Regulation of zebrafish embryogenesis by the RNA-binding protein Igf2bp3

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Declaration

This thesis is my original work and has been written by me, and all sources or collaboration have been acknowledged where applicable.

This thesis has not been submitted for consideration of any degree in any other university previously

Abstract

During development, establishment of the germline is an early event that is essential for ensuring the future embryo has reproductive capability. Precursors of the germline originate as primordial germ cells, which are specified far from their destination where the gonads will develop, and these cells require cues to migrate, proliferate and sustain their fates.

In zebrafish, development of the germline is controlled by maternally contributed factors known as germplasm, a collection of RNA that is tightly regulated by proteins, forming ribonucleoprotein complexes, and cells receiving this material are fated to become the PGCs; and misregulation of the germplasm leads to defects in the germline.

In this study, we have examined the role of the *igf2bp* family of RNA-binding proteins, which are implicated in many functions in RNA metabolism, such as localisation, stability and translation, with disruption of these genes *in vivo* leading to developmental consequences in the soma. Using transgenic insertion alleles and Cas9 mutant alleles for *igf2bp3,* we characterised the role of *igf2bp3* in zebrafish development.

Contrary to previous studies, maternal-zygotic *igf2bp3* mutants did not show any overt defects in the soma, developing to adulthood with no apparent disabilities. However, maternal *igf2bp3* mutants are developmentally delayed around blastula, exhibiting an expanded yolk syncytial layer, and otherwise continue developing with no further defects. Moreover, these maternal mutants also exhibit an aberrant germline, with PGCs displaying abnormal behaviour and are depleted in the gonadal ridge, leading to a skewed sex ratio.

These results point to a novel function for *igf2bp* genes in early development and germline formation, and future studies in uncovering the mechanism may show new insights for Igf2bp3 in regulating germplasm, by regulating the fates of directly bound transcripts, or as part of a ribonucleoprotein complex.

List of abbreviations

Abbreviations for model organisms

C. elegans	*Caenorhabditis elegans*
Drosophila	*Drosophila melanogaster*
Xenopus	*Xenopus laevis, Xenopus Africana*
Mice/Mouse	*Mus musculus*
Yeast	*Saccharomyces cerevisiae, schizosaccharomyces pombe*
Zebrafish	*Danio rerio*
Medaka	Oryzias latipes

Further abbreviations

5'/3' UTR	5'/3' untranslated region
APS	ammonium persulphate
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
Cas9	CRISPR-associated protein 9
CDS	coding sequence
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CRISPR	clustered regularly interspaced short palindromic repeats
DAPI	4′,6-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DTT	1,4-dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ENU	N-ethyl-N-nitrosourea
EVL	enveloping layer
GFP	green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpf	hours post fertilisation
HRP	horseradish peroxidase
kb	kilobase
kDa	kilodalton
LTR	long terminal repeat

m6A	N^6-methyladenosine
MAB	maleic acid buffer
MBT	midblastula transition
MMLV	moloney murine leukaemia virus
mRNA	messenger RNA
MZ	maternal zygotic
MZT	maternal zygotic transition
NLS	nuclear localisation sequence
nt	nucleotides
NTMT	alkaline phosphatase buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC	primordial germ cell
q(RT)-PCR	quantitative (reverse-transcription) polymerase chain reaction
RBP	RNA-binding protein
RIPA	radio immunoprecipitation assay
rNTPs	ribonucleoside triphosphate
RT-PCR	reverse-transcription polymerase chain reaction
RNA	ribonucleic acid
RNP	ribonucleoprotein
SDS	sodium dodecyl sulphate
sgRNA	single guide RNA
SSC	saline sodium citrate
TAE	tris acetate EDTA
TBS	tris buffered saline
Tg	transgene
TPM	transcripts per million
tRNA	torula yeast RNA or transfer RNA
YSL	yolk syncytial layer
YSN	yolk syncytial nucleus/nuclei
ZGA	zygotic genome activation

1. Chapter I - Introduction

In multicellular, eukaryotic organisms, development is a concerted process where a single diploid cell, the zygote, undergoes repeated cellular divisions to produce an embryo. This process is initially rapid and synchronous, and later, replication slows down as cells begin to differentiate and specialise into specific cell lineages that give rise to germ layers, paving the way for the development of organs and asymmetry in the embryo in a process known as gastrulation.

How cells in the embryo receive these instructions to diverge from the fates of surrounding cells is a topic of great interest, and significant advances have shown that some of these instructions have been established before the first cell division. An embryo uses many strategies to send messages to induce cells to adopt specific fates; using inherited components accompanying the nucleus, such as RNAs, proteins and a transport network that moves these instructions to where and when they are required. In some instances, extraembryonic signals can also induce cell specification.

As continuation of the life cycle is fundamental to evolution and perpetuation of a species, a unique lineage is specified early in development, known as primordial germ cells (PGCs), to facilitate this function. These cells are precursors of the future gametes and many aspects of their biology are topics of study, such as its proliferation, differentiation and migration, which has future implications in understanding diseases including infertility and germline tumours.

In this introduction, we will summarise the roles of inheritance in development, the importance of maternally inherited RNAs in early development of the germline, how the germline is regulated by RNA and RNA-binding proteins, and the use of zebrafish as a model to study germline development.

1.1. Parental contributions to embryonic development

In sexually reproducing animals, development of the zygote is initiated by fertilisation, in which the haploid gametes fuse to produce the diploid zygote. This process consists of more than simple merging of the sperm nucleus to the oocyte pronucleus, but also the inheritance of both maternal and paternal components to drive development in an organised and timely manner.

Paternally, few biological components are transmitted to the next generation, with the major components being the centrioles and the paternal DNA (Sathananthan *et al.*, 1996), although sperm RNA has been implicated in transgenerational inheritance (Ostermeier *et*

al., 2004) and appears to play a role in imprinting (Gapp *et al.*, 2014). This RNA is both coding (Ostermeier *et al.*, 2004) and non-coding, and require endogenous chemical modifications to be fully functional (Zhang *et al.*, 2018).

Paternal inheritance also extends to the methylome, as the paternal and maternal haploid genomes differ drastically, with the sperm methylome being inherited in early embryogenesis (Jiang *et al.*, 2013), a state that is hypermethylated in comparison to the oocyte methylome (Popp *et al.*, 2010). Together with modifications with retained histones in sperm DNA, both the methylome and the pattern of retained histones seem to have a functional outcome in development. (Denomme *et al.*, 2017; Ben Maamar *et al.*, 2018)

In contrast to paternal contribution, maternal inheritance is a well-established phenomenon that has been observed from invertebrates to mammals to control phenotypes such as egg colourations over a hundred years ago (Toyama, 1913) and several ways in which maternal inheritance has been noted in embryogenesis include polarity, patterning, oocyte development and the germline. And while the maternal genome is known to play a role in regulating development (Reik *et al.*, 1993), we shall focus on other maternally transmitted factors, such as RNAs and proteins.

Maternally deposited factors such as protein and RNAs are of particular importance during early embryogenesis, as the zygotic genome is initially transcriptionally quiescent, and maternal factors allow initial cell divisions to take place at a rapid and unrestricted rate until the midblastula transition (MBT), where zygotic transcription begins and is marked asynchrony of cell divisions, introduction of gap phases in the cell cycle and cell motility in a process that is controlled by the nucleocytoplasmic ratio (Kane and Kimmel, 1993).

In addition to activation of the zygotic transcription program, maternal proteins and RNAs start to be degraded in the soma until no maternal products remain and the embryo depends solely on zygotic transcription. This is known as the maternal-to-zygotic transition, and is a shared phenomenon from invertebrates to mammals (shown in Figure 1-1). In zebrafish, the timely progression of embryogenesis during the maternal-to-zygotic transition requires the degradation of maternal products and activation of the zygotic genome. This is in turn regulated by the availability of free histones (Joseph *et al.*, 2017) and transcription factors that regulate zygotic transcription and maternal degradation. For example, morpholino injections directed against maternally deposited transcription factors Pou5f3 (Joseph *et al.*, 2017), Nanog (C. Xu *et al.*, 2012) and Sox19b (Hu *et al.*, 2012) lead to embryonic arrest after the MBT. This embryonic arrest is correlated with reduced degradation of maternal RNA and failure to activate zygotic transcription in a process that is likely linked to increasing chromatin accessibility of zygotic genes (Pálfy *et al.*, 2019).

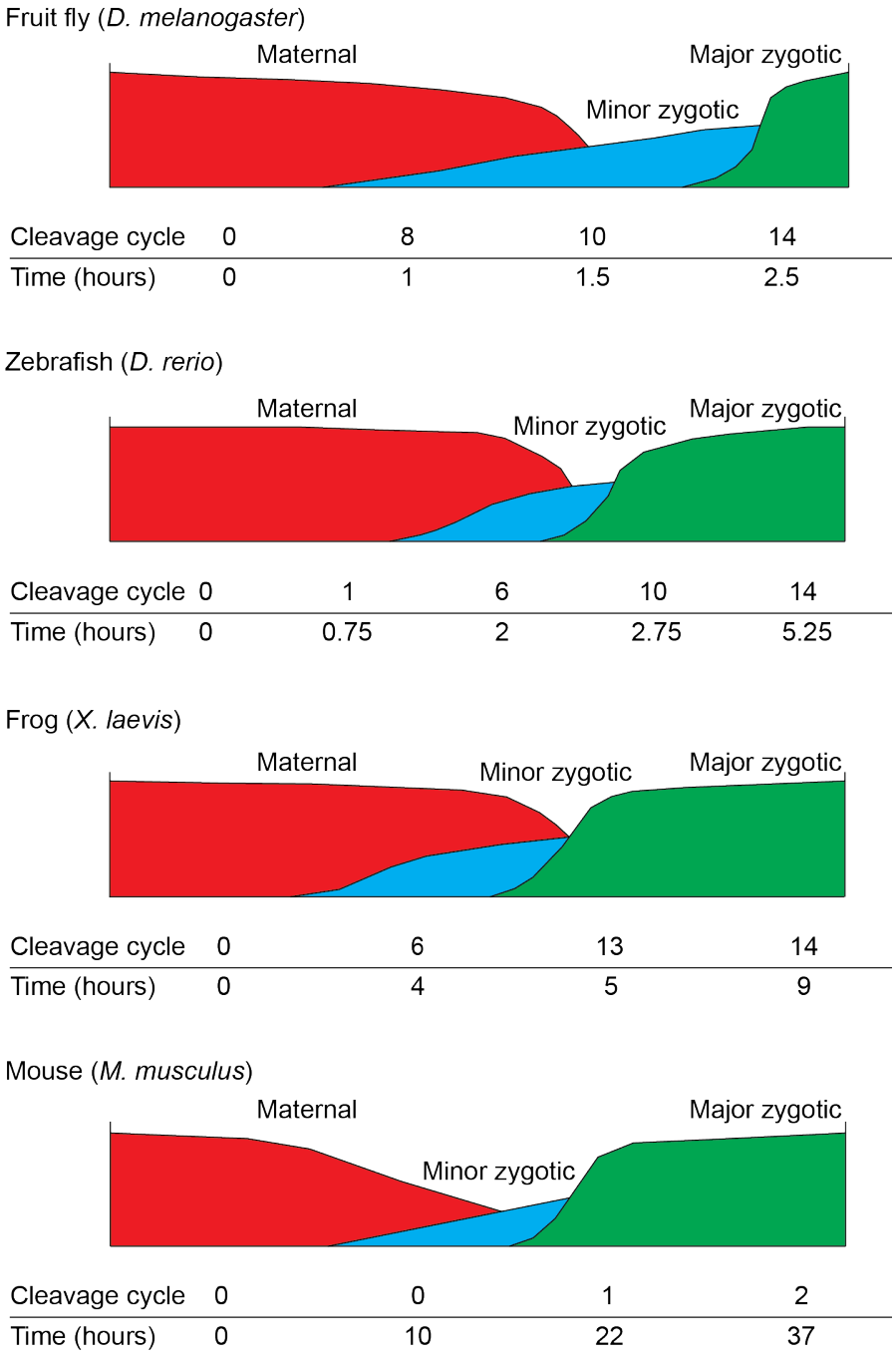


Figure 1-1. The maternal-to-zygotic transition illustrated in model organisms. The MZT in invertebrate (Drosophila), zebrafish, amphibian (Xenopus) and mammalian (mouse) models are illustrated, red area represents maternal transcription profile, and the blue and green areas represent the minor and major waves of zygotic transcription respectively. Adapted from Figure 2 from (Tadros and Lipshitz, 2009).

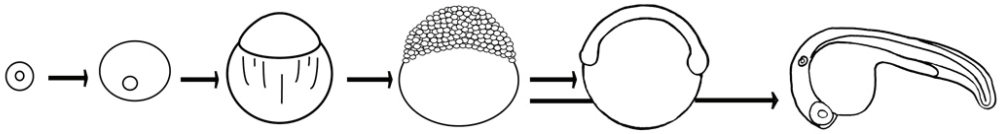
Maternal deposition is therefore required for timely progression of embryogenesis, and is not restricted to transcription factors. Further maternal factors are also well characterised, in *Ciona intestinalis*, maternal β-catenin in the vegetal hemisphere of the embryo suppresses genes expressed in the animal sphere and activates the transcription factor *FoxD* to specify

mesendodermal fates (Tokuhiro *et al.*, 2017), a mechanism that is also conserved in sea urchins (Logan *et al.*, 1999).

Evidence for maternal deposition for early patterning and axial coordination were also identified in *Drosophila*, where early works performed by (Bownes and Sander, 1976) showed that irradiation of the anterior pole of the embryo results in loss of the anterior axis. Later works by (Mlodzik, Fjose and Gehring, 1985; Lehmann and Nüsslein-Volhard, 1987; Driever and Nüsslein-Volhard, 1988; Wang and Lehmann, 1991) showed that anterior-posterior patterning of the *Drosophila* embryo is determined by maternal deposition of the *bicoid* and *nanos* mRNAs respectively, which, when translated during early cleavage, act to antagonise the translation of *hunchback* and *caudal* mRNAs, forming a concentration gradient of morphogens.

Axial patterning by maternal deposition is not exclusive to static depositions of RNAs in invertebrates, and active movement of mRNA to specific cells can also determine axis development. For example, the zebrafish dorsal axis is defined by the localisation of *sqt* mRNA (Gore *et al.*, 2005) in a microtubule-dependent process that is initiated by egg activation (Gore and Sampath, 2002).

Aside from axis specification, maternal deposition are also required much earlier for development, and some of these genes are exclusively required for development via maternal control (known as maternal effect genes), shown in Figure 1-2. The function of these maternal genes are critical for development, for example, the mouse maternal effect gene *Zar1* is crucial for oocyte-to-embryo transition and *Zar1*$^{-/-}$ oocytes cannot proceed beyond early cleavage (Wu *et al.*, 2003). Similarly, the zebrafish translational repressor, Ybx1, demonstrates a maternal effect and embryos fail to gastrulate (Kumari *et al.*, 2013), and *ybx1*$^{-/-}$ oocytes exhibit defective maturation and egg activation (Sun *et al.*, 2018), and the zebrafish *futile cycle* mutant, which cannot assemble mitotic machinery and produces anucleated cells (Dekens *et al.*, 2003).



Germ cell/oocyte development	Egg activation	Cleavage	Epiboly	Body plan
bucky ball *nanos* *over easy* *ruehrel* *souffle* *sunny side up* *zili*	*brom bones* *claustro* *emulsion* *jumpstart* *under repair*	*acytokinesis* *atomos* *aura* *barrette* *bo peep* *cellular atoll* *cellular island* *cobblestone* *dicer* *futile cycle* *golden gate* *indivisible* *irreducible* *kwal* *nebel* *screeching halt* *waldo* *weeble*	*bedazzled* *betty boop* *poky* *slow* *pou2*	*blistered* *ichabod* *hecate* *mission impossible* *pollywog* *pou2* *pug* *tokkaebi*

Figure 1-2. Maternal effect genes affecting zebrafish development at their respective stages. Genes identified with a maternal effect via genetic screens at specific stages of development. Figure adapted from Figure 1 from (Abrams and Mullins, 2009).

1.2. Development of the germline

During early embryogenesis, the small population of cells that are specified to become the primordial germ cells must undergo several processes into order to become the gametes. First, they must be able to maintain their potential to become committed to the germline lineage and be able to proliferate in order to populate their future niche in the gonadal ridge. Secondly, these unique, proliferating cells need to actively migrate from their site of specification to the gonadal ridge, and finally, the PGCs need to undergo meiosis and differentiation to become the future gametes: spermatogenesis for males, or oogenesis for females.

This inductive process can occur by two mechanisms, either by a preformative model, where early specification of germ cells occur by translocation of maternally provided determinants (Extavour and Akam, 2003). The second method for germ cell specification is known as epigenesis, where germ cells are specified later by inductive signals from surrounding tissues.

1.2.1. Germline development in zebrafish

Over the natural course of evolution, divergence has resulted in many variations in germline specification between species; and mechanistic studies in species utilising the preformative model of germline specification have shown that many core components have been conserved, such as in chick (Tsunekawa *et al.*, 2000), *Xenopus* (Whitington and Dixon, 1975), *Drosophila* (Illmensee and Mahowald, 1974), *C. elegans* (Sulston *et al.*, 1983) and teleost fish such as zebrafish. In these examples, specification of the PGC lineage requires fated cells to receive germplasm: cytoplasmic aggregates rich in mitochondria and granules of RNA and associated proteins.

Germplasm is a maternally contributed product that is deposited during oogenesis and accumulates in the Balbiani body during early oogenesis. The Balbiani body is an asymmetrically located, transient structure that is not membrane-bound and contains organelles such as the endoplasmic reticulum, mitochondria and Golgi components in addition to RNA and associated complexes (Marlow, 2010), its function and purpose is not clearly understood (Boke *et al.*, 2016), despite being highly conserved in evolution in models such as *Drosophila* (Cox and Spradling, 2003), mouse (Pepling *et al.*, 2007) and zebrafish (Bontems *et al.*, 2009).

In zebrafish, genetic screens identifying genes that perturb the formation of the Balbiani body appear to indicate that it is at least required to regulate embryo polarity (Dosch *et al.*, 2004; Marlow and Mullins, 2008; Gupta *et al.*, 2010; Escobar-Aguirre *et al.*, 2017) and organise the germplasm (Bontems *et al.*, 2009). It should be noted that the Balbiani body does not consist solely of germplasm, and many other mRNAs and proteins are collected in this structure prior to its dispersal, discussed later. Secondly, the criteria for a germplasm component are not defined solely by the gene, and the distinction between RNA and protein is important in this case: the protein for a gene may be a germplasm component but its corresponding mRNA may not. For example, the protein for the Balbiani body organiser, *bucky ball*, is a germplasm component. However, *bucky ball* mRNA is not colocalised with the germplasm and is degraded before the midblastula transition (Bontems *et al.*, 2009). An inverse example of this is *ddx4*, a germplasm gene where its mRNA is a germplasm component, but *ddx4* protein is not colocalised with germplasm and is quickly degraded from the soma (Wolke *et al.*, 2002). These are likely to be restrictive mechanisms to regulate the germline and prevent somatic cells from adopting aberrant fates, as overexpression of *bucky ball* produces a supernumerary number of primordial germ cells (Bontems *et al.*, 2009) and overexpression of *ddx4* can induce a meiotic cell fate (Medrano *et al.*, 2012).

Germplasm organisation occurs throughout oogenesis, and is initially deposited around the germinal vesicle during stage Ia oocytes. These begin to aggregate around the centrosome

during stage Ib to form the Balbiani body around the now established vegetal pole of the oocyte (Selman *et al.*, 1993). At the end of stage I oogenesis the Balbiani body is disassembled by Macf1 (Gupta *et al.*, 2010), and germplasm components previously assembled in the body are now distributed asymmetrically, such as *dazl* and *ddx4* in the vegetal pole and cortex respectively (Kosaka *et al.*, 2007). During this stage, organisation of the germplasm in the Balbiani body occurs concurrently with symmetry breakage, and the animal pole of the newly formed animal-vegetal axis begins to be populated with other mRNAs, such as *vg1* and *cyclin B* (Howley and Ho, 2000), shown in Figure 1-3A.

In stage II oogenesis, the distribution and accumulation of RNA at the animal and vegetal pole continues, for example, the germplasm RNA *ddx4* continues to be distributed cortically and *nanos* RNA becomes unlocalised from the vegetal cortex and other events in the oocyte begin to occur, such as formation of cortical granules (Selman *et al.*, 1993).

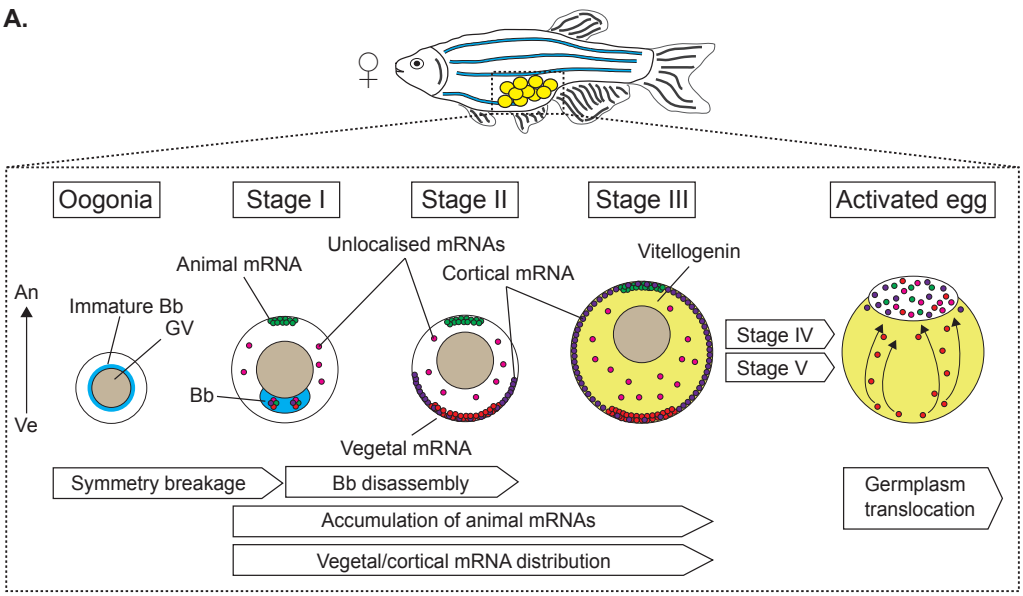
By stage III, the animal-vegetal polarity is further established by the formation of the micropylar cell at the animal pole (Selman *et al.*, 1993) and late pathway RNAs such as *bruno-like* become anchored to the vegetal cortex (Abrams and Mullins, 2009). Enlargement of the oocytes begins as vitellogenin accumulates and cortical granules begin to move towards the periphery of the oocyte (Selman *et al.*, 1993). In the final stages of oogenesis, Stage IV and V, the oocyte matures as the germinal vesicle migrates animally, followed by its disassembly and the formation of the polar body (Busby, Roch and Sherwood, 2010) and ends with the ovulation of a mature, fertilisable egg.

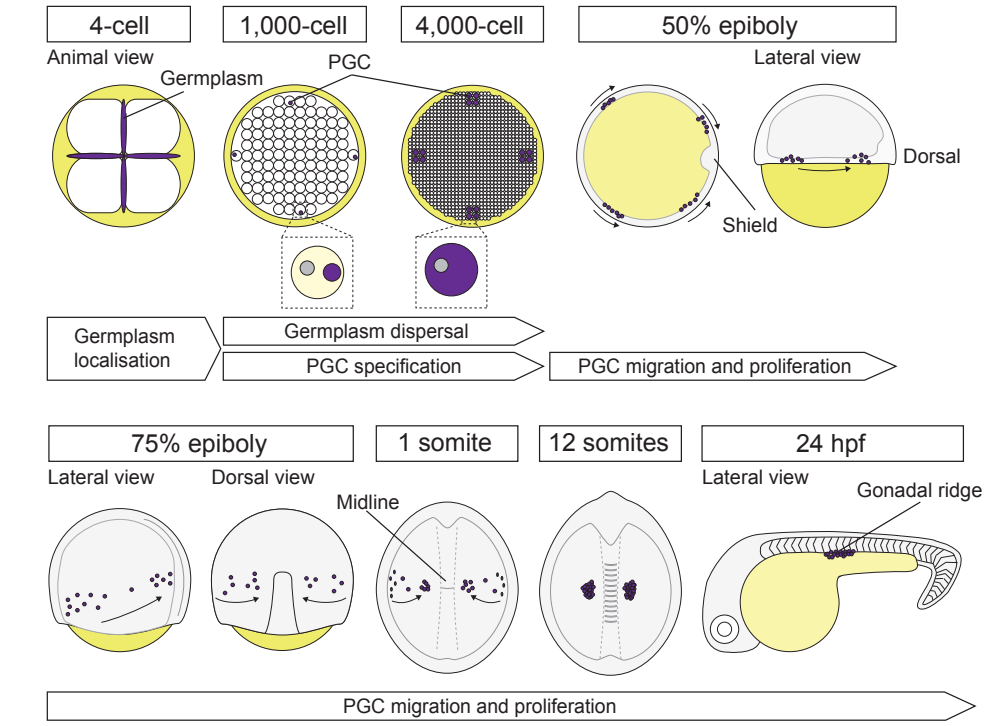
Following fertilisation, cytoplasm formation in the animal pole causes germplasm to be translocated to the blastoderm (Pelegri, Danilchik and Sutherland, 2017) and localise to the cleavage furrows of the 4-cell stage embryo (Yoon *et al.*, 1997; Braat *et al.*, 1999; Knaut *et al.*, 2000). The RNP granules present in the germplasm are homotypic (Eno, Hansen and Pelegri, 2019) and distinct from the cytoplasm from the cells receiving the germplasm, however, these structures dissolve and fill the cytoplasm during the end of the blastula stage to disperse their contents to multiple germline fated cells (Eno, Hansen and Pelegri, 2019). During gastrula, the PGCs proliferate and begin to converge dorsally towards the midline to form bilateral clusters around the trunk mesoderm (Weidinger, 1999) near the first somite. The majority of trailing clusters of PGCs continue to migrate up until the 6-somite stage, and posteriorly-located PGCs will continue migrating anteriorly until the 10-somite stage (Weidinger, 1999; Weidinger *et al.*, 2002) and ends by 24 hpf (Fig 1-3B).

The primary migratory cue for PGCs to move appears to be the chemokine *cxcl12a* and its cognate receptor *cxcr4b* (Molyneaux *et al.*, 2003), and disruption of a PGC's ability to sense or react to these migratory cues (Blaser *et al.*, 2006; Meyen *et al.*, 2015; Tarbashevich *et al.*, 2015) or inhibition of downstream signalling pathways (Dumstrei, 2004) is sufficient to

disrupt correct localisation. Many additional factors have been implicated in having a role in PGC migration, but crucially, loss of other germplasm components are required for correct migration, such as *dnd1* (Weidinger *et al.*, 2003), *nanos* (Köprunner *et al.*, 2001).

Upon conclusion of the migratory phase, the PGCs at the gonadal ridge remain stable during the first week of development, and exhibit dimorphic proliferation during the second week of larval development (Tzung *et al.*, 2015), where a subpopulation of larvae exhibit increased proliferation of the PGCs (Tzung *et al.*, 2015), and this appears to promote terminal differentiation into females (Tzung *et al.*, 2015; Ye *et al.*, 2019), as more PGCs are correlated with a female bias and vice versa (Fig 1-3C). Otherwise, the juvenile gonad remains bipotential until the 21 dpf but exhibits premature ovary-like features (Takahashi, 1977; Uchida *et al.*, 2002; von Hofsten and Olsson, 2005; Siegfried and Nüsslein-Volhard, 2008), which undergo degeneration to give rise to testis development (Uchida *et al.*, 2002).


A.
Oogonia
Stage I
Stage II
Stage III
Activated egg
An
Ve
Immature Bb
GV
Animal mRNA
Unlocalised mRNAs
Cortical mRNA
Vitellogenin
Bb
Vegetal mRNA
Stage IV
Stage V
Symmetry breakage
Bb disassembly
Accumulation of animal mRNAs
Vegetal/cortical mRNA distribution
Germplasm translocation


B.
4-cell
1,000-cell
4,000-cell
50% epiboly
Animal view
Germplasm
PGC
Lateral view
Dorsal
Shield
Germplasm localisation
Germplasm dispersal
PGC specification
PGC migration and proliferation
75% epiboly
1 somite
12 somites
24 hpf
Lateral view
Dorsal view
Midline
Lateral view
Gonadal ridge
PGC migration and proliferation

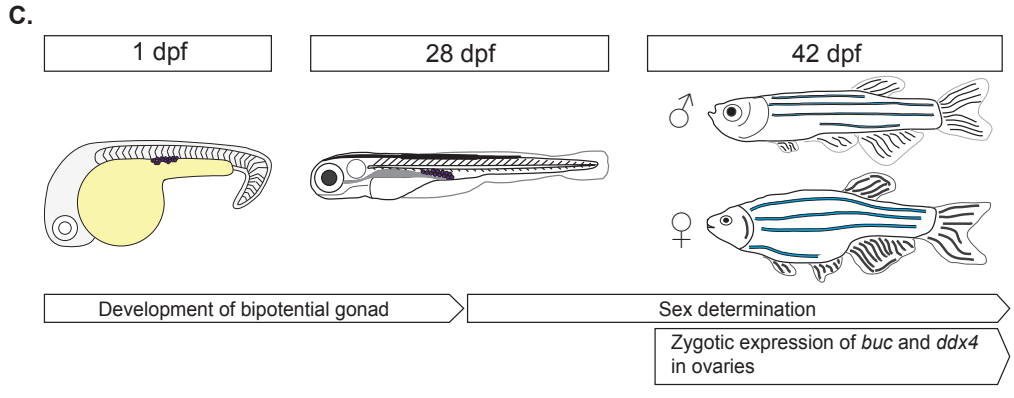

C.
1 dpf
28 dpf
42 dpf
Development of bipotential gonad
Sex determination
Zygotic expression of buc and ddx4 in ovaries

Figure 1-3. Overview of zebrafish germline development. A. Maternal RNA and germplasm aggregates asymmetrically during oogenesis. During oogonia, the immature Balbiani body (Bb) components surround the germinal vesicle (GV) and are asymmetrically located to the vegetal pole while the animal pole begins to be populated by mRNAs such as *ccnb1*. At stage I and II oogenesis, the Balbiani body forms and releases its contents in the vegetal pole, cortex, or disperses into the oocyte. Germplasm components are now localised vegetally (e.g. *dazl*), cortically (e.g. *ddx4)* or randomly (*nanos*). During stage II and III, continued organisation of the oocyte RNAs occurs and results in continued cortical spread of the RNA (e.g. *ddx4*), entry of late-pathway vegetal RNAs (e.g. *magoh*) or movement of RNA from the vegetal to animal pole (e.g. *buc*) until egg activation, where germplasm components begin to stream towards the blastodisc. **B. Germplasm localisation and movement in the first 24 hours of fertilisation.** Germplasm aggregates into the cleavage furrows generated in the first two cell divisions. Germplasm granules remain localised to four corners of the embryo inside a small number of cells until early gastrula, when the granules disperse uniformly into the cytoplasm of the specified PGCs. PGCs subsequently proliferate and migrate towards dorsally towards the midline until mid-somitogenesis, into the gonadal ridge of the 24 hpf embryo. **C. Development of the gonad and sex determination of the larval zebrafish.** PGCs located into the gonadal ridge begin to proliferate into a bipotential gonad until approximately 28 dpf, when sexual differentiation of the gonads into either testes or ovaries occurs. By 42 dpf, female zebrafish begin to zygotic expression of *buc* and *ddx4* occurs, beginning the next cycle of germplasm organisation during oogenesis. Bb = Balbiani body; GV = germinal vesicle; PGC; primordial germ cell. Figure adapted from Figure 6 from (Elkouby and Mullins, 2017), Figure 2 from (Abrams and Mullins, 2009), Figure 2 from (Dosch, 2015) and Figure 8.2 from (Howley and Ho, 2000).

1.2.2. Germline development in mouse

Mammalian specification of PGCs do not require germplasm (Eddy, 1976) but rather induction of signals to initiate transcription of germline specific genes in order to adopt the PGC fate. In mouse, *Wnt3* signalling from the extraembryonic ectoderm initially primes PGC precursors in the area of the epiblast that would later become the extraembryonic mesoderm to become competent in receive further signalling (Ohinata *et al.*, 2009). At approximately E5.5-6.5, cells become induced by signalling from BMP family members *Bmp2, Bmp4* and *Bmp8b* (Lawson *et al.*, 1999; Ying *et al.*, 2000; Ying and Zhao, 2001) and downstream effectors such as *Smad1* and *Smad5* (Chang and Matzuk, 2001; Tremblay, Dunn and Robertson, 2001; Hayashi *et al.*, 2002) to become presumptive PGCs. Together, these signals activates germline specific genes *Blimp1*, *Prdm14* (Yamaji *et al.*, 2008; Aramaki *et al.*, 2013) and whilst repressing expression of somatic genes (Mochizuki *et al.*, 2018) such as *hoxb1* (Frohman, Boyle and Martin, 1990), shown in Figure 1-4A, B.

At E6.5, *fragilis* expression induced into the proximal epiblast by the extraembryonic ectoderm marks the next step of germ cell competency and cells expressing high levels of *fragilis* subsequently express *stella* (Saitou, Barton and Surani, 2002; Sato *et al.*, 2002) and other germ-cell specification markers such as *Tnap* (MacGregor, Zambrowicz and Soriano, 1995).

After PGC specification by E7.5, they begin their migratory phase from the primitive streak into the hind-gut endoderm (Anderson *et al.*, 2000); and from E9, PGCs leave the hind gut and migrate towards the genital ridge. This migration utilises both repulsion/exclusion (Tanaka *et al.*, 2005) and attractive/pro-survival cues (Ara *et al.*, 2003; Molyneaux *et al.*, 2003; Stebler *et al.*, 2004) (Fig 1-4C).

Repulsive cues appear to come from the *fragilis* family of transmembrane proteins, which are required for migration from the endoderm to mesoderm as knockdown of *fragilis3* (expressed in the mesoderm surrounding the PGCs) causes the PGCs to remain in the mesoderm (Tanaka *et al.*, 2005). However, subsequent deletions in the *fragilis* locus and disruption of *fragilis3* did not repeat these phenotypes (Lange *et al.*, 2008).

Exclusive cues arises from the *c-kit* tyrosine kinase ligand Steel, which is expressed in the hindgut precursor (Motro *et al.*, 1991) and along the migratory path of the PGCs (Keshet *et al.*, 1991). Steel acts as a survival (Dolci *et al.*, 1991), proliferation (Godin *et al.*, 1991; Matsui *et al.*, 1991) and motility (Runyan *et al.*, 2006; Gu *et al.*, 2009) signalling factor for the PGCs, and gradual restriction of *Steel* expression from the migratory path causes apoptosis of PGCs that have failed to migrate towards the only remaining source of Steel at the genital ridge by E10.75 (Runyan *et al.*, 2006).

Attractive cues are also required for correct PGC migration, and the chemokine Cxcl12 (previously Sdf-1) produced near the genital ridge appears to act as a survival and motility promotion signal for PGCs to move through the endoderm to the genital ridge (Ara *et al.*, 2003; Molyneaux *et al.*, 2003; Stebler *et al.*, 2004). Corresponding deletions in Cxcr4, the cognate receptor for Cxcl12, have also reproduced this phenotype, and $Cxcr4^{-/-}$ PGCs die or remain in the hind-gut (Molyneaux *et al.*, 2003)

At the end of the migratory phase, the PGCs colonise the genital ridge by E10.5 (Molyneaux *et al.*, 2001), and expression of *Sry* from the sex chromosome Y between E10.5 - E12.5 at the genital ridge is the key initiator for dimorphic gonadogenesis (Koopman *et al.*, 1991; Hacker *et al.*, 1995; Morrish and Sinclair, 2002). Expression of *Sry* activates a signalling cascade involving *Sox9* (Sekido and Lovell-Badge, 2008; Li, Zheng and Lau, 2014) and upregulates masculinising genes such as *Dmrt1* (Raymond *et al.*, 2000) to induce nearby

somatic cells into differentiate into Sertoli cells and promote testes development (Griswold, 1998).

In the absence of the *Sry* expression (e.g. XX embryos), somatic cells in the genital ridge supporting the PGCs are fated to become granulosa cells by signalling from *Wnt4* (Jeays-Ward *et al.*, 2003; Ottolenghi *et al.*, 2007; Maatouk *et al.*, 2008), *Rspo1* (Parma *et al.*, 2006; Chassot *et al.*, 2008) and *FoxL2* (Elias *et al.*, 2009) by suppressing masculinising genes (Vainio *et al.*, 1999; Jeays-Ward *et al.*, 2003; Jordan *et al.*, 2003) and promoting ovary development via genes such as *Follistatin* (Yao *et al.*, 2004)

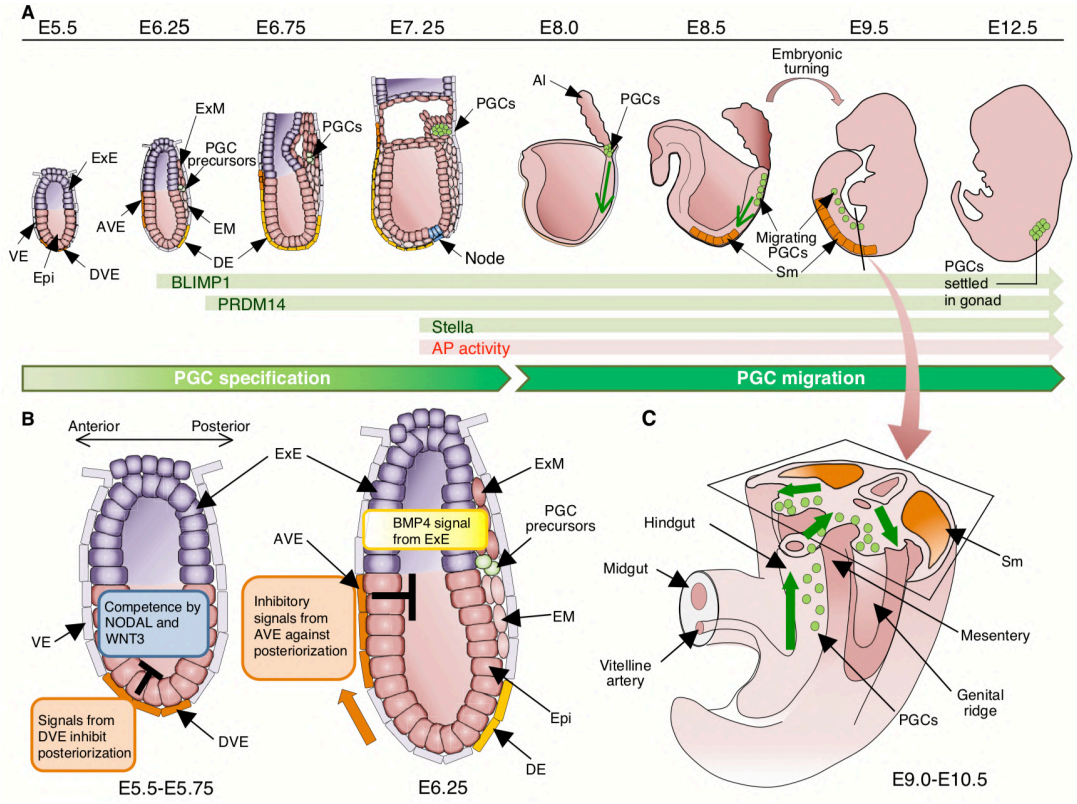


Figure 1-4. Specification and migration of the mouse primordial germ cells. A. Progression of germline development from induction to colonisation. Cells around the proximal epiblast are initially primed for PGC induction by BMP signalling (E5.5). Precursor PGCs begin expression of PGC-specific markers shown in rows below and begin their migratory path through the hindgut from the allantois and colonise the genital ridge at E12.5. **B. Induction of primordial germ cells is initiated by signalling between E5.5-6.25.** PGC competency requires signalling from BMP and Wnt family members. **C. Cross-section of the mouse embryo shows PGC migration from the hindgut to the genital ridges.** PGCs (green) migrate through the mesentery (paths in green arrows). Al = allantois; AVE = anterior visceral endoderm; DE = distal endoderm; DVE = distal visceral endoderm; EM = embryonic mesoderm; Epi = epiblast; ExE = extra-embryonic ectoderm; ExM = extra-embryonic mesoderm; PGC = primordial germ cell; Sm = somite; VE = visceral endoderm. Figure adapted from Figure 2 from (Saitou, Kagiwada and Kurimoto, 2012).

1.3. Use of zebrafish as a model to study germline development

As development of the germline is a process that occurs during early embryogenesis, a live animal model is required to reproduce conditions that cannot be replicated in cell culture, such as germplasm aggregation and translocation from the yolk to the embryo proper to form the germline.

The zebrafish system is a strong model for vertebrate embryogenesis for multiple reasons, the practical advantages of the embryo include: (1) the zebrafish embryo is relatively large, allowing microinjections of materials such as RNA, DNA or protein, (2) development occurs ex-vivo and the embryo is initially optically transparent, simplifying imaging without affecting embryonic development, (3) development occurs rapidly, shortening periods of observation required. Furthermore, many aspects of zebrafish development have been studied to great depth. Tools generated for fate mapping (Kimmel, Warga and Schilling, 1990) and lineage tracing (Mosimann *et al.*, 2011) have simplified the study of many aspects of development, and many fluorescent transgenic reporters are available to track germline development in zebrafish from germplasm to gonad (reviewed by (Kaufman and Marlow, 2016)).

In conjunction with the availability of imaging tools and reporter lines, multiple forward genetic screens with mutagens (Kimmel, 1989; Driever *et al.*, 1996; Haffter *et al.*, 1996) have made the mutant for any given gene is available for analysis, if not already generated in-house by directed mutagenesis such as Cas9.

Despite the advantages of zebrafish as a vertebrate model, genetic divergences between zebrafish and mammal such as mice can confound translatability between the two models. This is due to a third genome duplication event that occurred early in the teleost ancestry (Meyer and Schartl, 1999; Glasauer and Neuhauss, 2014), resulting in duplications of many genes that remain functional and are redundant (Kassahn *et al.*, 2009) or have sub-functionalised roles (McClintock, Kheirbek and Prince, 2002).

In the context of germline development, duplicated genes include the chemokine ligand that guides PGC *migration*, *Cxcl12*, which exists in mice as a single gene but is present as zebrafish paralogs *cxcl12a* and *cxcl12b,* although only *cxcl12a* appears to be required for PGC migration (Boldajipour *et al.*, 2011). The chemokine receptor for PGC migration, *Cxcr4* in mice, is also duplicated in zebrafish as *cxcr4a* and *cxcr4b* (Chong *et al.*, 2001), although only *cxcr4b* in zebrafish appears to play a role in PGC migration (Doitsidou *et al.*, 2002; Knaut *et al.*, 2003). However, mouse knockouts for *Cxcl12* or *Cxcr4* are lethal (Ma *et al.*, 1998; Tachibana *et al.*, 1998; Zou *et al.*, 1998) whereas mutants or morphants generated against zebrafish *cxcl12a/b* and *cxcr4a/b* are not (Doitsidou *et al.*, 2002; Knaut *et al.*, 2003; Chong *et al.*, 2007; Miyasaka, Knaut and Yoshihara, 2007; Boldajipour *et al.*, 2011).

An additional deviation between germline development in zebrafish and most vertebrate mammals such as mice that we have previously mentioned is that sex determination of these species. In most mammals and avians, sex determination is a monogenic system where heterogametic individuals can develop into males (e.g. XX/XY in mice), or females (e.g. ZZ/ZW system in chicken) (Smith *et al.*, 2009), as genes required for masculinisation or feminisation are located on these chromosomes, for example, the chicken *DMRT1* gene is a masculinising factor on the Z-chromosome, and two doses of this gene are required to induce a male sex in chick (Smith *et al.*, 2009).

In contrast, zebrafish sex determination is a polygenic system that appears to have arisen post-domestication of the species, as wild zebrafish appears to have a sex-linked loci with some plasticity to adopt male sex identity. Several sex-determinant-like genes have been identified in zebrafish, which, when knocked out, can induce masculinisation of the bipotential gonad, such as those required for ovarian development (Lau *et al.*, 2016), or rarely, genes inhibiting male sex determination will produce a female bias (Webster *et al.*, 2017).

Knockout or knockdown of genes that affect the primordial germ cells lead to the development of sterile males, such as *dnd* (Weidinger *et al.*, 2003) and *vasa* (Hartung, Forbes and Marlow, 2014). Although these genes are germplasm components, zygotic signalling factors such as *fgf24* also have a role in germline development (Leerberg, Sano and Draper, 2017). Zebrafish sex determination can also be manipulated environmentally, and perturbances such as heat (Ribas, Liew, *et al.*, 2017) and stress (Ribas, Valdivieso, *et al.*, 2017) are sufficient to induce masculinisation in wild-type populations.

In summary, sex determination in zebrafish is a multifactorial decision that appears to be influenced by both the germplasm via the PGCs, but also from the soma in response to environmental signals and more work is required to fully understand this process.

1.4. Overview of RNA regulation in development and germline specification

The molecular dogma of biology has traditionally dictated the flow of biological information to move forward from DNA to RNA via transcription, and to protein via translation (Crick, 1970). However, technical advances have shown that exceptions to this rule occur and biological information can be transferred backwards (e.g. DNA to RNA) and laterally (e.g. protein to protein).

Of these biomolecules, RNA is the most conserved and has prominent functions in all domains of life (e.g. rRNAs, tRNAs and mRNAs), preceding the appearance of DNA and

proteins (Robertson and Joyce, 2012). Therefore, it is unsurprising that RNAs have more complex functions than simply encoding for protein and can have non-coding functions.

Though some non-coding RNAs (rRNAs and tRNAs) are required for translation, further studies of ncRNAs have shown them to be essential for other functions. These can either be long and thus be categorised as “signals, decoys, guides and scaffolds” (Wang and Chang, 2011), or they can be short ncRNAs, which includes families of RNAs such as small interfering RNAs (siRNA), microRNAs (miRNA), piwi-interacting RNAs (piRNAs) and small nucleolar RNAs (snoRNAs). Furthermore, RNA may also have bifunctional roles (i.e. coding and non-coding regulatory functions) (Kumari and Sampath, 2015). In the following discussion we shall consider the roles of these small regulatory ncRNAs in the context of development only and not forward regulators of ncRNAs such as enhancer RNAs (Kim *et al.*, 2010) and promoter RNAs (Han, Kim and Morris, 2007).

1.4.1. Long non-coding RNAs in germline development

Long non-coding RNAs are a class of ncRNAs that are generally accepted to be more than 200 nt in length and may contain small open reading frames that can encode for small peptides or proteins (Anderson *et al.*, 2015; Zhang *et al.*, 2017). These function of lncRNAs are a controversial topic as deletions in some previously established lncRNAs have indicated that some are not necessary for development (Goudarzi *et al.*, 2019), and many screens have been performed to identify and assign putative functions to this class of RNA in humans and models such as zebrafish (Derrien *et al.*, 2012; Hu *et al.*, 2018). In comparison to short nc-RNAs, long ncRNAs are a highly complex and diverse class of regulatory RNAs that do not elicit a singular effect such as gene silencing, but rather a range of functions that is dependent on their individual characteristics, such as length, structure, subcellular localisation and origin in the genome (reviewed in (Dhanoa *et al.*, 2018)).

Nonetheless, some lncRNAs have been well characterised and their functions in disease, development and germline development have been studied. For example, the *H19* maternally imprinted lncRNA originally identified in mice (Bartolomei, Zemel and Tilghman, 1991; Leighton *et al.*, 1995) counteracts miRNAs such as let-7 (Kallen *et al.*, 2013) and is a master regulator for many other genes (Cao *et al.*, 2019), including chromatin modifiers. This has led to the observation that *H19* is misregulated in many disease states, such as cancer (Raveh *et al.*, 2015), atherosclerosis (Huang *et al.*, 2019) and also indirect maintenance of the germline (Lei *et al.*, 2019).

Other lncRNAs have been identified to have roles in driving sexual dimorphism by silencing the whole chromosome, such as *Xist*, which encodes a 17 kb long non-coding RNA that is

retained in the nucleus and silences the female X chromosome in *cis* (Brown *et al.*, 1992) via chromatin remodelling (Navarro *et al.*, 2005).

Currently, several lncRNAs have been identified to have roles in germline development, particular in spermatogenesis, these include *tsx* (Anguera *et al.*, 2011), *HongrES2* (Ni *et al.*, 2011) and *mrh1* (Ganesan and Rao, 2008), of which the latter two appear to have an additional subfunctional role as miRNAs after processing. An interesting example of trans-regulation via lncRNAs is *Dmr*/*Dmrt1. Dmrt1* is a sex determinant gene that can induce male sex determination in a dose-dependent response (Lambeth *et al.*, 2014; Zhao *et al.*, 2015), however, a *Dmrt1-related* gene (*Dmr*) on a different loci to *Dmrt1* can be trans-spliced into the 3' portion of *Dmrt1* transcripts (Zhang *et al.*, 2010), replacing the *Dmrt1* 3' UTR and truncating the protein (Zhang *et al.*, 2010), likely downregulating the effect of Dmrt1.

Long non-coding RNAs can also have coding functions, in *Drosophila*, *oskar* is a germplasm organiser analogous to zebrafish *buc* (Lehmann, 2016) that is required for pole cell formation and segmentation of the abdomen (Lehmann and Nüsslein-Volhard, 1986). The non-coding function of *oskar* arose when differences were observed between protein-null and RNA-null alleles, in which RNA-null mutant were sterile due to oocyte defects, but this phenotype could be rescued by introduction of the *oskar* 3' UTR only (Jenny *et al.*, 2006).

1.4.2. Short non-coding RNAs in germline development

Short non-coding RNAs are a group of RNAs that include miRNA, siRNAs and piRNAs. These RNAs act to silence gene expression in order to ensure genomic integrity against transposable elements (Carthew and Sontheimer, 2009) or fine tune gene expression (Sevignani *et al.*, 2006), shown in Figure 1-5.

miRNAs are initially transcribed as precursor miRNAs and are post-transcriptionally cleaved to produce smaller 22 nt ssRNA transcripts, initially by the nuclear RNAse Drosha (Lee *et al.*, 2003) and subsequently by Dicer (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Hutvágner *et al.*, 2001; Ketting *et al.*, 2001; Knight and Bass, 2001) to produce mature transcripts which directly bind to target RNAs by interactions to a consensus site (seed) on its target mRNAs in order to mark it for degradation by Argonaute family proteins (Lingel *et al.*, 2003; Song *et al.*, 2003; Yan *et al.*, 2003) in the RNA-induced silencing complex (RISC). miRNA genes are typically found as clusters (families) sharing the similar seed sequences and mediate several roles in development.

One example of miRNAs regulating development is in *C. elegans,* the *lin-4* miRNA family regulates the heterochronic gene *lin-14* (Lee, Feinbaum and Ambrost, 1993) and *lin-4* loss-

of-function mutants are identical to *lin-14* gain-of-function mutants (Ambros and Horvitz, 1987), characterised by developmental retardation.

The role of miRNAs in regulating the germline is also found in both vertebrates and invertebrates. In zebrafish, the miRNA-430 family is required to clear maternal RNAs through deadenylation (Giraldez *et al.*, 2006) in the soma but not in the germline (Mishima *et al.*, 2006), and its transcription represents the onset of zygotic genome activation (Chan *et al.*, 2019). In *C. elegans,* the *mir-35* family in *C. elegans* is a maternally supplied RNA that appears to be required for maintaining gender-naivety by downregulating RNAs that push sex determination prematurely (McJunkin and Ambros, 2017).

siRNAs are short RNA sequences that silence transcripts by annealing to their target RNAs, directly mediating degradation via Argonaute pathway proteins in a RISC as described previously (Carmell *et al.*, 2002), in order to maintain genome integrity from transposable elements (Ghildiyal *et al.*, 2008), protect the organism from viral infections (Ding and Lu, 2011), but also to directly tune gene expression by changing chromatin modifications (Fagegaltier *et al.*, 2009). A key divergence between siRNA and miRNA processing is the inducible nature of gene silencing via siRNA – degradation products from dsRNA are sufficient to induce the production of siRNAs (Fire *et al.*, 1998) due to amplification of more siRNA transcripts by the action of RNA-dependent RNA polymerases (Cogoni and Macino, 1999). The role of these short RNAs is not fully understood, as RdRP does not appear to exist in vertebrata (Pinzó *et al.*, 2019), but they appear to still be produced to maintain both male and female germlines in mammals (Stein *et al.*, 2003, 2015; Song *et al.*, 2011).

The final group of small non-coding RNAs are the piwi-interacting RNAs (piRNAs), which are distinct from both siRNA and miRNAs as they synthesised by a Dicer-independent mechanism (Parker, 2007) and loaded onto a specialised group Argonaute proteins, containing the Piwi domain group (Cox *et al.*, 1998). The roles of this class of RNAs appears to be diverse, with piRNAs acting in the soma to silence transposable elements through chromatin remodelling (Lee, 2015) but also being heavily influential in germline maintenance and development. Consistent with this hypothesis, knockdown of *Drosophila piwi* leads to accumulation of transposable element transcripts and subsequent sterility of females (Akkouche *et al.*, 2017).

In zebrafish, there are two Piwi-like family members (*piwi* and *zili*), which are expressed in the gonads and colocalise with the germplasm (Houwing *et al.*, 2007; Houwing, Berezikov and Ketting, 2008). Interestingly, *piwi* mutants are required for germ cell maintenance and germ cells undergo apoptosis during late larval and develop into sterile males (Houwing *et al.*, 2007), whereas mutations in *zili* affect germ cell differentiation and produce sterile females due to meiotic arrest (Houwing, Berezikov and Ketting, 2008)

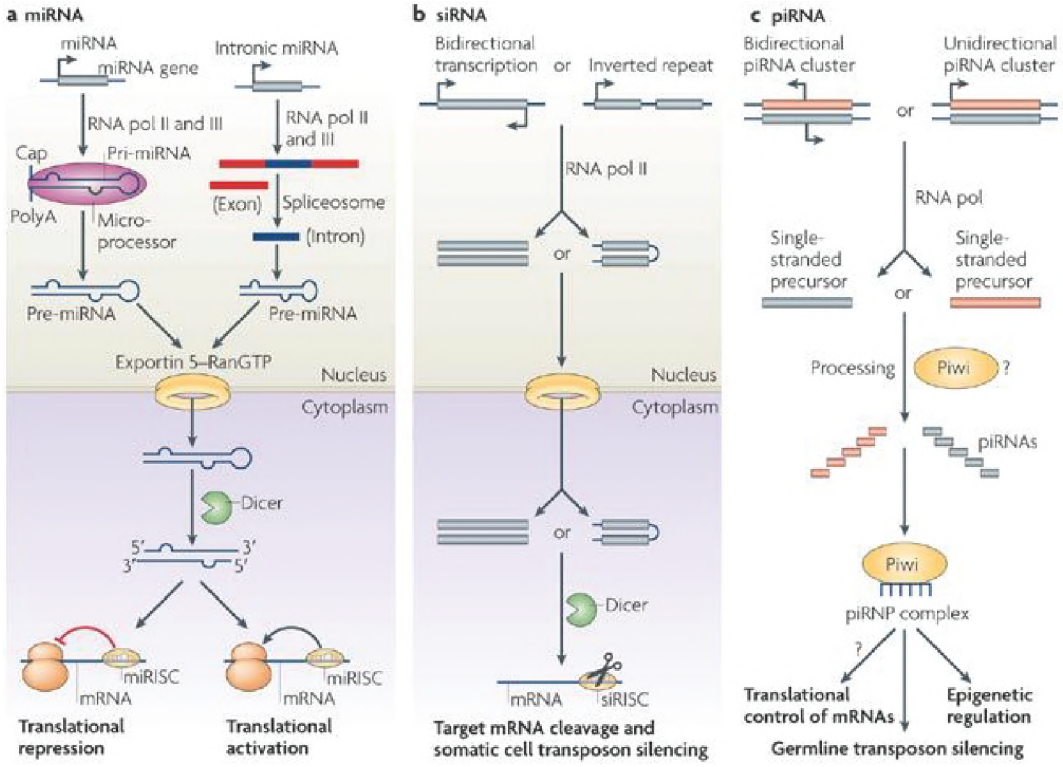


Figure 1-5. Overview of small ncRNA biogenesis and regulation. A. Biogenesis and regulation of RNA by miRNAs. miRNAs are transcribed from gene clusters in a Drosha-dependent (left) or independent fashion as pre-mRNAs are exported and processed by Dicer into short fragments to act on target mRNAs in the RISC. **B. Biogenesis and regulation of RNA by siRNAs.** siRNAs are transcribed bidirectionally or from an inverted repeat to generate a dsRNA precursor that is exported, processed by Dicer and acts on mRNAs in the RISC. **C. Biogenesis and regulation of RNA by piRNAs.** piRNAs are generated by bi- or unidirectional transcription and loaded onto Argonaute family Piwi proteins to mediate transcriptional silencing and translational control. Figure adapted from Box 1 from (Gangaraju and Lin, 2009).

1.4.3. RNA-binding proteins in germline development

We have now established that the development can be regulated by both coding and non-coding RNAs, and these RNAs can act in *cis* and *trans*, we shall now cover another indispensable *trans*-acting mechanism for RNA-regulation: the function of RNA-binding proteins in embryonic development and germline development.

In eukaryotes, nascent mRNA transcripts are bound into complexes of RNA-binding proteins known as heterogeneous nuclear ribonucleoproteins (hnRNPs), forming large complexes for further post-transcriptional regulation (Gall, 1956). These messenger ribonucleoprotein

complexes (mRNPs) are dynamically remodelled to influence the fate its cargos, and provide directionality for transcript maturation, with hnRNPs being involved in splicing (Guil *et al.*, 2003; Talukdar *et al.*, 2011), regulating capped mRNAs (Gamberi *et al.*, 1997), polyadenylation (Nazim *et al.*, 2016) and mRNA export (Nakielny and Dreyfuss, 1996). The interactivity of these proteins to their targets is altered by the contents of the RNA-binding domains, shown in Figure 1-6, with some domains showing specificity to single or double-stranded DNA and RNAs.

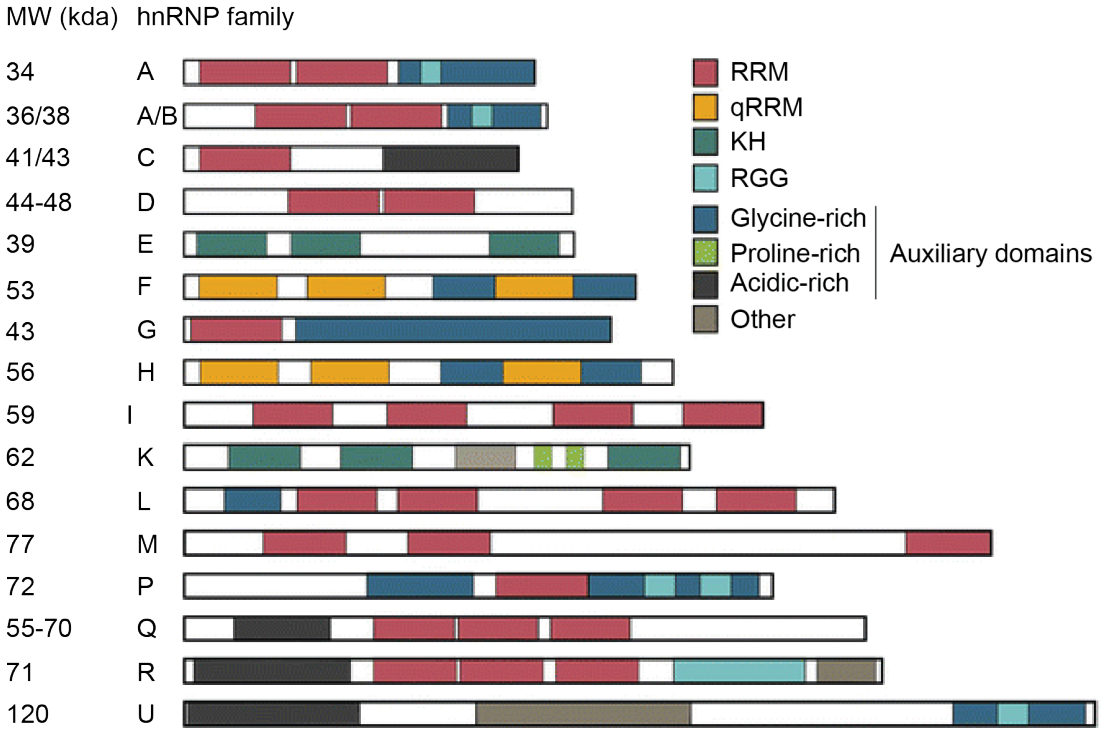


Figure 1-6. Structural arrangement and RNA-binding domains in the hnRNP family. The hnRNP family consists of many proteins consisting of different RNA-binding domains with different properties. MW = molecular weight, RRM = RNA recognition motif, KH = K-homology, RGG = arginine-glycine-glycine. Figure adapted from Figure 2 and Table 1 from (Geuens, Bouhy and Timmerman, 2016).

The role of these hnRNPs are also highly diverse, with disruptions in many family members leading to neuropathies, summarised in Table 1-1; and a further set of hnRNP members are essential for embryonic development in mice, such as hnRNPs A, C and E. These proteins are also regulators of the germline. In *Drosophila*, *nanos* RNA by regulated by several hnRNP family members, such as Rumpelstiltskin (hnRNP M) (Jain and Gavis, 2008) and Hrp38 (hnRNP A1). Hrp38 acts as a translational repressor for the germplasm mRNA *nanos* by interacting with GGG motifs in the 3' UTR (Ji and Tulin, 2016) and also translationally regulates E-cadherin mRNA (Ji and Tulin, 2012), which is essential for encapsulation of the germ cell and forms the interface between the gonad and the soma (Jenkins, McCaffery and Doren, 2003). Misregulation of Hrp38 activity by disrupting the gene or disrupting regulators

of Hrp38 such as Parg causes mislocalisation of the oocytes and failure to maintain the germ stem cell niche (Ji and Tulin, 2012).

Another hnRNP protein that regulates germline development is Dazap1 (Deleted in azoospermia associated protein 1) (Tsui, Dai, Roettger, *et al.*, 2000; Kurihara *et al.*, 2004; Akindahunsi, Bandiera and Manzini, 2005). Dazap1 is a multi-functional protein and has nucleocytoplasmic functions, interacting with profilin when complexed to localised mRNAs in *Xenopus* oocytes (Zhao *et al.*, 2001), and localising to the nucleus during transcription in sperm (Vera *et al.*, 2002; Lin and Yen, 2006). Dazap1 has further roles in transcriptional and translational regulation in the nucleus, where it acts as a splicing factor for target pre-mRNA transcripts (Chen, Yu and Yen, 2013; Choudhury *et al.*, 2014) and acts as a translational modulator, either in association with Daz family proteins (Tsui, Dai, Warren, *et al.*, 2000; Maegawa *et al.*, 2002; Collier *et al.*, 2005), or in a Daz-independent mechanism (Smith *et al.*, 2011). Consequently, Dazap1 is essential for germline development, with *dazap1*-null mice exhibiting severe infertility defects manifesting with hypogonadism, increased apoptosis in the gonads and arrested pre-meiotic cells (Dai *et al.*, 2001; Hsu *et al.*, 2008).

Table 1-1. Overview of the hnRNP family. The hnRNP family is represented, with structural and functional characteristics, with developmental consequences where applicable in mice. Figure adapted from (Geuens, Bouhy and Timmerman, 2016).

hnRNP	RNA-binding domains	Functions	Protein examples	Phenotype and disease associations	References
A	2x RRM, Gly-rich, RGG	Splicing, mRNA stability, translational regulation	hnRNP A1	Knockout embryonic lethal due to muscle defects. FTLD and cancer.	(Shan *et al.*, 2003; Park *et al.*, 2015; Mohagheghi *et al.*, 2016; Liu *et al.*, 2017)
A/B	2x RRM, Gly-rich, RGG	Splicing, mRNA stability,	hnRNP A2/B1	ALS/FTLD, Alzheimer's disease, cancer. Knockout mice neurons more susceptible to glutamate-induced excitotoxicity	(Hoek *et al.*, 1998; Shan *et al.*, 2003; Sinnamon *et al.*, 2012; QU *et al.*, 2015; Mohagheghi *et al.*, 2016)
C	RRM, Acid-rich	Splicing, translational	hnRNP C1/C2	Knockout embryonic lethal	(Choi *et al.*, 1986; Lee *et al.*, 2010;

		regulation, transcript sorting		Alzheimer's disease, Fragile X syndrome, cancer.	Anantha *et al.*, 2013; Borreca *et al.*, 2016)
D	2x RRM	mRNA decay, telomere, maintenance	hnRNP D	Knockout mice exhibit skin disease, premature aging and germline defects.	(Enokizono *et al.*, 2005; Fialcowitz *et al.*, 2005; Pont *et al.*, 2012)
E	3x KH	Translational regulation, transcriptional regulation, mRNA stability, splicing	hnRNP E1/E2/E3/E4	Knockout mice embryonic lethal. hnRNP E-deficient mice exhibit defects in heme biosynthesis.	(Ko and Loh, 2001; Meng *et al.*, 2007; Waggoner, Johannes and Liebhaber, 2009; Chaudhury *et al.*, 2010; Ghanem *et al.*, 2016; Ryu *et al.*, 2017)
F	3x qRRM, 2x Gly-rich	Splicing, telomere maintenance	hnRNP F	ALS/FLTD, cancer.	(de Silanes, d'Alcontres and Blasco, 2010; Lee *et al.*, 2013)
G	RRM, Gly-rich	Splicing	Rbmx	SMA, intellectual disability, sperm-specific germline defect.	(Shashi *et al.*, no date; Ehrmann *et al.*, 2008; Moursy, Allain and Cléry, 2014)
H	3x qRRM, 2x Gly-rich	Splicing	hnRNP H1	ALS/FLTD, cancer. Knockout mice exhibit decreased sensitivity to methamphetamine-induced behaviour.	(Lee *et al.*, 2013; Gautrey *et al.*, 2015; Ruan *et al.*, 2019)
I	4x RRM	Splicing, mRNA	PTB1	Knockout embryonic lethal,	(Bushell *et al.*, 2006; Söderberg, Raffalli-

		stability, transcriptional regulation		or develop hydrocephalus leading to lethality.	Mathieu and Lang, 2007; Shibayama *et al.*, 2009; Shibasaki *et al.*, 2013)
K	3x KH, Other	Translational regulation, transcriptional regulation, mRNA stability, splicing	hnRNP K	Knockout embryonic lethal, haploinsufficiency leads to numerous physiological defects. ALS/FLTD, cancer.	(STAINS *et al.*, 2005; Naarmann *et al.*, 2008; T. Fukuda *et al.*, 2009; Cao *et al.*, 2012; Gallardo *et al.*, 2015)
L	4x RRM, Gly-rich	Splicing, mRNA stability	hnRNP L	Knockout embryonic lethal.	(Melton *et al.*, 2007; Söderberg, Raffalli-Mathieu and Lang, 2007; Gaudreau *et al.*, 2012, 2016)
M	3x RRM	Splicing	hnRNP M	SMA, cancer.	(Cho *et al.*, 2014; Xu *et al.*, 2014)
P	2x Gly-rich, RRM, 2x RGG	Splicing	hnRNP P2	Knockouts perinatal lethal, neurodegenerative phenotype. ALS/FLTD.	(Hicks *et al.*, 2000; Vance *et al.*, 2009; Waibel *et al.*, 2010; Kino *et al.*, 2015; Scekic-Zahirovic *et al.*, 2016)
Q	3x RRM, Acid-rich	Splicing, translational regulation, translational repression	hnRNP Q1/Q2/Q3	SMA.	(Chen *et al.*, 2008; Svitkin *et al.*, 2013; Jung *et al.*, 2019)
R	3x RRM, Acid-rich, RGG, Other	Transcriptional regulation, translational regulation	hnRNP R	SMA.	(A. Fukuda *et al.*, 2009; Dombert *et al.*, 2014; Lee *et al.*, 2015)
U	Acid-rich, Other, Gly-rich, RGG	Splicing, transcriptional regulation	hnRNP U	Knockouts embryonic lethal or exhibit cardiac failure as juvenile.	(Roshon and Ruley, 2005; Bi *et al.*, 2013; Vu *et al.*, 2013; Ye *et al.*, 2015)

In addition to the hnRNP family of proteins, more RNA-binding proteins act in early development on maternal RNAs to guide their fates: translational repression, localisation, storage/decay or translational activation. The individual fates of these mRNAs are decided by cis-regulatory elements (CREs) or other post-transcriptional modifications that act to recruit specific RNA-binding proteins. These post-transcriptional modifications can be sequence modifications, such as splicing or polyadenylation, or chemical, such as 5' capping or m6A methylation. Examples of these cis-regulatory can be found in Table (1-2).

Table 1-2. List of cis-regulatory elements and modifications in mRNAs involved in development. Examples of known cis-regulatory elements/modifications involved in development are shown, left to right, the nature of the element, location of the element, mRNA containing this element, RNA-binding proteins recognising the element and the functional outcome of the interaction. Note that the list is not exhaustive, and many mRNAs may contain the same element, and may be bound by other RBPs for other functions). * = Elements/RBPs involved in germline development.

Cis-regulatory element/modification	Location	mRNA	RNA-binding protein (s)	Function	Reference
Bruno response element (BRE)*	3' UTR	*oskar*	Bruno/Cup	Translational repression	(Nakamura, Sato and Hanyu-Nakamura, 2004)
Dorsal localisation element (DLE)	3' UTR	*sqt*	Ybx1	Translational repression, mRNA localisation	(Kumari *et al.*, 2013)
Male sex lethal (MSL-2)*	5', 3' UTR	*msl-2*	Sex-lethal (SXL)	Translational repression	(Penalva and Sánchez, 2003)
Nanos translational control element (TCE)	3' UTR	*nanos, hunchback*	Smaug repressors	Translational repression	(Duchow *et al.*, 2005)
(U)-rich motif*	3' UTR	*mei-P26*	Ddx4	Translational activation	(Liu, Han and Lasko, 2009)
Vg1 localisation element (VLE)	3' UTR	*vg1*	Vg1 RBP	Localisation	(Git and Standart,

					2002)
Vg1 translation element (VTE)	3' UTR	*vg1*	ElrB	Translational repression	(Colegrove-Otero, Devaux and Standart, 2005)
5' capping	5' UTR		eIF4E	Translation	
3' polyadenylation	3' UTR		Poly (A) binding proteins	Translational repression/initiation	(Kühn and Wahle, 2004)
m6A*	5'UTR, 3'UTR, CDS(Chang, Yeh and Yong, 2017; Otsuka *et al.*, 2019)		Writers (Mettl3, Mettyl14), Readers (Igf2bp, Ythdf2, Ythdc2), Erasers (FTO, Alkbh5)	mRNA stability, degradation, translational enhancers, translational repression	(Tang *et al.*, no date; Liu *et al.*, 2014; Bailey *et al.*, 2017; Zhao *et al.*, 2017; Huang *et al.*, 2018; Ma *et al.*, 2018)

The regulation of maternal RNAs is particularly important in development, as the embryo must initially rely on these messages to direct development until the end of the maternal-zygotic transition, so, maternal messages must be received at the right place and the right time, utilising a network of proteins that transport RNA, and keep it stable and untranslated until it is required, summarised in Figure 1-7.

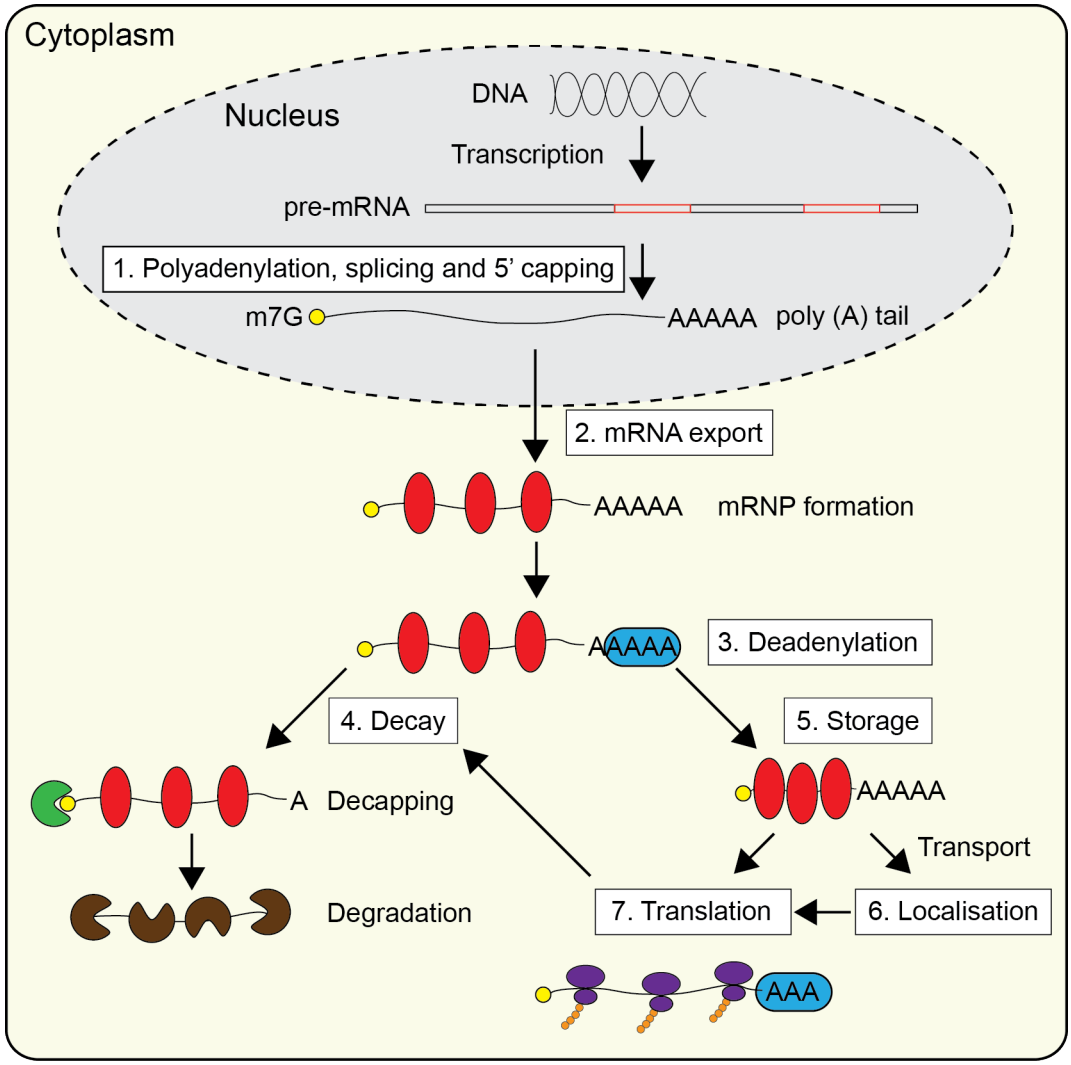


Figure 1-7. Overview of maternal RNA regulation by RNA-binding proteins. Upon transcription of an mRNA molecule, post-transcriptional modifications such as splicing, polyadenylation and the addition of the 5' cap are made to convert a pre-mRNA transcripts to a mature mRNA. mRNAs are subsequently exported from the nucleus and enters a network of RNA-binding proteins that control the fate of the mRNA (red circles), such as initial deadenylation. Subsequently, mRNAs can be sequestered into storage complexes for localisation, future translation or decay. Each step in this system with a white box requires RNA-binding proteins.

As such, many RNA-binding proteins identified to have a role in development are translational repressors and maintain stability of bound mRNAs. These are formed into ribonucleoprotein complexes are typically localised in cytosolic granules such as stress-granules or processing bodies (P-bodies) that share common components (Balagopal and Parker, 2009); in animals where utilising the preformative model of PGC specification, germplasm is organised into specialised ribonucleoprotein granules, known as P-granules or

germ granules, which contains other RNA-binding proteins not typically found in somatic ribonucleoprotein complexes.

Translational repressor and storage or maternal RNAs by RNA-binding proteins

Once a nascent mRNA molecule has been transcribed, it enters a network of proteins that influence its fate, such as splicing or polyadenylation. Polyadenylation is used as a mechanism for regulating the translational potential of a mRNA transcript, as polyadenylation of maternal mRNAs are required to unmask the mRNA for translation (De Moor and Richter, 1999), and deadenylation of maternal mRNAs below a critical threshold subsequently leads to decapping of the mRNA and degradation. Many cis-regulatory signals guide the adenylation state of a mRNA transcript, such as the AU-rich element (ARE) and the alternative polyadenylation signal (APA), and nucleases with specific 3' deadenylating activity mediate the removal of the adenylation signal, such as the *Xenopus* deadenylating nuclease (DAN) in oocyte maturation (Körner *et al.*, 1998).

In addition to 3' translational repression signals, 5' cap-dependent mechanisms also exist to repress translation at the initiation step. Internal chemical modifications of the 5' cap also repress the translational potential of the transcript, and increasing methylation of the m7G cap increases the efficiency of translation independently of the 3' polyadenylation status in maturing *Xenopus* oocytes (Gillian-Daniel *et al.*, 1998). Cap-dependent repression can also be produced by inhibiting the activity of the translation initiation factor, eIF4E, which can be blocked by proteins such as 4E-BP1 (Haghighat *et al.*, 1995) or Maskin (Minshall, Thom and Standart, 2001; Cao and Richter, 2002). Developmentally, the action of 4E-BP1 homologs are shown to produce gradients of protein activity in order to induce proper axis development: in *Drosophila*, the 4E-BP1 homolog d4EHP forms a complex with other classical translational repressors, Nanos and Pumilio (Forbes and Lehmann, 1998), to inhibit *hunchback* and *caudal* to establish the anterior-posterior axis.

Once an mRNA transcript has been fated for translational repression, these are shuttled to cytoplasmic ribonucleoprotein granules for further processing, a reversible state where an mRNA is kept in storage, protected from degradation and masked for translation until required, and several RNA-binding proteins are associated to these complexes. In *Xenopus* oocytes, such as mRNP can contain proteins such as Rap55, P54 and Prmt1 (Tanaka *et al.*, 2006; Matsumoto *et al.*, 2012).

Translational repressors localised to mRNP granules often have shared properties, for example, DEAD-box helicase proteins are found in both somatic granules (e.g. P54), and germ granules (e.g. Ddx4) (Minshall and Standart, 2004; Cordin *et al.*, 2006); and granule assembly proteins often have low-complexity or prion-like domains, such as zebrafish Bucky

ball, *Xenopus* Xvelo and *Drosophila* Oskar (Boke *et al.*, 2016; Lehmann, 2016). In the germline, these mRNP granules are actively remodelled to repress specific mRNAs depending on the sex fate, in *Drosophila* germline, germline mRNP granules are remodelled depending on sex, in males, three key proteins, Bam, Bgcm and Tut form complexes with *mei-P26* mRNA by binding to 3' UTR elements to prevent translation (McKearin and Spradling, 1990; Ohlstein *et al.*, 2000; Li *et al.*, 2013; Chen *et al.*, 2014). In females, Bam and Bgcm form an alternative complex with Sxl to Mei-P26 protein to promote differentiation of stem cells into the ovarian fate by repressing of *nanos* (Y. Li *et al.*, 2009; Chau, Kulnane and Salz, 2012; Li *et al.*, 2013).

Subsequently, derepression of maternal mRNA and germplasm mRNA are subsequently linked to severe developmental consequences. Loss of Bgcn in *Drosophila* leads to sterility (Mahowald and Wei, 1994) and corresponding mutants for Bgcn homologs in mice, Ythdc2, leads to hypogonadism and sterility from failure to undergo the mitotic to meiosis transition (Bailey *et al.*, 2017; Jain *et al.*, 2018). Similarly, mutants for translational repressors for *nanos* family leads to infertility (Köprunner *et al.*, 2001; Draper, McCallum and Moens, 2007; Park *et al.*, 2017) and loss of the Ybx family of translational repressors also leads to fertility defects in mice (Snyder *et al.*, 2015).

Localisation of maternal RNAs by RNA-binding proteins

Localisation of RNA is a conserved and powerful mechanism as it provides an additional method for the cell to compartmentalise and fine-tune the expression of a gene to where it is required on a subcellular level. This presents many advantages for a cell, as coupling localisation to translation allows protein to only be produced where it is needed, reducing the energetic cost to the cell. And, as mRNAs can be translated many times, it is more efficient to move an mRNA to where it is needed for translation than to utilise transport mechanisms moving the protein itself.

Localisation of mRNA can be observed in many instances where polarity and spatiotemporal regulation of protein expression is necessary. For example, migrating fibroblasts use microtubule network to shuttle mRNAs required for sustained cell migration to the leading edge of the cell, and this movement of RNAs utilises the RNA-binding protein APC in conjunction with detyrosinated microtubules, where the RNAs are anchored on the positive end (Mili, Moissoglu and Macara, 2008; Preitner *et al.*, 2014; T. Wang *et al.*, 2017).

In addition to APC-dependent mRNA transport, migrating cells also use a RNA-binding protein that binds to specific UTR sequences, known as zipcodes, to transport mRNA to the leading edge of the cell using actin or microtubule networks (Oleynikov and Singer, 2003), the most commonly studied instance of this interaction is the zipcode element in the β-actin

3’ UTR and its corresponding interaction with the zipcode-binding protein (Zbp-1) (Eom *et al.*, 2003) in chicken fibroblast cells. The *Drosophila* homologs of Zbp-1 is also found to bind to 3’ UTR of transcripts that direct the formation of F-actin and direct protein-expression in the growth cone of neurons (Hansen *et al.*, 2015; Vijayakumar *et al.*, 2019).

The purpose of RNA-binding proteins in localisation of RNAs serves not only to stabilise and protect their cargo during its transport to their destination, but also to attract molecular motors so that the cargo can be tethered to a transport network. In *Drosophila,* the RNA-binding protein Egalitarian binds to mRNA cargos such as *bicoid* and *gurken* and serves as a scaffold to attract the dynein adaptor Bicaudal D to transport cargo mRNAs to the oocyte anterior using dynein (Duncan and Warrior, 2002; Januschke *et al.*, 2002). In zebrafish, the dorsal axis is specified by cells receiving *sqt* RNA (Gore *et al.*, 2005). This RNA is kept in a translationally repressed state as it is functionally inactive due to being unspliced and non-polyadenylated (Lim *et al.*, 2012), and as it is bound by the translational repressor Ybx1 to a 3’ UTR element that also directs its localization from the yolk to the specified cells using microtubule networks (Gore and Sampath, 2002; Kumari *et al.*, 2013).

In the context of germplasm, localisation of RNAs is a more complicated affair compared to cytoplasmic localization of mRNA in somatic cells, where RNA is transcribed and translocated to where it is required upon the correct stimulus or patterned along gradients. For a germplasm mRNA such as *dazl*, localisation requires at least three cis-regulatory elements (Kosaka *et al.*, 2007) in its 3’ UTR; one for each stage of its translocation, first to the mitochondrial cloud during oogenesis, a second element for translocation to the vegetal cortex, and a third element for translocation to the early cleavage furrows (Kosaka *et al.*, 2007). For the tethering to the vegetal cortex from the mitochondrial cloud, the translocation mechanism is shared between zebrafish and *Xenopus,* and the mitochondrial cloud localisation element (MCLE) in *Xenopus* Xcat2 can be recognised by the zebrafish RNA-binding protein Hermes to direct its localisation to the vegetal cortex (Kosaka *et al.*, 2007).

Degradation of maternal RNAs by RNA-binding proteins

Like all mRNA, maternal mRNA transcripts are transient messages that have a limited lifetime upon fertilisation of the egg, and are eventually degraded from the zygote after they have served their purpose for coding proteins essential for development. Previously, we have discussed the roles of non-coding RNAs as maternal RNA clearance mechanisms, such as *miR-430* (Giraldez *et al.*, 2006). However, the action of micro-RNAs in RNA degradation also requires RNA-binding proteins with dsRNA nuclease activity such as Dicer in the RNA-induced silencing complex (RISC), and miRNA independent mechanisms for mRNA degradation also exist.

In the cytoplasm of a cell, degradation of mRNA typically occurs in mRNP granules called P-bodies, which are formed in response to gene silencing from RNA (Eulalio *et al.*, 2007). P-bodies are enriched in conserved core components that regulate mRNA degradation, such as decapping enzymes Dcp1p/Dcp2p, and decapping activators Dhh1p/RCK/p54, Pat1p, Scd6p, Edc3p and Lsm1p-7p and the 5' to 3' exonuclease Xrn1p (Parker and Sheth, 2007) (Franks and Lykke-Andersen, 2008). Together with miRNA, 5' decapping enzymes and Argonaute family proteins, deadenylation of the transcript is mediated by Ccr4-Not deadenylase and the Pan2-Pan3 complexes (Wahle and Winkler, 2013).

In addition to targeted degradation of transcripts by ncRNA, mRNA surveillance pathways in the cell act as quality control mechanisms to target mRNA transcripts with premature stop codons, such as those generated by aberrant splicing or frameshift mutations from random transcription errors or mutations in the genome, one such mechanism is known as the nonsense mediated decay pathway (NMD). The NMD pathway acts through a promiscuous ATP-dependent RNA-helicase with Upf1 (Leeds *et al.*, 1991), which binds to any available site on a mRNA molecule and, upon detection of premature stop codons in the presence of an exon junction complex, or an exceptionally long 3' UTR (Kurosaki, Popp and Maquat, 2019), interacts with eukaryotic release factors eRF1 and eRF3 to enhance termination and target the mRNA for degradation (Atkin *et al.*, 1995; Czaplinski *et al.*, 1998). The Smg1 kinase (in a complex with Smg8 and Smg9) is recruited to complexes of Upf1-eRF1-eRF3 to form the Smg1-Upf1-eRF1-eRF3 (SURF) complex that promotes the phosphorylation of Upf1, and irreversibly designates the mRNA for degradation by endonucleolytic decay by Smg6 or exonucleolytic decay by Smg5 and Smg7.

Targeted degradation of mRNA during development is an important process, and mutations in many core degradation components lead to developmental consequences or germline defects. In zebrafish, many mechanisms exist to modulate maternal RNA stability, uncommon codon usage can promote an mRNA for degradation, and longer 3' UTRs are associated with resistance to deadenylation (Mishima and Tomari, 2016). Disruption of the maternal degradation by blocking miRNA action or maternal mutations in Dicer both exhibit developmental defects (Giraldez, 2005; Giraldez *et al.*, 2006), female Dicer mutants in mice are sterile, due to a failure to metabolise maternal RNA in the oocytes (Tang *et al.*, 2007). Inhibition of the downstream CCR4-Not deadenylation complex also has consequences in development and the germline, in zebrafish, inhibition of the deadenylase activity results in defective somite segmentation (Fujino *et al.*, 2018). In *C. elegans,* knockdown in the deadenylase *ccr-4* and *ccf-1* results in either sterility or reduced brood size. In *Drosophila*, CCR4-Not deadenylases can be recruited by other translational repressors to direct the degradation of *Hsp83* (Semotok *et al.*, 2005, 2008).

More recently, it has been shown that many maternal RNAs have m6A modifications which appears to mark them for degradation during oocyte maturation (Ivayla Ivanova *et al.*, 2017) or the midblastula transition (Zhao *et al.*, 2017) in mice and zebrafish respectively. Mutants of the RNA-binding proteins recognising m6A modification, Ythdf2, leads to defective oocytes and subsequent sterility of the females but not the males (Ivayla Ivanova *et al.*, 2017). However, in zebrafish, the inverse phenotype is observed and homozygous males appear to have defective sperm and maternal mutants have a delay in cell division during the midblastula transition (Zhao *et al.*, 2017). Therefore, these modifications have diverse functions in both the soma and the germline, and can influence the fate of their transcripts depending on the biological context, shown in Figure 1-8.

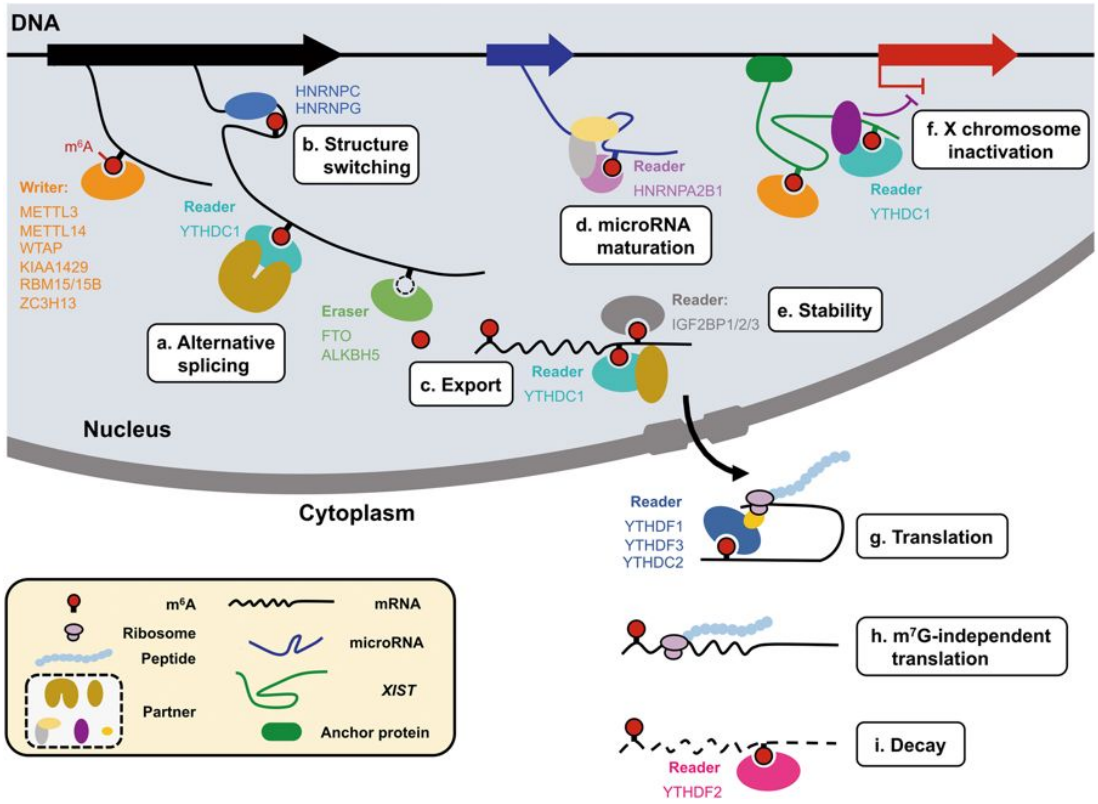


Figure 1-8. Functions of m6A modifications in RNA to direct fate. m6A modifications are controlled by writers (orange) and erasers (green), and are recognised by an array of reader proteins (blue, grey, pink and cyan), which directs its fate accordingly. This includes alternative splicing (a), secondary structure switching (b), export control (c), pri-miRNA processing (d), modulating transcript stability (e), lncRNA activity such as *Xist*-dependent X chromosome inactivation (f), enhancing translation in the cytosol (g,h) and decay (i). Figure adapted from Figure 1 from (Y. Yang *et al.*, 2018).

Translational activation of maternal RNAs by RNA-binding proteins

We have previously covered the role of maternally deposited proteins and RNAs, and various mechanisms to repress, localise and degraded maternal messages, one further consideration is the activation of these messages when they are required, both for development and germline function.

The adenylation status of an mRNA is known to affect its stability and translational potential; and a longer poly (A) tail is linked to translational activation of a specific mRNA (Vassalli *et al.*, 1989; Salles *et al.*, 1994), cytoplasmic polyadenylation of the maternal transcript is stimulated by the cytoplasmic polyadenylation element (CPE), which attracts CPE-binding proteins (Hake and Richter, 1994). The mRNA-CPEB complex recruits other proteins that controls the adenylation state of an mRNA, Symplekin acts as a scaffold for attachment of modulators such as the poly (A) ribonuclease (PARN) deadenylase (Copeland and Wormington, 2001), cleavage and polyadenylation specificity factor (CPSF) (Dickson *et al.*, 1999) and germ-line-development factor 2 (Gld2) (Barnard *et al.*, 2004; ROUHANA *et al.*, 2005; Rouhana and Wickens, 2007). Activation of maternal mRNA via polyadenylation signals have significant roles in development, in *Xenopus* and mouse, translational activation of the maternal *c-mos* mRNA is a polyadenylation-dependent process that requires two CPEs in the *c-mos* 3' UTR (Gebauer *et al.*, 1994; Sheets, Wu and Wickens, 1995). In *Xenopus, c-mos* is the one of the earliest activated transcripts and acts to promote further maturation signals in the oocyte (de Moor and Richter, 1997; Peter *et al.*, 2002); and oocyte maturation is marked by the poly (A) extension of many maternal mRNAs (Fox, Sheets and Wickens, 1989; McGrew *et al.*, 1989).

Transcriptional competence and zygotic genome activation also appears to be dependent on the polyadenylation-dependent translational activation of maternal RNAs in 1-cell mouse embryos (Oh *et al.*, 2000; Aoki, Hara and Schultz, 2003). In *Drosophila,* mutations in genes required for maternal poly (A)-dependent mRNA activation such as *cortex* and *grauzone* result in early embryonic arrest.

Translational activation of maternal RNAs in the germplasm is used to determine and maintain the germline and several germplasm genes perform functions to translationally activate other germplasm mRNAs, providing an autoregulatory mechanism to suppress somatic fates and promote the germ line. In *C. elegans*, *nanos* family genes such as *nos-1* and *nos-2* (Subramaniam and Seydoux, 1999) are germplasm mRNA components that are restricted to the primordial germ cells and is translationally silenced by RNA-binding proteins Oma-1, Oma-2, Mex-4 and Spn-4 (Jadhav, Rana and Subramaniam, 2008). This translational silencing is derepressed by Pie-1 and Pos-1 (Tenenhaus *et al.*, 2001; Jadhav, Rana and Subramaniam, 2008), which suppresses somatic genes (Hayashi, Hayashi and Kobayashi, 2004). In *Xenopus*, *dazl* protein stimulates translation of germplasm mRNAs by recruiting poly (A) binding proteins to promote translation (Collier *et al.*, 2005). Similarly in

zebrafish, Dazl promotes adenylation of its target mRNAs and antagonises miRNAs that mediate maternal clearance such as miR-430 (Takeda *et al.*, 2009). In *Drosophila, ddx4* interacts with uridine-rich motifs in the 3' UTR of *mei-P26* mRNA to activate its translation (Liu, Han and Lasko, 2009).

In summary, RNA-binding proteins act as post-transcriptional regulation of gene expression spatiotemporally, by repression, storage, degradation and translation of their targets. These mechanisms are particularly important during embryogenesis where multiple transition states occur, such as maturation of the oocyte and utilisation of maternal RNAs until the activation of the zygotic genome. More importantly, this is important for development of a heterogeneous cell population such as the germline as its fate is fundamentally divergent from the rest of the soma.

1.5. Overview of Igf2bp proteins

The *insulin-like growth factor II mRNA binding protein* (*igf2bp*) family of oncofetal genes are a group of RNA-binding proteins that are clinically linked to diseases such as diabetes [reviewed by (Christiansen *et al.*, 2009)] and cancers [reviewed by (Bell *et al.*, 2013). These genes were initially identified independently through biochemical screens attempting to identify binding elements for oncogenic growth factors, cell cycle and cytoskeletal regulators and have previously been reviewed as VICKZ (Vg1 RBP, Imp-1/2/3, Crd-bp, Koc, Zbp-1) proteins due to their original names (Yisraeli, 2005), however, we will retain the nomenclature of referring to them as Igf2bp proteins where possible to prevent confusion. In this section, we will primarily focus on the roles of these genes in development, influencing cell behaviours and the molecular basis for their actions.

The Igf2bp RNA-binding proteins are highly conserved in development, typically containing a canonical structure of two RNA-recognition motifs (RRMs) and four K-homology (KH) domains (Nielsen et al., 1999), arranged as two pairs of KH didomains (i.e. KH1-2, KH3-4) (Yisraeli, 2005), shown in Figure 1-9. As these proteins appear to be required to export RNA from the nucleus (Sim et al., 2012), they are nucleocytoplasmic and are able to leave the nucleus via conserved nuclear export signals in the second and fourth KH domains (Nielsen *et al.*, 2003) and mutations in these domains leads to nuclear accumulation (Wächter *et al.*, 2013). The mechanism for Igf2bp proteins to enter the nucleus is unknown, as they do not have a distinct nuclear localisation sequence and are otherwise too large to enter by passive diffusion, however, as the RRM is involved in the import of RNA-binding proteins (Cassola, Noé and Frasch, 2010), it is likely that nuclear import of Igf2bp proteins acts via this mechanism.

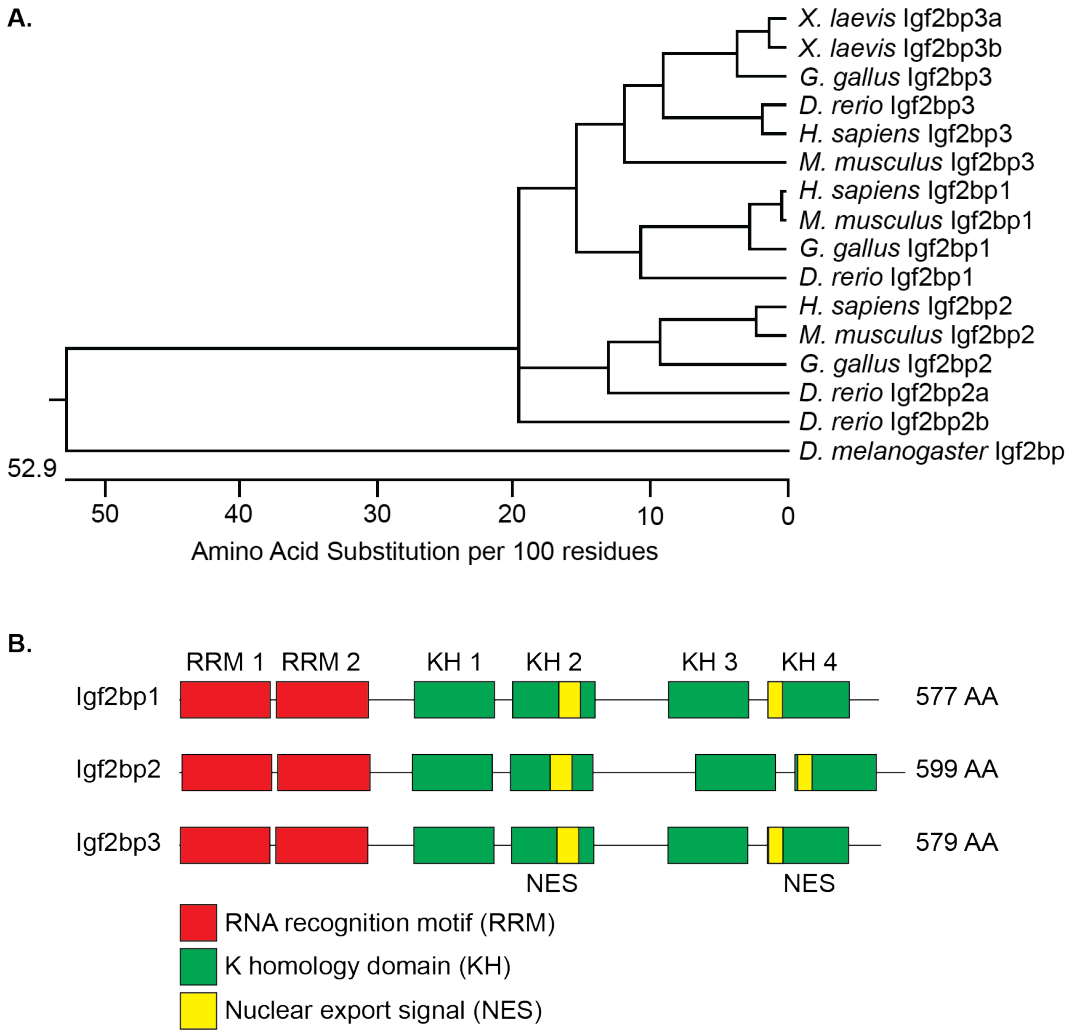


Figure 1-9. Evolutionary conservation of Igf2bp proteins. A. Phylogenetic tree of Igf2bp family proteins from *Xenopus*, chick, zebrafish, human, mouse and *Drosophila*. Igf2bp1, Igf2bp2 and Igf2bp3 proteins from several model organisms were aligned with Clustal W to show the phylogenetic conservation of these proteins. **B. Schematic of human Igf2bp1, Igf2bp2 and Igf2bp3.** Human Igf2bp proteins representative of the canonical structure of mammalian Igf2bp proteins were represented.

Traditional literature on the requirement for an RNA molecule to be bound and recognised by Igf2bp proteins has been focused on sequence identity rather than structural or chemical requirements. In *Xenopus*, where Igf2bp proteins were initially identified as Vg1 RBP due to its ability to localise Vg1 RNA to the vegetal pole of the oocyte, the sequence has been defined to a series UUCAC motifs within a 340 nt sequence in the 3' UTR (Kwon *et al.*, 2002) and all four KH domains are needed for this interaction (Git and Standart, 2002). It is likely that Vg1 RBP/Igf2bp localises many other RNAs in the oocyte, as vegetally localised RNAs in the oocyte are generally enriched in this motif (Betley *et al.*, 2002).

Evidence for a motif-based interactivity were also reproduced by with other Igf2bp paralogs in other organisms, for example, repeated UUUAY motifs in the *oskar* 3' UTR is the recruitment signal for *Drosophila* Igf2bp to colocalise with *oskar* transcripts (Munro *et al.*, 2006) and this is also conserved in mammalian sequences, where Igf2bp proteins show enhanced specificity for CACA-like motifs (Conway *et al.*, 2016), this observation was also initially observed in the chicken Igf2bp homolog (Zbp-1), where the sequence in the *β-actin* 3'UTR required to recruit Igf2bp was strongly associated with a ACACCC motif (A F Ross *et al.*, 1997) and an CGGAC motif (Nicastro *et al.*, 2017).

Subsequent studies on the properties of Igf2bp have revealed the requirements of the proteins to bind RNA, currently, studies conducted on the RRMs have indicated that it appears to be dispensable (Git and Standart, 2002; Nielsen *et al.*, 2002; Chao *et al.*, 2010) and that the KH domains are more important functionally. Biochemical analyses with chick Igf2bp1 have shown that the KH 3-4 didomain are required to bind its known mRNA targets such as *actin, CD44* and *c-myc* (Farina *et al.*, 2003; Wächter *et al.*, 2013; Barnes *et al.*, 2014; Nicastro *et al.*, 2017). Structural dissection and kinetic studies of the KH didomain interactions with human Igf2bp1 on *c-myc* RNA by (Dagil *et al.*, 2019) have shown that the KH 1-2 didomain has a higher association rate with RNA, but the KH 3-4 didomain interaction is more stable.

In vivo, Igf2bp1 coalesces into granules of RNP complexes (Farina *et al.*, 2003; Barnes *et al.*, 2014); site directed mutagenesis on the KH-domain specific GxxG motifs required for RNA-binding (Hollingworth *et al.*, 2012) has shown this ability to form granules is only compromised when the KH 1-2 domains are mutated but not KH 3-4 (Barnes *et al.*, 2014).

Recent studies in the role of m6A modifications in RNA have shown that Igf2bp proteins show preferential binding to m6A modified sites (Huang *et al.*, 2018), which improves the stability of bound transcripts from degradation, either by shuttling to P-bodies for storage (Huang *et al.*, 2018) or by protection from miRNA mediated degradation (Müller *et al.*, 2019), shown in Figure 1-10. The requirement for an adenine residue in RNA to be methylated is that it must be part of a RRACH motif (R = A/G, H = A/C/U), for reasons unknown, these motifs are preferentially methylated near the stop codon or in the 3' UTR (Huang *et al.*, 2018), and bears similar *c-myc* CGGAC motif previously reported (Nicastro *et al.*, 2017). The role of m6A appears to be intricately tied to the function of Igf2bp proteins and several lines of evidence have reproduced findings by previous studies: RNA-pull downs with methylated and unmethylated *c-myc* RNA probes against KH-domain mutated Igf2bp proteins indicate that the KH 3-4 didomain is essential for recognising *c-myc* (Huang *et al.*, 2018). Secondly, phenotypes of Igf2bp KO cell lines (reduced cell proliferation and colony formation) could be rescued by the expression of WT Igf2bp but not KH 3-4 mutants, and could also be rescued by ectopic *c-myc* expression (Huang *et al.*, 2018).

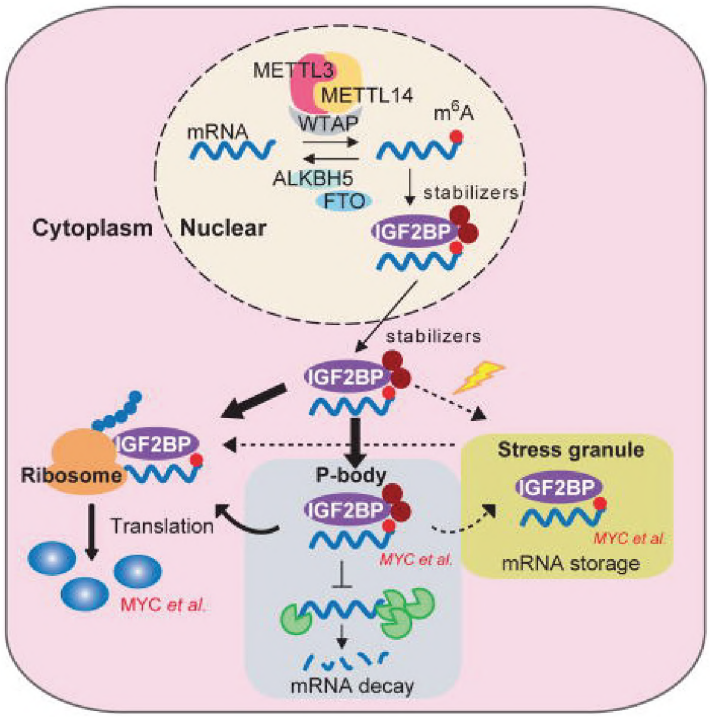


Figure 1-10. Igf2bp proteins as m6A binding proteins. Putative model for the role of Igf2bp proteins as m6A interacting proteins. mRNA transcripts can be methylated by the m6A writer complex containing Mettl3, Mettl14 and WTAP and demethylated by m6A erasers ALKBH5 and FTO. Methylated transcripts are bound by nuclear Igf2bp and acts to recruit other modulators such as RNA stabilisers that regulate the fate of these transcripts. Upon translocation to the cytoplasm, Igf2bp-containing ribonucleoprotein complexes are sequestered to storage, such as P-bodies or stress granules, for degradation or later translation. Adapted from Figure 7 in (Huang *et al.*, 2018).

1.5.1. In vivo functions of igf2bp proteins

Currently, few complete loss-of-function analyses have been conducted on the consequences of Igf2bp disruption *in vivo*. However, multiple *in vitro* studies have been consistent in identifying *igf2bp* genes in sharing common targets, allowing some inference as to their putative function *in vivo*, which appears to correlate with mutant or morpholino studies.

In humans, the link between the *igf2bp* group of RNA-binding proteins and regulation of *igf2* was identified by (Nielsen *et al.*, 1999), who showed that human *igf2bp1, igf2bp2* and *igf2bp3* were able to translationally repress *igf2* transcripts with specific leader elements. Further works by (Müeller-Pillasch *et al.*, 1997; Mueller-Pillasch *et al.*, 1999) also identified the homologs independent by screens for KH-containing RNA-binding proteins

overexpressed in cancer (*Koc)*. Many studies conducted on the role of human Igf2bp proteins *in vitro* have shown them to be upregulated in many cancer cell lines (P. Wang *et al.*, 2017; Shi *et al.*, 2017; Müller *et al.*, 2019; Waly *et al.*, 2019), or are required to induce oncogenic phenotypes such as migration, cell proliferation and invasion (Mancarella *et al.*, 2018) and appear to be driven by preventing miRNA-mediated degradation of Igf2bp targets (Müller *et al.*, 2018) and post-transcriptionally enhances target oncogenes in an m6a-dependent manner (Ennajdaoui *et al.*, 2016; Müller *et al.*, 2019) and additional co-immunoprecipitation studies have identified several proteins with RNA-stabilising functions that form a core complex to prevent decay of proto-oncogenic transcripts such as *c-myc* (Weidensdorfer *et al.*, 2008). Furthermore, the oncogenic role of Igf2bp proteins as miRNAs that target *igf2bp* transcripts are downregulated in multiple cancers, leading to de-suppression of *igf2bp* translation, these miRNAs include *let7* (Kugel *et al.*, 2016; Müller *et al.*, 2018; Waly *et al.*, 2019), *miR1243p* (P. Wang *et al.*, 2017), *miR-873* (Wang *et al.*, 2015) and *miR-29* (J. Yang *et al.*, 2018).

In mice, *igf2bp* genes were identified due to their ability to bind the coding region of *c-myc* mRNA and designated *coding region determinant binding protein* (*Crd-bp*) (Leeds *et al.*, 1997). These genes appear to be expressed biphasically (Nielsen *et al.*, 2000; C. Nielsen, Jacob Nielsen, Jan Chri, 2001), with expression as apparent as two-cell stage but sharply peaking at E12.5 (Nielsen *et al.*, 1999). Whilst *igf2bp* expression can be detected in adult tissues (Nielsen *et al.*, 1999), embryonically, their expression appears ubiquitous, with *igf2bp1* expressed in major organs such as the lung and intestine in addition to the basal epidermis, muscle, tail and limb buds (Nielsen *et al.*, 1999; Hansen *et al.*, 2004). Similarly, mouse *igf2bp2* is expressed in the major organs and mouse *igf2bp3* appears to be additionally enriched in the CNS (Sakakibara *et al.*, 2002; Yaniv and Yisraeli, 2002). This suggests that while there is a temporal element to *igf2bp* expression, their localisation does not appear to be restricted to specific germ layers or lineages. Furthermore, the function of individual *igf2bp* genes in mice appears to be divergent, mouse *igf2bp1* is required to bind mRNA transcripts for correct localisation and timely translation of mRNA in neurons (Donnelly *et al.*, 2011; Perycz *et al.*, 2011; Welshhans and Bassell, 2011; Lepelletier *et al.*, 2017), cell migration (Katz *et al.*, 2012) and maintenance of pluripotency (Nishino *et al.*, 2013). Deletion of *igf2bp1* appears to be catastrophic, and mutant mice exhibit significant perinatal mortality as a result of physiological defects in the intestine, physical retardation (Hansen *et al.*, 2004) and abnormal neurological development (Nishino *et al.*, 2013). These phenotypes are not observed in *igf2bp2* mutant mice, where a metabolic role in maintaining energy expenditure (Dai *et al.*, 2015; Regué *et al.*, 2019). Currently, mouse *igf2bp3* mutants have not been characterised. As the phenotypes of *igf2bp* knockouts do not overlap, it suggests that they likely play different roles in development or are redundant to some extent.

The chick *igf2bp* homolog was identified from characterising 3' UTR binding proteins required for the localisation of *actin* mRNA identified the *zipcode-binding protein* (*Zbp-1*) (Anthony F Ross *et al.*, 1997). Morphant knockdown of all three chicken *igf2bp* paralogs leads to delamination in the neural tube (Shoshkes *et al.*, 2015) and specific knockdown of Igf2bp2 lead to defective axonal projections, concordant with a neurological role in mice.

In *Xenopus*, the *igf2bp* ortholog was identified as a *Vg1* RNA-binding protein (*Vg1 RBP)* that is required to localize *Vg1* RNA to the vegetal pole of the oocyte (Melton, 1987; Yisraeli and Melton, 1988; Kwon *et al.*, 2002) through a 340 nt 3' UTR element (Mowry and Melton, 1992) requiring microtubule mediated transport (Elisha *et al.*, 1995; Havin *et al.*, 1998); this protein also appears to be a transcription factor for TFIIIA in oocytes (Griffin *et al.*, 2003). Whilst human, mice and chick have three *igf2bp* paralogs, only two appear to be present in *Xenopus*: *igf2bp3-a* and *igf2bp3-b.* The expression pattern of *Xenopus* appears to be conserved in fish (Zhang *et al.*, 1999) and morpholino injections disrupting one of the two homologs leads to defects in the neural crest and cell migration (Yaniv *et al.*, 2003) and axon arborisation in neurons (Kalous *et al.*, 2014), which appears to be consistent with similar observations in chick. Interestingly, an knockdown of *Xenopus Vg1 RBP* also leads to impaired endoderm, with morphants failing to induce proper pancreas or gut (Spagnoli and Brivanlou, 2006), which is also concordant with apparent endodermal defects in mice *igf2bp1* mutants.

In zebrafish, there are four *igf2bp* genes, *igf2bp1*, *igf2bp2a, igf2bp2b* and *igf2bp3.* The presence of a second *igf2bp2* gene appears to be due to a duplication of *igf2bp2a*. The function of this family is poorly characterised in zebrafish, although *igf2bp1* and *igf2bp3* appear to be expressed ubiquitously (Zhang *et al.*, 1999; Gaynes *et al.*, 2015). Morphants generated in *igf2bp1* exhibit a range of phenotypes, such as defective axonal projections, increased apoptosis, heart and trunk defects (Gaynes *et al.*, 2015), however, unpublished works with *igf2bp1* morphants from other researchers have not reported this phenotype (Van Rensburg, 2014). Morphants generated in *igf2bp2a* do not appear to present with a phenotype (O'Hare *et al.*, 2016) whereas unpublished data in *igf2bp2b* morphants reported phenotypes in the eyes and notochord (Li *et al.*, 2011). In the current literature, no report has been produced on disrupting *igf2bp3* function in zebrafish embryogenesis, although overexpression and injection of α-Igf2bp3 antibody appears to affect oocyte maturation (Takahashi *et al.*, 2014).

In invertebrates such as, *Drosophila* only appears to have one *igf2bp* homolog, which appears to have two peaks of expression (Nielsen *et al.*, 2000) and is present in the nervous system (Adolph *et al.*, 2009) and the pole cells (Nielsen *et al.*, 2000), which is later expressed in the ovaries (Boylan *et al.*, 2008) and testes (Toledano *et al.*, 2012). Moreover, as zygotic *igf2bp* mutants are lethal (Munro *et al.*, 2006), this appears to suggest that at

least one *igf2bp* homolog is required for development. Further analysis on the role of *Drosophila igf2bp* implicates it to have a role in axonal projections (Medioni *et al.*, 2014), synaptic development (Boylan *et al.*, 2008), and germ cell maintenance via miRNA (Toledano *et al.*, 2012) and Notch signalling regulation (Fic, Faria and St Johnston, 2019).

1.6. Objectives of this study

The regulation of maternal RNA during embryogenesis is required for many processes, previous mass-spectrometry experiments were carried out to define *sqt* RNA binding proteins using *in vitro* transcribed aptamers, which identified several putative RNA-binding proteins that are likely to bind to *sqt* RNA during early embryogenesis.

Questions raised by these previous results are:

- Do these RNA-binding proteins play a role in development, such as in the regulation of *sqt* RNA?
- How do these RNA-binding proteins regulate their targets?
- What is the requirement for these RNA-binding proteins to interact with their target mRNAs?

In this study, genetic deletions were generated by CRISPR-Cas9 for these proteins of interest, and we have retrieved several mutants to homozygosity and have characterised them in the context of early development.

2. Chapter 2 - Materials and Methods

2.1. Materials

2.1.1. Chemicals

Common chemicals (e.g. salts, buffers), or communally provided reagents by the department (e.g. LB, antibiotics, PBS) are not listed. Other chemicals were purchased from VWR, Thermo Fisher Scientific or Sigma Aldrich.

Chemical	Catalogue number	Supplier
Phenol-chloroform-isoamyl alcohol	77617	Sigma-Aldrich
Chloroform	C/4960/17	VWR
Ethanol	20821.330	VWR
Isopropanol	P/7500/PB17	VWR
Agarose	A9539	Sigma-Aldrich
Low melting agarose	1613111	Bio-Rad
Diethyl pyrocarbonate	D5758	Sigma-Aldrich
Glycerol	G0650/17	VWR
Paraformaldehyde	P6148	Sigma-Aldrich
Ethyl 3-aminobenzoate methanesulfonate (Tricaine/MS-222)	E10521	Sigma-Aldrich
30% acrylamide/bis solution	#1610154	Bio-Rad
Sodium dodecyl sulphate	L3771	Sigma-Aldrich
Ammonium persulphate	A3678	Sigma-Aldrich
1,4-Dithiothreitol	10197777001	Sigma-Aldrich
Bromophenol Blue	B0126	Sigma-Aldrich
Tris base	2922190090	Melford
Glycine	BP381-1	Fisher Scientific

Table 2-1. List of chemicals used.

2.1.2. Biological reagents and kits

Reagent/Kit	Catalogue number	Supplier
mMESSAGE mMACHINE SP6 Transcription Kit	AM1340	Thermo Fisher Scientific
HiScribe T7 High Yield RNA Synthesis Kit	E2040S	New England Biolabs
SuperScript IV Reverse	18090010	Thermo Fisher Scientific

Transcriptase		
Phusion High-Fidelity DNA Polymerase	M0530S	New England Biolabs
QIAquick Gel Extraction Kit	28506	Qiagen
QIAprep Spin Miniprep Kit	27104	Qiagen
100 mM dNTPs	10297018	Thermo Fisher Scientific
T4 DNA Ligase (1 U/μL)	15224017	Thermo Fisher Scientific
Riboprobe Combination Systems	P1450	Promega
DIG RNA Labeling Mix	11277073910	Sigma Aldrich
RNaseOUT Recombinant Ribonuclease Inhibitor	10777019	Thermo Fisher Scientific
qPCRBIO cDNA Synthesis Kit	PB30.11-10	PCR Biosystems
PCRBIO SyGreen Blue Mix Lo-ROX	PB20.15-05	PCR Biosystems
Monarch Total RNA Miniprep Kit	T2010S	New England Biolabs
TRIzol Reagent	15596026	Thermo Fisher Scientific
QIAprep Spin Miniprep Kit	27104	Qiagen
QIAquick PCR Purification Kit	28104	Qiagen
Restriction Enzymes		New England Biolabs
DNA ladders		New England Biolabs
RNA ladders		Invitrogen
Proteinase K		Sigma-Aldrich
5X GoTaq Reaction Buffer	M7911	Promega
T4 DNA Ligase	15224017	Invitrogen
BM Purple	11442074001	Roche
Bovine Serum Albumin	421501J	VWR
cOmplete, EDTA-free Protease Inhibitor Cocktail	11873580001	Sigma-Aldrich
Clarity Western ECL Substrate	1705061	Bio-Rad
DAPI (4',6-Diamidino-2-Phenylindole, Dilactate)	D3571	Thermo Fisher Scientific

Table 2-2. List of biological reagents and kits used.

2.1.3. Antibodies

Antibody	Catalogue	Supplier	Purpose	Dilution

	number			
α-Igf2bp3 (rabbit polyclonal)	PA5-46704	Thermo Fisher Scientific	Western blot	1:1000 (5% milk in TBSTw)
α-Igf2bp1 (rabbit polyclonal)	ab82968	Abcam	Western blot	1:1000 (5% milk in TBSTw)
α-β-catenin (rabbit polyclonal)	C2206	Sigma Aldrich	Immunofluorescence	1:500 (1% DMSO, 1% FBS in PBSTw)
α-rabbit Alexa Fluor 488 conjugated secondary antibody (goat)	A-11008	Thermo Fisher Scientific	Immunofluorescence	1:1000 (1% DMSO, 1% FBS in PBSTw)
α-actin HRP conjugated antibody (rabbit polyclonal)	sc-1615	Santa Cruz Biotechnology	Western blot	1:1000 (5% milk in TBSTw)
α-tubulin (rabbit monoclonal)	2125	Cell Signaling Technology	Western blot	1:1000 (5% milk in TBSTw)
α-rabbit HRP conjugated antibody (goat)	7074	Cell Signaling Technology	Western blot	1:3000 (5% milk in TBSTw)
α-acetylated tubulin (mouse monoclonal)	6-11B-1	Sigma Aldrich	Immunofluorescence	1:200 (1% DMSO, 1% BSA, 1% FBS in PBSTw
α-mouse Alexa Fluor 488 conjugated secondary antibody (donkey)	A-21202	Thermo Fisher Scientific	Immunofluorescence	1:1000 (1% DMSO, 1% FBS in PBSTw)

Table 2-3. List of antibodies used.

2.1.4. Software and bioinformatic tools

Software	Purpose	Reference
Ensembl Genome Browser	Genomic/Transcriptomic database	http://www.ensembl.org/index.htr
DNASTAR	Protein alignment and phylogeny analysis	https://dnastar.com
UCSC Genome Browser	Genomic database	http://genome.ucsc.edu/
A plasmid Editor (ApE) –	Molecular biology software for	http://biologylabs.utah.edu/jorg

v2.0.55	sequence alignments and plasmid map constructions	ensen/wayned/ape/
SnapGene	Molecular biology software for sequence alignments and plasmid map constructions	https://www.snapgene.com
Blast Local Alignment Search Tool (BLASTn/p)	Alignment tool for DNA/RNA/protein	http://blast.ncbi.nlm.nih.gov/
Clustal Omega	Alignment tool for DNA/RNA/protein	https://www.uniprot.org
T-coffee	Alignment tool for DNA/RNA/protein	http://tcoffee.crg.cat/apps/tcoffee/do:regular
BoxShade	Shading tool for DNA/RNA/protein alignments	https://embnet.vital-it.ch/software/BOX_form.html
CHOPCHOP v2	CRISPR-Cas9 sgRNA predictor tool	http://chopchop.cbu.uib.no
The Zebrafish Information Network (ZFIN)	Zebrafish reference database	https://zfin.org
ImageJ	Imaging analysis software	https://imagej.nih.gov
Integrative Genomics Viewer 2.3.92	User interface for visualising genomic and epigenomic data	https://software.broadinstitute.org/software/igv/
MxPro 3005P qPCR software	qPCR analysis software	
GraphPad Prism 8	Illustrative software	https://www.graphpad.com
Adobe Illustrator CS6	Illustrative software	https://www.adobe.com/

Table 2-4. List of software and bioinformatic tools used and their accessible locations.

2.1.5. PCR Primers (Oligonucleotides)

The following PCR primers were used in this study, primers were procured from IDT or Sigma-Aldrich pre-diluted to 100 µM in water and diluted to 10 µM in DEPC-treated water before use.

2.1.5.1. Genotyping primers

Gene	Primer name	Sequence (5'-3')	Amplicon length (bp)	Designed by
raver1	raver1 TSS forward primer	CTCAGCCAGCTGGGTTAAAA	1060	LV
raver1	raver1 TSS	ATGTCTACGATGACCTGGCA	442	LV

	forward primer WT only			
raver1	raver1 forward new	TAAACTGGCCAAAGCGAACGAG	299	LV
raver1	raver1 TSS reverse primer	TCCAAACGACTGGCAATCTC		LV
raver1	raver1 F EcorI PCR geno	ATCGGAATTCTAAACTGGCCAAAGCGAACGAG	299	LV
raver1	raver1 R EcorI PCR geno	ATCGGAATTCTCCAAACGACTGGCAATCTC		LV
syncrip	syncrip TSS forward primer	CCATGGTCTCTAGAGCACCTT	1299	LV
syncrip	syncrip TSS forward primer WT only	TTCTGGAGTTTAACGGTGGC	478	LV
syncrip	syncrip TSS F NEW	CACAGCAAGAAGGTCACTTG	894	LV
syncrip	syncrip TSS F WT ONLY NEW	TACAAAGTAATTCTGGCACG	352	LV
syncrip	syncrip TSS R NEW	CCGGTAAAATGAAGCCCTCT		LV
syncrip	syncrip TSS reverse primer	GTGTTGAAGTGGTCAGAATGGG		LV
syncrip	Syncrip Exon 5 F	GTTTGGACATTCCTGACTTA	305	LV
syncrip	Syncrip Exon 5 R	GGTAAACTGTCAGCGTAACAAC		LV
syncripl	syncripl TSS forward primer	GAATGAATTGTGTGGAACCCTG	1241	LV
syncripl	syncripl TSS forward primer WT only	GAGAAACCCCGTACAACC	394	LV
syncripl	syncripl TSS reverse primer	AAAGCACACAATGACTCCGC		LV
syncripl	syncripL Exon 2 F	GGTTTATCCTGCAGCGTTTC	388	LV
syncripl	syncripL Exon 2 F WT ONLY	ATGGCCACGGAGCATATAAA	270	LV
syncripl	syncripL Exon 2	ACTCAGTTTGATCAGTGGTG		LV

	R			
syncripl	syncrip like exon 3 forward	GGTGAAATGTGTTGGCAGATTG	395	LV
syncripl	syncrip like exon 3 reverse	CACCAAAAGGCCATTTTCAC		LV
syncripl	Syncripl Exon 4 Forward	TGGTTCTGATGCGGTCTCAGTT	305	LV
syncripl	Syncripl Exon 4 Reverse	GAAGCGGCACACAGAAAAGCAC		LV
syncripl	syncripl exon 5 f	TGTCTGTTCTGTCCAGCCGCTG	298	LV
syncripl	syncripl exon 5 r	GACCCCTTGCGGTTCTAGTGTT		LV
syncripl	syncripl e5 F EcorI PCR geno	ATCGGAATTCTGTCTGTTCTGTCCAGCCGCTG	298	LV
syncripl	syncripl e5 R EcorI PCR geno	ATCGGAATTCGACCCCTTGCGGTTCTAGTGTT		LV
igf2bp3	igf2bp3 tss forward	AGTTGCGCTTGTGGTCTCAGAT	1062	LV
igf2bp3	igf2bp3 forward NEW	CGCCTACACTAACGTGTGTG	496	LV
igf2bp3	igf2bp3 tss/exon1 reverse	CTGAAAGCGTGTCAATGGCCCT		LV
igf2bp3	igf2bp3 f atg ex1	ATGAATAAGCTGTACATCGGG	279	LV
igf2bp3	igf2bp3 reverse NEW	GCACACAATCCCTGCTTTGCAT		LV
igf2bp3	igf2bp3 geno/seq PCR F	ATCCCATGGATGAATAAGCTGTACATCGGG	279	LV
igf2bp3	igf2bp3 geno/seq PCR R	GATCATATGGCACACAATCCCTGCTTTGCAT		LV
igf2bp3	igf2bp3 F EcorI PCR geno	ATCGGAATTCATGAATAAGCTGTACATCGGG	279	LV
igf2bp3	igf2bp3 R EcorI PCR geno	ATCGGAATTCCTGAAAGCGTGTCAATGGCCCT		LV
igf2bp3	igf2bp3 nested F	GCTCTCTACAAACACTGCCCAT	500	LV
igf2bp3	igf2bp3 nested R	TTTGCCCTGCATACACAAGC		LV
igf2bp3	5071	GATGAGGGCCATTGACACGC	800/248	(1)

	(la0202659.gF)			
igf2bp3	5070 (la020659.gR)	GCAACGCTGCAACTTTCATAGACC		(1)
igf2bp3	5069 (la010361.gR)	GTCTCGCTGCTCGTGGCTAGG	900/339	(1)
igf2bp3	5068 (la010361.gR)	CTTAGGGACAGAGTGCTCCACTTC		(1)
igf2bp3	3' LTR F22	AAAGACCCCACCTGTAGGTTTG		(1)
igf2bp1	igf2bp1 nested F	CGTTAGGCTACTGTTAGTCG	568	LV
igf2bp1	igf2bp1 nested R	AGGCTTGCATTGCTCTGACTTG		LV
igf2bp1	igf2bp1 T7E1 EcorI F	ATCGGAATTCCTGTGGTTGTCTTGGTTCTT	330	LV
igf2bp1	igf2bp1 T7E1 EcorI R	ATCGGAATTCAAGAAACCCGGCGAATAAGC		LV
sqt	sqt 3'UTR ECorI T7E1 seq F	ATCGGAATTCAGTCTTTTGAAGCTGCACCA	334	LV
sqt	sqt 3'UTR T7E1 seq R	ATCGGAATTCCAGATAAGGCAAACACGCAA		LV

Table 2-5. List of genotyping primers used.

2.1.5.2. Primers for generating constructs

Gene	Primer name	Sequence (5'-3')	Amplicon length (bp)	Designed by
raver1	raver1 cdna forward	TAGGTGAATTCTAATGGCGGCCGCAGTGTCCG	2178	LV
raver1	raver1 cdna reverse	GCTACTCGAGTCAAAAGATGCGCTTGCGTT		
raver1	pmal raver1 ECorI F	TAGGTGAATTCATGGCGGCCGCAGTGTCCG		
raver1	pmal raver1 XbaI R	ATCTAGATCAAAAGATGCGCTTGCGTT		
syncrip	syncrip cdna	TCGGCGAATTCTAATGGCCACTGAACATATTAATG	1893	

	forward			
syncrip	syncrip cdna reverse	TGCGGCCGCCTACTTCCACTGTTGCCCAAAAG AAT		
syncrip	pmal syncrip EcorI F	TCGGCGAATTCATGGCCACTGAACATATTAATG		
syncrip	pmal syncrip XbaI R	ATCTAGACTACTTCCACTGTTGCCCAA		
syncripl	syncripl cdna forward	TCGGCGAATTCTAATGGCCACGGAGCATATAAA TG	1683	
syncripl	syncripl cdna reverse	TGCGGCCGCTCATACTACCTGGTCAGGACCA		
syncripl	pmal syncripl EcorI F	TCGGCGAATTCATGGCCACGGAGCATATAAAT G		
syncripl	pmal syncripl XbaI R	ATCTAGATCATACTACCTGGTCAGGAC		
igf2bp3	igf2bp3 cdna cloning F	TCGGCGAATTCTAATGAATAAGCTGTACATCGG GA	1749	
igf2bp3	igf2bp3 cdna cloning R	TGCGGCCGCCTATTTCCTCCTGGCGACTGGT		
igf2bp3	pgex4t1 igf2bp3 cloning F	GGGGAATTCATGAATAAGCTGTACATCGGGA		
igf2bp3	pmal-c2x igf2bp3 R	GATGGATCCCTATTTCCTCCTGGCGACTG		
igf2bp3	igf2bp3 3 UTR F	ATCGCTCGAGAGCCACTGCGTCTTCTCGGA	584	
igf2bp3	igf2bp3 3 UTR R	ATCTAGACTGAGGTACTCTAGCCTGAG		
igf2bp1	igf2bp1 cDNA cloning	ATCGGATCCTAATGAACAAGCTATACATTGG	1797	

	pet28c F			
igf2bp1	igf2bp1 cDNA cloning pet28c R	CGATCTCGAGTCACTTCCTCCTGGGCTCTT		
igf2bp1	pmal igf2bp1 EcorI F	ATCGGATCCATGAACAAGCTATACATTGG		
igf2bp1	pmal igf2bp1 XbaI R	ATCTAGATCACTTCCTCCTGGGCTCTT		
ybx1	Ybx1 pet28c F BamHI	ATCGGATCCTAATGAGCAGCGAGGCCGAGAC	930	
ybx1	Ybx1 pet28c R XhoI	CGATCTCGAGTTAATCTGCTCCGCCCTGTT		

Table 2-6. List of primers used to generate constructs.

2.1.5.3. Sequencing primers

Gene	Primer name	Sequence (5'-3')	Designed by
raver1	Raver1 cdna sequencing f	GCAACAGTTCGAGGAGCTAGTT	LV
raver1	Raver1 cdna sequencing r	GGGATACACCAACATCTTTCGG	LV
syncrip	Syncrip cdna sequencing f	TGAAAAGGCAGGGCCGATCTGG	LV
syncrip	Syncrip cdna sequencing r	CATACATTTGTGTCTTCGCTGC	LV
syncripl	Syncripl cdna sequencing f	AGAAAGCCGGACCCATCTGGGA	LV
syncripl	Syncripl cdna sequencing r	GCCGAACTGACAGAACGTCTTC	LV
igf2bp3	Igf2bp3 cdna sequencing f	CAAGGACCAAGCCAGAGAAGCA	LV
igf2bp3	Igf2bp3 cdna sequencing r	CCCCAAATGATTGATATCCAGC	LV
	M13F	TGTAAAACGACGGCCAGT	Generic

			primer
	M13R	CAGGAAACAGCTATGAC	Generic primer
	pGEX 3’	CCGGGAGCTGCATGTGTCAGAGG	Generic primer
	pMAL F	GATGAAGCCCTGAAAGACGC	Generic primer
	pGEX F	CTGGCAAGCCACGTTTGGTG	Generic primer
	SP6	ATTTAGGTGACACTATAG	Generic primer
	T7	TAATACGACTCACTATAGGG	Generic primer
	T7 terminal	GCTAGTTATTGCTCAGCGG	Generic primer

Table 2-7. List of sequencing primers used.

2.1.5.4. RT-PCR and qPCR primers

Gene	Primer name	Sequence (5’-3’)	Amplicon length (bp)	Designed by
18S	18S qPCR F	TCGCTAGTTGGCATCGTTTATG	62	
	18S qPCR R	CGGAGGTTCGAAGACGATCA		
igf2bp1	igf2bp1 14F	GTGAATGAACTGCAGAACCT	274	L.V
	igf2bp1 cDNA cloning pet28c R	CGATCTCGAGTCACTTCCTCCTGGGCTCTT		L.V
igf2bp2a	igf2bp2a 14F	GCGCAGGGCAGGATATTTGG	264	L.V
	igf2bp2a 16R	GATTTTCCTCTGTGCAGTCT		L.V
igf2bp2b	igf2bp2b 12F	GCACAGGGGAGGATATATGG	246	L.V
	igf2bp2b 13R	CTGACTGGCAAAGAAATGTC		L.V
igf2bp3	igf2bp3	ATTGCGCCTGCTGATGGAAT	207	L.V

	exon 13 RT-PCR F			
	igf2bp3 exon 14 RT-PCR R	CGTTTTGCCTCCTTTACCAATG		L.V
mxtx2	mxtx2 forward	TCTGATCTGCAAGCAACACC	232	1
	Mxtx2 reverse	TGTCCCAAAATGCAGAATCA		1
hhex	hhex forward	ACCATCGAGCTGGAGAAGAA	186	1
	hhex reverse	GTCCTCCGCTTCCCTTTTAC		1
mixl1	bonnie/mixer forward	GAGAACTTACAAAGAACCTCAACATTTAC	173	1
	bonnie/mixer reverse	ACACTCAGGTGATCAGTTTTGATG		1
cldnE	claudin E forward	AGAGATTTCTACAATCCTCTGCTCA	163	1
	claudin E reverse	GCTGGGAGTATTTCATGTTGTATTT		1
buc	buc forward	GTAAATCAATCCACCAGCAAAGG	216	1
	buc reverse	ACATACAGTCAAGAACAGTGTCC		1
cxcl12a	cxcl12a forward	ATGACCTGATTCTGCTGAGCGTGA	146	1
	cxcl12a reverse	TGGCTTCACTTGAAGGGTCGATTG		1
cxcr4a	cxcr4a forward	GGCTTATTACGGACACATCGTC	340	1
	cxcr4a reverse	CATGAACCCTCCAAAGTACCAGTC		1
cxcr4b	cxcr4b f forward	GGACTTGTGGTGCTTGTGATG	403	1
	cxcr4b r sequence	GGTAAGTAAGCTCGCAGATGG		1
tdrd1	tdrd1 forward	CCACCAGGGCAACTTAAGGTG	334	1
	tdrd1 reverse	CATCTCCTCGCACTGACAGTG		1

tdrd7a	tdrd7a forward	CATTGCTGGAGAGGTGATGTG	318	1
	tdrd7a reverse	TCAGGCACTGGTGATTTCCTG		1
gdf3	Vg1 forward	GCAAACAGACCGTTAATGACC	181	1
	Vg1 reverse	AACGAGAGTTGCTCAACCTCC		1
dazl	dazl forward	TACCCGTGTGCCTGATATGTGG	374	1
	dazl reverse	TGACACTGACCGAGAACTTCGC		1
dnd1	dead end forward	AGATGGACTTCCTTCTCCAAGTC	230	1
	dead end reverse	ATCAGCTCATTTCTTGACATTATGG		1
ddx4	vasa forward	CAACAGCAAGGAAAATATAGTCCA	192	1
	vasa reverse	ATCTAGTTCTGGATGAAGCAGACAG		1

Table 2-8. List of RT-PCR and qPCR primers used.

2.1.5.5. CRISPR sgRNA primers

Primer name	Gene targeted	Sequence (5' – 3')	Designed by
Oligo 2	None	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	(Varshney *et al.*, 2015)
raver1 TSS S3	*raver1*	AATTAACCCTCACTAAAGGCTCGAAACTCAAAAAAAAAGAGGGTTTTAGAGCTAGAAATAGC	L.V
raver1 TSS E1	*raver1*	AATTAACCCTCACTAAAGGGGCGGCCGCCATGTCTCCGTGTGGGTTTTAGAGCTAGAAATAGC	L.V
syncrip TSS S2	*syncrip*	AATTAACCCTCACTAAAGGAACCCACACCTCACCAACACAGGGTTTTAGAGCTAGAAATAGC	L.V
syncrip TSS E1	*syncrip*	AATTAACCCTCACTAAAGGTGGCCACTGAACATATTAATGGGGTTTTAGAGCTAGAAATAGC	L.V
syncripl TSS S1	*syncripl*	AATTAACCCTCACTAAAGGAGTTTAGAAATAATGATTCAAGGGTTTTAGAGCTAGAAATAGC	L.V
syncripl TSS	*syncripl*	AATTAACCCTCACTAAAGGGGGCACTACAGCAG	L.V

S2		AAGATTATGGGTTTTAGAGCTAGAAATAGC	
syncripl TSS E1	*syncripl*	AATTAACCCTCACTAAAGGAGAGGGAAACGTAT TGAAAGAGGGTTTTAGAGCTAGAAATAGC	L.V
syncripl TSS E2	*syncripl*	AATTAACCCTCACTAAAGGCGTATTGAAAGAGG ATAAAGTGGGTTTTAGAGCTAGAAATAGC	L.V
syncripl exon 2 T1	*syncripl*	TAATACGACTCACTATAGGGCTGCACAGGTCAC GGAGACATGGGTTTTAGAGCTAGAAATAGC	L.V
raver1 exon 1 T3	*raver1*	TAATACGACTCACTATAGGGCAGTAATTACGAC CAAACAGCGGGTTTTAGAGCTAGAAATAGC	L.V
igf2bp3 exon 1 T13	*igf2bp3*	TAATACGACTCACTATAGGGCTCCCTTCCTCGT AAAAAGTGGGTTTTAGAGCTAGAAATAGC	L.V
igf2bp3 exon 1 T2	*igf2bp3*	TAATACGACTCACTATAGGGTCATCGCTACCTT CTCGTCGGGGGTTTTAGAGCTAGAAATAGC	L.V
Syncripl Exon 2 T7	*syncripl*	TAATACGACTCACTATAGGGATTTCCATTTATAT GCTCCGTGGGTTTTAGAGCTAGAAATAGC	L.V
Syncripl Exon 2 T15	*syncripl*	TAATACGACTCACTATAGGGCCTTACATCTGCA CAGGTCACGGGTTTTAGAGCTAGAAATAGC	L.V
Syncripl Exon 4 T2	*syncripl*	TAATACGACTCACTATAGGGTGATGAAGACGTA CAGGCAGGTTTTAGAGCTAGAAATAGC	L.V
igf2bp3 Exon 1	*igf2bp3*	TAATACGACTCACTATAGGGCTCCCTTCCTCGT AAAAAGGTTTTAGAGCTAGAAATAGC	L.V
raver1 T3 sgRNA	*raver1*	TAATACGACTCACTATAGGGCAGTAATTACGAC CAAACAGGTTTTAGAGCTAGAAATAGC	L.V
raver1 T4 sgRNA	*raver1*	TAATACGACTCACTATAGGGAGGACGAGTGTTC ACCGAGTTTTAGAGCTAGAAATAGC	L.V
Syncripl Exon 5 sgRNA 1	*syncripl*	TAATACGACTCACTATAGGGTGGGCTGCACTCC GGCATGTTTTAGAGCTAGAAATAGC	L.V
Syncripl Exon 5 sgRNA 2	*syncripl*	TAATACGACTCACTATAGGGTATACGCTTGACG TGACGACGTTTTAGAGCTAGAAATAGC	L.V
Syncrip Exon 5 sgRNA 1	*syncrip*	TAATACGACTCACTATAGGGTGTAGACGGACTC TGGCGGGTTTTAGAGCTAGAAATAGC	L.V
Syncrip Exon 5 sgRNA 2	*syncrip*	TAATACGACTCACTATAGGGACAGGCGCTCAG CCCACCGTGTTTTAGAGCTAGAAATAGC	L.V
Sqt DLE sgRNA 1	*sqt*	TAATACGACTCACTATAGGGCTAGAGTTGAGTT CCTTTGGTTTTAGAGCTAGAAATAGC	L.V

Sqt DLE sgRNA 7	*sqt*	TAATACGACTCACTATAGGGAACTCAACTCTAG CACTTGTTTTAGAGCTAGAAATAGC	L.V
Sqt DLE sgRNA 8	*sqt*	TAATACGACTCACTATAGGGAATACATATTTTTG GGGTCGGTTTTAGAGCTAGAAATAGC	L.V
Sqt DLE sgRNA 9	*sqt*	TAATACGACTCACTATAGGGTTCTTAAATACATA TTTTTGGTTTTAGAGCTAGAAATAGC	L.V
igf2bp1 sgRNA 7	*igf2bp1*	TAATACGACTCACTATAGGGCTATGCGTTTGTT GATTGCCGTTTTAGAGCTAGAAATAGC	L.V
igf2bp1 sgRNA 6	*igf2bp1*	TAATACGACTCACTATAGGGTCATTGCCCATTG GTCGTCCGTTTTAGAGCTAGAAATAGC	L.V
Igf2bp1 sgRNA 14	*igf2bp1*	TAATACGACTCACTATAGGGACAGTTTCTCATG AAAACGTTTTAGAGCTAGAAATAGC	L.V

Table 2-9. List of sgRNAs designed as oligonucleotide primers used.

2.1.6. Constructs

The following constructs were generated or used in this study

Plasmid	Purpose	Size (kb)	Source
pET-28c: raver1	Protein expression	7.5	LV
pGEX-5X1: raver1	Protein expression	7.1	LV
pET-28c: syncrip	Protein expression	7.2	LV
pET-28c: syncripl	Protein expression	7	LV
pET-28c: igf2bp3	Protein expression	7.1	LV
pGEX-4T1: igf2bp3	Protein expression	6.7	LV
pMAL-c2x: igf2bp3	Protein expression	8.4	LV
pET28c: igf2bp1	Protein expression	7.1	LV
pGEX-5X1: igf2bp1	Protein expression	6.7	LV
pET-28c: ybx1	Protein expression	6.3	LV
pCS2: raver1	Capped mRNA/whole *in situ* hybridisation probe synthesis	6.3	LV
pCS2: syncrip	Capped mRNA/whole *in situ* hybridisation probe synthesis	6	LV
pCS2: syncripl	Capped mRNA/whole *in situ* hybridisation probe synthesis	5.8	LV
pCS2: igf2bp1	Capped mRNA/whole *in situ* hybridisation probe synthesis	5.9	LV
pCS2: igf2bp3	Capped mRNA/whole *in situ* hybridisation probe synthesis	5.8	LV

pCS2: igf2bp3 3' UTR	Capped mRNA/whole *in situ* hybridisation probe synthesis	6.4	LV
pT3TS: nCas9n	Capped mRNA	7.3	(Jao, Wente and Chen, 2013)
pT3TS: cas9 nos1 3' UTR	Capped mRNA	8.8	(Moreno-Mateos *et al.*, 2015)
pSP64: GFP-nos1 3' UTR	Capped mRNA		(Köprunner *et al.*, 2001)
pSP64: F' eGFP-nos1 3' UTR	Capped mRNA		(Weidinger *et al.*, 2002)
S6 (sox17)	Whole *in situ* hybridisation probe synthesis		
S17 (shha)	Whole *in situ* hybridisation probe - synthesis		
S40 (southpaw)	Whole *in situ* hybridisation probe synthesis		
V1 (vg1)	Whole *in situ* hybridisation probe synthesis		
V4 (ddx4)	Whole *in situ* hybridisation probe synthesis		
D11 (dazl)	Whole *in situ* hybridisation probe synthesis		
E10 (eve1)	Whole *in situ* hybridisation probe synthesis		
F12 (foxA3)	Whole *in situ* hybridisation probe synthesis		
G4 (gsc)	Whole *in situ* hybridisation probe synthesis		

Table 2-10. List of constructs used.

2.2. Methods

2.2.1.Zebrafish maintenance and embryo manipulations

2.2.1.1. Zebrafish lines

The following stable zebrafish lines were generated or used in this study

Line	Description	Background	Source
TU (WT)	Tubingen wild-type strain.	TU	

SG (WT)	Singapore wild-type strain.	SG	
igf2bp3^{la020659Tg}	Mutant line generated by retroviral insertion mutagenesis; GT transgenic construct inserted in intron one.	SG, isogenic	(Varshney *et al.*, 2013)
igf2bp3^{la010361Tg}	Mutant line generated by retroviral insertion mutagenesis; GT transgenic construct inserted in intron one.	SG, isogenic	(Varshney *et al.*, 2013)
igf2bp3$^{\Delta 7\ bp}$	Mutant line generated by Cas9 mutagenesis, 7 bp deletion in exon one.	TU	LV
igf2bp1$^{\Delta 10\ bp}$	Mutant line generated by Cas9 mutagenesis, 10 bp deletion in exon one.	TU	LV
igf2bp1$^{\Delta 13\ bp}$	Mutant line generated by Cas9 mutagenesis, 13 bp deletion in exon one.	TU	LV
igf2bp1$^{\Delta 11\ bp}$	Mutant line generated by Cas9 mutagenesis, 11 bp deletion in exon one.	TU	LV
igf2bp1$^{\Delta (+) 5\ bp}$	Mutant line generated by Cas9 mutagenesis, 5 bp insertion in exon one.	TU	LV
raver1$^{\Delta 73\ bp}$	Mutant line generated by Cas9 mutagenesis, 73 bp deletion in exon one.	TU	LV
raver1$^{\Delta 4\ bp}$	Mutant line generated by Cas9 mutagenesis, 4 bp deletion in exon one.	TU	LV
raver1$^{\Delta 20\ bp}$	Mutant line generated by Cas9 mutagenesis, 20 bp deletion in exon one.	TU	LV
raver1$^{\Delta 11\ bp}$	Mutant line generated by Cas9 mutagenesis, 11 bp deletion in exon one.	TU	LV

Table 2-11. List of zebrafish lines generated or used.

2.2.1.2. Zebrafish and embryo care

All adult zebrafish were kept at the ambient temperature in the animal facility in compliance to institutional animal care regulations (Westerfield, 2007), and embryos were collected from pair-wise or pooled intercrosses set up the previous evening using plastic mating tanks, transferred to plastic petri dishes (Thermo Fisher Scientific) and incubated at 28.5°C in 0.3X Danieau's solution with methylene blue. Solutions were made from 30X Danieau's solution (1.74 M NaCl, 21 mM KCl, 150 mM HEPES buffer (pH 7.2), 18 mM $Ca(NO_3)_2$, 12 mM $MgCl_2$) 1:100 in pure RO water or distilled water. Where required, embryos were dechorionated with a pair of Dumont Tweezers #5.

Fish dissected for extraction of tissues for further analysis were first euthanized according to institutional regulations and permission by rapid cooling and decapitation, tissues extracted

for analysis were snap frozen in liquid nitrogen before further processing (see Molecular Biology techniques).

2.2.1.3. Generation of mutant zebrafish by Cas9 mutagenesis

Cas9 mutagenesis was used to generate mutant lines in this study; all mutants were generated in the TU background from adult crosses. The targeted loci were previously sequenced to ensure minimal mismatches between the sgRNA and target.

Evaluation of the genes of interest were first performed to identify the best site to create mutations, mRNA and protein isoforms were aligned to identify regions conserved in all variants and domain functions required for protein activity, sequences upstream of these areas were targeted for mutagenesis by inputting these sequences into ChopChop (Labun *et al.*, 2016) and results were used to create sgRNAs targeting these regions.

Mutagenesis was performed by coinjection of the Cas9 mRNA and corresponding sgRNA into the yolk of 1-cell stage (20 mpf) embryos. Titrated dosages were typically performed with 100-150 pg of Cas9 mRNA and 25-50 pg of sgRNA if lethality was observed. In order to calculate the efficiency of sgRNA, injected embryos were collected in pools of 3-5 embryos, lysed and used as PCR templates to generate amplicons spanning the targeted regions. These were used for T7 endonuclease analysis.

Upon selection of an efficient sgRNA, adult matings were set up again and embryos collected were injected with Cas9 mRNA with the *nos1* 3'UTR to minimize somatic mutations, these were bleached at 24 hpf and raised to adulthood according to institutional protocols.

2.2.1.4. Genotyping mutants

Genotyping was performed with genomic DNA obtained from lysis of tail-fin clips or embryos. The following alleles were regularly genotyped and screened. Other mutants derived from Cas9 mutagenesis screens were identified from Sanger sequencing.

igf2bp3

igf2bp3^{la020659Tg}

The *igf2bp3^{la020659Tg}* insertion allele was genotyped by the use of three primers in a single PCR reaction, a forward/reverse primer flanking the insertion and a second reverse primer specific to the long terminal repeats in the retroviral construct. This generates a 250 bp WT

product and a ~800 bp product for the insertion allele. PCR products were analysed on a 2% agarose gel, shown in Figure 2-1.

igf2bp3^{la010361Tg}

The *igf2bp3*^{la010361Tg} insertion allele was genotyped by the use of three primers in a single PCR reaction, a forward/reverse primer flanking the insertion and a second forward primer specific to the long terminal repeats in the retroviral construct. This generates a 350 bp WT product and a ~900 bp product for the insertion allele (Fig. 2-1).

igf2bp3$^{\Delta\ 7\ bp}$

The *igf2bp3*$^{\Delta\ 7\ bp}$ deletion allele contains a continuous 7 bp deletion in exon 1. This mutation generates a BssαI restriction site. PCR products digested by BssαI were analysed on a 3% agarose gel.

igf2bp1

igf2bp1$^{\Delta(+)\ 5\ bp}$

The *igf2bp1*$^{\Delta(+)\ 5\ bp}$ insertion allele contains a non-continuous 5 bp insertion in exon 1, consisting of a 3 bp insertion, followed by a 2 bp insertion with a 3 bp space. This mutation generates a DdeI restriction site. PCR products digested by DdeI were analysed on a 3% agarose gel.

igf2bp1$^{\Delta\ 10\ bp}$

The *igf2bp1*$^{\Delta\ 10\ bp}$ deletion allele contains a continuous 10 bp deletion in exon 1. This mutation generates an AlwnI restriction site. PCR products digested by AlwNI were analysed on a 3% agarose gel.

igf2bp1$^{\Delta\ 5\ bp}$*; igf2bp3*^{la020659Tg}

The *igf2bp1*$^{\Delta\ 5\ bp}$ deletion allele contains a continuous 5 bp deletion in exon 1. This mutation loses a BspHI restriction site that is found in the WT allele. PCR products digested by BspHI were analysed on a 3% agarose gel. This allele was generated in the *igf2bp3*^{la020659Tg} homozygous background and genotyped as previously described.

raver1

raver1$^{\Delta\ 73}$ bp

The *raver1*$^{\Delta\ 73\ bp}$ deletion allele contains a continuous 73 bp deletion in exon 1. PCR products from this allele were analysed on a 3% agarose gel and directly distinguishable from the WT-sized product.

***raver1*$^{\Delta\ 20\ bp}$**

The *raver1*$^{\Delta\ 20\ bp}$ deletion allele contains a continuous 20 bp deletion in exon 1. This mutation generates an AlwNI restriction site. PCR products digested by AlwNI were analysed on a 3% agarose gel.

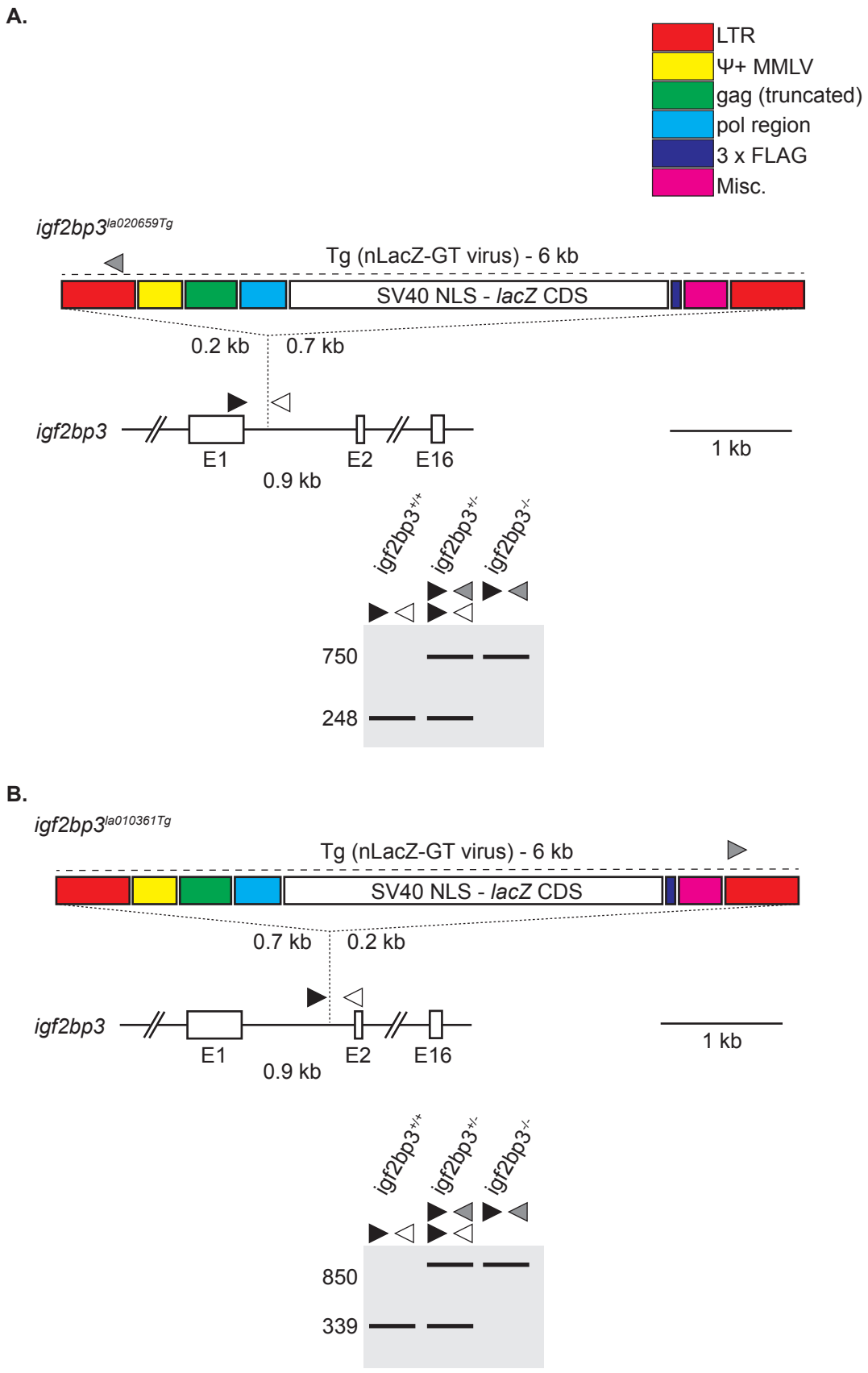


Figure 2-1. Strategy used to genotype *igf2bp3* transgenic insertion mutants. A. Schematic of the *igf2bp3*^{la020659Tg} allele. This allele contains the 6 kb Tg (nLacZ-GT virus) construct inserted approximately 200 bp into intron 1. Primer pairs (triangles) can be used to

distinguish between the WT and mutant alleles when analysed by gel electrophoresis (lower). **B. Schematic of the *igf2bp3*^{la010361Tg} allele.** This allele contains the Tg (nLacZ-GT virus) construct inserted approximately 700 bp into intron 1; different WT primers were used for this allele (shown in triangles) which can be used to distinguish between WT and mutant alleles in gel electrophoresis (bottom). The different elements in this construct are colour coded (upper right).

2.2.1.5. Calculations for overexpression analysis

The CDS for the genes of interest were cloned into the pCS2 vector to produce capped mRNA for overexpression analysis. To ensure dosages injected represented true overexpression, the amount of endogenous gene expression was approximated with the following protocol.

Firstly, the amount of total RNA in a single zebrafish embryo must be approximated, previous research by (Peterson and Freeman, 2009) indicates that approximately 15 µg of total RNA can be extracted from 50 embryos, resulting in approximately 300 ng of RNA per embryo. This value is roughly concordant with values obtained from results in this study (values between 200-500 ng of total RNA/embryo).

However, as the majority of total RNA is comprised of ribosomal RNA or transfer RNAs, these must be excluded in order to maximise the sampling of polyadenylated mRNAs in the population, it has been previously reported by (Detrich and Yergeau, 2004) that approximately 2-5% of total RNA is polyadenylated, which means that the amount of polyadenylated RNA per zebrafish embryo is approximately 6-15 ng.

As an overexpression experiment requires the introduction of transcript that is more than the endogenous concentration, the maximum expression of the genes of interest in this study were recorded using RNAseq data available from (White *et al.*, 2017), values were recorded as transcripts per million (TPM), and the sum of all transcripts were also recorded at the relevant stages. As the RNAseq data produced from (White *et al.*, 2017) here were enriched for polyadenylated transcripts, no further exclusion from ribosomal RNA were accounted for in the following calculations.

Hence, the proportion of gene of interest at its highest expression is presented as:

$$Proportion\ of\ GOI\ =\ Transcipts\ per\ million\ \div\ Sum\ Transcripts$$

As RNAseq analysis data is normalised per transcript lengths, the data produced by (White *et al.*, 2017) were inputted into Biomarts via Ensembl. The lengths of transcripts were

computed containing both the CDS and the UTRs and the median transcript length was derived. The median transcript length was used, as the transcript is not normally distributed but a largely positively skewed distribution, shown in Figure 2-2.

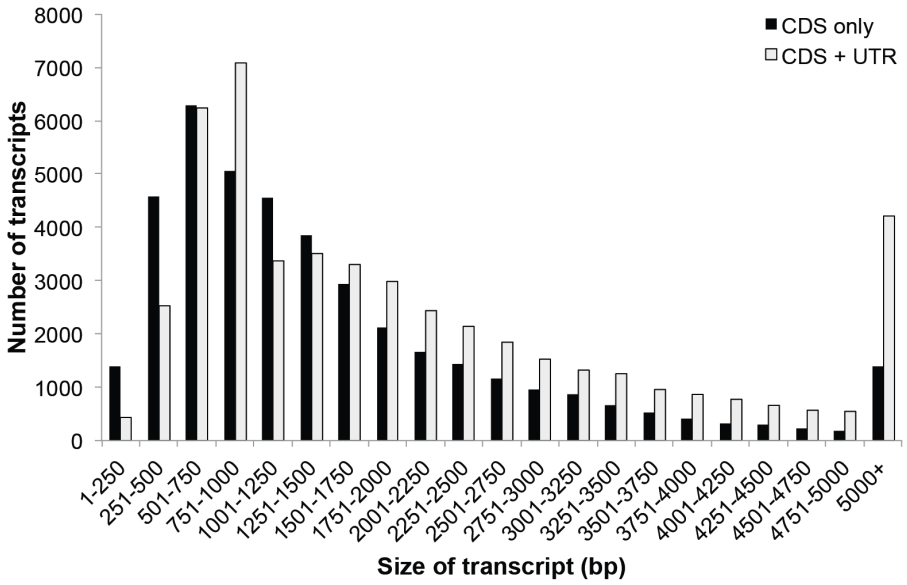


Figure 2-2. Distribution of RNA transcript length in zebrafish embryogenesis. RNAseq data set from early zebrafish embryogenesis were curated and the lengths of transcripts consisting of only the CDS and the CDS with the 5' and 3' UTRs were binned and plotted. Data obtained from (White *et al.*, 2017).

The scaling factor was then produced as follows:

$$Scaling\ factor\ =\ CDS\ of\ gene\ of\ interest\ \div\ Median\ transcript\ length$$

The final calculation used to estimate the amount of endogenous transcript present for each gene of interest is as follows:

$$Mass\ total\ RNA\ per\ embryo\ \ (ng) =\ 300$$

$$Percent\ polyadenylated\ RNA\ =\ \ 2-5\%$$

$$Mass\ polyadenylated\ RNA\ per\ embryo\ (ng)\ =\ 300\ \times\ 0.02-0.05$$

$$G.O.I\ RNA\ present\ (pg)$$
$$= Proportion\ of\ GOI\ \times\ Mass\ polyadenylated\ RNA/embryo$$
$$\times\ Scaling\ factor\ \times\ 1000$$

Using these formulae, the amount of endogenous RNA for the genes where overexpression experiments were performed were as follows:

Gene of interest	mRNA/embryo, lower (pg)	mRNA/embryo, upper (pg)
igf2bp3	1.44	3.60
igf2bp1	1.91	4.78
raver1	3.05	7.62

Table 2-12. Approximate RNA present per embryo for each gene of interest at its peak of expression.

2.2.2. Molecular Biology techniques

2.2.2.1. Generation of constructs

Plasmid constructs were generated in this study for the production of recombinant protein (i.e. pET-28c, pMAL-c2x, pGEX-4TI), capped mRNA or antisense labelled RNA (i.e. pCS2+). This was performed by cloning the respective cDNAs by reverse transcription of total RNA obtained by TRIzol extraction (Thermo Fisher Scientific) and column purification of RNA (NEB) following the manufacturer's instructions. Total RNA was sourced from dissection of zebrafish ovaries or 32-cell stage embryos. Reverse transcription was performed using the Superscript IV (Thermo Fisher Scientific) kit assembled in a 20 µL reaction as follows:

Total RNA	~ 5 µg
10 mM dNTPs	1 µL
Random hexamers (250 ng/µL)	1 µL
Nuclease-free water	to 13 µL

Following mixing and centrifugation, samples were heated at 65°C for 5 minutes and incubated on ice for 1 minute and the following reagents were added.

5X Superscript IV buffer	4 µL
100 mM DTT	1 µL
RNAse OUT RNAse Inhibitor	1 µL
Superscript IV Reverse Transcriptase	1 µL

Following mixing and centrifugation, samples were incubated at 25°C for 10 minutes and cDNA synthesis was initiated by incubation at 55°C for 1 hour and terminated by incubation at 85°C for 5 minutes.

After generating cDNA templates, high fidelity PCR was used to ensure amplicons were of the correct sequence by using Phusion High-Fidelity DNA Polymerase (NEB) assembled in a 50 µL reaction as follows:

5X Phusion HF buffer	10 µL
10 mM dNTPs	1 µL
10 µM forward primer	2.5 µL
10 µM reverse primer	2.5 µL
Phusion Taq polymerase	0.5 µL
cDNA template	1 µL

The following PCR program was typically used to amplify the cDNA for the genes of interest:

1.	98°C	30 second
2.	98°C	5 seconds
3.	66°C	30 seconds
4.	72°C	30 s/kb
5.	Step 2	39 times
6.	72°C	5 minutes
7.	4°C	Indefinitely

Following amplification, PCR products by purified by excision from bands obtained by gel electrophoresis (Qiagen) and restriction digested with enzymes corresponding to the overhangs (NEB), following the manufacturer's instructions.

PCR products were repurified from the restriction digest and cloned into linearised plasmids by ligation with T4 Ligase (Thermo Fisher Scientific) following the manufacturer's instructions using a molar ratio of 3:1 of insert to vector. Approximately 1 µL of this reaction was used to transform chemically competent XL-1 blue *E. coli* cells. Colonies were selected and amplified by colony PCR and amplicons from colonies with the correct size were miniprepped (Qiagen) and Sanger sequenced to ensure correct fidelity of the insert (Eurofins Genomic).

2.2.2.2. Production of capped mRNA, DIG-labelled anti-sense probes and sgRNA

Capped mRNA was generated from linearised plasmids using the SP6 mMessage mMachine kit (Thermo Fisher Scientific) assembled in 20 µL reactions as follows:

Linearised plasmid	0.1-1 µg

2X NTP/Cap mix	10 µL
10X reaction buffer	2 µL
SP6 enzyme mix	1 µL
Nuclease-free water	to 20 µL

Following 2 hours of incubation at 37°C, the reaction was stopped with 10 µL of stop solution (5 M ammonium acetate, 100 mM EDTA), 2 U of Turbo DNase I and diluted to 100 µL final volume. The mix was incubated for a further 15 minutes at 37°C to digest DNA templates and purified with phenol-chloroform extraction followed by precipitation with isopropanol for ~ 1 hour at -80°C.

Following precipitation by centrifugation, the RNA pellet was washed 80% ethanol in DEPC-treated water and dissolved in nuclease-free water, RNA concentrations were verified with spectrophotometry (N60, Implen) and with gel electrophoresis following denaturation with formamide and aliquoted before storage in -80°C.

Antisense DIG-labelled probes for *in situ* hybridisation were generated from linearised plasmids in 20 µL reactions assembled using Promega reagents with DIG-labelling mix (Sigma Aldrich) as follows:

Linearised plasmid	1 µg
10X DIG-labelling mix	2 µL
5X Optimised Transcription buffer	4 µL
100 mM DTT	2 µL
SP6/T3/T7 RNA polymerase	1 µL
RNAse OUT RNAse Inhibitor	1 µL
Nuclease-free water	to 20 µL

Following 3-4 hours of incubation at 37°C, DNA templates were digested with 2 U of Turbo DNase I and diluted to 50 µL final volume and incubated for a further 15 minutes at 37°C. The reaction was stopped, and RNA precipitated by addition of 1 volume of lithium chloride precipitation solution (7.5 M lithium chloride, 50 mM EDTA) at -80°C for ~ 1 hour. RNA was recovered by precipitation and ethanol washes followed by verification as previously described.

2.2.2.2.1. Production of Cas9 mRNA and sgRNAs

The template for Cas9 mRNA with the *Xenopus globin* 3' UTR or with the *nos1* 3' UTR was obtained by linearising the pT3TS-nCas9n or pCS2 T3 Cas9 nos1 3' UTR with XbaI and NotI (NEB) respectively, column purified (Qiagen) and the mMESSAGE mMACHINE Kit

(Thermo Fisher Scientific) components were used with T3 polymerase (Promega) following the manufacturer's instructions.

CRISPR-Cas9 sgRNA targets were predicted using CHOPCHOP v2 algorithm(Montague *et al.*, 2014; Labun *et al.*, 2016) by providing the targeting sequence and templates produced following protocols previous described by Burgess et al(Varshney *et al.*, 2015). Briefly, sgRNA templates for in vitro transcription were assembled by annealing a primer consisting of a T7 promoter sequence (with an additional GG if not included in the sgRNA sequence) followed by a 20 nucleotide targeted gDNA sequence and the crRNA/tracrRNA sequence:

5'-TAATACGACTCACTATA(GG)[20 nt sgRNA]GTTTTAGAGCTAGAAATAGC-3'

A second primer was used to complete the crRNA/tracrRNA sequence as follows:

5'-
AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTT
GCTATTTCTAGCTCTAAAAC-3'

The primers were annealed, and overhangs filled by a one cycle PCR reaction assembled as follows:

5X GoTaq PCR Buffer	20 µL
10 mM dNTPs	2 µL
10 µM Primer 1	4 µL
10 µM Primer 2	4 µL
Taq polymerase	2 µL
Nuclease-free water	to 100 µL

The following PCR protocol was used:

1.	95°C	3 minutes
2.	50°C	10 minutes
3.	72°C	10 minutes
4.	4°C	Indefinitely

Products were verified by gel electrophoresis and column purified (Qiagen) and concentration verified by spectrophotometry. This was used for in vitro transcription with the T7 HiScribe High Yield RNA Synthesis kit (NEB) as follows:

Template DNA	1 µg
10X Reaction buffer	2 µL

rNTP mix (100 mM)	8 µL
T7 polymerase mix	2 µL
Nuclease-free water	~ 20 µL

The mix was incubated for 2 hours at 37°C and the RNA was purified and verified with phenol-chloroform extraction as previously described.

2.2.3. Genomic DNA isolation from tissues

DNA was isolated from embryos at 24 hpf by incubating with lysis buffer (10 mM Tris pH 8.3, 50 mM KCl) with proteinase K (200 µg/mL) at 55°C for 3 hours, followed by inactivation at 95°C for 10 minutes. Fin clips were incubated for up to 16 hours for complete lysis and lysates were used directly for PCR.

2.2.3.1. Genomic DNA isolation from whole mounted embryos from in situ hybridisation

After embryos were imaged from in situ hybridisation, two washes in PBSTw (0.1%) were performed, followed by a wash in 100% methanol and a subsequent wash in distilled water. Embryos were transferred to a PCR tube and 20 µL of alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA) were added, digestion was performed by heating samples at 95°C for 30 minutes, followed by neutralisation with an equal volume of 40 mM Tris HCl pH 5.5. Lysates were directly used for nested PCR and approximately 2 µL of the nested PCR reaction was used for the final PCR reaction to genotype embryos.

2.2.4. CRISPR-Cas9

2.2.4.1. T7 endonuclease assay

Efficiency of sgRNAs were assessed from pooled embryonic lysates coinjected with Cas9 mRNA/sgRNAs, lysed as previously described at 24 hpf, and used for templates for PCR to amplify the locus. Approximately 5 embryos were used per pool.

PCR products were subsequently annealed by mixing 5 µL of PCR products with 4 µL H_2O and 1 µL 10X NEBuffer 2 (500 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1mM DTT, pH 7.9) to form the heteroduplex with the following thermocycler conditions:

95°C, 5 minutes.
95°C – 85°C, decreasing at -2°C/s.
85°C – 25°C, 0.1°C/s.

Following heteroduplex formation, the T7 endonuclease mix was added:

H_2O	8.25 µL
10X NEBuffer 2	1 µL
T7 endonuclease	0.75 µL

This mix was incubated for a further 30 minutes at 37°C. Digested products were loaded on a 3% 1X TAE agarose gel for electrophoresis and resolved band intensities were analysed using ImageJ to calculate mutagenesis previously described by (Lim *et al.*, 2013).

The T7 endonuclease assay uses the T7 endonuclease I, a DNA endonuclease that detects and cleaves mismatches in DNA strands. Templates for this are generated by PCR flanking the putative indel and products are subsequently annealed to ensure homogeneity and correct formation of the dsDNA with minimal secondary structures.

After digestion, digested and non-digested bands on the PCR gel must be made clearly distinct through by lengthy resolution at a low voltage and high percentage of agarose in order to produce reliable and accurate quantification of mutagenesis. Gels were imaged and the colours inverted to produce better visualisation of the cleaved bands (see Figure 2-3). The intensities of the bands were quantified on ImageJ (Analyze > Gels) by plotting two identical boxes across the undigested control and the digested lanes (Fig. 2-1, Image analysis). The peaks observed in the intensity patterns corresponding to the cleaved mutant products were isolated, adjusted for the background observed in the undigested controls, and the area under the peaks calculated by the Wand tool. Peaks for remaining WT products in both treated and untreated samples were also gathered.

The mutagenesis was calculated from these values using the following formulae. The sum of all bands were calculated in the treated samples.

$$Total\ Band\ Intensity = Uncleaved\ product + Cleaved\ products$$

Then the fraction of sample that was cleaved was derived as a proportion of the total products as follows:

$$Fraction\ Cleaved = Cleaved\ products\ /\ Total\ Band\ Intensity$$

Finally, the gene modification was calculated as:

$$Gene\ modification\ (\%) = 1 - \sqrt{(1 - Fraction\ Cleaved)}$$

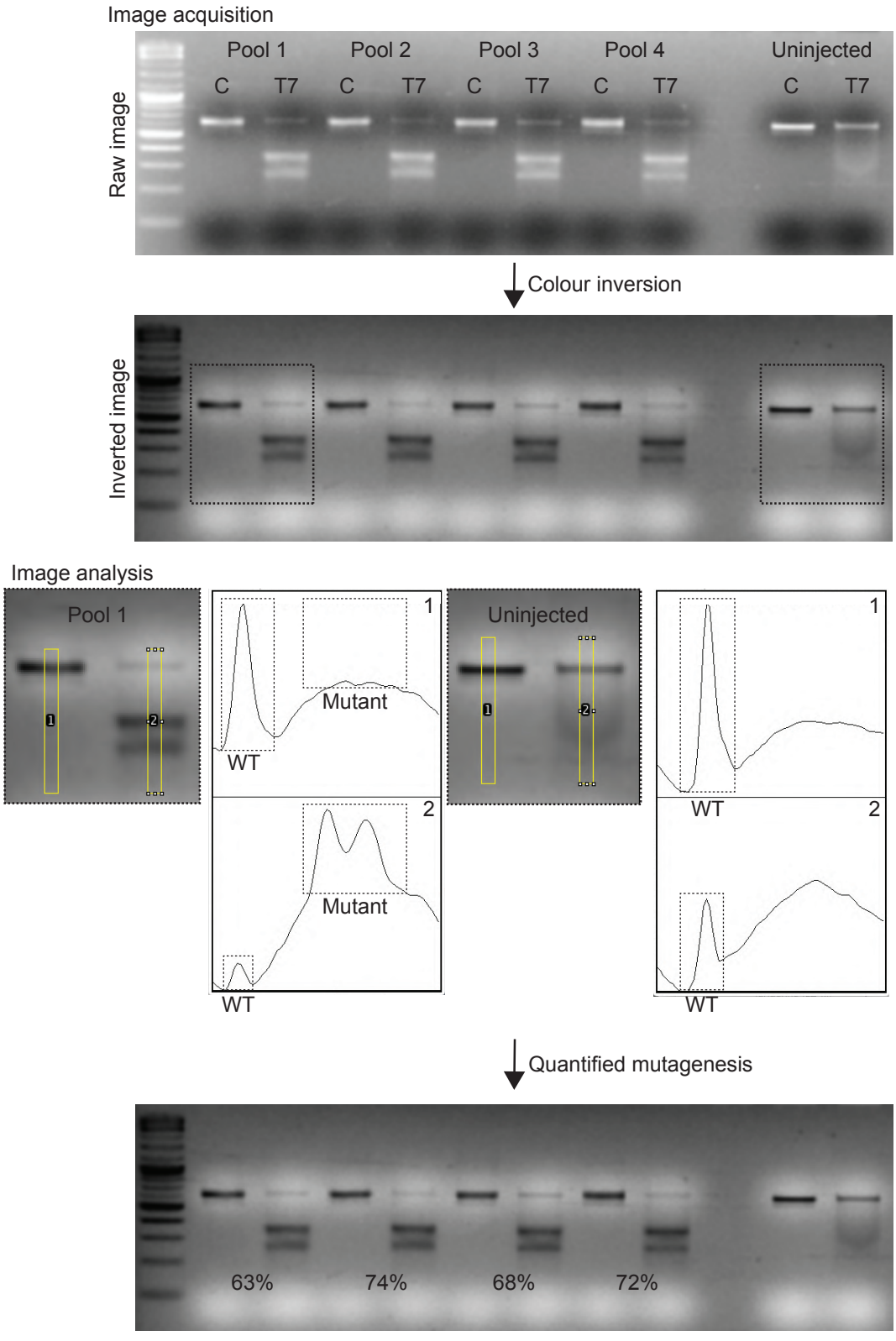


Figure 2-3. Methodology for quantification of Cas9 mutagenesis from gDNA extracted from whole embryos. Mutagenesis assessment was performed in three stages, acquisition of the image (upper), post-processing of the image by colour inversion and (middle) and

subsequent analysis of the gel band profiles (below). A representative image used to illustrate these steps.

The efficiencies of the sgRNAs were evaluated and reported below

Gene	sgRNA name	Efficiency (%)
Raver1	Raver1 TSS S3	0
	Raver1 TSS E1	0
	Raver1 T3 sgRNA	0
	Raver1 T4 sgRNA	45
Syncrip	Syncrip TSS S2	0
	Syncrip TSS E1	0
	T7 syncrip exon 2 ATG	0
	Syncrip exon 5 sgRNA 1	12
	Syncrip exon 5 sgRNA 2	9
Syncripl	Syncripl TSS S1	0
	Syncripl TSS S2	0
	Syncripl TSS E1	0
	Syncripl TSS E2	0
	Syncripl exon 2 T1	0
	Syncripl exon 2 T7	0
	Syncripl exon 2 T15	0
	Syncripl exon 4 T2	0
	Syncripl exon 5 sgRNA 1	31
	Syncripl exon 5 sgRNA 2	0
Igf2bp3	Igf2bp3 exon 1 T13	22
	Igf2bp3 exon 1 T2	0
Igf2bp1	Igf2bp1 sgRNA 7	0
	Igf2bp1 sgRNA 6	0
	Igf2bp1 sgRNA 14	23
Sqt	Sqt 3’ UTR DLE sgRNA 1	56
	Sqt 3’ UTR DLE sgRNA 7	69
	Sqt 3’ UTR DLE sgRNA 8	0

Table 2-13. List of sgRNA efficiencies.

2.2.4.2. Analysis of somatic mutations from Cas9 mutagenesis

Targeted loci were amplified from isolated genomic DNA described previously via PCR with primers designed 100-300 bp upstream and downstream of the expected target site and analysed with T7 endonuclease assay described previously.

Samples from fish identified as carriers for somatic mutations were then purified, restriction digested (NEB), cloned into the pGEM-T Easy vector (Promega) with DNA T4 Ligase (Thermo Fisher Scientific) and transformed into XL1-Blue chemically competent cells and plated with ampicillin resistance.

Transformants were screened with colony PCR to identify positive clones, the multiple cloning site of positive colonies to retrieve the cloned fragment using M13 universal primers, PCR purified and sequenced with Sanger sequencing (Eurofins genomic) and reads were aligned with the WT amplicon in ApE and predicted transcripts and translation products were generated in SnapGene to confirm whether these were null alleles, and whether a genotyping strategy could be produced.

2.2.5. qRT-PCR and analysis

RNA extractions were initiated with TRIzol, and followed by the Monarch Total RNA Miniprep kit following the manufacturer's instructions. cDNA synthesis was performed with the PCRBIO cDNA synthesis kit following the manufacturer's instructions with approximately 300-500 ng per 20 µL reaction.

qPCR samples were made with the PCRBIO SyGreen Blue Mix Lo-ROX, following the manufacturer's instructions. 1 µL of cDNA was used as input for each replicate.

qPCR was performed with the Stratagene MX3005P, and subsequent analysis was done with the MxPro 3005P qPCR software.

The efficiencies of the primers were evaluated with standard curves, performed using ten-fold titrations of cDNA, reported below

Gene	R^2	Efficiency (%)
18S	0.995	109.4
cldnE	0.995	109
hhex	0.994	95.8
mixl1	0.984	104.7
mxtx2	0.986	129.2
igf2bp1	0.996	110.9
igf2bp2a	0.907	80.1

igf2bp2b	0.992	151.2
igf2bp3	0.996	141.1
buc	0.996	132.5
dazl	0.997	100.8
vg1	0.999	113.8
ddx4	0.994	111.1
cxcl12a	1	94.1
cxcr4a	0.991	107
cxcr4b	0.972	100.7
tdrd1	1	85.5
tdrd7	0.994	97.9

Table 2-14. List of qPCR and primer efficiencies.

2.2.6. Biochemistry

2.2.6.1. Protein gel electrophoresis and Western blot

SDS-PAGE gels were prepared using the Bio-Rad protein electrophoresis systems, 10% and 12% separating gels were used in this study. To produce lysates, zebrafish embryos were homogenised in RIPA (50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA, 0.1% (w/v) SDS) lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich) using a syringe and needle. Following homogenisation, lysates were briefly centrifuged for 30 seconds at 1,000 x g, the supernatant was collected into a new tube and mixed with 4X loading buffer (200 mM Tris-HCl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue and 40% glycerol). Samples were mixed before being heated at 95°C for 2 minutes and centrifuged for 5 minutes at 12,000 x g. Supernatants were retained for loading, approximately 40-60 µL were used for each well, corresponding to approximately 20-30 embryos.

Electrophoresis was performed in 1X protein gel running buffer (25 mM Tris, 19.2 mM glycine, 0.1% SDS) using a Bio-Rad PowerPac Basic at 25 mA per gel until clear resolution of the ladder bands were obtained. Transfer of proteins from the gel was performed using an Enduro power supply (CS-300V, Appleton Woods) for 1 hour at 13 V to a nitrocellulose blotting membrane (10600004, Amersham Protran) that were sandwiched between 6 layers of Whatman paper (3030-917, GE Healthcare) soaked in 1X semidry transfer buffer (48 mM Tris, 39 mM glycine, 0.00375% SDS).

After transfer of proteins, membranes were rinsed with TBSTw once and blocked in 5% skimmed-milk powder in TBSTw for 1 hour before incubation with primary antibody

overnight. After incubation, membranes were rinsed 4X in TBSTw for 5 minutes and transferred to secondary antibody for 4 hours at room temperature, excess antibody subsequently removed by a further 4 washes in TBSTw for 5 minutes before detection with ECL Western blotting reagent (Bio-Rad) following manufacturer's instructions.

Signal detection was performed using a ChemiDoc MP Imaging system (Bio-Rad) or with CL-XPosure Film (34089, Thermo Fisher Scientific).

2.2.7. Microscopy and imaging techniques

2.2.7.1. RNA in-situ hybridisation

Fixed embryos were processed for whole mount *in situ* hybridisation using digoxygenin (DIG) labelled anti-sense RNA probes.

Embryo fixation

Briefly, embryos were fixed in fish fix buffer (4% paraformaldehyde, 4% sucrose, 120 µM $CaCl_2$ in 0.1M phosphate buffer at pH 7.2) or 4% PFA in PBS overnight at 4°C, embryos were subsequently dechorionated, washed with PBSTw (0.1%) and dehydrated in methanol series (25%, 50%, 75% in PBS) before storage in 100% methanol in -20°C.

Hybridisation

Hybridisation of embryos were achieved after rehydration in methanol series (75%, 50%, 25%) before washing in PBSTw for 4 times at 5 minutes. Embryos were digested with Proteinase K (20 µg/mL in PBSTw) if required as follows:

Bud and before	No digestion
1-18 somites	1 minute
18 somites – 24hpf	2 minutes
24 hpf to 72 hpf	30 minutes

After proteinase K digestion, embryos were post-fixed in fish fix or 4% PFA in PBS and washed for a further 4 times in PBSTw before pre-hybridisation in pre-warmed hybridisation buffer (60% formamide, 5X SSC, 1 mg/mL tRNA, 100 µg/mL heparin, 1X Denhardt's solution, 0.1% CHAPS, 10 mM EDTA, 0.1% Tween-20, pH 6.0-6.5) for 4 hours at 65°C. Pre-hybridisation solution was exchanged with hybridisation buffer containing probes at 1 ng/µL, and at incubated overnight at 65°C.

Following hybridisation, the following post-hybridisation washes were carried out at 65°C. Embryos were washed twice with solution 1 (50% formamide, 1X SSC, 0.1% Tween) for 30

minutes each, then solution 2 once (2X SSC, 0.1% Tween) for 15 minutes and solution 3 twice (0.2X SSC, 0.1% Tween 20) for 30 minutes.

Detection

After post-hybridisation, embryos were then washed with MABTw at room temperature 4 times for 5 minutes, before being blocked with blocking buffer (10% FBS, 1% Roche Blocking Reagent in MABTw) at room temperature for 2 hours before incubation with 1:4000 α-DIG antibody (Sigma-Aldrich) diluted in blocking buffer overnight at 4°C. Unbound antibody was removed from the embryos with 8 washes of MABTw at 15 minutes each.

Staining

For colourimetric detection, embryos were equilibrated in NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 1% Tween-20) followed by incubation in darkness with the alkaline phosphatase substrate, BM Purple (Roche) until complete staining was observed. Staining was stopped by addition of stop buffer (1 mM EDTA in PBSTw pH 5.5), washed in PBSTw before clarification in glycerol series before storage in 4°C.

2.2.7.2. Membrane and nuclei staining for immunofluorescence

Embryos prepared for immunofluorescence were collected at the 3 hpf and 4.5 hpf, fixed in 4% PFA in PBS overnight before dechorionation and dehydration in methanol as described previously. After rehydration in PBSTw, embryos were incubated in blocking solution (1% DMSO, 1% BSA in PBSTw) for 2 hours at room temperature before incubation in primary antibody (1:500 in blocking solution) overnight at 4°C. Embryos were subsequently washed 6X with PBSTw for 15 minutes each before secondary antibody was applied (1:1000 in blocking solution) overnight at 4°C. The unbound antibody was removed as previously described and incubated with DAPI (1 µg/mL) in PBS overnight at 4°C. Excess DAPI was removed by 4X washes of PBSTw for 15 minutes each before embryos were gradually transferred into 100% glycerol in gradients of 25%, 50% and 75%.

2.2.7.3. PGC labelling for spinning-disk confocal microscopy

In order to track migration statistics, PGCs were labelled with a fluorescent reporter by microinjecting 150 pg of GFP-nos1 3'UTR (Köprunner *et al.*, 2001) as 2 nL at 75 ng/µL into the yolk of the 1-cell embryo. The capped mRNA encoded by the construct (pSP64-mmGFP5-nos1-3'UTR) was generated after linearization with SacII and transcription with the mMessage mMachine kit.

In order to label the membrane of the PGCs to analyse filopodia dynamics, PGCs were labelled with a fluorescent reported by microinjecting 150 pg of EGFP-F-nos1-3′UTR

(Weidinger *et al.*, 2002) as 2 nL at 75 ng/μL into the yolk of the 1-cell embryo.. The capped mRNA encoded by the construct (pSP64-eGFP-F-nos1-3'UTR) was generated after linearization with NotI and transcription with the mMessage mMachine kit.

2.2.7.4. Microscopy

Time-lapsed embryos were imaged by brightfield microscopy using a Nikon Eclipse Ni upright microscope using an ORCA-Flash 4.0 LT (Hamamatsu) digital CMOS camera with a Nikon CFI Plan Fluor 10X (0.3 NA) objective. Embryos were mounted in 0.6% low melting agarose into a 35 mm glass bottom dish (Thermo Fisher Scientific).

Live embryos and stained embryos from *in situ* hybridisation were mounted in 2% methylcellulose and 100% glycerol respectively, and imaged with a Nikon SMZ18 stereomicroscope equipped with a DS-Fi2 (Nikon) camera. Live embryos imaged 24 hpf and later were anaesthetised in 0.016% Tricaine prior to imaging.

β-catenin and DAPI-stained embryos for immunofluorescence were mounted in 1% low melting agarose in PBS, and imaged with a Zeiss LSM 880 scanning confocal microscope, using the 25X Zeiss Plan-Neofluar 25X/0.8 NA and 40X Plan-Neofluar 40X/1.3 NA objectives. Live mounted embryos to image PGCs during somitogenesis were mounted in 0.6-0.8% low melting agarose.

Embryos imaged for live tracking of PGCs were imaged with an Andor Revolution Spinning Disk system, based on a Nikon Ni-E PFS inverted microscope equipped with a Yokogawa CSU-X1 spinning disk unit, and captured with a iXon Ultra 888 EMCCD camera. Images were captured using either a Nikon Plan Apochromat 20X/0.75 NA or the Nikon Apochromat 60X/1.49 NA oil immersion objectives. For the green fluorescent channel, excitation was generated by a 488 nm laser. Images were acquired with the Andor iQ3 software. Embryos were kept at 28.5°C using a heated stage.

2.2.7.5. Image analysis

RGB images generated from whole *in situ* hybridisation were not manipulated post-exposure although acquisition settings such as aperture and exposure time may be slightly adjusted between captures.

Time-lapsed live imaging from widefield microscopy was post-processed in the following method, z-stacks were captured encompassing the entire embryo at each timepoint and select slices from each timepoint where the embryo was in focus were used and

concatenated to generate a time-lapsed movie. Images were minimally adjusted for brightness/contrast but embryos were equally manipulated in Fiji/ImageJ.

Live imaging from line-scanning confocal microscopy was post-processed in the following method, z-stacks were captured encompassing the entire embryo for each developmental stage and stacks were presented as maximum intensity projections. Images were minimally adjusted for brightness/contrast in Fiji/ImageJ.

Immunofluorescence imaging for β-catenin/DAPI staining from line-scanning confocal microscopy was post-processed in the following method, z-stacks were captured from the first visible yolk syncytial nuclei until no further YSN could be observed in the field of view. Using Fiji/ImageJ, z-stacks were presented as maximum intensity projections and a 300 μm window across the centre of the embryo was drawn and YSN in this area were quantified.

Live imaging from spinning-disk confocal microscopy was post-processed in the following method. For time-lapsed imaging of migrating PGCs, z-stacks were generated with the 20X objective with 1 μm step-sizes at 1 minute intervals for 1 hour. Using ImageJ/Fiji, these were subsequently presented as maximum intensity projections and the MTrackJ (Meijering, Dzyubachyk and Smal, 2012) plugin was used as follows, PGCs were tracked at each timepoint by manually selecting the nuclei (or the centre of the cell where possible) until the end of the period track measurements were obtained through the plugin. These were computed into Excel to calculate Speed and Straightness by extracting the Length and Displacement (D2S) using the following equations:

$$Speed\ (\mu m/mins) = Length\ (\mu m)/60\ (mins)$$

$$Straightness = Displacement\ (\mu m)/\ Length\ (\mu m)$$

For live imaging of the PGC to analyse filopodia dynamics, z-stacks were generated with the 60X objective with 0.5 μm step-sizes at 10 second intervals for 2-10 minutes. Using ImageJ/Fiji, maximum intensity projections were generated and the filopodia numbers per PGC were recorded by counting the filopodia for a single timepoint. The persistence of the filopodia were calculated by counting the number of consecutive frames a single filopodium was present for. The length of a filopodia was calculated as the average length of a filopodium over its observable lifetime.

3. Chapter 3 - Results

3.1. Expression profile of *igf2bp* genes in zebrafish embryogenesis

To investigate the possible functions of *igf2bp3* in development, the identity of all *igf2bp* family genes in zebrafish were collated to determine if these had conserved features that were consistent with previous literature on these proteins.

In invertebrates such as *C. elegans* and *D. melanogaster*, only one homolog of *igf2bp* exists; and in amphibians such as frogs, the number of existing homologs is unclear. Previous work in identifying the function of *Xenopus* identified two highly similar cDNAs (~97% identity) that were attributed to allelic variants from the same locus (Deshler *et al.*, 1998; Havin *et al.*, 1998; Mueller-Pillasch *et al.*, 1999; Yaniv *et al.*, 2003), *vg1 RBP D* and *vg1 RBP B.* Whilst these may be allelic variants, alignment searches against the *X. laevis* proteome suggests this is unlikely and two Vg1 RBP variants (hereafter referred to as Igf2bp3A and Igf2bp3B) can be matched (UniProt ID O73932 and O57526), with a similar sequence identity of 97%. Igf2bp3A and Igf2bp3B are unlikely to be allelic variants of Igf2bp3 arising from the same locus, as they map to independent chromosomes (chromosomes 6 and 4 respectively), a key feature of vertebrate *igf2bp* synteny. Interestingly, a further search for *igf2bp3* in the closely related *X. tropicalis* only revealed one *igf2bp3* gene, found in chromosome 6. As the *X. laevis* genome is allotetraploid compared to the diploid nature of the *X. tropicalis* genome, the presence of a second *igf2bp3* gene in *X. laevis* but not in *X. tropicalis* strengthens the possibility that this is a genuine observation and the ancestral members in the *Xenopus* genus likely only contain one *igf2bp* gene.

In higher vertebrates such as chick, mouse and humans, three *igf2bp* genes exist, *igf2bp1*, *igf2bp2* and *igf2bp3*, located on independent chromosomes, with the canonical domain arrangement of two RRMs and four KH domains.

In our search for *igf2bp* genes in zebrafish using previously published RNAseq analysis (White *et al.*, 2017), four *igf2bp* genes could be identified, *igf2bp1, igf2bp2a, igf2bp2b* and *igf2bp3,* shown in Figure 3-1A. The presence of a second *igf2bp2* gene is probably due to a small scale duplication of *igf2bp2a* (Fig. 3-1B). The conservation of these *igf2bp* genes were assessed by sequence alignments of the proteins (Q08CK7, A0A0B4J1B0, A0A0R4IVY2 and Q9PW80). Based on predictions, it initially appeared that only Igf2bp1, Igf2bp2b and Igf2bp3 retained their RRMs, although Igf2bp2b has no RRMs. However, when alignments of these proteins were made, Igf2bp2a also has the full canonical structure of the Igf2bp protein, shown in Figure 7-1.

The expression of these genes appears to be biphasic, with *igf2bp1* and *igf2bp3* being the most dominantly expressed genes during embryogenesis: *igf2bp3* appears to be strongly expressed initially, and its downregulation is subsequently followed by the upregulation of *igf2bp1*, matching previous observations in the literature on the biphasic expression of *igf2bp* in development (Nielsen *et al.*, 2000).

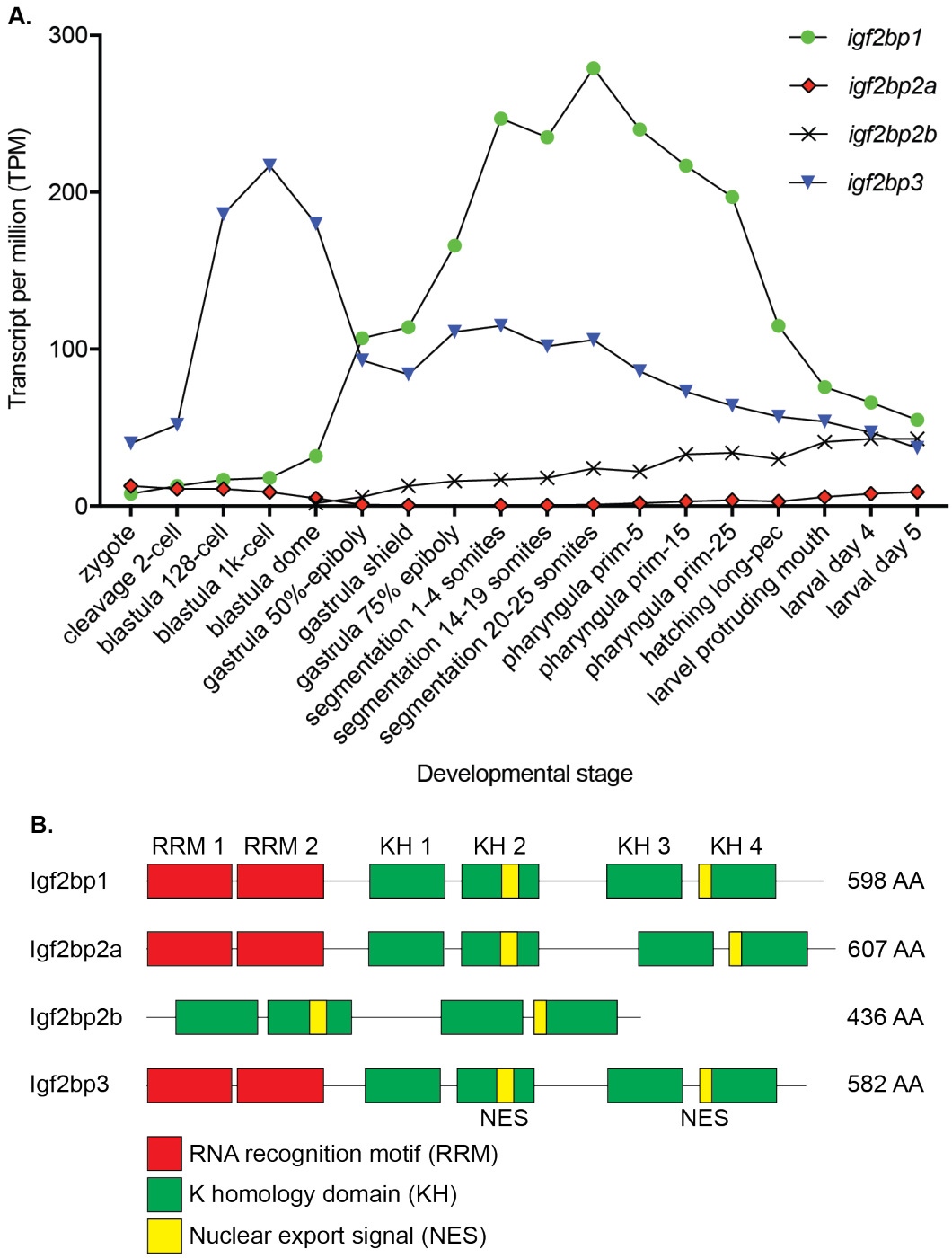


Figure 3-1. Expression profile and structural alignment of zebrafish *igf2bp* genes during development. A. RNAseq data for zebrafish *igf2bp1, igf2bp2a, igf2bp2b* and *igf2bp3* during embryogenesis. Expression data for all four zebrafish *igf2bp* homologs were plotted over the course of development (1-cell to 5 dpf). **B. Structural alignment of zebrafish Igf2bp proteins.** Zebrafish Igf2bp proteins were arranged and drawn according to

their RRM and KH domains, along with putative nuclear export signals from mammalian homologs. Data obtained from (White *et al.*, 2017).

After curating previously published RNAseq data and aligning the zebrafish Igf2bp sequences according to sequence identity and divergence (shown in Figure 7-2), Igf2bp1 and Igf2bp3 are the highest expressed *igf2bp* genes with the highest sequence identity, with approximately 72% sequence identity.

As Igf2bp1 and Igf2bp3 have a high sequence identity, are strongly expressed and have peak expressions that are exclusive, it was more likely that these genes are essential in development, as shown in previous mice and *Drosophila* mutants. Therefore, whole *in-situ* hybridisation were performed on early stage embryos up to 24 hpf (Figure 3-2) to determine if the expression patterns of these genes could provide indications as to where these genes are likely to act. However, as both *igf2bp1* and *igf2bp3* were ubiquitously and very strongly when probed for *igf2bp1* and *igf2bp3*, they are not spatially restricted in development and do not particularly appear to be enriched in specific tissues.

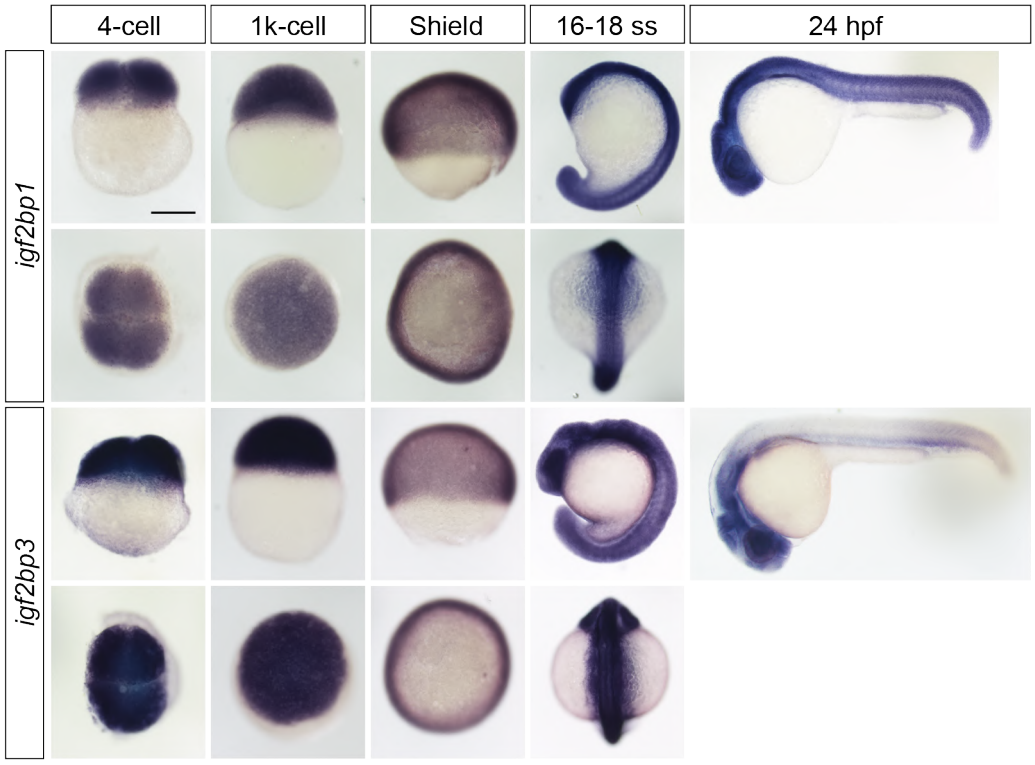


Figure 3-2. Expression of *igf2bp1* and *igf2bp3* during early zebrafish development. WT embryos were probed for *igf2bp1* and *igf2bp3* during cleavage, blastula, gastrula, somitogenesis and 24 hpf. Scale bar = 200 µm.

3.2. Overexpression of *igf2bp1* and *igf2bp3* does not affect early zebrafish embryogenesis

Whilst *igf2bp1* and *igf2bp3* are not spatially restricted in embryogenesis, the abundance of its expression suggests that these genes may play a role in development in regulating its targets; several lines of evidence suggest that regulation of mRNA targets by Igf2bp-complexed proteins are dose-dependent. Direct evidence from overexpression of Igf2bp in cell lines indicate that target mRNA targets are much less susceptible to stress-induced degradation (Huang *et al.*, 2018), and clinical analysis of patient carcinomas indicated that upregulation of *igf2bp* genes in cancers were linked to poor prognoses due to aggressive cell behaviours (Hsu *et al.*, 2015), and, in *Drosophila,* overexpression of Igf2bp or another mRNP complex protein Syncrip (Weidensdorfer *et al.*, 2008; McDermott *et al.*, 2012) leads to misregulation of its target mRNAs (Geng and Macdonald, 2006), such as the dorso-ventral determinant, *gurken*.

To explore the possibility that overexpression of Igf2bp proteins could also influence zebrafish development, the *igf2bp1* and *igf2bp3* cDNAs were cloned into the pCS2+ vectors to generate capped mRNAs and injected into 1-cell WT zebrafish embryos, shown in Figure 3-3. The phenotypes of these injected embryos could not be distinguished from uninjected controls, and we could not observe any gross or obvious defects leading to lethality in these embryos, suggesting that transient overexpression of *igf2bp1* and *igf2bp3* do not appear to affect early zebrafish development.

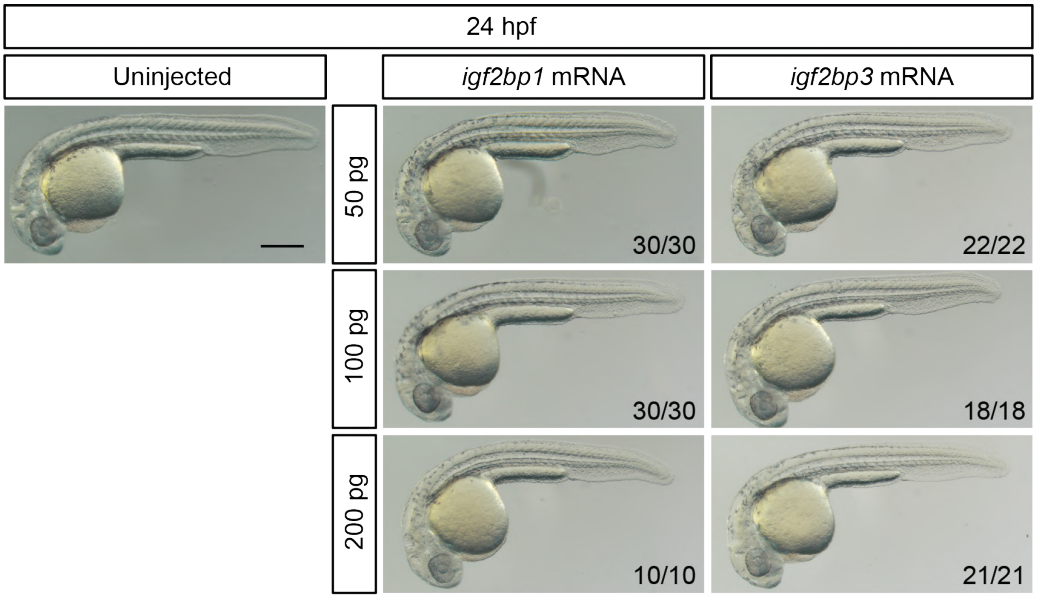


Figure 3-3. Overexpression of *igf2bp1* and *igf2bp3* does not affect early zebrafish embryogenesis. The *igf2bp1* and *igf2bp3* CDS were transcribed as synthetic capped mRNA and injected into WT 1-cell stage embryos at 50 pg, 100 pg and 200 pg before imaging at 24 hpf. Scale bar = 200 μm.

3.3. An *igf2bp3* transgenic insertion allele does not produce detectable Igf2bp3

After investigation of the *igf2bp* gene family in zebrafish, the presence of maternally provided *igf2bp1* and *igf2bp3* transcripts are clear and we subsequently attempted to perturb Igf2bp3 function in zebrafish. As maternal Igf2bp3 protein is present in abundance in zebrafish embryos (Bontems *et al.*, 2009), morpholinos would not be effective in blocking Igf2bp3 function, especially as the mutant phenotype has not been characterised.

In order to study the mutant phenotypes, the ZFIN database was checked for previously generated mutant alleles that could be retrieved for analysis and we found four transgenic insertion alleles and four point mutation alleles. As the point mutation alleles produce premature stop codons or splice site mutations in the 3' end of the transcript, we focused our attention on the transgenic insertion alleles.

These alleles contained the *Tg(nLacZ-GTvirus)* construct produced from a murine leukaemia virus (MLV) based mutagenesis screen (Varshney *et al.*, 2013), and two alleles contained the retroviral insertion in intron 1, which are *igf2bp3*^{la010361Tg} and *igf2bp3*^{la020659Tg}, hereafter referred to as *igf2bp3*$^{-659\ Tg}$ and *igf2bp3*$^{-361\ Tg}$. These alleles were procured as we hypothesised the earliest possible integrations in the locus would lead to a higher possibility of these alleles being stronger loss-of-function alleles for *igf2bp3*.

Therefore, fish harbouring the *igf2bp3*$^{-659\ Tg}$ and *igf2bp3*$^{-361\ Tg}$ alleles were outcrossed over at least two generations and intercrossed to homozygosity, shown in Figure 3-4A. These fish did not show any obvious defects in development, and we could not observe any issues with fertility or fecundity in the zygotic mutants, although not statistically tested.

As the functional consequence of these alleles was not documented, we used RT-PCR to validate the consequence of the insertion. RT-PCRs were performed with ovarian tissue samples extracted from WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ siblings to determine if the allele disrupts molecular events such as splicing and/or transcription. As the integration of the retroviral construct occurs in intron 1, we performed RT-PCR with primers spanning the exon 1-2 and exon 1-LTR junction (see Fig. 3-4A, B), to determine if splicing events were affected. We could not observe products in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PCR products with either primer sets, suggesting that the retroviral insertion is not transcribed. Further RT-PCRs were performed with primers spanning the *igf2bp3* exon 13-14 junction to check whether remaining transcripts could be observed. As exons 13 and 14 are retained in all *igf2bp3* mRNA transcripts that are not affected by nonsense-mediated decay, this junction was used as a proxy to determine if alternative splicing or transcription from an alternative transcriptional start site was present that could produce transcripts skipping exon 1-2 that were capable of

producing functional Igf2bp3. Similarly, we were not able to observe any bands with RT-PCRs against this junction.

However, as transgenic insertion alleles are often hypomorphic, we could not conclude that reduction or loss of *igf2bp3* transcripts in this allele meant it was a protein-null allele, and we used Western blots to verify whether the loss of *igf2bp3* transcript would produce a loss of detectable protein. Using a commercial α-Igf2bp3 antibody raised against the mammalian Igf2bp3 KH 1 domain, corresponding to exons 6 and 7 in the zebrafish *igf2bp3* cDNA. Lysates from 1-cell to early gastrula from WT and *igf2bp3*$^{-/-}$ stage embryos were probed for the presence of Igf2bp3 (see Fig. 3-4C), and no detectable bands could be observed in mutant lysates compared to WT controls, confirming that Igf2bp3 is deposited maternally and the *igf2bp3* transgenic insertion alleles do not produce detectable Igf2bp3 protein.

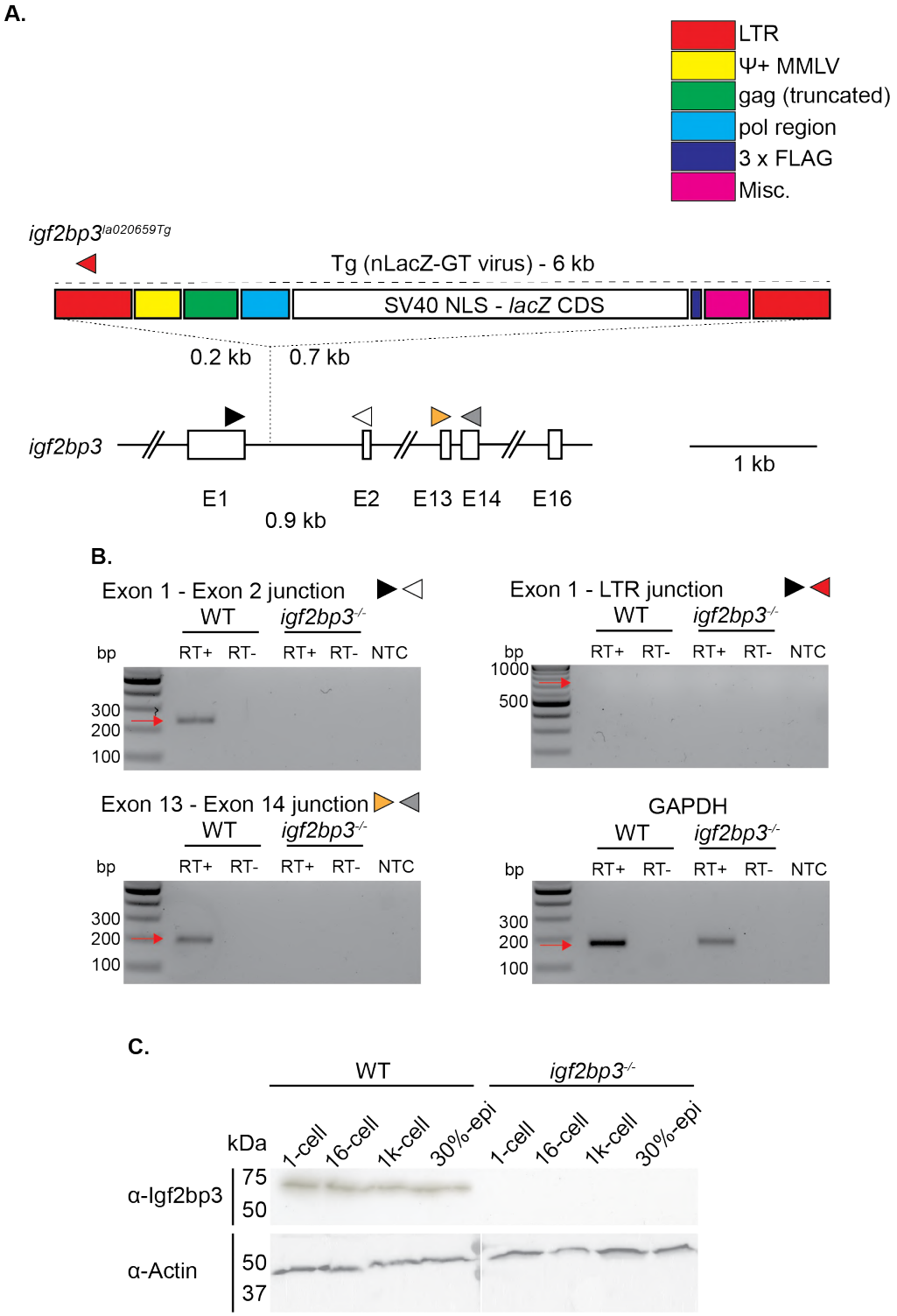


Figure 3-4. *igf2bp3* transgenic insertion mutants do not produce detectable Igf2bp3 protein. A. Schematic of the *igf2bp3*$^{-659\ Tg}$ insertion. The *igf2bp3*$^{-659\ Tg}$ insertion allele contains the *Tg (nLacZ-GT virus)* construct, of ~6 kb, in intron one, ~0.9 kb. **B. RT-PCR of the WT and *igf2bp3*$^{-659\ Tg}$ allele.** RT-PCR performed on ovary cDNA in WT and *igf2bp3*$^{-659\ Tg}$ mutants for exon 1-2 junction, exon 1-LTR junction, exon 13-14 junction and a GAPDH control with annotated primers (triangles in **A.**), red arrow indicates expected cDNA product size. **C. Western blots for Igf2bp3 and β-actin in WT and *igf2bp3*$^{-659\ Tg}$ embryonic**

lysates. Embryonic lysates were probed with α-Igf2bp3 and α-Actin antibodies to confirm whether Igf2bp3 protein is present in transgenic insertion mutants.

3.4. Loss of *igf2bp3* does not appear to lead to genetic compensation during early gastrula

As preliminary observations with the *igf2bp3*$^{-659\ Tg}$ allele did not produce any observable phenotype or defects, we considered the possibility that genetic compensation could occur in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants by upregulation of the remaining members of the *igf2bp* family, particularly those with the next highest expression and sequence identity such as *igf2bp1.*

To test this hypothesis, qRT-PCRs were performed on 1-cell, 1k-cell, Dome, 50%-epiboly, 75%-epiboly and Bud stage embryos against *igf2bp1*, *igf2bp2a, igf2bp2b* and *igf2bp3* with primers targeting exon-spans found in all transcript variants of these genes, shown in Figure 3-5. CT values for *igf2bp2b* were excluded for 1-cell and 1k-cell as expression of *igf2bp2b* occurs from Dome, and values, if any, were towards the maximum cycles of the qPCR protocol and were unlikely to be genuine observations or accurate measures of transcription. *18S* was used as the housekeeping control for gene expression and all subsequent qRT-PCR experiments, as initial tests with the housekeeper gene *GAPDH* was unreliable as *GAPDH* expression appeared to be reduced in the *igf2bp3*$^{-/-}$ embryos.

Nonetheless, the expression of remaining *igf2bp* genes does not appear to be consistently upregulated in the *igf2bp3*$^{-/-}$ mutants, with expression of *igf2bp1* and *igf2bp2a* being comparable at all stages tested, *igf2bp2b* expression was also comparable, except for 75%-epiboly and Bud stage (p-values < 0.05 and 0.094 respectively), where some upregulation appears to be observed. Interestingly, qRT-PCR for *igf2bp3* reveals that the expression in the *igf2bp3*$^{-659\ Tg}$ allele is reduced to approximately ~5% of the WT levels, suggesting this allele is at least severely hypomorphic for *igf2bp3*.

Together, it is unlikely that genetic compensation is occurring in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants from remaining *igf2bp* genes.

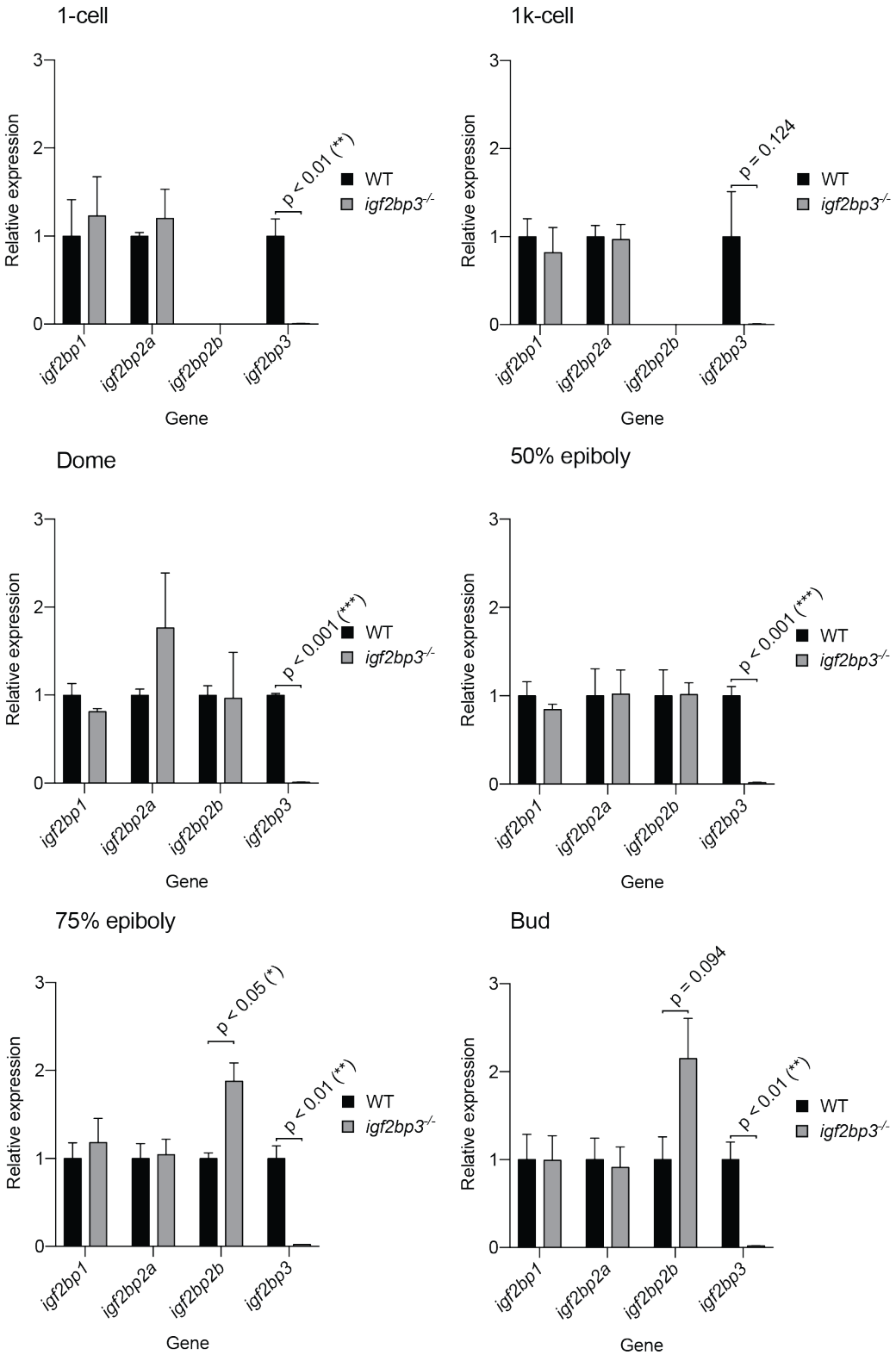


Figure 3-5. qRT-PCR of *igf2bp* genes in *igf2bp3* mutants. qRT-PCR were performed on WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants at 1-cell, 1k-cell, Dome, 50%-epiboly, 75%-epiboly and Bud for relative expression of *igf2bp1, igf2bp2a, igf2bp2b* and *igf2bp3* to the housekeeping gene *18S.* Note that *igf2bp2b*s is not expressed maternally and is not represented at 1-cell and 1k-cell. Statistical analysis performed with two-tailed unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.5. Axis formation and early endoderm development does not appear to be significantly affected in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants

As we had initially identified Igf2bp3 through proteomic screens for *sqt* mRNA binding proteins, we hypothesised that loss of Igf2bp3 could lead to misregulation to *sqt* and defects is axis development and/or cell migration, which would be consistent with the *Drosophila IMP* mutant (Geng and Macdonald, 2006).

Initially, we attempted to gauge defects in the early dorso-ventral axis by measuring the expression arcs of WT and *igf2bp3*$^{-/-}$ embryos at 30% and 50% epiboly with the dorsal axis marker *goosecoid* (*gsc*), shown in Figure 3-6A. However, this was not significant, and we also quantified the expression arc of the ventral axis marker, *even-skipped-like-1* (*eve1*), at 30%-epiboly; which appeared to be reduced in the *igf2bp3*$^{-/-}$ embryos (Fig. 3-6B).

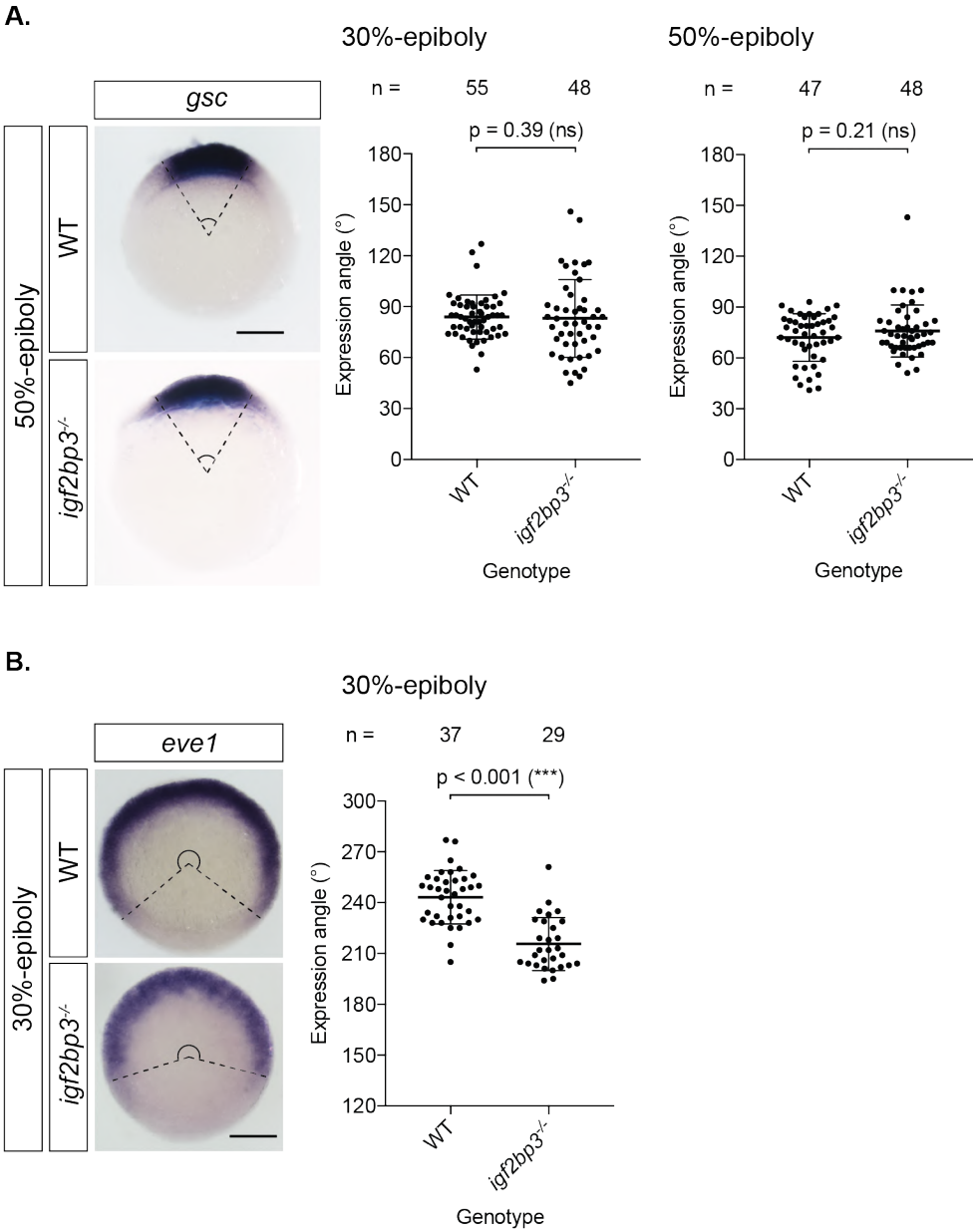


Figure 3-6. Dorso-ventral axis does not appear to be significantly affected in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants. A. Expression arcs of WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos for *gsc*. Embryos were probed for *gsc* expression (left) and the arcs quantified (right) at 30%- and 50%-epiboly. Statistical analysis was performed with two-tailed unpaired t-test. **B. Expression arcs of WT and *igf2bp*$^{-659\ Tg/-659\ Tg}$ embryos for *eve1*.** Embryos were probed for *eve1* expression (left) and the arcs quantified (right) at 30%-epiboly. Statistical analysis was performed with two-tailed unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Scale bar = 200 µm.

In addition to the dorso-ventral axis, we assessed left-right asymmetry with whole *in-situ* hybridisation with the nodal-related gene, *southpaw* (*spaw*), which, in WT populations, has a strong preference being expressed in the left lateral plate mesoderm (LPM) during

somitogenesis, shown in Figure 3-7. In WT populations, the expression of *spaw* in the left KPM is above typically 90%.

After scoring WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$, the difference between the genotypes is only marginally affected, with leftward expression of *spaw* in the LPM being 100% and 95% between the WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ respectively.

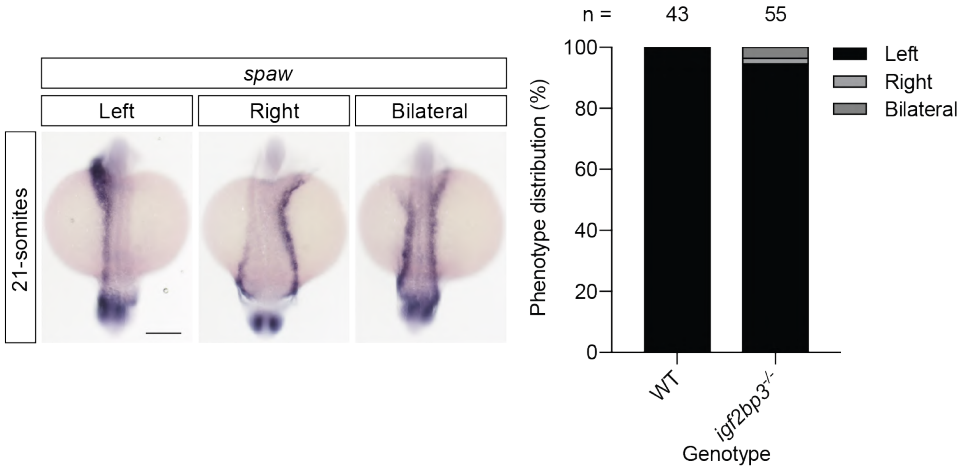


Figure 3-7. Left-right asymmetry is marginally affected in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants. WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos were temperature shifted at 60%-epiboly until 21-somites and probed for *spaw*. Phenotypes (left) were scored according to expression and the distribution quantified (right). Scale bar = 200 μm.

As we have not observed any significant defects in the dorso-ventral axis or left-right patterning, we decided to explore whether cell migration was defective by monitoring the movement of the dorsal forerunner cells (DFCs) during epiboly. The DFCs are precursors of the Kupffer's vesicle, a ciliated structure that generates asymmetric nodal flow, defects in these cells can infer potential issues in organ laterality or endoderm development. Furthermore, as these cells strongly express *sox17*, their behaviour can be easily visualised.

To test this hypothesis, WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ were probed at 70%-epiboly for *sox17* and the dispersion of the DFCs scored, shown in Figure 3-8. However, we were not able not to discern any differences between the two genotypes, with the distribution of the cells falling in class I and class II phenotypes at roughly approximate proportions (60% and 40% for both classes and genotypes respectively), which represent the distribution of the cells as either a distinct cluster or a dispersion in two or more smaller clusters. We did not observe more severe classes previously reported in the literature such as anterior movements or failure of the DFCs to coalesce (Zhang *et al.*, 2016) and there did not appear to be any obvious perturbances in the endodermal cells around the blastoderm.

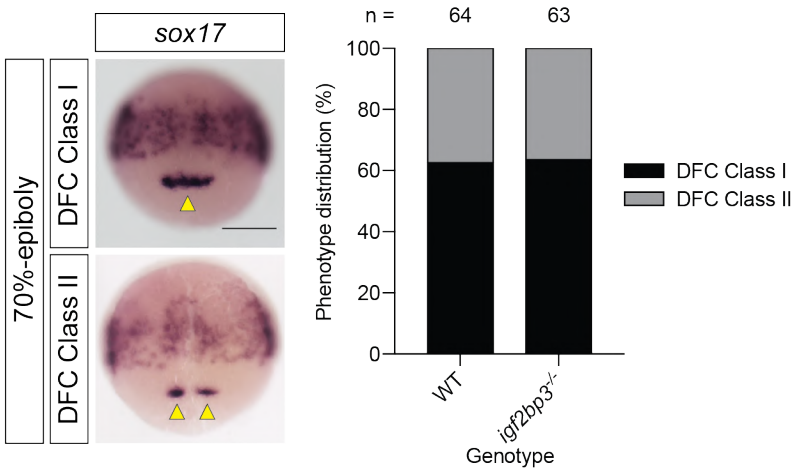


Figure 3-8. Endoderm marker expression is similar between WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants. WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos were probed for *sox17* to score the migration of the dorsal forerunner cells (yellow triangle) and the phenotypes were scored accordingly (right). Scale bar = 200 µm.

3.6. Maternal *igf2bp3* mutants are transiently delayed during early blastula and the yolk syncytial later appears to be expanded in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants

In the course of monitoring and collecting WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos for analysis, the progression of these embryos broadly followed the patterns previously established by (Kimmel *et al.*, 1995).

However, WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos laid at approximately the same time did not remain synchronous and were consistently delayed by gastrulation, in which *igf2bp3*$^{-659\ Tg/-659\ Tg}$ appeared to be delayed around 3 hpf for approximately one hour before continuing development without further defects, shown in Figure 3.9, which appears to occur around the midblastula transition. This delay does not appear to lead to any further phenotype and is requires only maternal mutants in *igf2bp3*, embryos generated from crossing homozygous *igf2bp3* males to WT females do not produce any delay.

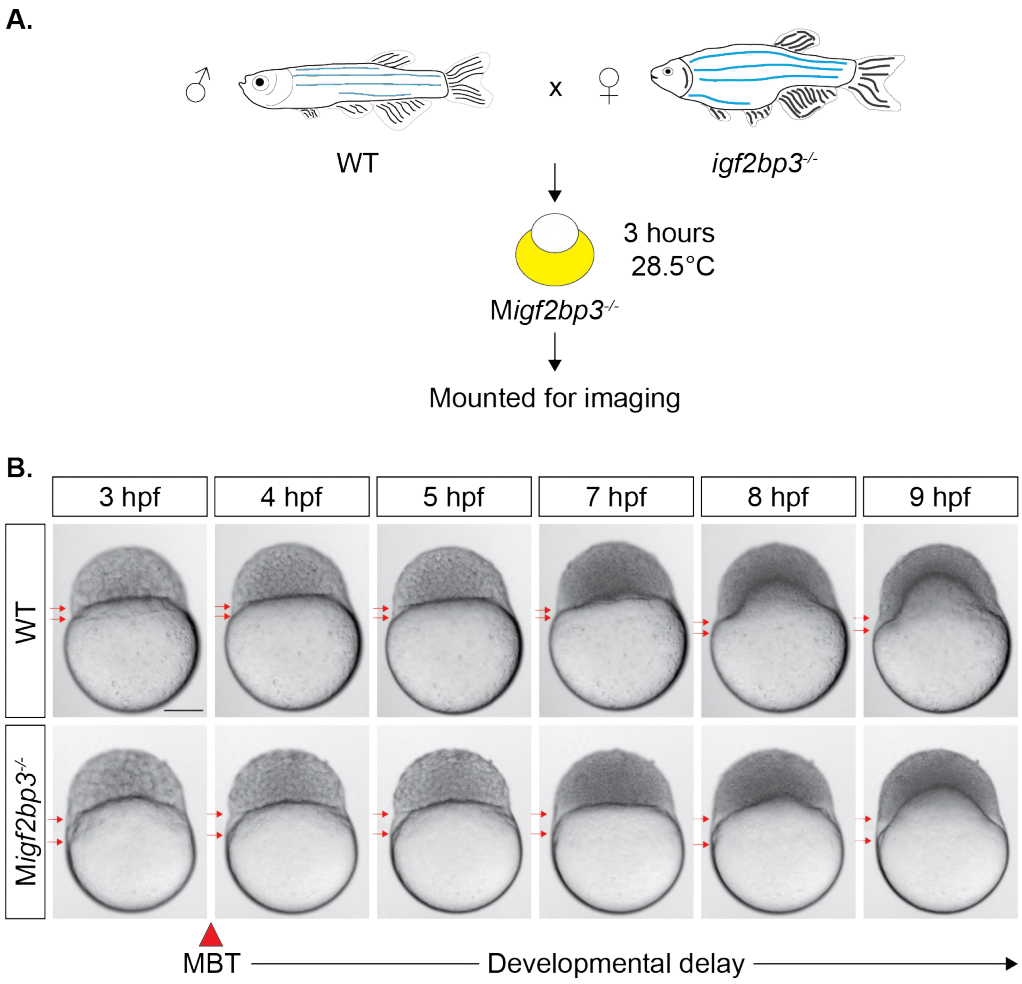


Figure 3-9. Maternal *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants are delayed during early embryogenesis. A. Schematic for generating and imaging maternal *igf2bp3*$^{-659\ Tg/-659\ Tg}$

embryos. WT males were mated with either WT or *igf2bp3*$^{-659\ Tg/-659\ Tg}$ females, incubated at 28.5°C until 1k-cell and dechorionated for imaging. **B. Maternal *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants are delayed during blastula.** Maternal *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants are delayed between 3-4 hpf and remain delayed throughout embryogenesis, which appears to exhibit an expanded YSL (arrows). Scale bar = 200 μm.

Many factors are required to maintain timely progression of embryogenesis around this time. Classically, loss of transcription factors such as *nanog, sox19b* and *pou5f3* that are required to activate zygotic genes are sufficient to cause embryonic arrest around this period (Pálfy *et al.*, 2019) and the nucleocytoplasmic ratio also influence the length of cell divisions during the MBT (Kane and Kimmel, 1993). More recently, it has been demonstrated that loss of m6A readers also causes a very similar phenotype, where the maternal mutants are embryonically delayed around 3 hpf for a short period before normal progression without further defects (Zhao *et al.*, 2017).

In addition to activation of zygotic genes by transcription factors, maintaining the correct ratio of cytoplasm to nucleus, clearance of maternal RNAs by m6A readers, timely progression of embryogenesis is also affected by induction of the yolk syncytial layer, an extraembryonic layer of cells arising from fusion of the marginal blastomeres into the yolk to form the yolk syncytial layer (YSL) (Kimmel and Law, 1985), the formation of the YSL is hypothesised to contribute to mechanical forces required to drive epiboly movement (Solnica-Krezel and Driever, 1994). Induction of the YSL from the Nanog-Mxtx2-Nodal pathway drives transcriptions of endoderm inducing factors such as *sox32* and *mixl1* from these marginal blastomeres (C. Xu *et al.*, 2012) and deregulation of the nodal pathway causes an expansion of the YSL and failure of the embryo to progress beyond epiboly (Kumari *et al.*, 2013).

To investigate the possibility that the yolk syncytial layer is contributing to the embryonic delay of the maternal *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos, we used immunofluorescence to label the blastoderm membranes with a β-catenin antibody and counterstained the nuclei of the embryos with DAPI. This allowed us to view the yolk syncytial nuclei (YSN) relative to the blastoderm, in which we quantified the number of YSN within a 300 μm span in the field of view and the distance between the blastoderm and the furthest YSN.

At 3 hpf, mutant *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos generally appear to have two or more tiers of YSN (Figure 3-10A) that are spaced further from the blastoderm compared to WT embryos, which usually only have one tier in close association to the blastoderm. The difference between the YSN and the blastoderm membrane is approximately ~35 μm in WT embryos and ~50 μm in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants. This increase in the layers of YSN also results in a slight increase in the number of YSN, from a median of 8 in WT embryos to 10 in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants.

However, by 4.5 hpf, this behaviour is not as apparent, and the spacing of the YSN and the blastoderm appears approximately equivalent (mean distances of ~57 μm and ~52 μm in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ respectively), although there is a mild decrease in the number of YSN by this stage, from a median of 34 YSN in WTs to 28 in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos.

We also conducted qPCR analysis for the enveloping layer marker *claudinE* (*cldnE*), and YSL markers that are in the Nanog-Mxtx2-Nodal signalling pathway, *haematopoietically expressed homeobox* (*hhex*), *mix-type homeobox gene 2* (*mxtx2*) and *mix paired-like homeobox* (*mixl1*), at 1k-cell and 50%-epiboly (Fig. 3-10B). These results were inconclusive, and we could not correlate the mild increase of the YSL to upregulation of YSL markers or downstream signalling components.

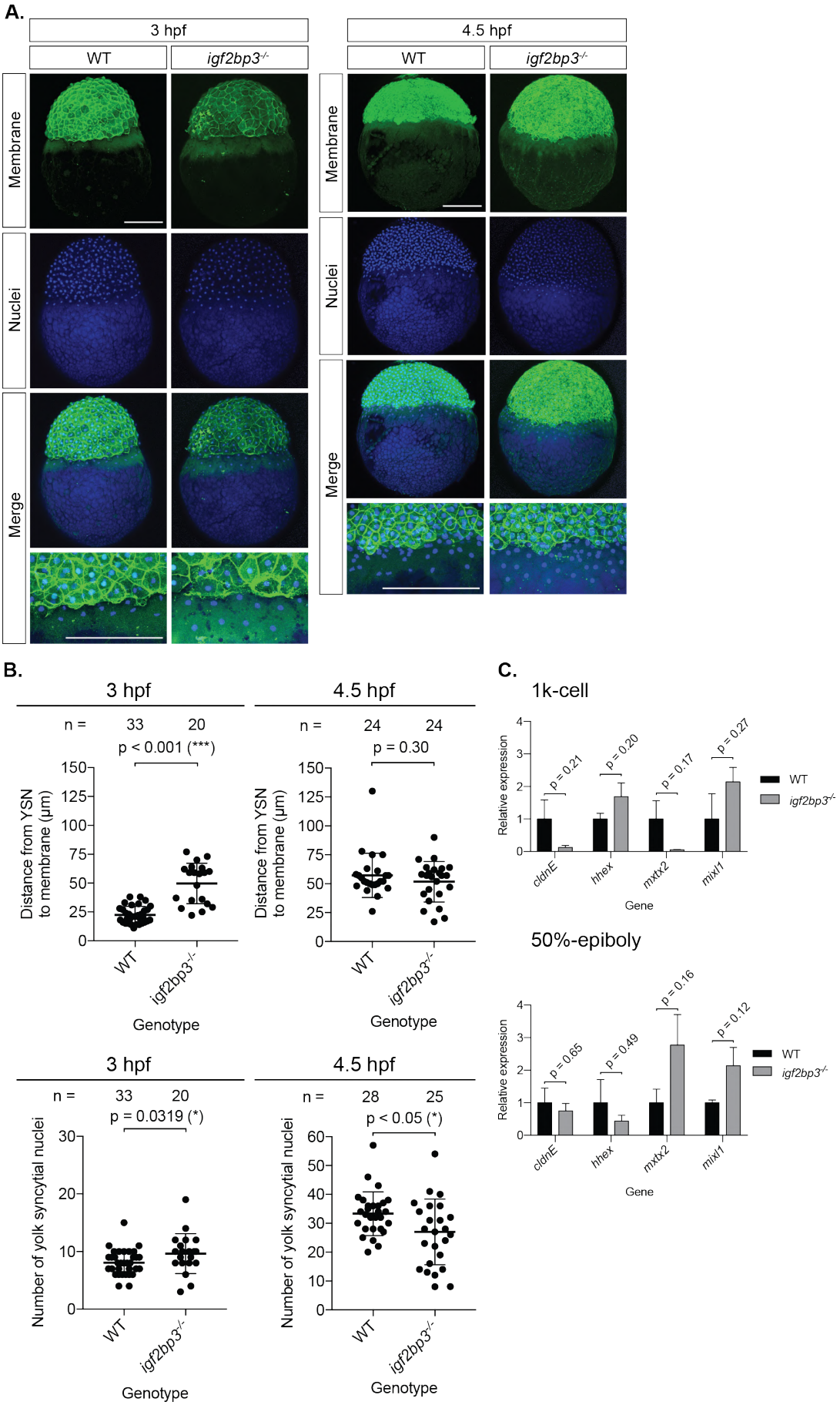

A.
3 hpf
4.5 hpf
WT
igf2bp3-/-
Membrane
Nuclei
Merge
B.
3 hpf
4.5 hpf
n = 33 20
p < 0.001 (***)
n = 24 24
p = 0.30
Distance from YSN to membrane (µm)
Genotype
n = 33 20
p = 0.0319 (*)
n = 28 25
p < 0.05 (*)
Number of yolk syncytial nuclei
C.
1k-cell
50%-epiboly
Relative expression
p = 0.21
p = 0.20
p = 0.17
p = 0.27
p = 0.65
p = 0.49
p = 0.16
p = 0.12
cldnE
hhex
mxtx2
mixl1
Gene

Figure 3-10. The yolk syncytial layer appears to be marginally expanded in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants. A. Immunofluorescence of WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos. WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos were stained for membranes (β-catenin) and nuclei (DAPI) at 3 hpf (left) and 4.5 hpf (right). **B. Quantification of yolk syncytial layer expansion.** The distance of the furthest yolk syncytial nuclei (YSN) from the blastoderm, and the number of yolk syncytial nuclei were compared between WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos. Statistical analyses performed two-tailed unpaired t-test. **C. qRT-PCR for enveloping layer and yolk syncytial layer markers.** qRT-PCR were performed at 1k-cell and 50% epiboly from WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos for enveloping layer marker *cldnE* and yolk syncytial layer markers *hhex, mxtx2* and *mixl1* relative to the housekeeping gene *18S*. Statistical analysis performed with two-tailed unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Scale bar = 200 μm.

3.7. Oocyte polarity is not affected in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants

As Igf2bp3 is a well-conserved protein that has a maternal component, we considered earlier events in embryogenesis that could be misregulated as a consequence of loss of Igf2bp3, considering an embryonic delay was observed around early blastula. As Igf2bp3 is a Balbiani body component and *Xenopus* Igf2bp3 is required to localise *vg1* mRNA transcripts to the vegetal pole of the oocyte (Git and Standart, 2002; Bontems *et al.*, 2009), we explored the possibility of a polarity defect in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos or a possible requirement for Igf2bp3 to localise or regulate *vg1* (now known as *growth differentiation factor*, *gdf3*) transcripts.

To test this, 1-cell stage embryos were probed with animal and vegetal polarity markers, *gdf3* and *deleted in azoospermia-like* (*dazl*) respectively, *igf2bp3*$^{-659\ Tg/-659\ Tg}$ were also probed for *gdf3* expression at 50%-epiboly and somitogenesis, where it stains the lateral plate mesoderm and heart primordium. Analyses from these embryos did not reveal any differences between the two genotypes in staining, and correct organisation of the animal and vegetal poles were apparent, in addition to the correct staining of the whole embryo and lateral plate mesoderm and heart primordium in later stages.

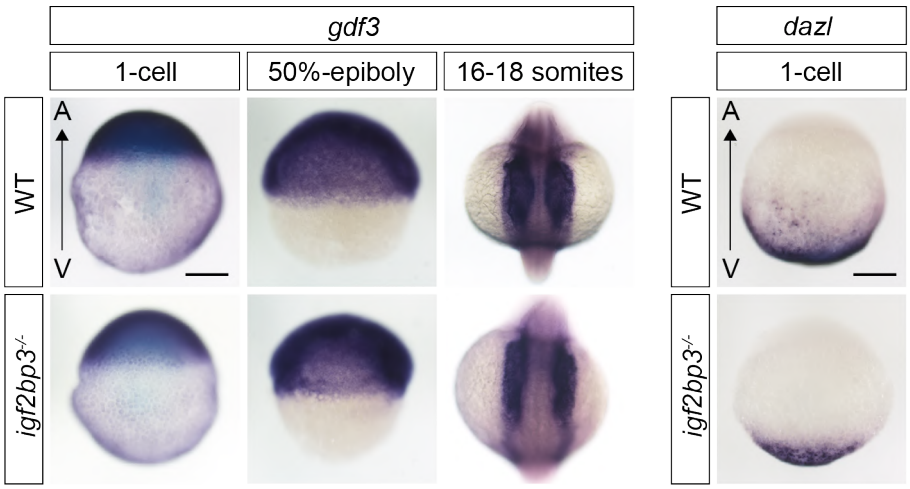


Figure 3-11. Animal-vegetal axis and left-right axis is not affected in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants. WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos were probed for the animal pole marker *gdf3* (left) at 1-cell, 50%-epiboly and 16-18 somites and the vegetal pole marker *dazl* at 1-cell. Scale bar = 200 µm.

3.8. *igf2bp3* is required for correct primordial germ cell maintenance

As the roles of Igf2bp3 were unclear and did not match expectations from the literature, we decided to examine the possible functions that Igf2bp3 could have by comparing mutants made in other organisms and/or related proteins, prompting an investigation of a possible requirement for germ line function of Igf2bp3.

Several lines of evidence suggest that Igf2bp3 may be required for germ line development: immunofluorescence of the Balbiani body shows Igf2bp3 to be colocalised in this structure, which organises germplasm components prior to dispersal in later oogenesis (Bontems *et al.*, 2009), mass-spectrometry of *Xenopus* oocytes have also confirmed this observation, which shows Igf2bp3 is specifically enriched in this structure (Boke *et al.*, 2016). Moreover, in *Drosophila*, the Igf2bp homolog is a marker for the pole cells (Adolph *et al.*, 2009), which are precursors for the adult germ cells and finally, other m6A-regulating proteins such as the readers *ythdf2* (Zhao *et al.*, 2017)*, ythdc2* (Bailey *et al.*, 2017) and writer *mettl3* (Xia *et al.*, 2017) also have germ line defects, leading to poor fertility.

Experimental evidence that *igf2bp3* may have a role in germ line function in zebrafish became apparent when we generated a CRISPR-Cas9 mutant allele for *igf2bp3.* Exon 1 of *igf2bp3* was targeted with the Cas9 system, and, despite exhaustive screening of over twenty founders, we were only able to retrieve a single founder that transmitted a 7 bp deletion allele (hereafter referred to as *igf2bp3*$^{\Delta\ 7\ bp}$) that could be genotyped by restriction digest, as the vast majority of remaining alleles were 3 bp deletions (that did not produce a protein-null), or much less frequently, a 2 bp allele that was not genotypable with restriction digest.

Nonetheless, we were able to produce a stable line for the *igf2bp3*$^{\Delta\ 7\ bp}$ allele, which we predicted to target all transcripts of *igf2bp3* (shown in Figures 7-3 and 7-4), which should encode a frameshift in exon 1 of the transcript, leading to a missense at residue 35 and premature stop codon at residue 46, shown in Figure 3-12A-B. Zygotic mutants for *igf2bp3*$^{\Delta\ 7\ bp}$ were stable, were retrieved according to Mendelian ratios as adults ($p = 0.34$) (Fig. 3-12C), and we did not observe any differences between WT, heterozygous or homozygous siblings at 5 dpf when adults heterozygous were intercrossed (Fig. 3-12D). Contrary to our expectations, we were not able to retrieve homozygous females in our first generation of heterozygous intercrosses, even though the sex ratios for the other genotypes were approximately equivalent. Within our first generation, all the homozygous adults developed into fertile males, and, in our second generation of heterozygous intercrosses, we were able to retrieve homozygous females, albeit with a strong male bias in this generation (3:1 male/female sex ratio).

As depletion of primordial germ cells (PGCs) typically manifests as a male sex bias in adult zebrafish, there was a strong possibility that *igf2bp3* mutants would also have a defect in the PGCs. As we did not retrieve homozygous females in time for complete analysis, we have analysed the defect in PGCs in the context of the *igf2bp3*$^{-659\ Tg}$ and *igf2bp3*$^{-361\ Tg}$ alleles only.

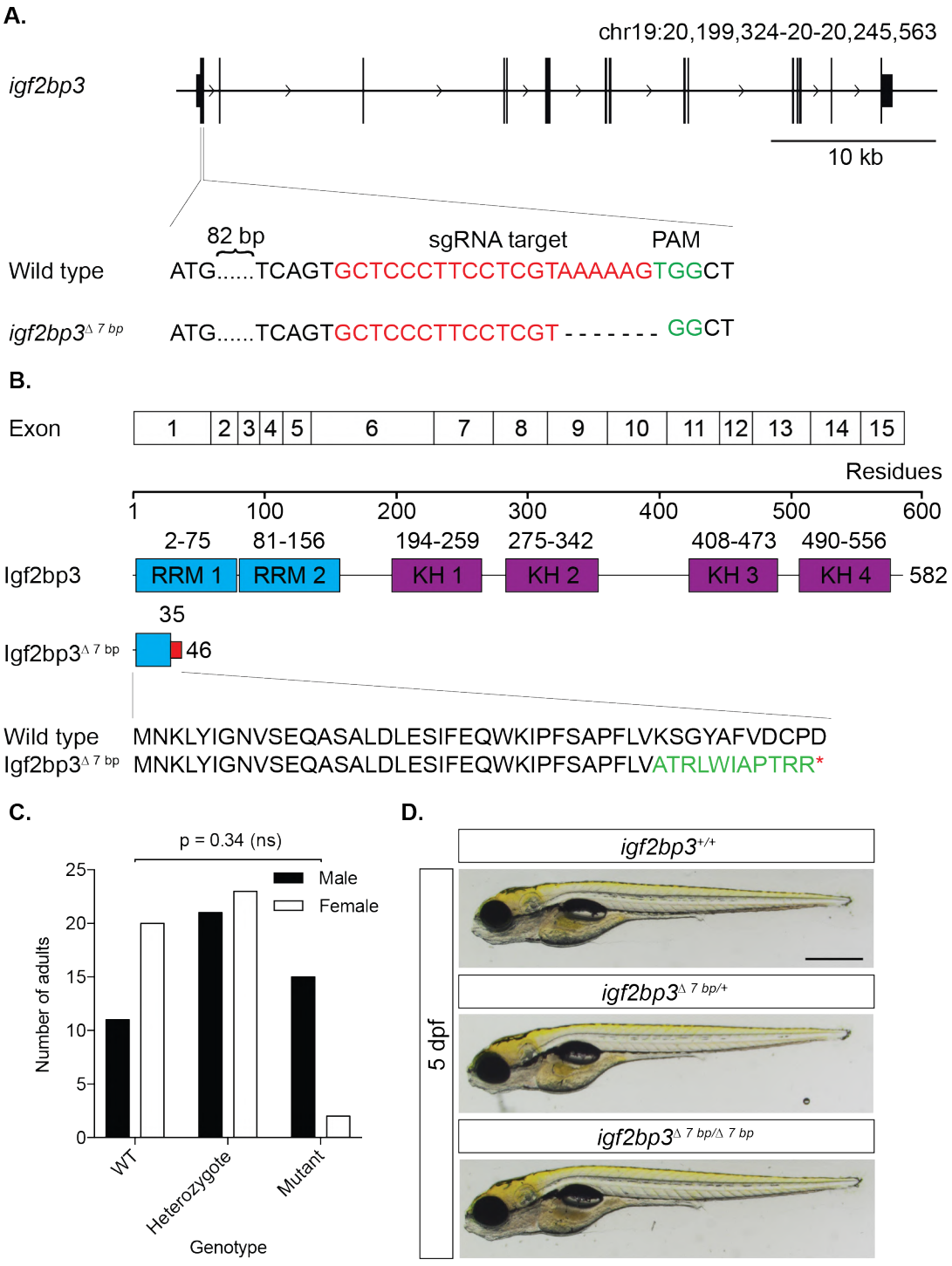


Figure 3-12. Generation of the igf2bp3 CRISPR-Cas9 mutant. A. Targeting strategy for *igf2bp3.* CRISPR-Cas9 was used to target exon 1 of *igf2bp3,* resulting in a 7 bp deletion. **B. Protein prediction of the *igf2bp3* CRISPR-Cas9 allele.** The *igf2bp3*$^{\Delta\ 7\ bp}$ allele, encoding a

frameshift at residue 35 and premature stop codon at the residue 46, is predicted to produce a truncated Igf2bp3 polypeptide that does not encode for a full RNA-binding motif. **C. Sex and Mendelian inheritance ratio of *igf2bp3*$^{\Delta 7\,bp/\Delta 7\,bp}$ adults.** Adults from an *igf2bp3*$^{\Delta 7bp/+}$ intercross were genotyped and sexed. Statistical analysis of Mendelian ratio performed with Chi-squared test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. **D. Phenotype of *igf2bp3*$^{\Delta 7\,bp/\Delta 7\,bp}$ embryos at 5 dpf.** Embryos from an *igf2bp3*$^{\Delta 7\,bp}$ intercross were incubated to 5 dpf, imaged and genotyped. Scale bar = 1 mm.

To test whether *igf2bp3* played a role in germ line development, WT and *igf2bp3*$^{-659\,Tg/-659\,Tg}$ embryos were probed for *DEAD box polypeptide 4* (*ddx4*, previously known as *vasa*) to label the PGCs, shown in Figure 3-13A. The *igf2bp3*$^{-659\,Tg/-659\,Tg}$ embryos showed a strong reduction in the number of PGCs and we observed ectopic PGCs at a higher proportion; to dissect the genetic basis for this PGC defect, we compared the number of PGCs in embryos generated from a WT mating, homozygous male to WT female (paternal heterozygote), WT male to homozygous female (maternal mutant) and homozygous parents (maternal-zygotic mutant) (Fig. 3-13B). From these analyses, we observed a statistically significant reduction in the number of PGCs across all genotype combinations with the *igf2bp3*$^{-361\,Tg}$ allele, with mean PGC values of 35, 24, 13 and 15 for WTs, paternal heterozygotes, maternal mutants and maternal-zygotic mutants respectively. The strongest reduction in the number of PGCs occurred in the maternal and maternal-zygotic mutants, which lost more than 50% of their PGCs.

This data indicated a clear maternal role for *igf2bp3*, as the number of PGCs between the maternal and maternal-zygotic mutants were comparable and lead to the highest reduction in the number of PGCs. Interestingly, a reduction in the number of PGCs were also observed in the paternal heterozygotes, suggesting that *igf2bp3* might play a zygotic role in regulating the germline and that haploinsufficiency can induce a phenotype. Another possibility that might explain this reduction of PGCs could be that this allele may have some dominant negative effect, perhaps by encoding for truncated polypeptides that are not detectable but can interfere with normal RNA regulation from Igf2bp3, such as by binding target RNAs, or by blocking Igf2bp3 from being incorporated into mRNP complexes.

In addition to PGC depletion, maternal and maternal-zygotic *igf2bp3*$^{-659\,Tg/-659\,Tg}$ also exhibited a further defect in the dispersion of the PGCs, and many PGCs appeared to be ectopically distributed throughout the embryo. PGCs were roughly plotted according to their anterior-posterior axial coordinates at 24 hpf embryos (Fig. 3-13C), and the number of embryos with an ectopic number of PGCs was quantified (Fig. 3-13E), defined as the number of embryos with more than 4 ectopic PGCs.

As zygotic loss of *igf2bp3* appeared to be sufficient to cause a reduction in the number of PGCs, adults from an *igf2bp3*$^{-659\,Tg/+}$ intercross were sexed and genotyped (Fig 3-13D),

however, these did not show any sex bias and were retrieved according to Mendelian ratios (p = 0.83). Homozygous adults were also intercrossed, and the subsequent progeny appeared to have a male sex bias (7:2 male/female sex ratio).

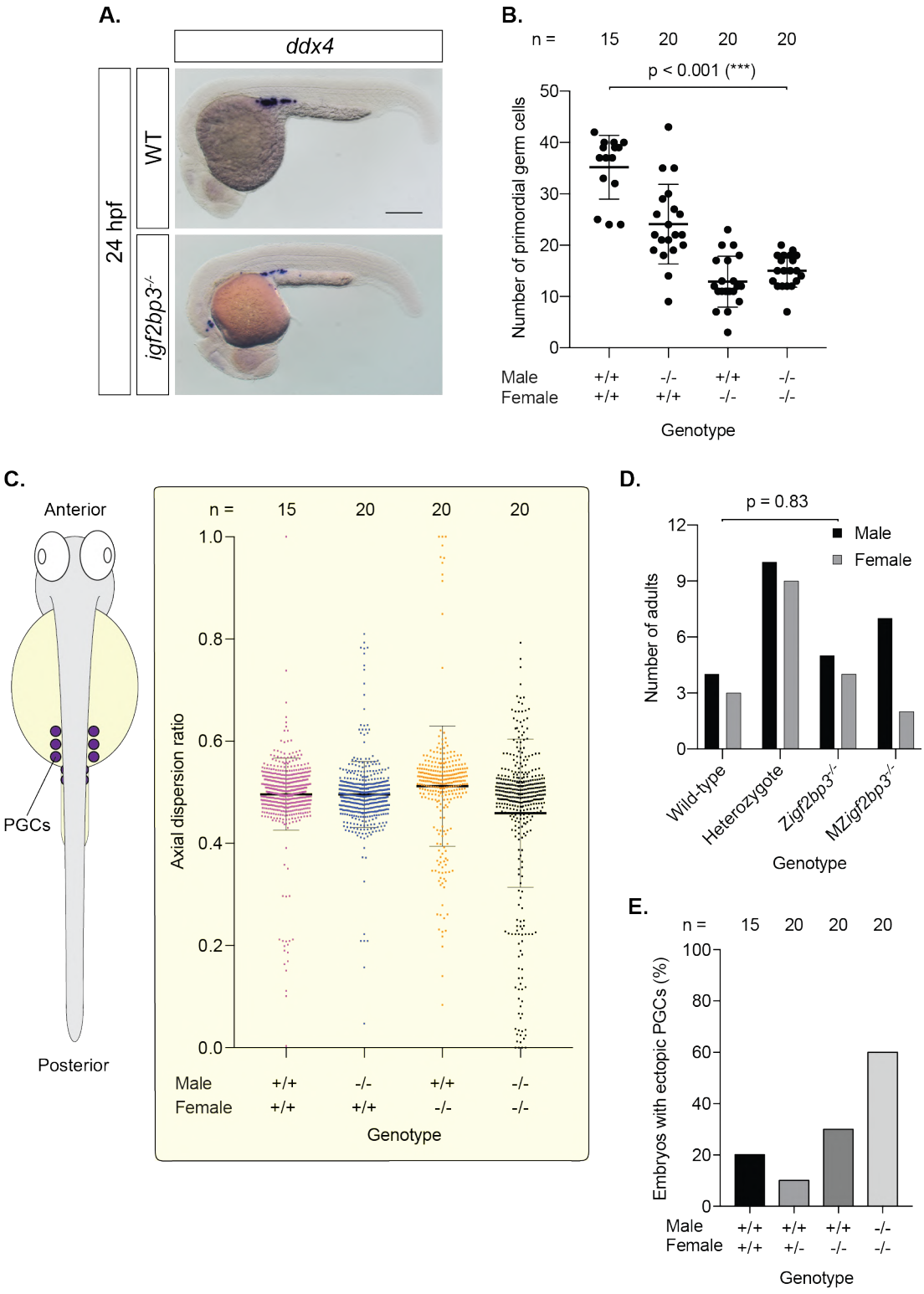


Figure 3-13. *igf2bp3* is required for primordial germ cell maintenance. A. Primordial germ cells are misregulated in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants at 24 hpf. Embryos were probed at 24 hpf with the PGC marker *ddx4*. **B. Quantification of primordial germ cells at**

24 hpf. PGCs from WT, paternal heterozygotes, maternal mutants and maternal-zygotic mutants were quantified. Statistical analysis performed with two-tailed t-test. **C. Quantification of axial PGC dispersion at 24 hpf.** The anterior-posterior dispersion of PGCs in each genotype was calculated. **D. Sex and Mendelian inheritance ratios of *igf2bp3*$^{-659\ Tg/-659\ Tg}$ adults.** Adults from an *igf2bp3*$^{+/-659\ Tg}$ and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ intercross were genotyped and sexed. **E. Loss of *igf2bp3* induces PGC mismigration.** The number of ectopic PGCs were quantified between WT, heterozygote, maternal mutant, and maternal-zygotic mutants. Statistical analysis of Mendelian ratio performed with Chi-squared test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Scale bar = 200 μm.

3.9. A second *igf2bp3* transgenic insertion allele also has an aberrant germ line

Preliminary evidence from both the *igf2bp3*$^{\Delta\ 7\ bp}$ and the *igf2bp3*$^{-659\ Tg}$ allele points towards a possible role in regulating germ line development by *igf2bp3*, we decided to strengthen our data by confirming these observations with our third allele, the *igf2bp3*$^{-361\ Tg}$ allele, which also contains the *Tg(nLacZ-GTvirus)* insertion in intron 1, shown in Figure 3-14A. Maternal-zygotic mutants from this allele also showed a significant reduction in the number of PGCs (Fig 3-14B), with mutants having approximately 10 PGCs to 29 PGCs in the WT controls. Furthermore, these mutant embryos also have a similar mismigration defect that was observed in the *igf2bp3*$^{-659\ Tg}$ allele, and we confirmed with Western blots that this allele also did not produce detectable Igf2bp3 protein (Fig. 3-14C).

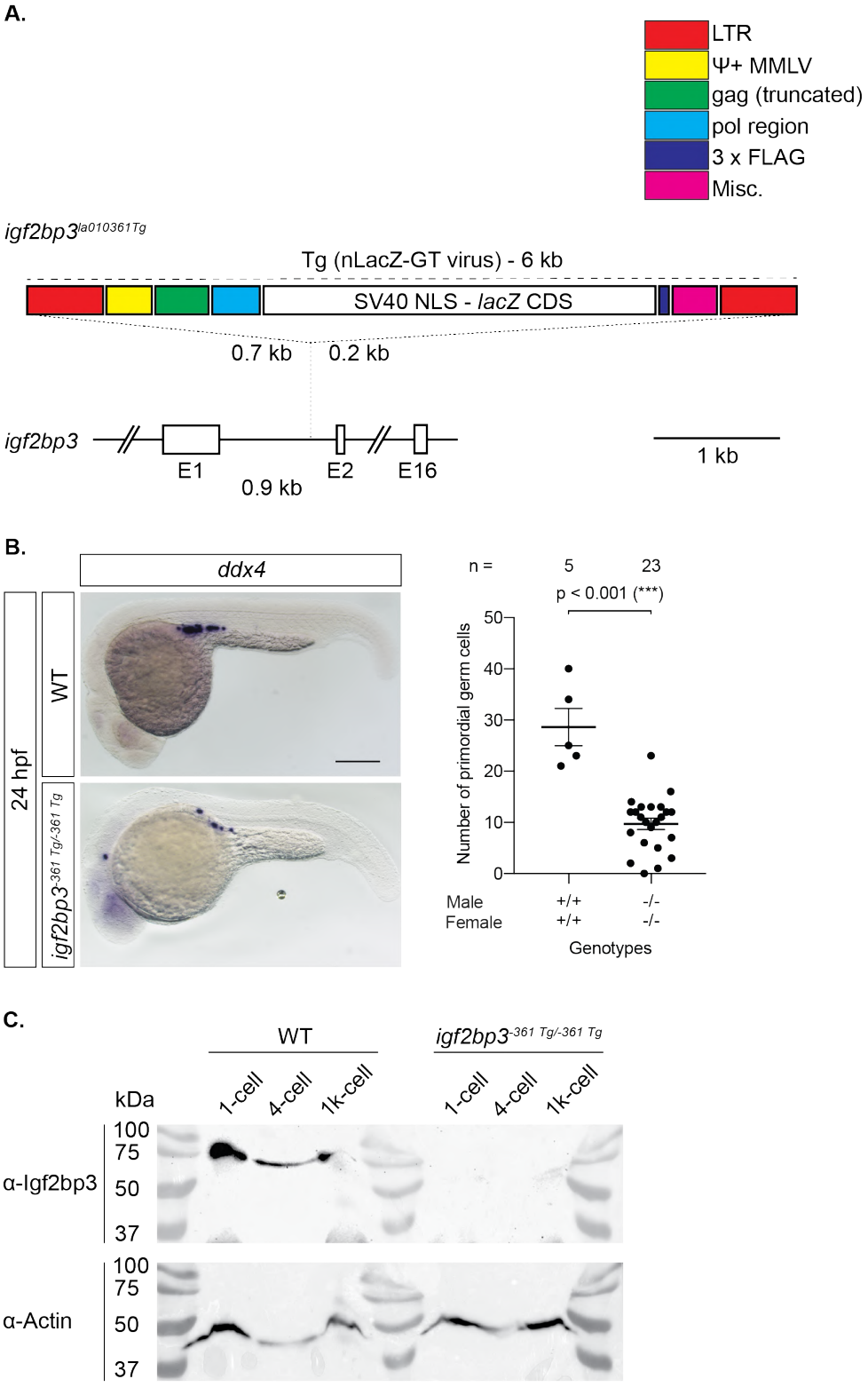


Figure 3-14. *igf2bp3* transgenic insertion mutants have a germline defect by 24 hpf. A. Schematic of the *igf2bp3*$^{-361\ Tg}$ transgenic insertion allele. The *igf2bp3*$^{-361\ Tg}$ insertion allele contains the *Tg (nLacZ-GT virus)* construct, of ~6 kb, in intron one, ~0.9 kb. **B. Visualisation of the primordial germ cells at 24 hpf.** WT and maternal-zygotic *igf2bp3*$^{-361\ Tg/-361\ Tg}$ embryos were probed for the PGC marker, *ddx4*, imaged (left) and their PGCs

quantified (right). **C. Western blots for Igf2bp3 and β-actin in WT and *igf2bp3*$^{-361\ Tg}$ - embryonic lysates.** Embryonic lysates were probed with α-Igf2bp3 and α-Actin antibodies to confirm whether Igf2bp3 protein is present in transgenic insertion mutants.

3.10. The germ line is misregulated by Shield in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos

As we have some robust data that *igf2bp3* is required to maintain primordial germ cells, in which we have identified a mismigration defect, as PGCs mismigrate, and PGCs are partially depleted in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos, although whether this is due to failure to proliferate or survive is unclear.

This phenotype appears to have a strong maternal component and we used live imaging to specify when PGC begin to be misregulated. Using the GFP-nos1 3' UTR construct (Köprunner *et al.*, 2001) to generate capped mRNA encoding GFP with the *nos1* 3'UTR, the PGCs can be labelled for live imaging beginning from late gastrulation, shown in Figure 3-15A. To image the PGCs during segmentation, WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ were injected with GFP-nos mRNA, incubated to Bud, and imaged throughout somitogenesis and 24 hpf to confirm that whole *in-situ* hybridisation analysis conducted previously. PGCs appeared to be depleted throughout somitogenesis, and a reduction was clear by 1-somite and mismigration by 25-somites. It is clear then, that aberrations in the germline must occur prior before the end of gastrula.

In addition to moving backwards in developmental time to establish the first period of PGC misregulation, we also tested whether *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos recovered their PGCs at a later stage or whether depletion of PGCs continues after 24 hpf. Whole *in-situ* hybridisation of 48 hpf embryos (Fig. 3-15B) also revealed a reduction in the PGCs, and, whilst some mismigration is still apparent along the extended yolk extension, we did not observe the same mismigration of PGCs to the head or embedded in the trunk, suggesting that these PGCs are lost, possibly by apoptosis or transdifferentiation from their PGC state to another cell type.

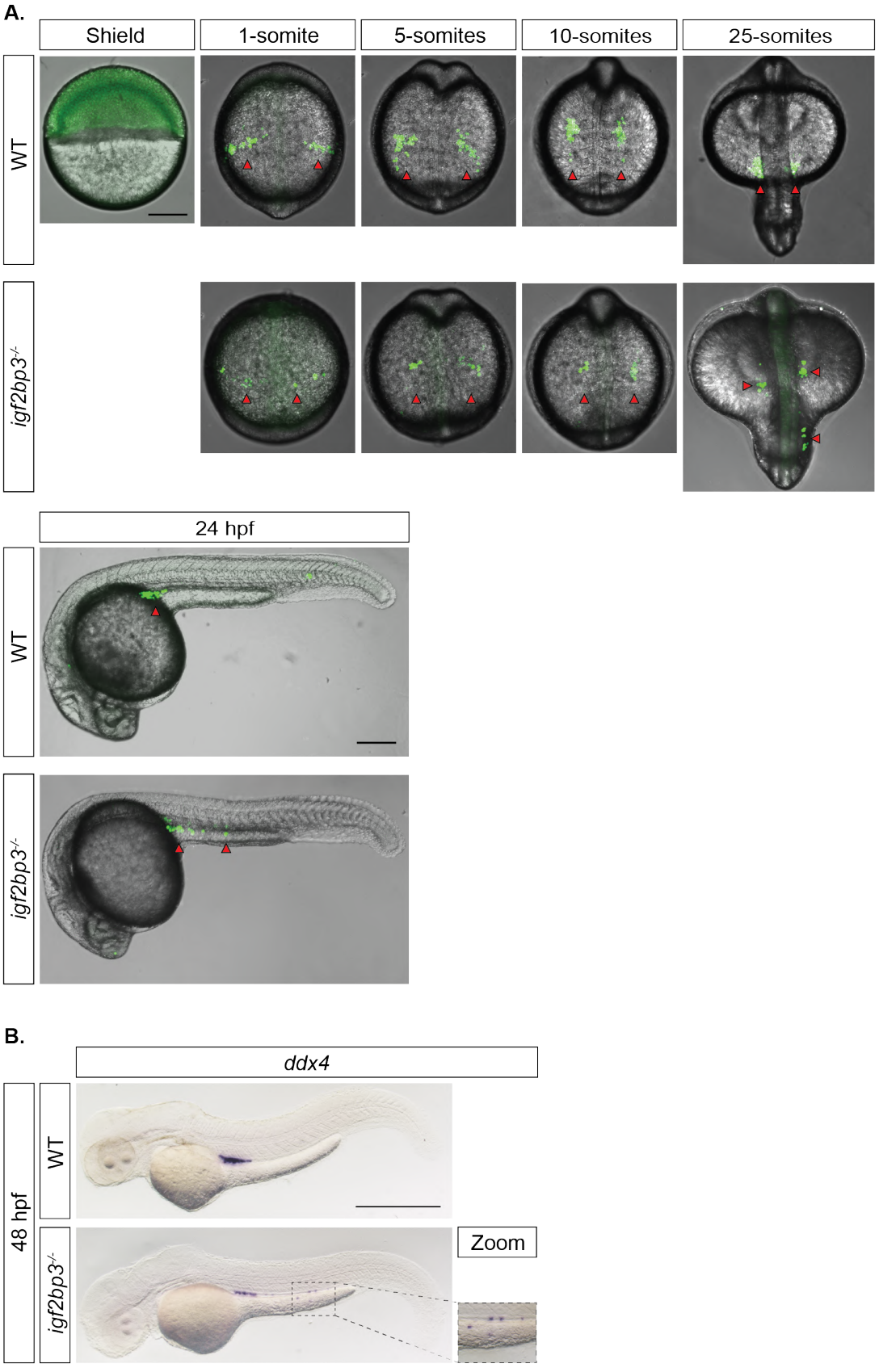


Figure 3-15. Visualisation of PGCs before and after 24 hpf also shows abolished primordial germ cell development. A. Live visualisation of primordial germ cells during somitogenesis. WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos were injected with GFP-nos1

3'UTR mRNA and imaged at Bud, 1-somite, 5-somites, 10-somites, 25-somites and 24 hpf. Scale bar = 200 µm. **B. Visualisation of primordial germ cells at 48 hpf.** WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos were probed for the PGC marker *ddx4* and imaged. Scale bar = 1 mm.

Nonetheless, to provide further specificity to the first period in which PGCs exhibit abnormal behaviour, we performed further whole *in-situ* hybridisations with *ddx4* at 4-cell, 1k-cell, Shield and Bud to validate our findings, shown in Figure 3-16A,B. Concordant with our expectations, germplasm was correctly localised to the cleavage furrows of the 4-cell stage embryos, and was correctly distributed to four corners of the embryo at 1k-cell stage. Remarkably, we began to observe aberrant PGC behaviour at approximately Shield stage. During late blastula, PGCs enter a motile phase and are found in close association with the hypoblast at the edge of the embryo and are embedded in a relatively deep position (Braat *et al.*, 1999). However, in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos, PGCs become dispersed and ectopic localised anteriorly in the embryo, suggesting misregulation of the germ line occurs during the motility phase of PGC development that occurs before Shield.

During somitogenesis, PGCs are initially aligned in two trails in either side of the midline, and migrate laterally towards the midline to form two clusters in the gonadal mesoderm. To test whether PGC depletion at Bud matched our observations with live-imaged embryos, double whole *in-situ* hybridisations were performed with *ddx4* and *sonic hedgehog a* (*shha*), which labels the notochord at Bud, and allows us to visualise the relative positions of the PGCs to the destination (Fig. 3-16B). As WISH produces more reliable staining of all PGCs, we quantified the number of PGCs in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos and found a significant reduction in the number of cells, with a median of 49 PGCs in WTs compared to 23 in the mutants. We further observed small clusters or individual cells that appeared to be on the posterior side of the embryo, beyond the typical range that we might normally expect a migrating PGC to appear in WT controls.

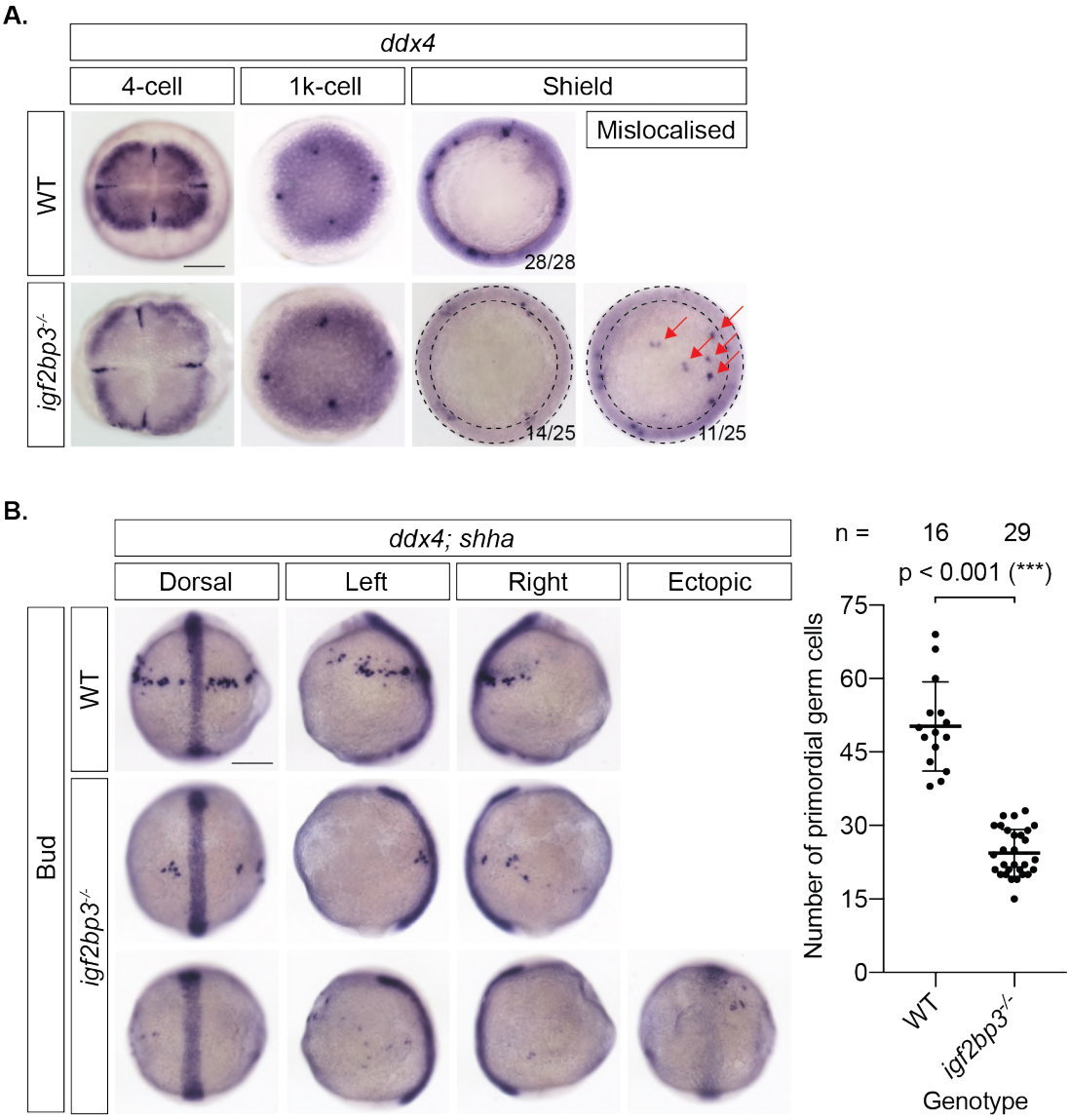


Figure 3-16. Misregulation of primordial germ cells in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryo is apparent by gastrula. A. Primordial germ cells are mislocalised by Shield in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutant. WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos were probed for the PGC marker *ddx4* at 4-cell, 1k-cell and Shield, embryos with abnormal PGC localisation from the blastoderm margin were scored accordingly. **B. Loss of primordial germ cells is apparent by Bud stage.** PGCs in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ visualised relative to the midline with *shha* (left). The number of PGCs was quantified (right). Statistical analysis performed with two-tailed unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Scale bar = 200 µm.

3.11. Some germplasm components are downregulated in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants

In both preformative and inductive mechanisms of germ line specification, RNA-binding proteins are essential to regulate PGCs, and many germplasm components are either RNA-binding proteins or encode RNA-binding proteins as transcripts. As Igf2bp3 is a maternally deposited RNA-binding protein that is required for correct germline development, we reasoned that loss of *igf2bp3* would result in a reduction of maternal or germplasm mRNAs.

To test this hypothesis, we performed qRT-PCR at 1-cell, 50%-epiboly, Bud and 24 hpf in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos, to determine if the loss of germplasm components might be progressive over the course of development and whether the loss of germplasm components might correlate with our imaging observations, and we used the panel of the following genes: *buc* (*bucky ball*)*, cxcl12a* (*chemokine ligand 12a*), *cxcr4a* (*chemokine receptor 4a*), *cxcr4b* (*chemokine receptor 4b*), *dazl, ddx4*, *tdrd1* (*tudor domain containing 1*), *tdrd7a* (*tudor domain containing 7a*), and *gdf3/vg1*.

As maternal *buc* mRNA is degraded over the course of early development and is not maintained, perturbances in *buc* levels might suggest global issues with maternal RNA regulation, however, this was not significant across the tested stages. We further tested *gdf3/vg1* as Igf2bp3 is known in *Xenopus* to bind *vg1* mRNA in the oocytes, possibly providing some stabilising effect, similarly, this was also not significant.

The Cxcl12-Cxcr4 signalling axis contributes to the migratory patterns of many cell types, and well-studied examples include the migration of endodermal cells and the PGCs (Molyneaux *et al.*, 2003; Mizoguchi *et al.*, 2008). We tested whether the chemokine signalling pathway was misregulated by assessing the expression levels of the ligand *cxcl12a* and its corresponding receptors *cxcr4a* and *cxcr4b*. The levels of the receptors remained comparable at all stages, whilst *cxcl12a* expression appeared to be slightly reduced in at 50%-epiboly only.

We also tested the germplasm components, *dazl*, *ddx4*, *tdrd1* and *tdrd7a.* Surprisingly, *ddx4* appeared to be consistently reduced when tested, except at Bud, with mutant expression being approximately 25% of the WT levels whereas other germplasm components tested did not appear diminished at 1-cell, 50%-epiboly and Bud. However, *dazl* and *tdrd1* were also significantly reduced at 24 hpf, with the expression of these genes being reduced to 4% and 14% respectively, suggesting that loss of Igf2bp3 leads to a progressive degradation of some germplasm mRNAs.

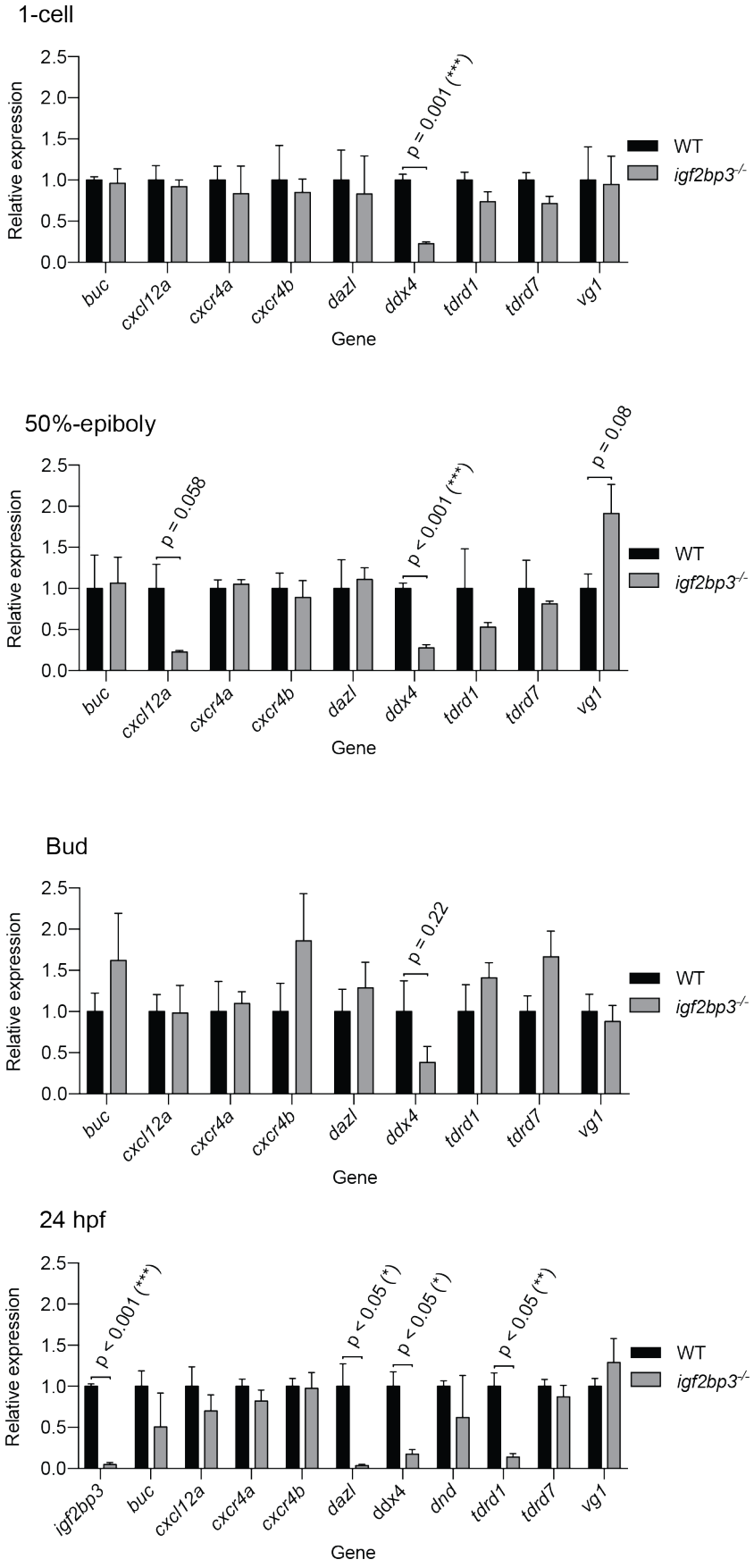


Figure 3-17. qRT-PCR on germplasm genes in *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutants. qRT-PCR were performed on 1-cell, 50%-epiboly, Bud and 24 hpf in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$

embryos on maternal RNAs and germplasm linked components relative to the housekeeping gene *18S*. Statistical analysis performed with two-tailed unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.12. Analysis of PGC migration and behaviour with live fluorescent reporters

Misregulation of the PGCs by interruptions in signalling and depletion of germplasm components leads to changes in behaviour that can be observed in live-embryos. Therefore, we took advantage of the ease of live-imaging early zebrafish with fluorescent reporter constructs in order to visualize the behaviour of these cells more closely.

To measure PGC migration, embryos were injected with GFP-nos mRNA (Köprunner *et al.*, 2001) at 1-cell and imaged at Bud, a stage at the end of gastrula where the PGCs can be seen moving rapidly towards the midline, which is apparent as a thickening of the tissue forming the midline along the anterior-posterior axis. WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGC migration tracks were traced, shown in Figure 3-18A, and we were not able to notice clear differences in the tracks in actively migrating PGCs of both genotypes. However, we also noticed some fluorescent cells in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos that appeared to be further away from the midline than expected (ref. Fig. 3-16B), and, after imaging of these cells, they appeared to move in random patterns and did not acquire significant displacement from the start of their tracks (Fig. 3-18A, B). These PGCs are subsequently designated as ectopic PGCs and track statistics were compiled and we compared the displacement, speed and straightness index of the PGCs (Fig. 3-18B). Comparison of displacement was not significant between the two genotypes (91 μm and 93 μm for WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$) respectively, although ectopic PGCs had a significantly lower displacement (46 μm). The speed of the PGCs was also comparable (2.1 μm/minute for both genotypes and 1.92 μm/minute for ectopic *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGCs). The straightness ratio, defined as the displacement over distance travelled, of the WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGCs was calculated as 0.72 and 0.73 for WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ respectively, and for ectopic *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGCs, this was 0.38.

During the course of imaging the *igf2bp3*$^{-659\ Tg/-659\ Tg}$, we were able to image several instances in which PGCs appeared to display apoptotic behaviour (Fig. 3-18C), we observed two instances of this occurring in two independent embryos imaged on separate days, suggesting that some PGCs may be lost during the migratory path due to apoptosis than mismigration. Another possibility is that the PGCs in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos fail to proliferate, however, we have also observed instances of mitosis in these embryos, (Fig 7-5), and this does not seem to be the case, although the rate of proliferation has not been measured.

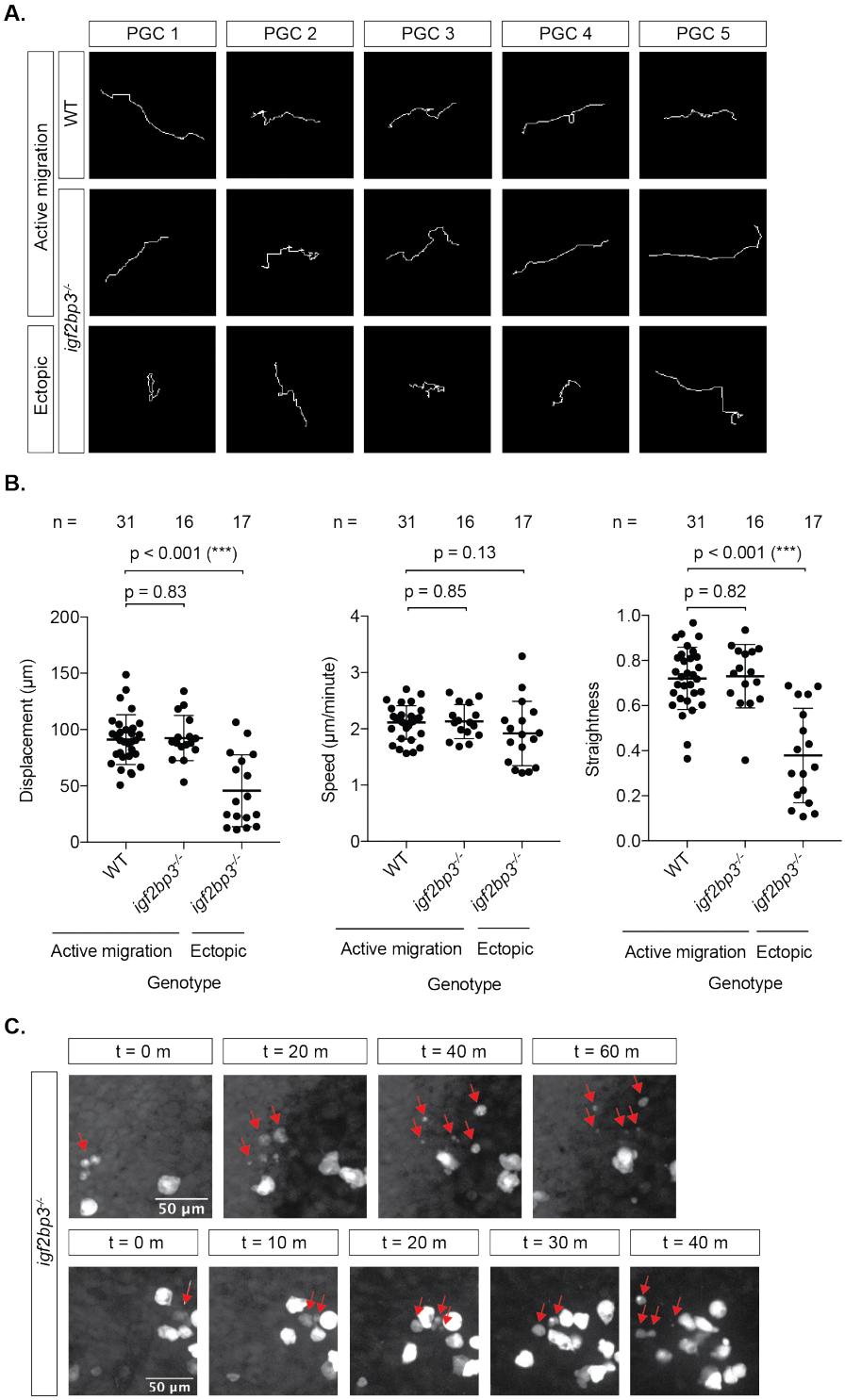


Figure 3-18. Cell movement in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ primordial germ cells shows heterogeneity in the *igf2bp3*$^{-/-}$ embryos. A. Migration tracks of WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGCs. PGCs expressing a live fluorescent reporter were tracked over an hour and the migration path traced as a track. **B. Quantification of migration statistics in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGCs.** The displacement, speed and straightness of the PGCs were calculated to compare their migration dynamics. Statistical analysis performed with two-tailed unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. **C. Apoptotic behaviour of**

***igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGCs during migration.** Examples of apoptotic PGCs in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ were captured during its migratory phase. Arrows show fragmenting PGCs.

Chemokine signalling is also component of PGC migration, and the ability to sense and interpret chemical cues is required for directional movement of PGCs; to facilitate the ability of a cell to sense these cues, cells will often send projections from the surface of the cell to its surrounding environment. These actin-based projections, termed filopodia, extend to the direction of its migration and inhibiting filopodia formation is directly compromises a migrating cell's ability to move (Meyen *et al.*, 2015).

To test whether the defects in the germline were an autonomous- or host- effect, we utilised a farnesylated eGFP mRNA construct that labels the membranes of the PGCs (Weidinger *et al.*, 2002) and more importantly, allowed visualisation and quantification of the filopodia with fluorescent live imaging. WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ embryos were imaged from Bud stage as performed previously and whole PGCs were imaged over several minutes to capture the behaviour of individual filopodium over their lifetime, shown in Figure 3-19A. We did not observe any unusual behaviour in the PGCs in both genotypes, and the morphology of the PGCs as a whole appeared unremarkable (Fig 7.5). As the PGCs were able to generate filopodia with no apparent difficulty, we post-processed the filopodia movies by noting their position relative to the midline and calculated the angles of their filopodia projections (Fig 3-19A). As expected, a greater frequency of filopodia were directed towards their intended destination in the WTs, although this pattern was not as clear in the *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGCs, suggesting that there could be perturbances in either the chemokine environment or the sensory state in the PGCs, although this is a rough approximation of filopodia analysis.

Further quantification of filopodia behaviour was performed and we calculated the number of filopodia per PGC, the persistence (lifetime) of each filopodium, its average length over its lifetime and its maximum length during its lifetime. The dynamics of the filopodia appeared to be consistent in all measures, with the mean number of filopodia, (12.1, 12.6 in WT, *igf2bp3*$^{-659\ Tg/-659\ Tg}$ respectively), persistence time (110s, 106s in WT, *igf2bp3*$^{-659\ Tg/-659\ Tg}$ respectively), average filopodium length (median length 3.8 μm in both genotypes), and maximum filopodium length (median length 6.4 μm, 5.7 μm in WT, *igf2bp3*$^{-659\ Tg/-659\ Tg}$ respectively) being comparable.

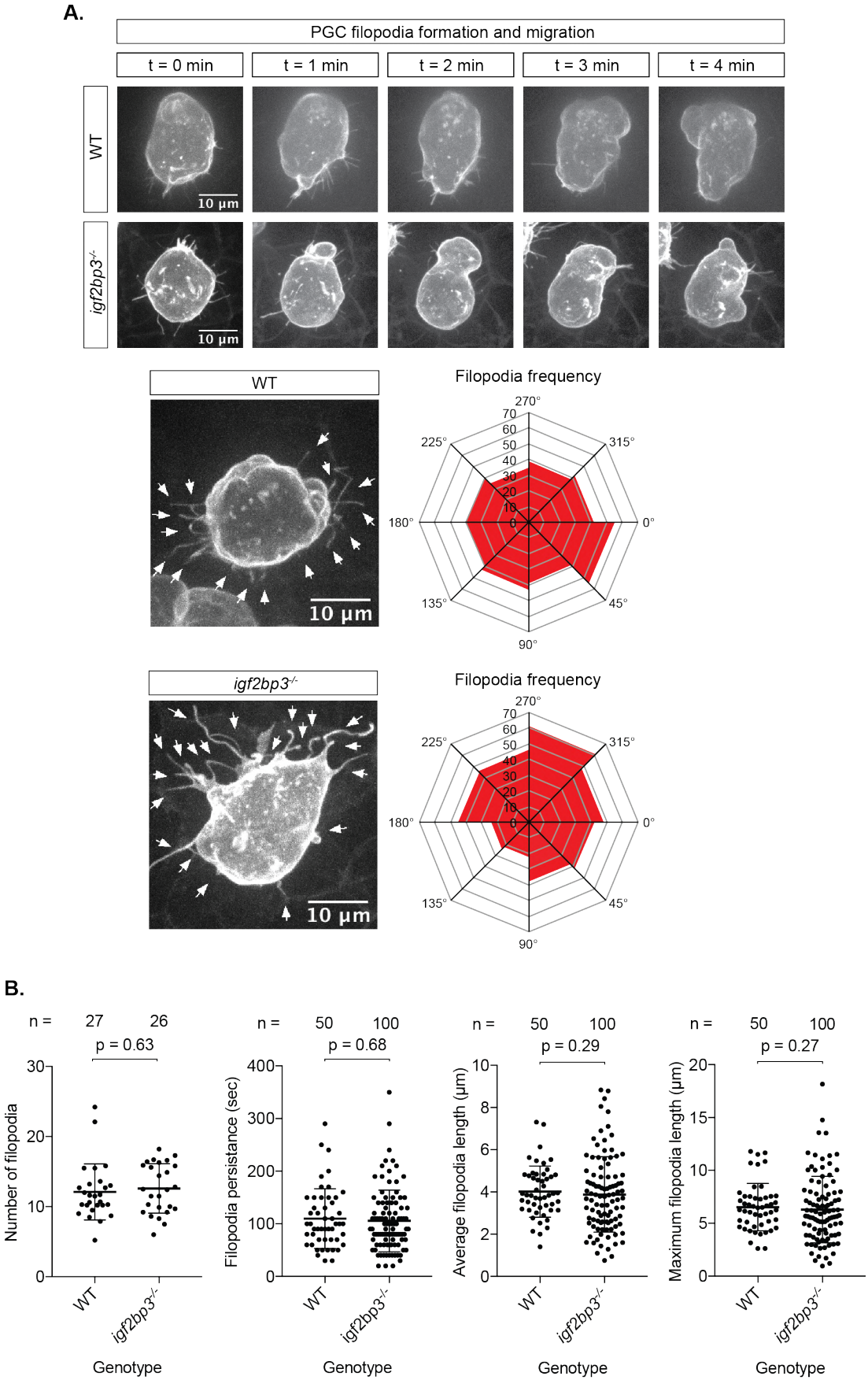


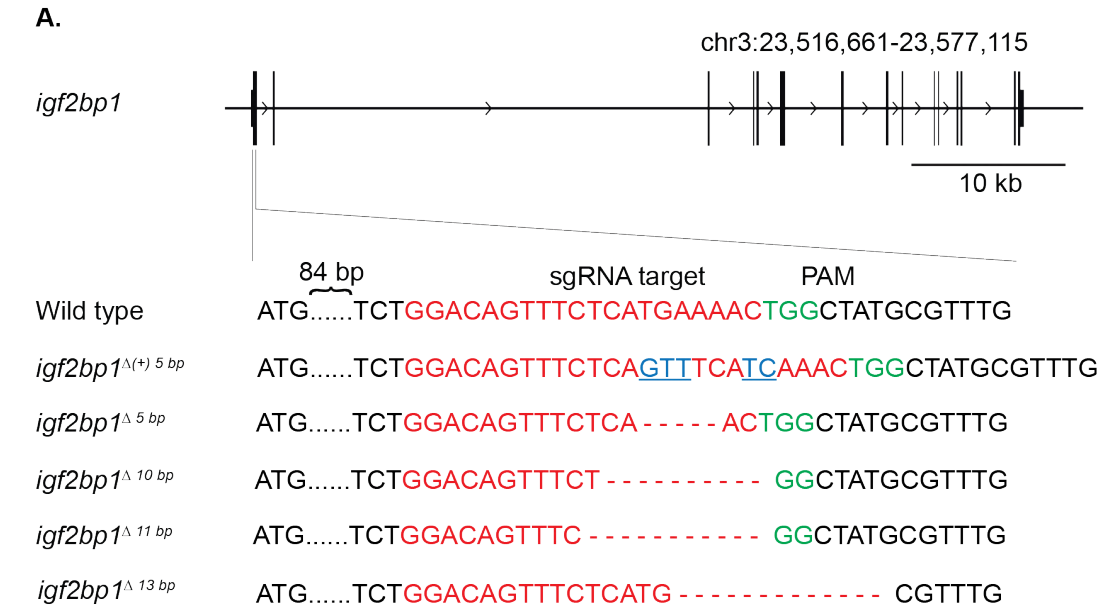
Figure 3-19. Filopodia analysis in the primordial germ cells of WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ mutant embryo. A. Filopodia protrusions in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$. WT and

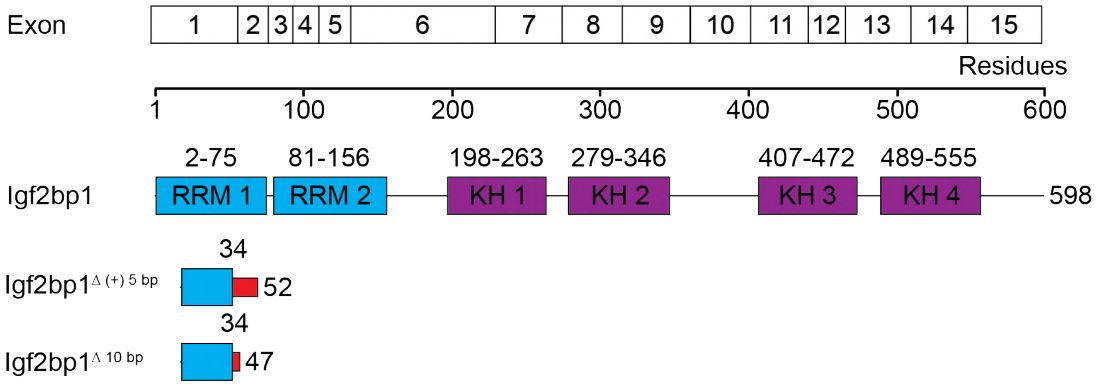
igf2bp3$^{-659\ Tg/-659\ Tg}$ PGCs were imaged between 2-10 minutes and the representative images of PGCs (upper) and the angle of protrusions relative to the midline (bottom) are shown. **B. Quantification of filopodia dynamics in WT and *igf2bp3*$^{-659\ Tg/-659\ Tg}$ PGCs.** The number of filopodia per PGC, its persistence time, average length over its lifetime and maximum length were calculated. Statistical analyses were performed with two-tailed unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.13. The *igf2bp1* CRISPR-Cas9 mutant does not have any overt defect in embryogenesis

Igf2bp1 is another Igf2bp protein family member that shares high homology with Igf2bp3, has a strong expression profile and is coexpressed with *igf2bp3* when probed by *in-situ* analysis (see Fig 3-1, 3-2), we considered duplication as a possible compensation mechanism for the lack of a strong phenotype that were previously reported in other organisms.

Therefore, we used CRISPR-Cas9 to generate indel alleles in exon 1 of the *igf2bp1* locus, which is found in all transcript variants and isoforms of zebrafish Igf2bp1 (Fig. 7-6, 7-7). Several indel alleles were retrieved that encode frameshifts leading to premature stop codons, and preliminary analysis were performed with a 10 bp deletion allele, *igf2bp1*$^{\Delta\ 10\ bp}$ and a discontinuous 5 bp insertion allele, *igf2bp1*$^{\Delta\ (+)\ 5\ bp}$, shown in Figure 3-20A, B. Contrary to our expectations, zygotic *igf2bp1*$^{\Delta\ 10\ bp/\Delta\ 10\ bp}$ mutants did not appear to exhibit any phenotype, and we were able to retrieve homozygous adults according to Mendelian ratios ($p = 0.44$) without any sex bias (Fig 3-20C) and maternal-zygotic *igf2bp1*$^{\Delta\ 10\ bp/\Delta\ 10\ bp}$ embryos also did not have any gross defects in development (Fig. 3-20D).


A.
chr3:23,516,661-23,577,115
igf2bp1
10 kb
84 bp
sgRNA target
PAM
Wild type ATG.....TCTGGACAGTTTCTCATGAAAACTGGCTATGCGTTTG
igf2bp1Δ(+) 5 bp ATG.....TCTGGACAGTTTCTCAGTTTCATCAAACTGGCTATGCGTTTG
igf2bp1Δ 5 bp ATG.....TCTGGACAGTTTCTCA - - - - - ACTGGCTATGCGTTTG
igf2bp1Δ 10 bp ATG.....TCTGGACAGTTTCT - - - - - - - - - - GGCTATGCGTTTG
igf2bp1Δ 11 bp ATG.....TCTGGACAGTTTC - - - - - - - - - - - GGCTATGCGTTTG
igf2bp1Δ 13 bp ATG.....TCTGGACAGTTTCTCATG - - - - - - - - - - - - - CGTTTG


B.
Exon
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Residues
1 100 200 300 400 500 600
2-75 81-156 198-263 279-346 407-472 489-555
Igf2bp1
RRM 1 RRM 2 KH 1 KH 2 KH 3 KH 4
598
34
Igf2bp1Δ (+) 5 bp
52
34
Igf2bp1Δ 10 bp
47
Wild type MNKLYIGNLNEKVTAEDLVKTFEDYKIPYSGQFLMKTGYASVDCPDDQWA
Igf2bp1Δ (+) 5 bp MNKLYIGNLNEKVTAEDLVKTFEDYKIPYSGQFLSFIKLAMRLLIARTTNGQ*
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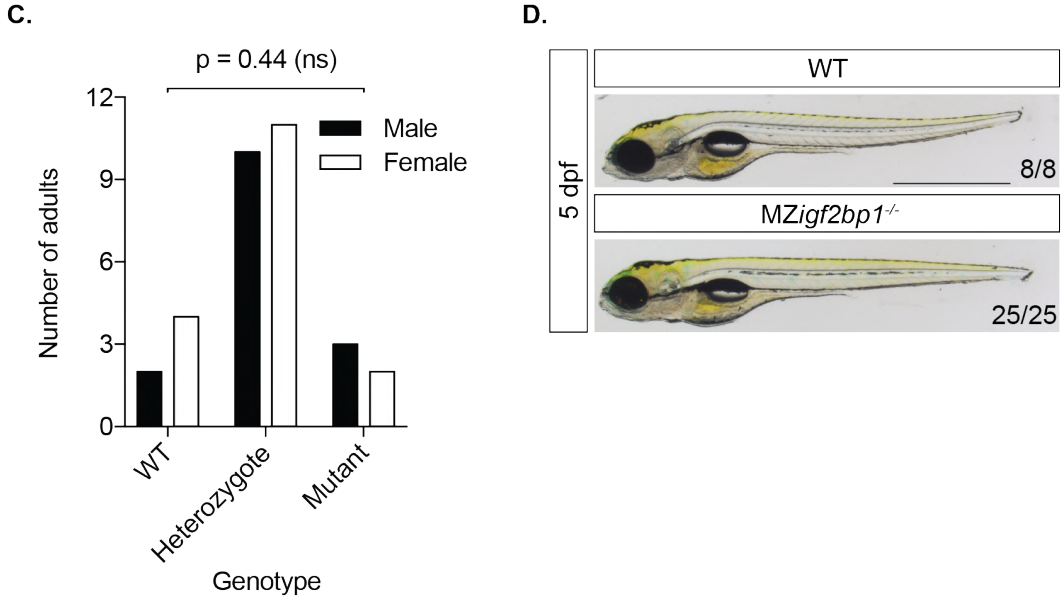
C.
p = 0.44 (ns)
Male
Female
Number of adults
12
9
6
3
0
WT
Heterozygote
Mutant
Genotype
D.
WT
5 dpf
8/8
MZigf2bp1-/-
25/25

Figure 3-20. Generation of the *igf2bp1* CRISPR-Cas9 mutant. A. Targeting strategy for *igf2bp1.* CRISPR-Cas9 was used to target exon 1 of *igf2bp1,* resulting in several recovered indel alleles. **B. Protein prediction of the *igf2bp1* CRISPR-Cas9 alleles.** The *igf2bp1*$^{\Delta (+) 5 bp}$ and *igf2bp1*$^{\Delta 10 bp}$ alleles encoding frameshifts at residue 34, are predicted to produce a truncated Igf2bp1 polypeptide that does not encode for a full RNA-binding motif. **C. Sex and Mendelian ratio of the *igf2bp1*$^{\Delta 10 bp/+}$ intercross.** Adults from an *igf2bp1*$^{\Delta 10 bp/+}$ intercross were genotyped and sexed. Statistical analysis of Mendelian ratio performed with Chi-squared test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. **D. Phenotype of maternal-zygotic *igf2bp1*$^{\Delta 10 bp/\Delta 10 bp}$ embryos at 5 dpf.** Embryos from an *igf2bp1*$^{\Delta 10 bp/\Delta 10 bp}$ intercross were incubated to 5 dpf and imaged. Scale bar = 1 mm.

We further considered whether *igf2bp1* may also play a role in PGC development and we quantified PGCs stained by *in-situ* hybridisation for *ddx4* at 24 hpf, shown in Figure 3-21A. A mild decrease in the number of PGCs was observed, with 42 and 29 PGCs in the WT and maternal-zygotic *igf2bp1*$^{-/-}$ mutants respectively. No change in the distribution of PGCs were observed, and there was no apparent difference in the number of ectopic PGCs. As PGCs appeared to be reduced, qRT-PCR were performed at 24 hpf to assess whether maternal or germplasm mRNA components were reduced (Fig 3-21B), gene expression did not appear to be perturbed for all genes tested, although *igf2bp1* was reduced by 80% in the *igf2bp1*$^{-/-}$, suggesting that the indel alleles are producing transcripts that are degraded by nonsense mediated decay.

As *igf2bp1* is strongly expressed (Fig. 3-2) during late gastrula and peaks during segmentation, and *igf2bp1* transcripts appeared to be deposited maternally, we considered whether Igf2bp1 protein was also present during this time and we utilised a commercial antibody directed against the RRM 1 of human Igf2bp1, shown in Figure 3-21C. Whilst we were able to observe positive bands corresponding to Igf2bp1 in mouse trophoblast stem cells, we were unable to find corresponding bands of the expected molecular weight in early stage embryos, where we expected Igf2bp1. At 24 hpf, a single band corresponding to the expected molecular weight of Igf2bp1 could be observed and the identity of the persistent band observed at ~50 kDa is unclear, as Igf2bp1 and Igf2bp3 do not have isoforms that are this molecular weight, one possibility is that these bands correspond to Igf2bp2a, which has several isoforms that are approximately these sizes.

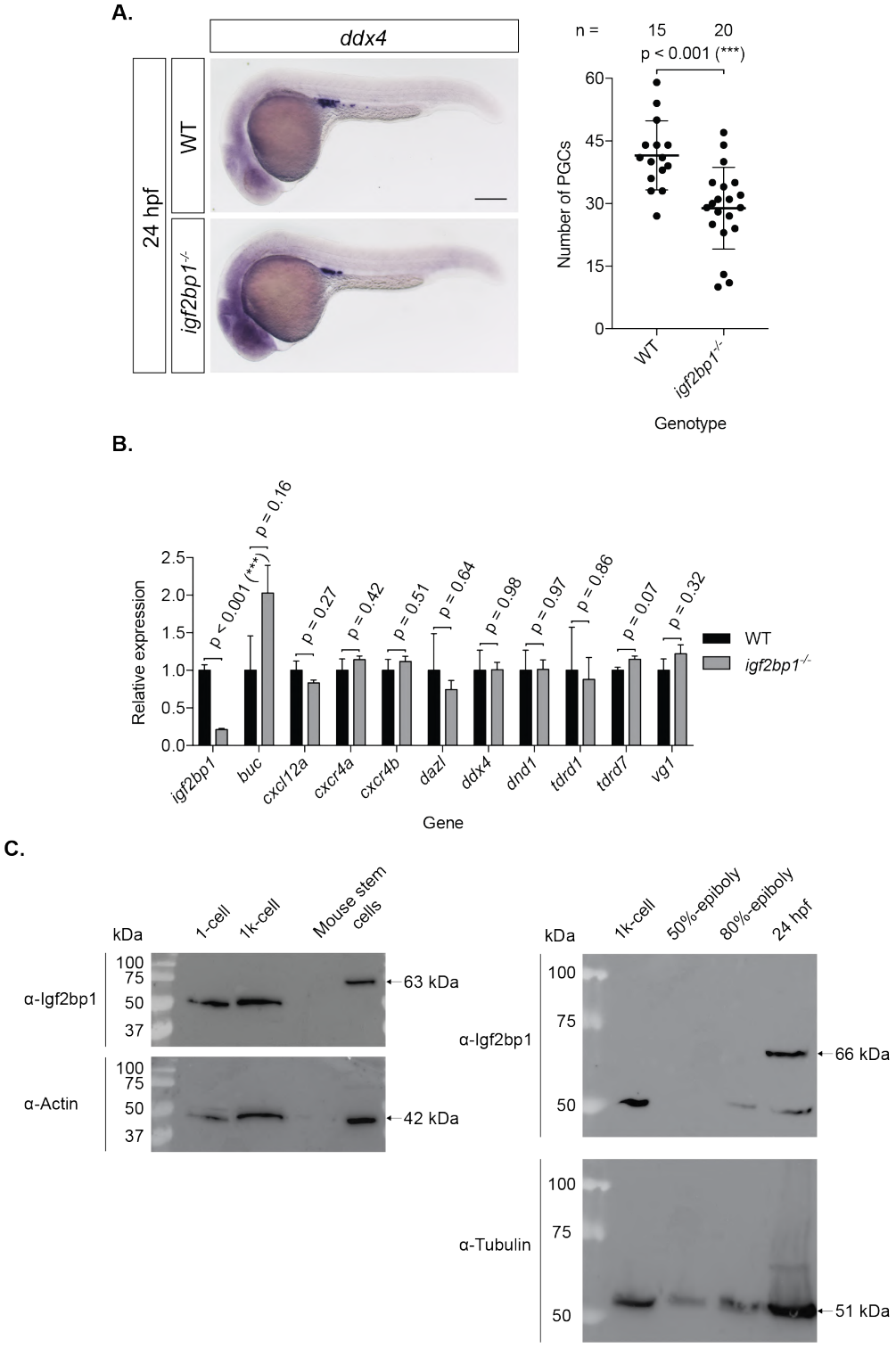

Figure 3-21. Preliminary characterisation of the *igf2bp1* mutant in primordial germ cell development. A. Loss of *igf2bp1* reduces the number of PGCs. WT and maternal-zygotic *igf2bp1* mutants were probed for *ddx4* at 24 hpf (left) and the number of PGCs quantified (right). Statistical analysis performed with two-tailed unpaired t-test. **B. qRT-PCR**

of germplasm genes in *igf2bp1*$^{-/-}$ mutants. qRT-PCR was performed on a panel of germplasm and germplasm-linked genes at 24 hpf relative to the housekeeping gene *18S*. Statistical analysis performed with two-tailed unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. **C. Validation of an Igf2bp1 antibody in zebrafish lysates.** Zebrafish lysates at 1-cell, 1k-cell in conjunction with a positive control, mouse trophoblast stem-cells (left), and subsequently repeated at 1k-cell, 50%-epiboly, 80%-epiboly and 24 hpf. Scale bar = 200 µm.

3.14. *igf2bp1; igf2bp3* mutants are unviable as they fail to inflate the swim bladder

igf2bp1 and *igf2bp3* have been described as having a biphasic expression during development, where the peak expression of these genes occur consecutively (Nielsen *et al.*, 2000), to establish the genetic basis for this observation, we generated attempted to generate *igf2bp1*$^{\Delta (+) 5 bp/(+) 5 bp}$*; igf2bp3*$^{-659 Tg/-659 Tg}$ mutants by intercrossing *igf2bp1*$^{\Delta (+) 5 bp/+}$*; igf2bp3*$^{-659 Tg/-659 Tg}$ adults, however, we were not able to retrieve double mutants ($p < 0.01$), shown in Figure 3-22A, as these adults appear to be unviable. As we were not unable to retrieve *igf2bp1*$^{\Delta (+) 5 bp/(+) 5 bp}$*; igf2bp3*$^{-659 Tg/-659 Tg}$ adults through this method, there was a corresponding increase in the obtained number of remaining genotypes.

These adults were intercrossed again, and the embryos monitored closely throughout development, and did not appear distinguishable between the genotype combinations initially. However, by 5 dpf, we observed a significant number of embryos that failed to inflate the swim bladder (Fig 3-22B), and upon genotyping of these embryos, these were the zygotic *igf2bp1*$^{-/-}$ mutants generated in the MZ*igf2bp3*$^{659 Tg}$ background, suggesting that combined loss of two *igf2bp* genes is not viable.

The genetic basis for this phenotype is difficult to dissect, as both MZ*igf2bp1*$^{\Delta 10 bp}$ and MZ*igf2bp3*$^{-659 Tg}$ mutants alone do not exhibit any defects in the swimbladder, however, as heterozygous *igf2bp1* mutants in the MZ*igf2bp3*$^{-659 Tg}$ background (*igf2bp1*$^{\Delta (+) 5 bp/+}$*; igf2bp3*$^{-659 Tg/-659 Tg}$) did not have any swimbladder defect, this suggests that paternal or maternal transmission of the *igf2bp1*$^{\Delta (+) 5 bp}$ allele alone does not control inflation of the swim bladder, but points to a zygotic role in *igf2bp1.*

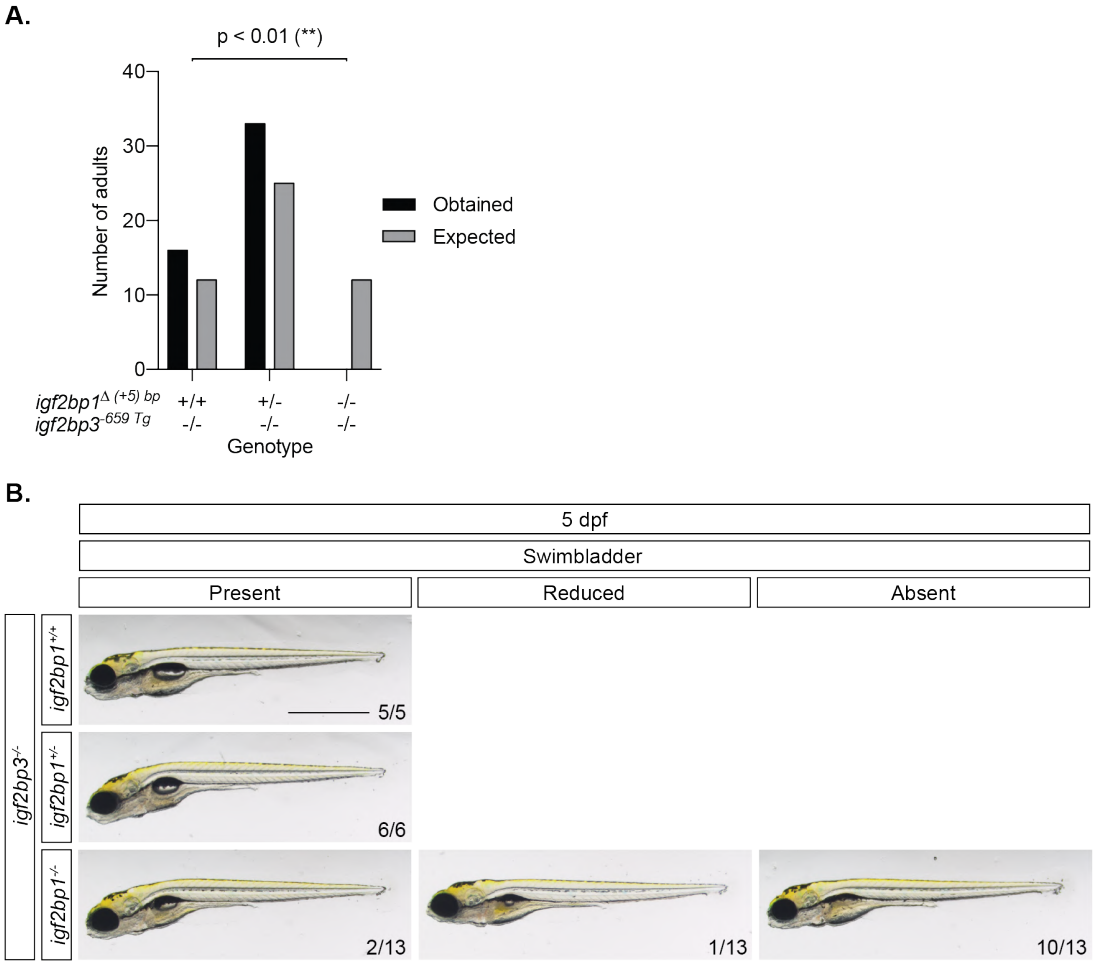

Figure 3-22. Zygotic *igf2bp1* mutants are unviable in the maternal-zygotic *igf2bp3*$^{-659\ Tg}$ background. A. Genotype of adults generated from an *igf2bp1*$^{\Delta\ (+5)\ bp/+}$; *igf2bp3*$^{-659\ Tg/-659\ Tg}$ intercross. Adults were genotyped and their genotype for *igf2bp1* scored. Statistical analysis performed with Chi-squared test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001. **B. The swim bladder fails to inflate in the *igf2bp1*$^{\Delta\ (+5)\ bp/\Delta\ (+5)\ bp}$; *igf2bp3*$^{-659\ Tg/-659\ Tg}$ double mutant.** *igf2bp1*$^{\Delta\ (+5)\ bp/+}$; *igf2bp3*$^{-659\ Tg/-659\ Tg}$ adults were intercrossed and embryos imaged and genotyped at 5 dpf, with the swimbladder phenotype scored. Scale bar = 1 mm.

Failure to inflate the swimbladder is a common phenotype in many genetic perturbances, and many mutants or morphants are known to fail to develop the swim bladder, and defects in circulation often lead to failure to inflate the swim bladder (Winata *et al.*, 2010; Rawnsley *et al.*, 2013). To investigate this, we looked at major organs such as the heart, liver and pancreas with the *myl7* (*myosin, light chain, 7)* and *foxA3* probes, shown in Figure 3-23. Embryos from an *igf2bp1*$^{\Delta\ (+)\ 5\ bp/+}$; *igf2bp3*$^{-659\ Tg/-659\ Tg}$ intercross were probed at 24 and 50 hpf for *myl7* to visualise the heart for looping and jogging defects, and we were not able to find any instances where the mutants of all genotype combination deviated from the norms established in the literature (left-sided bias). Similarly, our stainings for *foxA3* were also within the expected range, showing the correct normal laterality of organ arrangements.

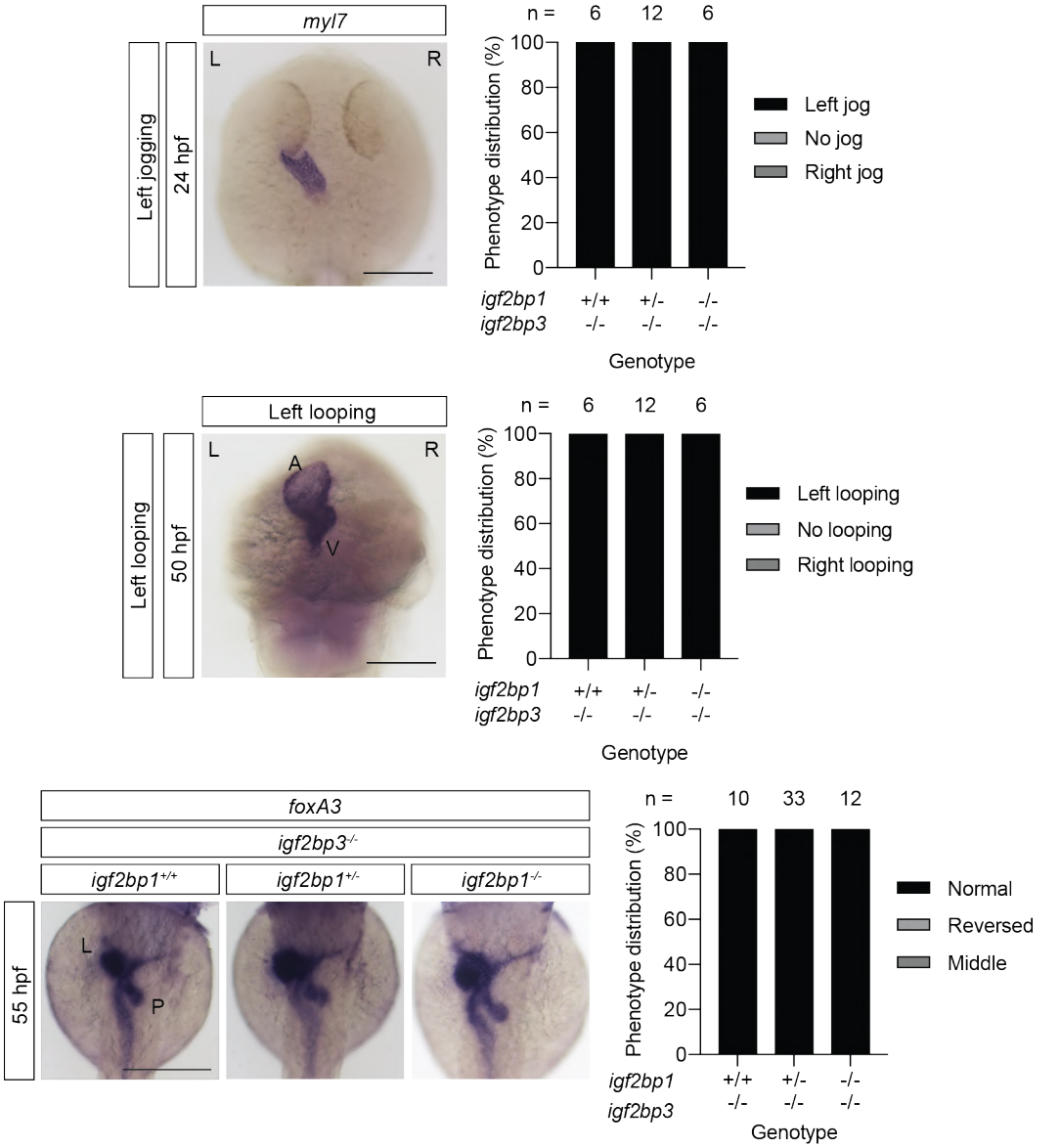

Figure 3-23. Organ formation and laterality is not affected in the *igf2bp1* and *igf2bp3* mutants. Adult *igf2bp1*$^{\Delta\ (+5)\ bp/+}$*; igf2bp3*$^{-659\ Tg/-659\ Tg}$ fish were intercrossed and the progeny probed for *myl7* at 24 hpf (upper) and 50 hpf (middle) to assess heart jogging and looping. Embryos were also probed for *foxA3* at 55 hpf to assess laterality and formation of the liver and pancreas. Scale bar = 200 µm.

As the swimbladder did not inflate in our *igf2bp*$^{\Delta\ (+5)\ bp/\Delta\ (+5)\ bp}$*;* igf2bp3$^{-659\ Tg/-659\ Tg}$ embryos, we tested whether a swimbladder was present in these embryos by probing them with *shha*, which labels the swimbladder, notochord and gut, shown in Figure 3-24. Swimbladders could be seen in all genotype combinations observed, and the failure to inflate the swimbladder in the *igf2bp*$^{\Delta\ (+5)\ bp/\Delta\ (+5)\ bp}$*;* igf2bp3$^{-659\ Tg/-659\ Tg}$ is not due to a failure to form the swimbladder. There also appeared to be no defects observed in the notochords, or gut, and we cannot find any evidence of any defects in these organs/tissues.

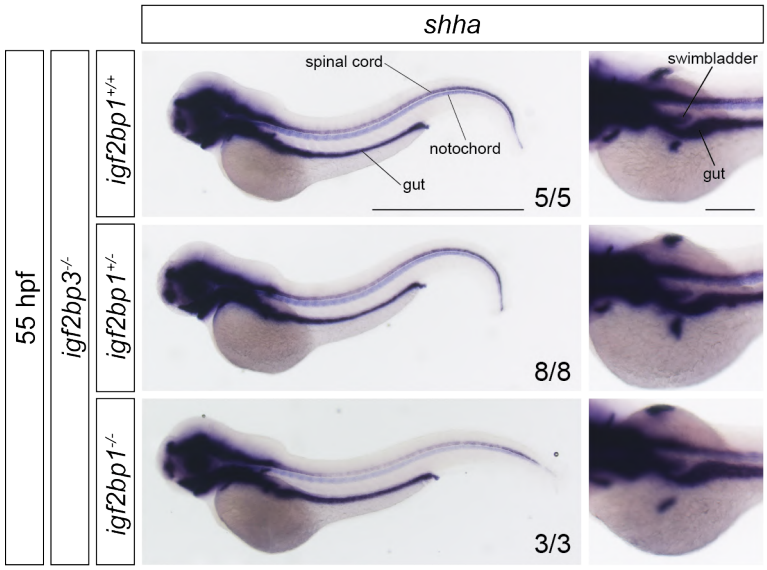

Figure 3-24. Development of swim bladder is not affected by the loss of *igf2bp1* or *igf2bp3*. Adult *igf2bp1*$^{\Delta\ (+5)\ bp/+}$*; igf2bp3*$^{-659\ Tg/-659\ Tg}$ fish were intercrossed and the progeny probed for *shha* at 55 hpf to visualise the swimbladder. Scale bar = 1 mm (left) and 200 µm (right).

As loss of *igf2bp1* and *igf2bp3* can cause a reduction in the number of PGCs, we considered whether further depletion of PGCs could be achieved by the loss of both *igf2bp1* and *igf2bp3*. As we could not generate double maternal-zygotic *igf2bp*$^{\Delta\ (+5)\ bp/\Delta\ (+5)\ bp}$*;* igf2bp3$^{-659\ Tg/-659\ Tg}$ mutants, we continued intercrossing heterozygous *igf2bp1* adults in the maternal-zygotic *igf2bp3* background and quantified the PGCs before genotyping the imaged embryos, shown in Figure 3-25. Loss of *igf2bp1* in any combination in the maternal-zygotic *igf2bp3* background did not reduce or enhance the loss of PGCs, with the average number of PGCs in the *igf2bp1*$^{+/+}$*, igf2bp1*$^{+/-}$ and *igf2bp1*$^{-/-}$ genotypes being 10, 9 and 11 respectively.

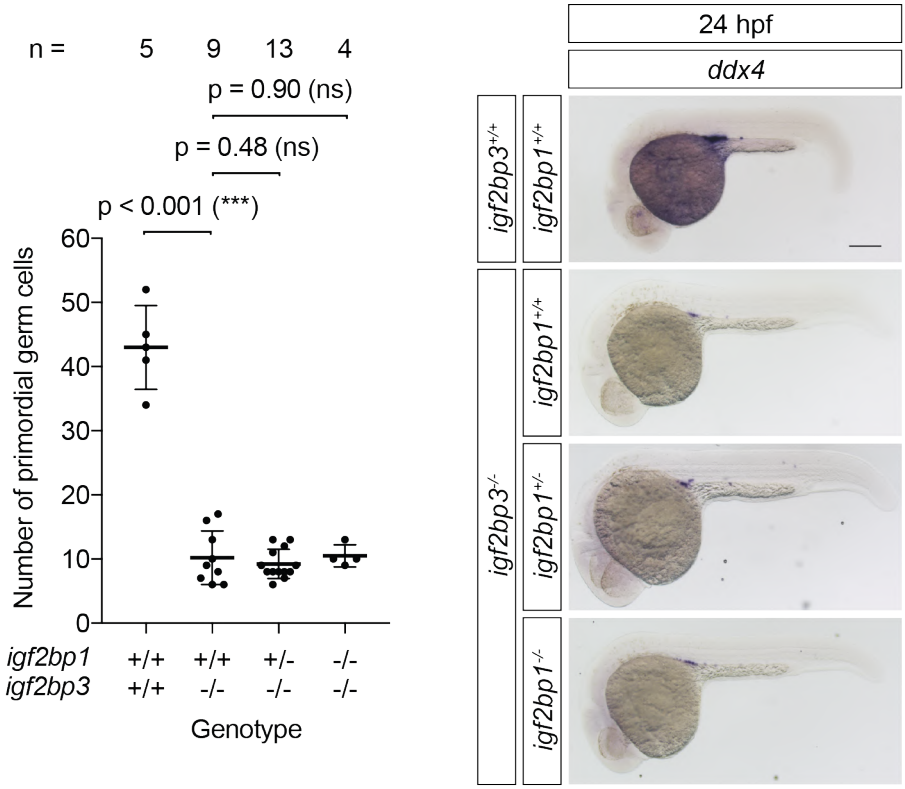

Figure 3-25. Loss of zygotic *igf2bp1* does not enhance loss of PGCs in the maternal-zygotic *igf2bp3* background. Adult *igf2bp1*$^{\Delta (+5) bp/+}$; *igf2bp3*$^{-659 Tg/-659 Tg}$ fish were intercrossed and the progeny probed for *ddx4* to visualise the PGCs (right). The number of PGCs were quantified (left). Statistical analysis performed with two-tailed t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Scale bar = 200 µm.

4. Chapter 4 – Generation of mutants in RNA-binding proteins

In addition to identifying Igf2bp3 as a putative *sqt* RNA-binding protein, proteomic screens also identified the Raver1, Syncrip and Syncripl as possible *sqt* binding proteins, and we attempted to generate mutants in these genes to determine whether they had a role in zebrafish development.

4.1. Role of the RNA-binding protein Raver1

Raver1 is an RNA-binding protein that was initially identified from yeast two-hybrid screens directed against C-terminal ends of metavinculin to identify new ligands and is a nucleocytoplasmic protein that appears to localise to the nucleus and cellular focal adhesions *in vivo* (Hüttelmaier *et al.*, 2001), mammalian Raver1 contains three RNA-recognition motifs and has several isoforms. Raver1 also appears to form complexes with the hnRNP I family proteins, the polypyrimidine tract-binding proteins (PTBP) 1 and 2 (Hüttelmaier *et al.*, 2001; Gromak *et al.*, 2003; Spellman *et al.*, 2005; Rideau *et al.*, 2006; Gooding *et al.*, 2013; Keppetipola *et al.*, 2016; Wongpalee *et al.*, 2016). The genetic loci of Raver1 and PTBP 1 appears to share common promoter regulatory elements (Romanelli *et al.*, 2007) and the colocalisation of Raver1 with PTBP 1 and PTBP 2 seem to control alternative splicing and splicing repression of target transcripts such as *α-tropomyosin* (Gromak *et al.*, 2003; Gooding *et al.*, 2013) and *vinculin* (Lee *et al.*, 2012).

The consequence of Raver1 loss is unclear, *in vivo*, as although *raver1* is expressed in many tissues, such as the brain, heart, muscle and intestines (Lahmann *et al.*, 2008) and Raver1 is found in muscle myofibrils, the nuclei and enriched and cellular focal adhesion points (Zieseniss *et al.*, 2007; Lee *et al.*, 2009), loss of *raver1* does not appear to generate an phenotype in KO-mice when cell morphology and splicing of PTBP regulated exons were tested (Lahmann *et al.*, 2008). Interestingly, hippocampal slices examined in *raver1* mutant mice for long-term potentiation in long-term depression were not comparable (Lahmann *et al.*, 2008), implicating Raver1 in synaptic plasticity. More recently, Raver1 has been shown to be able to partially rescue the loss of another RNA-binding protein, TDP-43, when Raver1 was fused to the RNA recognition domains of TDP-43 (Donde *et al.*, 2019). Raver1 has also been implicated in other cellular processes, and Raver1 appears to modulate innate anti-viral responses (Chen *et al.*, 2013) and also interacts with the CCR4-NOT complex in conjunction with proteins such as the aforementioned TDP-43 (Miyasaka *et al.*, 2008).

4.2. Preliminary analysis on the role of *raver1* in embryogenesis and generation of CRISPR-Cas9 mutants for *raver1.*

In vertebrates, two *raver* homologs are present, *raver1* and *raver2*, that retain similar functional features (Keppetipola *et al.*, 2012) although their expression differs significantly, *raver1* is expressed very highly early during blastula stages, shown in Figure 4-1A, whereas *raver2* is expressed at very basal levels.

To explore the spatial expression of *raver1* in zebrafish development, whole *in-situ* hybridisation were performed with *raver1* probe against WT embryos up to 24 hpf, and we able to observe strong and ubiquitous staining of the embryos (Fig. 4-1B). Previously, overexpression of *raver1* in cell lines were able to trigger alternative splicing of *tropomyosin* mRNA (Gromak *et al.*, 2003), we performed overexpression experiments by injecting capped mRNA generated from cloning the *raver1* CDS into the pCS2+ vector (Fig. 4-1C), these embryos did not show any lethality and we could not observe any defects in development from overexpression of *raver1*.

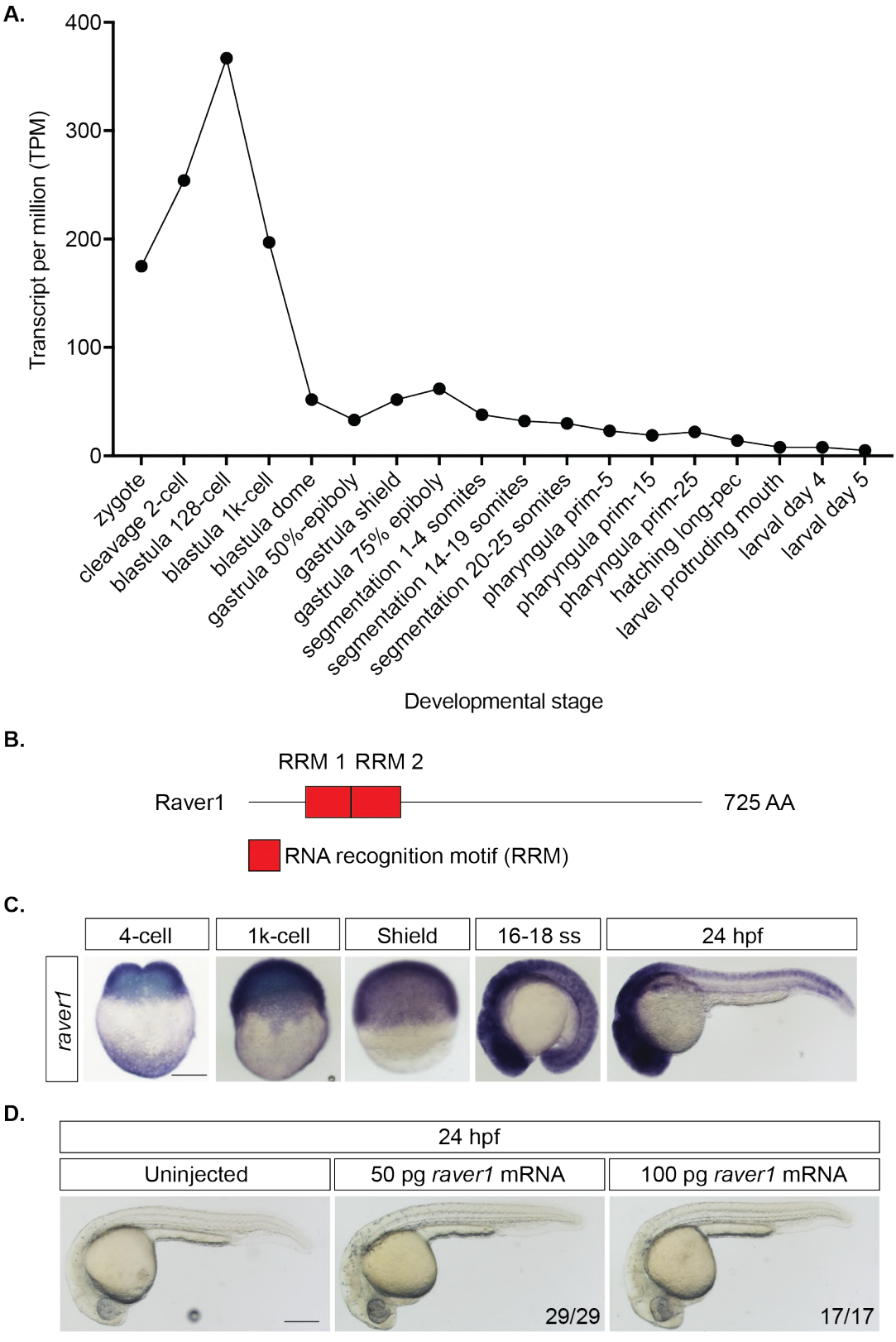

Figure 4-1. Expression of *raver1* during embryogenesis. A. RNAseq data for zebrafish *raver1* during embryogenesis. Expression data for *raver1* were plotted over the course of development (1-cell to 5 dpf). **B. Structural alignment of Raver1 protein.** Zebrafish Raver1

was arranged and drawn according to their RRM domains. **C. Spatial expression of *raver1*.** WT embryos were probed with *raver1* to visualise expression during early embryogenesis. **D. Overexpression of *raver1* in WT embryos.** WT embryos were injected with synthetic capped *raver1* mRNA and imaged at 24 hpf. Scale bar = 200 μm. Data from Figure 4-1A extracted from (White *et al.*, 2017).

4.3. Generation of the *raver1* CRISPR-Cas9 mutant

Since Raver1 was identified from proteomic screens and *raver1* transcripts are present in abundance maternally, we generated CRISPR-Cas9 mutants for *raver1* by targeting exon 1 of the locus, and we retrieved several indel alleles predicted to encode premature stop codons before the first RRM of Raver1, shown in Figure 4-2A. The lesion is predicted to target all isoforms and transcripts of *raver1* (Fig. 7-8, 7-9).

Despite our attempts, we were not able to procure a Raver1 antibody that has reactivity in zebrafish, so we intercrossed the allele with the earliest frameshift and truncation, *raver1*$^{\Delta 73\ bp}$, to homozygosity, and these fish did not present with any obvious defects in development, as we retrieved them according to Mendelian ratios ($p = 0.95$) and no sex bias (Fig. 4-2B).

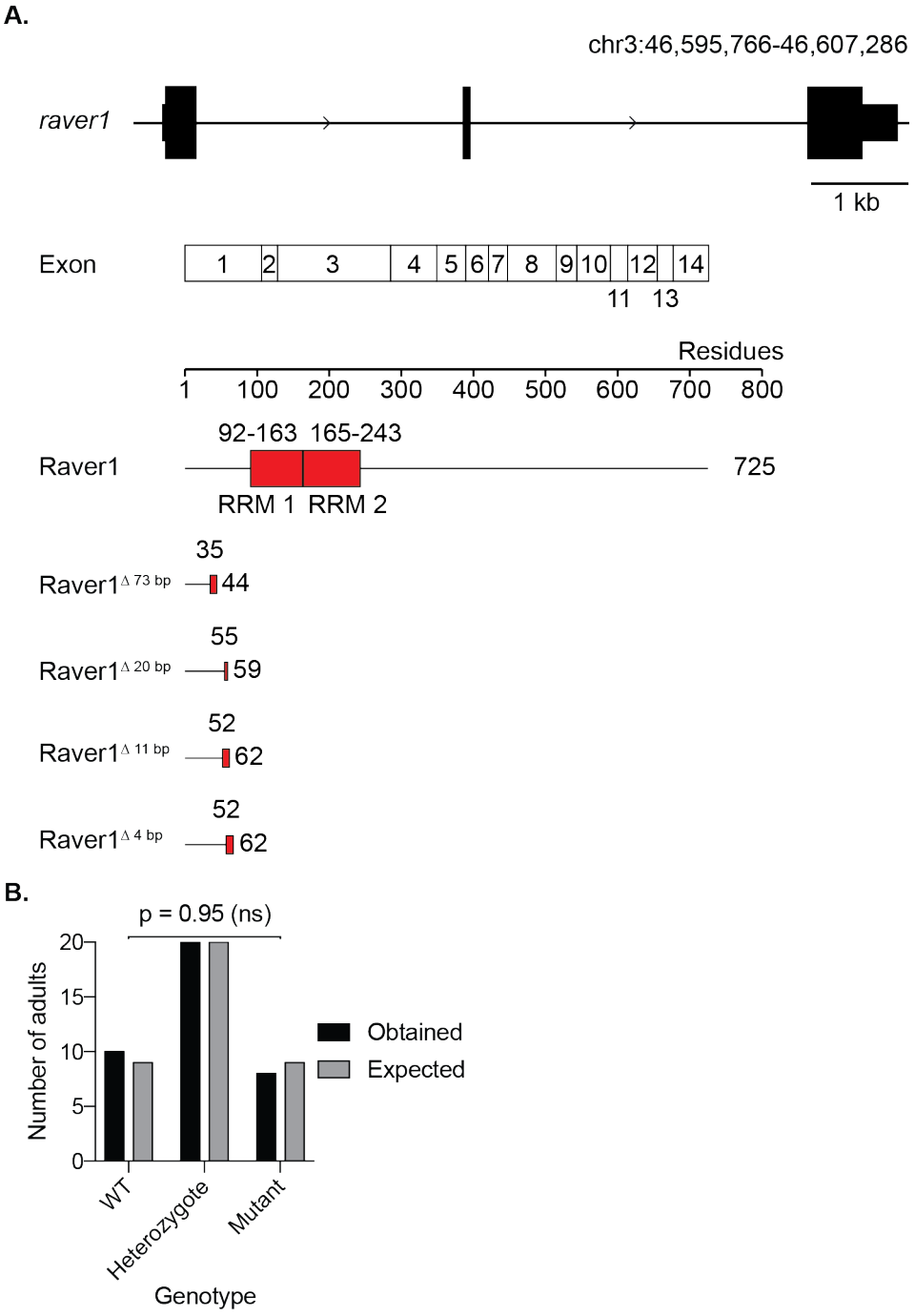

Figure 4-2. Generation of the raver1 CRISPR-Cas9 mutant. A. Genomic locus of *raver1* and predicted polypeptides from *raver1* CRISPR-Cas9 alleles. The genomic locus of *raver1* (upper) and the arrangement of the cDNA to the WT Raver1 protein (middle) are shown relative to the predicted translated products from the indel alleles generated from the CRISPR-Cas9 screen. **B. Mendelian ratios of the *raver1*$^{\Delta 73\ bp/+}$ intercross.** Heterozygous *raver1*$^{\Delta 73\ bp}$ adults were intercrossed and grown to adulthood and subsequently sexed and genotyped. Statistical analysis performed with Chi-squared test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.4. Role of the RNA-binding protein Syncrip

Another group RNA-binding protein identified from proteomic screens for *sqt* binding proteins is Syncrip and Syncrip-like (Syncripl). These proteins, originally referred to as Synaptotagmin cytoplasmic RNA interacting protein, are members of the hnRNP Q family of the proteins preferentially bind to polyadenylated RNAs (Mizutani *et al.*, 1997, 2000), which affects its phosphorylation state (Hresko and Mueckler, 2002) and subsequent ability to regulate other RNA-binding proteins such as Apobec1 (Quaresma *et al.*, 2006) and Smn (Survival of motor neuron) (Mourelatos *et al.*, 2001).

Syncrip has three RNA-recognition motifs for binding RNA molecules, and methylation of the protein modulates its ability to bind target transcripts (Woodsmith *et al.*, 2018) and methylation of the C-terminus controls its subcellular localisation to the nucleus (Passos, Quaresma and Kobarg, 2006). Syncrip has been associated with enhancing translation (Kim *et al.*, 2004; Cho *et al.*, 2007; Vincendeau *et al.*, 2013), translational inhibition by directly interacting with the UTRs of transcripts (Kim *et al.*, 2013; Svitkin *et al.*, 2013; Kulkarni *et al.*, 2017) splicing (Kabat, Barberan-Soler and Zahler, 2009; Beuck *et al.*, 2016) and post-transcriptional stabilisation of mRNAs (Moser *et al.*, 2007; Weidensdorfer *et al.*, 2008).

The function of Syncrip as an RNA-binding protein is not well established *in vivo*, yeast two-hybrid screens have found a spliced isoform of Syncrip to be a component of the RNA editing complex (Blanc *et al.*, 2001; Lau, Chang and Chan, 2001; Quaresma *et al.*, 2006). Neuronally, Syncrip is expressed ubiquitously in rat brains (Tratnjek, Živin and Glavan, 2017) and several studies have found Syncrip to be localised to RNA transport granules to the neurons (Bannai *et al.*, 2004; Kanai, Dohmae and Hirokawa, 2004; Duning *et al.*, 2008; Chen *et al.*, 2012).

In *Drosophila*, Syncrip binds to *gurken* and *oskar* mRNA and acts as an localisation protein and translational repressor in conjunction with Smn to regulate axis specification (McDermott *et al.*, 2012; Aquilina and Cauchi, 2018), and further studies in *Drosophila* have identified Syncrip as a regulator of neuronal differentiation and diversity (Syed, Mark and Doe, 2017) by regulating expression of transcription factors, such as Chinmo, that are involved in neuronal patterning (Liu *et al.*, 2015). *Drosophila* Syncrip also fine tunes expression of genes such as BMP signalling pathway components (Halstead *et al.*, 2014) to direct the morphology of the neuromuscular synapse and regulate synaptic plasticity (McDermott *et al.*, 2014).

In higher organisms, the role of Syncrip is less clear, several screens for neurodegenerative diseases and behavioural disorders such as Alzheimer's and autism have found a correlation between mutations in Syncrip and risk of acquiring these diseases (Guttula,

Allam and Gumpeny, 2012; Lelieveld *et al.*, 2016; Bakkar *et al.*, 2018; Guo *et al.*, 2019), which is consistent with a role in neurological development, which may be linked to Smn, as overexpression of Syncrip can rescue knockdown of *smn-1* in *C. elegans* (Rizzo *et al.*, 2019). Syncrip also has been documented to be involved in carcinogenesis, deletion of Syncrip is associated with acceleration and delayed onset of carcinogenesis in different contexts (Vu *et al.*, 2017; Gachet *et al.*, 2018).

4.5. Generation of the *syncrip* and *syncripl* CRISPR-Cas9 mutant

To generate CRISPR-Cas9 mutants for *syncrip* and *syncripl,* we were not able to produce any mutagenesis with sgRNAs directed against exons 2, 3 and 4 for either genes, and we were able to produce mutagenesis with sgRNAs directed against exon 5, although stable lines for these genes have not been retrieved yet. The alleles from this sgRNA are predicted to target all transcripts of *syncrip* and *syncripl* (Fig. 7.10, 7-11).

Furthermore, we have also attempted to detect zebrafish Syncrip and Syncripl with an antibody directed against the mammalian homologs, but Western blots with this antibody were unsuccessful.

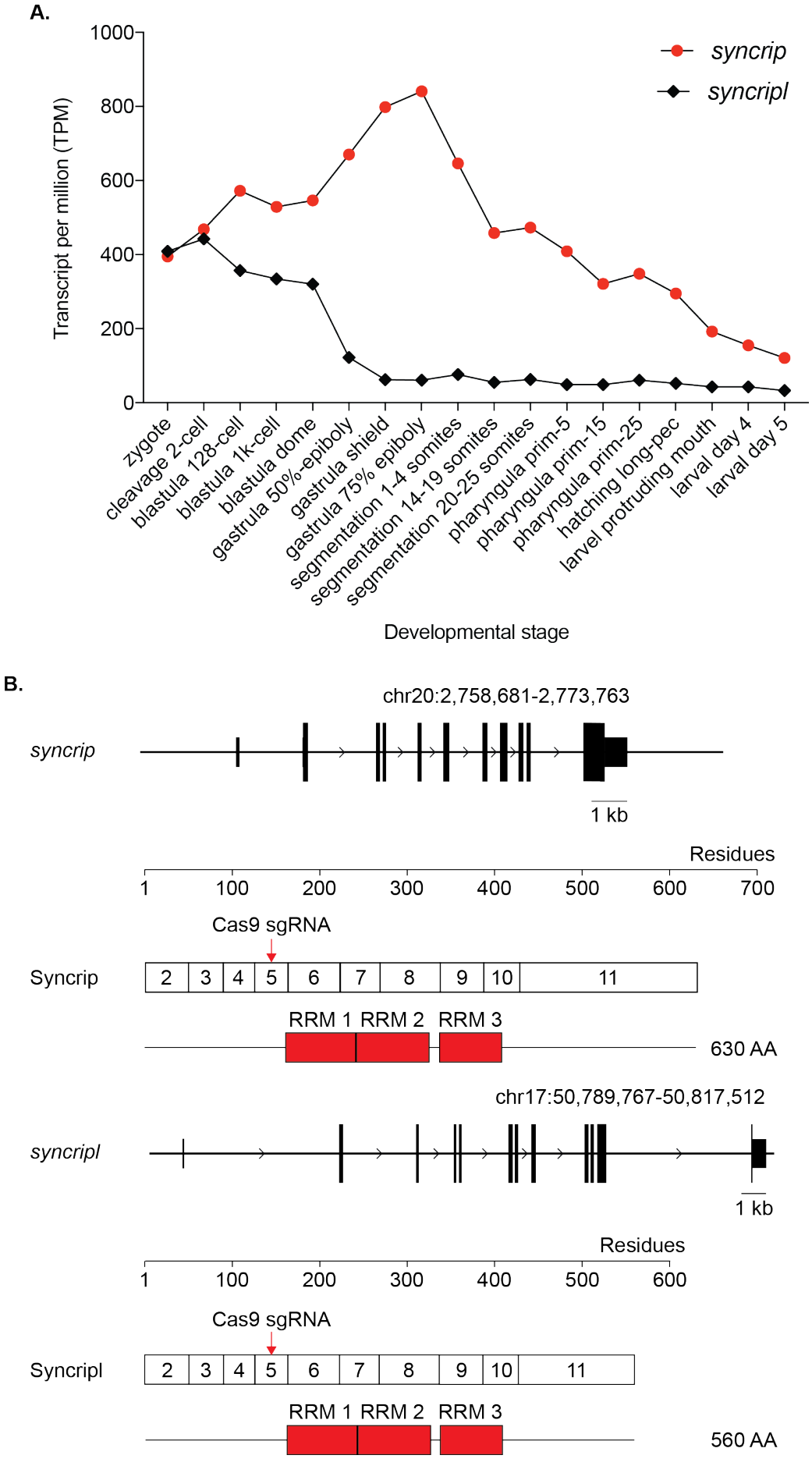
A.
1000
800
600
400
200
0
Transcript per million (TPM)
syncrip
syncripl
zygote
cleavage 2-cell
blastula 128-cell
blastula 1k-cell
blastula dome
gastrula 50%-epiboly
gastrula shield
gastrula 75% epiboly
segmentation 1-4 somites
segmentation 14-19 somites
segmentation 20-25 somites
pharyngula prim-5
pharyngula prim-15
pharyngula prim-25
hatching long-pec
larvel protruding mouth
larval day 4
larval day 5
Developmental stage
B.
chr20:2,758,681-2,773,763
syncrip
1 kb
Residues
1
100
200
300
400
500
600
700
Cas9 sgRNA
Syncrip
2
3
4
5
6
7
8
9
10
11
RRM 1
RRM 2
RRM 3
630 AA
chr17:50,789,767-50,817,512
syncripl
1 kb
Residues
1
100
200
300
400
500
600
Cas9 sgRNA
Syncripl
2
3
4
5
6
7
8
9
10
11
RRM 1
RRM 2
RRM 3
560 AA

Figure 4-3. Expression profile and generation of the zebrafish syncrip and syncripl CRISPR-Cas9 mutants. A. RNAseq data for zebrafish *syncrip* and *syncripl* during embryogenesis. Expression data for *syncrip* (red) and *syncripl* (black) were plotted over the course of development (1-cell to 5 dpf). **B. Targeting strategy for generating zebrafish CRISPR-Cas9 *syncrip* and *syncripl* mutants.** The genomic loci for *syncrip* and *syncripl* are shown (upper), followed by the cDNA (middle) and protein domain arrangements (bottom) for each gene and the targeting sgRNA used to generate putative mutants. Data from Figure 4-3A extracted from (White *et al.*, 2017).

4.6. Summary

Currently, we have produced further mutants for RNA-binding proteins which were found in RNA pull-downs for *sqt* interacting factors. The roles of these proteins in a complex with *sqt* are unclear, and further analysis of maternal mutants in the future will provide information to their roles in development, and how they function in the *sqt* mRNP complex.

5. Chapter 5 - Discussion

5.1. The role of Igf2bp3 in development

Maternal contribution to development begins during oocyte maturation, when maternal proteins and RNAs are deposited for later activation to ensure proper development. One such RNA that is maternal contributed and is essential to early development is the Nodal signalling factor, *sqt*, which acts as a dorsal and mesendoderm inducing morphogens (Feldman *et al.*, 2000). Correct post-transcriptional regulation of these RNAs is an essential process for development, and interruption of this regulation by loss or disruption of RNA-binding proteins that activate, localise, degrade or repress maternal RNAs leads to disastrous consequences.

Previously, screens for *sqt* RNA interacting factors identified an RNA-binding protein, Ybx1, that acts as a translational repressor, and prevents precocious *sqt* translation (Kumari *et al.*, 2013); Ybx1 also acts as a global repressor of translation in the early embryos (Sun *et al.*, 2018). Further screens conducted on zebrafish embryonic lysates to find *sqt* interacting proteins identified several proteins, in particular, Igf2bp3. Therefore, we characterised the role of Igf2bp3 in regulating zebrafish development and discovered a role for Igf2bp3 in germline maintenance.

In *Xenopus,* Igf2bp3 was originally identified to interact with *Vg1* mRNA, which contributes to Nodal signalling (Chen *et al.*, 2005; Pelliccia, Jindal and Burdine, 2017) and localises *vg1* transcripts to the vegetal pole of the oocytes (Schwartz *et al.*, 1992), suggesting that Igf2bp3 may also have a similar role in zebrafish to coordinate axial patterning or oocyte polarity somehow, although this was not affected when we examined the expression patterns of *vg1* in zebrafish embryos up to somitogenesis.

However, using transgenic insertion mutants for *igf2bp3,* we did not observe any issues with embryonic patterning in our *igf2bp3*$^{-/-}$ mutants when we assessed dorsal and endoderm expression markers or left-right asymmetry markers, suggesting that *igf2bp3* does not play a role in early axis development. The animal-vegetal polarity of the embryos were not affected, although we have not checked the distribution of cortical and unlocalised RNAs such as *vasa* and *nanos* respectively (Braat *et al.*, 1999; Abrams and Mullins, 2009). Although, as the Balbiani body is essential for polarity of the oocyte, correct axis formation in the *igf2bp3*$^{-/-}$ mutants suggests that Igf2bp3 is not required to form the Balbiani body, despite being localised in this structure (Bontems *et al.*, 2009).

In zebrafish, *igf2bp3* is expressed ubiquitously and strongly throughout early development, and we were not able to induce morphological defects by overexpression experiments, nor

did we observe any failures to develop into adulthood, suggesting that the abundance of Igf2bp3 is not essential for the development of the soma. This is not concordant with the knockdown phenotypes reported in *Xenopus,* where knockdown of *igf2bp3* results in severe abnormalities in cell migration such deficiencies in the neural crest and loss of the pancreas (Yaniv *et al.*, 2003; Spagnoli and Brivanlou, 2006), suggesting an underlying genetic mechanism is affecting the phenotypes observed.

5.2. The use of genetic mutants to remove maternal Igf2bp3 function

In this study, we have focused on the characterisation of a maternal function for Igf2bp3 in development. To this end, we used or generated both transgenic insertion alleles and a Cas9 indel allele for this study. Other options to disrupt gene function could include the use of morpholinos, however, as there is a considerable amount of maternal Igf2bp3 protein, which is organised into the Balbiani body during oogenesis, it is likely that the maternal protein is essential for development, which would not be affected with morpholinos. The use of morpholinos in this instance would also not provide robust data as the mutant phenotype was, prior to this study, not established (R Stainier *et al.*, 2017).

Injecting antibodies raised against Igf2bp3 into embryos to block maternal Igf2bp3 protein would be an alternative, however, most antibodies raised against Igf2bp3 are not based on the zebrafish homolog and we have not had success with using the commercial Igf2bp3 antibody with immunofluorescence, and results from this are unlikely to be conclusive, especially if Igf2bp3 may be acting during early oogenesis.

One observation made with the use of genetic mutants to study loss-of-gene function is that genetic compensation arises from similar genes via nonsense mediated decay (El-Brolosy *et al.*, 2019; Ma *et al.*, 2019). In our transgenic insertion mutants, we have observed a strong decrease in transcript of over 95%, suggesting that this allele blocks transcription rather than blocking splicing, and is hypomorphic such that gene expression is below the detection range of Western blots. Measuring the gene expression of other *igf2bp* genes, especially *igf2bp1*, did not reveal any strong expression of other genes, which would support this hypothesis. However, one discrepancy between our Cas9 and transgenic insertion alleles seems to be in our sex ratios of the zygotic mutants, where our Cas9 allele shows a heavy male sex-bias in the zygotic mutants, which is not observed in our transgenic insertion allele. This is unexpected, as it would be expected that the Cas9 allele has the greater chance of inducing genetic compensation, however, one explanation could be that as the alleles were generated in different strains, the genetic background of these alleles could play a role in influencing sex determination.

In the future, should the phenotypes of these alleles be incomparable in the maternal-zygotic mutants, a full-locus deletion of *igf2bp3* to complete remove transcript and protein could be considered. A GFP-tagged transgene for *igf2bp3* can also be used to confirm whether this can rescue the PGC defect, providing further evidence that our phenotypes are due to genuine deficiencies in Igf2bp3, and also provide spatiotemporal information as to where Igf2bp3 is produced *in vivo.*

5.3. Igf2bp3 is required to ensure timely progression through blastula and restrict expansion of the YSL

The first ten cell divisions in the zebrafish embryo occurs in a rapidly and synchronous manner until the midblastula transition (Kane and Kimmel, 1993). During the MBT, introduction of gap phases and asynchrony in cell divisions marks the beginning of major expression of zygotic genes and further degradation of maternal products (Tadros and Lipshitz, 2009).

Recently, m6A readers have previously been shown to be required for timely development during late blastula by ensuring rapid clearance of m6A-modified maternal RNAs (Zhao *et al.*, 2017), and, in the absence of an apparent phenotype in the *igf2bp3*$^{-/-}$ mutants, we followed maternal *igf2bp3*$^{-/-}$ embryos through this period and found these mutants were similarly delayed, suggesting that the cause for this delay is shared between the two genes: delayed degradation of maternal RNAs, hypothesised in Figure 5-1.

Our observations in the *igf2bp3*$^{-/-}$ embryos show that they are transiently delayed for an hour and the YSL appears to be expanded, as shown by immunostaining. We have yet to further explore the basis of this phenotype, and many factors have been shown to affect timely progression of embryos. During the midblastula transition, the length of the cell cycle is linked to the ratio of nucleus to cytoplasm (Kane and Kimmel, 1993). Defects in structural organisation of the yolk cells and microtubules can also lead to a delay during this period (Du *et al.*, 2012), and the embryonic delay of the *igf2bp3*$^{-/-}$ mutants are somewhat reminiscent of the MZ*eomesa* mutants, in which doming is delayed by approximately one hour (Du *et al.*, 2012). In addition embryonic development post-MBT being controlled by clearance of maternal RNAs, activation of the zygotic transcription program is also required to continue development, and loss of zygotic transcription factors are known to cause arrests in development, such as *nanog, sox19b* and *pou5f3* (Pálfy *et al.*, 2019).

An embryonic delay is also linked to a phenotype in the YSL, and *nanog* induces the expression of *mxtx2*, which in turn induces the expression of YSL genes (C. Xu *et al.*, 2012). The YSL acts as a source for expression of Nodal signals such as *sqt* (Erter, Solnica-Krezel and Wright, 1998; Feldman *et al.*, 1998; C. Xu *et al.*, 2012) and overexpression or

depression of *sqt* RNA causes an arrest in gastrulation and expansion of the YSL (Hagos and Dougan, 2007; Kumari *et al.*, 2013). Furthermore, as the YSL generates mechanical forces (Trinkaus, 1993; Solnica-Krezel and Driever, 1994), expansion or inhibition of YSL formation reduces the rate of epiboly progression and also delays embryogenesis (Chen and Kimelman, 2000).

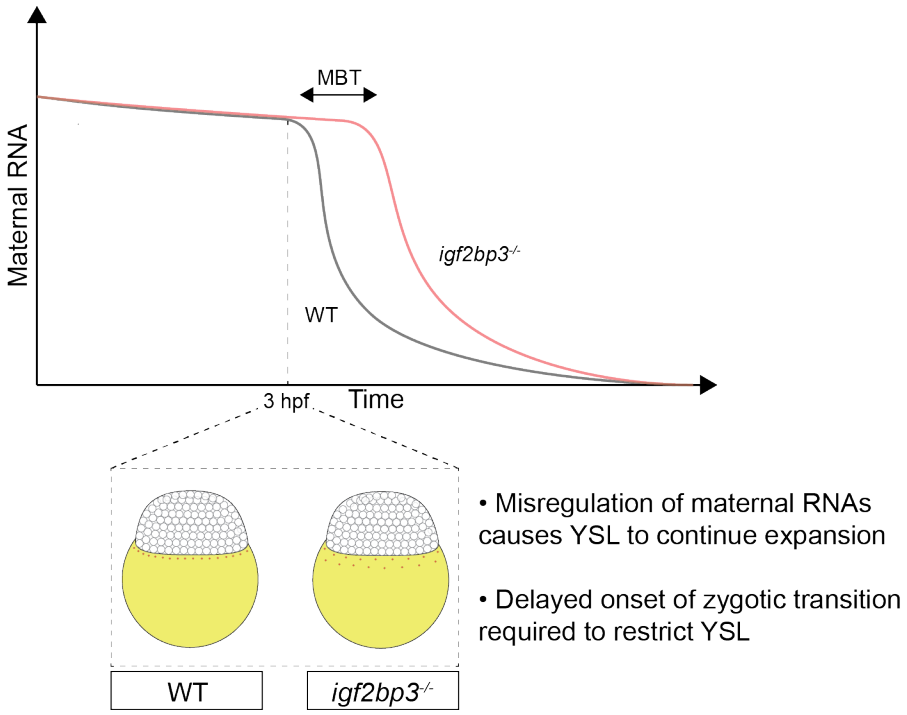

Figure 5-1. Hypothesis for *igf2bp3* for timely gastrulation and restriction of the yolk syncytial layer. Maternal Igf2bp3 degrades maternal RNAs prior to the midblastula transition, causing a delay in embryonic progression as zygotic activation is delayed (upper), causing misregulation of genes required for expansion and restriction of the YSL (below).

5.4. Igf2bp3 is required for germline development in early zebrafish embryogenesis

5.4.1. Igf2bp3 is required for correct localisation and migration of PGCs during germline development

Over the course of characterising the mutants for *igf2bp3*, we have found a role for Igf2bp3 to regulate the germline and produce the PGCs, which are progenitors of the gametes during early development. As the aggregation of the germplasm to the cleavage furrows were not affected in our 4-cell and 1k-cell stage embryos, germline defects do not seem to be associated with recruitment or assembly of germplasm, such as in the *kif5ba* (Campbell *et al.*, 2015) or *tdrd6a* (Roovers *et al.*, 2018) mutants.

While examining the PGCs using the expression marker *ddx4*, the earliest instance of a PGC defect was observed at Shield, where PGCs are found mislocalised at the embryonic dome, this appears to resemble the *cftr* (*cystic fibrosis transmembrane receptor*) mutant previously shown to cause PGC mislocalisation (Liao *et al.*, 2018), suggesting a motility/migration defect, summarised in Figure 5-2.

Many factors can influence the migration and motility of the zebrafish PGCs, which can be cell-autonomous or non cell-autonomous effects. As migration of the PGCs has a strong chemotactic component, we tested the expression levels of the main signalling ligand *cxcl12a* and their corresponding receptors *cxcr4a* and *cxcr4b*, as interruptions with *cxcl12-cxcr4* causes strong phenotypes in PGC migration (Doitsidou *et al.*, 2002; Knaut *et al.*, 2003; Takeuchi *et al.*, 2010; Boldajipour *et al.*, 2011). The level of *cxcl12a* remained comparable to WT levels at three out of the four stages tested and the expression of the chemokine receptors were comparable at all stages. Together with the gross morphology of the mutants, this could be expected as the *cxcl12-cxcr4* signalling axis is utilised by both mesodermal and endodermal cells during gastrulation (Mizoguchi *et al.*, 2008), and downregulation of this signalling axis leads to endoderm and mesoderm defects, such as absent organ development or muscular and circulatory defects (Chong *et al.*, 2007; Valentin, Haas and Gilmour, 2007; Mizoguchi *et al.*, 2008; Siekmann *et al.*, 2009). However, the relatively unperturbed expression of the *cxcl12-cxcr4* signalling axis would not be concordant with the observations in the *cftr* mutants, where upregulation of these genes were found (Liao *et al.*, 2018).

Even though the migration of the PGCs utilises chemotactic cues generated by the soma, it must also be considered that a functional or otherwise intact chemokine environment does not preclude issues with signal reception or transduction, and some germplasm genes are required for the motility (Blaser, 2005) or polarity (Tarbashevich *et al.*, 2015) of PGCs. In the PGCs, polarity is generated in response to the Cxcl12 chemokine gradient that directs cell movement, and polarity of the PGCs is an autonomous effect that is controlled by an increase of pH by the activity of the germplasm gene *ca15b* (*carbonic anhydrase*) (Tarbashevich *et al.*, 2015). The induction of polarity in the PGCs causes an increase in F-actin, and importantly, an increase of the Rho-GTPase Rac1 towards the chemokine gradient that is essential for cell polarity (H. Xu *et al.*, 2012; Tarbashevich *et al.*, 2015). This polarised behaviour is not limited to intracellular signalling, but also leads to an increased frequency of filopodia towards the signalling source (Meyen *et al.*, 2015), suggesting that the cell membrane plays a role in PGC migration, likely by hosting transmembrane proteins that are required for receiving and transducing signals. Indeed, inhibition of HMGCoA reductase activity, which generates prenylation precursors for farnesylation and geranylgeranylation, also results in migration defects (Thorpe *et al.*, 2004). Unsurprisingly, Rac1 is a target for

such modifications (Kinsella, Erdman and Maltese$, 1991) and it is possible that other transmembrane chemokine receptors aside from Cxcr4a/Cxcr4b are also receptive to Cxcl12a signalling, as *cxcl12a* knockdown can generate more severe phenotypes than knockdown/knockout of *cxcr4b* (Miyasaka, Knaut and Yoshihara, 2007) and further receptors likely exist to modulate Cxcl12a, such as the receptor Cxcr7, which acts as a decoy receptor (Naumann *et al.*, 2010).

To explore the migration defect in the *igf2bp3*$^{-/-}$ PGCs, we used live fluorescent reporters to mark the cytoplasm and cell membranes of the PGCs separately to observe their migration behaviour and filopodia dynamics. Analysis of the migration tracks shows that actively migrating PGCs that move towards the midline in both WT and mutant embryos do so without any apparent restriction, however, we seem to observe a small population of PGCs in the *igf2bp3*$^{-/-}$ embryos that do not migrate with this correct dynamic, and move in apparently random motions. As these PGCs do not appear to reach the midline within the span of our imaging, it is possible that these are the source for the ectopic PGCs we observe in the 24 hpf embryos, although it is unclear why this variation in the PGC population exists, and it could be linked to the mislocalised PGCs we observe by Shield. To test this, a transgenic line that marks the PGCs from their initial moment of specification through to gastrulation could be used in the future to determine the source and destination of ectopic PGCs in the *igf2bp3*$^{-/-}$ mutants.

We have also assessed the formation of the filopodia in the PGCs to determine if the dynamics could point to a defect in sensing chemokine signalling, and could not find any obvious deficiencies in the frequency, persistence, and length of the filopodia, suggesting that, on a superficial level, mismigration and depletion does not appear to be correlated to cellular protrusions, and the ability of the PGCs to sense chemokines using cellular projections is not affected. However, we have not looked at other factors that are related to PGC migration, such as F-actin distribution, intracellular pH gradient inside the cell, or intracellular signalling components.

Therefore, it is yet unclear how *igf2bp3* acts to regulate the migration of the PGCs, and there are many factors that remain yet unexplored that are unconnected to chemokine sensing or signal transduction, for example, depletion of the chaperone Hsp90 compromises the cell cycle in the PGCs, also leading to a mismigration defect (Pfeiffer *et al.*, 2018), and, disruption of other RNA-binding proteins such as Stau1 and Stau2 can also cause similar defects in PGC migration and survival (Ramasamy *et al.*, 2006)

5.4.2. Igf2bp3 is required for survival of PGCs in germline development

In addition to an early mislocalisation phenotype, we have observed further aberrant phenotypes in the germline, namely an increased dispersion and depletion of the PGCs that is apparent up to 48 hpf. As the strongest phenotypes can be produced in the maternal mutants, which is comparable to the maternal-zygotic mutant, this suggests that Igf2bp3 is likely acting on a maternal component such as germplasm.

Therefore, as germplasm is essential for the development and maintenance of the zebrafish germline, we considered that Igf2bp3 must impart some form of stabilising effect to germplasm, more specifically, its RNA components, leading to a cell-autonomous defect, such as apoptotic behaviours or failure to proliferate. Using live imaging of fluorescent reporters, we have observed some instances of apoptosis as PGCs migrate towards their destination in the presumptive gonadal ridge, however, we have only collected very few movies of this. One reason for this could be that the PGCs are already quite depleted by the end of gastrulation, and therefore, most apoptotic behaviour (if this is the case) has already occurred. A second possibility that results in a depletion of PGCs is failure to proliferate, as with apoptosis, this is difficult to assess without a transgene as capped mRNA only begins to show good resolution of the PGCs from mid gastrula onwards, making it difficult to determine whether the failure to proliferate occurs before this point. With the live-imaging of PGCs at Bud, we did observe proliferative activity of PGCs in the *igf2bp3*$^{-/-}$ embryos, suggesting that the loss of PGCs is not due to an absolute failure to proliferate, but we cannot yet rule out whether the loss of PGCs is due to a reduction in the rate of proliferation.

5.4.3. Some germplasm components are targets of Igf2bp3 regulation

In species that use the preformative model of germline specification, germplasm is an indispensable component for the formation of the germline (Hashimoto *et al.*, 2004) and we investigated a putative role for Igf2bp3 to regulate the RNA components of the germplasm by performing qPCRs against germplasm genes such as *ddx4*, *dazl*, *tdrd1* and *tdrd7*.

In our results, we reported a consistent downregulation of the germplasm gene *ddx4*, which is apparent as early as 1-cell, and further genes are regulated by 24 hpf. In our other germplasm genes, we did not detect any significant downregulation until 24 hpf, at which point *dazl* and *tdrd1* are also downregulated. These results are contrary to our expectations, as one should expect a loss of PGCs to lead to a global loss of all germplasm marker if this were the limiting factor, that some germplasm markers remain at WT levels suggests that the germplasm itself is largely intact and the lack of PGCs is due to failure to proliferate than limitations due to death or having sufficient germplasm to maintain the fate of PGCs.

The loss of *ddx4*, *tdrd1* and *dazl* then, may explain the disruption of the germline and the subsequent male sex-bias that we have observed in our maternal-zygotic mutants. Ddx4

(previously Vasa) is an DEAD box ATP-dependent RNA-helicase (Hay, Jan and Jan, 1988; Lasko and Ashburner, 1988) and its expression in the gonads is highly conserved in evolution (Fujiwara *et al.*, 1994; Komiya and Tanigawa, 1995; Gruidl *et al.*, 1996; Ikenishi and Tanaka, 1997; Olsen, Aasland and Fjose, 1997; Yoon *et al.*, 1997; Tsunekawa *et al.*, 2000). Ddx4 is required for germ-line differentiation into the gonads, and mutants for Ddx4 manifests in fertility defects (Styhler *et al.*, 1998; Tanaka *et al.*, 2000; Spike *et al.*, 2008; Hartung, Forbes and Marlow, 2014). In zebrafish, *ddx4* mutants develop into sterile males but does not appear to be required for initial formation of the germline (Braat *et al.*, 2001; Hartung, Forbes and Marlow, 2014), although *ddx4* may be required for PGC migration in other teleosts (M. Li *et al.*, 2009). Therefore, it is difficult to conclude whether reduction of *ddx4* in the *igf2bp3*$^{-/-}$ mutants is the cause of the germline defect. This is because we have not assessed the levels of maternal Ddx4 protein, which is stable in zygotic *ddx4* mutants up to 10 dpf (Hartung, Forbes and Marlow, 2014), although blocking maternal Ddx4 protein by injecting antibodies raised against Ddx4 appears to inhibit PGC formation (Ikenishi and Tanaka, 1997). Furthermore, as knockdown of *ddx4* does not seem to affect the initial formation of the germline (Braat *et al.*, 2001), the depletion phenotype would not be adequately addressed in the *igf2bp3*$^{-/-}$ mutant. Whether the loss of *ddx4* in the *igf2bp3*$^{-/-}$ mutants plays a role in the sex bias we have observed in the maternal-zygotic mutants is unclear, as these mutants are not sterile and haploinsufficiency of *ddx4* does not appear to influence sex determination (Hartung, Forbes and Marlow, 2014).

Another germplasm gene that is regulated in our *igf2bp3*$^{-/-}$ mutants is *tdrd1*. Tdrd1 belongs to the Tudor-domain containing family of proteins (CALLEBAUT and MORNON, 1997; Ponting, 1997), which interacts with other proteins by binding to methylated residues such as arginine and lysine (Brahms *et al.*, 2001; Friesen *et al.*, 2001; Sattler *et al.*, 2001). The Tudor domain also plays a role in RNA metabolism, for example, the Tudor domain in Smn mediates splicing by recruiting spliceosomal proteins (Fischer, Liu and Dreyfuss, 1997; Pellizzoni *et al.*, 1998), many Tudor-domain containing proteins are localised to stress granules during stress, such as Tdrd3 and Tudor-SN (Goulet *et al.*, 2008; Linder *et al.*, 2008; Gao *et al.*, 2010), which interacts with translational regulators such as FMRP (Linder *et al.*, 2008). The Tudor domain is also involved in small RNA processing, and the previously mentioned Tudor and nuclease domain containing Tudor-SN is a conserved component of the RISC (Caudy *et al.*, 2003). Many components of the piRNA pathway, such as the Piwi proteins, have methylated arginines (Chen *et al.*, 2009; Kirino *et al.*, 2009; Nishida *et al.*, 2009), acting as targets for recruitment by Tudor (Vagin *et al.*, 2009; H. Liu *et al.*, 2010; K. Liu *et al.*, 2010). The piRNA pathway plays a role in regulating the germline against transposable elements, and many Tudor-domain containing proteins are subsequently found in germline granules such as nuage and germplasm, where Tudor proteins are essential to assemble these complexes and produce piRNAs (Boswell and Mahowald, 1985; Anne, 2010; Liu *et al.*, 2011; Anand and Kai, 2012). Therefore, Tdrd1 is essential for germline

maintenance and interacts with Piwi proteins (Wang *et al.*, 2009; Huang *et al.*, 2011), and mutants in Tdrd1 show reductions in nuage formation and defects in the germ cells (Chuma *et al.*, 2006; Huang *et al.*, 2011). In zebrafish, the role of *tdrd1* in establishment of the germline is unclear, as although *tdrd1* is expressed in the PGCs, Tdrd1 protein is only detectable from 4 dpf (Huang *et al.*, 2011), so it is unlikely that downregulation of *tdrd1* would affect the PGCs in the 24 hpf embryos we have studied.

Our third downregulated germplasm gene is *dazl*, a member of the DAZ repeat containing family of proteins (Reijo *et al.*, 1995), which includes other proteins such as Daz and Boule (Xu, Moore and Pera, 2001). Originally identified from genetic screens in men with infertility (Reijo *et al.*, 1995, 1996), DAZ family proteins have been established to be essential for germline development, with mutations producing defects in the gametes (Eberhart, Maines and Wasserman, 1996; Shan *et al.*, 1996; Ruggiu *et al.*, 1997; Kadyk *et al.*, 1998; Mita and Yamashita, 2000; Stone *et al.*, 2009) at the meiotic or post-meiotic stage, and mutants in the DAZ family genes produces germ cells that fail to transition into the meiotic phase and overexpression of DAZ genes can rescue meiotic defects (Rilianawati *et al.*, 2003; Xu *et al.*, 2003; Lin *et al.*, 2008; VanGompel and Xu, 2010). As DAZ family proteins also contain an RNA recognition motif, they are RNA-binding proteins and regulate the germ line by interacting with the 3' UTR of its target transcripts and act as translational activators (Sousa Martins *et al.*, 2016; Li *et al.*, 2019), repressor and stabiliser (Fukuda *et al.*, 2018; Zagore *et al.*, 2018; Yang *et al.*, 2019). The role of *dazl* in zebrafish development is not well studied, however, similar studies in medaka have shown that knockdown of *dazl* does not reduce Dazl levels due to the abundance of maternal protein and does not appear to affect PGC formation (Li *et al.*, 2016), but injection of an antibody raised against Dazl depletes PGC formation that blocks Dazl function leads to a depletion of the PGCs (Li *et al.*, 2016).

Together, we have shown that Igf2bp3 regulates germplasm RNAs that are essential for development for the germline. However, the connection between reduction of the RNA and the defects in the germline is unclear, as the levels of the protein counterparts are not known in the *igf2bp3*$^{-/-}$ mutants. Further analysis in the germline phenotype will uncover any bias and specificity of the regulation of germplasm in Igf2bp3, and further likely candidates that are essential for early establishment, maintenance and motility of the germline include *nanos1, nanos3, and dnd1*.

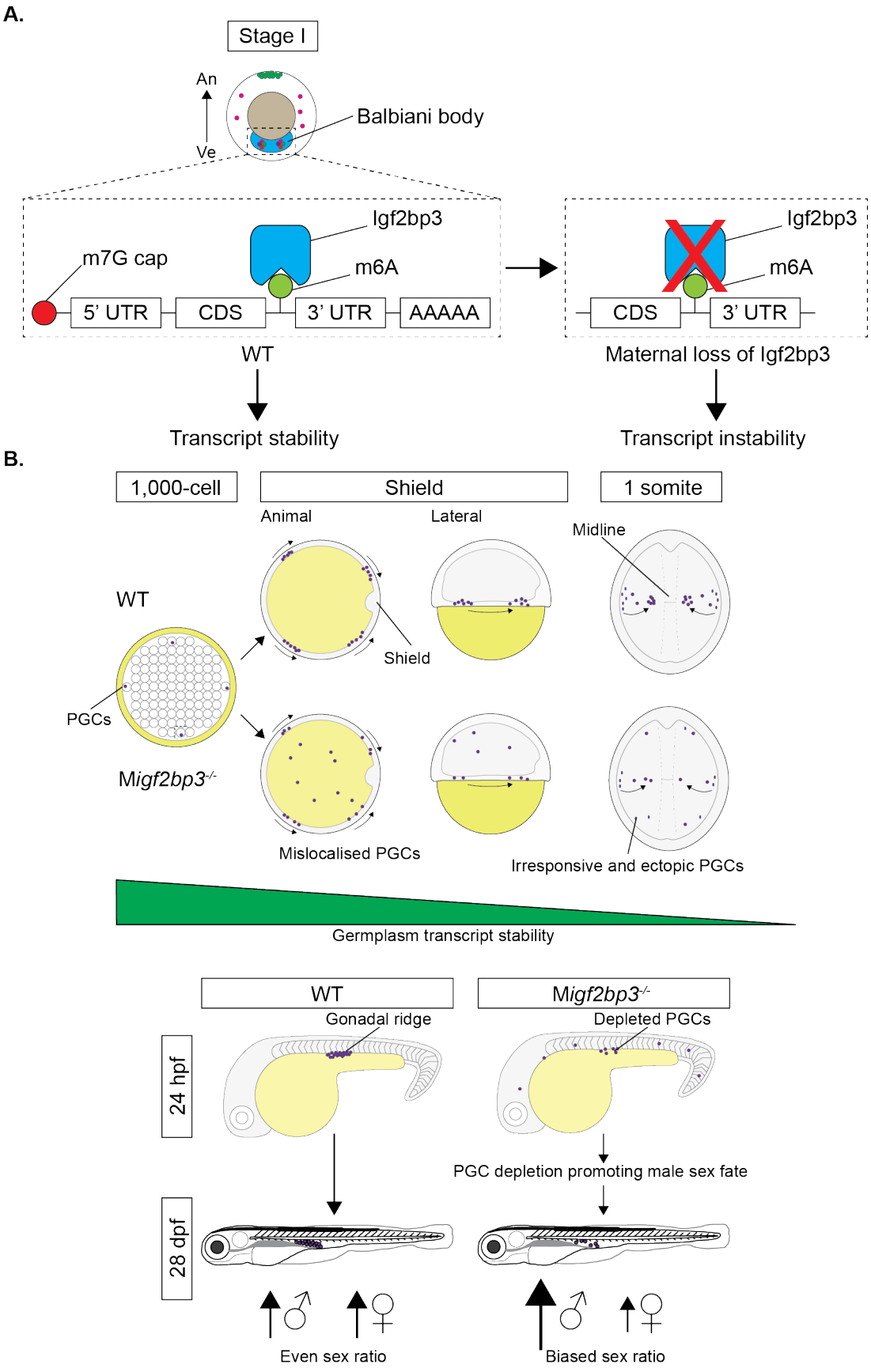

Figure 5-2. Hypothesis for requirement of Igf2bp3 in germline maintenance. A. Igf2bp3 begins germline regulation by regulating germplasm. Maternal Igf2bp3 is likely to act on

germplasm during oogenesis. As Igf2bp3 and germplasm are aggregated together during oogenesis, it is likely to be bound in these mRNPs prior to its dispersal, possibly by binding to m6A modifications in RNAs. **B. Germplasm RNA instability over time causes a depletion of PGCs.** As germplasm RNAs are more severely affected at 24 hpf, germplasms are likely being destabilised over time, causing a depletion of the PGCs and promoting the male sex fate.

5.4.4. Igf2bp3 as an RNA-binding protein with m6A-interacting specificity

More recently, many previous identified RNA-binding proteins that have roles in development have now been shown to bind to chemical modifications in RNA, such as N6-methyladenosine, and can typically either directly modify RNA to methylate adenosines (writers), recognise and interact with these modifications (readers), or erase these modifications (erasers). N6-methyladenosine modifications are found in over 36% of maternal genes and 40% of mRNA transcripts in early development (Zhao *et al.*, 2017).

These proteins include WTAP (WT1 associated protein), initially identified as an oncogene (Little, Hastie and Davies, 2000) but now known to be a regulatory subunit of the m6A methyltransferase complex (Liu *et al.*, 2014; Ping *et al.*, 2014). Bgcn (Benign gonial cell neoplasm), a tumour suppressor identified in *Drosophila* to be required for differentiation of germ cells (Gateff, 1982), closely related to the m6A reading protein Ythdc2 in mice (Hsu *et al.*, 2017; Soh *et al.*, 2017; Jain *et al.*, 2018). Another protein, FTO (Fat mass and obesity associated), is correlated to metabolic disorders such as diabetes (Frayling *et al.*, 2007), is now known as an m6A eraser (Gerken *et al.*, 2007; Meyer *et al.*, 2012)

The role of Igf2bp3 as an m6A reader is (Huang *et al.*, 2018) supports our findings that it is required for germline development, as mutations in the m6A writers and readers leads to germline defects, suggesting a conserved role for m6A in regulation of the germline, we curated previous published m6A-seq and m6A-CLIPseq data to determine whether germplasm genes were methylated published by (Zhao *et al.*, 2017), which would provide some mechanistic basis for these genes to be regulated by Igf2bp3. From the search, we have found *dazl*, *ddx4*, *tdrd1*, *tdrd7* to all be methylated during early development, we have also found *nos3* to be methylated whereas *nos1* is not conclusive, suggesting *nos3* could also be a target of Igf2bp3 regulation to be considered in future analysis.

5.4.5. Igf2bp3 as an RNA-binding protein in multiple mRNPs

A second possibility for Igf2bp3 in regulating germplasm RNAs is by interacting with structural elements such as a sequence or motif, and Igf2bp proteins have been shown to

bind to such elements such as the zipcode in the *actin* 3'UTR (Anthony F Ross *et al.*, 1997) or the *vg1* VLE (Git and Standart, 2002), whether such a shared sequence exists in germplasm RNAs that can be recognised by Igf2bp3 is unknown.

We identified Igf2bp3 originally in *sqt* mRNP complexes using *sqt* aptamer pulldowns, and the presence of Igf2bp3 in a complex with germplasm suggest that it is a component of several mRNP complexes, acting as a modular component. Our rationale for this is that previous works have shown that *sqt* mRNA is recognised by Ybx1 by a combination of both sequence and motif elements, termed the YBE (Y-box binding element) (Kumari *et al.*, 2013; Zaucker *et al.*, 2018) and experiments performed by Dr. Lavanya Sivashanmugam have shown that Igf2bp3 can be co-immunoprecipitated from Ybx1. Further unpublished data have shown that YBEs are found in other UTRs in the zebrafish transcriptome, and in some germplasm genes such as *tdrd1*, and Ybx1 also appears to contribute to germline development, shown in unpublished data by Dr. Andreas Zaucker.

5.5. Future directions and experiments

This study has reported two phenotypes linked to loss of maternal *igf2bp3* in zebrafish embryogenesis. A transient delay during gastrula, which appears to be linked to an expansion of the YSL, and a germline defect, characterised by mismigration and depletion of the PGCs.

In the future, dissection of these phenotypes to produce a molecular basis for these effects would provide further insight on the function of these proteins. The transient delay in gastrula appears to be reminiscent of two reported mutants in the literature, the MZ*eomesa* and M*ythdf2* mutants. However, more information to when the delay in these embryos occur and the cellular behaviour is needed, this can be achieved by the use of a transgenic nuclei marker or injection of a fluorescent dye can be done to view the YSL and to pinpoint the cell division which becomes arrested in the *igf2bp3*$^{-/-}$ embryos. Biochemical analysis on the m6A-levels in the embryo can also provide some explanation for the transient delay.

In our second phenotype, the defect in the germline is likely to be linked to m6A modifications in RNA that is appears to require Igf2bp3, and many lines of investigation are possible to produce data to strengthen this conclusion and answer remaining questions in this study.

Does Igf2bp3 interact with germplasm during its assembly?

Whilst we have an Igf2bp3 antibody that detects zebrafish Igf2bp3 in Western blots, it does not seem to have good reactivity for immunofluorescence or immunoprecipitation.

Furthermore, we have struggled to produce recombinant Igf2bp3 protein during this study, making *in vitro* analysis difficult.

Therefore, to initially establish a connection between germplasm and Igf2bp3 in zebrafish, the *Tg(buc: buc-egfp)* transgene, which tags the germplasm organiser Bucky ball (Riemer *et al.*, 2015) would be a useful tool. This transgene can be used to immunoprecipitate GFP and perform co-immunoprecipitation for Igf2bp3 to establish whether the localisation of Igf2bp3 in the Balbiani body is a true interaction with germplasm.

Is the interaction of Igf2bp3 and germplasm specific?

If a connection between Igf2bp3 and germplasm exists, the specificity of this interaction can be studied. Whilst it is possible to extract RNA from immunoprecipitation of the germplasm organiser to analyse gene expression, this would not adequately address the requirement of Igf2bp3.

An alternative approach would be to generate tagged constructs of Igf2bp3 (e.g. myc, FLAG, or GFP), inject these into WT embryos, and perform Western blots against the tag to confirm expression and immunoprecipitate these tags to capture the targets of Igf2bp3 regulation. As I have no information to the spatial localisation of Igf2bp3 protein, it is inferred that Igf2bp3 is likely ubiquitous due to the unrestricted expression of *igf2bp3*, so this approach would not be extremely beyond biological context. If relative enrichment of germplasm genes can be found in these immunoprecipitations, deletion constructs for the RNA-interaction domains to determine the requirement for these RNAs to be bound by Igf2bp3 could be made, and, if m6A is required for this interaction, then deletion of the N-terminal KH domains should abolish Igf2bp3 interactions with germplasm RNAs.

How does loss of Igf2bp3 affect the translation and stability of m6A-modified transcripts in vivo?

Within this study, we have shown that Igf2bp3 has a stabilising effect on some germplasm RNAs, however, whether Igf2b3 affects the translational stability of these transcripts, methylated or otherwise, is unclear.

To answer this question, capped mRNA fluorescent reporters of the PGCs could be made *in vitro* with and without m6A-modified adenosine ribonucleotides, and inject these into WT and *igf2bp3*$^{-/-}$ embryos to provide a proxy for translation efficiency with these modifications in the germline; and provide insights as to whether m6A modifications are functional in the context of mRNA dynamics.

5.6. The role of Igf2bp1 in development

In addition to generating and analysing the *igf2bp3* mutant, we also generated an *igf2bp1* mutant for preliminary analysis. We generated these mutants as we observed a biphasic expression of *igf2bp1* and *igf2bp3*, which is conserved in evolution (Nielsen *et al.*, 2000), suggesting the expression of these genes in development might serve a function. Secondly, as Igf2bp1 and Igf2bp3 are highly similar, they could act redundantly to compensate for each other; finally, genetic mutations in genes resulting in premature termination codons have been shown to cause upregulation of homologs with similar sequences (El-Brolosy *et al.*, 2019; Ma *et al.*, 2019), and we hypothesised that Igf2bp1 would be the source of any genetic compensation arising from the *igf2bp3* mutants.

Therefore, we produced *igf2bp1* mutants by creating indel lesions in exon 1 of the *igf2bp1* locus, and we were able to retrieve zygotic mutants to adulthood with no apparent difficulty. This is contrary to our expectations from the literature, as zygotic *igf2bp1* mutants in mice show a high level of perinatal mortality and numerous physiological phenotypes (Hansen *et al.*, 2004); further morphants generated in *igf2bp1* have also previously reported several neurological and gross morphological defects (Gaynes *et al.*, 2015), although the phenotype of this morphant has not been reproducible (Van Rensburg, 2014), suggesting some off-target or non-specific effects have been reported instead.

One explanation to why the phenotype observed in the zebrafish is not observed in mice could be that they perform separate functions in these models, another possibility is that there is genetic compensation or redundancy arising from *igf2bp3.* Nonetheless, as we were also able to raise maternal-zygotic *igf2bp1* mutants to 5 dpf with no obvious phenotype, we conclude that zygotic *igf2bp1* is dispensable for development.

Interestingly, when maternal-zygotic *igf2bp1* mutants were analysed for a PGC defect, we observed a slight reduction in the number of PGCs at a stage when we have confirmed Igf2bp1 protein to be expressed, suggesting that *igf2bp1* may also play a role in germline development, albeit a much milder one. It is also possible that a germline defect may manifest later in development in the maternal-zygotic mutants, although further analysis needs to be performed.

5.6.1. A redundant role for *igf2bp* genes in development

In the course of this study, we attempted to generate *igf2bp1; igf2bp3* double mutants by intercrossing adults heterozygous for *igf2bp1* in the homozygous *igf2bp3*$^{-659\ Tg}$ background, of which we could not retrieve double mutants for, the reasons for this are unclear, as zygotic and maternal-zygotic adults for both of these genes alone do not appear to produce

any phenotype. Whilst we did observe upregulation of *igf2bp2* in the *igf2bp3* mutants, we did not make mutants for these due to time constraints. However, it is interesting to note that *igf2bp2* mutants in mice do not produce an unconditional detrimental phenotype.

The phenotype we have observed in the *igf2bp1*$^{-/-}$*; igf2bp3*$^{-/-}$ larva is failure to inflate the swimbladder by 5 dpf, and these larvae presumably die due to failure to swim and feed. A small proportion of *igf2bp1*$^{-/-}$*; igf2bp3*$^{-/-}$ larvae do inflate the swimbladder, and as we haven't been able to retrieve these to adulthood, it's possible there are further disabilities in the double mutants that we have yet to characterise.

Failure to inflate the swimbladder is a common phenotype observed in many zebrafish genetic screens (Amsterdam *et al.*, 1999; McCune and Carlson, 2004), as misregulation of many genes can cause such a phenotype, and our investigation of the organs including the heart, liver, pancreas, swim bladder and gut did not reveal any discernable abnormalities in the overall shape or positioning, strengthening our previous data that these genes are not required for axis specification.

Finally, we quantified the PGCs in the progeny of the *igf2bp1*$^{+/-}$*; igf2bp3*$^{-659\ Tg}$ intercross, and found no differences in the number of PGCs between all three genotype combinations, suggesting that, either zygotic *igf2bp1* does not affect germline development by 24 hpf, or that *igf2bp1* and *igf2bp3* are regulating the same targets, since maternal-zygotic *igf2bp1* mutants have a mild reduction of PGCs.

5.7. Future implications of this study

The Igf2bp family of proteins have been studied in various contexts, *in vivo* and *in vitro*, and these studies have shown that these RNA-binding proteins have a range of specificities against many targets to direct cell behaviour and RNA metabolism, and the extent at which Igf2bp regulates the transcriptome has expanded with its discovery as an m6A reader.

Igf2bp proteins have been implicated in metabolic disorders such as diabetes (Rodriguez *et al.*, 2010) and carcinogenesis, by altering cellular behaviour, often resulting in poorer outcomes, such as aggressive behaviours and chemoresistance (Ellis *et al.*, 1998; Hsu *et al.*, 2015). As Igf2bp proteins as upregulated in these disorders and are otherwise basally expressed in adult tissues (Bell *et al.*, 2013), insights into these genes may provide potential as a future therapeutic target.

To date, the role of *igf2bp* genes in development has poorly characterised, and our use of genetic mutants to unravel the role of two *igf2bp* genes have shown a new role for these genes in regulating the germline, a phenotype that has previously not been shown in other

models and whether *igf2bp* genes also regulate the germline in other model organisms is a question that would be interesting to address in future studies.

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7. Supplementary

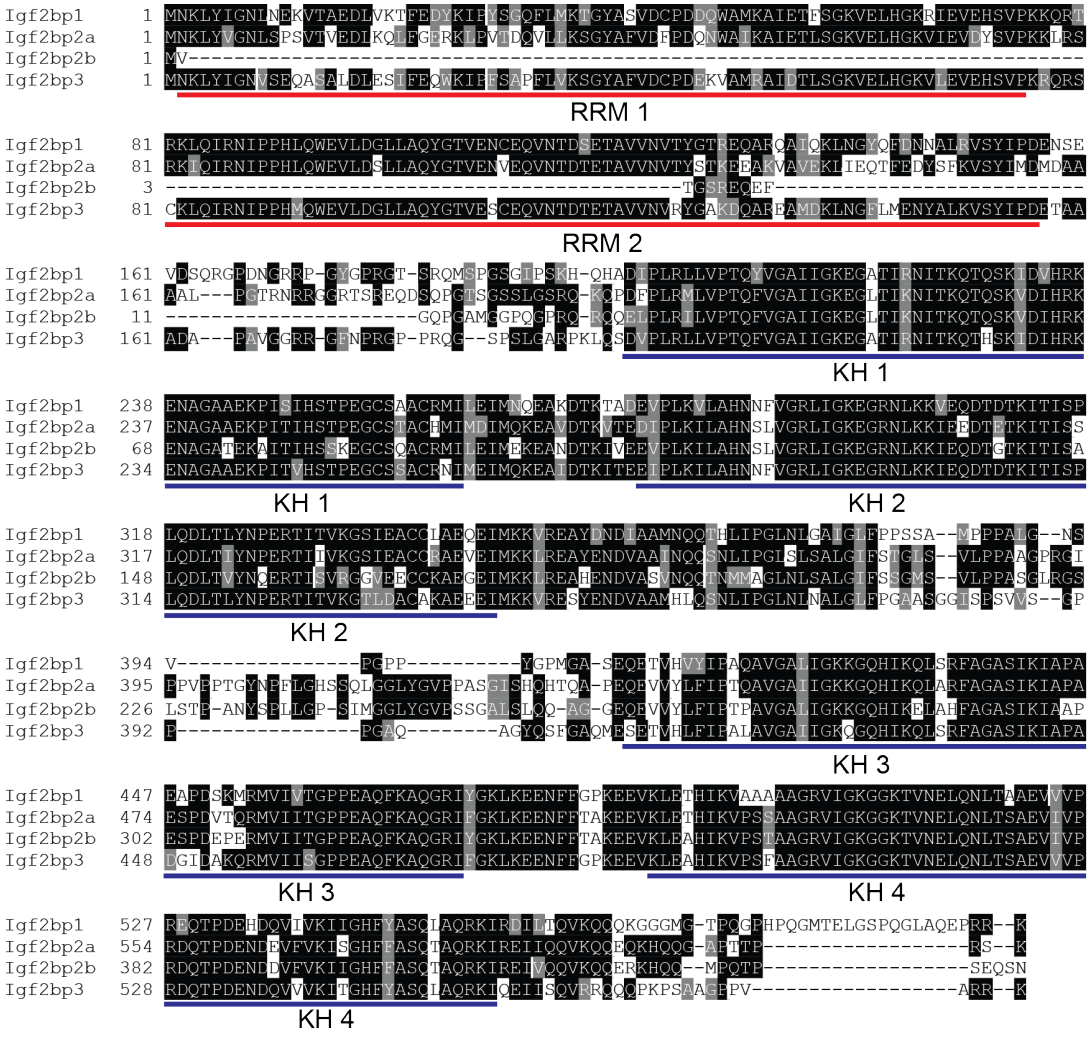

Figure 7-1. Alignment of zebrafish Igf2bp proteins. Zebrafish Igf2bp1, Igf2bp2a, Igf2bp2b and Igf2bp3 were aligned and their RNA recognition motifs (red) and K-homology domains (blue) highlighted.

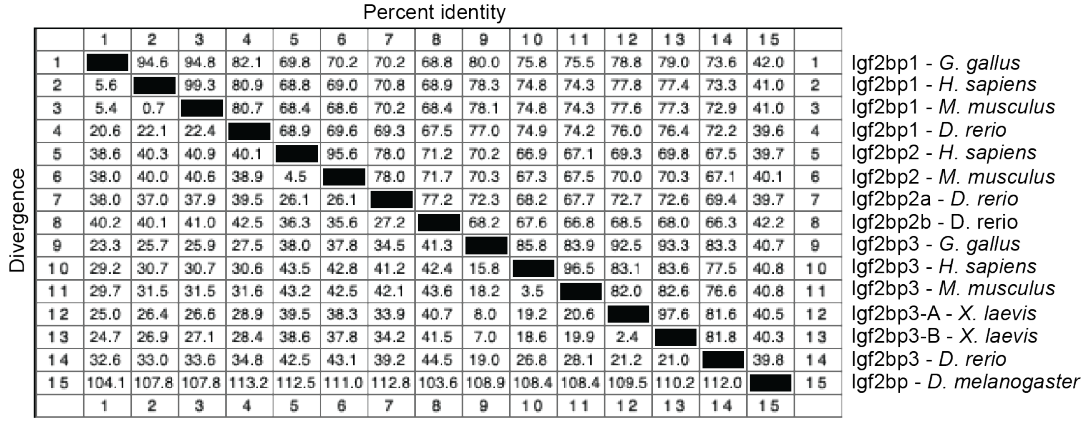

Figure 7-2. Sequence identity and divergence matrix of Igf2bp proteins. *G. gallus, H. sapiens, M. musculus, D. rerio, X. laevis* and *Drosophila* Igf2bp were aligned to produce an

alignment report. The following UniProt codes were used as follows (1-15): O42254, Q9NZI8, O88477, Q08CK7, Q9Y6M1, Q5SF07, A0A0B41K1B0, A0A0R4IVY2, Q5ZLP8, O00425, Q9CPN8, O73932, O57526, Q9PW80, M9NF14.

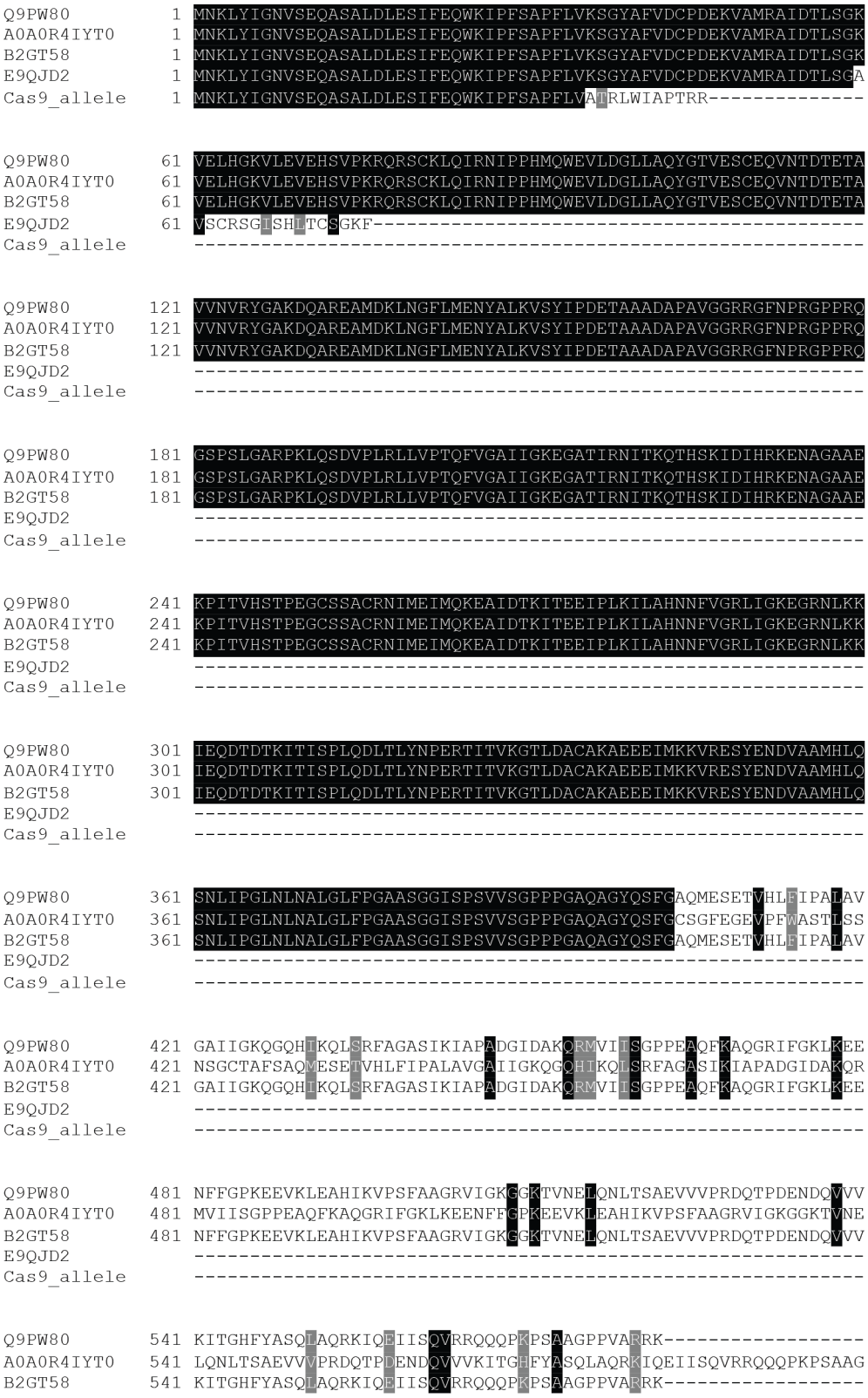

Figure 7-3. The *igf2bp3*$^{\Delta 7\ bp}$ CRISPR-Cas9 allele targets all isoforms of Igf2bp3 protein. All isoforms of Igf2bp3 were aligned against the predicted truncation product of the *igf2bp3*$^{\Delta 7\ bp}$ transcript (Cas9_allele).

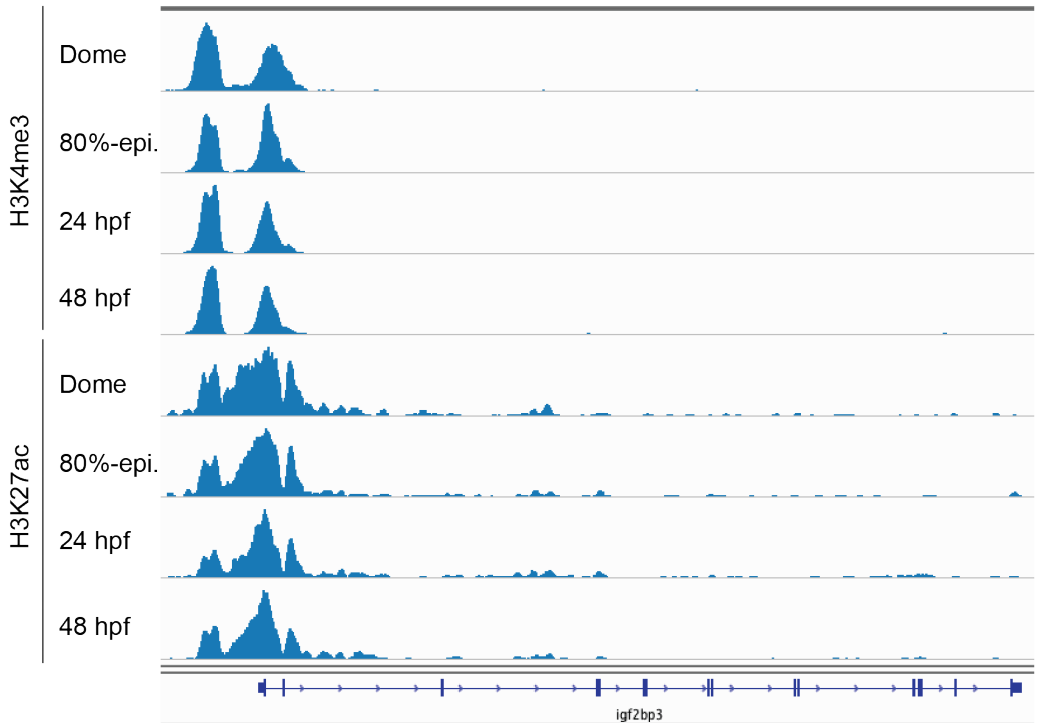

Figure 7-4. Curation of chromatin enhancer marks identifies only one 5' transcriptional start site for *igf2bp3*. Chromatin modifications linked to active transcription (H3K4me3 and H3K27ac) were curated to determine likely transcriptional start sites in *igf2bp3*. Data extracted from (Bogdanovic *et al.*, 2012).

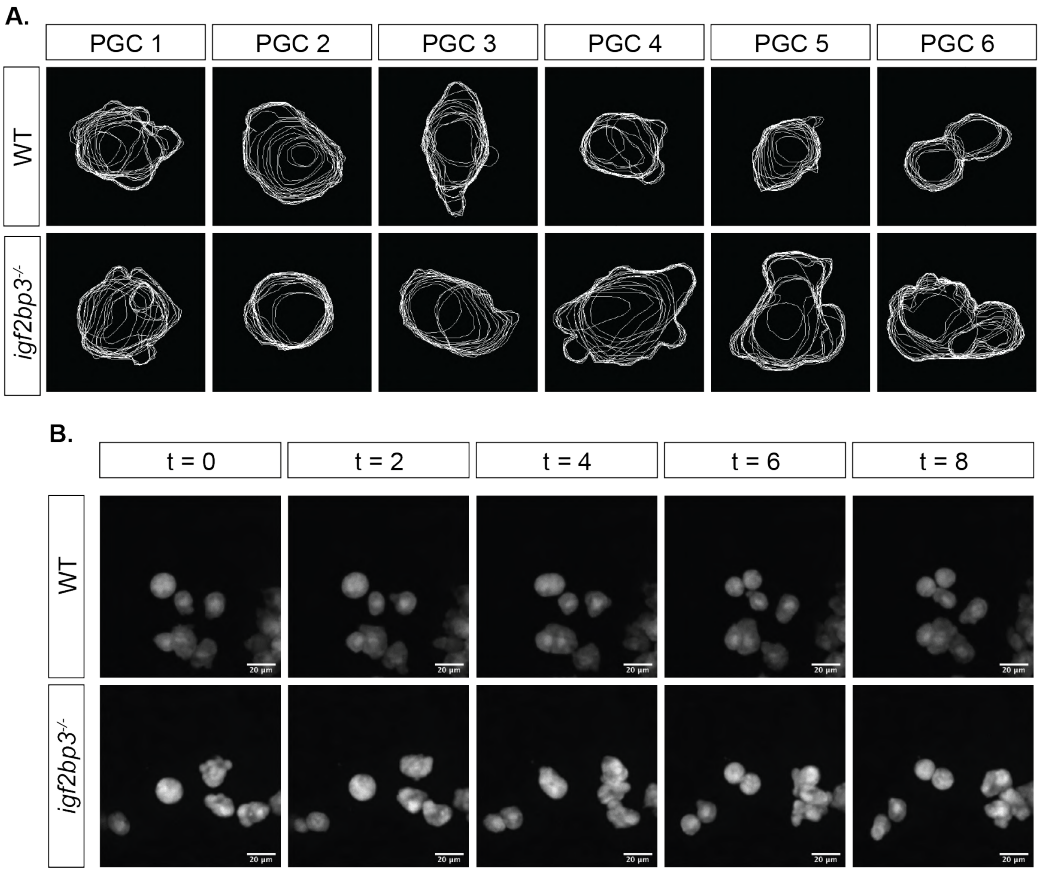

Figure 7-5. Cell morphology and division in WT and *igf2bp3*$^{-/-}$ PGCs. A. Outlines of WT and *igf2bp3*$^{-/-}$ PGCs. PGC morphology was extracted by tracing the outlines of cells labelled with a fluorescent membrane reporter. **B. PGCs in both WT and *igf2bp3*$^{-/-}$ embryos exhibit mitotic behaviour.** PGC tracks were analysed for instances of mitotic behaviour when labelled with a fluorescent cytoplasmic reporter. Scale bar = 10 μm.

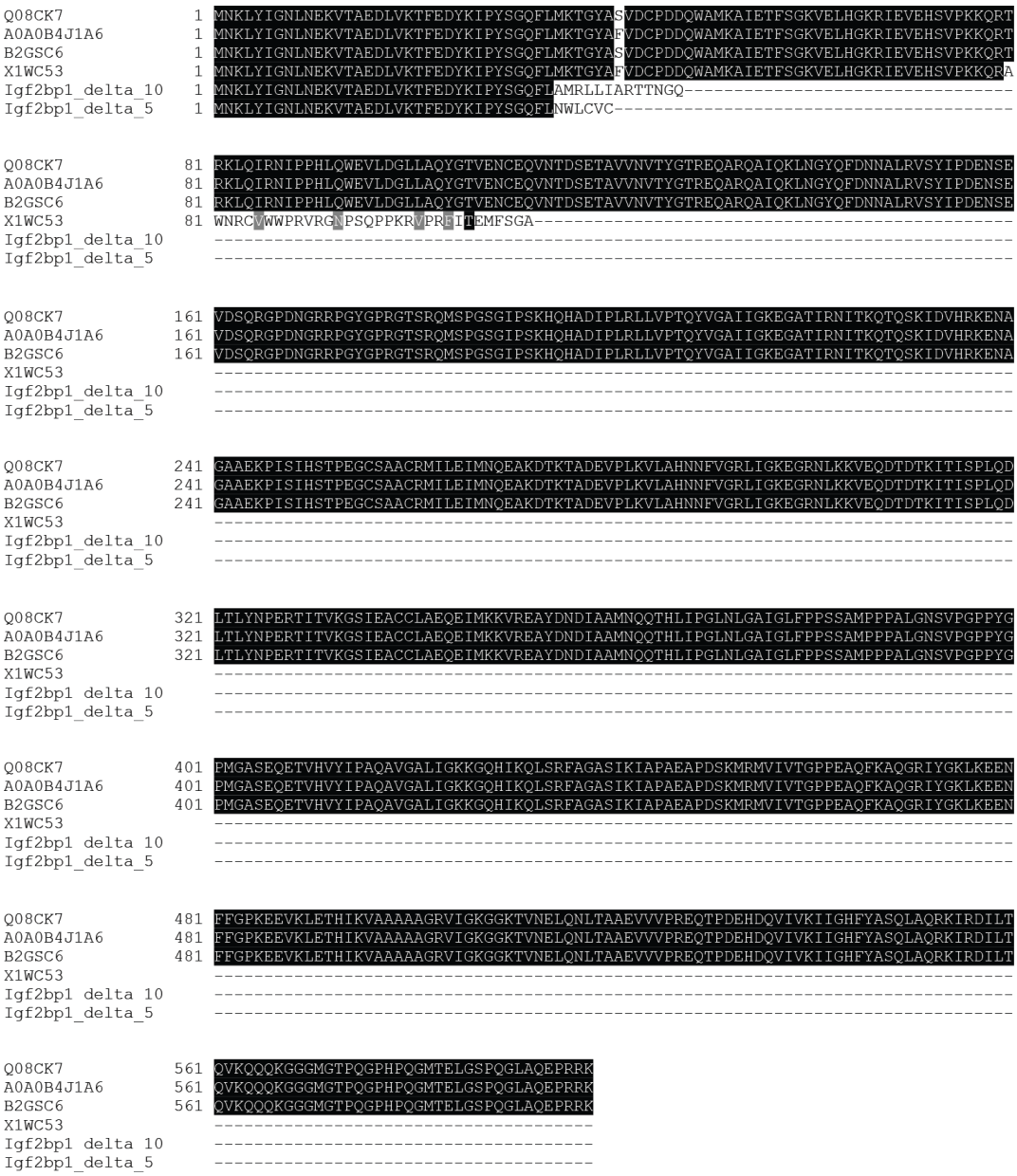

Figure 7-6. The *igf2bp1* indel alleles target all isoforms of Igf2bp1 protein. All isoforms of Igf2bp3 were aligned against the predicted truncation products of the *igf2bp3*$^{\Delta 10\ bp}$ and *igf2bp1*$^{\Delta\ 5\ bp}$ transcripts.

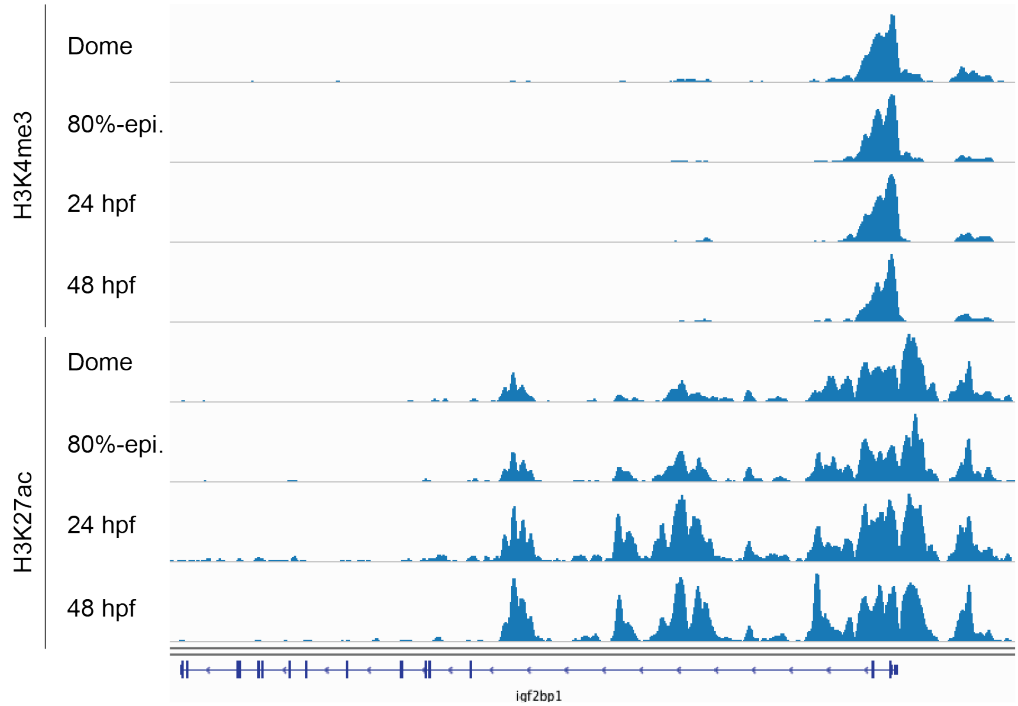

Figure 7-7. Curation of *igf2bp1* chromatin enhancer marks does not provide conclusive evidence of a single transcriptional start site. Chromatin modifications linked to active transcription (H3K4me3 and H3K27ac) were curated to determine likely transcriptional start sites in *igf2bp1*. Data extracted from (Bogdanovic *et al.*, 2012).

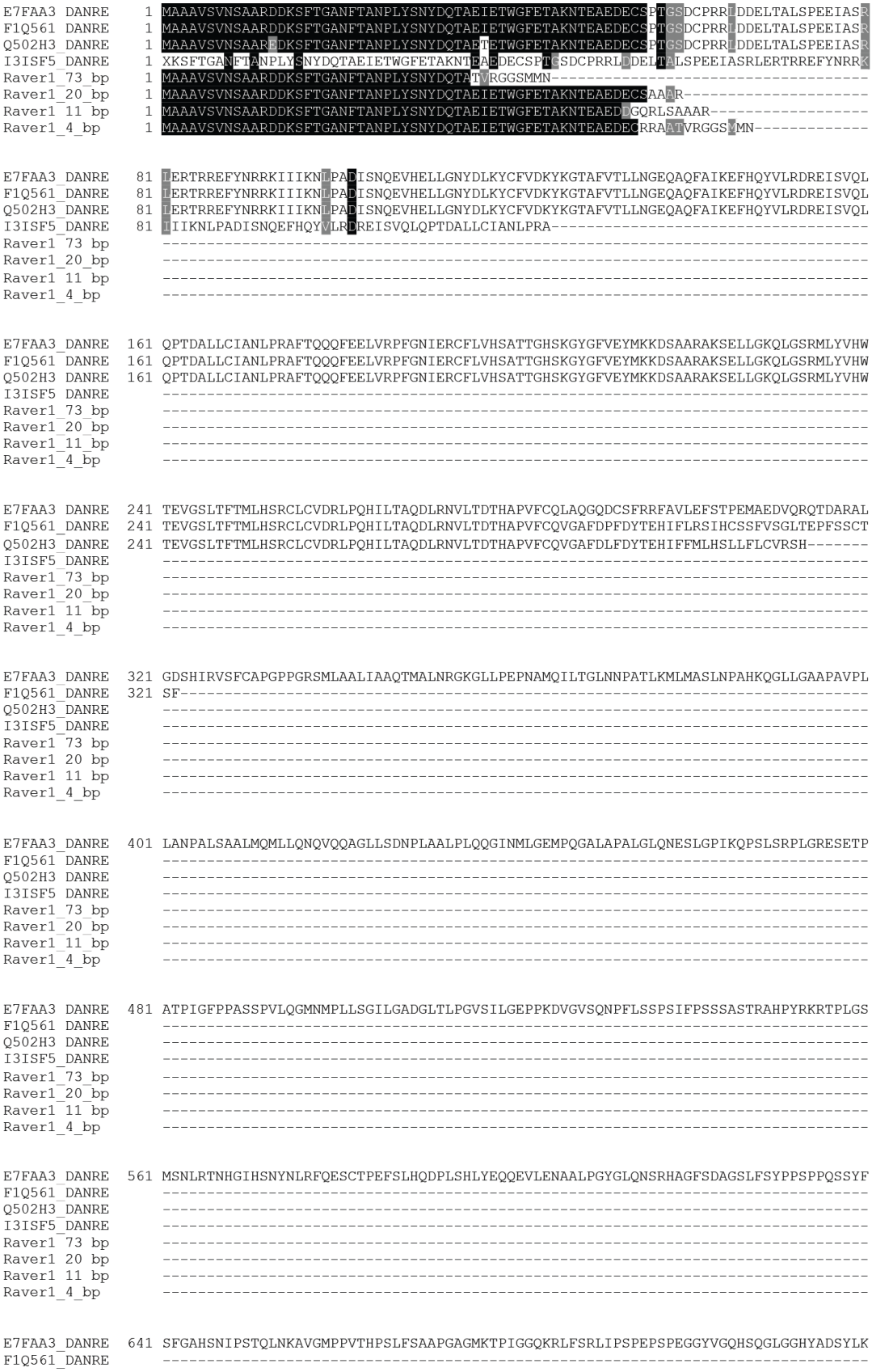

Figure 7-8. The *raver1* indel alleles target all isoforms of Raver1 protein. All isoforms of Raver1 were aligned against the predicted truncation products of the *raver1*$^{\Delta\ 73\ bp}$, *raver1*$^{\Delta\ 20\ bp}$, *raver1*$^{\Delta\ 11\ bp}$ and *raver1*$^{\Delta\ 4\ bp}$ transcripts.

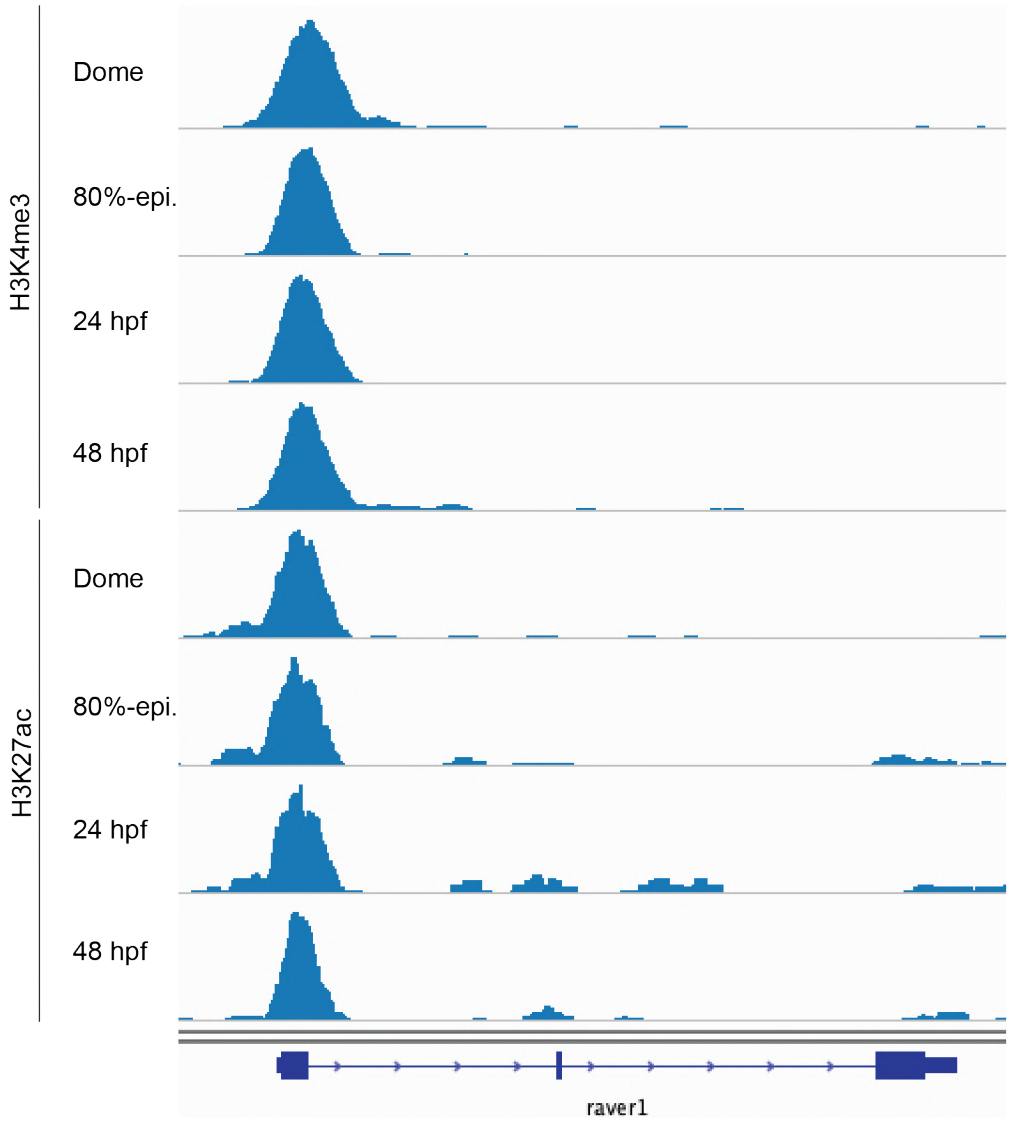

Figure 7-9. Curation of chromatin enhancer marks identifies only one 5' transcriptional start site for *raver1.* Chromatin modifications linked to active transcription (H3K4me3 and H3K27ac) were curated to determine likely transcriptional start sites in *raver1.* Data extracted from (Bogdanovic *et al.*, 2012).

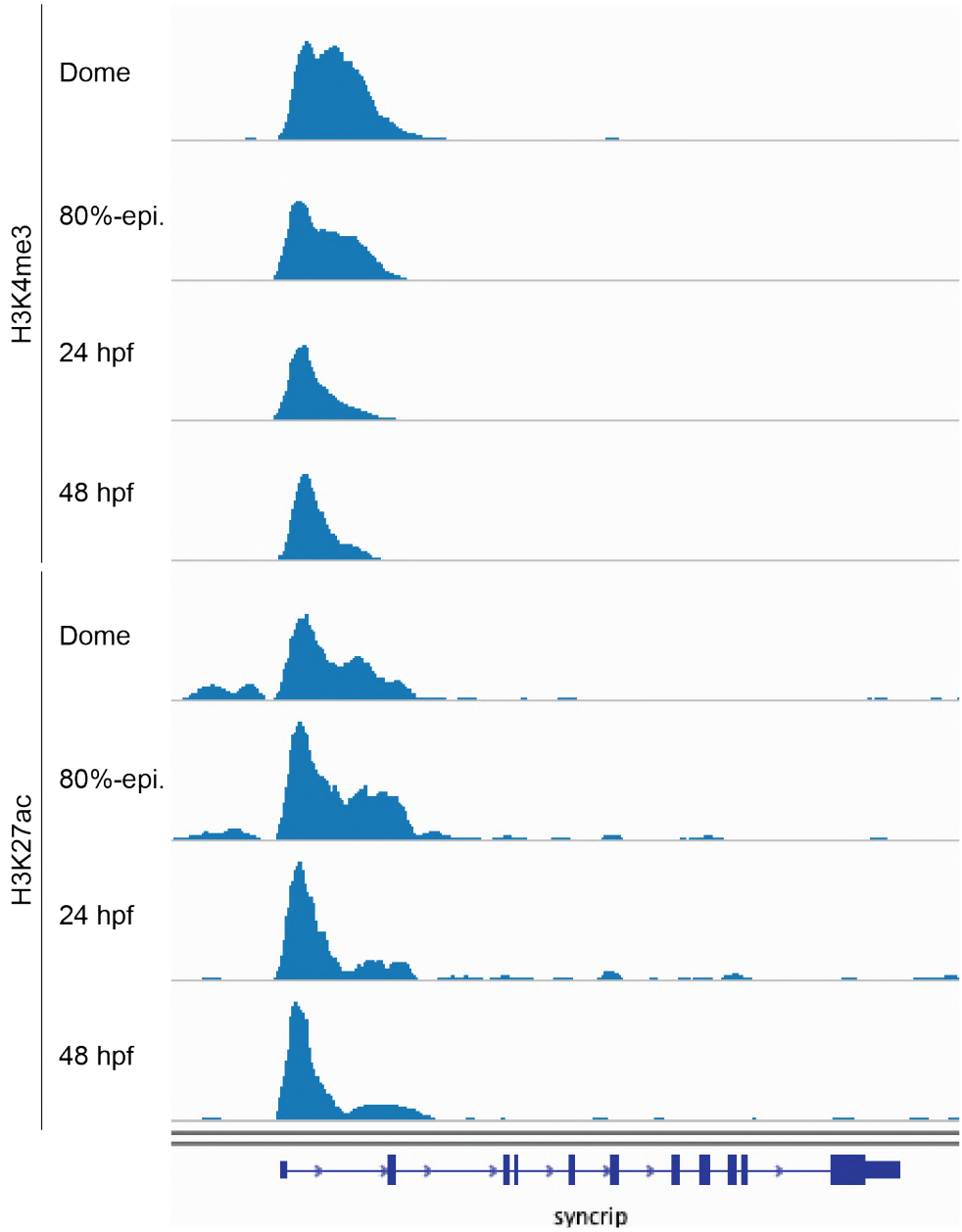

Figure 7-10. Curation of chromatin enhancer marks identifies only one 5' transcriptional start site for *syncrip*. Chromatin modifications linked to active transcription (H3K4me3 and H3K27ac) were curated to determine likely transcriptional start sites in *syncrip*. Data extracted from (Bogdanovic *et al.*, 2012).

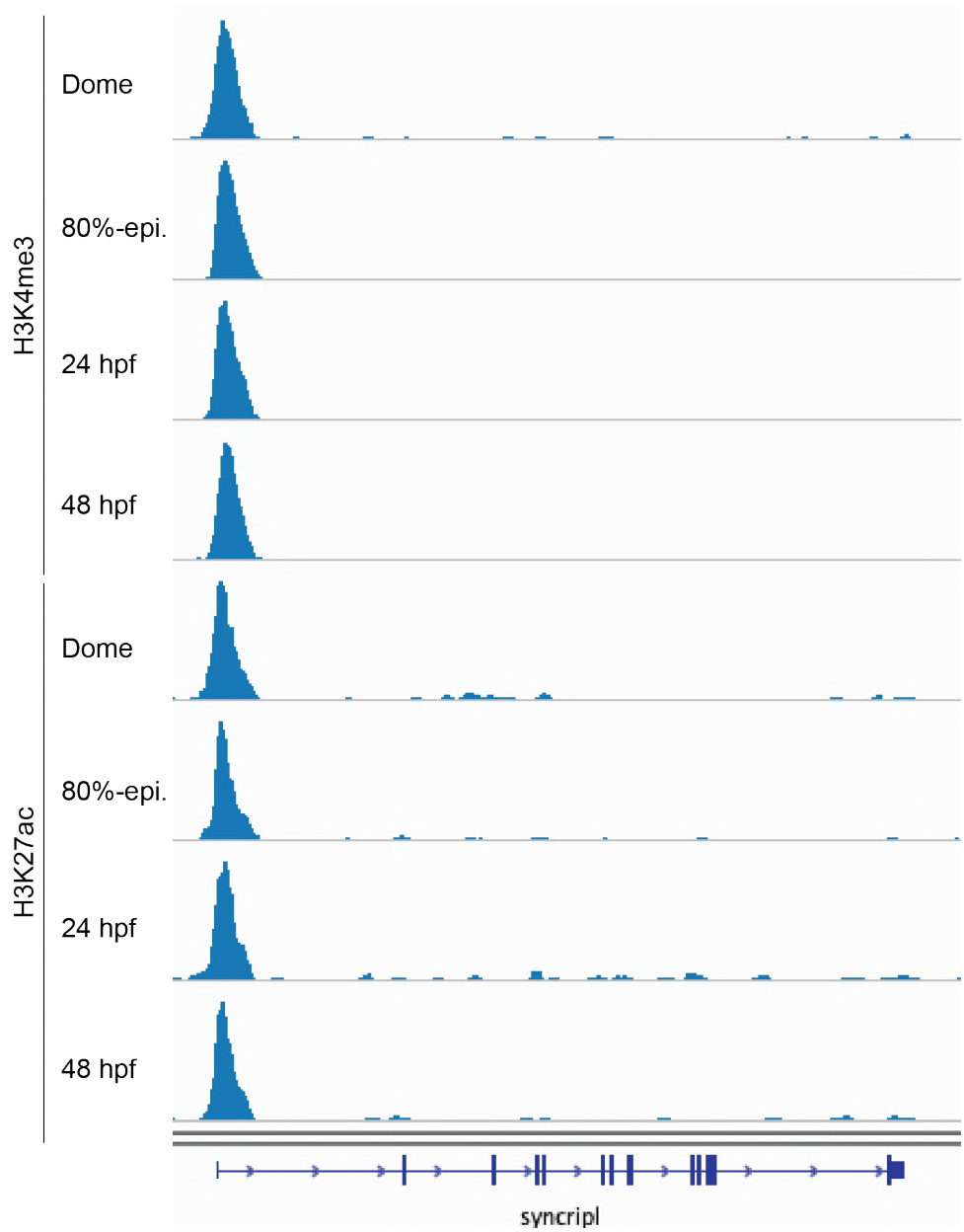

Figure 7-11. Curation of chromatin enhancer marks identifies only one 5' transcriptional start site for *syncripl.* Chromatin modifications linked to active transcription (H3K4me3 and H3K27ac) were curated to determine likely transcriptional start sites in *syncripl.* Data extracted from (Bogdanovic *et al.*, 2012).