Mol Genet* 2018 27:1533–1544.
3. Park K-S, Mitra A, Rahat B, Kim B, Pfeifer K. Loss of imprinting mutations define both distinct and overlapping roles for misexpression of *IGF2* and of *H19* lncRNA. *Nucleic Acids Res* 2017 45:12766–12779.
4. Mitra A, Park K-S, Pfeifer K. Circular RNAs and competing endogenous (ceRNA) networks. *Transl Cancer Res* 2018 7:S624–628.
5. Geng H, Bu HF, Liu F, Wu L, Pfeifer K, Chou PM, Wang X, Sun J, Lu L, Pandey A, Bartolomei MS, De Plaen IG,

Wang P, Yu J, Qian J, Tan XD. In inflamed intestinal tissues and epithelial cells, interleukin 22 signaling increases expression of H19 long noncoding RNA, which promotes mucosal regeneration. *Gastroenterology* 2018 155:144–155.

Collaborators

- Judy Kassis, PhD, *Section on Gene Expression, NICHD, Bethesda, MD*
- Beth Kozel, MD, PhD, *Cardiovascular & Pulmonary Branch, NHLBI, Bethesda, MD*
- Danielle A. Springer, VMD, Dipl ACLAM, *Animal Program, NHLBI, Bethesda, MD*

Contact

For more information, email pfeiferk@nih.gov or visit <http://pfeiferlab.nichd.nih.gov>.

Rare Genetic Disorders of Cholesterol Homeostasis and Lysosomal Diseases

We study the molecular, biochemical, and cellular processes that underlie genetic disorders resulting from impaired cholesterol homeostasis and from lysosomal dysfunction. The disorders include malformation/cognitive impairment syndromes resulting from inborn errors of cholesterol synthesis and neurodegenerative disorders resulting from impaired intracellular cholesterol and lipid transport. Human malformation syndromes attributable to inborn errors of cholesterol synthesis include the Smith-Lemli-Opitz syndrome (SLOS), lathosterolosis, desmosterolosis, X-linked dominant chondrodysplasia punctata type 2 (CDPX2), and the CHILD syndrome. We also study Niemann-Pick disease type C (NPC), as well as Juvenile Batten disease caused by pathogenic variants of *CLN3* (CLN3 disease). Both NPC and CLN3 are lysosomal diseases that result in progressive neurodegeneration. We conduct both basic and clinical research. Our research group uses basic, translational, and clinical research approaches with the ultimate goal of developing and testing therapeutic interventions for these rare genetic disorders. Our basic research uses induced pluripotent stem cells (iPSC)-derived neuronal, zebrafish, and mouse models of these genetic disorders to understand the biochemical, molecular, cellular, and developmental processes that underlie the birth defects and clinical problems encountered in affected patients. Our clinical research focuses on translating basic findings to the clinic. Natural history trials of SLOS, CLN3, and NPC1 are ongoing. We have large cross-sectional and longitudinal collections of biomaterial from individuals whose disease course and phenotype are known, and such samples can be used for both for biomarker discovery and validation. Therapeutic trials have been conducted for SLOS and NPC1. A therapeutic trial for CLN3 disease has been initiated. Also, in collaboration with NCATS (the National Center for Advancing Translational Sciences), our research group has been involved in a multicenter trial of creatine transporter deficiency.

Inborn errors of cholesterol synthesis

SMITH-LEMLI-OPITZ SYNDROME (SLOS)

SLOS is an autosomal recessive, multiple-malformation syndrome characterized by dysmorphic facial features, cognitive impairment, hypotonia, poor growth, and various structural anomalies of heart, lungs, brain, limbs, gastrointestinal tract, and genitalia. The SLOS



Forbes D. Porter, MD, PhD,
*Head, Section on Molecular
Dysmorphology*

Niamh Cawley, PhD, *Staff Scientist*

Chris A. Wassif, PhD, *Staff Scientist*

Nicole M. Farhat, CPNP, *Nurse
Practitioner*

Insung Kang, PhD, *Visiting Fellow*

Tristan Furnary, BA,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Ana Johnson-Escauriza, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Rachel Luke, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Anna Lyons, BS, *Postbaccalaureate
Intramural Research Training
Award Fellow*

Kyli McKee, BS, *Postbaccalaureate
Intramural Research Training
Award Fellow*

Katerina Melnyk, BA,
*Postbaccalaureate Intramural
Research Training Award Fellow*

(continued)

phenotype is extremely variable. At the severe end of the phenotypic spectrum, infants often die as result of multiple major malformations, while mild SLOS combines minor physical malformations with behavioral and learning problems. The syndrome is the result of an inborn error of cholesterol biosynthesis that blocks the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol.

Our laboratory initially cloned the human 3beta-hydroxysterol delta7-reductase gene (*DHCR7*) and demonstrated mutations of the gene in SLOS patients. Together with others, we have so far identified over 100 mutations in *DHCR7*. We also used gene targeting in murine embryonic stem cells to produce several SLOS mouse models, including a null deletion and a hypomorphic point mutation. Mouse pups homozygous for the null mutation (*Dhcr7*^{delta3-5/delta3-5}) exhibit variable craniofacial anomalies, are growth-retarded, appear weak, and die during the first day of life because they fail to feed. Thus, we were not able to use them to study postnatal brain development, myelination, or behavior or to test therapeutic interventions. For this reason, we developed a missense allele (*Dhcr7*^{T93M}). The T93M mutation is the second most common mutation found in SLOS patients. *Dhcr7*^{T93M/T93M} and *Dhcr7*^{T93M/delta3-5} mice are viable and demonstrate SLOS with a gradient of biochemical severity (*Dhcr7*^{delta3-5/delta3-5} greater than *Dhcr7*^{T93M/delta3-5} and greater than *Dhcr7*^{T93M/T93M}). We used *Dhcr7*^{T93M/delta3-5} mice to test the efficacy of therapeutic interventions on tissue sterol profiles. As expected, dietary cholesterol therapy improved the sterol composition in peripheral tissues but not in the central nervous system. Treatment of mice with the statin simvastatin improved the biochemical defect in both peripheral and central nervous system tissue, suggesting that simvastatin therapy may be used to treat some of the behavioral and learning problems in children with SLOS. Most recently,

Andrea Munoz, BS,
*Postbaccalaureate Intramural
 Research Training Award Fellow*

Rebecca Telese, BS,
*Postbaccalaureate Intramural
 Research Training Award Fellow*

Julia Yerger, BS, *Postbaccalaureate
 Intramural Research Training
 Award Fellow*

Cristian White, BA,
*Postbaccalaureate Intramural
 Research Training Award Fellow*

Emelin Hernandez, *Animal
 Technician*

Research Group

Research Group
 Members (left to right)

Top row: Samar Rahal,
 Denny Porter, Chris
 Wassif, Niamh Cawley,
 An Dang Do

Middle row: Andrea
 Munoz, Nicole Farhat,
 Derek Alexander,
 Kisha Jenkins, Andrea
 Gutierrez Maria, Insung
 Kang, Dorie Scordilis

Bottom row: Tristan
 Furnary, Rachel Luke,
 Andrew Smith, Kyli
 McKee, Katie Scott





FIGURE 1.

Dr. Porter and one of our patients. Neurological exams in children frequently involve 'playing' with the child.

we developed a zebrafish model for SLOS that will allow us to study the impact of aberrant cholesterol synthesis on behavior. Characterization of iPSCs from SLOS patients demonstrated a defect in neurogenesis, which results from inhibition of Wnt signaling owing to a toxic effect of 7-DHC.

We are conducting a longitudinal Natural History trial. Given that SLOS patients have a cholesterol deficiency, they may be treated with dietary cholesterol supplementation. To date, we have evaluated over 125 SLOS patients. The studies are now focused on defining the phenotype of older adolescents and young adults with SLOS. We are also working to identify cerebrospinal-fluid biomarkers that may provide insight into neurological dysfunction.

A laboratory observation that SLOS fibroblasts can develop an NPC-like cellular phenotype with endolysosomal storage of unesterified cholesterol is leading to development of a therapeutic trial. This is a prime example of how our research group translates basic research to clinical research.

One reason for studying rare genetic disorders is to gain insight into more common disorders. Most patients with SLOS exhibit autistic characteristics. We are currently collaborating with other NIH and extramural groups to further evaluate this finding.

Niemann-Pick disease type C1

Niemann-Pick disease type C1 (NPC1) is a neurodegenerative disorder that results in ataxia and dementia. In view of the dementia, it has been referred to as childhood Alzheimer's disease. The disorder is caused by a defect in intracellular lipid and cholesterol transport. Initially, as part of a bench-to-bedside award, we began a clinical protocol to identify and characterize biomarkers that could be used in a subsequent therapeutic trial. The project also received support from the Ara Parseghian Medical Research Foundation, Dana's Angels Research Trust, and Together Strong. We have enrolled over 135 individuals with NPC1 in a longitudinal Natural History trial. The goals of the trial are to identify (1) a blood-based diagnostic/screening test, (2) biomarkers that can be used as tools to facilitate development and implementation of therapeutic trials, and (3) clinical symptoms/signs that may be used as efficacy outcome measures in a therapeutic trial.

Currently, the average time from first symptom to diagnosis, the 'diagnostic delay,' in our cohort of NPC patients is on the order of four to five years. In collaboration with Daniel Ory, we found elevated levels of non-enzymatically produced oxysterols in NPC1 patients. Testing for oxysterols or bile-acid derivatives has now become a standard method of diagnosis, and they are a potential biomarker that may be used to follow therapeutic interventions. We are now involved in a collaboration to implement newborn screening for NPC.

FIGURE 2. Gliosis in NPC1 mouse cerebellum

Immuno-staining of a sagittal section from the cerebellum of an NPC1-mutant mouse. Cerebellar Purkinje neurons are stained for calbindin 28K, and the expected loss of anterior Purkinje neurons is readily apparent. Expression of GFAP and IBA1 are used to detect astrogliosis and microgliosis, respectively. Nuclei are stained with Hoechst 3342.



In addition to our Natural History study, we completed a randomized, placebo-controlled, cross-over trial to investigate the safety and efficacy of *N*-acetyl cysteine (NAC) in NPC1. The goal was to determine whether NAC treatment would reduce oxidative stress and subsequently lower levels of the non-enzymatically produced oxysterols. We also tested the safety and efficacy of the histone deacetylase (HDAC) inhibitor vorinostat in adult NPC1 patients. In collaboration with the Therapeutics of Rare and Neglected Disease Program of NCATS, we completed a phase 1/2a therapeutic trial of lumbar intrathecal cyclodextrin (VTS-270, adraβetadex) therapy in NPC1. We participated in a multicenter, international phase 2b/3 of adraβetadex and investigated the safety and efficacy of combined intrathecal and intravenous adraβetadex. In collaboration with investigators at St. Louis Children's Hospital, we study the efficacy of cyclodextrin to ameliorate liver disease in infants with NPC. We are now collaborating with multiple groups in order to advance gene therapy.

To complement the clinical work, we have begun to apply molecular and proteomic approaches to both mouse and human biomaterials in order to identify biological pathways disrupted in NPC1. We identified several blood and CSF (cerebral spinal fluid) proteins and are in the process of validating the biomarkers as potential outcome measures to be used as tools in the development of therapeutic interventions. In collaboration with investigators from NHGRI and Scripps, we are obtaining genomic sequences on a large cohort of well phenotyped individuals with NPC1. Utilizing novel machine learning/artificial intelligence techniques, we are attempting to gain insight into genetic modifiers of the NPC1 phenotype.

Development of NPC1-induced pluripotent stem cell lines that can be efficiently differentiated into neurons has permitted us to initiate a number of studies in collaboration with NCATS to find drugs/genes that reduce endolysosomal cholesterol storage in NPC1 neurons.

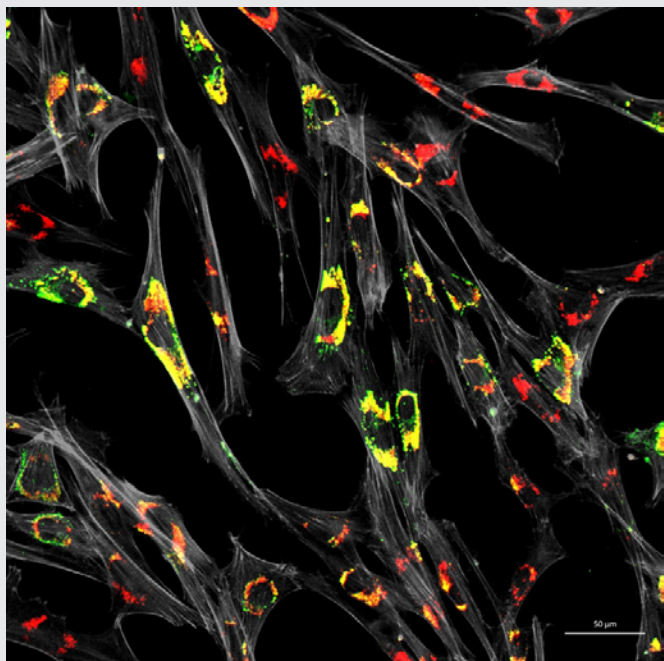


FIGURE 3. Accumulation of unesterified cholesterol in NPC1 patient fibroblasts

Human NPC1 fibroblasts were immuno-stained for Lamp1 (*green*) and stained with filipin (*red*); filipin stains unesterified cholesterol, which accumulates in the Lamp1-positive endolysosomal compartment. Cell structure was outlined by immuno-staining for actin (*gray*).

CLN3 disease

CLN3 disease (juvenile Batten disease) is an autosomal recessive, progressive neurodegeneration arising from mutation of *CLN3*, the gene encoding the lysosomal/endosomal protein battenin. The function of the battenin is not known, but its absence leads to a lysosomal storage disorder. Children with CLN3 disease typically first lose vision, followed by progressive cognitive and motor impairment. Similar to the other disorders that we study, our goal is to conduct a natural history study in order to facilitate studies designed to understand the pathology underlying these disorders as well as to develop therapeutic interventions. A major effort in our laboratory is to identify biomarkers that provide insight into CLN3 pathology and facilitate therapeutic trials. A diagnostic metabolite, especially one that could be used in a newborn screen, would be a major advance in the field.

To complement our clinical research, we are also studying CLN3 in our laboratory. We have developed an induced pluripotent stem cell line that can be efficiently induced to form CLN3-deficient neurons. The neurons are being used in genome-wide CRISPRi screens to identify genes that modify the CLN3 neuronal phenotype. The induced pluripotent stem cells (iPSC)-derived neurons will also be used to screen for potential therapeutic drugs.

Creatine transport deficiency

In collaboration with NCATS, we initiated a natural history trial of creatine transport deficiency (CTD). CTD is an X-linked disorder arising from mutation of *SLC6A8* (which encodes solute carrier family 6 Member 8, a sodium- and chloride-dependent creatine transporter). Individuals with CTD manifest significant developmental delay and have frequent seizures. The work on CTD is a multicenter trial conducted in collaboration with NCATS and Ultragenyx. Our goal is to obtain detailed natural history data, establish a biorepository, find biomarkers, and identify potential clinical outcome measures in preparation for a therapeutic trial. A major clinical finding of this natural history trial was the detailed characterization of prolonged electrocardiographic QTc in many of the individuals with CTD, which has led to specific clinical recommendations.

Additional Funding

- Ara Parseghian Medical Research Fund, University of Notre Dame
- Together Strong NPC Foundation
- NCL-Stiftung Award
- Ultragenyx Cooperative Research and Development Agreement (CRADA)
- Mandos Health CRADA
- Amicus CRADA
- Beyond Batten Disease Foundation CRADA
- SOAR NPC
- FireFly Fund

Publications

1. Francis KR, Ton AN, Xin Y, O'Halloran PE, Wassif CA, Malik N, Williams IM, Cluzeau CV, Trivedi NS, Pavan WJ, Cho W, Westphal H, Porter FD. Modeling Smith-Lemli-Opitz syndrome with induced pluripotent stem cells reveals a causal role for Wnt/beta-catenin defects in neuronal cholesterol synthesis phenotypes. *Nat Med* 2016 22:388–396.
2. Ory DS, Ottinger EA, Farhat NY, King KA, Jiang X, Weissfeld L, Berry-Kravis E, Davidson CD, Bianconi S, Keener L, Rao R, Soldatos A, Sidhu R, Walters KA, Xu X, Thurm A, Solomon B, Pavan WJ, Machielse BN, Kao M, Silber SA, McKew JC, Brewer CC, Vite CH, Walkley SU, Austin CP, Porter FD. Intrathecal 2-hydroxypropyl- β -cyclodextrin decreases neurological disease progression in Niemann-Pick disease, type C1: an ad-hoc analysis of a non-randomized, open-label, phase 1/2 trial. *Lancet* 2017 390(10104):1758–1768.
3. Cougnoux A, Yerger JC, Fellmeth M, Serra-Vinardell J, Martin K, Navid F, Iben J, Wassif CA, Cawley NX, Porter FD. Single cell transcriptome analysis of Niemann-Pick disease, type C1 cerebella. *Int J Mol Sci* 2020 21(15):5368.
4. Solomon BI, Smith AC, Sinaii N, Farhat N, King MC, Machielse L, Porter FD. Association of miglustat with swallowing outcomes in Niemann-Pick disease, type C1. *JAMA Neurol* 2020 77(12):1564–1568.
5. Dang Do AN, Sinaii N, Masvekar R, Baker E, Thurm A, Soldatos A, Bianconi S, Bielekova B, Porter FD. Neurofilament light chain levels correlate with clinical measures in CLN3 disease. *Genet Med* 2020 23(4):751–757.

Collaborators

- William Balch, PhD, *The Scripps Research Institute, La Jolla, CA*
- Elizabeth Berry-Kravis, MD, PhD, *Rush University Medical Center, Chicago, IL*
- Juan Bonifacino, PhD, *Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD*
- Stephanie Cologna, PhD, *University of Illinois at Chicago, Chicago, IL*
- Cristan Davidson, PhD, *Support Of Accelerated Research for Niemann-Pick C (SOAR-NPC), Washington, DC*
- Patricia Dickson, MD, *St. Louis Children's Hospital, Washington University School of Medicine, St. Louis, MO*
- Paul Helquist, PhD, *University of Notre Dame, Notre Dame, IN*
- Fang Liu, PhD, *University of Notre Dame, Notre Dame, IN*
- Judith Miller, PhD, *Children's Hospital of Philadelphia, Philadelphia, PA*
- Daniel Ory, MD, *Institute of Clinical and Translational Sciences, Washington University in St. Louis, St. Louis, MO*
- William J. Pavan, PhD, *Genetic Disease Research Branch, NHGRI, Bethesda, MD*

- Ninet Sinaii, PhD, *Biostatistics and Clinical Epidemiology Service, NIH Clinical Center, Bethesda, MD*
- David Sleat, PhD, *Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, New Jersey*
- Beth Solomon, MS, CCC-SLP, *Rehabilitation Medicine Department, NIH Clinical Center, Bethesda, MD*
- Audrey Thurm, PhD, *Neurodevelopmental and Behavioral Phenotyping Service, NIMH, Bethesda, MD*
- Charles H. Vite, DVM, PhD, *University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA*
- Michael E. Ward, MD, PhD, *Inherited Neurodegenerative Diseases Unit, NINDS, Bethesda, MD*
- Jill Weimer, PhD, *Sanford Research, Sioux Falls, SD*
- Wei Zheng, PhD, *Therapeutics for Rare and Neglected Diseases Program, NCATS, Bethesda, MD*

Contact

For more information, email fdporter@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/porter>.

Three-Dimensional Organization of the Genome as a Determinant of Cell-Fate Decisions

Our lab seeks to understand cell-lineage differentiation, gene regulation, and how non-coding DNA elements and the 3D architecture of chromosomes contribute to such processes during development and disease. We are also interested in early mammalian development as a system in which to decipher how cells make lineage decisions and how gene-regulatory networks are established.

Eukaryotic cells need to deal with the biophysical constraints of packaging two meters of DNA inside a tiny nucleus (2–10 microns) and still retain the ability to access both its coding and non-coding elements to precisely orchestrate gene expression programs. Research over the past decade has begun to elucidate the mechanisms through which DNA condensation and organization in the nucleus are achieved. The results of such research suggest that the processes are tightly controlled and are themselves critical components of gene regulation. Our long-term goal is to understand how such processes occur *in vivo* and how their regulation dictates cell identity and cell-fate decisions in mammals.

To do so, we combine the robustness of mouse-genome editing and genetics with cutting-edge sequencing-based genomic techniques such as ATAC-Seq (assay for transposase-accessible chromatin using sequencing), ChIP-Seq (chromatin-immuno-precipitation DNA-sequencing), and Hi-C (high-throughput chromosome conformation capture technique), as well as live-imaging approaches. We believe that the early mouse embryo is an ideal model system in which to determine how nuclear architecture is regulated in the context of an organism and how that impacts cell behavior and identity.

Fertilization is the ultimate reprogramming experiment, where two highly differentiated cells (oocyte and sperm) fuse to form a zygote with totipotent potential. This involves a massive rearrangement of epigenetic modifications, both at the level of the DNA and of the histones, and the activity of many transcriptional regulators. Our studies aim to understand how 3D chromatin structures are established during this period and how they impact future developmental decisions.

Following fertilization and within a few cell divisions, the first cell



Pedro Rocha, PhD, Head, Unit on Genome Structure and Regulation

Nina Kopitchinski, *Lab Manager*

Shreeta Chakraborty, PhD, *Postdoctoral Fellow*

Joyce J. Thompson, PhD, *Postdoctoral Fellow*

Zhenyu Zuo, PhD, *Postdoctoral Fellow*

Representative image of the lab's research

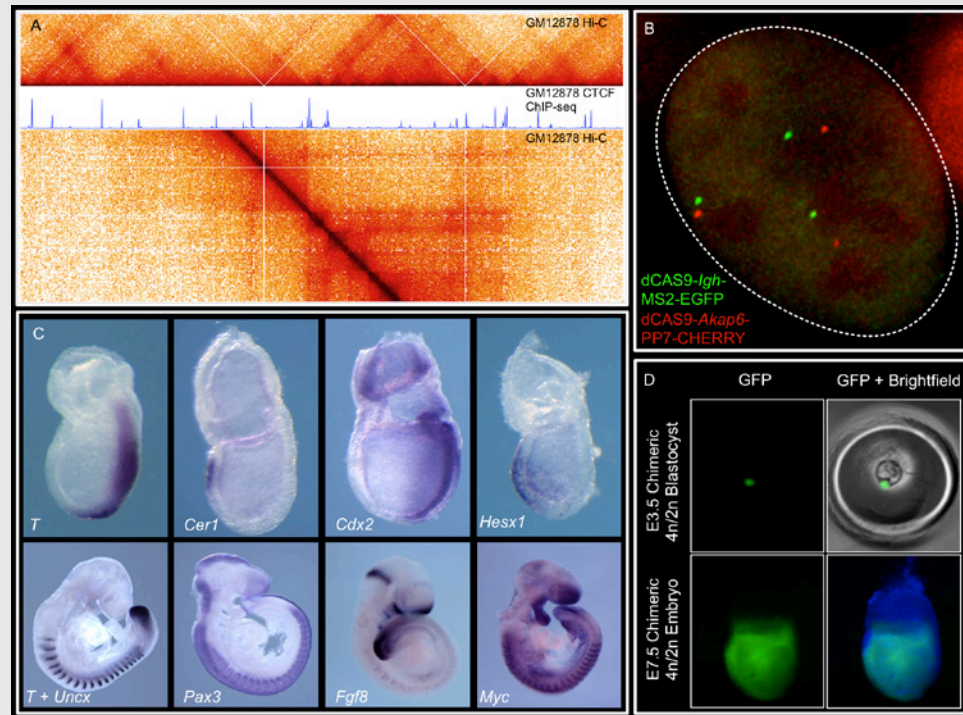
We combine imaging techniques in both fixed and living cells with sequencing-based genomic techniques that assess DNA-DNA interactions.

A. Hi-C and CTCF ChIP-Seq of GM12878 cells, which allow characterization of chromatin structure and identification of binding sites of an important architectural protein.

B. dCAS9 MCP-EGFP and PCP-CHERRY live imaging of the *Igh* and *Akap6* loci. The mouse embryo is an unparalleled system for understanding how tissue-specific gene expression is achieved.

C. Whole-mount *in situ* hybridization for patterning markers in mid and late gastrulating embryos.

D. Tetraploid aggregation with GFP (green fluorescent protein)-labeled ES (embryonic stem) cells allows generation of fully ES cell-derived embryos.



lineages are established and different gene-expression programs are put into action. In mammals, the result is the formation of the blastocyst, a structure that contains three different cell types, each with a defined differentiation potential. The trophoctoderm is responsible for forming the placenta, the primitive endoderm leads to the yolk sac, and the epiblast gives rise to all remaining embryonic tissues. We will build on decades of lineage-fate experiments and precisely characterized signaling pathways known to regulate early mouse development to understand the contribution of nuclear organization to gene regulation during these early cell fate decisions.

We are also interested in understanding not only how DNA organization impacts cell behavior, and ultimately animal development and health, but also the mechanisms through which DNA folding itself is established and regulated, and which proteins are involved in these processes. To broadly address such questions, we will employ several high-throughput technologies that we have established in the lab, in combination with genome-wide CRISPR screens. Ultimately, we will fully characterize *in vivo* candidates identified this way to stringently determine their impact on gene regulation during mammalian development.

Publications

1. Chakraborty S, Kopitchinski N, Zuo Z, Eraso A, Awasthi P, Chari R, Mitra A, Tobias IC, Moorthy SD, Dale RK, Mitchell JA, Petros TJ, Rocha PP. Enhancer-promoter interactions can bypass CTCF-mediated boundaries and contribute to phenotypic robustness. *Nat Genet* 2022 1–12.
2. Thompson JJ, Lee DJ, Mitra A, Frail S, Dale RK, Rocha PP. Extensive co-binding and rapid redistribution of NANOG and GATA6 during emergence of divergent lineages. *Nat Commun* 2022 13:4257.
3. Kurotaki D, Kikuchi K, Cui K, Kawase W, Saeki K, Fukumoto J, Nishiyama A, Nagamune K, Zhao K, Ozato K, Rocha PP, Tamura T. Chromatin structure undergoes global and local reorganization during murine dendritic cell development and activation. *Proc Natl Acad Sci U S A* 2022 119:e2207009119.
4. Beck DB, Basar MA, Asmar AJ, Thompson JJ, Oda H, Uehara DT, Saida K, Pajusalu S, Talvik I, D'Souza P, Bodurtha J, Mu W, Baranano KW, Miyake N, Wang R, Kempers M, Tamada T, Nishimura Y, Okada S, Kosho T, Dale R, Mitra A, Macnamara E, Matsumoto N, Inazawa J, Walkiewicz M, Ounap K, Tifft CJ, Aksentijevich I, Kastner DL, Rocha PP, Werner A. Linkage-specific deubiquitylation by OTUD5 defines an embryonic pathway intolerant to genomic variation. *Sci Adv* 2021 7(4):eabe2116.
5. Rhodes C, Mitra A, Lee D, Lee D, Zhang Y, Thompson J, Rocha P, Dale R, Petros T. Single cell chromatin accessibility reveals regulatory elements and developmental trajectories in the embryonic forebrain. *Nat Commun* 2022 13:4196.

Collaborators

- Bradley Bernstein, MD, PhD, *Broad Institute and Dana-Farber Cancer Institute, Cambridge, MA*
- Stefan Feske, MD, *New York University School of Medicine, New York, NY*
- Daniel Herranz, PharmD, PhD, *Cancer Institute of New Jersey, Rutgers University, New Brunswick, NJ*
- Mark Lewandoski, PhD, *Cancer and Developmental Biology Laboratory, Center for Cancer Research, NCI, Frederick, MD*
- Timothy Petros, PhD, *Unit on Cellular and Molecular Neurodevelopment, NICHD, Bethesda, MD*
- Achim Werner, PhD, *Stem Cell Biochemistry Unit, NIDCR, Bethesda, MD*

Contact

For more information, email pedro.rocha@nih.gov or visit <http://rochalab.nichd.nih.gov>.

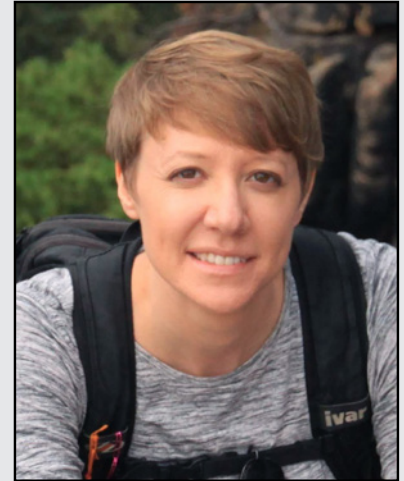
Decoding Developmental Signaling in Vertebrate Embryos

To create the different tissues required in healthy adults, embryos must activate fate-specifying genes in a variety of stereotyped patterns (Figure 1A), a process that is mediated by signaling molecules that spread through embryonic tissues. It is thought that signaling levels, dynamics, and combinations regulate gene expression, and that position-specific signaling differences underlie the diverse gene expression patterns required for normal development. However, it is not clear what signaling features are 'decoded' by genes and how those features are converted into differential gene expression during vertebrate embryogenesis.

We investigate how signaling molecules spread through embryonic tissues, how signaling levels and dynamics are decoded, and how many pathways cooperate to pattern the body plan (Figure 1B). To directly examine these processes, we use molecular optogenetics approaches that offer tunable, reversible experimental manipulations with excellent temporal (seconds) and spatial (subcellular) resolution. Using the microscopy-friendly zebrafish embryo as a vertebrate model system, our lab harnesses established optogenetic approaches and develops new ones to understand how cells decode signaling during embryogenesis.

How do signaling molecules move through tissues?

The distribution of signaling molecules within developing tissues helps determine patterns of gene expression. Competing models have been proposed to explain how signaling-molecule distributions are established: signals may diffuse away from producing cells through the extracellular space, move through cells (transcytosis), or be confined to the producing cells themselves. We will develop optogenetic tools to probe how extracellular diffusion and transcytosis, among others, affect signaling-molecule distribution, and we will use these with *in vivo* methods, including FRAP (fluorescence recovery after photobleaching) and FDAP (fluorescence decay after photoactivation) (Figure 2), to directly measure signaling-molecule mobility and stability. This will help determine how signaling-molecule distribution is regulated during zebrafish embryogenesis.



Katherine W. Rogers, PhD, *Head, Unit on Developmental Signaling*
William K. Anderson, BS, *Research Technician*

Daria Lukasz, PhD, *Postdoctoral Fellow*

Catherine E. Rogers, BS, *Postbaccalaureate Fellow*

Allison J. Saul, BS, *Postbaccalaureate Fellow*

What information is encoded in signaling gradients?

Signaling gradients are found in developing tissues from the fly wing precursor to the mammalian neural tube. The classic morphogen model proposes that the precisely graded distribution of signaling is important because genes are activated by different signaling levels. Alternatively, a simple signaling asymmetry may suffice to pattern tissues in some contexts. The relatively subtle signaling perturbations required to distinguish between these models can be difficult to achieve *in vivo*. We will develop optogenetic approaches and use

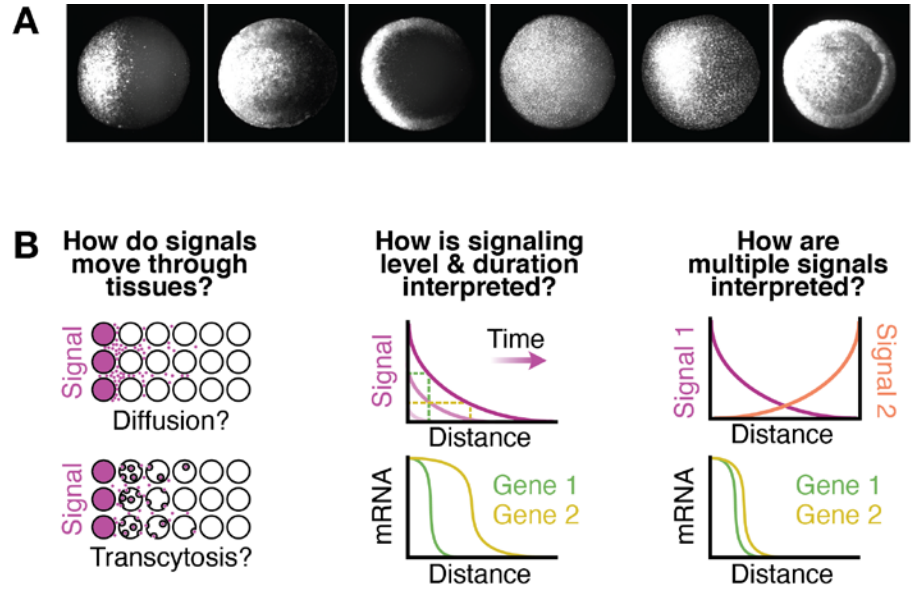


FIGURE 1.

A. Gene expression in the zebrafish embryo;

B. outstanding developmental questions

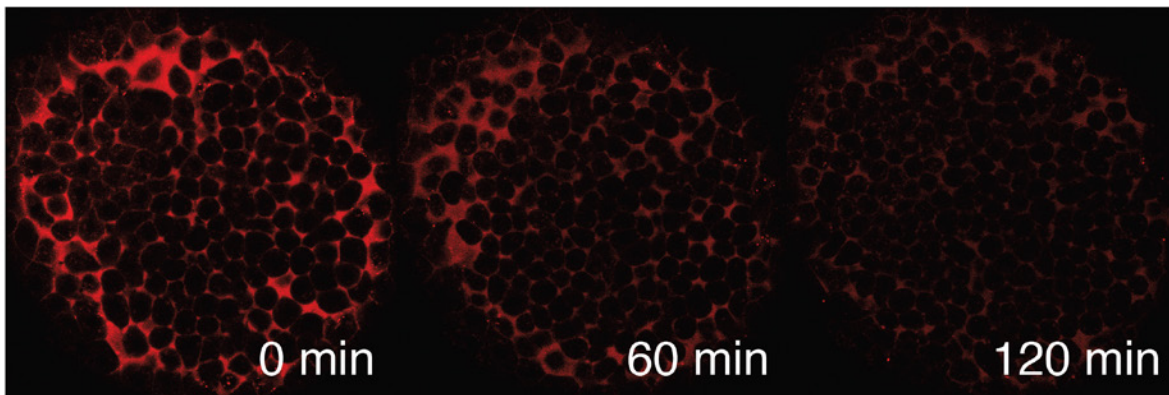
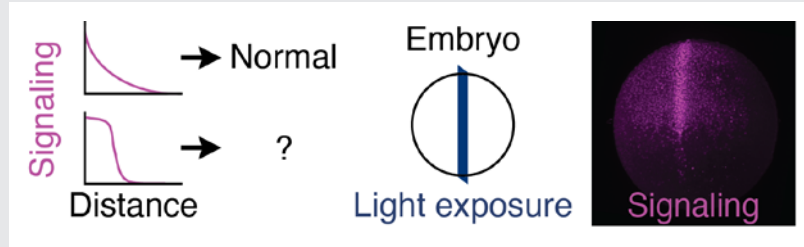


FIGURE 2.

Measuring signaling-molecule stability *in vivo* using FDAP

FIGURE 3.
Spatial gradient manipulation

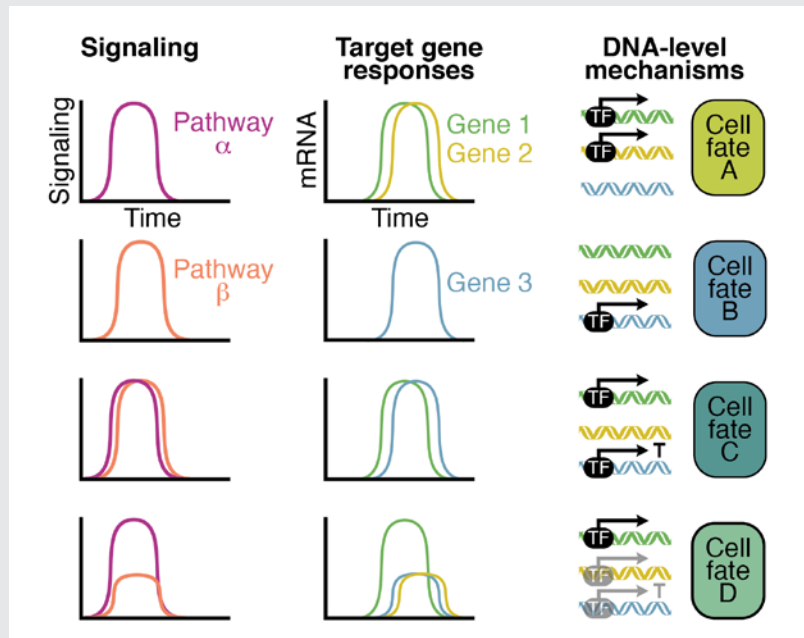


them with a digital micromirror device to introduce novel signaling distributions in zebrafish embryos and assess patterning consequences (Figure 3). This will determine the spatiotemporal signaling requirements for normal tissue patterning during early zebrafish development.

How are signaling levels, dynamics, and combinations interpreted in the embryo?

Cells in developing tissues experience a variety of signaling levels and dynamics, as well as simultaneous signaling from several pathways. We investigate how different genes respond to these inputs and we seek to determine the input/output relationship between signaling and gene expression during early vertebrate embryogenesis. To achieve this, we are developing orthogonal optogenetic tools to manipulate signaling levels and dynamics in zebrafish embryos. We will characterize gene responses and investigate the DNA-level mechanisms responsible for differential responses (Figure 4). This will help elucidate which features of signaling encode information and explain how the diverse gene expression patterns needed to produce healthy adults are robustly generated.

FIGURE 4.
Orthogonal opto-signaling experiment



Publications

1. Rogers KW, ElGamacy M, Jordan BM, Müller P. Optogenetic investigation of BMP target gene expression diversity. *eLife* 2020 e58641.
2. Rogers KW, Müller P. Optogenetic approaches to investigate spatiotemporal signaling during development. *In: Gradients and Tissue Patterning. Small S, Briscoe J, eds, 2020 37–77.*
3. Rogers KW, Lord ND, Gagnon JA, Pauli A, Zimmerman S, Aksel DC, Reyon D, Tsai SQ, Joung JK, Schier AF. Nodal patterning without Lefty inhibitory feedback is functional but fragile. *eLife* 2017 e28785.
4. Pomreinke AP, Soh GH, Rogers KW, Bergmann JK, Bläßle AJ, Müller P. Dynamics of BMP signaling and distribution during zebrafish dorsal-ventral patterning. *eLife* 2017 e25861.
5. Preiß H, Kögler AC, Mörsdorf D, Čapek D, Soh GH, Rogers KW, Morales-Navarrete H, Almuedo-Castillo M, Müller P. Regulation of Nodal signaling propagation by receptor interactions and positive feedback. *eLife* 2022 e66397.

Contact

For more information, email katherine.rogers@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/rogers>.

Mechanisms of Disease in Preterm Labor and Complications of Prematurity; Prenatal Diagnosis of Congenital Anomalies

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide, and two-thirds of all preterm births occur after the onset of preterm labor. The cost of prematurity in the United States alone was estimated at \$26 billion per year in 2007. Therefore, the important goals are to understand the mechanisms of disease responsible for preterm birth and fetal injury and to improve the prediction and prevention of preterm birth. The Pregnancy Research Branch proposed that preterm parturition is a syndrome caused by multiple pathologic processes (Romero R *et al. Science* 2014;345:760). The Branch developed methods for the rapid diagnosis of intra-amniotic infection/inflammation and showed that such pathologic processes can be treated successfully. In addition, the current approach to predict and prevent spontaneous preterm birth in clinical obstetrics is based on the work of the Branch (the PREGNANT trial and subsequent meta-analyses). The research team continues to study the physiology of pregnancy and parturition to inform studies of spontaneous preterm labor, and, in particular, the use of high-dimensional, post-genomic tools, such as transcriptomics, proteomics, and the analysis of parturition at single-cell resolution.

Imaging is a powerful instrument for scientific discovery, which has changed the practice of obstetrics and maternal-fetal medicine. The single most important step that has made fetal medicine a discipline is the transformation of the fetus from an invisible to a visible subject through the use of imaging techniques, in particular, ultrasound. This technology has allowed the definition of fetal anatomy, biometry, and growth as well as the study of physiologic parameters, e.g., cardiac function, fetal sleep, and breathing. We use different imaging modalities to examine the diagnosis of anomalies and obstetrical syndromes. Such modalities include ultrasound (2-dimensional, 3-dimensional), magnetic resonance imaging, and optical methods. We have also utilized other imaging techniques to study the human placenta.

Understanding the mechanisms of human term and preterm parturition through transcriptomics

A SINGLE-CELL ATLAS OF THE MYOMETRIUM IN HUMAN PARTURITION

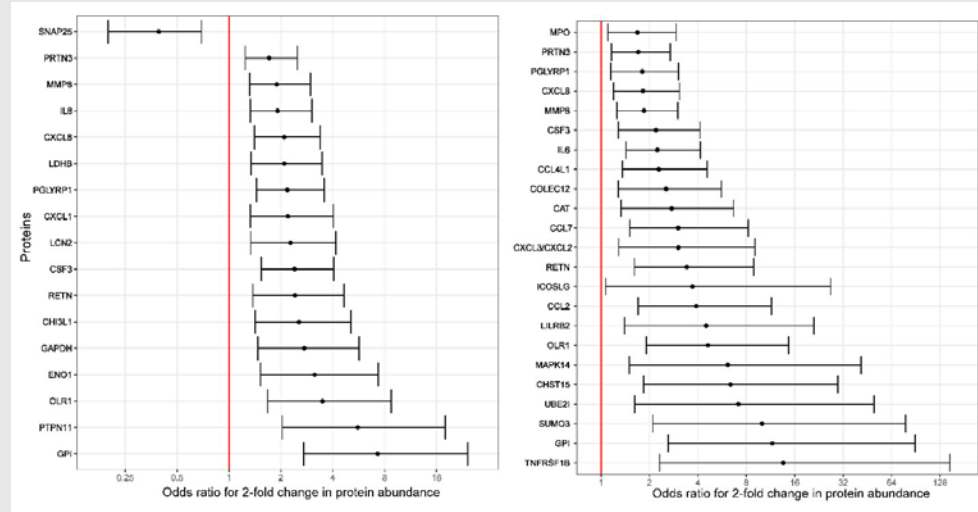
Few biological processes as central to the survival of viviparous



Roberto Romero, MD, DMedSci,
*Chief, Pregnancy Research
Branch*

FIGURE 1. Odds ratios (and 95% confidence intervals) for the association between amniotic fluid protein abundance and imminent preterm delivery among asymptomatic women with a short cervix

Odds ratios are shown for delivery within two weeks (*left*) and within one week from the amniocentesis (*right*). The odd ratios are calculated for a two-fold change in protein abundance and are adjusted for cervical length.



species are so incompletely understood as parturition. Yet, there is an inadequate understanding of the physiology of normal labor and the pathophysiology of labor disorders, preterm and term. The Branch used bulk transcriptomics to understand the components of the common pathway of parturition, which include myometrial contractility, cervical remodeling, and membrane/decidual activation, and we characterized differentially expressed genes and biological processes enriched during parturition. We leveraged single-cell technology to assemble a human cell atlas of parturition and analyzed the placenta and chorioamniotic membranes. However, the engine of labor is the myometrium, which is inaccessible for study. Therefore, one of our goals has been to determine whether it is possible to monitor cellular activity in the myometrium non-invasively through maternal blood. We performed RNA single-cell sequencing (scRNA-Seq) and generated the first single-cell atlas of the human myometrium during labor as well as the first map of cell type-specific transcriptomic activity modulation. This information provides the foundation for studying labor disorders. Integrating scRNA-Seq information with transcriptomic data derived from bulk analyses of the myometrium, we characterized the contributions of smooth muscle cells and inflammation during labor. Importantly, we showed that myometrium-derived single-cell signatures could be detected and quantitated in maternal blood. The result provides evidence that it is possible to monitor myometrial biology non-invasively by interrogating maternal blood. Indeed, we developed a transcriptomic signature of labor in maternal blood in term and preterm labor.

The proteome of human pregnancy and preterm delivery

THE AMNIOTIC-FLUID PROTEOME IN NORMAL PREGNANCY AND IMPENDING PRETERM DELIVERY

We studied the amniotic-fluid proteome to establish a systems-biology approach for pregnancy complications. We first characterized the amniotic fluid proteome and found that about 25% (320/1310) of proteins changed significantly in abundance with gestational age. Intersecting gestational age-modulated proteins and their corresponding mRNAs, previously reported in the maternal blood, identified neutrophil-related protein/mRNA pairs modulated in the same direction. Our observations have implications for the discovery of biomarkers to diagnose obstetrical and fetal disorders.

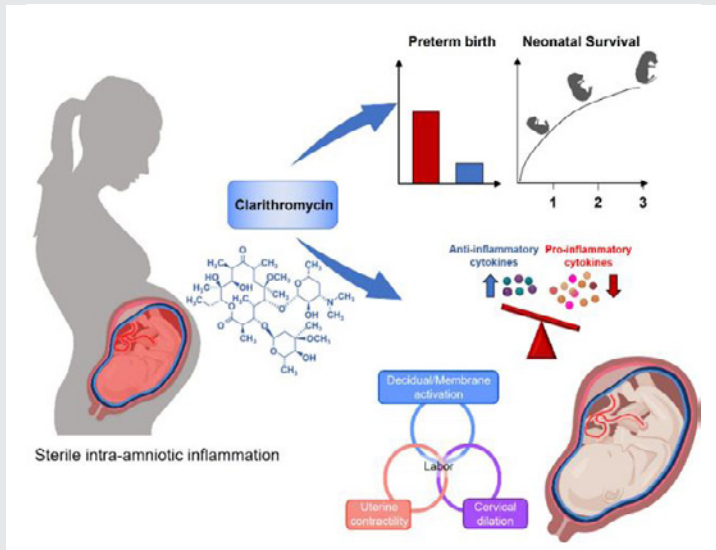


FIGURE 2.

Clarithromycin prevents preterm birth and neonatal mortality by dampening alarmin-induced maternal-fetal inflammation in mice.

derived from cervical length alone. We have already developed rapid point-of-care tests for determining CXCL8 and MMP-8 in amniotic fluid. These observations have clinical implications, given the recent evidence generated by our group that intra-amniotic inflammation can be eradicated, leading to prolongation of pregnancy. In addition, the diagnosis of silent intra-amniotic inflammation has implications for the management of the patient presenting with an asymptomatic short cervix.

Novel molecular and cellular treatments to prevent premature labor and fetal injury

TREATMENT OF STERILE INTRA-AMNIOTIC INFLAMMATION WITH CLARITHROMYCIN PREVENTS PRETERM DELIVERY AND REDUCES NEONATAL MORBIDITY THROUGH MODULATION OF INFLAMMATORY RESPONSES IN MATERNAL AND FETAL TISSUES.

Sterile intra-amniotic inflammation has emerged as the most frequent identifiable etiology of spontaneous preterm labor with intact membranes and cervical insufficiency. Our recent clinical studies demonstrated that successful treatment of sterile intra-amniotic inflammation can be achieved with clarithromycin, an antimicrobial agent with strong anti-inflammatory properties. We conducted several studies to determine the mechanisms by which clarithromycin prolongs gestation and improves neonatal outcomes. Using a previously established animal model of sterile intra-amniotic inflammation generated in our laboratory, we found that clarithromycin prolonged gestation, reduced the rate of preterm birth, and improved neonatal survival. Clarithromycin also exhibited potent anti-inflammatory effects in the placental and fetal tissues, which may contribute to the improved neonatal outcomes. The findings provide mechanistic evidence that clarithromycin can be administered to prevent preterm birth and to improve neonatal survival in the context of sterile intra-amniotic inflammation [Galaz J. *et al. BMC Pregnancy Childbirth* 2022;22:503].

A sonographic short cervix, the most powerful predictor of spontaneous preterm birth, is an indication for treatment with vaginal progesterone to prevent preterm birth. However, only a fraction of women respond to progesterone. Therefore, markers are required to determine which patients with a sonographic short cervix will progress to imminent delivery. We conducted a study to identify proteomic signatures of impending preterm delivery in patients with an asymptomatic sonographic short cervix (less than 25 mm) at 16 to 32 weeks of gestation. A combination of four amniotic fluid proteins (CXCL8, SNAP25, PTPN11, and MMP8) had a sensitivity of 79%, with a 10% false-positive rate, for the prediction of delivery within two weeks. This was a substantial improvement over the prediction

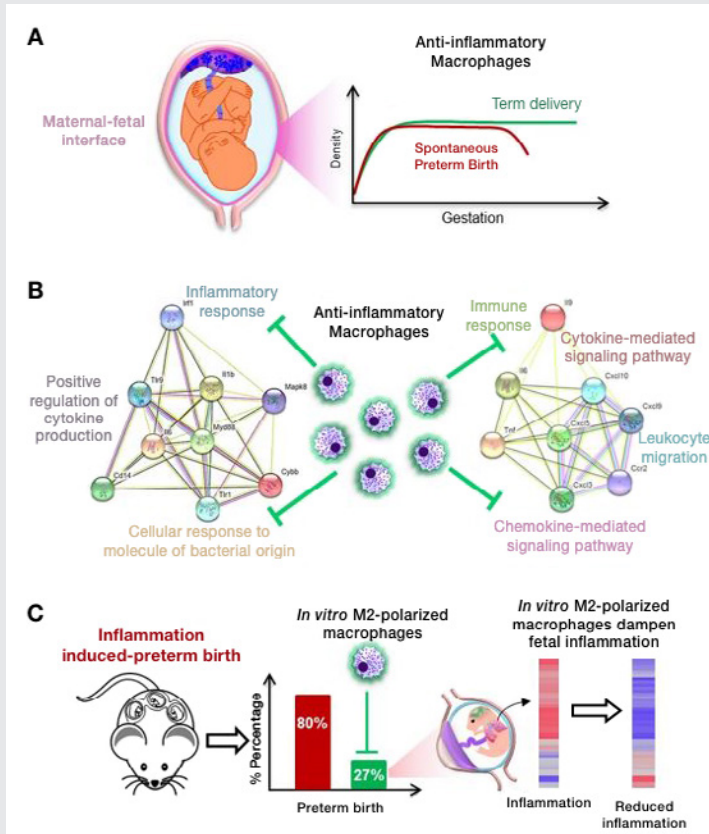


FIGURE 3. Macrophages exert homeostatic actions in pregnancy to protect against preterm birth and fetal inflammatory injury.

The role of maternal macrophages in the maintenance of pregnancy and the potential use of M2-polarized macrophages as cellular therapy to protect against inflammation-induced premature labor and fetal injury

Macrophages participate in the mechanisms of preterm and term labor by amplifying inflammation; however, there is a considerable body of evidence that a subset of macrophages, typically referred to as M2 macrophages, have powerful anti-inflammatory effects. We undertook a series of experiments to study the role of macrophages in pregnancy and observed that: (1) women in labor had a reduced number of M2-like macrophages at the maternal-fetal interface; 2) depletion of maternal CD11b⁺ myeloid cells led to preterm labor and adverse neonatal outcomes, which was ameliorated by replacement of

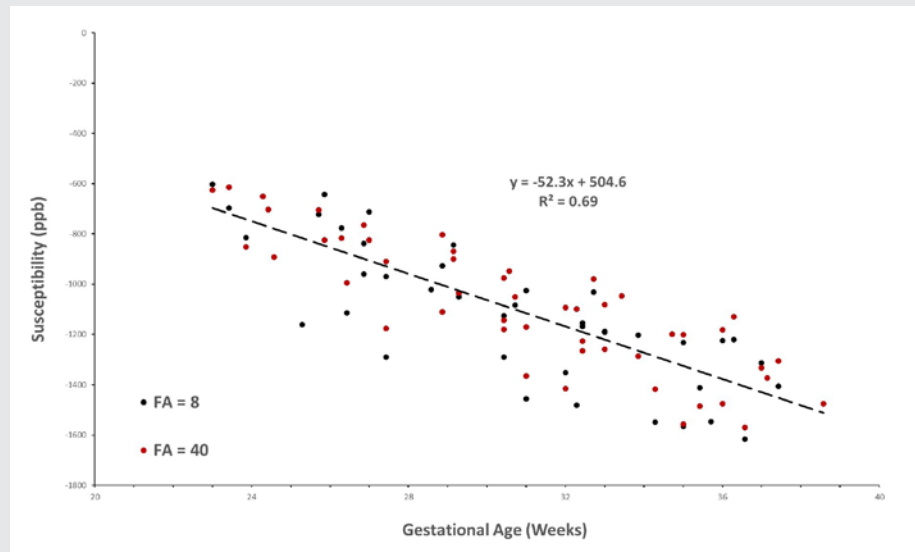
wild-type macrophages; (3) adoptive transfer of M2-polarized macrophages *in vitro* reduced the incidence of preterm birth and improved neonatal survival in a model of intra-amniotic inflammation; and (4) M2-polarized macrophages downregulated the inflammatory response in the fetal brain and lungs (major target organs in the fetal inflammatory response syndrome). The results demonstrate a hitherto unappreciated homeostatic role for macrophages in the physiology of pregnancy and a therapeutic role for M2-polarized macrophages for the treatment of premature labor and of the fetal inflammatory response syndrome.

Refining the use of progesterone for the prevention of preterm birth

Our previous studies showed that vaginal progesterone reduces the rate of preterm birth in women with a sonographic short cervix in the mid-trimester and reduces neonatal morbidity. Some professional organizations have proposed that vaginal progesterone should also be used in patients who have a history of preterm birth, regardless of cervical length. Therefore, we conducted two meta-analyses and showed that vaginal progesterone is not efficacious in patients with a prior history of preterm birth or in those without a short cervix, which has immediate implications for clinical practice.

FIGURE 4. Average susceptibility (associated with calcium) of the vertebrae plotted as a function of gestational age

The decreasing susceptibility with gestational age indicates that the *in vivo* theory behind increasing fetal calcium content during fetal development.



Quantifying calcium changes in the fetal spine using quantitative susceptibility mapping as extracted from STAGE imaging

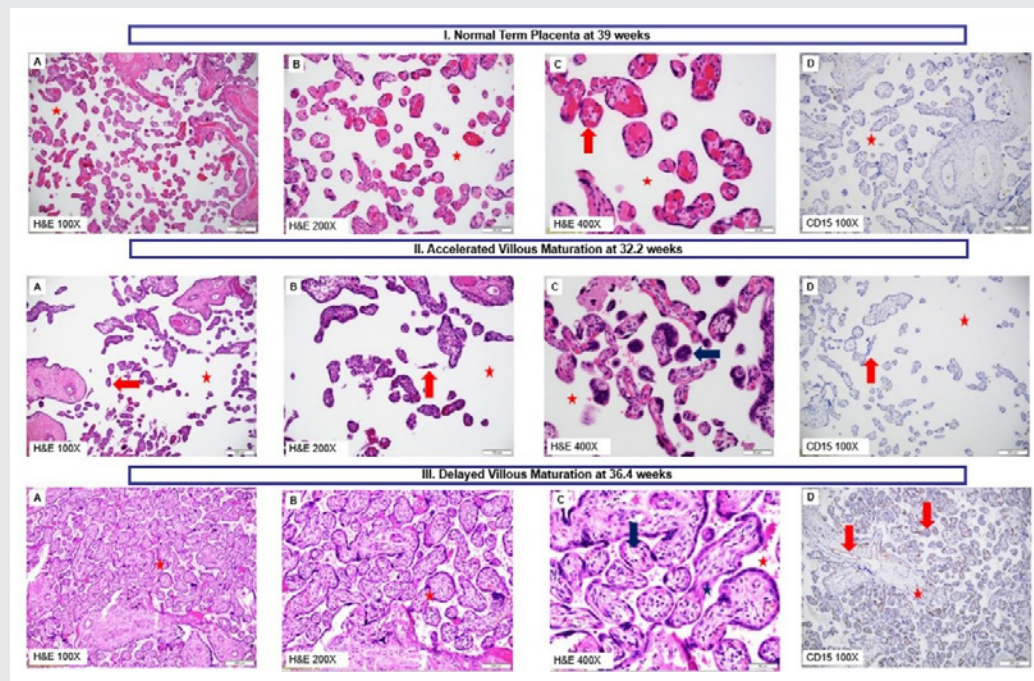
Even though ultrasound is the standard method to image the fetus, it has limitations, e.g., for characterizing the state of mineralization of the fetal spine. Computer tomography (CT) could be considered an alternative, but it carries the risk of ionizing radiation to the fetus and the mother. Fetal skeletal bone development during pregnancy has been qualitatively described by different imaging modalities, yet there is a paucity of quantitative evidence of bone development as a function of gestational age in the second and third trimesters. Similar to iron, calcium in the bones also induces severe-phase dephasing at longer echo times. However, as opposed to iron, which is paramagnetic with positive susceptibility, calcium is diamagnetic, meaning that it shows a negative susceptibility. Quantitative susceptibility mapping (QSM) has also been introduced as an *in vivo* non-ionizing imaging alternative to CT from which bone calcification can be monitored and quantified longitudinally over the course of pregnancy. We successfully evaluated the susceptibility of the fetal spine and demonstrated a strongly decreasing susceptibility with gestational age. For the first time, our results have been able to evaluate changes in fetal bone calcification over time.

The role of the placenta in spontaneous preterm labor and delivery with intact membranes

Preterm birth is the leading cause of death in children younger than five years of age. It accounts for 35% of deaths in neonates and affects 10.6% of live births. Infants who survive a preterm birth have a higher rate of long-term morbidity, including neurologic and developmental disabilities, and a shorter life expectancy than infants born full term. Fetal nutrition depends on the blood flow and its circulation on the maternal and fetal sides of the placenta. Therefore, an important pathway whereby the placenta causes non-infection/inflammation-related complications of pregnancy must involve abnormalities of the fetoplacental circulation that impair the exchange function of the placenta. We conducted a study to determine whether placental vascular pathology and impaired placental exchange (resulting from maturational defects) are involved in the etiology of spontaneous preterm labor and delivery in cases without infection/inflammation, i.e.,

FIGURE 5.

Panel I: Normal Term Placenta (39 weeks). A–C. Normal chorionic villi that are mature and appropriate for gestational age with normal intervillous space (white space; red star); C. Mature terminal villi with arrows pointing towards vasculo-syncytial membranes formed by the apposition of syncytiotrophoblasts with villous capillary endothelium (normal 3–5 per terminal villi); terminal villi with conspicuous capillaries and barely discernible stroma; D. Chorionic villous capillary endothelium negative for CD-15 staining (absence of brown staining). A–C: H&E-100X, 200X, and 400X; D: CD-15-100X



Panel II: Placenta at gestational age 32.2 weeks showing accelerated villous maturation. A–C. Chorionic villi displaying histology resembling that of term villi (see Panel I) with considerably increased intervillous space (white space; red star) for the gestational age; the terminal villi appear slender, hypermature, hypoplastic (red arrows). C. Hypermature villi with villous syncytial knotting (blue arrow). F. CD-15 negative chorionic villous capillary endothelium (absence of brown staining). A–C: H&E-100X, 200X, and 400X; D: CD-15-100X.

Panel III: Placenta at gestational age 36.4 weeks showing delayed villous maturation. A–C. Chorionic villi appear crowded, immature, affecting more than 50% of the placental villous population and resembling the histology of second trimester villi. Intervillous space is considerably diminished to virtually absent (red star). The villi display more stroma (blue star), centralized vessels (red arrows), and paucity of vasculo-syncytial membranes, as compared with a normal pregnancy (see Panel I). D. CD15-positive (brown staining) capillary endothelium in mature intermediate chorionic villi and stem villi. A–C: H&E-100X, 200X, and 400X; D: CD-15-100X.

without histologic acute chorioamnionitis. An evaluation was performed for 184 cases of pregnancies resulting in spontaneous preterm labor and delivery (less than 37 weeks of gestation) and for 2,471 controls in uncomplicated pregnancies that delivered fetuses at term (37–42 weeks of gestation). We found that the frequency of maternal blood flow abnormalities, represented pathologically as lesions of maternal vascular malperfusion, was greater in the placentas of patients with preterm labor than in the control group, i.e., 14.1% (26/184) vs. 8.8% (217/2471). Likewise, placental developmental abnormalities, termed as disorders of villous maturation, were more frequent in the group with preterm labor (41.1%; 39/95) than in the control group: delayed villous maturation, 31.6% (30/95) of cases vs. 2.5% (13/519) of controls; accelerated villous maturation, 9.5% (9/95) of cases vs. 0% of controls. We demonstrated that maturational defects of placental villi contributed to unexplained spontaneous preterm labor and delivery of appropriate-for-gestational-age fetuses in the absence of acute inflammatory lesions of the placenta.

Publications

1. Motomura K, Romero R, Plazyo O, Garcia-Flores V, Gershater M, Galaz J, Miller D, Gomez-Lopez N. The alarmin S100A12 causes sterile inflammation of the human chorioamniotic membranes as well as preterm birth and neonatal mortality in mice. *Biol Reprod* 2021 1494–1509.
2. Garcia-Flores V, Romero R, Xu Y, Theis KR, Arenas-Hernandez M, Miller D, Peyvandipour A, Bhatti G, Galaz J, Gershater M, Levenson D, Pusod E, Tao L, Kracht D, Florova V, Leng Y, Motomura K, Para R, Faucett M, Hsu CD, Zhang G, Tarca AL, Pique-Regi R, Gomez-Lopez N. Maternal-fetal immune responses in pregnant women infected with SARS-CoV-2. *Nat Commun* 2022 1–20.
3. Pique-Regi R, Romero R, Garcia-Flores V, Peyvandipour A, Tarca AL, Pusod E, Galaz J, Miller D, Bhatti G, Para R, Kanninen T, Hadaya O, Paredes C, Motomura K, Johnson JR, Jung E, Hsu CD, Berry SM, Gomez-Lopez N. A single-cell atlas of the myometrium in human parturition. *JCI Insight* 2022 1–21.
4. Oh KJ, Romero R, Kim HJ, Jung E, Gotsch F, Suksai M, Yoon BH. A role for intraamniotic inflammation in threatened midtrimester miscarriage. *Am J Obstet Gynecol* 2022 1.e1–1.e13.
5. Romero R, Jung E, Chaiworapongsa T, Erez O, Gudicha DW, Kim YM, Kim JS, Kim B, Kusanovic JP, Gotsch F, Taran AB, Yoon BH, Hassan SS, Hsu CD, Chaemsaitong P, Gomez-Lopez N, Yeo L, Kim CJ, Tarca AL. Toward a new taxonomy of obstetrical disease: improved performance of maternal blood biomarkers for the great obstetrical syndromes when classified according to placental pathology. *Am J Obstet Gynecol* 2022 615.e1–615.e25.
6. Purandare N, Kunji Y, Xi Y, Romero R, Gomez-Lopez N, Fribley A, Grossman LI, Aras S. Lipopolysaccharide induces placental mitochondrial dysfunction in murine and human systems by reducing MNRR1 levels via a TLR4-independent pathway. *iScience* 2022 25:105342.

Collaborators

- Tinnakorn Chaiworapongsa, MD, *Wayne State University School of Medicine, Detroit, MI*
- Agustin Conde-Agudelo, MD, *Wayne State University School of Medicine, Detroit, MI*
- Mark Haacke, PhD, *Wayne State University School of Medicine, Detroit, MI*
- Leonid Margolis, PhD, *Section on Intercellular Interactions, NICHD, Bethesda, MD*
- Adi L. Tarca, PhD, *Wayne State University, Detroit Medical Center, Detroit, MI*
- Lami Yeo, MD, *Wayne State University School of Medicine, Detroit, MI*
- Bo Hyun Yoon, MD, PhD, *Seoul National University, Seoul, Korea*

Contact

For more information, email romeror@mail.nih.gov or visit <http://irp.nih.gov/pi/roberto-romero>.

Regulation of Mammalian Iron Metabolism and Biogenesis of Iron–Sulfur Proteins

Our goal is to understand how mammals regulate intracellular and systemic iron metabolism to support processes that require iron and iron-sulfur clusters cofactors. Iron-regulatory proteins 1 and 2 (IRP1 and IRP2) regulate the expression of numerous proteins involved in iron metabolism. In iron-depleted cells, IRP proteins bind to RNA stem-loops in transcripts known as iron-responsive elements (IRE). IRP binding stabilizes the mRNA that encodes the transferrin receptor and represses the translation of transcripts that contain IREs near the 5' end of the ferritin H and L chains. IRP1 is an iron-sulfur protein that functions as a cytosolic aconitase in iron-replete cells. IRP2 is homologous to IRP1 but undergoes iron-dependent degradation in iron-replete cells. In mouse models, loss of IRP2 results in mild anemia, erythropoietic protoporphyria, and adult-onset neurodegeneration, all phenotypes that are likely caused by functional iron deficiency. Using this mouse model of neurodegeneration, we found that the antioxidant Tempol works by activating the latent IRE-binding activity of IRP1, which partially restores normal iron homeostasis. Given that mitochondrial energy production is required to maintain axonal integrity and that motor neurons have the longest and most vulnerable axons, we hypothesized that mitochondrial dysfunction resulting from iron deficiency causes axonal degeneration. We described and reported a case series of three unrelated patients who develop neonatal and progressive neurodevelopmental delays attributable to complete loss of IRP2 function (IREB2^{-/-}), to microdeletions, or to missense mutations.

Our ongoing work on iron-sulfur cluster biogenesis has led to new insights into how mammalian iron-sulfur clusters are synthesized and transferred to specific recipient proteins [Maio N, Rouault TA. *Trends Biochem Sci* 2020;45:411–426]. Several human diseases are now known to be caused by deficiencies in the iron-sulfur cluster biogenesis machinery. We developed an anti-sense treatment for the rare disease ISCU (iron-sulfur cluster assembly enzyme) myopathy. By identifying a tripeptide motif common to many iron-sulfur recipient proteins, we proposed an algorithm that facilitates discovery of previously unrecognized mammalian iron-sulfur proteins, work that led us to suggest that there are hundreds of previously unrecognized mammalian iron-sulfur proteins. Identification of iron-sulfur cofactors will lead to breakthroughs in several research areas



Tracey A. Rouault, MD, Head,
*Section on Human Iron
Metabolism*

Nunziata Maio, PhD, *Staff Scientist*

Deliang Zhang, PhD, *Staff Scientist*

Christina Porras, PhD,
Postdoctoral Fellow

Anna Santamaria, PhD,
Postdoctoral Fellow

Yang Zhang, PhD, *Postdoctoral
Fellow*

Audrey Heffner, BS, *Doctoral
Student*

Hayden Ollivierre-Wilson, *Animal
Care Technician*

Daryl Watson, BA, *Program
manager*

Anamika Singh, MS, *Technical
Contract Worker*

Gennadiy Kovtunovych, PhD,
Special Volunteer

involving DNA repair, ribosomal biogenesis, mRNA translation, intermediary metabolism, and the regulation of the growth and energy-sensing pathways that are critical for determining the fates of many cell types. In 2021, we discovered that SARS-CoV-2 contains iron-sulfur cofactors in its replicase, which can be inactivated by treatment with the stable nitroxide Tempol, resulting in attenuation of infection in tissue culture and also in Golden Syrian hamster models. We are working to develop an antiviral therapy that will be effective against many coronaviral infections by inactivating viral replication.

The molecular basis for the regulation of intracellular iron metabolism in mammals

In previous years, our laboratory identified and characterized the *cis* and *trans* elements mediating iron-dependent alterations in the abundance of ferritin and of the transferrin receptor. IREs (iron-responsive elements) are RNA stem-loops found in the 5' end of ferritin mRNA and the 3' end of transferrin receptor mRNA. We cloned, expressed, and characterized the two essential iron-sensing proteins IRP1 and IRP2. IRPs bind to IREs when iron levels are depleted, resulting in either inhibition of translation of ferritin mRNA and of other transcripts that contain an IRE in the 5' untranslated regions (UTR) or stabilization of the transferrin receptor mRNA and possibly other transcripts that contain IREs in the 3' UTR. The IRE-binding activity of IRP1 depends on the presence of an iron-sulfur cluster (see "Mammalian iron-sulfur cluster biogenesis" below). IRP2 also binds to IREs in iron-depleted cells but, unlike IRP1, in iron-replete cells it is selectively ubiquitinated and then degraded by the proteasome.

To approach questions about the physiology of iron metabolism, we generated loss-of-function mutations of IRP1 and IRP2 in mice through homologous recombination in embryonic cell lines. In the absence of provocative stimuli, we initially observed no abnormalities in iron metabolism associated with loss of IRP1 function. *Irp2*^{-/-} mice develop a progressive neurologic syndrome characterized by gait abnormalities and axonal degeneration. Ferritin overexpression occurs in affected neurons and in protrusions of oligodendrocytes into the space created by axonal degeneration. *Irp2*^{-/-} animals develop iron-insufficiency anemia and erythropoietic protoporphyria. In animals that lack IRP1, IRP2 compensates for loss of IRP1's regulatory activity in most cell types, but we discovered several cell types and accompanying phenotypes in which *Irp2* expression cannot be sufficiently increased to compensate. Animals that lack both IRP1 and IRP2 die as early embryos. The adult-onset neurodegeneration of adult *Irp2*^{-/-} mice is exacerbated when one copy of *Irp1* is also deleted. *Irp2*^{-/-} mice offer a unique example of spontaneous adult-onset, slowly progressive neurodegeneration; analyses of gene expression and iron status at various stages of disease are ongoing. Dietary supplementation with Tempol mitigates neurodegeneration; the treatment appears to work by recruiting the IRE-binding activity of IRP1. We found that motor neurons were the most adversely affected neurons in *Irp2*^{-/-} mice and that neuronal degeneration accounted for the gait abnormalities. In collaboration, we identified two *IRP2*^{-/-} patients who suffered from severe neurodegenerative disease in infancy and died before adolescence or were bed-ridden. A third infant with choreoathetosis established IRP2 deficiency as a cause of infantile and childhood neurodevelopmental disease.

We discovered that a transcript of the iron exporter ferroportin that lacks the IRE at its 5' end is important in intestinal iron uptake. It allows ferroportin to permit iron to cross the duodenal mucosa in iron-deficient animals and also to prevent developing erythroid cells from retaining high amounts of iron. Our findings explain why microcytic anemia is usually the first physiological manifestation of iron deficiency in humans.

Unexpectedly, we discovered that ferroportin is an abundant protein on mature red cells, where, as our work showed, it is needed to export free iron released from heme by oxidation. Using erythroid ferroportin knockout animals, we showed that the absence of ferroportin results in accumulation of intracellular iron, increased oxidative stress, and reduced viability of cells in circulation.

Upon realizing that ferroportin is key to reducing free iron levels in red cells, we analyzed the Q248H mutation of ferroportin, which confers gain of function and reduces iron abundance in red cells. The Q248H mutation underwent positive selection in malarious regions of Africa, and we hypothesized that it conferred resistance to malaria by diminishing iron available to support growth of the malaria parasite in red cells. Upon infecting mice that lacked erythroid ferroportin with several malaria strains, we demonstrated that the mice experienced increased morbidity and mortality, likely because iron concentrations in red cells were high and supported parasite growth well. We noted that more than 8% of African Americans carry this allele, which has the potential to cause tissue iron overload in liver and kidney, perhaps accounting for some of the morbidities to which African Americans are unusually predisposed.

We recently discovered that loss of IRP1 causes polycythemia and pulmonary hypertension by derepressing hypoxia-inducible factor 2-alpha (HIF2a) translation in the renal interstitium through the IRE-IRP system. We confirmed that overexpression of HIF2a drives production of erythropoietin and polycythemia in a mouse model of Chuvash polycythemia (an autosomal recessive form of erythrocytosis, which is endemic in patients from Chuvashia, an autonomous republic within the Russian Federation), and we discovered that we could reverse disease by activating *Irp1* to repress HIF2a translation using Tempol, which converts IRP1 from the aconitase to the IRE-binding form. Phlebotomy has not been a very helpful therapy to the thousands of patients with Chuvash polycythemia in Russia, and we propose that oral Tempol supplementation could constitute a good therapeutic intervention. We also are conducting experiments with HIF2alpha inhibitors, which reveal that the drugs reverse polycythemia and pulmonary hypertension in our *Irp1*^{-/-} and Chuvash polycythemia models.

We also elucidated the pathophysiology of intravascular hemolysis and hyposplenism in animals that lack heme oxygenase 1 (HMOX1). Their tissue macrophages die because they cannot metabolize heme after phagocytosis of red cells. To mitigate or reverse disease, we performed bone marrow transplants from wild-type animals to supply animals with functional macrophages, transplants that were successful. We then discovered that the transplant was not necessary by demonstrating that exogenously expanded wild-type macrophages can repopulate the reticuloendothelial system of *Hmox1*^{-/-} mice, restore normal erythrophagocytosis, and reverse renal iron overload and anemia. Five human *HMOX1*^{-/-} patients have been identified, but we believe that this represents an underdiagnosed and often misdiagnosed rare human disease. We are evaluating results from a large experiment on transcript expression in macrophages after red cell phagocytosis.

Mammalian iron-sulfur cluster biogenesis

Our goal in studying mammalian iron-sulfur biogenesis is to understand how iron-sulfur prosthetic groups are assembled and delivered to target proteins in the various compartments of mammalian cells, including mitochondria, the cytosol, and the nucleus. We also seek to understand the role of iron-sulfur cluster assembly in the regulation of mitochondrial iron homeostasis and in the pathogenesis of diseases such as Friedreich's ataxia and sideroblastic anemia, which are both characterized by incorrect regulation of mitochondrial iron homeostasis.

The iron-sulfur protein IRP1 is related to mitochondrial aconitase, a citric acid cycle enzyme, and it functions as a cytosolic aconitase in iron-replete cells. Regulation of the RNA-binding activity of IRP1 involves a transition from a form of IRP1 in which a [4Fe-4S] cluster is bound to a form that loses both iron and aconitase activity. The [4Fe-4S]-containing protein does not bind to IREs. Controlled degradation of the iron-sulfur cluster and mutagenesis reveal that the physiologically relevant form of the RNA-binding protein in iron-depleted cells is an apoprotein. The status of the cluster appears to determine whether IRP1 binds to RNA.

We identified numerous mammalian enzymes of the iron-sulfur cluster assembly that are homologous to those encoded by the *NIFS*, *ISCU*, and *NIFU* genes, which are implicated in bacterial iron-sulfur cluster assembly, and we observed that mutations in several iron-sulfur cluster biogenesis proteins cause disease. Loss of frataxin, a protein that promotes the biosynthesis of heme and the assembly and repair of iron-sulfur clusters by enhancing early steps of iron-sulfur cluster biogenesis, causes Friedreich's ataxia, which is characterized by a progressive compromise of balance and of cardiac function. In a cohort of patients of Swedish descent, we found that loss of the iron-sulfur cluster assembly enzyme ISCU causes skeletal myopathy. To explain the tissue specificity of the ISCU myopathy, we studied myoblasts and other patient-derived tissue samples and cell lines. We discovered that many factors contribute to insufficiency of ISCU (iron-sulfur cluster assembly enzyme) in skeletal muscle, including more pronounced abnormal splicing and unusual sensitivity of ISCU to degradation upon exposure to oxidative stress. Thus, oxidative stress may impair the ability of tissues to repair damaged iron-sulfur clusters by directly damaging a key component of the biogenesis machinery. We discovered that antisense therapy would likely work as a treatment for ISCU myopathy patients, as we were able to correct the causal splicing defect in patient myoblasts using stable antisense RNAs that were manufactured by high-quality techniques suitable for use in patients. In one patient, we found that a splicing abnormality of glutaredoxin 5 was associated with sideroblastic anemia. In the affected tissues, mitochondrial iron overload is a feature common to all three diseases.

We identified a tripeptide motif, LYR, in many apoproteins that are recipients of nascent iron-sulfur clusters. The co-chaperone HSC20 binds to HSPA9, its partner HSP70-type chaperone, and the chaperone complex binds to ISCU bearing a nascent iron-sulfur cluster that is delivered to iron-sulfur cluster-recipient proteins. We identified several direct iron-sulfur-recipient proteins in a yeast two-hybrid assay, using HSC20 as bait. By studying one known iron-sulfur recipient, succinate dehydrogenase subunit B (SDHB), we discovered that several LYR motifs of the SDHB primary sequence engage the iron-sulfur transfer apparatus by binding to the C-terminus of HSC20, facilitating delivery of the three iron-sulfur clusters of SDHB. We further discovered that the assembly factor SDHAF1 also engages the iron-sulfur cluster transfer complex to facilitate transfer of iron-sulfur clusters to SDHB. The discovery of the LYR motif will aid in the identification of unknown iron-sulfur proteins, which are likely to be much more common in mammalian cells than had been previously appreciated. More recently, we discovered that, through recognition of LYR-like motifs in these recipient proteins, HSC20 is responsible for the delivery of iron-sulfur clusters to respiratory chain complexes I–II. Using informatics, we predicted that amino levulinic acid dehydratase (ALAD), a heme-biosynthetic enzyme, is a previously unrecognized iron-sulfur protein, and we identified more unrecognized iron-sulfur proteins by using the LYR motif to analyze candidate proteins. Using informatics, over-expression of candidate proteins, and iron detection with ICP-MS (inductively coupled mass spectrometry), we identified many more iron-sulfur proteins that are involved in a wide range of metabolic pathways, ranging from intermediary metabolism, DNA repair, and RNA synthesis, and possibly to regulation of cellular growth. Iron-sulfur proteins will prove to be integral to the functioning and sensing of numerous pathways important in cellular functions.

We discovered that the mitochondrial protein ABCB7 (ATP-binding cassette sub-family B member 7) forms a complex with dimeric ferrochelatase, which binds ABCB10 to the other half of the ferrochelatase dimer. Our preliminary results suggest that ABCB7 could represent a mitochondrial heme exporter. We discovered that the intermediary scaffold protein NFU1 acquires its iron-sulfur clusters from ISCU2 and the iron-scaffold assembly protein ISCA1 to form a cubane iron-sulfur cluster that is delivered directly to lipoic acid synthase. We are working to shed light on the complex use of such secondary iron-sulfur scaffold proteins to deliver iron-sulfur clusters to many recipient proteins in the cell.

Using informatics, we identified several potential iron sulfur proteins encoded by SARS-CoV-2. We demonstrated that the SARS CoV-2 replicase Nsp12 ligates two cubane iron sulfur clusters, one of which is needed for primer extension, whereas the other is needed for full assembly formation. Using Tempol to degrade the iron sulfur clusters, we stopped viral replication in tissue culture and greatly mitigated COVID19 disease in a Syrian Golden hamster model. Other coronaviruses also require iron sulfur cofactors for function, including the original SARS and MERs, and likely, three causes of the common cold. We are actively pursuing studies and treatments for coronaviral disease based on these insights.

Additional Funding

- COVID-19 NIH funding

Publications

1. Liu G, Sil D, Maio N, Tong WH, Bollinger JM, Krebs C, Rouault TA. Heme biosynthesis depends on previously unrecognized acquisition of iron-sulfur cofactors in human amino-levulinic acid dehydratase. *Nat Commun* 2020 11:6310.
2. Jain A, Singh A, Maio N, Rouault TA. Assembly of the [4Fe-4S] cluster of NFU1 requires the coordinated donation of two [2Fe-2S] clusters from the scaffold proteins, ISCU2 and ISCA1. *Hum Mol Genet* 2020 29:3165–3182.
3. Maio N, Lafont BAP, Sil D, Li Y, Bollinger JM, Krebs C, Pierson TC, Linehan WM, Rouault TA. Fe-S cofactors in the SARS-CoV-2 RNA-dependent RNA polymerase are potential antiviral targets. *Science* 2021 373:236–241.
4. Maio N, Cherry S, Schultz DC, Hurst BL, Linehan WM, Rouault TA. TEMPOL inhibits SARS-CoV-2 replication and development of lung disease in the Syrian hamster model. *iScience* 2022 25:105074.
5. Maio N, Zhang DL, Ghosh MC, Jain A, SantaMaria AM, Rouault TA. Mechanisms of cellular iron sensing, regulation of erythropoiesis and mitochondrial iron utilization. *Semin Hematol* 2021 58:161–174.
6. Maio N, Saneto RP, Steet R, Sotero de Menezes MA, Skinner C, Rouault TA. Disruption of cellular iron homeostasis by IREB2 missense variants causes severe neurodevelopmental delay, dystonia and seizures. *Brain Commun* 2022 23:1–14.

Collaborators

- J. Martin Bollinger, PhD, *Penn State University, University Park, PA*
- Carsten Krebs, PhD, *Penn State University, University Park, PA*
- W. Marston Linehan, MD, *Urologic Oncology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Russell Saneto, PhD, DO, *Seattle Children's Hospital and University of Washington, Seattle, WA*

- Grace Yoon, MD, *The Hospital for Sick Children, Toronto, Canada*

Contact

For more information, email rouault@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/rouault>.

Mechanisms of Synapse Assembly and Homeostasis

The purpose of our research is to understand the mechanisms of synapse development and homeostasis. The chemical synapse is the fundamental nervous-system communication unit, which connects neurons to one another and to non-neuronal cells, and is designed to mediate rapid and efficient transmission of signals across the synaptic cleft. This transmission forms the basis for the biological computations that underlie and enable our complex behavior. Crucial to the function is the ability of a synapse to change its properties, so that it can optimize its activity and adapt to the status of the cells engaged in communication and/or to the larger network comprising them. Consequently, synapse development is a highly orchestrated process coordinated by intercellular communication between the pre- and postsynaptic compartments and by neuronal activity itself. Our long-term goal is to elucidate the molecular mechanisms that regulate the formation of functional synapses during development and that fine-tune them during plasticity and homeostasis. We focus on four key processes in synaptogenesis: (1) trafficking of components to the proper site; (2) organizing those components to build synaptic structures; (3) maturation of the synapse to optimize its activity; and (4) homeostatic mechanisms that restore synapse activity to a set point after perturbations. We address the molecular mechanisms underlying these processes using a comprehensive set of approaches, which include genetics, biochemistry, molecular biology, super-resolution imaging, and electrophysiology recordings in live animals and in reconstituted systems.

Because of its many advantages, we study these events in a powerful genetics system, *Drosophila melanogaster*, and use the neuromuscular junction (NMJ) as a model for glutamatergic synapse development and function. The fact that individual NMJs can be reproducibly identified from insect to insect and are easily accessible for electrophysiological and optical analysis makes them uniquely suited for *in vivo* studies on synapse assembly, growth, and plasticity. In addition, the richness of genetic manipulations that can be performed in *Drosophila* permits independent control of individual synaptic components in distinct cellular compartments. Importantly, the fly NMJ relies entirely on kainate-type receptors, a family of ionotropic glutamate receptors that impact synaptic transmission and neuronal excitability in the mammalian central nervous system but remain poorly understood.



Mihaela Serpe, PhD, Head,
*Section on Cellular
Communication*

Peter Nguyen, *Biological
Laboratory Technician*

Tae Hee Han, PhD, *Staff Scientist*
Rosario Vicidomini, PhD, *Research
Fellow*

Wen Chieh Hsieh, PhD, *Visiting
Fellow*

Tho Huu Nguyen, PhD, *Visiting
Fellow*

Debabrata Sinha, PhD, *Visiting
Predoctoral/Postdoctoral Fellow*

Sijia Chen, BSc, *Postbaccalaureate
Fellow*

Thomas Brody, PhD, *Special
Volunteer*

The *Drosophila* NMJ can thus be used to analyze and model defects in the structural and physiological plasticity of glutamatergic synapses, which are associated with a variety of human pathologies, from learning and memory deficits to autism. *Drosophila* has long served as a source of insight into human genetics, development, and disease, and the basic discoveries of our laboratory in the fly are likely to serve our overarching goal of understanding how chemical synapses are assembled and sculpted during development and homeostasis.

Cellular diversity in the *Drosophila* third instar larval ventral cord revealed by single-cell transcriptomics

In flies as in vertebrates, neuronal activity induces input-specific changes in synaptic strength; at the larval NMJ, the postsynaptic sensitivity is primarily modulated via synapse-specific recruitment of postsynaptic glutamate receptors. Robust homeostatic mechanisms keep synapses within an appropriate dynamic range, such that the evoked potentials measured in the muscle remain constant from embryo to third instar larvae. Reduced postsynaptic sensitivities (i.e., reduced glutamate receptor activity) trigger a compensatory increase in quantal content (QC), i.e., the number of vesicles released by the motor neuron (MN), which is referred to as presynaptic homeostatic potentiation (PHP). To learn how MNs respond to various signals from muscles or compensate for perturbations in NMJ activity, we took a transcriptomic approach. We performed single-cell RNA sequencing (scRNA-Seq) of larval ventral nerve cords (VNCs), the fly equivalent of the mammalian spinal cord. However, MNs represent a relatively small fraction (less than 2%) of the cells within the larval VNC. To recognize the MN-specific transcriptomes and to begin comparing them under different conditions/genotypes, we first had to assemble a larval VNC atlas that captured and defined the cellular diversity within the *Drosophila* third instar larvae. During this process, we adapted and developed new protocols for dissociating single cells from fly larvae, then assembled a custom multistage analysis pipeline that integrates modules contained in different R packages, in order to ensure flexible, high-quality RNA-Seq data analysis. The work was conducted with support from the NICHD [Genomics Core](#) and in collaboration with Steve Coon, Fabio Rueda Fauz, and James Iben.

We dissected third instar larvae VNCs, dissociated the cells, and sequenced about 31,000 high-quality single cells. Using un-supervised clustering algorithms, we clustered the cells into distinct populations. We then assigned the populations to specific cell types using known markers. Through a series of reiterative processes, we first identified eight different glia subtypes, each with distinct metabolic pathways. Secondly, based on the expression of neuroblast genes and the temporal determinant genes, we revealed a developmental trajectory leading from neural precursors to newborn neurons. We also detected novel, differentially expressed genes along this trajectory. Thirdly, we identified over 40 types of clearly differentiated interneurons, each expressing unique combinations of transcription factors and neurotransmitters (*VGlut*, *Gad1*, *VAcHT*, *DAT*). We found that most interneuron subtypes express many GPCRs (G protein-coupled receptors) and cell-recognition molecules, suggesting that many sensory modalities converge onto single cells to elicit specific motor functions/behaviors. We also identified a large MN cluster in which all cells express *VGlut* (encoding a glutamate transporter) but not the other neurotransmitter transporters or markers (*Gad1*, *VAcHT*, *DAT*). As expected, the MN transcriptomes are enriched in *futsch* (encoding a microtubule-binding protein involved in the formation of synaptic boutons at the neuromuscular junctions), *proctolin* (*proc*, encoding a neurohormone that modulates NMJ function through unknown mechanisms), and *target of wit* (*twit*, a BMP transcriptional target previously implicated in NMJ function).

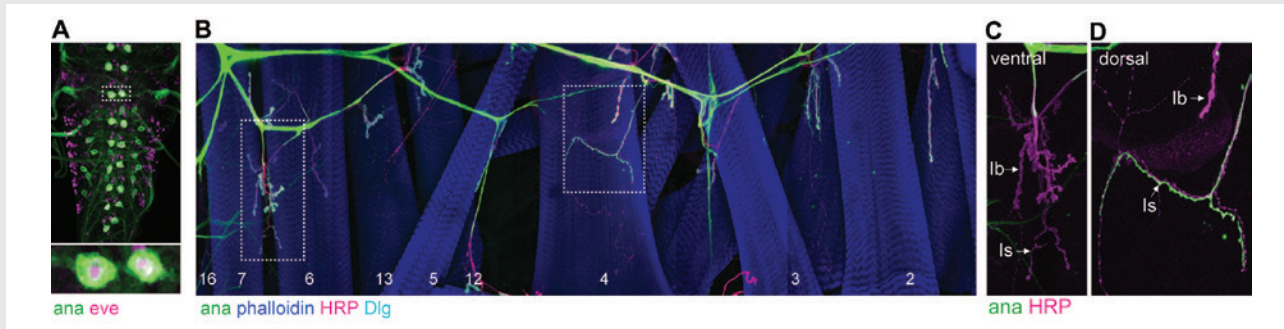


FIGURE 1. *ana*, encoding a secreted glycoprotein, is specifically expressed in dorsal type I-s motor neurons.

Confocal images of ventral nerve cord (A) or muscle fields (B–D) from third instar larvae illustrating specific expression of *ana* (*ana-Gal4>UAS-CD4::GFP*) (green) in only two type-I-s motor neurons per larval segment. The ventral cord (A) is co-labeled for *eve* (magenta), a transcription factor that marks dorsally projecting motor neurons. The NMJ fields are stained for phalloidin (blue) (B), which marks the body-wall muscles, for Horseradish Peroxidase (HRP) (magenta), which labels an epitope of the neuron cell membrane and for Discs-large (Dlg) (cyan), a scaffold protein that accumulates predominantly around the type I-b terminals. The membrane-attached GFP, driven by the *ana-Gal4* promoter, specifically labels dorsal (D) but not ventral (C) type I-s motor neurons.

The fly larval/primary MNs are probably the most studied and best understood neurons, as their accessibility and stereotyped morphology has facilitated in-depth studies for almost 50 years. However, to date there is no systematic transcriptome analysis or class-specific characterization the larval MNs. Each abdominal hemisegment has 30 body-wall muscles innervated by about 36 larval MNs of four classes: type I-b (tonic) and type I-s (phasic) glutamatergic MNs, type II octopaminergic and glutamatergic, and type III peptidergic neurons. To improve our ability to examine the heterogeneity within the MN cluster isolated in our scRNA-seq data, we marked the MNs with a *twit-Gal4* promoter (and *UAS-nls-GFP*), dissociated VNCs from *twit-GFP* third instar larvae, and FACS-sorted the GFP-positive MNs. We accomplished the FACS sorting of the small *Drosophila* MNs in collaboration with Dragan Maric. The additional enrichment step allowed us to generate high quality scRNA-Seq data for over 1,200 MNs. This large pool of MNs was then subdivided in 28 clusters. Several of the clusters are very well isolated and correspond to neurosecretory cells (expressing orcokinin, leukokinin, or GPa), type II MNs (expressing *Tdc2* and *Vmat*) and type III/peptidergic neurons (expressing the neurohormone bursicon and the neuropeptide crustacean cardioactive peptide, CCAP). Our dataset reveals new markers for these types of neurons. For example, the AMPA-type glutamate receptor subunits *GluRIA* and *GluRIB* are primarily expressed in type II and type III neurons, suggesting that the modulatory activity of these neurons is regulated by glutamatergic input.

Drosophila has two type I MNs: tonic I-b neurons (with large synaptic boutons), which innervate single, dedicated muscles; and phasic I-s neurons, with small boutons and innervation spanning up to 7–8 muscles. Each larval hemisegment contains two I-s MNs, one projecting dorsally and one ventrally, and about 30 type I-b MNs, organized in distinct bundles that innervate subsets of body-wall muscles. Among the 28 MN clusters, two adjacent ones correspond to I-s MNs; they both express *DIP-alpha* (encoding a cell-surface molecule of the immunoglobulin superfamily), with the transcription factor gene *eve* marking the dorsally projecting I-s cluster. We found that *anachronism* (*ana*, encoding a secreted glycoprotein) specifically marks dorsally projecting I-s and confirmed this restricted expression using an *ana-Gal4* (*CRIMIC*) line (Figure 1). We also recognized

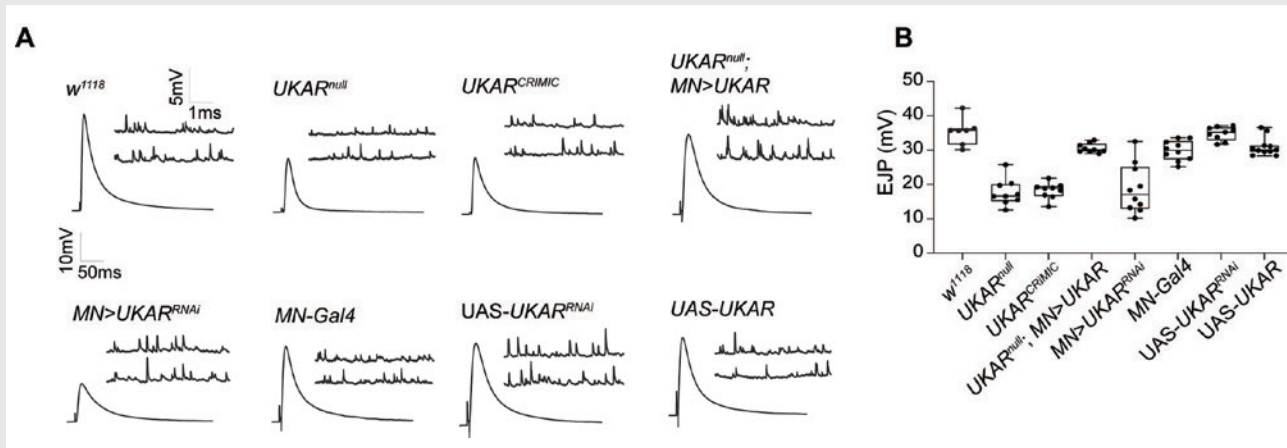


FIGURE 2. UKAR, a novel kainate receptor subunit, modulates basal neurotransmission at larval NMJ.

A. Representative traces for miniature excitatory junction potentials (mEJPs) (which represent the postsynaptic response to the spontaneous release of single synaptic vesicles) and evoked excitatory junction potentials (EJPs) from muscle 6 of the indicated genotypes.

B. Bar graph illustrating the EJP amplitudes in various genotypes.

different I-b MN bundles, projecting dorsally, ventrally, or laterally. We already have a distinct set of markers so that all types of larval MNs can be unequivocally identified, irrespective of genetic background or synapse activity. Current work focuses on increasing the granularity of MN transcriptomic analysis and describing each of the MN bundles that innervate the larval body-wall muscles.

Our studies on the transcriptomes of larval MNs together with the assembly of a larval VNC atlas have already uncovered new molecules critical for synapse development and function. One example of a novel glutamate receptor will be discussed below. In addition, the larval VNC will provide a valuable resource for future studies on neuronal development and behavior.

A novel kainate receptor subunit modulates basal neurotransmission and homeostasis at the *Drosophila* NMJ

In flies, as in humans, the interplay between different postsynaptic receptor subtypes with different channel properties controls synapse strength and plasticity. The *Drosophila* NMJ utilizes two types of postsynaptic receptors, types A and B, which contain either GluRIIA or GluRIIB subunits, plus GluRIIC, GluRIID and GluRIIE; these receptors mediate the postsynaptic response to neurotransmitter. In addition, a presynaptic autoreceptor containing the KaiR1D subunit controls basal neurotransmission. At excitatory synapses, autoreceptors provide a feedback mechanism that modulates neurotransmitter release and ensures stable neuronal network activities. Phylogenetic analysis indicates that all these pre- and postsynaptic glutamate receptor subunits are closely related to the vertebrate kainate receptors, although the *Drosophila* receptors have strikingly different ligand-binding profiles. Like vertebrate kainate receptors, *Drosophila* kainate-type receptors are modulated by a member of the Neto (Neuropilin and Tolloid-like) family of auxiliary proteins (see below).

While characterizing larval MN transcriptomes, we noted that all type I MNs show high expression of a novel gene, *CG11155*, predicted to encode a kainate-type receptor subunit. The transcript was also abundant in larval interneurons (our dataset) as well as in many cells of the adult fly brain (recently published); this unusual, ubiquitous expression prompted us to refer to this gene as *ubiquitous kainate receptor (ukar)*. To search for the role for UKAR during MNs development and function, we used CRISPR/Cas9 and RNAi methodologies to generate null mutants and tissue-specific knock downs. We found that UKAR functions in MNs to ensure normal basal neurotransmission; in the absence of UKAR, basal neurotransmission is reduced to half of the control (Figure 2), a loss-of-function phenotype that is reminiscent of the *KaiRID*^{null} mutants. Given that glutamate receptors function as heterotetramers, i.e., usually dimers of dimers, our data suggest that KaiRID and UKAR represent the two subunits of a presynaptic autoreceptor that controls basal neurotransmission at the larval NMJ.

Reduction in postsynaptic receptor activities causes reduced frequency and amplitude of miniature excitatory junction potentials (mEJPs), but such NMJs have normal evoked potentials (EJPs) owing to a compensatory increase in neurotransmitter release, a phenomenon referred to as presynaptic homeostatic potentiation (PHP). For example, application of sub-blocking concentrations of philanthotoxin (PhTx), a polyamine toxin derived from wasp venom, to semi-intact larval preparations triggers a fast reduction in quantal size and an increase in quantal content (QC), so that the basal neurotransmission recovers within minutes. Loss of presynaptic KaiRID (or Neto) renders such NMJs unable to express PHP. Likewise, loss of UKAR (in *ukar*^{null} mutants) or knockdown of *ukar* specifically in MNs, induced loss of ability to express PHP at these NMJs: upon toxin application the mini amplitudes were reduced but the basal neurotransmission never recovered. Expression of a *ukar* transgene in larval MNs effectively restored basal neurotransmission and PHP to normal levels. Together, our results indicate that KaiRID and UKAR share multiple activities at larval NMJ and that they probably function as a heterodimer autoreceptor.

Neto, a highly conserved auxiliary subunit that modulates kainate-type receptors

We previously discovered that an obligatory auxiliary protein, Neto, is absolutely required for clustering of postsynaptic glutamate receptors and for NMJ functionality. Neto belongs to a family of highly conserved auxiliary proteins that share an ancestral role in the formation and modulation of glutamatergic synapses. Vertebrate Neto1 and Neto2 were shown to modulate the properties of selective glutamate receptors, in particular the kainate-type receptors. Our previous investigations unveiled essential roles for *Drosophila* Neto during NMJ development and strongly support the notion that trafficking of both postsynaptic and the presynaptic glutamate receptors, their synaptic recruitment and stabilization, and their function are tightly regulated by Neto. We found that the fly Neto directly engages the glutamate receptor complexes, as well as other intracellular and extracellular proteins, to selectively regulate the distribution of postsynaptic glutamate receptor subtypes, the recruitment of postsynaptic proteins, and the organization of postsynaptic structures. In recent studies, we focused on how Neto modulates the gating properties of post- and pre-synaptic receptors.

Drosophila *neto* encodes two isoforms, Neto- α and Neto- β , which share the extracellular and transmembrane domains but have distinct intracellular parts. Neto- β is the predominant isoform at the NMJ and functions in the muscle to recruit glutamate receptors and other postsynaptic components. Neto- α acts predominantly in the motor neurons to ensure normal basal neurotransmission. To study the biophysical properties of *Drosophila* NMJ receptors, we are using fast agonist application on outside-out patches from HEK293 cells

transfected with various combinations of receptors with or without Neto isoforms/variants. Neto is critical for the functional reconstitution of both postsynaptic receptors (type-A: GluRIIA/C/D/E and type-B: GluRIIB/C/D/E): 100% of the more than 100 patches yielded no currents in the absence of Neto proteins. Furthermore, the two isoforms, Neto- α and Neto- β , differentially modulate the desensitization and deactivation rates for postsynaptic receptors/channels. The desensitization rates are similar for type-A or type-B channels in complexes with Neto- β or Neto- Δ CTD (which lacks any intracellular domain), but are significantly reduced when Neto- α is co-transfected with either postsynaptic receptor complex. Neto is absolutely required for the function of type-A and type-B receptors, but not for the KaiR1D homotetramer channels. When transfected by itself, KaiR1D forms rapidly activating and desensitizing channels. When KaiR1D was co-transfected with Neto proteins, the receptor's expression increased (from 36% to 61.8% success rate for KaiR1D/Neto- α complexes) and the gating kinetics were altered. We found non-significant changes in the deactivation rates, but the desensitization time constants increased two-fold from KaiR1D alone to KaiR1D in combination with Neto- α , Neto- β , or Neto- Δ CTD. The CUB1 domain of Neto proteins is absolutely required for any Neto-dependent modulation. This is probably because this domain was predicted by Cryo-EM studies to form strong interactions, an anchor point, with the amino-terminal domain of the receptor complexes. The intracellular domains of Neto had differential effects on various receptors/channels. Pre-treatment with Concanavalin A increases the single-channel open times for all postsynaptic and presynaptic receptors, whereas extracellular philanthotoxin blocks the channels to various extents. These channels are differentially regulated by addition of intracellular polyamines (spermine) at physiological concentration, which changes the rectification profiles for each channel. Our studies reveal that Neto is not only required for the function of selected channels but also increases the diversity of the receptor properties.

Novel split methodology to image, track, and reconstitute complex proteins

Synaptic proteins such as Neto and glutamate receptors are notoriously difficult to track and study because of their low abundance and the high density of functional domains. Studies using transgenic lines that express selective tagged isoforms/variants often cannot generate definitive conclusions owing to heterologous promoters and overexpression artifacts. To facilitate structure-function studies and accomplish reliable detection of low-abundant synaptic proteins in different tissues, we turned to a recently described cell-biology tool, the ALFA system. The system consists of a synthetically designed epitope tag of only 14 amino acids, the ALFA tag (AT), with no homology in the animal kingdom, and a nanobody (NbALFA) that binds to ALFA-tagged proteins with picomolar affinities. The high affinity of ALFA tag/NbALFA binding and the intrabodies capabilities of NbALFA (that is binding ALFA-tagged proteins when expressed in living cells) have prompted the development of a variety of *in vitro* cell-biology applications, from super-resolution to live detection of tagged proteins.

To probe whether the methodology is suitable for *in vivo* application, we chose a case-study protein, *Drosophila* Neurexin-1 (Nrx-1). Neurexins are key adhesion proteins that coordinate extracellular and intracellular synaptic assemblies. Neurexins are also notoriously difficult to track and study because of their low abundance and the high density of functional domains. These proteins are crucial for synapse assembly and function; however, the role of some of their domains (for example the C-terminal PDZ binding motifs) has only been inferred from *in vitro* studies. Guided by phylogenetic analysis and secondary structure prediction, we generated ALFA-tagged Nrx-1 variants, including an endogenously tagged *Nrx-1-AT* allele, which is indistinguishable from the wild-type control, and a *Nrx*^{DPDZ}-AT allele that resembles the *Nrx-1*^{null} mutant. Using a combination of classic genetics and cell biology and electrophysiology approaches, we found that *Nrx-1-AT* NMJs have normal morphology and

function, whereas the *Nrx*^{DPDZ}-AT mutants have smaller NMJs with much reduced basal neurotransmission, reminiscent of *Nrx-1*^{null} mutant. Similar to untagged *Nrx-1*, endogenously edited *Nrx-1*-AT localizes at presynaptic sites. Remarkably, the ALFA system enabled detection of endogenous *Nrx-1*-AT in only one immunohistochemistry step, using the monovalent binder NbALFA conjugated to two fluorophores (FluoTag-X2 anti-ALFA). Using cytosolic NbALFA-mScarlet intrabody, we also tracked live *Nrx-1* transport along the motor neuron axons and described fast anterograde and slow retrograde synaptic transport vesicles. These data confirm the expectation that the ALFA system ensures high-affinity binding, linear (monovalent) signals with respect to target molecule, with no amplification by polyclonal secondaries, and virtually no background in animal tissues.

In addition, we found that the PDZ binding motif is key to *Nrx-1* *in vivo* surface expression and synaptic localization: the *Nrx*^{DPDZ}-AT variant was trapped in the ER, unable to traffic to the cell surface. This explained why the *Nrx*^{DPDZ}-AT allele had NMJ defects similar with the *Nrx-1*^{null} mutant. Given that the ALFA system is very compact and has high binding affinity, both inside and outside the cells, we next investigated whether the system could deliver the missing PDZ binding motif *in trans*, facilitating the reconstitution of functional *Nrx-1*. To this end, we generated a genetically encoded NbALFA-PDZ binding motif chimera (*UAS-Nb-PDZ*) and expressed it in the *Nrx*^{DPDZ}-AT neurons. The resulting animals were viable and fertile and had normal NMJ morphology and function, indicating that a PDZ-binding motif provided *in trans* fully restored the synaptic localization and function of *Nrx*^{DPDZ}-AT. The ability to use the ALFA system as a split system to reconstitute and track functional proteins *in vivo* opens up a new realm of possibilities for functional studies in specific cells/tissues and during defined developmental windows. We anticipate that this methodology will pave the way towards dissecting functional domains of complex proteins *in vivo*.

Publications

1. Vicidomini R, Serpe M. Local BMP signaling: a sensor for synaptic activity that balances synapse growth and function. *Curr Topics Dev Biol* 2022 50:211–246.
2. Nguyen TH, Vicidomini R, Choudhury S, Coon SL, Iben J, Brody T, Serpe M,. Single-cell RNA sequencing analysis of the Drosophila larval ventral cord. *Curr Protoc* 2021 e38.
3. Vicidomini R, Nguyen TH, Choudhury S, Brody T, Serpe M. Assembly and exploration of a single cell atlas of the Drosophila larval ventral cord. Identification of rare cell types. *Curr Protoc* 2021 e37.
4. Han TH, Vicidomini R, Ramos CI, Wang Q, Nguyen P, Jarnik M, Lee CH, Stawarski M, Hernandez RX, Macleod GT, Serpe M. Neto-alpha controls synapse organization and homeostasis at the Drosophila neuromuscular junction. *Cell Rep* 2020 32:107866.
5. Nguyen TH, Han TH, Newfeld SJ, Serpe M. Selective disruption of synaptic BMP signaling by a Smad mutation adjacent to the highly conserved H2 helix. *Genetics* 2020 216:159–175.

Collaborators

- Steven Coon, PhD, *Molecular Genetics Core, NICHD, Bethesda, MD*
- James Iben, PhD, *Molecular Genetics Core, NICHD, Bethesda, MD*
- Gregory T. Macleod, PhD, *Florida Atlantic University, Jupiter, FL*
- Dragan Maric, PhD, *Flow and Imaging Cytometry Core, NINDS, Bethesda, MD*
- Stuart Newfeld, PhD, *School of Life Sciences, Arizona State University, Tempe, AZ*

- Felipe Opazo, PhD, *Center for Biostructural Imaging of Neurodegeneration, Göttingen, Germany*
- Fabio Rueda-Faucz, PhD, *Molecular Genetics Core, NICHD, Bethesda, MD*

Contact

For more information, email mihaela.serpe@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/Serpe>.

Vascular Malformations

The primary goal of our translational research group is to develop efficacious therapies for patients with complex lymphatic anomalies. To do this, we seek to understand the molecular etiologies of these complex lymphatic malformations, how these molecular etiologies alter molecular signaling, and how this affects the cellular mechanisms regulating normal lymphatic development. Ultimately, these answers will allow us to develop novel therapies.

Complex lymphatic anomaly is a term that encompasses four different complex lymphatic malformations: central conducting lymphatic anomaly (CCLA), generalized lymphatic anomaly (GLA), Kaposiform lymphangiomatosis (KLA), and Gorham Stout disease (GSD). Patients suffer from symptoms such as pleural effusions, pericardial effusions, ascites, and bone lesions, which can cause significant morbidity and even death. Currently, there is only one medication approved for patients with complex lymphatic anomalies caused by *PIK3CA*. Similar, precision-medicine approaches are needed for patients with other complex lymphatic anomalies.

Research in our lab will combine patient studies and genomics with the zebrafish model to identify novel therapies. The zebrafish model allows for us to manipulate the genetics rapidly to create patient-based models, image the developing vasculature, understand cellular dynamics *in vivo*, and perform drug screening.

Natural history study of lymphatic disorders

We are developing a prospective natural history study for patients with lymphatic anomalies to systematically evaluate the disease phenotypes and long-term outcomes. This will allow us to provide improved prognostication to families, establish screening/monitoring guidelines, determine best practices for genetic diagnosis, and explore family opinions and fertility for those on long-term medication management. The study will allow us to identify novel end-points for future clinical trials.

Genotype-phenotype correlations in central conducting lymphatic anomaly

Central conducting lymphatic anomalies (CCLA) occur when there is a



Sarah E. Sheppard, MD, PhD, MST, Head, Unit on Vascular Malformations

Scott Paulissen, PhD, *Research Specialist IV*

Georgia Krikorian, BA, *Postbaccalaureate Fellow*

Dhyanam Shukla, BA, *Postbaccalaureate Fellow*

Kristina Woodis, BS, *Postbaccalaureate Fellow*

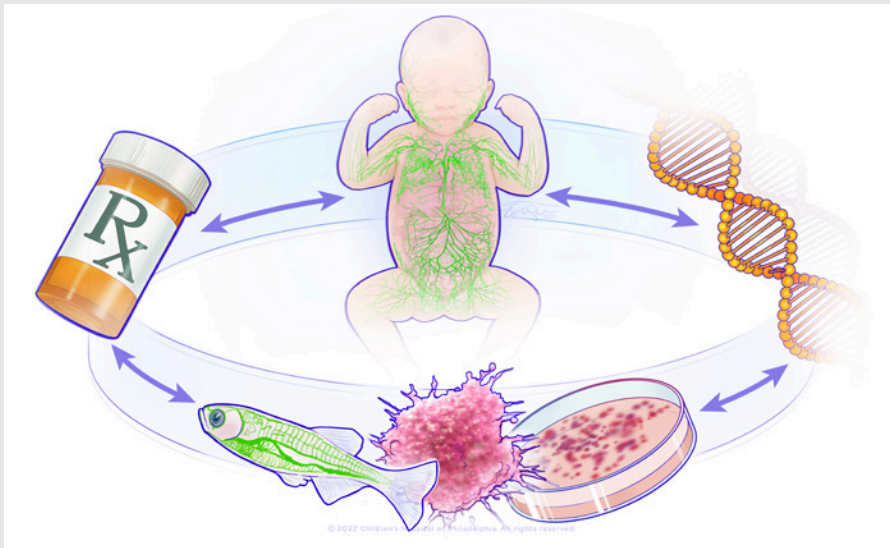


FIGURE 1. A bedside-to-bench precision medicine program for lymphatic anomalies

The figure shows a circle with the major components of the research in the lab: a child with abnormal lymphatics, a DNA molecule, a representation of a zebrafish model and organoid model, and a pill bottle. This represents a bedside-to-bench-to-bedside program using organoid and zebrafish to model patient's lymphatic anomalies and develop therapies that will be translated back to the patients.

disruption of central lymphatic flow resulting in complications such as non-immune fetal hydrops, chylothorax, chylous ascites, protein-losing enteropathy, other effusions, or lymphedema. The heterogeneity of CCLA complicates diagnosis, treatment, and prognostication. Understanding the molecular etiology of a patient's disease can drive medical care, including novel treatment strategies. However, few genetic causes have been identified for CCLA. Clinical geneticists use distinct facial features to assist in diagnosis of rare disorders. Given the recent advances in lymphatic imaging, we sought to understand whether we could use features identified by dynamic contrast magnetic resonance imaging for diagnosing CCLA. We discovered that only about a quarter of patients with CCLA have an underlying genetic diagnosis that can be identified by routine clinical evaluation. We also demonstrated that germline RASopathies, mosaic KRASopathies, *PIEZO1*-related lymphatic dysplasia, and Trisomy 21 have distinct central lymphatic flow phenotypes.

Cellular and molecular mechanism of lymphatic disorders

Previously, I demonstrated that activating variants in *KRAS* can drive lymphatic malformations in the zebrafish, which can be treated with MEK inhibitors. We identified several genetic causes of CCLA. We will evaluate these novel potential causes to understand their effect on the cellular and molecular mechanisms driving lymphatic development and identify new therapies.

Additional Funding

- NIH Distinguished Scholars Program

Publications

1. Liu M, Smith CL, Biko DM, Li D, Pinto E, O'Connor N, Skraban C, Zackai EH, Hakonarson H, Dori Y, Sheppard SE. Genetics etiologies and genotype phenotype correlations in a cohort of individuals with central conducting lymphatic anomaly. *Eur J Hum Genet* 2022 30(9):1022–1028.

FIGURE 2. Clinical imaging of lymphatic anomalies according to genotype

T2 space and dynamic contrast MR lymphangiography (DCMRL) from seven different genotypes, illustrating lymphatic conduction abnormalities.

A. Mosaic *BRAF* (p.Val600Glu): T2 space shows significant edema in the intercostal, mesentery, and liver lymphatics (*left panel*) (*arrows*) that correlates with abnormal perfusion patterns on intrahepatic DCMRL (*right*). Also note the abnormal lymphatic thoracic vessels with absence of a normal thoracic duct.

B. Mosaic *KRAS* (p.Gly12Asp): There is edema on T2 space within the mediastinum and lungs (*arrows*). Patient also with cystic right kidney (*asterisk*). Intrahepatic DCMRL demonstrates correlation with mediastinal, pulmonary, and supraclavicular edema, with perfusion of dilated lymphatic structures. Of note, this patient has a central thoracic duct (*arrow heads*), but it was not patent to the venous circulation on ultrasound contrast imaging.

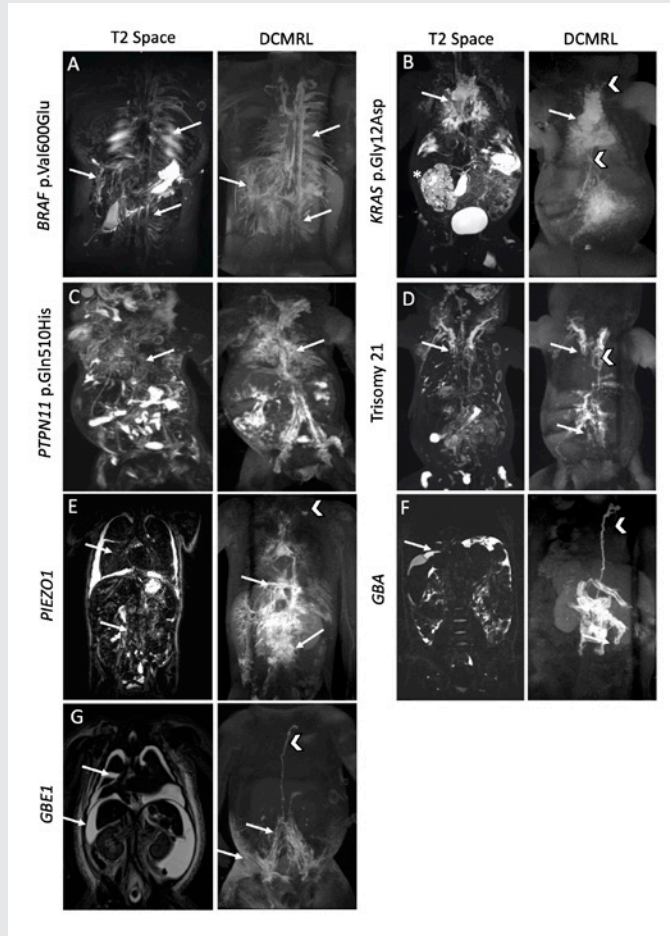
C. Noonan syndrome (*PTPN11* p.Gln510His): T2 space imaging demonstrating mediastinal and intercostal edema predominately. With intranodal DCMRL, these areas correlate with abnormal perfusion (*arrows*). Again, note there is no central thoracic duct, but persistent pulmonary and intercostal perfusion.

D. Trisomy 21: T2 space imaging with edema in the supraclavicular and superior mediastinal lymphatics (*arrows*). On intrahepatic DCMRL, there is retrograde flow into retroperitoneal lymphatics, intercostal, mediastinal, pulmonary, and supraclavicular perfusion (*arrows*). There is a patent thoracic duct that courses to the left venous angle (*arrowhead*).

E. *PIEZO1*: T2 space shows bilateral pleural effusions and pulmonary and retroperitoneal edema (*arrows*). Intrahepatic DCMRL shows extensive flow to the hepatic capsular lymphatics, with extension into the mediastinum and pulmonary lymphatics (*arrows*). There is also retrograde flow into the retroperitoneal lumbar and mesenteric lymphatics. There is a small thoracic duct seen coursing to the left venous angle (*arrow head*), patent on follow-up imaging.

F. Gaucher's disease Type III: T2 space notable for ascites. Intrahepatic DCMRL shows retrograde perfusion to retroperitoneal lumbar lymphatics and mesentery (*arrows*). The thoracic duct is mildly dilated and tortuous as it courses to the left venous angle (*arrowhead*).

G. Andersen's disease: T2 space imaging with significant ascites, pleural effusions, and anasarca (*arrows*). With intranodal DCMRL, there is extensive dermal perfusion and dilated retroperitoneal lymphatics. A thoracic duct is present and mildly dilated and tortuous (*arrowhead*). Figure and caption from [Reference 1].



- Byrne AB, Brouillard P, Sutton DL, Kazenwadel J, Montazaribarforoushi S, Secker GA, Oszmiana A, Babic M, Betterman KL, Brautigan PJ, White M, Piltz SG, Thomas PQ, Hahn CN, Rath M, Felbor U, Korenke GC, Smith CL, Wood KH, Sheppard SE, Adams DM, Kariminejad A, Helaers R, Boon LM, Revencu N, Moore L, Barnett C, Haan E, Arts P, Vikkula M, Scott HS, Harvey NL. Pathogenic variants in *MDFIC* cause recessive

central conducting lymphatic anomaly with lymphedema. *Sci Transl Med* 2022 4(634):eabm4869.

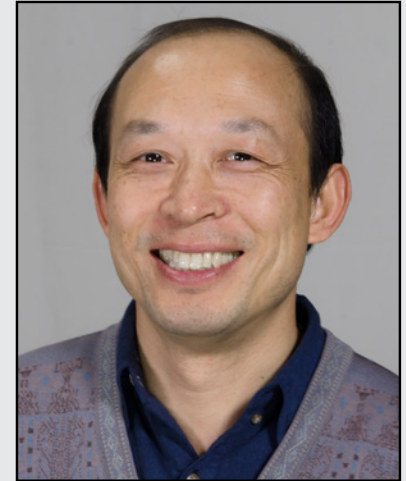
3. Szigety KM, Crowley TB, Gaiser KB, Chen EY, Priestley JRC, Williams LS, Rangu SA, Wright CM, Adusumalli P, Ahrens-Nicklas RC, Calderon B, Cuddapah SR, Edmondson A, Ficicioglu C, Ganetzky R, Kalish JM, Krantz ID, McDonald-McGinn DM, Medne L, Muraresku C, Pyle LC, Zackai EH, Campbell IM, Sheppard SE. Clinical effectiveness of telemedicine-based pediatric genetics care. *Pediatrics* 2022 150(1):e2021054520.

Contact

For more information, email sarah.sheppard@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/sheppard>.

Thyroid Hormone Regulation of Vertebrate Postembryonic Development

The laboratory investigates the molecular mechanisms of thyroid hormone (TH) function during postembryonic development, a period around birth in mammals when plasma TH levels peak. The main model is the metamorphosis of *Xenopus laevis* and *X. tropicalis*, two highly related species, which offer unique but complementary advantages. The control of this developmental process by TH offers a paradigm for the study of gene function in postembryonic organ development. During metamorphosis, different organs undergo vastly different changes. Some, like the tail, undergo complete resorption, while others, such as the limb, are developed *de novo*. The majority of larval organs persist through metamorphosis but are dramatically remodeled to function in a frog. For example, tadpole intestine is a simple tubular structure consisting primarily of a single layer of larval epithelial cells. During metamorphosis, it is transformed into an organ with a multiply folded adult epithelium surrounded by elaborate connective tissue and muscles, through specific larval epithelial cell death and *de novo* development of the adult epithelial stem cells, followed by their proliferation and differentiation. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis both *in vivo* by using genetic approaches or hormone treatment of whole animals, and *in vitro* in organ cultures offer an excellent opportunity to: (1) study the developmental function of TH receptors (TRs) and the underlying mechanisms *in vivo*; and (2) identify and functionally characterize genes that are critical for organogenesis, particularly the formation of the adult intestinal epithelial stem cells, during postembryonic development in vertebrates [Reference 1]. A major recent focus has been to make use of the TALEN and CRISPR/Cas9 technologies to knock out the endogenous genes for functional analyses. In addition, the recent improvements in the annotation of the *Xenopus tropicalis* genome allow us to carry out RNA-Seq and chromatin-immunoprecipitation (ChIP)-Seq analyses at the genome-wide level. It also allows us to adapt single-cell sequencing technology to study how TH induces cell transformations during vertebrate development. We complement our frog studies by investigating the genes found to be important for frog intestinal stem-cell development and in developing mouse intestine, by making use of the ability to carry out conditional knockout.

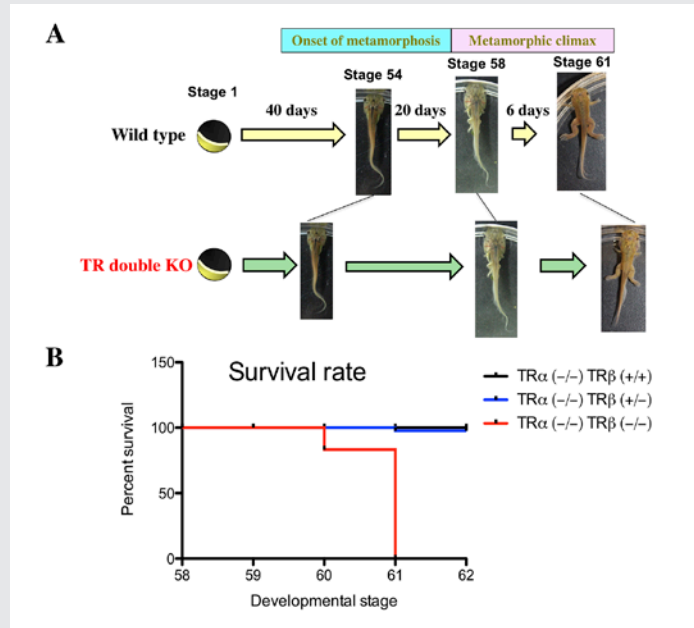


Yun-Bo Shi, PhD, *Head, Section on Molecular Morphogenesis*
Liezhen Fu, PhD, *Staff Scientist*
Nga Luu, MS, *Biologist*
Lingyu Bao, PhD, *Visiting Fellow*
Wonho Na, PhD, *Visiting Fellow*
Yuta Tanizaki, PhD, *Visiting Fellow*
Shouhong Wang, PhD, *Visiting Fellow*
Zhaoyi Peng, BS, *Graduate Student*

FIGURE 1. Effects of TR double KO on developmental rate. TR double KO leads to premature initiation of metamorphosis but slows metamorphic progress and causes lethality at the metamorphic climax.

A. TR double KO animals take a shorter time to reach the onset of metamorphosis (stage 54), indicating accelerated premetamorphic development. Once metamorphosis begins, the KO animals take longer to reach the beginning of metamorphic climax (stage 58) and also develop more slowly during the climax stages, between stages 58 and 61. The length of each stage indicates the relative time needed for development between two adjacent stages.

B. Tadpoles without any TR die during the climax of metamorphosis. The tadpoles of mixed genotypes at stage 58 were allowed to develop to stage 62 and were genotyped at stage 62 or when they died during this developmental period. The survival rate for each of the three genotypes, $tra^{-/-}tr\beta^{+/+}$, $tra^{-/-}tr\beta^{+/-}$, and $tra^{-/-}tr\beta^{-/-}$, was thus obtained and plotted. Note that no double knockout tadpoles developed to stage 62 and that a single copy of $tr\beta^{-/-}$ was sufficient for the animal to complete metamorphosis and develop into a reproductive adult.



Thyroid hormone receptor α (TR α) controls the hindlimb metamorphosis by regulating cell proliferation and WNT signaling pathways in *Xenopus tropicalis*.

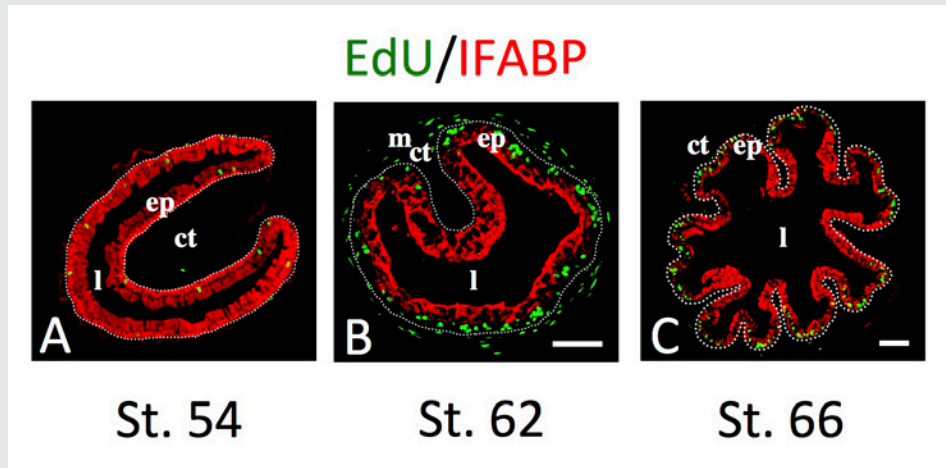
We have been studying *Xenopus tropicalis* metamorphosis as a model for postembryonic human development, and we demonstrated that TR α knockout induces precocious hindlimb development. To reveal the molecular pathways regulated by TR α during limb development, we performed chromatin immunoprecipitation and RNA sequencing on the hindlimb of premetamorphic wild-type and TR α knockout tadpoles and identified over 700 TR-bound genes up-regulated by TH treatment in wild-type but not in TR α knockout tadpoles [Reference 2]. Interestingly, most of these genes were expressed at higher levels in the hindlimb of premetamorphic TR α knockout tadpoles than in stage-matched wild-type tadpoles, suggesting their de-repression upon TR α knockout. Bioinformatic analyses revealed that the genes were highly enriched with cell-cycle- and WNT signaling-related genes. Furthermore, cell-cycle and WNT signaling pathways were also highly enriched among genes bound by TR in wild-type but not in TR α knockout hindlimb. The findings suggest that direct binding of TR α to target genes related to cell-cycle and WNT pathways is important for limb development: first by preventing precocious hindlimb formation by repressing these pathways as unliganded TR before metamorphosis; and later by promoting hindlimb development during metamorphosis by mediating TH activation of the pathways.

Transcriptome profiling reveals gene regulation programs underlying tail development in the Ornamented Pygmy frog *Microhyla fissipes*.

For comparative studies on the role of TH in anuran metamorphosis, we had previously carried out RNA-Seq analysis of the TH-induced gene expression program during tail resorption in *Microhyla fissipes* tadpoles.

FIGURE 2. Intestinal metamorphosis involves the formation of clusters of proliferating, undifferentiated epithelial cells at the climax.

Tadpoles at premetamorphic stage 54 (A), climax, stage 62 (B), and the end of metamorphosis, stage 66 (C) were injected with 5-ethynyl-2'-deoxyuridine (EdU) one hour before sacrifice. Cross-sections of the intestine from the resulting tadpoles were double-stained by EdU labeling of newly synthesized DNA and by immunohistochemistry of IFABP (intestinal fatty acid-binding protein), a marker for differentiated epithelial cells. The dotted lines depict the epithelium-mesenchyme boundary. Note that there are few EdU-labeled proliferating cells in the epithelium and that they express IFABP at premetamorphosis (A) and increase in the form of clustered cells (proliferating adult stem cells), which lack IFABP at the climax of metamorphosis (B). At the end of metamorphosis, EdU-labeled proliferating cells are localized mainly in the troughs of the epithelial folds, where IFABP expression is low (C).



Parallel to the metamorphic study, we also analyzed the tail at different developmental stages. Tadpole tail develops from the tailbud, an apparently homogenous mass of cells at the posterior of the embryo. While much progress has been made in understanding the origin and the induction of the tailbud, its subsequent outgrowth and differentiation have received much less attention, particularly with regard to global gene expression changes. By using RNA-Seq with single-molecule real time (SMRT) sequencing and further analyses, we revealed the transcriptome profiles at four key stages of tail development, from a small tailbud to the onset of feeding (S18, S19, S21, and S28) in *Microhyla fissipes* [Reference 3]. We obtained 48,826 transcripts and discovered 8,807 differentially expressed transcripts (DETs, $q < 0.05$) among these four developmental stages. We functionally classified the DETs by using GO (gene ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses and revealed 110 significantly enriched GO categories and six highly enriched KEGG pathways (protein digestion and absorption; ECM-receptor interaction; pyruvate metabolism; fatty acid degradation; valine, leucine, and isoleucine degradation; and glyoxylate and dicarboxylate metabolism) that are likely critically involved in developmental changes in the tail. In addition, analyses of DETs between any two individual stages demonstrated the involvement of distinct biological pathways/GO terms at different stages of tail development. Furthermore, the most dramatic changes in gene expression profile are those between S28 and any of the other three stages. The upregulated DETs at S28 are highly enriched in “myosin complex” and “potassium channel activity,” which are important for muscle contraction, a critical function of the tail that the animal needs by the end of embryogenesis. Additionally, many DETs and enriched pathways during tail development, which we discovered, such as the HDAC1, Hes1, and Hippo signaling pathway, have also been reported to be vital for the tissue/organ regeneration, suggesting conserved functions between development and regeneration.

The sperm-associated antigen 7 gene (*spag7*) is activated by TH during *Xenopus tropicalis* metamorphosis via a thyroid hormone-response element within the first intron.

We previously identified *spag7* as a candidate TH target gene that is potentially involved in adult stem cell development and/or proliferation during intestinal metamorphosis. To investigate whether TH regulates *spag7* directly at the transcriptional level via TR, we first conducted qRT-PCR to analyze its expression during natural and TH-induced metamorphosis and found that *spag7* was up-regulated during natural metamorphosis in the intestine, tail, brain, and hindlimb, peaking at the climax of metamorphosis in all those organs, and upon TH treatment of premetamorphic tadpoles. Next, we demonstrated that an intronic thyroid hormone-response element (TRE) in *spag7*, first identified through bioinformatic analysis, could bind to TR *in vitro* and *in vivo* during metamorphosis. A dual luciferase assay utilizing a reconstituted frog oocyte transcription system showed that the TRE could mediate promoter activation by liganded TR. The results indicate that *spag7* expression is directly regulated by TH through the TRE in the first intron during metamorphosis, implicating a role for *spag7* early during TH-regulated tissue remodeling and resorption [Reference 4].

Thyroid hormone receptor α controls larval intestinal epithelial cell death by regulating the CDK1 pathway.

We have been studying intestinal remodeling during *Xenopus tropicalis* metamorphosis as a model in which to study TR function in adult organ development. By using ChIP (chromatin immunoprecipitation)-Seq, we identified over 3,000 TR-bound genes in the intestine of premetamorphic wild type or TR α (the major TR expressed during premetamorphosis)-knockout tadpoles [Reference 5]. Surprisingly, cell cycle-related GO (gene ontology) terms and biological pathways were highly enriched among TR target genes, even though the first major event during intestinal metamorphosis is larval epithelial cell death, and TR α knockout drastically reduced this enrichment. More importantly, treatment of tadpoles with cell-cycle inhibitors blocked TH-induced intestinal remodeling, especially larval epithelial cell death, suggesting that TR α -dependent cell cycle activation is important for TH-induced apoptosis during intestinal remodeling.

Additional Funding

- Japan Society for the Promotion of Science (JSPS) fellowship for Yuta Tanizaki
- FY22 NICHD Early Career Award for Yuta Tanizaki

Publications

1. Shi Y-B, Shibata Y, Tanizaki Y, Fu L. The development of adult intestinal stem cells: Insights from studies on thyroid hormone-dependent anuran metamorphosis. *Vitam Horm* 2021 116:269–293.
2. Tanizaki Y, Shibata Y, Zhang H, Shi Y-B. Thyroid hormone receptor α controls the hindlimb metamorphosis by regulating cell proliferation and Wnt signaling pathways in *Xenopus tropicalis*. *Int J Mol Sci* 2022 23(3):1223.
3. Wang S, Liu L, Shi Y-B, Jiang J. Transcriptome profiling reveals gene regulation programs underlying tail development in the Ornamented Pygmy frog *Microhyla fissipes*. *Cell Biosci* 2021 26:1001–12.
4. Fu L, Crawford L, Tong A, Luu N, Tanizaki Y, Shi Y-B. Sperm associated antigen 7 (SPAG7) is activated by T3 during *Xenopus tropicalis* metamorphosis via a thyroid hormone response element (TRE) within the first intron. *Dev Growth Differ* 2022 64:48–58.

5. Tanizaki Y, Zhang H, Shibata Y, Shi Y-B. Thyroid hormone receptor α controls larval intestinal epithelial cell death by regulating the CDK1 pathway. *Commun Biol* 2022 5:112.

Collaborators

- Steven Coon, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Eiichi Hinoi, PhD, *Kanazawa University Graduate School, Kanazawa, Japan*
- James Iben, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Jianping Jiang, PhD, *Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China*
- Tianwei Li, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Susan Mackem, MD, PhD, *Cancer and Developmental Biology Laboratory, Center for Cancer Research, NCI, Frederick, MD*
- Bingyin Shi, MD, *Xi'an Jiaotong University School of Medicine, Xi'an, China*
- Hari Shroff, PhD, *Section on High Resolution Optical Imaging, NIBIB, Bethesda, MD*
- Guihong Sun, PhD, *Wuhan University School of Medicine, Wuhan, China*
- Peter Taylor, PhD, *University of Dundee, Dundee, United Kingdom*
- Henry Zhang, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*

Contact

For more information, email shi@helix.nih.gov or visit <http://smm.nichd.nih.gov>.

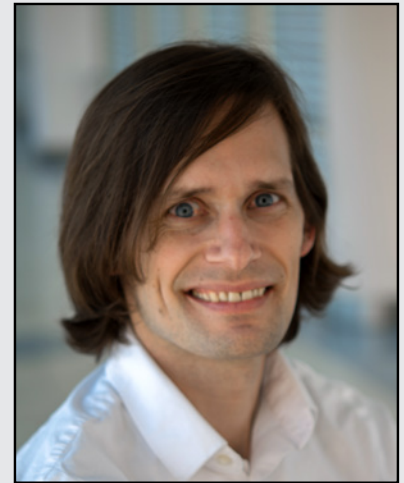
Modeling the Biophysics of Cellular Membranes

The integrity of lipid membranes is essential for life. They provide spatial separation of the chemical contents of the cell and thus make possible the electrical and chemical potential differences that are used to transmit signals and perform work. However, the membrane must be broken frequently to form, for example, new membrane structures in the cell. The simplest structure is a vesicle to transport cargo. Such vesicles are constantly cycled between organelles and the outer plasma membrane. Thus, there is a careful balance between boundary-establishing membrane fidelity and the necessary ability of the cell to change these boundaries.

The challenge in studying the membrane is its complexity. The membrane is a thin sheet of small molecules, i.e., lipids. There are hundreds of types of lipids in the cell. Each lipid changes the properties of the membrane in its vicinity, sometimes making the sheet stiffer, sometimes softer, and sometimes acting to bend the membrane into a ball or tube. Furthermore, the lipids are constantly jostling and tangling, both with each other and with proteins embedded in the membrane. To predict of how membranes are reshaped thus requires not only knowing how lipids affect the properties of the membrane surface, but also the location of specific lipids.

The question as to how molecular scale features influence extensive biological processes must be answered in the language of physical laws. Physics is the language of mechanism at the molecular scale. The challenge is linking physics to the 'big' processes that happen in life. Our lab uses detailed physics-driven molecular simulation to 'build up' models that can be applied at the much larger level of the cell, which requires retaining important information and eliminating irrelevant details. The software our lab develops is based on the models that we are building. Thus, a broad objective of our research is to create a publicly available software package that can be used either as a stand-alone application for analyzing membrane-reshaping processes or as a library for cellular-scale modeling packages for which the role of the membrane may be unclear or unanticipated.

Another key component of our research is to seek the best possible validation of our models. Few techniques are able to yield molecular information about lipids. Recent breakthroughs that break the



Alexander J. Sodt, PhD, Head,
*Unit on Membrane Chemical
Physics*

Andrew Beaven, PhD,
Postdoctoral Fellow

Amirali Hossein, PhD, *Postdoctoral
Fellow*

Henry H. Lessen, PhD,
Postdoctoral Fellow

Kayla Sapp, PhD, *Postdoctoral
Fellow*

diffraction-limit barrier are typically only applicable to static structures much larger than a molecular dye. In contrast, lipids are small and dynamic. Our group is making a sustained effort to validate our simulation findings by applying neutron scattering techniques. This year, our lab initiated new collaborations on the basis of our previous years' work developing methodology for predicting the scattering signal from our simulations.

The projects use the NIH computing resources, including the Biowulf cluster, to run simulations and models. We use molecular dynamics software (such as NAMD and CHARMM) to conduct molecular simulations. In-house software development for public distribution is a key element of the lab's work.

A critical analysis of the crowding mechanism of membrane reshaping

Cellular membranes are reshaped by various mechanisms. A principal mechanism is for a scaffold of curved proteins to bind to the membrane, enforcing their own shape. Proteins and lipids may also directly modify the properties of the surface in which they are embedded, for example, by 'wedging' into the surface to which they apply a force to bend. A third mechanism is that of 'crowding,' in which a surface, packed with large proteins, curves to lower the density of proteins. In our article [Reference 1], which analyzes the physical forces that are part of crowding, our revision of the theory makes clear that the literature explanation is not valid at the lower (closer to physiological) regime of protein coverage. Instead, we suggest that there is likely an additional interaction of bound proteins with the membrane surface that is independent of crowding.

The biophysics of membrane reshaping in fusion and fission

A key point of our work this year touches on the physics of lipids in membrane fusion and fission, i.e., the process of creating and absorbing vesicles (tiny lipidic spheres) that transport proteins between organelles.

The key physical descriptor of how hard it is to reshape a piece of membrane into a vesicle is the bending modulus, which is an elastic constant similar to what engineers use to describe macroscopic materials. It describes the flexibility of the nanometer-thick sheet of lipids that form a bilayer membrane in the cell. Intuitively, the stiffness of cells' membranes is determined by how thick they are, with saturated fatty tails and cholesterol typically leading to thicker, stiffer membranes. We showed how molecular simulations can identify mechanisms by which lipids can make membranes softer, and thus easier to reshape [Reference 4]. The methodology has the ability to characterize how nanometer-scale patches of lipids can determine the properties of complex biological membranes and thus explain how disorders in lipid synthesis affect the many processes that require membranes to be reshaped.

Our study has guided current projects on how small changes in sterol chemistry lead to significant differences in the outcomes of clathrin-mediated endocytosis. In collaborations, the lab has contributed theory to describe the shape and stability of membranes as they are reshaped by clathrin cages [Reference 2], as well as molecular simulations of amphipathic helices of the immune system protein IFITM3, as part of a project exploring the mechanism of its action blocking viral fusion with our cells [Reference 3].

Publications

1. Sapp KC, Sodt AJ. Observed steric crowding at modest coverage requires a particular membrane-binding scheme or a complementary mechanism. *Biophys J* 2022 121:430–438.

2. Guo SK, Sodt AJ, Johnson ME. Large self-assembled clathrin lattices spontaneously disassemble without sufficient adaptor proteins. *PLoS Comput Biol* 2022 18:e1009969.
3. Rahman K, Datta SAK, Beaven AH, Jolley AA, Sodt AJ, Compton AA. Cholesterol binds the amphipathic helix of IFITM3 and regulates antiviral activity. *J Mol Biol* 2022 434:167759.
4. Lessen HJ, Sapp KC, Beaven AH, Ashkar R, Sodt AJ. Molecular mechanisms of spontaneous curvature and softening in complex lipid bilayer mixtures. *Biophys J* 2022 121:3188–3199.

Collaborators

- Rana Ashkar, PhD, *Virginia Tech, Blacksburg, VA*
- Alex A. Compton, PhD, *HIV Dynamics and Replication Program, Center for Cancer Research, NCI, Frederick, MD*

Contact

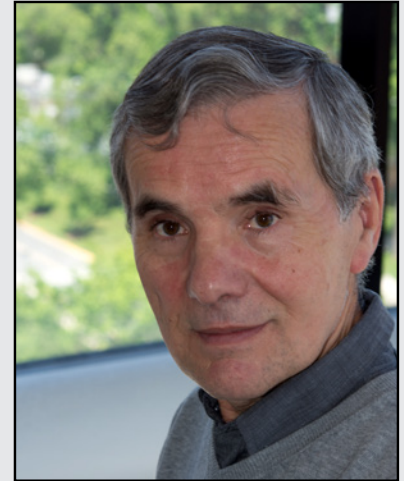
For more information, email alexander.sodt@nih.gov or visit <http://sodtlab.nichd.nih.gov>.

Signaling and Secretion in Neuroendocrine Cells

The main goal of the research in our Section is to examine cell-signaling cascades, gene expression, and hormone secretion in neuroendocrine cells from the hypothalamus and pituitary gland during development. We place special emphasis on the characterization of individual cells, using fluorescence imaging, patch-clamp recordings, simultaneous membrane potential/calcium and current/calcium recordings, electrophysiological and imaging recordings of single-cell exocytic events, single-cell RNA sequencing (scRNA-Seq), and single-cell quantitative reverse transcription polymerase chain reaction (RT-PCR). Our recent and ongoing work has focused on the following: signaling, transcription, and secretion in the pituitary gland specific for age, sex, and tissue structure; pituitary cell heterogeneity, reflecting their postnatal gene expression; the role of 1-phosphatidylinositol 4-kinases and protein receptor tyrosine phosphatase N2 in postnatal proliferation and maintenance of pituitary lineages; and cell-specific electrical activity and exocytic pathways. Current and proposed studies depend in part on the use of equipment in NICHD's [Microscopy and Imaging Core Facility](#) and the [Molecular Genomics Core Facility](#).

Role of PI4-kinase alpha in calcium signaling and prolactin secretion

We continue investigations on genes expressed in pituitary cells and their roles in signaling and hormone secretion, focusing on the transcriptome profiles of secretory and non-secretory cell types, using scRNA-Seq of freshly dispersed pituitary cells from adult female rats. Among others, the studies revealed that all hormone-producing cells express the phosphatidylinositol (PI) kinase genes *Pi4ka*, *Pi4kb*, *Pi4k2a*, *Pi4k2b*, *Pip5k1a*, *Pip5k1c*, and *Pik3ca*, as well as *Pikfyve* and *Pip4k2c*. In a recent study, we analyzed the contribution of phosphatidylinositol kinases to calcium-driven prolactin (PRL) release in pituitary lactotrophs: PI4Ks, which control PI4P production; PIP5Ks, which synthesize PI(4,5)P₂ by phosphorylating the D-5 position of the inositol ring of PI4P; and PI3Ks, which phosphorylate PI(4,5)P₂ to generate PI(3,4,5)P₃. We used common and PIK-specific inhibitors to evaluate the strength of calcium-secretion coupling in rat lactotrophs. Wortmannin, a PI3K and PI4K inhibitor, but not LY294002, a PI3K inhibitor, blocked spontaneous action potential-driven PRL release with a half-time of about 20 minutes when applied in 10 μ M



Stanko S. Stojilkovic, PhD, Head, Section on Cellular Signaling
Stephanie Constantin, PhD, Staff Scientist

Rafael M. Previde, PhD, Visiting Fellow

Kosara Smiljanic, PhD, Visiting Fellow

Srdjan J. Sokanovic, PhD, Visiting Fellow

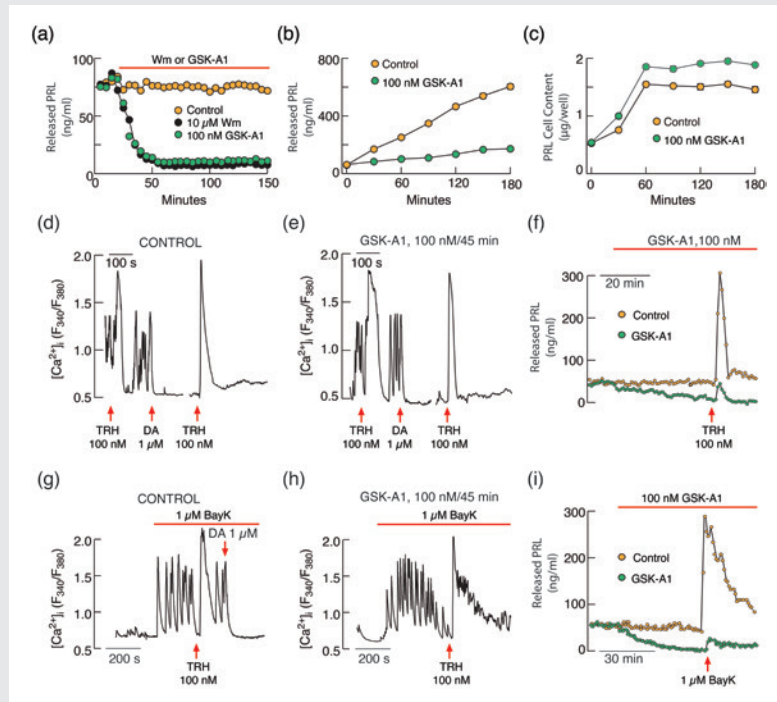
FIGURE 1.

PI4-kinase controls basal and receptor-stimulated exocytosis in pituitary lactotrophs independently of PI(4,5)P2.

(a–c) Inhibition of basal prolactin (PRL) release by wortmannin (Wm) and GSK-A1 in perfused (a) and static pituitary cells (b) without affecting *de novo* PRL synthesis (c).

(d–f) GSK-A1 does not inhibit thyrotropin-releasing hormone (TRH)-stimulated calcium signaling in pituitary lactotrophs (d vs e) but inhibits basal and TRH-stimulated PRL release in perfused pituitary cells (f).

(g–h) GSK-A1 also does not affect stimulated voltage-gated calcium influx by the L-type calcium-channel agonist BayK 8644 (BayK) in pituitary lactotrophs (g vs h) but inhibits BayK-stimulated PRL release in perfused pituitary cells.



concentration, leading to accumulation of intracellular PRL content. Wortmannin also inhibited the increase in PRL release by high potassium, the calcium channel agonist Bay K8644, and calcium-mobilizing thyrotropin-releasing hormone, without affecting accompanying calcium signaling. GSK-A1, a specific inhibitor of PI4KA, also inhibited calcium-driven PRL secretion without affecting calcium signaling or *Prl* gene expression. In contrast, PIK93, a specific inhibitor of PI4KB, and ISA201B and UNC3230, specific inhibitors of PIP5K1A and PIP5K1C, respectively, did not affect PRL release (Figure 1). These experiments revealed a key role of PI4KA in calcium-secretion coupling in pituitary lactotrophs downstream of voltage-gated and PI(4,5)P2-dependent calcium signaling [Reference 1].

Pituitary gonadotroph-specific patterns of gene expression and hormone secretion

Contrary to the hypothesis of pituitary cell plasticity published by several groups, gonadotrophs appear as a single cluster of homogeneous cells, uniquely expressing *Fshb*, *Lhb*, and *Gnrhr*. These cells also specifically express other genes, including *Chrna4*, *Cnga1*, *Dmp1*, *Dusp15*, *Icam5*, *Lama1*, *Nhlh2*, *Nr5a1*, *Pitx3*, *Spp1*, *Tgfb3l*, and *Vash2*. Some of the genes are clearly expressed in a sex-specific manner, like *Dmp1* and its sister gene *Spp1*. In general, the specific roles of these genes in gonadotroph functions have not yet been elucidated. Our recent studies point to the gonadotroph-specific patterns of gene expression and hormone secretion. The luteinizing hormone (LH) secretory profiles appear to reflect depletion of prestored LH in secretory vesicles by regulated exocytosis. In contrast, follicle-stimulating hormone (FSH) is predominantly released by constitutive exocytosis, and secretory activity reflects the kinetics of *Fshb* gene expression controlled by gonadotropin-releasing hormone (GnRH), activin, and inhibin. Consistent with the role of activin and inhibin on *Fshb* expression, pituitary cells express three inhibin subunit genes: *Inha* is expressed in all hormone-producing cell types and folliculostellate cells (FSCs) in the anterior pituitary and pituicytes in the posterior pituitary; *Inhba* is

expressed only in FSCs and pituicytes, and *Inhbb* is expressed in gonadotrophs, corticotrophs, FSCs, pituicytes, and pituitary endothelial cells [Reference 2].

Our ongoing work in this project focuses on the roles of the neuroendocrine marker genes *Ptprn* and *Ptprn2*, which encode the protein tyrosine phosphatase receptors N and N2, on pituitary gonadotroph function. To do this, we analyzed the effects of their double knockout (DKO) in mice on the hypothalamic-pituitary-gonadal axis. In DKO females, delayed puberty and lack of ovulation were observed, complemented by changes in ovarian gene expression and steroidogenesis. In contrast, testicular gene expression, steroidogenesis, and reproductive organ development were not significantly affected in DKO males. However, in both sexes, pituitary *Lhb* gene expression and LH levels were reduced, as well as the *Fshb* gene, while the calcium-mobilizing and LH secretory actions of GnRH were preserved. Hypothalamic *Gnrh1* and *Kiss1* gene expression was also reduced in DKO females and males. In parallel, a significant reduction in the density of immunoreactive GnRH and kisspeptin fibers was detected in the hypothalamic arcuate nucleus of DKO females and males, while kisspeptin immunoreactivity in the rostral periventricular region of the third ventricle was lowered only in DKO females. These experiments in progress indicate a critical role of *Ptprn* and *Ptprn2* in kisspeptin–GnRH neuronal function and sexual dimorphism in the threshold levels of GnRH required to preserve reproductive function.

A transcriptomics perspective of pituitary corticotrophs

Our scRNA-Seq studies using rat pituitary cells revealed that the corticotroph transcriptome profile was most comparable to that of melanotrophs, and generally agrees with previous basic and clinical work with these cells. However, data on pituitary scRNA-Seq from several species indicate homogeneity of postnatal corticotrophs, forming a distinct cluster of cells, compared with melanotrophs, other hormone-producing cells, and non-hormonal pituitary cells. Genes specific for corticotrophs include *Clrn1*, *Chrna1*, *Adh1*, *Angptl8*, *Hspb3*, *Lmx1a*, *Scube2*, and *Trdn*. The roles of these genes in corticotroph functions have not been characterized. Certainly, the most critical genes for corticotroph functions are *Crhr1* and *Avpr1*, encoding the G protein-coupled receptors CRHR1 and AVPR1b, activated by hypothalamic corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP), respectively. *Avpr1b* is specifically expressed in corticotrophs, whereas *Crhr1* has been detected in some melanotrophs as well. Other genes are specific for melanotrophs, including *Oacyl*, *Pax7*, *Esm1*, and *Pcsk2*. Moreover, scRNA-Seq data provide a wealth of new information regarding the expression of several common genes encoding other G protein-coupled receptors and enzyme-linked plasma membrane receptors, and their signal transduction pathways, which have not previously been reported to be expressed in the pituitary gland. The expression pattern of these receptors and their ligands highlight the importance of autocrine/paracrine regulation of pituitary cell function and the modulating role of peripheral glands through nuclear receptors [Reference 3].

We also contributed to the work of our collaborator Prashant Chittiboina on scRNA-Seq studies of human hormone-producing pituitary adenomas causing Cushing's disease (CD). The analysis included over 25,000 cells and identified unique CD adenoma transcriptomic signatures compared with adjacent normal cells, with validation by bulk RNA-Seq, DNA methylation, qRT-PCR, and immunohistochemistry. CD adenoma cells include a subpopulation of proliferating, terminally differentiated corticotrophs. In CD adenomas, we found recurrent promoter hypomethylation and transcriptional upregulation of PMAIP1 (encoding proapoptotic BH3-only bcl-2 protein noxa) but paradoxical noxa downregulation. Using primary CD adenoma cell cultures and a corticotroph-enriched mouse cell line, we found that selective proteasomal inhibition with bortezomib stabilizes noxa and induces apoptosis, indicating its utility as an anti-tumor agent [Reference 4].

FIGURE 2. Identification of two FSC subtypes

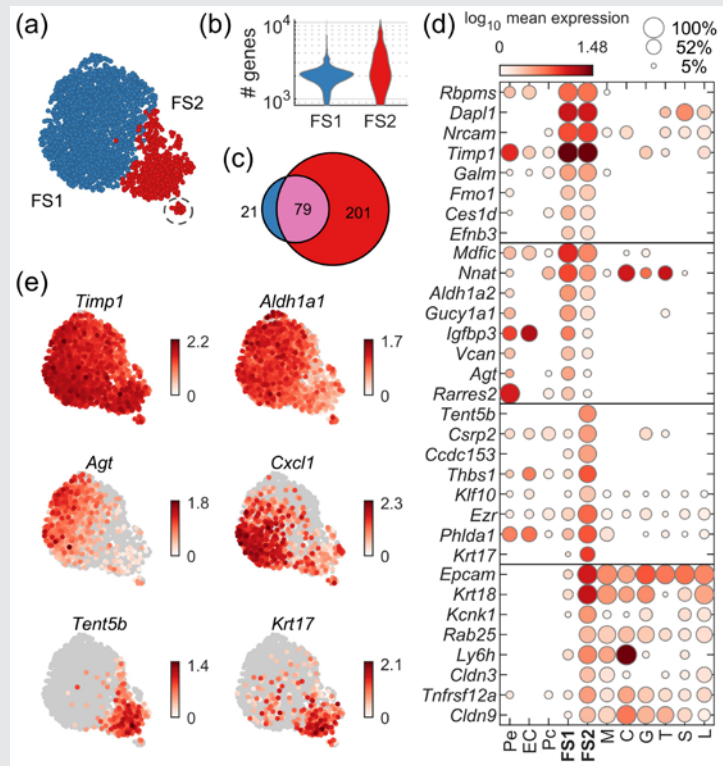
(a) UMAP embedding showing unsupervised clustering of FSCs into two subtypes. A small portion of FS2 cells (circled) express cell cycle markers.

(b) Violin plot of the distributions of number of genes per cell in FS1 and FS2, with median values of 2,099 and 3,137, respectively.

(c) Venn diagram showing the number of genes identified as FS1-dominant, FS2-dominant, and common to both, relative to both cell types except pituicytes.

(d) Dot plot showing selected top FSC-expressed genes: dominant in both subtypes (first group), FS1-dominant (second group), FS2-dominant (third group), and co-expressed in FS2 and HPC (fourth group).

(e) UMAP scatter plots showing the expression patterns of selected genes from panel d.



The astroglial and stem cell functions of adult pituitary folliculostellate cells

Recently, we presented scRNA-Seq and immunohistofluorescence analyses of pituitary cells of adult female rats, with a focus on the transcriptomic profiles of nonhormonal cell types. Samples obtained from whole pituitaries and separated anterior and posterior lobe cells contained all expected pituitary-resident cell types and lobe-specific vascular cell subpopulations. FSCs and pituicytes expressed *S100B*, *ALDOC*, *EAAT1*, *ALDH1A1*, and *VIM* genes and proteins, as well as other astroglial marker genes, some common and some cell-type specific. We also found that the *SOX2* gene and protein were expressed in about 15% of pituitary cells, including FSCs, pituicytes, and a fraction of hormone-producing cells, arguing against its stem-cell specificity. FSCs comprised two *Sox2*-expressing subclusters; one larger (FS1, 1,877 cells) and one smaller (FS2, 511 cells). Interestingly, FS2 cells expressed a much greater diversity of genes than did FS1 cells, with a median of 3,137 and 2,099 genes detected per cell, respectively. The finding that 80% of FS1-upregulated genes are also upregulated in FS2 suggests that FS1 and FS2 cells are closely related cells. In contrast, only 30% of FS2-upregulated genes were pan-FSC genes, suggesting that these cells have additional activated gene-expression programs. We also noticed that FSCs shared expression of several genes with hormone-producing cells. However, a majority of these were dominantly expressed by FS2 compared with FS1 (Figure 2). We also found that FS1 cells were randomly distributed in the anterior and intermediate lobes, while FS2 cells were localized exclusively in cells of the marginal zone between the anterior and intermediate lobes. These data indicate that the identity of the FSCs are specialized anterior pituitary-specific astroglia, with FS1 cells representing differentiated cells, with transcriptomes consistent with classical FSC roles, and FS2 cells exhibiting additional stem cell-like features [Reference 5].

Publications

1. Kucka M, Gonzales-Iglesias A, Tomic M, Previde RM, Smiljanic K, Sokanovic SJ, Fletcher PA, Sherman A, Balla T, Stojilkovic SS. Calcium-prolactin secretion coupling in female rat pituitary lactotrophs is controlled by PI4-kinase alpha. *Front Endocrinol* 2021 12:790441.
2. Constantin S, Bjelobaba I, Stojilkovic SS. Pituitary gonadotroph-specific patterns of gene expression and hormone secretion. *Curr Opin Pharmacol* 2022 66:102274.
3. Stojilkovic SS, Previde RM, Sherman AS, Fletcher PA. Pituitary corticotroph identity and receptor-mediated signaling: A transcriptomics perspective. *Curr Opin Endocr Metab Res* 2022 25:100364.
4. Asuzu DT, Alvarez R, Fletcher PA, Mandal D, Johnson K, Wu W, Elkahloun A, Clavijo P, Allen C, Maric D, Ray-Chaudhury A, Rajan S, Abdullaev Z, Nwokoye D, Aldape K, Nieman LK, Stratakis C, Stojilkovic SS, Chittiboina P. Pituitary adenomas evade apoptosis via noxa deregulation in Cushing's disease. *Cell Rep* 2022 40:111223.
5. Fletcher PA, Smiljanic K, Prévide RM, Constantin S, Sherman AS, Coon SL, Stojilkovic SS. The astroglial and stem cell functions of adult rat folliculostellate cells. *Glia* 2022 10:1002.

Collaborators

- Tamás Balla, MD, PhD, *Section on Molecular Signal Transduction, NICHD, Bethesda, MD*
- Prashant Chittiboina, MD, PhD, *Neurosurgery Unit for Pituitary and Inheritable Diseases, NINDS, Bethesda, MD*
- Patrick A. Fletcher, PhD, *Laboratory of Biological Modeling, NIDDK, Bethesda, MD*
- Arthur Sherman, PhD, *Laboratory of Biological Modeling, NIDDK, Bethesda, MD*

Contact

For more information, email stojilks@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/stojilkovic>.

Chemosensory Coding and Decoding by Neuron Ensembles

All animals need to know what is going on in the world around them. Brain mechanisms have thus evolved to gather and organize sensory information to build transient and sometimes enduring internal representations of the environment.

Using relatively simple animals and focusing primarily on olfaction and gustation, we combine electrophysiological, anatomical, behavioral, computational, optogenetic, and other techniques to examine the ways in which intact neural circuits, driven by sensory stimuli, process information. Our work reveals basic mechanisms by which sensory information is transformed, stabilized, and compared, as it makes its way through the nervous system.

We use three species of insects, each with specific and interlocking experimental advantages, as our experimental preparations: locusts, moths, and fruit flies. Compared with the vertebrate, the insect nervous system contains relatively few neurons, most of which are readily accessible for electrophysiological study. Essentially intact insect preparations perform robustly following surgical manipulations, and insects can be trained to provide behavioral answers to questions about their perceptions and memories. Ongoing advances in genetics permit us to target specific neurons for optogenetic or electrophysiological recording or manipulations of activity. Furthermore, the relatively small neural networks of insects are ideal for tightly constrained computational models that test and explicate fundamental circuit properties.

Response heterogeneity and adaptation in olfactory receptor neurons

The olfactory system, consisting of relatively few layers of neurons, with structures and mechanisms that appear repeatedly in widely divergent species, provides unique advantages for the analysis of information processing by neurons. Olfaction begins when odorants bind to olfactory receptor neurons, triggering them to fire patterns of action potentials. Recently, using new electrophysiological recording tools, we found that the spiking responses of olfactory receptor neurons are surprisingly diverse and include powerful and variable history dependencies. Single, lengthy odor pulses elicit patterns of



Mark Stopfer, PhD, *Head, Section on Sensory Coding and Neural Ensembles*

Zane Aldworth, PhD, *Staff Scientist*

Alejandra Boronat Garcia, PhD, *Postdoctoral Fellow*

Yu-Shan Hung, PhD, *Postdoctoral Fellow*

Subhasis Ray, PhD, *Postdoctoral Fellow*

Bo-Mi Song, PhD, *Postdoctoral Fellow*

Kui Sun, MD, *Technician*

Brian Kim, BS, *Graduate Student*

Leah Pappalardo, BS, *Graduate Student*

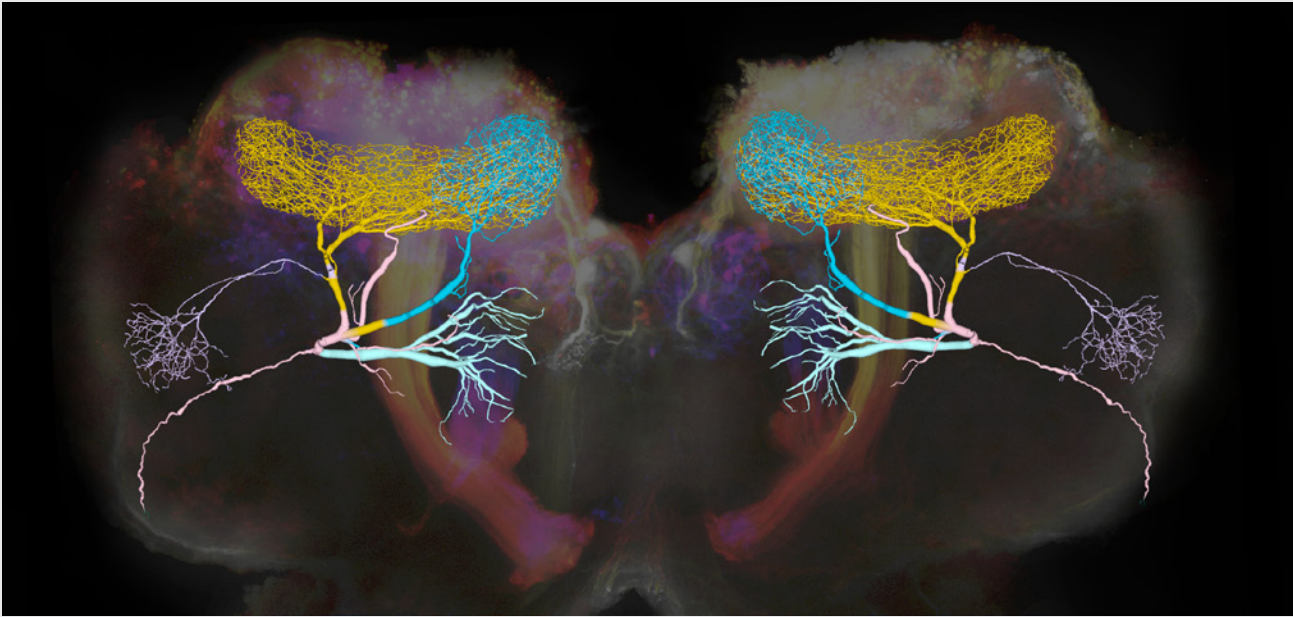


FIGURE 1. Giant GABAergic neurons regulate olfactory responses in the locust brain.

The composite image shows the structure of a compartmental computational model of the giant GABAergic neurons (GGNs) superimposed on dextran-dyed mushroom bodies in the locust brain. Different branches of GGN are shown in different colors. GGNs, only one on each side of the brain, regulate the firing of tens of thousands of olfactory neurons through feedback inhibition.

excitation and inhibition that cluster into four basic types. Different response types undergo different forms of adaptation during lengthy or repeated stimuli. A computational analysis showed that such diversity of odor-elicited spiking patterns helps the olfactory system efficiently encode odor identity, concentration, novelty, and timing, particularly in realistic environments.

Feedback inhibition and its control in an insect olfactory circuit

Inhibitory neurons play critical roles in regulating and shaping olfactory responses in vertebrates and invertebrates. In insects, such roles are performed by relatively few neurons, which can be interrogated efficiently, revealing fundamental principles of olfactory coding. With electrophysiological recordings from the locust and a large-scale biophysical model, we analyzed the properties and functions of the giant GABAergic neuron (GGN), a unique neuron that plays a central role in structuring olfactory codes in the locust brain (Figure 1). Analysis of our *in vivo* recordings and simulations of our model of the olfactory network suggest that the GGN extends the dynamic range of Kenyon cells (high-order neurons in a brain area analogous to the vertebrate piriform cortex, which fire spikes when the animal is presented with an odor pulse), which leads us to predict the existence of a yet undiscovered olfactory pathway. Our analysis of GGN-intrinsic properties, inputs, and outputs, *in vivo* and *in silico*, reveals basic new features of this critical neuron and the olfactory network that surrounds it. Together, results of our *in vivo* recordings and large-scale realistic computational modeling provide a more complete understanding of how different parts of the olfactory system interact.

Spatiotemporal coding of individual chemicals by the gustatory system

Four of the five major sensory systems (vision, olfaction, somatosensation, and audition) are thought to be encoded by spatiotemporal patterns of neural activity. The exception is gustation. Gustatory coding by the nervous system is thought to be relatively simple, i.e., every chemical ('tastant') is associated with one of a small number of basic tastes, and the presence of a basic taste, rather than the specific tastant, is represented by the brain. In mammals as well as insects, five basic tastes are usually recognized: sweet, salty, sour, bitter, and umami. The neural mechanism for representing basic tastes is unclear. The most widely accepted postulate is that, in both mammals and insects, gustatory information is carried through labeled lines of cells sensitive to a single basic taste, that is, in separate channels from the periphery to sites deep in the brain. An alternative proposal is that the basic tastes are represented by populations of cells, with each cell sensitive to several basic tastes.

Testing these ideas requires determining, point-to-point, how tastes are initially represented within the population of receptor cells and how this representation is transformed as it moves to higher-order neurons. However, it has been highly challenging to deliver precisely timed tastants while recording cellular activity from directly connected cells at successive layers of the gustatory system. Using a new moth preparation, we designed a stimulus and recording system that allowed us to fully characterize the timing of tastant delivery and the dynamics of the tastant-elicited responses of gustatory receptor neurons and their monosynaptically connected second-order gustatory neurons, before, during, and after tastant delivery.

Surprisingly, we found no evidence consistent with a basic taste model of gustation. Instead, we found that the moth's gustatory system represents individual tastant chemicals as spatiotemporal patterns of activity distributed across the population of gustatory receptor neurons. We further found that the representations are transformed substantially, given that many types of gustatory receptor neurons converge broadly upon follower neurons. The results of our physiological and behavioral experiments suggest that the gustatory system encodes information not about basic taste categories but rather about the identities of individual tastants. Furthermore, the information is carried not by labeled lines but rather by distributed, spatiotemporal activity, which is a fast and accurate code. The results provide a dramatically new view of taste processing.

Argos: a toolkit for tracking multiple animals in complex visual environments

Understanding how neural mechanisms drive behaviors often requires a rigorous analysis of those behaviors, a task that can be arduous and time-consuming. Many recent software utilities can automatically track animals in homogeneous, uniformly illuminated scenes with constant backgrounds, using traditional image-processing algorithms, and recently, neural networks have improved animal tracking and pose-estimation software. However, these tools are optimized to analyze experiments in unrealistically simplified laboratory environments, for example, by using thresholding steps to detect contiguous binary objects in a video frame, and then using this information to automatically train a neural network to identify the animals. The thresholding-based detection can fail if the background changes over time or is cluttered with objects of size and contrast similar to target animals. Some multi-animal pose-estimation tools can identify individual animals even with inhomogeneous backgrounds, but such tools require either large manually annotated datasets for training or are practically limited to a few animals. We focused on the task of tracking multiple animals, especially in inhomogeneous or changing environments, without visual identification or pose estimation.

To facilitate capturing video, training neural networks, tracking several animals, and reviewing tracks, we

developed Argos, a software toolset that incorporates both classical and neural net-based methods for image processing, and provides simple graphical interfaces to control parameters used by the underlying algorithms. Argos includes tools for compressing videos based on animal movement, for generating training sets for a convolutional neural network (CNN) to detect animals, for tracking many animals in a video, and for facilitating review and correction of the tracks manually, with simple graphical user interfaces. Argos can help reduce the amount of video data to be stored and analyzed, speed up analysis, and permit analyzing difficult and ambiguous conditions in a scene. Thus, Argos supports several approaches to animal tracking suited for varying recording conditions and available computational resources. Together, these tools allow the recording and tracking of movements of many markerless animals in inhomogeneous environments over many hours. The tools thus provide many benefits to researchers.

Identification and analysis of odorant receptors expressed in two main olfactory organs: antennae and palps of a model organism, the locust *Schistocerca americana*

Olfaction allows animals to detect, identify, and discriminate among hundreds of thousands of odor molecules present in the environment. It requires a complex process to generate the high dimensional neural representations needed to characterize odorant molecules, which have different sizes, shapes and electrical charges, and are often organized into chaotic and turbulent odor plumes. Understanding the anatomical organization of the olfactory system at the cellular and molecular levels has provided important insights into the coding mechanisms underlying olfaction, and studies performed in insects have contributed substantially to our knowledge of odor processing. Further, mechanisms that allow the olfactory system to generate representations for odors have been shown to be widely conserved among very divergent species. Extending our understanding of olfaction requires knowledge of the molecular and structural organization of the olfactory system.

Odor sensing begins with olfactory receptor neurons (ORNs), which express odorant receptors (ORs). In insects, ORNs are housed, in varying numbers, in olfactory sensilla. Because the organization of ORs within sensilla affects their function, it is essential to identify the ORs they contain. Using RNA sequencing, we identified 179 putative ORs in the transcriptomes of the two main olfactory organs, antenna and palp, of the locust *Schistocerca americana*. Quantitative expression analysis showed most putative ORs (140) are expressed in antennae, while only 31 are in the palps. Further, one OR was detected only in palps, and seven are expressed differentially by sex. An *in situ* analysis of OR expression revealed at least six classes of sensilla in the antenna. A phylogenetic comparison of OR-predicted protein sequences revealed homologous relationships among two other Acrididae species. Our results provide a foundation for understanding the organization of the first stage of the olfactory system in *S. americana*, a well studied model for olfactory processing.

Additional Funding

- NICHD Career Development Award to Dr. Bo-Mi Song

Publications

1. Ray S, Aldworth Z, Stopfer M. Feedback inhibition and its control in an insect olfactory circuit. *eLife* 2020 9:e53281.

2. Gupta N, Singh SS, Stopfer M. Oscillatory integration windows in neurons. *Nat Commun* 2016 7:13808.
3. Reiter S, Campillo Rodriguez C, Sun K, Stopfer M. Spatiotemporal coding of individual chemicals by the gustatory system. *J Neurosci* 2015 35:12309–12321.
4. Ray S, Stopfer M. Argos: a toolkit for tracking multiple animals in complex visual environments. *Methods Ecol Evolution* 2022 13:3:585–595.
5. Aldworth ZN, Stopfer M. Insect neuroscience: filling the knowledge gap on gap junctions. *Curr Biol* 2022 32(9):R420–R423.

Collaborators

- Maxim Bazhenov, PhD, *Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, CA*

Contact

For more information, email stopferm@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/stopfer> or <https://dir.ninds.nih.gov/Faculty/Profile/mark-stopfer.html>.

Regulatory Small RNAs and Small Proteins

The group currently has two main interests: identification and characterization of small noncoding RNAs; and identification and characterization of small proteins of less than 50 amino acids. Both small RNAs and small proteins have been overlooked because they are not detected in biochemical assays, and the corresponding genes are missed by genome annotation and are poor targets for genetic approaches. However, both classes of small molecules are being found to have important regulatory roles in organisms ranging from bacteria to humans.

Identification and characterization of small regulatory RNAs

During the past 20 years, we have carried out several different systematic screens for small regulatory RNAs in *Escherichia coli*. The screens included computational searches for conservation of intergenic regions and direct detection after size selection or co-immunoprecipitation with RNA-binding proteins. Most recently, we have been using deep sequencing approaches to map the 5' and 3' ends of all transcripts to further extend our identification of small RNAs in a range of bacteria species [Reference 1]. The work showed that sRNAs are encoded by diverse loci, including sequences overlapping mRNAs.

A major focus for the group has been to elucidate the functions of the small RNAs that we and others identified. Early on, we showed that the OxyS RNA, whose expression is induced in response to oxidative stress, acts to repress translation through limited base-pairing with target mRNAs. We discovered that OxyS action is dependent on the Sm-like Hfq protein, which acts as a chaperone to facilitate OxyS RNA base-pairing with its target mRNAs. Follow up studies allowed us to learn more about the mechanism by which the Hfq protein facilitates base-pairing through multiple RNA binding domains [Reference 2]. We also started to explore the role of ProQ, a second RNA chaperone in *E. coli* and, by comparing the sRNA-mRNA interactomes by deep sequencing, found that ProQ and Hfq have overlapping as well as competing roles in the cell. It is likely that still other RNA binding proteins, such as KH domain proteins, are involved in small RNA-mediated regulation in bacteria [Reference 3].



Gisela Storz, PhD, Head, Section on Environmental Gene Regulation

Aixia Zhang, PhD, *Staff Scientist*

Jordan J. Aoyama, PhD, *Postdoctoral Fellow*

Aisha Burton Okala, PhD, *Postdoctoral Fellow*

Rajat Dhyani, PhD, *Postdoctoral Fellow*

Shuwen Shan, PhD, *Postdoctoral Fellow*

Narumon Thongdee, PhD, *Postdoctoral Fellow*

Lauren R. Walling, PhD, *Postdoctoral Fellow*

Rilee D. Zeinert, PhD, *Postdoctoral Fellow*

Aoshu Zhong, PhD, *Postdoctoral Fellow*

Miranda Alaniz, BS, *Postbaccalaureate Fellow*

Juwaan Douglas-Jenkins, BS, *Postbaccalaureate Fellow*

Kyle D. Rekedal, BS, *Postbaccalaureate Fellow*

Tiara D. Tillis, BA, *Postbaccalaureate Fellow*

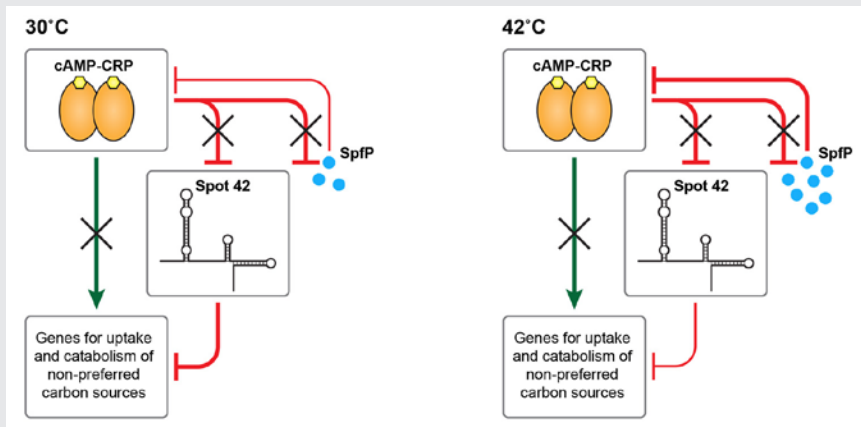


FIGURE 1. The small protein SpfP reinforces the multi-output feedforward loop between CRP and Spot 42.

For cells grown in the absence of glucose, CRP directly increases transcription of targets and represses Spot 42. When glucose is available (*shown here*), the Spot 42 RNA represses CRP-activated targets through base pairing, particularly at lower temperatures (*left*), and the small protein SpfP blocks CRP-dependent activation, particularly at higher temperature (*right*).

Hfq-binding small RNAs, which act through limited base-pairing, are integral to many different stress responses in *E. coli* and other bacteria, as well as during the interaction between bacteria and bacteriophage. For example, we showed that the Spot 42 RNA, whose levels are highest when glucose is present, plays a broad role in catabolite repression by directly repressing genes involved in central and secondary metabolism, redox balancing, and the consumption of diverse non-preferred carbon sources. Similarly, we discovered that MicL, a transcription factor Sigma(E)-dependent small RNA, which is transcribed from a promoter located within the coding sequence of the *cutC* gene, represses synthesis of the lipoprotein Lpp, the most abundant protein in the cell, to oppose membrane stress. We found that the copper-sensitivity phenotype, previously ascribed to inactivation of the *cutC* gene, is actually derived from the loss of MicL and elevated Lpp levels. Most recently, we showed that a small RNA derived from the 3' UTR (untranslated region) of the *glnA* gene, encoding glutamine synthetase, impacts *E. coli* growth under low nitrogen conditions by modulating the expression of genes that affect carbon and nitrogen flux [Reference 4]. As more and more sRNAs encoded by 5' or 3' UTRs or internal to coding sequences are being found, our observations raise the possibility that other phenotypes currently attributed to protein defects are the result of deficiencies in previously unidentified regulatory RNAs.

One interesting recent observation is that some small RNAs have dual functions in that they act by both base-pairing and by encoding a small, regulatory protein. For example, we discovered that the Spot 42 RNA also encodes a 15-amino acid protein (denoted SpfP) [Reference 5]. Overexpression of just the small protein from a Spot 42 derivative deficient in base-pairing activity, or just the base-pairing activity from a Spot 42 derivative with a stop codon mutation both prevented growth on galactose, revealing that the small protein and the small RNA impact the same pathway. Co-purification experiments showed that SpfP binds to the CRP (cyclic AMP receptor protein) transcription factor, affecting the kinetics of induction when cells are shifted from a glucose to a galactose medium. Thus, as shown in Figure 1, the small protein reinforces the feedforward loop regulated by the base-pairing activity of the Spot 42 RNA. As a second example, we found a 164-nucleotide RNA previously shown to encode a 28-amino acid protein (denoted AzuC) also base-pairs with the *cadA* and *galE* mRNAs to block expression [Reference 6]. Interestingly, AzuC translation interferes with the observed repression of *cadA* and *galE* by the RNA, and base-pairing interferes with AzuC translation, demonstrating that the translation and base-pairing functions compete. We hypothesize that many more dual-function RNAs remain to be discovered and suggest that they can be exploited to control gene expression at many levels.

We successfully constructed a functional synthetic dual-function regulator from a small protein and a small protein encoded by adjacent genes, and used this synthetic construct to study the functional organization of dual-function RNAs [Aoyama JJ, Raina M, Storz G. *J Bacteriol* 2022;204:e00345–21].

In addition to small RNAs that act via limited base pairing, we have been interested in regulatory RNAs that act by other mechanisms. For instance, early work showed that the 6S RNA binds to and modulates RNA polymerase by mimicking the structure of an open promoter. In another study, we discovered that a broadly conserved RNA structure motif, the yybP-ykoY motif, found in the 5' UTR of the *mntP* gene, which encodes a manganese exporter, directly binds manganese, resulting in a conformation that liberates the ribosome-binding site.

Further studies to characterize other Hfq- and ProQ-binding RNAs and their physiological roles and evolution as well as regulatory RNAs that act in ways other than base-pairing are ongoing.

Identification and characterization of small proteins

In our genome-wide screens for small RNAs, we found that a number of short RNAs actually encode small proteins. The correct annotation of the smallest protein genes is one of the greatest challenges of genome annotation. Further, there is limited evidence that proteins are synthesized from annotated and predicted short ORFs. Although these proteins have largely been missed, the few small proteins that have been studied in detail in bacterial and mammalian cells have been shown to have important functions in regulation, signaling, and in cellular defenses [Gray T, Storz G, Papenfort K. *J Bacteriol* 2022;204:e0034121]. We thus established a project to identify and characterize proteins of fewer than 50 amino acids.

We first used sequence conservation and ribosome binding-site models to predict genes encoding small proteins of 16–50 amino acids in the intergenic regions of the *E. coli* genome. We tested expression of these predicted, as well as previously annotated small proteins, by integrating the sequential peptide affinity tag directly upstream of the stop codon on the chromosome and assaying for synthesis, using immunoblot assays. This approach confirmed that 20 previously annotated and 18 newly discovered proteins of 16 to 50 amino acids are synthesized. We also carried out a complementary approach, based on genome-wide ribosome profiling of ribosomes arrested on start codons, to identify many additional candidates; we confirmed the synthesis of 38 of these small proteins by chromosomal tagging. Our studies, together with the work of others, documented that *E. coli* synthesizes over 150 small proteins of fewer than 50 amino acids.

Many of the initially discovered proteins were predicted to consist of a single transmembrane alpha-helix and were found to be in the inner membrane in biochemical fractionation. Interestingly, assays of topology-reporter fusions and strains with defects in membrane-insertion proteins, revealed that, despite their diminutive size, small membrane proteins display considerable diversity in topology and insertion pathways. Additionally, systematic assays for the accumulation of tagged versions of the proteins showed that many small proteins accumulate under specific growth conditions or after exposure to stress. We also generated and screened bar-coded null mutants and identified small proteins required for resistance to cell envelope stress and acid shock.

We now are using the tagged derivatives and information about synthesis and subcellular localization, and are employing many of the approaches the group has used to characterize the functions of small regulatory RNAs, to elucidate the functions of the small proteins. The combined approaches are beginning to give insights into

how the small proteins are acting in *E. coli*. We first discovered that the 49–amino acid inner-membrane protein AcrZ, whose synthesis increases in response to noxious compounds such as antibiotics and oxidizing agents, associates with the AcrAB–TolC multidrug efflux pump, which confers resistance to a wide variety of antibiotics and other compounds. Co-purification of AcrZ with AcrB, two-hybrid assays, and suppressor mutations indicate that this interaction occurs through the inner-membrane protein AcrB. Mutants lacking AcrZ are sensitive to many, but not all, the antibiotics transported by AcrAB–TolC, resulting from AcrZ effects on the conformation of the AcrB drug-binding pocket. We also found that synthesis of a 42–amino acid protein, MntS, is repressed by high levels of manganese and the MntR transcription factor. The lack of MntS leads to reduced activities of manganese-dependent enzymes under manganese-poor conditions, while overproduction of MntS leads to very high intracellular manganese and bacteriostasis under manganese-rich conditions. These and other phenotypes led us to propose that MntS modulates intracellular manganese levels, possibly by inhibiting the manganese exporter MntP. Additionally, we showed that the 31–amino acid inner-membrane protein MgtS, whose synthesis is induced by very low magnesium and the PhoPQ two-component system, acts to increase intracellular magnesium levels and maintain cell integrity upon magnesium depletion. Upon development of a functional tagged derivative of MgtS, we found that MgtS interacts with MgtA to increase the levels of this P-type ATPase magnesium transporter under magnesium-limiting conditions. Correspondingly, the effects of MgtS upon magnesium limitation are lost in an *mgtA* mutant, and MgtA overexpression can suppress the *mgtS* phenotype. MgtS stabilization of MgtA provides an additional layer of regulation of this tightly controlled magnesium transporter. Unexpectedly, we found that MgtS also interacts with and modulates the activity of a second protein, the PitA cation–phosphate symporter, to further increase intracellular magnesium levels.

A limited number of transcripts encoding both a small protein and possessing base-pairing activity, dual-function RNAs, are being identified. For example, the 109–nucleotide Spot 42 RNA, which is one of the best characterized base-pairing small RNAs (sRNAs) in *E. coli* encodes a 15–amino acid protein (denoted SpfP). As mentioned above, overexpression of just the Spot 42 base-pairing activity or just the SpfP small protein prevented growth on galactose, indicating that the sRNA and protein impact the same pathway [Reference 5]. SpfP binding to CRP blocks the ability of the transcription factor to activate specific genes, reinforcing the feedforward loop regulated by the base-pairing activity of the Spot 42 RNA (Figure 1). A second example is the 164–nucleotide RNA previously shown to encode a 28–amino acid, amphipathic-helix protein (denoted AzuC). We discovered that the membrane-associated AzuC protein interacts with GlpD, the aerobic glycerol-3-phosphate dehydrogenase, and increases dehydrogenase activity [Reference 6]. The observations that an overexpression defect was still observed for a stop-codon mutant derivative and that the RNA (denoted AzuR) can also base-pair with the *cadA* and *galE* mRNAs documented that AzuCR is a dual-function RNA. Interestingly, the MgtS protein mentioned above is encoded divergent from the MgrR small regulatory RNA, which is also important for bacterial adaptation to low magnesium. To investigate the competition between protein-coding and base-pairing activities, we constructed synthetic dual-function RNAs comprising MgrR and MgtS [Aoyama JJ, Raina M, Storz G. *J Bacteriol* 2022;204:e00345–21]. The constructs allowed us to probe how the organization of the coding and base-pairing sequences and the distance between the two components contribute to the proper function of both activities of a dual-function RNA. By understanding the features of natural and synthetic dual-function RNAs, future synthetic molecules can be designed to maximize their regulatory impact.

Our work, together with our ongoing studies of other small proteins and related findings by others in eukaryotic cells, supports our hypothesis that small proteins are an overlooked class of proteins.

Additional Funding

- NICHD Early Career Award
- NIGMS Postdoctoral Research Associate (PRAT) Program

Publications

1. Adams PP, Baniulyte G, Esnault C, Chegireddy K, Singh N, Monge M, Dale RK, Storz G, Wade JT. Regulatory roles of Escherichia coli 5' UTR and ORF-internal RNAs detected by 3' end mapping. *eLife* 2021 10:e62438.
2. Kavita K, Zhang A, Tai CH, Majdalani N, Storz G, Gottesman S. Multiple in vivo roles for the C-terminal domain of the RNA chaperone Hfq. *Nucleic Acids Res* 2022 50:1718–1733.
3. Olejniczak M, Jiang X, Basczok MM, Storz G. KH-domain proteins: another family of bacterial RNA matchmakers? *Mol Microbiol* 2022 117:10-19.
4. Walling LR, Kouse AB, Shabalina SA, Zhang H, Storz G. A 3' UTR-derived small RNA connecting nitrogen and carbon metabolism in enteric bacteria. *Nucleic Acids Res* 2022 50:10093–10109.
5. Aoyama JJ, Raina M, Zhong A, Storz G. Dual-function Spot 42 RNA encodes a 15-amino acid protein that regulates the CRP transcription factor. *Proc Natl Acad Sci USA* 2022 119:e2119866119.
6. Raina M, Aoyama JJ, Bhatt S, Paul BJ, Zhang A, Updegrove TB, Miranda-Ríos J, Storz G. Dual-function AzuCR RNA modulates carbon metabolism. *Proc Natl Acad Sci USA* 2022 119:e2117930119.

Collaborators

- Shantanu Bhatt, PhD, *Department of Biology, Saint Joseph's University, Philadelphia, PA*
- Ryan K. Dale, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*
- Caroline Esnault, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*
- Susan Gottesman, PhD, *Laboratory of Molecular Biology, Center for Cancer Research, NCI, Bethesda, MD*
- Todd Gray, PhD, *Wadsworth Center, New York State Department of Health, Albany, NY*
- Xiaofang Jiang, PhD, *National Library of Medicine, NIH, Bethesda, MD*
- Nadim Majdalani, PhD, *Laboratory of Molecular Biology, Center for Cancer Research, NCI, Bethesda, MD*
- Juan Miranda-Rios, PhD, *Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico*
- Mikolaj Olejniczak, PhD, *Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland*
- Kai Papenfort, PhD, *Institute of Microbiology, Friedrich-Schiller-Universität, Jena, Germany*
- Brian J. Paul, PhD, *International Flavors & Fragrances Inc., Wilmington, DE*
- Svetlana A. Shabalina, PhD, *National Library of Medicine, NIH, Bethesda, MD*
- Chin-Hsien Tai, PhD, *Laboratory of Molecular Biology, Center for Cancer Research, NCI, Bethesda, MD*
- Taylor B. Updegrove, PhD, *Laboratory of Molecular Biology, Center for Cancer Research, NCI, Bethesda, MD*
- Joseph T. Wade, PhD, *Wadsworth Center, New York State Department of Health, Albany, NY*
- Henry Zhang, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*

Contact

For more information, email storzg@mail.nih.gov or visit <https://storz.nichd.nih.gov>.

Evaluation of Hypothalamic and Pituitary Disorders

The goal of our research is to phenotype patients with rare hypothalamic and pituitary disorders and to identify biomarkers for disease diagnosis and of presentation and progression. We are also continuing to investigate the genetic mechanisms underlying pituitary tumorigenesis, which, along with novel biomarkers, will ultimately provide new therapeutic targets.

Phenotyping of patients with hypothalamic and pituitary disorders

Hypothalamic and pituitary tumors are rare disorders, which may present with hormone excess or hormone deficiencies. Hormone dysregulation or mass effects may lead to various complications. For example, patients with hypercortisolemia manifest with, among other, growth stagnation, obesity, and hyperglycemia. Under the protocol [97-CH-0076](#), we are evaluating patients with pituitary disorders/adenomas/tumors. We are collecting clinical and biochemical data, as well as research biospecimens, to advance our knowledge of the genetic etiology, pathophysiology, presentation, diagnosis, and treatment of pituitary disorders. Previous results from this protocol have assisted in evaluation and management of these patients.

Molecular genetics of pituitary disorders

We continue our investigation of the genetic mechanisms of pituitary tumorigenesis. Previously, in collaboration with Constantine Stratakis, we reported that chromosomal aberrations may lead to more aggressive behavior of pituitary tumors. We are currently offering genetic testing for all patients with proven pituitary tumors, in a collaboration with the National Institute of Allergy and Infectious Diseases (NIAID). Additionally, we continue to pursue studies to understand novel candidate gene function and effect in pituitary tumors.

Survivors of pediatric Cushing's disease

Meg Keil is leading a research protocol on the long-term effects of pediatric hypercortisolemia ([19-CH-0051](#)) to identify residual clinical and biochemical findings after cure of pediatric Cushing's syndrome. Patients are eligible for the protocol if they were diagnosed with ACTH-secreting pituitary adenomas (Cushing's disease) before the



Christina Tatsi, MD, MHSc, PhD,
Head, Unit on Hypothalamic and Pituitary Disorders

Meg Keil, CRNP, *Nurse Practitioner*

Lola Saidkhodjaeva, RN, BSN, CMSRN, *Research Nurse*

Samah Agabein, MBBS, CCRC, *Research Coordinator*

Matthew Nguyen, BA, *Postbaccalaureate Fellow*

Jessica Weinberg, BA, *Postbaccalaureate Fellow*

Meghan Waters, BA, *Lab Technician*

age of 21 and are in biochemical remission. Patients are studied at five-year intervals until 20 years after cure. Anthropometric, clinical, biochemical, imaging, and other data are collected.

Immunologic effects of hypercortisolemia

Glucocorticoids have many effects on the immune system, which makes them one of the most commonly prescribed medications. Patients with ACTH-secreting pituitary adenomas present with endogenous hypercortisolemia and are a model for the evaluation of the immune effects of glucocorticoids, especially in the pediatric population, where other confounding co-existing morbidities are rare. We started our investigation on the immune effects of hypercortisolemia by looking at the complete blood count (CBC) of these patients, and we reported that simple inflammatory biomarkers can be used for the diagnosis of these patients. We further collaborated with Sergio Rosenzweig's group to describe specific cytokine patterns that may be involved in the pathophysiology of glucocorticoid-related immunosuppression. Luis Franco's group is currently conducting a study to further characterize the immune phenotype of our patients.

Therapeutic approaches for patients with rare sellar/suprasellar disorders

Gigantism is a condition characterized by excessive growth hormone (GH) secretion, which is commonly caused by GH-secreting pituitary adenomas, or by other rare disorders of dysregulated GH secretion. Although surgery remains the first line of treatment for these patients, up to 50% of patients will not achieve remission and will require further medical treatment or radiation. Currently, all therapeutic options we have for children derive from studies in adults and selected case reports. Under the protocol [19-CH-0071](#), we study the safety and efficacy of pegvisomant, a GH-receptor antagonist, in children and adolescents with growth hormone excess, a protocol that is open to enrolment.

Publications

1. Wurth R, Rescigno M, Flippo C, Stratakis CA, Tatsi C. Inflammatory biomarkers in the evaluation of pediatric endogenous Cushing syndrome. *Eur J Endocrinol* 2022 186(4):503–510.
2. Tatsi C, Bompou ME, Flippo C, Keil M, Chittiboina P, Stratakis CA. Paediatric patients with Cushing disease and negative pituitary MRI have a higher risk of nonremission after transsphenoidal surgery. *Clin Endocrinol (Oxf)* 2021 95:856–862.
3. Tatsi C, Saidkhodjaeva L, Flippo C, Stratakis CA. Subclinical hemorrhage of ACTH-secreting pituitary adenomas in children and adolescents changes their biochemical profile. *J Endocr Soc* 2022 6:bvac080.

Collaborators

- Prashant Chittiboina, MD, *Neurosurgery Unit for Pituitary and Inheritable Diseases, NINDS, Bethesda, MD*
- Ryan Dale, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*
- Luis M. Franco, MD, *Functional Immunogenomics Section, NIAMS, Bethesda, MD*
- Jamie Morton, PhD, *Biostatistics and Bioinformatics Branch, NICHD, Bethesda, MD*
- Sergio D. Rosenzweig, MD, PhD, *Department of Laboratory Medicine, NIH Clinical Center, Bethesda, MD*
- Constantine A. Stratakis, MD, D(med)Sci, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Magdalena A. Walkiewicz-Yvon, PhD, *Division of Intramural Research, NIAID, Bethesda, MD*

Contact

For more information, email christina.tatsi3@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/tatsi>.

Point of Care and Wearable Biophotonics for Characterizing Tissue Composition and Metabolism

By advancing models, methods, and devices that utilize the interaction of light with biological tissue, we strive to develop non-invasive techniques that can help guide therapy and aid in clinical decision making. The techniques are used to perform real-time quantitative measurements of clinically relevant information, including tissue blood flow, oxygen extraction, and body/tissue composition. Our research seeks to move such technologies from 'bench to bedside,' where they can be applied to clinical problems, including vascular and metabolic diseases.

Optical characterization of vascular health in sickle cell disease

Sickle cell disease (SCD) is an inherited hemoglobinopathy that disproportionately impacts minority populations in the United States and affects about 100,000 individuals. The substitution of valine by glutamic acid on the 6th amino acid of beta globin results in an abnormal hemoglobin variant (HbS), which polymerizes once deoxygenated and alters both the structure and function of red blood cells (RBCs), distorting them into a 'sickle' shape. Sickled RBCs have $\frac{1}{6}$ th of the lifespan of normal RBCs, resulting in chronic hemolytic anemia; they are also rigid and can obstruct microvascular blood flow, causing recurring, unpredictable cycles of acute vaso-occlusive pain, commonly referred to as vaso-occlusive crises (VOC). Recurrent VOCs lead to hypoxia-reperfusion injury and, together with the intravascular hemolysis, promote inflammation and vascular endothelial damage. The systemic vasculopathy affects many organs, leading to cardiovascular complications, chronic pain, and cerebral and kidney impairment. Given that current treatment options are not universally effective, there is a significant, unmet need for new technologies that can quantitatively characterize SCD physiology and provide new insights for optimizing therapeutic impact in SCD patients.

Given the impairments to microvascular flow and endothelial dysfunction associated with SCD, advanced quantitative NIRS (near-infrared spectroscopy) is an attractive candidate to provide comprehensive hemodynamic evaluations in point-of-care settings. We are optically characterizing tissue composition, metabolism, and perfusion in several SCD studies, led by our collaborators Swee Lay Thein, Arun Shet, and Courtney Fitzhugh:



Bruce Tromberg, PhD, *Head, Section on Biomedical Optics, Director of the National Institute of Biomedical Imaging and Bioengineering*

Timothy Quang, PhD, *Staff Scientist*

Brian Hill, MS, *Biomedical Engineer*

Yun He, PhD, *Visiting Fellow*

Elise Berning, BS, *Postbaccalaureate Fellow*

Sully Chen, BS, *Postbaccalaureate Fellow*

Careniena Opem, BS, *Postbaccalaureate Fellow*

Eric Gallagher, *Student Fellow*

Kathryn Jaroszynski, *Student Fellow*

- [NCT04610866](#) – Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of Long-term Mitapivat Dosing in Subjects With Stable Sickle Cell Disease: An Extension of a Phase I Pilot Study of Mitapivat
- [NCT05604547](#) – Exploring Near Infrared Spectroscopy (NIRS) Technologies for Assessment of Muscle Physiology, Tissue Oxygenation, and Blood Flow in Patients With Sickle Cell Disease (SCD)
- [NCT04514510](#) – Fixed Dose Flavonoid Isoquercetin on Thrombo-Inflammatory Biomarkers in Subjects With Stable Sickle Cell Disease
- [NCT05213572](#) – Observational Study to Deeply Phenotype Major Organs in Sickle Cell Disease After Curative Therapies

Our group is assessing the sensitivity of various optical devices to hemodynamic changes induced by SCD treatments and evaluating whether the changes correlate with what is observed from blood chemistry. Our latest preliminary findings are noted in Figures 1 and 2.

Development of a wearable point-of-care monitoring device for pediatric obstructive sleep apnea

Obstructive sleep apnea (OSA) is the most common type of sleep apnea, in which the blockage of the airway causes the patient to stop breathing involuntarily for ten seconds or more throughout the night during sleep. When breathing stops, the oxygen level in the blood falls, sometimes waking the sleeper. Pediatric obstructive sleep apnea (POSA) can be particularly concerning, with several associated morbidities, which can have long-term effects extending into adulthood, including adverse changes in cardiovascular, metabolic, and developmental health. Onset usually occurs between ages 2–8, as tonsils reach their peak growth. Unfortunately, pediatric OSA remains largely under-diagnosed because of a lack of education about symptoms and the limited availability of sleep-medicine physicians. Early diagnosis and treatment are imperative for preventing many of these morbidities.

FIGURE 1. Average brain (pink) and forearm (blue) StO_2 values compared with average blood Hb level over the first three months of a Mitapivat study (n = 9 pts)

Comparison of the mean blood hemoglobin (Hb) level acquired from blood draws to the mean forehead StO_2 and mean forearm StO_2 , measured by time-domain near-infrared spectroscopy (TD-NIRS) across the first three months of treatment. We calculated optical hemodynamic metrics such as mean resting baseline concentrations of $[O_2Hb]$, $[HHb]$, and $[THb]$ as well as tissue oxygen saturation (StO_2) in the forearm and forehead for all 45 measurement visits; one visit was excluded as the result of poor data quality. On average, forehead StO_2 acquired by TD-NIRS trended similarly to the Hb level acquired by blood draws over the first three months. We observed that 7 of 9 patients exhibited a sustained increase in blood Hb compared with baseline; the mean Hb increase for all patients compared with baseline was 1.2 g/dL at the end of three months. Similarly, forehead StO_2 also showed a similar, sustained increase. The mean forehead StO_2 increase after three months over baseline was 4.0%.

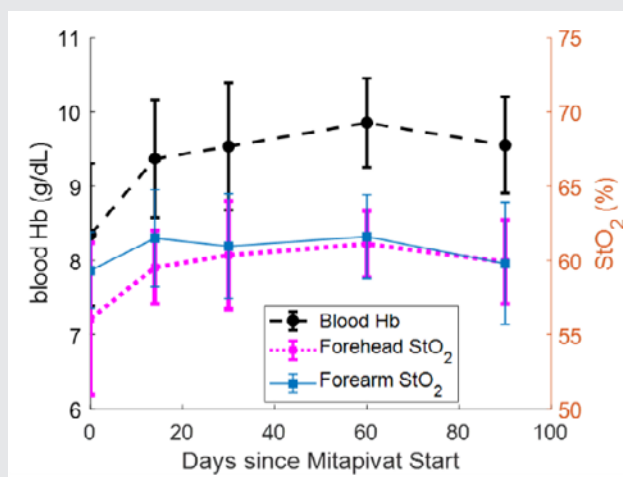
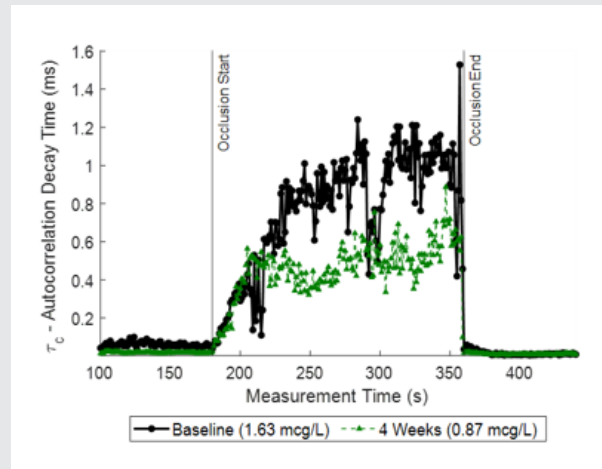


FIGURE 2. Change in autocorrelation decay time during brachial cuff occlusion during baseline and four-week follow-up visits for a single Isoquercetin patient; D-dimer levels are noted in the legend.

Change in autocorrelation decay time (τ) during the entire occlusion measurement at the baseline (*black*) and four-week follow-up (*green*) visits for one patient. The decay time τ is recovered using diffuse correlation spectroscopy (DCS) and reflects the dynamic properties of the blood. We can observe a reduction in τ (1.01 ms vs 0.57 ms), which corresponds with a reduction in D-dimer levels from 1.63 $\mu\text{g}/\text{mL}$ to 0.87 $\mu\text{g}/\text{mL}$. Additionally, we observe that τ plateaus faster at the follow-up visit than at the baseline visit.

In this preliminary analysis of 10 patients with baseline and follow-up visits, we found agreement between the mean τ and slope to D-dimer levels. Three patients showed opposing trends in τ and d-dimer levels and one patient showed a reduction in τ and no change in d-dimer levels. Further analysis will evaluate whether measures of vascular reactivity correlate with biomarker of endothelial activity such as VCAM-1 (vascular cell adhesion protein-1).



Additionally, the mechanisms of OSA that explain OSA-related outcome measures involving brain health and degeneration are still largely unknown. Approximately 50% of patients with OSA are excessively sleepy during the day, and many develop cardiovascular disease, cerebrovascular disease, and/or cognitive impairment, particularly if untreated. Traditional OSA measures do not predict sleepiness, and the apnea-hypopnea index (AHI) and oxygen desaturation index (ODI), measured through standard pulse oximetry, do not typically explain health outcomes, although both hypoxemia and sleep fragmentation are mildly associated.

NIRS is a non-invasive technology that is well suited for measuring cerebral hemodynamics during sleep. NIRS uses near-infrared light to penetrate human tissue and measure changes in oxygenation. There is extensive literature documenting the utility of NIRS in sleep studies of both normal and sleep-disordered breathing (SDB). In normal sleep, NIRS has been used to investigate cerebral hemodynamics in sleep stages and transitions between stages. For sleep apnea applications, NIRS can detect drops in cerebral oxygenation, as well as respiration, heart rate, and blood flow changes that occur due to apnea. Previous work in NIRS sleep analysis showed that transient rises in cerebral deoxyhemoglobin are prominent feature of apneas and hypopneas, and autoregulatory mechanisms have been shown to fail in preventing hypoxia during severe obstructive events. NIRS is uniquely suited to capture such breathing-induced cerebrovascular disturbances with high sensitivity and temporal resolution.

[NCT05052216](#) – DEVELOPMENT OF A WEARABLE POINT OF CARE MONITORING DEVICE FOR PEDIATRIC OBSTRUCTIVE SLEEP APNEA

In collaboration with Ashura Buckley and the NIMH Sleep and Neurodevelopment Service (SNS), we are currently enrolling children aged 3–8 to investigate how optical signals change throughout sleep by comparing physiological signals derived from NIRS with data from polysomnography. Tissue hemodynamics of a healthy child during a pause in breathing are shown in Figure 3.

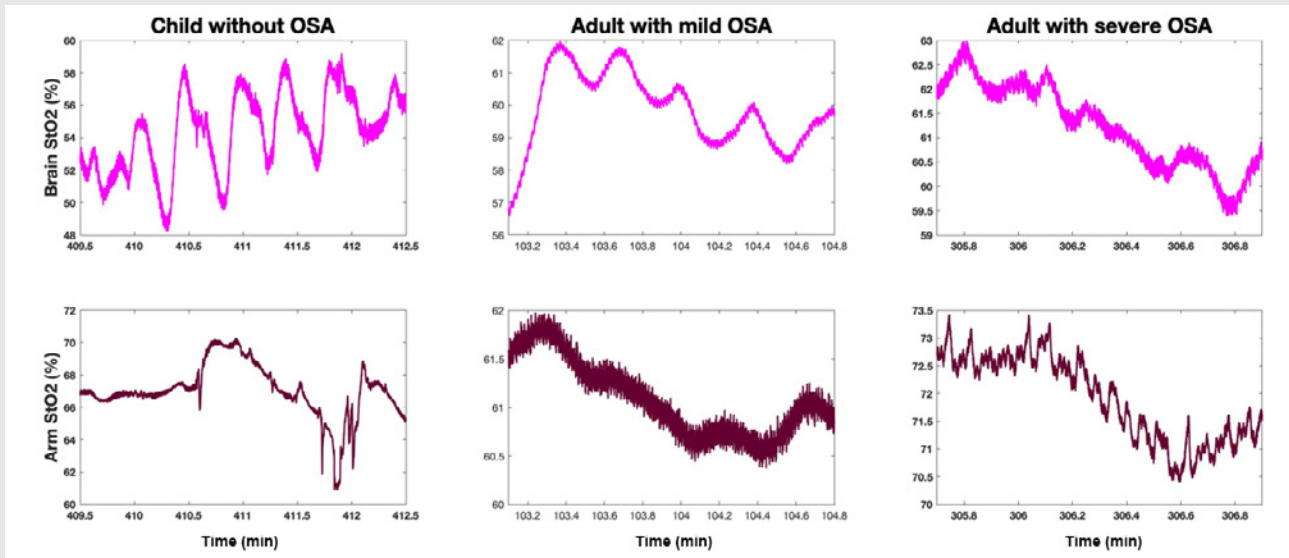


FIGURE 3. Change in arm and cerebral tissue oxygenation during a breathing stoppage for a healthy child, adult with mild OSA, and adult with severe OSA

Comparison of hemodynamic response during sleep to a breathing stoppage for a healthy child, adult with mild OSA, and adult with severe OSA. While response in the arm is similar across the three cases, with tissue oxygenation dropping shortly after the pause in breathing, there is a stark difference in the cerebral hemodynamic response. While adults with OSA also see a drop in cerebral oxygen saturation following the breathing stoppage, no net reduction is seen in the healthy child. The results suggest that OSA leads to impaired autoregulatory mechanisms, mechanisms that serve to protect the brain from hypoxia.

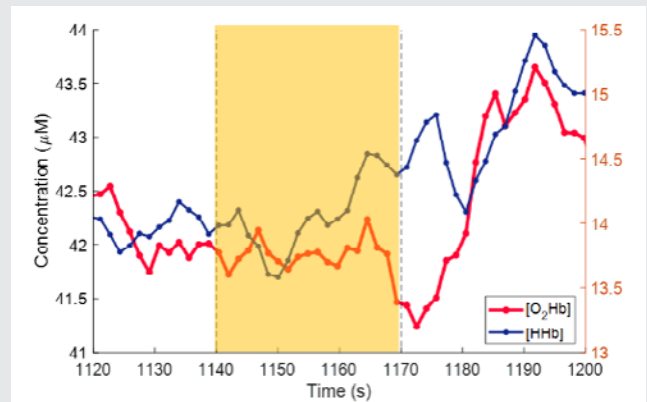
Vascular diseases driven by genetic alterations and COVID infection

The study and management of diseases with unknown vascular phenotypes is challenging. Given the importance of the vascular network to every organ system, understanding the extent and severity of vascular complications is necessary in order to develop effective treatments. Monogenic vascular diseases are characterized by a single genetic mutation, which can have deleterious effects on protein structure, function, or synthesis, and can be associated with severe complications, with high mortality and morbidity rates. While each individual disease is rare, these diseases can encompass a wide array of vascular phenotypes affecting any part of the body. Infectious diseases are another pathway for vascular complications, which can either manifest directly by viral/bacterial infection of the endothelial lining or indirectly as a result of damage triggered by inflammatory responses to the pathogen.

For this project, we aim to develop multi-modal NIRS assessment methods that are sensitive to the hemodynamic impairments of two patient cohorts: (1) patients with rare monogenic vascular disease; and (2) patients recovering from COVID-19. This consists of the selection of both the appropriate optical modality and measurement challenge to characterize the afflicted area. Our goal is to perform comprehensive clinical evaluations of patients recruited into the study, in order to better understand the disease pathology, heterogeneity of symptoms within the disease population, and progression of the various, rare vascular diseases over time. In collaboration with the Translational Vascular Medicine Branch (TVMB) at the NHLBI,

FIGURE 4. TD-NIRS trace of [O₂Hb] and [HHb] concentrations of a CADASIL patient during a breath hold

Trace of the [O₂Hb] (red) and [HHb] (blue) concentrations in the forehead and forearm of a CADASIL patient during a breath hold (highlighted in yellow). During each breath hold, there does not appear to be a discernable increase in [O₂Hb]. Three breath holds were performed for each patient for a total of nine breath holds across the three patients recruited. The median slope of [O₂Hb] during the breath hold period was $0.0029 \pm 0.06 \mu\text{M/s}$. Previous literature evaluating NIRS as a candidate for assessing cerebrovascular reactivity reports a gradual increase in [O₂Hb] during a breath hold. This lack of a cerebrovascular response can be observed in the other two patients in the cohort. Prior literature has suggested that cerebrovascular reactivity could be a predictive test of disease progression. To date, three patients have been enrolled in the study.



three specific disease cohorts will be explored: ACDC (arterial calcification due to CD73 deficiency), CADASIL (cerebral autosomal dominant arteriopathy and leukoencephalopathy), and COVID-19.

[NCT03538639](#) – VASCULAR DISEASE DISCOVERY PROTOCOL

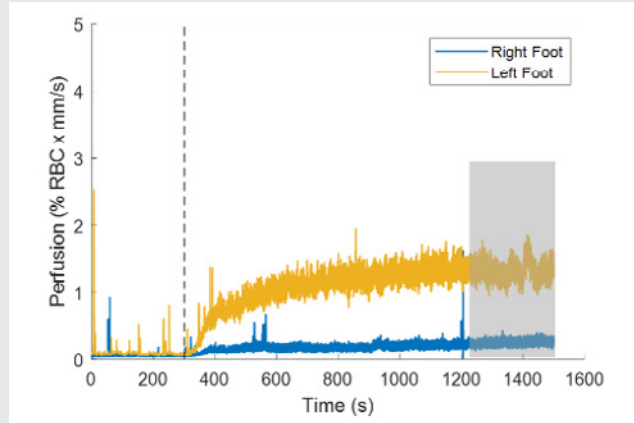
ACDC is characterized by progressive vascular calcification, typically affecting arteries of the lower limbs, and manifests clinically as debilitating lower extremity pain resulting from the lower limb claudication and ischemia. For the ACDC cohort, we will evaluate vascular reactivity and endothelial function in the lower limbs using a hyperthermia challenge with an optical probe combining diffuse reflectance spectroscopy (DRS) and laser doppler flowmetry (LDF) (Perimed AB, Sweden) to evaluate vascular reactivity in skin. The probe measures hemodynamic parameters such as hemoglobin (Hb) concentration, tissue oxygen saturation (StO₂), and tissue perfusion. Measures of vascular reactivity and endothelial function in the lower limbs can provide a rapid and direct method for assessing the extent of severity. To date, three patients have been recruited for the study.

[NCT05072483](#) – NATURAL HISTORY STUDY OF CADASIL

CADASIL is a monogenic small-vessel disease that affects the arteries in the brain. Clinical manifestations include migraines, recurrent strokes, and progressive white matter degeneration. Current diagnosis for CADASIL is done through MRI imaging of the white matter, genetic testing, or through family history. Previous literature reported impaired cerebrovascular reactivity detected by MRI and transcranial doppler. NIRS is an attractive alternative method for assessing cerebrovascular reactivity because of its compact size and penetration depth. For the CADASIL cohort, optical probes will be affixed to the forehead and forearm to acquire information from the prefrontal cortex and skeletal muscle respectively. The optical measurement consists of a five-minute baseline period followed by a five-minute brachial cuff occlusion and a five-minute recovery period. A series of three end-exhalation breath holds follow the recovery period; patients will be asked to exhale and hold their breaths for either 30 seconds or for as long as they can tolerate. We anticipate that these assessments of cerebrovascular compared with skeletal muscle reactivity could provide a predictive test of disease progression. To date, five patients have been enrolled in the study.

FIGURE 5. Perfusion on the right (*blue*) and left (*yellow*) foot of a patient with ACDC during a thermal hyperemia challenge. Black dotted line denotes when probe temperature was increased to 44°C.

Total perfusion measured on both feet from a patient with ACDC. Following the baseline period, the perfusion response can be observed in two phases. The first phase is a rapid increase within the first few minutes of the temperature rise, followed by a more gradual increase that eventually plateaus towards the end of the measurement. We define a mean perfusion level during the last five minutes of the measurement in the plateau region as the plateau perfusion. We compared the plateau perfusion in the ACDC cohort with previous literature, which compared the plateau perfusion in a patient with diabetes with age-matched healthy volunteers. Plateau perfusion responses in the ACDC cohort ($n = 3$, $0.91 \pm 0.50\%$ RBC \times mm/s) were more similar in magnitude to the diabetic cohort ($1.09 \pm 0.50\%$ RBC \times mm/s) than the healthy volunteer cohort ($1.60 \pm 0.98\%$ RBC \times mm/s). To date, three patients have been recruited for the study, and additional patients with ACDC are anticipated.



[NCT04595773 - COVID-19 - CHRONIC ADAPTATION AND RESPONSE TO EXERCISE \(COVID-CARE\): A RANDOMIZED CONTROLLED TRIAL](#)

While COVID-19 is mainly perceived as a respiratory illness, there is also significant evidence of vascular complications, especially in those with severe symptoms and long-term effects. In collaboration with NHLBI's TVMB and the NIH Clinical Center's Rehabilitation Medicine Department, we have performed optical hemodynamic assessments in a cohort of patients recovering from COVID. The measurements are one component of a larger randomized clinical study evaluating the efficacy of targeted exercise intervention to improve recovery from COVID. Patients recruited for this study are randomized into either a control or treatment arm. Patients in the treatment arm undergo a ten-week exercise regimen after their baseline visit, followed by a follow-up visit. Patients in the control arm continue with their typical daily routine for ten weeks after the baseline visit and return for a follow-up visit; patients then begin the ten-week exercise regimen followed by another follow-up visit. Patients recruited into the study undergo a brachial cuff occlusion and also participate in a set of breath holding exercises. In a separate measurement session, patients undergo an exercise test during which optical measures are acquired before and after exercise. As the study is ongoing, analysis is currently limited to comparing optical measures to established clinical metrics. We anticipate that a more comprehensive analysis will be performed upon conclusion of the study.

Continuous blood pressure and vascular monitoring technologies for optimizing maternal health

Pre-eclampsia is a pregnancy-specific disorder responsible for more than 70,000 maternal deaths and 500,000 fetal deaths worldwide every year. While high blood pressure is the main clinical phenotype, pre-eclampsia can trigger several hemodynamic changes, including increased vascular stiffness and reduced vascular reactivity. While delivery can resolve most symptoms, they can persist postpartum and continue to impact the mother. Early diagnosis combined with treatment remains a key component to improving patient outcomes. However, the transition from gestational hypertension to mild pre-eclampsia to severe pre-eclampsia is a dynamic process; early diagnosis would ideally necessitate continuous, heightened surveillance. There is a need for

technologies that can easily monitor the hemodynamic state of the mother and aid in the detection and risk stratification of pre-eclampsia.

Optical spectroscopic techniques (i.e., NIRS) can provide a broad, point-of-care hemodynamic evaluation by continuously measuring hemodynamic parameters such as vascular reactivity, blood flow, and pulse transit time. There are a limited number of studies evaluating NIRS technologies in pre-eclampsia; Guerchi *et al.* [*Crit Care Med* 2014;42:2379] measured significantly impaired baseline cerebral oxygenation in patients with severe pre-eclampsia and demonstrated the utility of NIRS in monitoring cerebral oxygen saturation during the administration of magnesium sulfate (MgSO₄). A combination of ECG and photoplethysmography (PPG) has been proposed to measure pulse transit time (PTT), the time needed for an arterial pressure wave to pass between two sites. PTT is inversely related to blood pressure and is a potential approach for continuous and cuff-free blood pressure monitoring.

Affixed transmission speckle analysis (ATSA), introduced by our lab in 2018, is a promising candidate to measure blood flow, vascular stiffness, and pulse transit time in a compact form-factor. To recover blood flow information, ATSA sends coherent light through a peripheral digit to measure intensity fluctuations caused by moving red blood cells. The high acquisition speed of ATSA enables the recovery of a pulsatile waveform originating from blood flow, referred to as the speckle plethysmograph (SPG). While previous work with pulse transit time evaluated the feasibility of PPG-based approaches, the SPG offers better signal quality than the PPG, with SPG estimations of heart rate variability having improved accuracy over PPG estimations.

We are developing a compact, multi-modal optical platform that provides continuous, comprehensive hemodynamic assessments at the bedside. The sensor would both acquire information pertaining to cerebral oxygenation as well as PTT derived from the SPG signal. We are first developing a method for calculating PTT with the SPG signal and comparing its ability to estimate BP to PTT calculated with a PPG signal.

In collaboration with [Roberto Romero](#), we recently conducted a pilot study to compare hemodynamic differences between patients with pregnancy complications and patients without complications. Additionally, we monitored a set of patients with pregnancy complications undergoing treatment for their symptoms. These data could help to distinguish patients with preeclampsia from normal physiological variation in healthy controls. The optimal NIRS technique can then be adopted and applied for disease monitoring in pre-eclampsia. Analysis of this study is now under way.

Publications

1. Warren RV, Bar-Yoseph R, Hill B, Reilly D, Chiu A, Radom-Aizik S, Cooper DM, Tromberg BJ. Diffuse optical spectroscopic method for tissue and body composition assessment. *J Biomed Opt* 2022 27:6.

Collaborators

- Ruth Benca, MD, PhD, *Wake Forest School of Medicine, Winston-Salem, NC*
- Manfred Boehm, MD, *Laboratory of Cardiovascular Regenerative Medicine, NHLBI, Bethesda, MD*
- Ashura Buckley, MD, *Office of the Clinical Director, NIMH, Bethesda, MD*
- Leighton Chan, MD, MPH, *Rehabilitation Medicine, Clinical Center, NIH, Bethesda, MD*

- Courtney Fitzhugh, MD, *Cellular and Molecular Therapeutics Branch, NHLBI, Bethesda, MD*
- Ingemar Frederiksson, PhD, *Perimed Instruments, Järfälla-Stockholm, Sweden*
- Amir Gandjbakhche, PhD, *Section on Translational Biophotonics, NICHD, Bethesda, MD*
- Ramy Khayat, MD, *School of Medicine, University of California, Irvine, CA*
- Thomas J. Pohida, MS, *Instrumentation Development and Engineering Application Solutions, NIBIB, Bethesda, MD*
- Forbes Porter, MD, *Office of the Clinical Director, NICHD, Bethesda, MD*
- Randall Pursley, *Instrumentation Development and Engineering Application Solutions, NIBIB, Bethesda, MD*
- Samar Rahhal, MD, *Office of the Clinical Director, NICHD, Bethesda, MD*
- Roberto Romero, MD, DMedSci, *Perinatology Research Branch, NICHD, Detroit, MI*
- Adam Schiffenbauer, MD, *Clinical Research Branch, NIEHS, Bethesda, MD*
- Arun Shet, MD, PhD, *Sickle Cell Branch, NHLBI, Bethesda, MD*
- Hiroaki Suzuki, PhD, *Hamamatsu Photonics K.K., Hamamatsu, Japan*
- Swee Lay Thein, MB, FRCP, FRCPATH, DSc, *Sickle Cell Branch, NHLBI, Bethesda, MD*

Contact

For more information, email bruce.tromberg@nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/tromberg>.

Organ and Tissue Formation during Development

The major focus of the Section is to understand how the elaborate networks of blood and lymphatic vessels arise during vertebrate development. Blood vessels supply every tissue and organ with oxygen, nutrients, and cellular and humoral factors. Lymphatic vessels drain fluids and macromolecules from the interstitial spaces of tissues, returning them to the blood circulation, and they play an important role in immune responses. Our studies on the formation of blood and lymphatic vessels are of great clinical interest because of the roles that both types of vessel play in pathologies such as cancer and ischemia.

The zebrafish (*Danio rerio*), a small tropical freshwater fish, possesses a unique combination of features that make it particularly suitable for studying vessel formation. Zebrafish are genetically tractable vertebrates with externally developing, optically clear embryos, which are readily accessible to observation and experimental manipulation, features that permit observation of every vessel in the living animal and simple, rapid screening for even subtle vascular-specific defects (Figure 1). Our current studies use genetic screening, experimental analysis, and imaging to examine cues directing vascular patterning and morphogenesis, regulation of vascular integrity, assembly of the lymphatic system, and the roles of novel vascular-associated cells.

As a second major effort in addition to our work on vessel development, we are pursuing studies on the role of epigenetics during early development, in particular how DNA methylation and other epigenetic mechanisms help coordinate cell, tissue, and organ specification and differentiation, using a novel 'EpiTag' epigenetic reporter line and the first large-scale genetic screen for tissue-specific epigenetic regulators in a vertebrate organism.

Specification and patterning of developing blood vessels

We are working to elucidate the cellular and molecular mechanisms responsible for the specification, patterning, and differentiation of blood vessels during development. Blood vessels are ubiquitous and vital components of vertebrate animals, innervating and supplying every tissue and organ with oxygen and nutrients. Many of the recent insights into mechanisms of blood vessel formation have come from



Brant M. Weinstein, PhD,
*Head, Section on Vertebrate
Organogenesis*

Van Pham, BS, *Scientific Technician*

Leah Greenspan, PhD,
Postdoctoral Fellow

Aurora Kraus, PhD, *Postdoctoral
Fellow*

Miranda Marvel, PhD,
Postdoctoral Fellow

Jong Park, PhD, *Postdoctoral
Fellow*

Laura Pillay, PhD, *Postdoctoral
Fellow*

Kiyohito Taimatsu, PhD,
Postdoctoral Fellow

Marina Venero Galanternik, PhD,
Postdoctoral Fellow

Keith Ameyaw, BS,
Postbaccalaureate Fellow

John Prevedel, BS,
Postbaccalaureate Fellow

Yehyun Kim, BS,
Postbaccalaureate Fellow

Celia Martinez-Aceves, BS,
Postbaccalaureate Fellow

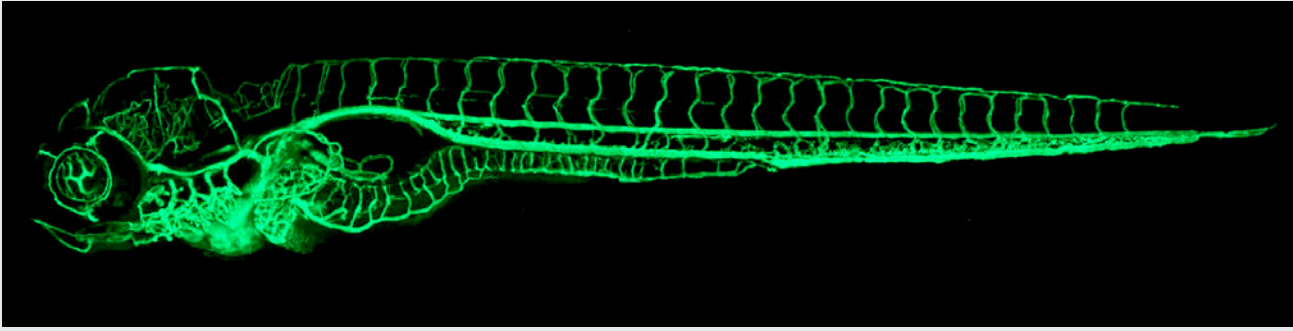


FIGURE 1. The zebrafish vascular system

Confocal micro-angiogram of the vascular system of a 4½-day-old zebrafish larva labeled by injecting fluorescent microspheres. The transparency of zebrafish larvae makes it possible to use high-resolution optical imaging methods to visualize the entire vasculature in exquisite detail.

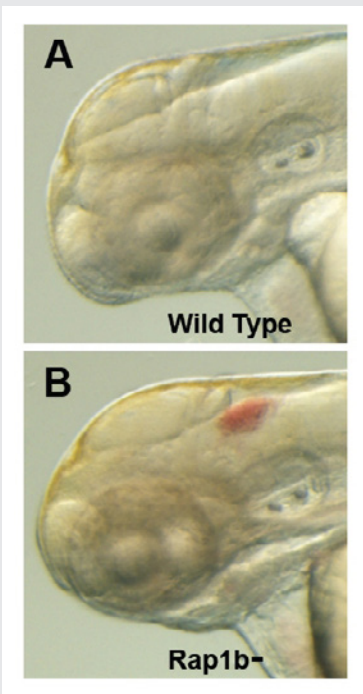


FIGURE 2. Intracranial hemorrhage (ICH) in the developing zebrafish

The clarity of zebrafish larvae also makes it straightforward to screen for animals with intracranial hemorrhage, as is evident in comparing lateral views of a 2-day-old wild-type larva (A) with a hemorrhage-prone larva deficient in *rap1b* (B).

studies in model organisms, including the zebrafish. Using the fish, we are carrying out several related projects, which are described below.

NEW TOOLS FOR EXPERIMENTAL ANALYSIS OF VASCULAR DEVELOPMENT

We generate novel transgenic lines for visualizing different types of endothelial and perivascular cells, and for driving gene expression or performing molecular profiling of mRNAs and microRNAs in these cell populations.

GENETIC ANALYSIS OF VASCULAR DEVELOPMENT

We have identified many novel mutants affecting vascular development in our transgene-assisted forward-genetic screens and are currently characterizing the phenotypes and molecular basis for several of the mutants.

ANALYSIS OF VASCULAR SPECIFICATION, PATTERNING, MORPHOGENESIS, AND FUNCTION

We are studying the development and function of several vascular beds, including the vasculature of the gills, the fish equivalent of the mammalian lungs, which contains unique endothelial cell populations and plays important roles in gas exchange.

Regulation of vascular integrity

We are using the zebrafish to understand the cellular and molecular mechanisms responsible for proper vessel morphogenesis and for the generation and maintenance of vascular integrity. Disruption of vascular integrity is associated with hemorrhagic stroke, a severe and

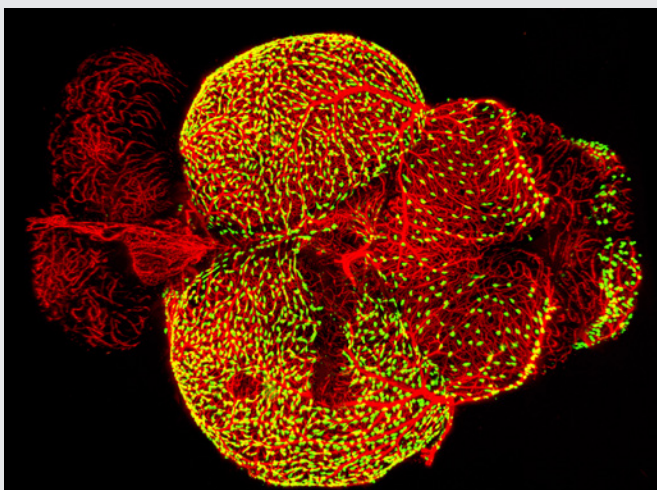


FIGURE 3. Novel perivascular cells on the zebrafish brain

Confocal micrograph of fluorescent granular perithelial cells (FGPs, *green*) adhering to the outside of meningeal blood vessels (*red*) on the brain of a *Tg(mrc1a:egfp);Tg(kdrl:cherry)* double-transgenic adult zebrafish. We recently showed that FGPs are unique endothelium-derived perivascular cells with unusual scavenging properties that are likely to be critical for brain homeostasis.

debilitating form of stroke associated with high morbidity and mortality. Meningeal vascular dysfunction is also associated with neuro-cognitive deficits and neuro-degenerative disease. Many of the recent insights into the molecular mechanisms regulating vascular integrity have come from studies in model organisms such as the zebrafish. We are pursuing several related projects.

GENES REGULATING VASCULAR INTEGRITY

With forward-genetic screens we identify new zebrafish mutants that disrupt cranial vascular integrity in the zebrafish (Figure 2), using next-gen sequencing methods to accomplish higher throughput cloning of mutants. We already characterized the role of *GDF6* (growth differentiation factor 6, also known as *BMP13*) in vascular integrity, demonstrating that the gene promotes maintenance of vascular integrity by suppressing excess VEGF (vascular endothelial growth factor) signaling. We recently characterized the role of RHOA in vascular integrity and angiogenesis. This small GTPase-regulatory protein has been shown to be involved in cytoskeletal dynamics, transcription, cell-cycle progression, and cell transformation, and a precisely calibrated level of RHOA signaling is required for proper vascular growth and function, with either too little or too much RHOA signaling resulting in vascular-integrity and angiogenesis defects.

REVASCULARIZATION AFTER INJURY

Vascular regrowth and remodeling are critical for proper wound healing, and defective vessel growth in cutaneous wounds is associated with delayed wound repair and/or chronic open wounds susceptible to infection. We developed a new zebrafish model of vascular regrowth and repair after cutaneous injury in adult zebrafish, and we are using this model to understand the cellular and molecular changes in that lead to defective vascular responses to injury and poor wound healing in aging or diabetes, with an eye toward uncovering new therapeutic targets to promote improved revascularization and healing in these contexts.

VASCULATURE AND VASCULAR-ASSOCIATED CELLS IN THE MENINGES

The meninges are an external, enveloping connective tissue that encases the brain, producing cerebrospinal fluid, acting as a cushion against trauma, nourishing the brain via nutrient circulation, and removing waste. Despite its importance, the cell types present in the meninges and its function and embryonic origins are

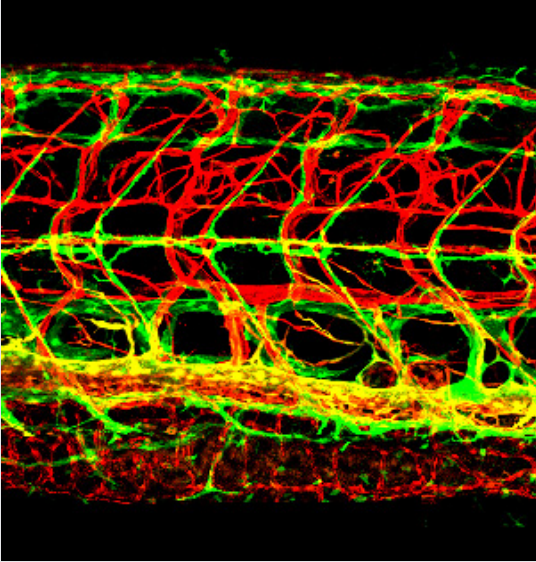


FIGURE 4. Novel lymphatic vascular reporter

Lateral view confocal image of the trunk of a 12 dpf (days post-fertilization) *Tg(kdrl:cherry); Tg(mrc1a:egfp)* double-transgenic zebrafish with red fluorescent blood vessels and green fluorescent lymphatics. See Jung HM, *et al.* *Development* 2017;144:2070 for additional details.

still not well understood. We recently discovered and characterized fluorescent granular perithelial cells (FGPs) in the zebrafish, a novel endothelium-derived perivascular cell population closely associated with meningeal blood vessels, which is likely to play a critical role in meningeal function (Figure 3). As discussed further below, we also discovered a *bona fide* meningeal lymphatic vascular network in the zebrafish. We are currently carrying out additional comprehensive anatomical and molecular studies to understand the structure and cellular composition of the zebrafish meninges and the function of FGPs, meningeal lymphatics, and other novel meningeal cell populations.

Specification and patterning of the lymphatic system

The lymphatic system is a vascular system completely separate from the blood circulatory system consisting of an elaborate blind-ended tree of vessels that extends through most of the body, emptying lymph fluid into the venous blood vascular system via several evolutionarily conserved drainage points.

The lymphatic system is essential for immune responses, fluid homeostasis, and fat absorption, and

is involved in many pathological processes, including tumor metastasis and lymphedema. However, progress in understanding the origins and early development of the system has been hampered by difficulties in observing lymphatic cells *in vivo* and performing defined genetic and experimental manipulation of the lymphatic system in currently available model organisms. Our ground-breaking studies demonstrated that zebrafish possess a lymphatic system that shares many of the morphological, molecular, and functional characteristics of lymphatic vessels found in other vertebrates, providing a powerful model for the purposes of imaging and studying lymphatic development. We are currently pursuing further study of the formation of the lymphatic system through several ongoing projects.

1. We generated new transgenic lines that permit direct, specific visualization, and tissue-specific molecular profiling of developing lymphatic vessels and are using these transgenic animals to further characterize lymphatic development (Figure 4).
2. We carried out forward-genetic ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis screens, using our lymphatic reporter transgenic lines to identify lymphatic-specific mutants with defects in novel genes that play important roles in lymphatic development.
3. We characterized and studied novel microRNAs expressed in the lymphatic endothelium and how these small regulatory RNAs influence lymphatic gene expression and lymphatic development.
4. We discovered a previously unreported lymphatic network in the dural meninges of the zebrafish (Figure 5). Like similar recently discovered meningeal lymphatics surrounding the mammalian brain, the zebrafish network is likely to play critical role in maintaining homeostasis and protecting the brain

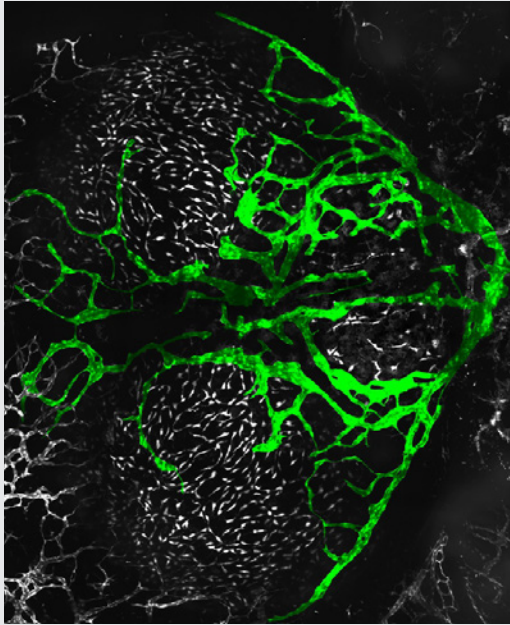


FIGURE 5. Adult zebrafish meningeal lymphatic network

Confocal image of the dorsal head of a *Tg(mrc1a:egfp)^{v251}, casper* adult zebrafish, with *mrc1a*+FGPs and superficial lymphatics in grey and intracranial meningeal lymphatics pseudocolored green. Anterior is to the left, right is up.

from mechanical trauma and infection, and we are carrying out a detailed analysis of the development, form, and function of these critical vessels and their role in immune cell interaction and trafficking.

The results of our studies, combining the genetic and experimental tools available in the zebrafish with the ability to perform high-resolution microscopic imaging of developing vascular structures in living animals, will continue to lead to important new insights into the origins and growth of the lymphatic system and molecular mechanisms that are critical in lymphatic development and lymphatic pathologies.

Epigenetics of development

We are using the genetically and experimentally accessible zebrafish and Mexican tetra (*Astyanax mexicanus*, also known as the blind cave fish) models to uncover the molecular basis for organ- and tissue-specific epigenetic regulation during development in the following interrelated projects:

FORWARD-GENETIC SCREEN FOR EPIGENETIC REGULATORY FACTORS

Genetic screens carried out in *Drosophila* and the nematode *Caenorhabditis elegans* have been highly successful in identifying genes regulating cell type-

specific epigenetic gene regulation in invertebrates, but the molecular mechanisms involved in organ- and tissue-specific epigenetic regulation in vertebrates are still relatively unknown. We developed a novel zebrafish transgenic reporter line that allows us to monitor dynamic changes in epigenetic regulation in intact animals during development. Using the transgenic line, we performed the first large-scale F3 genetic screen in a vertebrate to identify recessive mutants in regulators of epigenetic gene silencing or activation (Figure 6). Among other mutants, the screen yielded epigenetic regulators of liver development, pharynx development, and arterial differentiation, and we are currently pursuing follow-up studies on these mutants.

MOLECULAR MEDIATORS OF GLYCEMIC MEMORY IN DIABETIC VASCULOPATHY

The global burden of diabetes has risen dramatically, with projections that more than 600 million adults will be affected by 2030. Micro- and macrovascular complications in patients with diabetes are the major causes of cardiovascular mortality, renal failure, blindness, and non-traumatic amputations. Diabetes-related complications can emerge even many years after the blood sugar level levels have been brought under control, a phenomenon known as 'glycemic memory.' Although the cause of the phenomenon remains to be elucidated, epigenetic alterations in endothelial cells (ECs) may be responsible for the perdurance of diabetic vascular effects. We are using the zebrafish as an *in vivo* model to examine whether short-term exposure to hyperglycemia results in persistent transcriptomic and epigenomic changes in endothelial cells, even

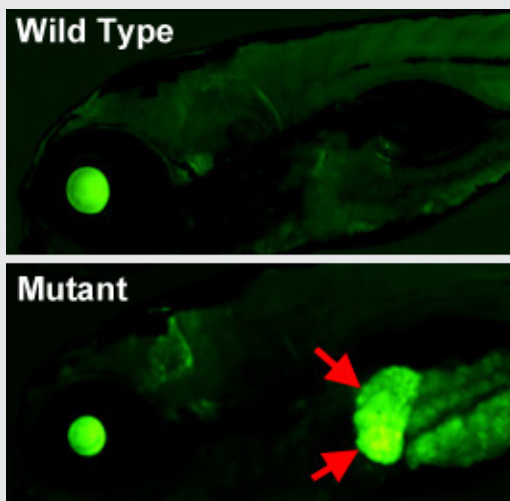


FIGURE 6. An epigenetic silencing mutant in the zebrafish

Lateral views of the head and anterior trunk of a wild-type (*top*) and tissue-specific epigenetic silencing mutant (*bottom*) zebrafish. The mutation causes loss of epigenetic silencing specifically in the liver (*red arrows*), as visualized with a novel transgenic reporter line developed in our lab, which permits dynamic, tissue-specific visualization of epigenetic silencing in living animals.

after return to normo-glycemic conditions. We identified several genes with significantly altered endothelial transcription and methylation levels during hyperglycemia that persist during the memory phase. We are currently carrying out further investigation of these 'glycemic memory loci' by a variety of methods. Unveiling the epigenetic and transcriptomic landscape of glycemic memory in ECs may lead to better identification of molecular targets and, potentially, to the design of personalized, epigenetic-based therapies to alleviate the enormous burden of diabetic vasculopathy.

EPIGENETIC REGULATION OF FAT AND MUSCLE DEVELOPMENT IN CAVEFISH

In addition to eye and pigment loss and other adaptations, *Astyanax* cavefish (Figure 7) have extreme and unusual metabolic adaptations that allow them to survive chronic and long-term food deprivation,

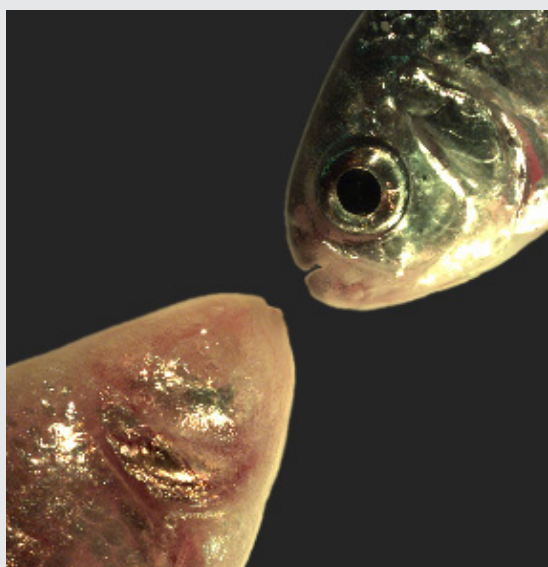


FIGURE 7. Mexican tetra cave- and surface fish

The Mexican tetra *Astyanax mexicanus* is a freshwater fish native to parts of southern Texas and eastern and central Mexico, which exists in both surface-dwelling ('surface morphs,' *top right*) and very closely related cave-dwelling ('cave morphs,' *bottom left*) populations. Cave morphs have a series of uniquely evolved adaptations, including loss of eyes and pigment, dramatically altered metabolism, altered vascular function, and altered sleep regulation and behavior. Results from our laboratory suggest that altered DNA methylation and resulting coordinated changes in expression of large sets of genes have helped drive at least some of this rapid evolutionary change.

including excess fat deposition, altered liver function, and resistance to metabolic disease. We hypothesize that, in a similar manner to loss of eyes, changes in epigenetic gene regulation may also underlie cavefish metabolic adaptations. We are using single-cell profiling to investigate differences in adipocytes and other cell types in the muscles (where in cavefish there are large amounts of stored fat) and livers of cavefish and surface fish. We are also performing whole-genome bisulfite sequencing and RNA-Seq from surface and cavefish muscles and livers to identify differentially expressed and methylated genes. We will follow up on these findings to elucidate how differential DNA methylation influences fat metabolism and obesity.

Additional Funding

- K99/R00 Award (to M. Venero Galanternik)
- Japan Society for the Promotion of Science (JSPS) Award (to K. Taimatsu)
- NICHD Intramural Research Fellowship (to Miranda Marvel)
- NICHD Intramural Research Fellowship (to Jong Park)
- NICHD Early Career Award (to Miranda Marvel)
- NICHD Early Career Award (to Leah Greenspan)
- NICHD Early Career Award (to Kiyohito Taimatsu)

Publications

1. Castranova D, Samasa B, Jung HM, Venero Galanternik M, Weinstein BM. Live imaging of intracranial lymphatics in the Zebrafish. *Circ Res* 2020 128:42–58.
2. Ma L, Ng M, Shi J, Gore AV, Castranova D, Weinstein BM, Jeffery WR. Maternal control of visceral asymmetry evolution in *Astyanax* cavefish. *Sci Rep* 2021 11:10312.
3. Pillay LM, Yano JJ, Davis AE, Butler MG, Ezeude MO, Park JS, Barnes KA, Reyes VL, Castranova D, Gore AV, Swift MR, Iben JR, Kenton MI, Stratman AN, Weinstein BM. In vivo dissection of Rhoa function in vascular development using zebrafish. *Angiogenesis* 2022 25:411–434.
4. Paulissen SM, Castranova DM, Krispin SM, Burns MC, Menéndez J, Torres-Vázquez J, Weinstein BM. Anatomy and development of the pectoral fin vascular network in the zebrafish. *Development* 2022 149:dev199676.
5. Castranova D, Samasa B, Venero Galanternik M, Gore AV, Goldstein AE, Park JS, Weinstein BM. Long-term imaging of living adult zebrafish. *Development* 2022 149:dev199667.

Collaborators

- Andreas Baxeavanis, PhD, *Computational and Statistical Genomics Branch, NHGRI, Bethesda, MD*
- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- George Davis, PhD, *University of Missouri-Columbia, Columbia, MO*
- Elisabetta Dejana, PhD, *The FIRC Institute of Molecular Oncology Foundation, Milan, Italy*
- James Iben, PhD, *Molecular Genomics Laboratory, NICHD, Bethesda, MD*
- Sumio Isogai, PhD, *Iwate Medical University, Morioka, Japan*
- William R. Jeffery, PhD, *University of Maryland, College Park, MD*
- Paul Liu, MD, PhD, *Genetics and Molecular Biology Branch, NHGRI, Bethesda, MD*
- Richard Maraia, MD, *Section on Molecular and Cell Biology, NICHD, Bethesda, MD*
- Yoh-suke Mukouyama, PhD, *Laboratory of Stem Cell and Neuro-Vascular Biology, NHLBI, Bethesda, MD*

- Lisa M. Price, PhD, *Division of Developmental Biology, NICHD, Bethesda, MD*
- Radu V. Stan, MD, PhD, *Geisel School of Medicine at Dartmouth, Lebanon, NH*

Contact

For more information, email weinsteb@mail.nih.gov or visit <https://www.nichd.nih.gov/research/atNICHD/Investigators/weinstein>.

Studies on DNA Replication, Repair, and Mutagenesis in Eukaryotic and Prokaryotic Cells

Under optimal conditions, the fidelity of DNA replication is extremely high. Indeed, it is estimated that, on average, only one error occurs for every 10 billion bases replicated. However, given that living organisms are continually subjected to a variety of endogenous and exogenous DNA-damaging agents, optimal conditions rarely prevail *in vivo*. While all organisms have evolved elaborate repair pathways to deal with such damage, the pathways rarely operate with 100% efficiency. Thus, persisting DNA lesions are replicated, but with much lower fidelity than in undamaged DNA. Our aim is to understand the molecular mechanisms by which mutations are introduced into damaged DNA. The process, commonly referred to as trans-lesion DNA synthesis (TLS), is facilitated by one or more members of the Y-family of DNA polymerases, which are conserved from bacteria to humans. Based on phylogenetic relationships, Y-family polymerases may be broadly classified into five subfamilies: DinB-like (pol IV/pol kappa-like) proteins are ubiquitous and found in all domains of life; in contrast, the Rev1-like, Rad30A (pol eta)-like, and Rad30B (pol iota)-like polymerases are found only in eukaryotes; and the UmuC (polV)-like polymerases only in prokaryotes. We continue to investigate TLS in all three domains of life: bacteria, archaea, and eukaryotes.

Prokaryotic studies

Pathogenic bacteria pose a major global threat through the precipitous emergence of *multidrug resistant strains*. Horizontal transfer of mobile genetic elements, including R-plasmids, integrative-conjugative elements (ICEs) and chromosomal instabilities, accompanied by high mutation rates, are among the key factors driving antibiotic resistance. Recent findings have provided new insights into sources of mutagenesis leading to drug resistance. Elevated mutagenesis accompanying the induction of the SOS stress response caused by exposure to antibiotics and, more generally, to a wide variety of exogenous DNA damage, have been linked to the development of bacterial antibiotic resistance. For example, exposure of clinical isolates of *E. coli* to ciprofloxacin or zidovudine and *Acinetobacter baumannii* to UV or the alkylating agent methyl methanesulfonate (MMS) resulted in the development of antibiotic resistance. There is a paucity of data documenting specific contributions of SOS-induced proteins, including pathogenic bacterial homologs of the *E. coli* LexA repressor RecA, and



Roger Woodgate, PhD, Head,
*Section on DNA Replication,
Repair and Mutagenesis*

John P. McDonald, PhD, *Biologist*

Mary McLenigan, BS, *Chemist*

Nicholas W. Ashton, PhD,
Postdoctoral Visiting Fellow

Marcella T. Latancia, PhD,
Postdoctoral Visiting Fellow

Natalia C. Moreno, PhD,
Postdoctoral Visiting Fellow

Mallory R. Smith, PhD,
*Postdoctoral Intramural
Research Training Award Fellow*

Dana A. D'Orlando, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Nickolas G. Moeckel, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

low-fidelity DNA polymerase V (pol V), toward the acquisition of drug resistance. Homologs of pol V have been identified in a variety of Gammaproteobacteria, many of which are pathogenic. In addition to homologs encoded chromosomally, many pol V homologs are found on mobile genetic elements that can be horizontally transferred between different bacterial species. We referred to generic pol V homologs encoded on the chromosomes of pathogens as Pathogen Encoded Pols, abbreviated as PEPols. Homologous enzymes encoded by mobile elements, particularly integrative conjugative elements (ICEs), were referred to as Mobile Element encoded Pols (MEPols).

In collaboration with Myron Goodman's laboratory, we used Rum pol (RumA'₂B), from the integrative conjugative element (ICE) R391, as a model mobile element-encoded polymerase (MEPol). The highly mutagenic Rum pol is transferred horizontally into a variety of recipient cells, including many pathogens. Moving between species, it is unclear whether Rum pol can function on its own or requires activation by host factors. To test this hypothesis, we investigated the biochemical and *in vivo* mutagenic behavior of Rum Mut assembled with RecA homologs purified from seven bacterial species; four clinical bacterial isolates in which the *rumAB* encoding R391/SXT family of ICE's have been previously identified (*E. coli*, *V. cholerae*, *P. rettgeri*, *K. pneumoniae*), and from three clinical isolates lacking *rumAB* genes (*P. aeruginosa*, *M. tuberculosis*, *S. aureus*).

First, we demonstrated that Rum pol biochemical activity requires the formation of a physical mutasomal complex, Rum Mut, containing RumA'₂B-RecA-ATP, with RecA being donated by each recipient bacteria. Interestingly, Rum Mut-specific activities *in vitro* and mutagenesis rates *in vivo* depended on the phylogenetic distance of host-cell RecA from *E. coli* RecA. We hypothesized that Rum pol, which is a highly conserved and effective mobile catalyst of rapid evolution, has the potential to generate a broad mutational landscape that could serve to ensure bacterial adaptation in antibiotic-rich environments, leading to the establishment of antibiotic resistance.

Eukaryotic studies

The Y-family DNA polymerases are responsible for copying damaged DNA during DNA replication in a process called translesion synthesis (TLS). These enzymes are highly specialized in order to accommodate different structural DNA distortions caused by a wide variety of DNA lesions. The Y-family is divided into six phylogenetically distinct subfamilies: two UmuC (pol V) branches; Rad30A/POLH (pol eta); Rad30B/POLI (pol iota); DinB (pol IV, Dpo4, pol kappa); and Rev. Across the different domains of life, Y-family polymerase subfamilies are found in various combinations. For example, UmuC orthologs are only detected in Gram-positive and Gram-negative bacteria, whereas Rev1 and Rad30A/B orthologs are only detected in eukaryotes. The DinB subfamily is the most evolutionarily conserved, with members scattered throughout all three domains of life, from unicellular bacteria to humans. However, differences in the distribution of Y-family DNA pols are present within each kingdom. For example, the eukaryote *Saccharomyces cerevisiae* contains neither a polk nor a poli gene. Indeed, it was originally assumed that poli was expressed only in higher eukaryotes. However, next-generation whole-genome sequencing revealed that pol iota orthologs are actually distributed throughout the whole Eukaryota domain. One example is the thermophilic fungus *Thermomyces lanuginosus*, which possesses all four eukaryotic Y-family subfamilies, much like humans, in contrast to its fungal relatives *S. cerevisiae* and *Schizosaccharomyces pombe*. We wished to determine whether there is a logic in a such seemingly random distribution of pol iota. Using phylogenetic analysis and comparing the biochemical characterization of Y-family pols from different species, we hoped to shed some light on this question.

To do so, we described the identification, purification, and characterization of thermostable eukaryotic orthologs of pol eta, pol iota, pol kappa, and Rev1 from *T. lanuginosus*. Biochemical characterization of TLS DNA pols eta, iota, kappa, and Rev1 included determination of the enzyme's fidelity, processivity, thermostability, metal ion requirements, and TLS specificity during bypass of cyclobutane pyrimidine dimers (CPDs), abasic sites, and benzo[*a*]pyrene diol epoxide (BPDE) adducts. Our findings serve as a basis for comparative analysis of the properties of proteins from different species and provided an important insight into the functional evolution of the Y-family polymerases.

Apart from the expected increased thermostability of the *T. lanuginosus* Y-family pols, their major biochemical properties are very similar to properties of their human counterparts. In particular, both Rad30B homologs (*T. lanuginosus* and human pol iota) exhibit remarkably low fidelity during DNA synthesis that is template sequence-dependent. It was previously hypothesized that higher organisms had acquired this property during eukaryotic evolution, but these observations imply that poli originated earlier than previously known, suggesting a critical cellular function in both lower and higher eukaryotes.

Publications

1. Vaisman A, McDonald JP, Smith MR, Aspelund SL, Evans TC Jr, Woodgate R. Identification and characterization of thermostable Y-Family DNA polymerases η , ι , κ and Rev1 from a lower eukaryote, *Thermomyces lanuginosus*. *Front Mol Biosci* 2021 8:778400.
2. Ojha D, Jaszczur MM, Sikand A, McDonald JP, Robinson A, van Oijen AM, Mak CH, Pinaud F, Cox MM, Woodgate R, Goodman MF. Host cell RecA activates a mobile element-encoded mutagenic DNA polymerase. *Nucleic Acids Res* 2022 50:6854–6859.

Collaborators

- Irina Bezsonova, PhD, *University of Connecticut, Farmington, CT*
- Anders R. Clausen, PhD, *Göteborgs Universitet, Göteborg, Sweden*
- Michael Cox, PhD, *University of Wisconsin, Madison, WI*
- Tom C. Evans, PhD, *New England Biolabs, Ipswich, MA*
- Iwona Fijlakowska, PhD, *Polish Academy of Sciences, Warsaw, Poland*
- Martín Gonzalez, PhD, *Southwestern University, Georgetown, TX*
- Myron F. Goodman, PhD, *University of Southern California, Los Angeles, CA*
- Karolina Makiela-Dzbenka, PhD, *Polish Academy of Sciences, Warsaw, Poland*
- Justyna McIntyre, PhD, *Polish Academy of Sciences, Warsaw, Poland*
- Andrew Robinson, PhD, *University of Wollongong, Wollongong, Australia*
- Ewa Sledziowska-Gojska, PhD, *Polish Academy of Sciences, Warsaw, Poland*
- Antoine Van Oijen, PhD, *University of Wollongong, Wollongong, Australia*
- Digby Warner, PhD, *University of Cape Town, Cape Town, South Africa*
- Wei Yang, PhD, *Laboratory of Molecular Biology, NIDDK, Bethesda, MD*

Contact

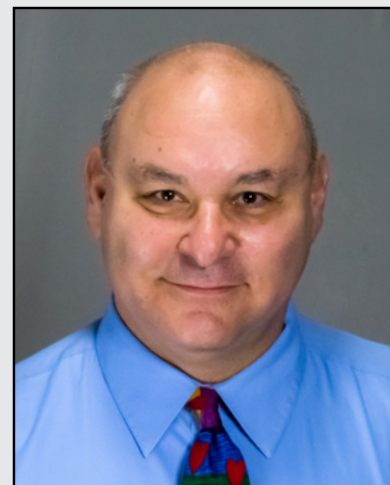
For more information, email woodgate@nih.gov or visit <http://sdrmm.nichd.nih.gov>.

Physiology, Psychology, and Genetics of Obesity

The prevalence of overweight and obesity in children and adults has tripled during the past 40 years. The alarming rise in body weight has likely occurred because the current environment affords easy access to energy-dense foods and requires less voluntary energy expenditure. However, such an environment leads to obesity only in those individuals whose body weight-regulatory systems are not able to control body adiposity with sufficient precision in our high calorie/low activity environment, which suggests there are subgroups in the U.S. with a uniquely high susceptibility to weight gain under the prevailing environmental conditions. Our primary goal is to elucidate the genetic underpinnings of the metabolic and behavioral endo-phenotypes that contribute to the development of obesity in children. Using our unique longitudinal cohorts of children who have undergone intensive metabolic and behavioral phenotyping, we examine genetic and phenotypic factors predictive of progression to adult obesity in children who have obesity or are in the “pre-obese” state, allowing characterization of phenotypes unconfounded by the impact of obesity itself. Once they are identified as linked to obesity, we intensively study genetic variants that impair gene function. We expect that these approaches will improve our ability to predict which children are at greatest risk for obesity and its comorbid conditions and will lead to more targeted, etiology-based prevention and treatment strategies for pediatric obesity.

Genetic factors important for childhood body-weight regulation

To identify gene variants affecting body composition, we have been examining polymorphisms in genes involved in the leptin signaling pathway. Such genes include the leptin receptor (*LEPR*), genes that appear to alter leptin receptor signal transduction such as those that are part of the BBSome (a protein complex of seven proteins), and those encoding proopiomelanocortin (*POMC*), the melanocortin 3 receptor (*MC3R*), the melanocortin 4 receptor (*MC4R*), and brain-derived neurotrophic factor (*BDNF*). We are currently studying a variant *MC3R* that is associated with adiposity in children and adults and appears to have functional significance for MC3R-signal transduction. Children and adults who were homozygous variant for both C17A and G241A polymorphisms have significantly greater fat



Jack A. Yanovski, MD, PhD, Head,
Section on Growth and Obesity

Anna Zenno, MD, *Endocrinology Fellow*

Sheila M. Brady, RN, FNP, *Nurse Practitioner*

Tusharkumar Patel, PhD, *Visiting Fellow*

Diana Elizondo, PhD, *Postdoctoral Fellow*

Robin Roberson, MS, *Technician*

Nasreen Moursi, BA, *Graduate Student Guest Researcher*

Megan Parker, MS, *Graduate Student Guest Researcher*

Brooke Arner, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

Kaitlin Ballenger, BA, *Postbaccalaureate Intramural Research Training Award Fellow*

Bess Bloomer, BA, *Postbaccalaureate Intramural Research Training Award Fellow*

Jocelyn Chen, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

(continued)

mass and higher plasma levels of insulin and leptin than unaffected or heterozygous children and appear to eat more at laboratory test meals (Figure 1). *In vitro* studies subsequently found that signal transduction and protein expression were significantly lower for the double mutant *MC3R*. Our ongoing studies attempt to understand the mechanisms by which these sequence alterations affect body weight. We therefore developed transgenic ‘knock-in’ mice expressing the human wild-type and human double-mutant *MC3R*. Using homozygous knock-in mouse models replacing murine *Mc3r* with wild type human (*MC3R^{hWT/hWT}*) and double-mutant (C17A+G241A) human (*MC3R^{hDM/hDM}*) *MC3R*, we found that *MC3R^{hDM/hDM}* mice have greater weight and fat mass (Figure 2), increased energy intake and feeding efficiency, but lower length and fat-free mass than *MC3R^{hWT/hWT}*. *MC3R^{hDM/hDM}* mice do not have increased adipose tissue inflammatory-cell infiltration or greater expression of inflammatory markers despite their greater fat mass. Serum adiponectin is elevated in *MC3R^{hDM/hDM}* mice and in *MC3R^{hDM/hDM}* human subjects (Figure 2). *MC3R^{hDM/hDM}* bone- and adipose tissue-derived mesenchymal stem cells (MSCs) differentiate into adipocytes that accumulate more triglyceride than do *MC3R^{hWT/hWT}* MSCs. *MC3R^{hDM/hDM}* thus impacts nutrient partitioning to generate increased adipose tissue that appears metabolically healthy. These data confirm the importance of *MC3R* signaling in human metabolism and suggest a previously unrecognized role for the *MC3R* in adipose tissue development. Ongoing studies continue to improve our understanding of the phenotype of such mice. We are investigating a novel role for *MC3R* in regulating hepatic autophagy, the role of *MC3R* in stem-cell fate, and how variations in *Mc3r* may alter signaling of several downstream signaling pathways. Using tissue-specific knockout and reactivation models [Reference 1], we are also studying the importance of hepatic and adipose tissue *MC3R* for whole body homeostasis.

Physiology, metabolism, and psychology of childhood body-weight regulation

Our studies are directed at understanding the physiological, psychological, and metabolic factors that place children at risk for undue weight gain. As part of these studies, we examined how best to measure eating-related psychopathology, insulin sensitivity, changes in body composition, energy intake, and energy expenditure in children, and we studied the short- and long-term stability of the components of metabolic syndrome. We previously found that leptin is an important predictor of weight gain in children, and we identified children with hyperleptinemia and proximal leptin signaling pathway mutations. We also found hyperleptinemia out of proportion with body fat mass in children with psychological loss of control (LOC) over eating. Such data suggest the importance of leptin resistance as a factor stimulating weight gain and have led to recent explorations of other syndromes associated with obesity that may cause dysregulation of leptin signaling, including WAGR (Wilms tumor, aniridia, genitourinary), Bardet-Biedl [Reference 2] and Alström syndromes. Current studies are directed at understanding additional genetic, physiological, and psychological

Jack Donahue, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Syeda Fatima, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Loie M. Faulkner, BA,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Suryaa Gupta, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Megan Hancock, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Melanie Hollis, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Arad Jain, BS, *Postbaccalaureate
Intramural Research Training
Award Fellow*

Celine Kisimba, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Esther Kwarteng, BA,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Julia Lazareva, BS,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Lucy Loch, BA, *Postbaccalaureate
Intramural Research Training
Award Fellow*

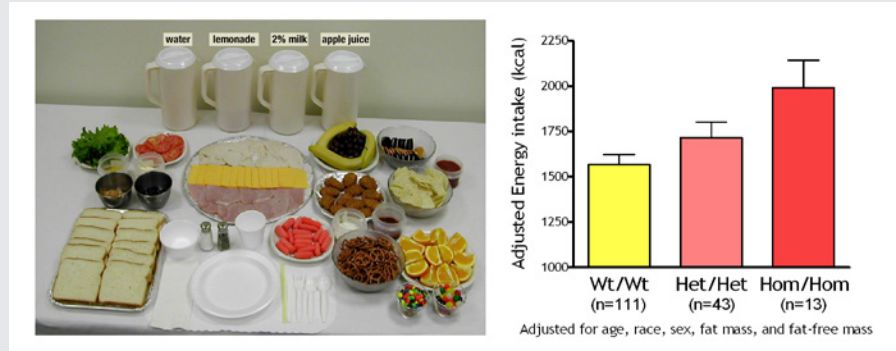
Ejike Nwosu, BA, *Postbaccalaureate
Intramural Research Training
Award Fellow*

Jennifer Te-Vazquez, BA,
*Postbaccalaureate Intramural
Research Training Award Fellow*

Hannah Haynes, BA, *Guest
Researcher*

FIGURE 1. Energy intake studied using free-access buffet meals of palatable foods

Children homozygous for two polymorphisms in the *MC3R* gene (Hom/Hom) consumed more at the buffet than heterozygotes (Het/Het) or than those with wild-type *MC3R* (Wt/Wt).



factors that place children at risk for undue weight gain, including humoral factors, sleep, food cravings, negative affective states such as depression and anxiety, weight-based teasing, alexithymia, executive functioning, and LOC eating. Some recent initiatives have targeted insulin resistance in girls at high risk for type 2 diabetes because of obesity and a family history of diabetes.

Our evaluations concentrating on binge-eating behaviors in children suggest that such behaviors also are associated with adiposity in children and with abnormalities in metabolism. We found that certain eating patterns may predict future weight gain in children at risk for obesity: children reporting binge-eating behaviors, such as LOC over eating gained, on average, an additional 2.4 kg of weight per year compared with non-binge-eating children. Our data also suggest that children endorsing binge eating consume more energy during meals. Actual intake during buffet meals averaged 400 kcal more in children with binge eating, but despite their greater intake, such children reported shorter-lived satiety than children without binge eating episodes. The ability to consume large quantities of palatable foods, especially when coupled with

FIGURE 2. Studies of a human *MC3R* variant containing two naturally occurring polymorphisms

The variant is associated with pediatric-onset obesity. We found that mice whose *Mc3r* was replaced by human versions of the gene were obese when they expressed the double-mutant gene (*MC3R^{hDM/hDM}*), with greater fat mass (a) and lower fat-free mass (b), but surprisingly greater adiponectin concentrations (c) than mice with the normal human *MC3R* (*MC3R^{hWT/hWT}*). Humans with the double-mutant receptor also showed greater adiponectin (d).

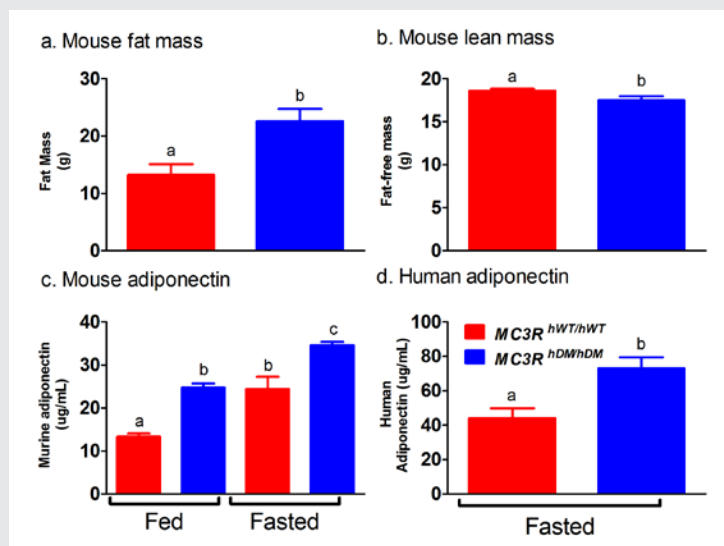
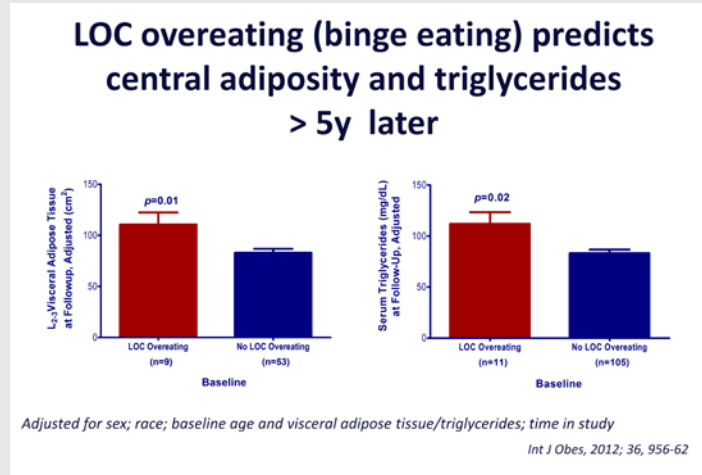


FIGURE 3. Loss of control (LOC) eating and metabolic complications in a longitudinal study

On average (\pm SE), children who engaged in binge eating at baseline had more visceral adipose tissue at the L₂₋₃ intervertebral space at follow-up than children who did not engage in binge eating at baseline, adjusting for sex, race, baseline age, baseline visceral adipose tissue at L₂₋₃, and time in study ($P = 0.01$). On average (\pm SE), children who engaged in binge eating at baseline had higher follow-up triglycerides than children who did not engage in binge eating at baseline, adjusting for sex, race, baseline age, body mass index (kg/m^2), baseline triglycerides, and time in study ($P = 0.02$).



reduced subsequent satiety, may play a role in the greater weight gain found in binge-eating children. We demonstrated, among cohorts of lean and obese youth, that youth with LOC eating had higher serum leptin and are at significantly greater risk for worsening of components of the metabolic syndrome than those without LOC episodes, even after adjusting for adiposity and other relevant covariates. Our data also suggest that anxiety symptoms may interact with LOC eating to become an important co-factor for excessive weight gain among children. These data also suggest that interventions targeting disordered eating behaviors may be useful in preventing excessive fat gain in children prone to obesity and have led to trials of preventative strategies related to binge eating.

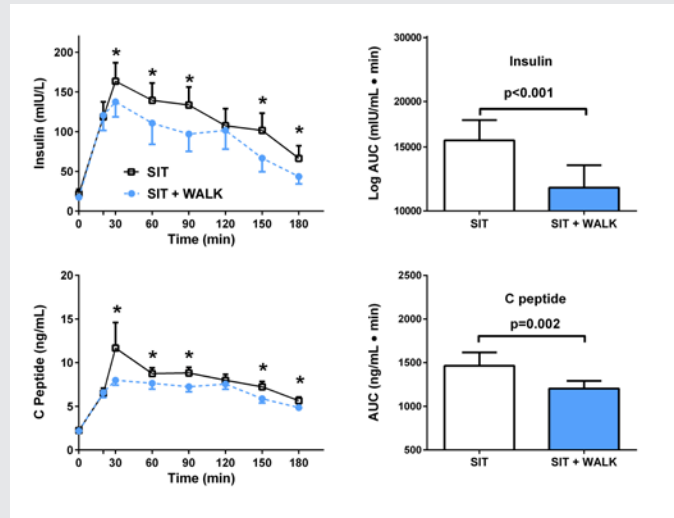
We study normal weight children and adolescents, children who already have obesity, and the non-obese children of parents with obesity, in order to determine the factors that are most important for developing the complications of obesity in youth. Body composition, leptin concentration, metabolic rate, insulin sensitivity, glucose disposal, energy intake at buffet meals, energy expenditure [Reference 3], and genetic factors believed to regulate metabolic rate and body composition are examined. Psychological and behavioral factors, such as propensity to engage in binge-eating behavior (Figure 3), and sleep are also studied. We are following children longitudinally into adulthood. In two protocols, we study actual food consumption of children during meals, to elucidate differences in the calorie and macronutrient content of meals and the circulating hormones related to hunger and satiety in those who either endorse binge eating or report no such behaviors. We found that eating in the absence of physiological hunger is a replicable trait that appears linked to obesity. We also investigated the role of sedentary behaviors, such as television watching, as a factor that alters metabolism. In a randomized, controlled, crossover trial (Figure 4) and in a 5-day parallel groups design, we found that glucose homeostasis was markedly improved in children with overweight or obesity who engaged in moderate activity for just three minutes every half hour, compared with those remaining sedentary [Reference 4].

Treatment of obesity and the co-morbid conditions associated with obesity

Given the rapid increase in the prevalence of obesity, the development of treatments for obesity in children and adults is urgently needed, yet current pharmacologic approaches are extremely limited, for both children

FIGURE 4. Effect of short, moderate-intensity walking breaks on children's glucose tolerance

Children with overweight or obesity who walked for three minutes every 30 minutes (*blue*) had lower insulin and C-peptide concentrations during an oral glucose tolerance test than when they sat without interruption for three hours (*black*).

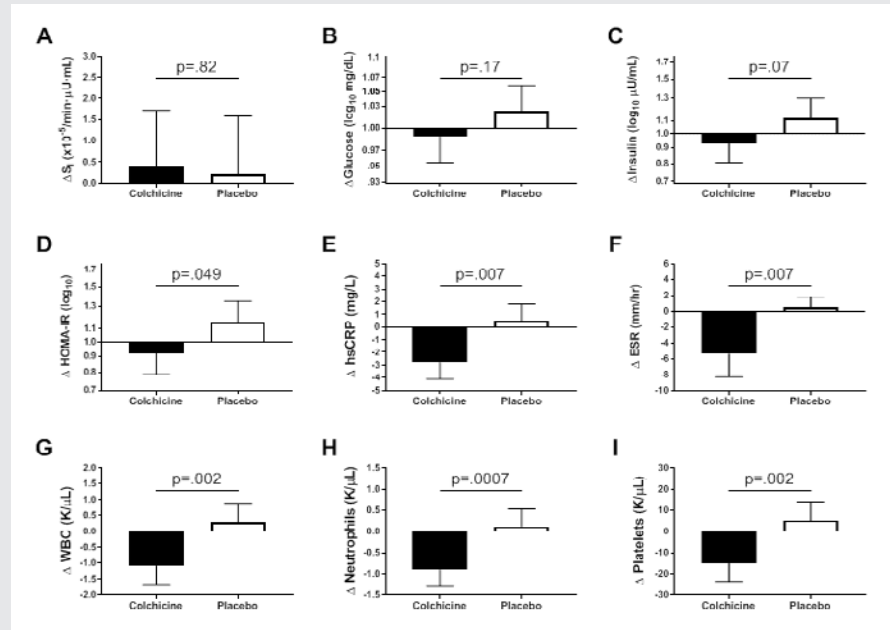


and adults. In several clinical protocols, we examined approaches for the prevention and treatment of excessive body weight. We completed a randomized controlled trial to examine the mechanism by which metformin may affect the body weight of younger children who have hyperinsulinemia and are therefore at risk for later development of type 2 diabetes. Compared with placebo-treated children, in those randomized to metformin, BMI, BMIz score (BMI standard deviation score for age), and body fat mass declined to significantly greater extents. Serum glucose and HOMA-IR (Homeostasis Model Assessment of Insulin Resistance) also decreased more in metformin-treated than in placebo-treated children. A second study compared prevention of weight gain using interpersonal therapy (IPT) with a control health education program (HE) in adolescents reporting loss-of-control eating behaviors. At three-year follow-up, baseline social-adjustment problems and trait-anxiety significantly moderated outcome. Among girls with high self-reported baseline social-adjustment problems or anxiety, IPT was significantly associated with the steepest declines in BMIz compared with those undergoing HE. For adiposity, girls with high or low anxiety in HE and girls with low anxiety in IPT experienced gains, while girls in IPT with high anxiety stabilized. Parent reports yielded complementary findings. The results have stimulated ongoing research to examine how anxiety may stimulate energy intake. We also published preliminary data from a third study examining IPT approaches in younger children, finding good tolerability for such a program. A fourth study examined whether reducing depressive symptoms could ameliorate insulin resistance in adolescents at risk for Type 2 Diabetes. Among girls with greater (moderate) baseline depressive symptoms (N = 78), those in CBT (cognitive behavioral therapy) developed significantly lower two-hour insulin than those in HE. Additional metabolic benefits of CBT were seen for this subgroup in *post hoc* analyses of post-treatment compared with one-year change. An ongoing study, based on lab data finding links between attentional biases to high-palatability foods in children with obesity, examines whether adolescents' attentional biases can be retrained. We also initiated a translational trial in patients with Bardet-Biedl syndrome studying the effects of modulation of the leptin signaling pathway with the melanocortin agonist setmelanotide [Reference 2]. Most recently, we initiated another study on the specific pharmacotherapy of using diazoxide for patients with Prader-Willi syndrome. These latest trials are examples of precision-medicine approaches to treat obesity. We also recently completed a novel randomized controlled pilot trial of colchicine to ameliorate the inflammation of obesity and thus improve its complications [Reference 5]. Adults with

FIGURE 5. Effects of colchicine on inflammatory and metabolic measures

Metabolic and inflammatory changes after three months of study medication in participants randomized to colchicine (N=21) or placebo (N=19).

- A. Insulin sensitivity (S).
- B. Fasting glucose.
- C. Fasting insulin.
- D. Homeostasis Model Assessment of Insulin Resistance (HOMA-IR).
- E. High sensitivity C-reactive protein (hsCRP).
- F. Erythrocyte sedimentation rate (ESR).
- G. White blood cell count (WBC).
- H. Neutrophil count.
- I. Platelet count. Data are presented as mean \pm SEM.



obesity and metabolic syndrome, but who did not have diabetes, were randomized to 0.6 mg colchicine or placebo capsules twice daily for three months. Compared with placebo, colchicine significantly reduced both C-reactive protein and erythrocyte sedimentation rate (Figure 5). The significant changes in homeostatic model assessment of insulin resistance, fasting insulin, and glucose effectiveness suggested metabolic improvements in the colchicine-treated group compared with the placebo group. We are now conducting a larger, adequately powered study to determine whether colchicine improves insulin resistance and other measures of metabolic health in at-risk individuals.

Additional Funding

- Rhythm Pharmaceuticals, Inc.: Setmelanotide (RM-493; Rhythm Pharmaceuticals, Inc.) phase 2 open-label treatment trials in patients with rare genetic disorders of obesity. 2017–2022
- Soleno Therapeutics, Inc. Grant support to fund an RCT testing diazoxide choline sustained release tablets in patients with Prader-Willi syndrome and hyperphagia. 2018–2022
- NIH Clinical Center “Bench-to-Bedside” Award: Liraglutide in Adolescents with Obesity After Sleeve Gastrectomy. 2021–2022
- NICHD Office of Education Fellows Recruitment Incentive Award. Funds postdoctoral fellowship for Diana Elizondo, PhD, 2022–2023

Publications

1. Wong AM, Patel TP, Altman EK, Tugarinov N, Trivellin G, Yanovski JA. Characterization of the adiponectin promoter + Cre recombinase insertion in the Tg(Adipoq-cre)¹Evd^r mouse by targeted locus amplification and droplet digital PCR. *Adipocyte* 2021 10:21–27.

2. Haws RM, Gordon G, Han JC, Yanovski JA, Yuan G, Stewart MW. The efficacy and safety of setmelanotide in individuals with Bardet-Biedl syndrome or Alstrom syndrome: Phase 3 trial design. *Contemp Clin Trials Commun* 2021 22(100780):1–5.
3. Pontzer H, Yamada Y, Sagayama H, Ainslie PN, Andersen LF, Anderson LJ, Arab L, Baddou I, Bedu-Addo K, Blaak EE, Blanc S, Bonomi AG, Bouten CVC, Bovet P, Buchowski MS, Butte NF, Camps SG, Close GL, Cooper JA, Cooper R, Das SK, Dugas LR, Ekelund U, Entringer S, Forrester T, Fudge BW, Goris AH, Gurven M, Hambly C, El Hamdouchi A, Hoos MB, Hu S, Joonas N, Joosen AM, Katzmarzyk P, Kempen KP, Kimura M, Kraus WE, Kushner RF, Lambert EV, Leonard WR, Lessan N, Martin C, Medin AC, Meijer EP, Morehen JC, Morton JP, Neuhouser ML, Nicklas TA, Ojiambo RM, Pietilainen KH, Pitsiladis YP, Plange-Rhule J, Plasqui G, Prentice RL, Rabinovich RA, Racette SB, Raichlen DA, Ravussin E, Reynolds RM, Roberts SB, Schuit AJ, Sjodin AM, Stice E, Urlacher SS, Valenti G, Van Etten LM, Van Mil EA, Wells JCK, Wilson G, Wood BM, Yanovski J, Yoshida T, Zhang X, Murphy-Alford AJ, Loechl C, Luke AH, Rood J, Schoeller DA, Westerterp KR, Wong WW, Speakman JR, Consortium IDD. Daily energy expenditure through the human life course. *Science* 2021 373(6556):808–812.
4. Broadney MM, Belcher BR, Ghane N, Sheni R, Jayson MJ, Trenchel RW, Collins SM, Brychta RJ, Davis EK, Brady SM, Yang SB, Courville AB, Smith KP, Rosing DR, Chen KY, Yanovski JA. Effects of interrupting daily sedentary behavior on children's glucose metabolism: a 6-day randomized controlled trial. *Pediatr Diabetes* 2022 23(8):1567–1578.
5. Levine JA, Sarrafan-Chaharsoughi Z, Patel TP, Brady SM, Chivukula KK, Miller E, Han JM, Periwai V, Wolska A, Remaley AT, Dagur PK, Biancotto A, Babyak A, Fantoni G, Yanovski JA, Demidowich AP. Effects of colchicine on lipolysis and adipose tissue inflammation in adults with obesity and metabolic syndrome. *Obesity* 2022 30(2):358–368.

Collaborators

- David Allison, PhD, *School of Public Health, Indiana University, Bloomington, IN*
- Jeffrey Baron, MD, *Section on Growth and Development, NICHD, Bethesda, MD*
- Britni Belcher, PhD, MPH, *Keck School of Medicine, University of Southern California, Los Angeles, CA*
- Karen Berman, PhD, *Section on Integrative Neuroimaging, NIMH, Bethesda, MD*
- Kong Chen, PhD, *Clinical Endocrinology Branch, NIDDK, Bethesda, MD*
- Ross Crosby, PhD, *Director of Biomedical Statistics, Neuropsychiatric Research Institute, Fargo, ND*
- Andrew P. Demidowich, MD, *Johns Hopkins University School of Medicine, Baltimore, MD*
- Joel Elmquist, DVM, PhD, *UT Southwestern Medical Center, Dallas, TX*
- Scott Engel, PhD, *University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND*
- I. Sadaf Farooqi, MD, *Cambridge Institute for Medical Research, Cambridge, United Kingdom*
- Oksana Gavrilova, PhD, *Mouse Metabolism Core Laboratory, NIDDK, Bethesda, MD*
- Joan C. Han, MD, *Le Bonheur Children's Hospital, Memphis, TN*
- Robert Haws, MD, *Marshfield Clinic Research Institute, Marshfield, WI*
- Steven B. Heymsfield, MD, *Pennington Biomedical Research Center, Baton Rouge, LA*
- Heidi Kong, MD, MHSc, *Cutaneous Microbiome and Inflammation Section, NIAMS, Bethesda, MD*
- Sergey Leikin, PhD, *Section on Physical Biochemistry, NICHD, Bethesda, MD*
- Joan C. Marini, MD, PhD, *Section on Heritable Disorders of Bone and Extracellular Matrix, NICHD, Bethesda, MD*
- Cara Olsen, PhD, *Uniformed Services University of the Health Sciences, Bethesda, MD*
- Vipul Periwai, PhD, *Laboratory of Biological Modeling, NIDDK, Bethesda, MD*
- Daniel Pine, MD, *Section on Development and Affective Neuroscience, NIMH, Bethesda, MD*

- Douglas Rosing, MD, *Cardiac Consultation Service, NHLBI, Bethesda, MD*
- Peter J. Schmidt, MD, *Section on Behavioral Endocrinology, NIMH, Bethesda, MD*
- Dale A. Schoeller, PhD, *University of Wisconsin, Madison, WI*
- Lauren B. Shomaker, PhD, *University of Colorado, Boulder, CO*
- Steven J. Soldin, PhD, *Department of Laboratory Medicine, Clinical Center, NIH, Bethesda, MD*
- John Speakman, PhD, *University of Aberdeen, Aberdeen, United Kingdom*
- Marian Tanofsky-Kraff, PhD, *Uniformed Services University of the Health Sciences, Bethesda, MD*
- B. Timothy Walsh, PhD, *Columbia University College of Physicians and Surgeons, New York, NY*
- Denise E. Wilfley, PhD, *Washington University School of Medicine, St. Louis, MO*
- Joshua Zimmerberg, MD, PhD, *Section on Integrative Biophysics, NICHD, Bethesda, MD*

Contact

For more information, email yanovskj@mail.nih.gov or visit <http://sgo.nichd.nih.gov>.

Membrane Mechanisms of Viral Spike Interactions and Packaging of Extracellular Vesicles

Eukaryotic life must create the many shapes and sizes of the system of internal membranes and organelles that inhabit the variety of cells in nature, membranes that must remodel for cells to repair damaged plasmalemma and deal with infectious agents such as viruses and parasites. Such basic membrane mechanisms must be highly regulated and highly organized in various hierarchies in space and time to allow the organism to thrive despite environmental challenges, genetic instability, unpredictable food supply, and physical trauma. We are using our expertise and the techniques we perfected over the years to address various biological problems that have in common the underlying regulation or disturbance of protein/lipid interactions. The overall goal of this project is to determine the physico-chemical mechanisms of membrane remodeling in cells. This year, we focused on enveloped viral assembly and cell entry, and the biology of extracellular vesicles.

Delta variant SARS-CoV-2 spike protein causes viral aggregation

Genetic variants of the severe acute respiratory syndrome coronavirus 2, SARS-CoV-2, continue to evolve as the virus circulates worldwide, and each variant holds the potential to evade acquired immunity and re-ignite the COVID-19 pandemic. SARS-CoV-2 is an enveloped RNA virus containing a single-stranded, positive-sense genome. Prominent, club-shaped spike glycoproteins (spikes) project from the viral envelope, mediating binding and fusion between the viral envelope and host-cell membranes to deliver the viral genome. Spikes are highly immunogenic, eliciting a robust neutralizing antibody response; the immunogen is encoded by the extremely efficacious mRNA vaccines. Given its essential role in viral entry and immunity, mutations occurring on the spike require careful genetic, structural, and functional surveillance.

The fully assembled, prefusion spike consists of a trimer of spike protomers, each of which is highly glycosylated. Proteolytic cleavage of the SARS-CoV-2 spike by furin during biosynthesis nicks the spike into two subunits, S1 and S2. The S1 subunit contains the receptor-binding domain (RBD), which can be in an up (receptor accessible) or down (receptor inaccessible) conformation, as well as the N-terminal domain



Joshua Zimmerberg, MD, PhD,
*Head, Section on Integrative
Biophysics*

Paul S. Blank, PhD, *Staff Scientist*

Svetlana Glushakova, MD, PhD,
Staff Scientist

Matthias Garten, PhD, *Visiting
Fellow*

Irene Jimenez Munguia, PhD,
Visiting Fellow

Yuto Kegawa, PhD, *Visiting Fellow*

Hang Waters, MS, *Biologist*

Jennifer Petersen, PhD, *Electron
Microscopist*

Elena Mekhedov, MA, *Contractor*

Tatyana I. Tenkova-Heuser, PhD,
Contractor

John E. Heuser, MD, *Senior
Biophysicist*

Glen Humphrey, PhD, *Guest
Researcher*

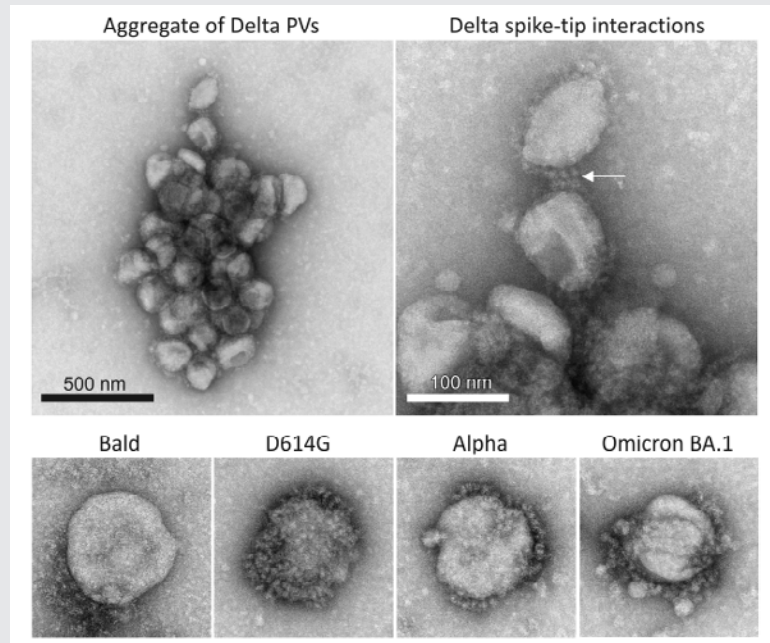
Adriana Golding, PhD,
*Postdoctoral Intramural
Research Training Award Fellow*

Garrett Tisdale, BA,
Postbaccalaureate Trainee

Jaquin Wallace, MS,
Postbaccalaureate Trainee

FIGURE 1. SARS-CoV-2 virus entry particles

Negative stain electron microscopy images of PVs bearing various SARS-CoV-2 spike proteins show that PVs bearing Delta spikes assemble into unique aggregates compared with PVs bearing other spike variants (D614G, Alpha, Omicron BA.1) or PVs bearing no spike protein (Bald), which exist mainly as single PVs. Interactions between the tips of Delta spike proteins may mediate Delta-specific aggregation.



(NTD) and two C-termini. S1 caps the S2 subunit, which harbors the membrane-fusion machinery. The spike is highly flexible owing to a hinged stalk, helping the RBD bind to angiotensin converting enzyme II (ACE2), the host-cell receptor. Once attached to the host cell by ACE2 binding, a second cleavage event occurs in S2 by a host-cell protease, either TMPRSS2 at the cell surface or cathepsin in the endosomal membrane, depending on cell type, protease availability, and viral variant. Upon S2 cleavage, the fusion peptide undergoes large conformational changes, which drive fusion between membranes.

Millions of SARS-CoV-2 genomic sequences have been cataloged since the original strain emerged in Wuhan, China, in December 2019. Amino acid substitutions or deletions in the spike that impart fitness benefits to the virus have evolved and sometimes converged independently in different geographical locations, giving rise to several variants designated Variants of Concern (VOCs). By May 2020, the D614G variant, designated Pango lineage B.1, rapidly supplanted the original Wuhan strain globally, and the D614G substitution is present in all subsequent variants. Next, the VOC Alpha (lineage B.1.1.7) emerged in the UK and became dominant worldwide by early 2021.

The Alpha variant prevailed until it was outcompeted by VOC Delta, a member of the B.1.617 lineage, which arose in India and became globally dominant in mid-2021. The Delta variant was considerably more transmissible than Alpha, infected individuals faster, produced earlier detection by PCR test with higher viral load, and was more pathogenic. The Delta variant's spike displayed a different combination of mutations than Alpha. Functional consequences of spike mutations can be tested using engineered mimics of enveloped viruses, called pseudotyped viral particles (PVs). PVs are produced by co-transfecting producer cells with plasmids encoding a capsid protein from a parental virus (typically a retrovirus or arbovirus capsid), the spike glycoprotein of interest, and a reporter gene that produces a fluorescent or luminescent protein signal upon host cell entry. The capsid core buds efficiently from the producer cell while incorporating the heterologous

spike and encapsulating the reporter gene. To search for an ultrastructural correlate to the increased initial infection rate of Delta PVs, we took advantage of our recent experience with PVs packaged with various SARS-CoV-2 spike variants into target cells, reported last year. PVs bearing the Delta spike (Delta PVs) drove markedly faster initial infection, and greater infection overall, than other variants, a phenotype shared by Delta SARS-CoV-2. Using four independent techniques (negative stain TEM, flow cytometry, NTA, and cryo-EM), we demonstrated that PVs bearing the Delta variant spike or the closely related Delta sublineage Delta AY.4.2 spike aggregate, whereas PVs bearing other spike variants and Bald PVs do not aggregate. Given that the PVs were prepared in parallel, handled under identical conditions, and factors that could promote aggregation such as pH changes, freeze-thaw cycles, and high-speed ultracentrifugation were avoided, the observed aggregation of Delta PVs most likely reflects a unique property of the Delta and Delta AY.4.2 spikes. The observation that Delta and Delta AY.4.2 PVs continued to aggregate in solution while stored at 4°C suggests that aggregation occurs after budding from the producer cell; however, interaction between PVs could also initiate during biosynthesis and budding from the producer cell [Reference 2].

Unlike SARS-CoV-2, which buds into the ER–Golgi intermediate compartment (ERGIC) during final assembly, retroviruses, including MLV, generally bud directly from the plasma membrane (but not always). Thus, it is not known when or whether Delta variant SARS-CoV-2 aggregates, and this is currently being investigated. Delta SARS-CoV-2 could aggregate while budding into the ERGIC, during egress through alkalized lysosomal organelles, after egress in the extracellular milieu, or even on the target-cell plasma membrane. The ability to aggregate may depend on the concentration of viral particles in each environment. The fact that Delta PVs continue to aggregate while stored at 4°C is consistent with a mass action mechanism for Delta PV aggregation. Measurements of the rate of aggregation of Delta PVs at temperatures ranging up to physiological temperature could shed light on the thermodynamic properties of Delta aggregation, and advance understanding of the mechanism of aggregation. Furthermore, to produce PVs, a 19 amino-acid C-terminal truncated version of each variant spike was expressed, which has been shown to increase the amount of spike incorporated into the PV envelope and raise PV infectivity. Truncations in the cytoplasmic tail could modify properties of the spike ectodomain structure and function. Although each variant spike possessed the same truncation, it is not impossible that the truncation uniquely affected the Delta spike ectodomain, conferring the aggregation property.

Examples of Delta PVs with apparent spike tip interactions were observed by negative staining, but these interactions did not extend to lateral aggregation of spike proteins on the surface of a PV. Ongoing cryo-electron tomography studies will reveal the nature of the interactions between aggregated PVs. Spike-mediated aggregation differs from antibody-driven aggregation of virions expected from polyvalent neutralizing antibodies, as those binding constants are expected to be much stronger. Thus, virions would be tightly packed and not likely to disaggregate at the cell surface. The spike tip interactions are more likely to come apart upon surface binding and receptor competition for the RBD at the tip of the spike.

Analysis of the mutations present in the Delta and Delta AY.4.2 spikes compared with other variants may provide clues as to their unique property to aggregate. Delta and Delta AY.4.2 amino-acid sequences are similar, except for four additional substitutions in Delta AY.4.2 (T95I, Y145H, A222V, and K458R). T95I is also present on the Omicron BA.1 spike, and residue Y145 is also substituted in Omicron BA.1 (Y145D). A222V and K458R are unique to Delta AY.4.2. Given that the presence of these mutations in Delta AY.4.2 does not abrogate or enhance the aggregation of Delta AY.4.2 compared with Delta, they appear to have no effect on aggregation.

Most of the other mutations on the Delta and Delta AY.4.2 spikes are shared with other variants. The D614G mutation is present in all variants, the substitution L452R, located in the RBD, is present in the Kappa variant (B.1.617.1), and a similar substitution L452Q occurs in the Lambda variant (C.37). A second RBD substitution at T478K is also found in Omicron BA.1 and BA.2. The mutation P681R is present in Kappa, and P681H exists in Alpha, Omicron BA.1, BA.2, and Mu (B.1.621). Substitution mutation D950N is also shared with Mu. Because the non-Delta variants studied here do not aggregate, it is unlikely that any of their Delta-shared mutations can be aggregation-dominant. There are, however, three residues, E156, F157, and R158, in the NTD of Delta and Delta AY.4.2 that are uniquely and identically mutated: substitution E156G, and deletions at F157 and R158. It is possible that these three mutations in the NTD are sufficient to bestow the aggregation property alone or in the context of the other Delta mutations.

The clustering of Delta PVs could account for the faster and larger initial infection observed in entry assays with the Delta PVs. Because the number of spike trimers is larger on an aggregate comprising multiple PVs, and the cell-surface contact area is larger for any collision between the aggregate and a target cell, the effective on-rate for aggregate binding should be larger, resulting in faster binding. Furthermore, the avidity of the aggregate for the target cell would be enhanced manyfold owing to the many potential binding partners on a single contacting surface. Moreover, the increased dwell time at that contact area will allow for diffusional and conformational motions of proteins and lipids to increase the chance of membrane fusion, as these factors are important for avoiding hemifusion and promoting full fusion. All these factors should lead to the relatively higher initial rate of PV entry into target cells from aggregated PVs. Whether or not aggregates could enable the simultaneous delivery of multiple copies of entry reporter genes to target cells is not clear, given that the PVs need not display ACE2 and may thus not fuse with each other, even in an endosome; thus, each virus in an aggregate may have to independently fuse with the endosomal membrane to place its genes to that cell's cytoplasm. Implicitly, there would be more overall binding events for unaggregated PVs, each at another site. However, if the probability of PV entry was low owing to unbinding, then the factors to increase PV avidity, discussed above, would tend to increase overall fusion and its rate.

In summary, an ultrastructural analysis of retrovirus pseudotyped viral particles bearing SARS-CoV-2 spike variants led to a serendipitous discovery of significant aggregation when the Delta variant spike was expressed, but not upon expression of three other variant spikes [Reference 2]. Viral aggregation can impart fitness benefits by protecting virions from environmental hazards and by effecting simultaneous delivery of multiple viral genomes, or collective infection. Notably, collective infection can favor initial infection in some contexts. Likely, the size of an aggregate, and therefore the number of virions per aggregate, is important. If an aggregate is too large, subsuming many virions, it would effectively reduce infectious units below a threshold. On the other hand, if there are too few virions in an aggregate, the benefit of collective infection is not gained. The unique property of the Delta spike to aggregate PVs may underlie the faster infection seen by Delta PVs. Furthermore, spike-mediated aggregation could be part of the molecular mechanism by which Delta variant SARS-CoV-2 achieves increased transmissibility and faster infection with a higher viral load. The continued aggregation of PVs over time indicates that clustering may be mediated by interactions between spike tips, which in turn may indicate an adhesivity of the viral surface recognized by the immune system, thus altering the balance of host antiviral response towards inflammation.

Control of RNA packaging into extracellular vesicles by CD47

Extracellular vesicles (EVs) are vesicles released by all cells that are loaded with specific cargoes, and they

mediate intercellular communication in physiological and disease states. CD47 is a signaling receptor for thrombospondin-1 (TSP1), the secreted extracellular matrix protein, which regulates a variety of cellular responses. CD47 is a component of extracellular vesicles (EVs) released by various cell types, including T cells. CD47 loads EVs with specific subsets of coding and non-coding RNAs that impart functional effects on target cells, including endothelial cells. How CD47 controls the loading of specific RNAs into EVs is unknown. Previous studies showed that EVs produced by CD47-knockout (KO) T cells lost the ability to modulate gene expression and signaling in recipient T cells and vascular endothelial cells, suggesting that the packaging of RNAs into specific subpopulations of EVs is regulated by CD47.

We examined the mechanisms by which CD47 directly or indirectly regulates which RNAs are packaged into T cell EVs. Mass spectrometry, biochemistry, light and electron microscopy, pharmacological manipulation, and genetic analyses were used to parse the mechanism by which CD47 controls the loading of EVs with specific RNAs. It was demonstrated that CD47 regulates nuclear/cytoplasmic transport of m7G-capped RNAs and their abundance in EVs via a physical interaction of CD47 and its cytoplasmic signaling adapter ubiquitin-1 with exportin-1 and several regulators of its nuclear export complex. The CD47/ubiquitin-1 complex regulates intracellular trafficking of capped miRNAs and mRNAs and their trafficking into EVs. These results establish TSP1/CD47 signaling as a regulator of nuclear/cytoplasmic RNA trafficking and the subsequent packaging and release of a subset of 5-7-methylguanosine-modified (m7G) RNAs in EVs. The relevance of m7G-cap-dependent RNA trafficking to the physiological functions of CD47 in cardiovascular disease, aging, cancer, and infection remain to be investigated [Reference 5].

Regulation of umbilical cord endothelial cells by extracellular vesicles

During pregnancy, umbilical cord endothelial cells engage in bidirectional communication that regulates angiogenesis. EVs play important roles in cell-cell communication by transferring bioactive molecules, including mRNAs and miRNAs, into recipient cells. EVs are taken up by recipient cells, where they modulate the fate of those cells, including altering gene expression. EVs produced by a given cell type are heterogeneous in their RNA content, but it is unclear how specific EV surface markers relate to their functional effects on target cells. Our previous work established that EVs bearing CD63, MHC-I, or CD47 surface markers contain distinct noncoding RNA populations. Thus, when applied to endothelial cells, EVs produced by wild-type (WT) T cells (that express CD47) compared with mutant T cells lacking CD47, lead to differential modulation of VEGF-induced cell proliferation, tube formation, and VEGFR2 phosphorylation, findings that reflect the divergent RNA compositions of the EVs produced by these different cells, leading to distinct functions in target cells [Reference 6].

In this project, we compared the effects on Human Umbilical Vein Endothelial cell (HUVEC) gene expression of CD63⁺ and MHC-I⁺ EV subsets derived from WT T cells and CD47-KO T cells. The goal was to identify several functional gene families in HUVECs that are differentially regulated by the two EV populations. The present study uses biochemical approaches, genetic sequencing, and electron microscopy to evaluate the EVs isolated from the cell types. The study reveals that CD63⁺ and MHC-I⁺ EVs from CD47-KO T cells are enriched in small non-coding RNAs compared with EVs from WT T cells. CD47-deficient T cells secrete more CD63⁺ and MHC-I⁺ EVs, but MHC-I⁺ EVs are selectively taken up more by HUVECs. Transcriptomics analysis of endothelial cells treated with CD63⁺ or MHC-I⁺ EVs showed surface marker- and CD47-dependent changes in gene expression in the target cells. Gene-set enrichment analysis identified effects of T-cell EVs on VEGF and inflammatory signaling, cell cycle, and lipid and cholesterol metabolism. Thus, subsets of T-cell EVs differentially regulate

endothelial cell metabolism and inflammatory and angiogenic responses. Future studies will focus on identifying mechanisms by which CD63 and MHC-I regulate EV uptake and the distinct functional effects of MHC-I⁺ EVs and CD63⁺ EVs on endothelial cells and other types of target cells. These studies may identify additional functions of EVs produced by T cells that regulate physiological angiogenic and inflammatory pathways and antitumor immunity.

Publications

1. Jiménez-Munguía I, Beaven AH, Blank PS, Sodt AJ, Zimmerberg J. Interferon-induced transmembrane protein 3 (IFITM3) and its antiviral activity. *Curr Opin Struct Biol* 2022 77:102467.
2. Petersen JD, Lu J, Fitzgerald W, Zhou F, Blank PS, Matthies D, Zimmerberg J. Unique aggregation of retroviral particles pseudotyped with the delta variant SARS-CoV-2 spike protein. *Viruses* 2022 14:1024.
3. Xu M, Pradhan M, Gorshkov K, Petersen JD, Shen M, Guo H, Zhu W, Klumpp-Thomas C, Michael S, Itkin M, Itkin Z, Straus MR, Zimmerberg J, Zheng W, Whittaker GR, Chen CZ. A high throughput screening assay for inhibitors of SARS-CoV-2 pseudotyped particle entry. *SLAS Discov* 2022 27:86–94.
4. Pfeiffer A, D Petersen JD, Falduto GH, Anderson DE, Zimmerberg J, Metcalfe DD, Olivera A. Selective immunocapture reveals neoplastic human mast cells secrete distinct microvesicle- and exosome-like populations of KIT-containing extracellular vesicles. *J Extracellular Vesicles* 2022 11:e12272.
5. Kaur S, Saldana AC, Elkahlon AG, Petersen JD, Arakelyan A, Singh SP, Jenkins LM, Kuo B, Reginald B, Jordan DG, Tran AD, Wu W, Zimmerberg J, Margolis L, Roberts DD. CD47 interactions with exportin-1 limit the targeting of m7G-modified RNAs to extracellular vesicles. *J Cell Commun Signal* 2022 16:397–419.
6. Kaur S, Elkahlon AG, Petersen JD, Arakelyan A, Livak F, Singh SP, Margolis L, Zimmerberg J, Roberts DD. CD63⁺ and MHC Class I⁺ subsets of extracellular vesicles produced by wild-type and CD47-deficient Jurkat T cells have divergent functional effects on endothelial cell gene expression. *Biomedicines* 2021 9:1705.

Collaborators

- Sergei Akimov, PhD, *Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, Russia*
- Oleg Batishchev, PhD, *Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, Russia*
- Andrew Beaven, PhD, *Unit on Membrane Chemical Physics, NICHD, Bethesda, MD*
- Christopher Karl Ernst Bleck, PhD, *Electron Microscopy Core Facility, NHLBI, Bethesda, MD*
- Wendy Fitzgerald, BA, *Section on Intercellular Interactions, NICHD, Bethesda, MD*
- Vadim Frolov, PhD, *Universidad del País Vasco, Bilbao, Spain*
- Timur Galimzyanov, PhD, *Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, Russia*
- Daniel Goldberg, MD, PhD, *Washington University St. Louis, St. Louis, MO*
- Samuel T. Hess, PhD, *University of Maine, Orono, ME*
- Sukbir Kaur, PhD, *Laboratory of Pathology, Center for Cancer Research, NCI, Bethesda, MD*
- Leonid Margolis, PhD, *Section on Intercellular Interactions, NICHD, Bethesda, MD*
- Doreen Matthies, PhD, *Unit on Structural Biology, NICHD, Bethesda, MD*
- Ana Olivera, PhD, *Mast Cell Biology Section, NIAID, Bethesda, MD*
- Richard Pastor, PhD, *Laboratory of Membrane Biophysics, NHLBI, Bethesda, MD*

- Annika Pfeiffer Daniels, PhD, *Laboratory of Allergic Diseases, NIAID, Bethesda, MD*
- Thomas S. Reese, MD, *Laboratory of Neurobiology, NINDS, Bethesda, MD*
- David D. Roberts, PhD, *Laboratory of Pathology, Center for Cancer Research, NCI, Bethesda, MD*
- Santimukul Santra, PhD, *Pittsburg State University, Pittsburg, KS*
- Anna Shnyrova, PhD, *Universidad del País Vasco, Bilbao, Spain*
- Alexander J. Sodt, PhD, *Unit on Membrane Chemical Physics, NICHD, Bethesda, MD*
- Niraj H. Tolia, MD, PhD, *Laboratory of Malaria Immunology and Vaccinology, NIAID, Bethesda, MD*
- Gary R. Whittaker, PhD, *College of Veterinary Medicine, Cornell University, Ithaca, NY*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*
- Wei Zheng, PhD, *Division of Preclinical Innovation, NCATS, Bethesda, MD*
- Fei Zhou, PhD, *Unit on Structural Biology, NICHD, Bethesda, MD*

Contact

For more information, email joshua.zimmerberg@nih.gov or visit <http://irp.nih.gov/pi/joshua-zimmerberg>.

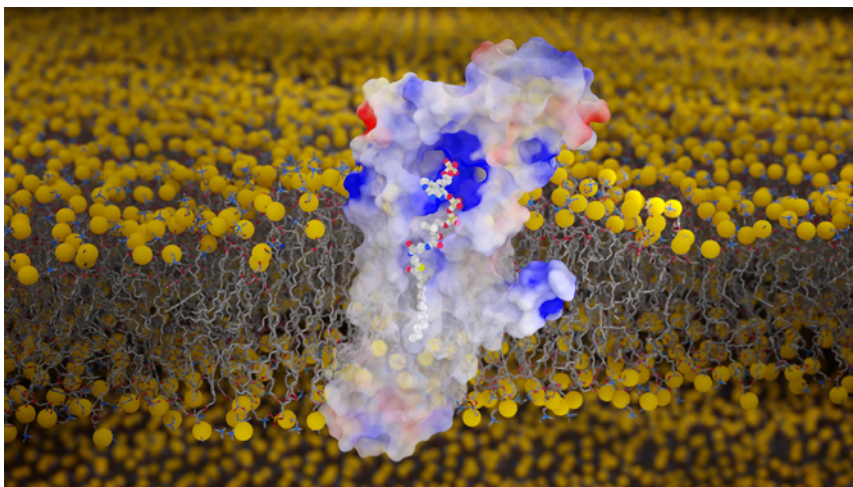
Colophon

About the Cover Image

Visualization of fatty acyl CoA bound to membrane-embedded human DHHC20.

Image created by Jeremy Swan for the [Banerjee lab](#) using [UCSF ChimeraX](#) and [Blender](#).

Read "[Visualizing a Key Protein Modification Process](#)" to learn more about this research.



Production Credits

Managing and Scientific Editor: Birgit An der Lan, PhD
Design and Layout: Nichole Swan
Application Development: Loc Vu
Head, Computer Support Services Core: Ryan Dale, PhD

For further information, contact:

Chris J. McBain, PhD, Scientific Director
Eunice Kennedy Shriver National Institute of Child Health and Human Development
10 Center Drive, Room 10D39
Bethesda, MD 20892

Phone: 301-594-5984

Email: NICHDsd@mail.nih.gov

Fax: 301-480-6480

Website: <https://www.nichd.nih.gov/about/org/dir>

An online version of this report can be found at <https://annualreport.nichd.nih.gov>.

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health