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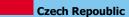
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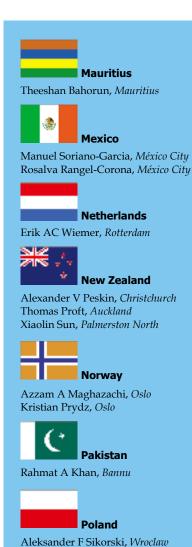






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FRONTIER

# Discovery and characterization of the first non-coding RNA that regulates gene expression, *micF* RNA: A historical perspective

#### Nicholas Delihas

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Author contributions: Delihas N initiated and participated in the previous research design, experiments, and data analysis, and wrote the current manuscript.

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#### Abstract

The first evidence that RNA can function as a regulator of gene expression came from experiments with prokaryotes in the 1980s. It was shown that *Escherichia coli micF* is

an independent gene, has its own promoter, and encodes a small non-coding RNA that base pairs with and inhibits translation of a target messenger RNA in response to environmental stress conditions. The micF RNA was isolated, sequenced and shown to be a primary transcript. In vitro experiments showed binding to the target ompF mRNA. Secondary structure probing revealed an imperfect *micF* RNA/*ompF* RNA duplex interaction and the presence of a non-canonical base pair. Several transcription factors, including OmpR, regulate micF transcription in response to environmental factors. micF has also been found in other bacterial species, however, recently Gerhart Wagner and Jörg Vogel showed pleiotropic effects and found micF inhibits expression of multiple target mRNAs; importantly, one is the global regulatory gene Irp. In addition, micF RNA was found to interact with its targets in different ways; it either inhibits ribosome binding or induces degradation of the message. Thus the concept and initial experimental evidence that RNA can regulate gene expression was born with prokaryotes.

Key words: Non-coding RNAs; RNA/RNA interaction; Regulation of gene expression; *micF* RNA; Trans-acting RNA gene

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**Core tip:** The original discovery and characterization of the first non-coding RNA gene and its transcript was with prokaryotes in the 1980s. At that time the *Escherichia coli micF* RNA gene was characterized in terms of properties, its promoter region, and activation by environmental stress conditions; and the *micF* RNA transcript structure as well as the *micF* RNA/target messenger RNA duplex interaction were elucidated. This occurred over 5 years before the discovery of the first eukaryotic regulatory miRNA, which is not generally recognized. Prokaryotic and eukaryotic non-coding RNAs greatly differ in terms of RNA



processing, but the basic principle of an RNA gene locus encoding a regulatory RNA that targets gene expression *in trans via* RNA/target RNA duplex formation is similar. Thus the concept and discovery of regulatory non-coding RNAs and their functions in messenger RNA inhibition originated with prokaryotes.

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#### INTRODUCTION

It is now recognized that regulation of gene expression by non-coding RNAs *via* RNA/mRNA interactions is a vastly widespread phenomena that occurs in all biological domains, including viruses, and it has become a basic principle in biology. Current research on non-coding RNAs has essentially "exploded"<sup>[1-7]</sup>. Yet before the 1980s, RNAs were thought of as macromolecules that primarily supported the protein synthesis machinery, were considered inert, and the concept of RNA as a regulator was unheard of.

In this review we focus on early history of the experimental work that showed that RNA is a regulator of gene expression, and this occurred approximately 30 years ago in the 1980s. *micF* RNA was the first regulatory RNA discovered<sup>[8-11]</sup>. This preceded the discovery of regulation of gene expression by RNAs in eukaryotes, which was in the 1990s<sup>[12]</sup>. Prokaryotic noncoding RNAs that regulate gene expression are termed sRNAs; the eukaryotic RNAs are termed microRNAs (miRNAs).

The second bacterial regulatory RNA discovered was DsrA. This RNA transcript was found to mediate *rpoS* expression in 1995<sup>[13]</sup> but its mechanism of action *via* RNA/RNA binding was not determined until 1998<sup>[14,15]</sup>.

# *ESCHERICHIA COLI* 6S RNA, SPOT 42, EUKARYOTIC 7SK RNA AND CRISPRS

The discovery that RNA can serve a regulatory role was in the 1980s, however, three regulatory RNAs were found before that but were uncharacterized: *Escherichia coli* (*E. coli*) 6S RNA, Spot 42 and the eukaryotic 7SK RNA. These RNAs were discovered and sequenced about 40 years ago but their functions were not elucidated until decades later. The 6S RNA was first detected in  $1967^{[16]}$ and was sequenced by George Brownlee in  $1971^{[17]}$ . Its function was determined approximately 30 years later in 2000 by Wassarman *et al*<sup>[18]</sup> who showed that 6S RNA binds the RNA polymerase-sigma70 complex and inhibits sigma factor-dependent gene transcription.

The second non-coding RNA found and also unchar-

acterized until decades later was the transcript termed Spot 42 encoded by the *E.coli spf* gene. It was first found in 1973 as a transcript on two-dimensional gel electrophoresis by Ikemura *et al*<sup>(19]</sup>, but its function as a regulatory non-coding RNA was not determined until 2002 when Valentine-Hansen's lab showed that Spot 42 binds the *galK* mRNA and inhibits the galactose operon<sup>[20]</sup>; however, more recently it has been shown to also target fourteen other operons<sup>[21]</sup>.

As to eukaryotic RNAs, in 1976 Gary Zieve and Sheldon Penman found several small RNAs in HeLa cells, one was the 7SK RNA, but its function and the function of the other RNAs were unknown at the time<sup>[22]</sup>. Almost 30 years later it was shown that 7SK RNA binds to a HEXIM1 protein complex, which then binds to the transcription elongation factor P-TEFb and inhibits transcription<sup>[23,24]</sup>.

There are parallels between the prokaryotic 6S RNA and the eukaryotic 7SK RNA - both were found decades before any functional roles were determined and both bind proteins resulting in inhibition of transcription. The majority of small non-coding RNAs regulate posttranscriptionally by binding target mRNAs.

Clustered regularly interspaced short palindromic repeats (CRISPRs) were discovered in *E.coli* in 1987 by Ishino *et al*<sup>(25]</sup>. CRISPRs are an array of genomic repeat sequences, which are separated by spacer sequences that originate from viral or plasmid DNA. They represent an acquired bacterial immune system. Similar to the RNAs discussed above, functions and mechanism of regulation of the CRISPR system as small RNA/target DNA inhibitors were not elucidated until about two to three decades later<sup>[26-29]</sup> (references shown represent only a partial list of contributors).

The bacterial CRISPR-specific RNA processing and targeting mechanism has been compared to the eukaryotic RNAi mechanism and they display striking similarities, although they are not homologous<sup>[30]</sup>. The CRISPR system currently represents one of the fastest moving fields in molecular biology, primarily because of its potential to alter gene structure and induce chromosomal rearrangements. On the other hand, Morange points out that some earlier pioneer work with the CRISPR system went under-appreciated<sup>[31]</sup>.

#### COLE1 RNAI

In 1981 two labs showed that antisense RNAs can inhibit plasmid replication in *E. coli*<sup>[32,33]</sup>. These are milestone experiments as they were the first to demonstrate a regulatory function for RNA. In addition, Tomizawa<sup>[34]</sup>, working with ColE1 plasmid replication, demonstrated that antisense RNA I binds to and inhibits the primer RNA II that is involved in initiating DNA replication.

Tomizawa<sup>[34]</sup> presented a model of antisense RNA/ primer RNA recognition by the so-called "kissing interaction". This involves a two-step process, whereby initially there is a loop-loop interaction *via* Watson-Crick base-pairing between the short stem loops of

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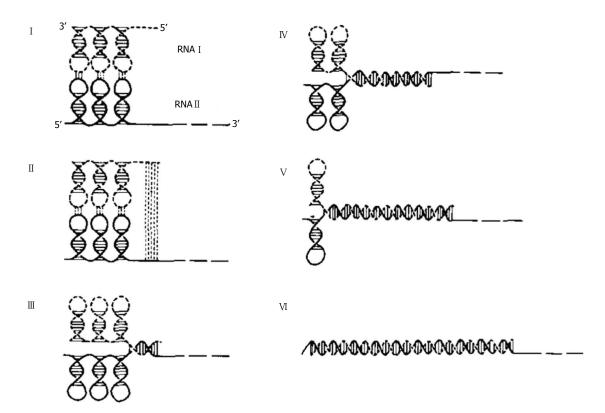


Figure 1 Model of two step interaction between antisense RNA I and sense RNA II-initial stem loop recognition followed by antisense/sense RNA/RNA duplex formation<sup>[34]</sup>.

RNA I and RNA II, followed by pairing of unstructured single-stranded segments of the two RNAs and eventual melting of the stem loops and formation of an antisense/sense RNA/RNA duplex (Figure 1).

The kissing interaction was another pioneer proposal by Tomizawa<sup>[34]</sup> whereby we now know there is board significance to the initial recognition of two RNAs *via* stem loop interactions<sup>[35-39]</sup>. The loop-loop interaction has also been predicted to occur in both the prokaryotic and eukaryotic non-coding RNA/target RNA recognition process<sup>[40-42]</sup>.

There were also other significant plasmid-related experiments at that time. In 1981 Rosen *et al*<sup>[43]</sup> found three RNA transcripts encoded by plasmids R100 and R1, one a counter transcript, but RNA functions were not fully elucidated. Additionally, Simons *et al*<sup>[44]</sup> and Kittle *et al*<sup>[45]</sup> showed that an antisense counter transcript from the insertion sequence of transposon IS10 inhibits transposase expression *via* RNA/RNA interactions and predicting an initial loop interaction.

#### **MICF RNA**

In 1984, Mizuno *et al*<sup>[8]</sup> were the first to present the concept of a regulatory RNA gene that controls expression of another gene *via* RNA/RNA base-pairing. This is the *E. coli micF* gene whose transcript inhibits the expression of the target gene *ompF*. This was based on multicopy plasmid repression of OmpF protein from a sequence upstream of the *ompC* gene. This sequence has

complementarity to *ompF* mRNA<sup>[8]</sup>. OmpF is an outer membrane protein, a porin protein that allows small molecules to diffuse passively through the cell. Thus it is an important protein to regulate in terms of protecting the cell from deleterious molecules.

At that time it was a rather bold and innovative proposal that an RNA can regulate gene expression, There were no role models for such a function, and RNA was for the most part still considered a passive macromolecule that participates in the protein synthesis process and formed the scaffolding for ribosomal proteins, notwithstanding the previous work on antisense RNA repression of plasmid replication<sup>[32,33]</sup>.

Dr. Inouye should be credited for his creativity in first proposing the existence of a regulatory RNA gene. But his lab had only multicopy plasmid effects and had not isolated the RNA transcript. An RNA molecular biologist was needed and I was asked to contribute to the work. Subsequently my lab isolated and sequenced the chromosomal-encoded *micF* RNA, found that *micF* is an independent gene with its own promoter, determined the regulatory RNA/target RNA base-pairing by structure probing, and determined its function in terms of regulation of target gene in response to environmental stress conditions<sup>[9,10]</sup>.

By using *in vivo* P<sup>32</sup> labeling, isolating low molecular weight RNAs and separation on gels, we found a 4.5S RNA transcript. Its sequence showed that it was *micF* RNA and that it was 93 nt long with a rho independent termination site<sup>[9]</sup>. To characterize the micF promoter

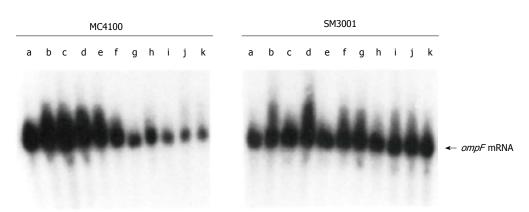


Figure 2 ompF mRNA levels after temperature shift from 24 °C to 42 °C. Lanes marked a-k correspond to time 0-120 min. Left, RNA from strain MC4100 that has the micF gene; right, strain SM3001 that is a micF deletion strain. Data from Andersen et al<sup>10</sup>. Reproduced with permission.

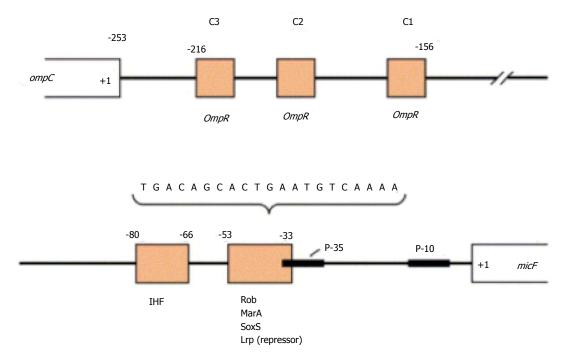


Figure 3 Schematic of micF gene, upstream P-10, P-35 promoter region and transcription factor binding sites. Diagram modified from Delihas et al<sup>61</sup>.

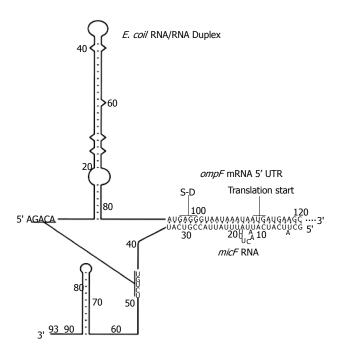
regions, lacZ was fused at different sites upstream of the *micF* gene and *micF* was found to have a strong promoter based on beta galactosidase activity.

# *MICF* RESPONSE TO STRESS CONDITIONS

A search for function was initiated by looking at the response of *micF* expression to various environmental factors. A major effect was found with temperature change and other factors. Levels of *micF* RNA increased dramatically in response to cell growth at high temperature<sup>[10]</sup>. The levels of *ompF* mRNA and the OmpF protein were also measured and found to decrease under these conditions. Figure 2 shows a marked decrease in *ompF* mRNA levels with temperature increase in the wild type strain, but no significant change in the *micF* deletion

strain (SM3001). The findings revealed that *micF* RNA plays a major role in the thermal regulation of *ompF* mRNA and OmpF protein. However, the mechanism of thermal regulation of *micF* expression is still an open question, but temperature has been shown to regulate DNA supercoiling that is associated with the induction of stress related genes<sup>[46-49]</sup>. *micF* expression was also found to respond to other environmental stress conditions such as osmolarity change and exposure to ethanol<sup>[10,50]</sup>.

The upstream *micF* regulatory region is complex in that it contains binding sites for a number of transcription factors; these regulate *micF* expression in response to different environmental stress factors<sup>[51]</sup>. Five transcription factors are known to bind in the upstream regulatory region (Figure 3). With respect to osmolarity change, there are three binding sites (C1-C3) for transcription factor OmpR that activates expression of *micF* in response



**Figure 4 Model of** *micF***RNA***/ompF***mRNA interaction.** Duplex based on secondary structure probing; the long range pairing of five bases at the 5' end of ompF mRNA with positions 45-49 of micF RNA was based on computational analysis<sup>[69]</sup>. Reproduced from Delihas *et al*<sup>[51]</sup>.

to osmolarity increase<sup>[50]</sup>. MarA responds to weak acids<sup>[52]</sup>, SoxS to oxidative stress<sup>[53]</sup>, Rob to peptide antibiotics<sup>[54,55]</sup> and Lrp to nutritionally poor media<sup>[56]</sup>. These four transcription factors share the same binding site on the *micF* promoter region (Figure 3). MarA, SoxS and Rob are related proteins that activate *micF*. Rowena Matthews' lab showed that Lrp represses *micF* transcription<sup>[56]</sup>; Lrp is part of the nucleoid-associated protein goup<sup>[57]</sup>. The crystal structure of transcription factor Rob bound to the *micF* promoter region has been elucidated (by Kwon *et al*<sup>[58]</sup>). The model shows one protein helix-turn-helix motif bound to the major groove of *micF* DNA. Additional biochemical studies showed a high affinity of binding<sup>[58]</sup>.

*micF* is also regulated at the post-translational level when cells are grown in a nutrient poor medium<sup>[59]</sup>. Dorman and co-workers hypothesized that Stp induces a conformational change in *micF* RNA that makes it susceptible to degradation by nucleases<sup>[59]</sup>. Stp is also a nucleoid-associated protein and a paralogue of H-NS<sup>[60,61]</sup>. Thus *micF* gene expression is regulated at both the transcriptional and post-transcriptional levels in response to different environmental conditions.

### MICF RNA/TARGET OMPF MRNA DUPLEX

It was demonstrated that a RNA/RNA duplex could be formed *in vitro* between *micF* RNA and the target *ompF* mRNA<sup>[62]</sup>. Full length *micF* RNA and a 150 nt 5' end fragment of *ompF* mRNA were synthesized *in vitro* and the RNA/RNA duplex was formed by annealing at 55  $^{\circ}$ C and 37  $^{\circ}$ C. Subsequently, *in vitro* formed duplexes

were used for secondary structure probing<sup>[63]</sup>. Single and double stranded specific RNases and chemical modification with a NiCR complex that is single strand G-specific<sup>[64]</sup> were used for probing. As nuclease probes can be sensitive to steric hindrance, chemical modification was performed using the NiCR reagent to try to gain additional information<sup>[63-65]</sup>. A schematic of the deduced *micF* RNA/*ompF* mRNA duplex base-pairing is in Figure 4.

The RNA/RNA interaction shows an imperfect duplex, and a non-canonical G<sup>99</sup>-G<sup>29</sup> base-pair that was determined from the absence of RNase T1 cleavage and NiCR reaction, but also the presence of double stranded specific RNase V1 cleavages<sup>[63,65]</sup>. The Shine-Dalgarno (S-D) ribosome binding site is blocked, which implies that *micF* RNA inhibits ribosome binding. *In vivo* experiments show that *ompF* mRNA levels decrease with temperature increase in an *E. coli* strain carrying *micF* (Figure 2); however, *micF* RNA has not been shown to directly participate in degradation of the message, but to block ribosome binding.

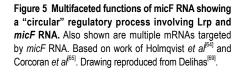
Long-range pairing based on the Maximum Weighted Matching computational program of Tabaska *et al*<sup>66]</sup> was predicted to also contribute to the RNA/RNA interaction (Figure 4). The pairing is supported by phylogenetic comparisons and for the most part, by structure probing. Long-range pairings should contribute to the three-dimensional structure of the interacting RNAs.

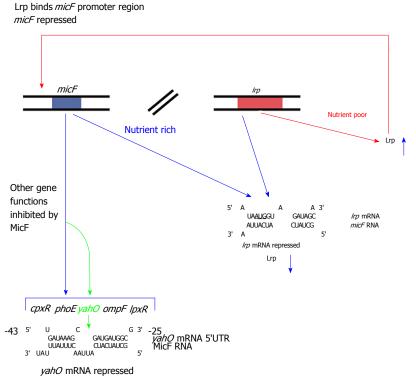
## MICF RNA HAS MULTIPLE TARGETS AND PARTICIPATES IN GLOBAL GENE REGULATION

Recently it has been shown that the functions of *micF* RNA are multifaceted and extend far beyond regulation of *ompF* expression. Gerhart Wagner's lab<sup>[67]</sup> showed that *micF* is part of a global regulatory network involving the leucine responsive protein (Lrp), and Jörg Vogel's lab<sup>[68]</sup> at the University of Würzburg in Germany showed that *micF* targets different mRNAs and base-pairs with these RNAs in different ways.

Approximately 10% of all genes in *E. coli* are controlled by Lrp, a transcription factor which responds to nutrient availability. Wagner's group<sup>[67]</sup> showed participation of *micF* RNA in a circular regulatory pathway, *i.e.*, Lrp regulates transcription of *micF* RNA and *micF* RNA regulate *lrp* expression post-transcriptionally, depending on the nutritional content of the cell growth media (Figure 5). Both Lrp and *micF* RNA function as repressors. *micF* RNA participates in this global regulatory network by regulating *lrp* mRNA; thus this greatly expands its role in regulation of gene expression and cell metabolism.

On the other hand, using *Salmonella* as a model, Vogel *et al*<sup>[68]</sup> demonstrated that *micF* RNA targets multiple mRNAs in addition to *ompF* mRNA and *lrp* mRNA (Figure 5). Interestingly, its mode of binding to different mRNAs is multifaceted. On the one hand, *micF* RNA can induce





destabilization of the *yahO* mRNA by unfolding a stemloop structure located at the 5'-terminus, and thus make the mRNA accessible to exonuclease action. With *lpxR* mRNA, *micF* RNA binds at two sites. One site is within the coding region of the message, and when *micF* RNA binds at this site, it induces a conformational change exposing an AU region that can be targeted by the endonuclease RNase E, which then degrades the message<sup>[68]</sup>. *yahO* encodes a periplasmic protein and *lpxR* encodes an outer membrane lipid A-modifying enzyme.

#### **HFQ PROTEIN**

*In vitro* studies showed that *micF* RNA binds to a protein based on mobility shifts of protein-RNA complexes on non-denaturing gels<sup>[62]</sup>. UV-cross-crosslinking experiments suggested that the protein was 80 kDa, however the protein was not further characterized. It is well known that many sRNAs bind the Hfq protein and that Hfq facilitates the binding of sRNAs to their target mRNAs, including *micF* RNA<sup>[70]</sup>. The molecular weight of Hfq is 11.2 KDa<sup>[71]</sup>, but the 80 kDa protein found to bind *micF* RNA<sup>[62]</sup> is inconsistent with this and there is a need for further investigation.

Hfq is a central component of the pairing of bacterial non-coding RNAs with their target messenger RNAs. The multifaceted ways that Hfq, together with sRNAs can participate in the inhibition of translation and/or mRNA degradation has been outlined<sup>[72]</sup>. Hfq also interacts with another protein, catalase HPII. The crystal structure of Hfq bound to catalase HPII has been solved<sup>[71]</sup>, but the crystal structure of an Hfq-sRNA complex has not been determined.

#### **OTHER NON-CODING RNA FUNCTIONS**

In this paper we focused on initial findings involving the regulation of gene expression by RNA, however, there is a wealth of discoveries with other functions of non-coding RNAs that also occurred during the 1980s, and it is important to mention this. Table 1 lists several initial findings that include enzymatic RNA, catalytic RNA and RNA functions in protein translocation. The work on regulation of gene expression by RNA and the studies outlined in Table 1 provide a breakthrough on understanding functions of RNAs whereby before the 1980s, RNAs were largely considered inert with no dynamic functions.

#### CONCLUSION

The initial discovery that RNA can function as a regulatory molecule occurred in the 1980s. Table 2 summarizes the chronology. Bacterial plasmid RNAs were first shown by two labs to regulate DNA replication via sense/ antisense RNA/RNA base-pairing<sup>[32,33]</sup> and the E. coli micF gene was the first found to regulate expression of another gene in trans via imperfect RNA/RNA basepairing, also by two labs<sup>[8-10]</sup>. These finding were major breakthroughs that came approximately 5-10 years before the discovery of eukaryotic microRNAs and their functions. Subsequently, numerous labs added to the molecular genetics of micF and to functional properties of the transcript that greatly advanced our knowledge of the role of this gene in cell survival. So it was essentially work with prokaryotes that first opened the door to a new form of regulation of gene expression by RNA

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| Non-coding RNADate discoveredFunctionRef.RNase P RNA1978, 1983Catalytic RNA[73,74]Tetrahymena intron1982RNA self-splicing[75]snRNA1982Involved in RNA[76] | Table 1 Discovery of other non-coding RNA functions |                 |                                                                             |              |  |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------|-----------------|-----------------------------------------------------------------------------|--------------|--|
| Tetrahymena intron 1982 RNA self-splicing [75]                                                                                                            | Non-coding RNA                                      | Date discovered | Function                                                                    | Ref.         |  |
| 7SL RNA 1982 splicing<br>Participates in protein [77]<br>translocation                                                                                    | Tetrahymena intron<br>snRNA                         | 1982<br>1982    | RNA self-splicing<br>Involved in RNA<br>splicing<br>Participates in protein | [75]<br>[76] |  |

#### Table 2 Discovery of regulatory non-coding RNA functions

| Non-coding RNA<br>(prokaryotic) | Date<br>determined to<br>be a regulator | Function                                          | Ref.    |
|---------------------------------|-----------------------------------------|---------------------------------------------------|---------|
| ColE I RNA I                    | 1981                                    | Inhibits DNA<br>replication                       | [32,33] |
| micF RNA                        | 1984/1987                               | Inhibits <i>ompF</i> mRNA + other mRNAs           | [8-10]  |
| 65 RNA                          | 2000                                    | Binds RNA polymerase<br>(transcription inhibitor) | [18]    |
| Spot 42                         | 2002                                    | Inhibits galK mRNA + other mRNAs                  | [20]    |
| Non-coding RNA                  |                                         |                                                   |         |
| (eukaryotic)                    |                                         |                                                   |         |
| lin 4                           | 1993                                    | Inhibits lin14                                    | [12]    |
| 7SK RNA                         | 2004                                    | Binds pTEFb<br>(transcription inhibitor)          | [23]    |

that is now found in all biological kingdoms, including viruses. However, there are many different types of regulatory RNAs that are found only in eukaryotes (*e.g.*, piRNA, siRNA, lncRNA), and the prospects of finding new and unique functions of RNAs may lie primarily with eukaryotic RNAs. Looking towards the future, there are tens of thousands of lncRNAs that have been detected, and for the most part are uncharacterized. But the few that have been characterized display very diverse and intricate functions<sup>[78-82]</sup>.

#### ACKNOWLEDGMENTS

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REVIEW

# Targeting amino acid metabolism in cancer growth and anti-tumor immune response

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#### Abstract

Recent advances in amino acid metabolism have revealed that targeting amino acid metabolic enzymes in cancer therapy is a promising strategy for the development of novel therapeutic agents. There are currently several drugs in clinical trials that specifically target amino acid metabolic pathways in tumor cells. In the context of the tumor microenvironment, however, tumor cells form metabolic relationships with immune cells, and they often compete for common nutrients. Many tumors evolved to escape immune surveillance by taking advantage of their metabolic flexibility and redirecting nutrients for their own advantage. This review outlines the most recent advances in targeting amino acid metabolic pathways in cancer therapy while giving consideration to the impact these pathways may have on the anti-tumor immune response.

**Key words:** Amino acid metabolism; T cells; Anti-tumor immune response; Tumor microenvironment; Cancer therapy

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**Core tip:** Amino acid metabolism has been a focus of increased attention by cancer researchers and immunologists due to its importance for the metabolic reprogramming of proliferating cells. Many amino acid enzymes are described as immunosuppressive in the tumor microenvironment and targeted for cancer therapy. This review addresses the metabolic control of tumor progression in the context of anti-tumor immunity and discusses current and future therapeutic approaches. Special emphasis is given to the emerging role of branched chain amino acid metabolism in cancer and immunity highlighting some recent work by our research group.

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#### INTRODUCTION

Two functionally distinct types of cells, cancer and T cells, undergo similar metabolic reprogramming during



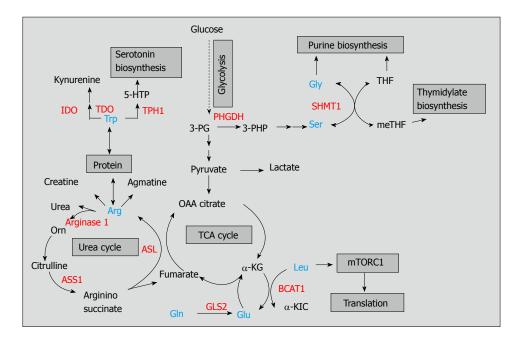


Figure 1 Schematic representation of amino acid metabolic pathways targeted in cancer therapy. The amino acid metabolism of Arg, Trp, Ser, Gly, Gln, Glu, and BCAAs interconnects with major catabolic and biosynthetic pathways shown in gray. Arg is an important precursor for creatine, urea, and agmatine synthesis while Trp is important for kynurenine and serotonin biosynthesis; Ser and Gly are major sources of methyl groups during purine and thymidylate biosynthesis; Leu is a known activator of complex 1 of mTOR pathway and Gln/Glu provide intermediates for the TCA cycle and restore glutathione to its reduced form. Metabolic enzymes catalyzing important steps in the metabolism of these amino acids are involved in clinical and/or preclinical cancer studies and are denoted in red. 5-HTP: 5-hydroxytrytophan; 3-PG: 3-phosphoglycerate; 3-PHP: 3-phosphohydroxy pyruvate; THF: Tetrahydrofolate; meTHF: Methyltetrahydrofolate; OAA: Oxaloacetate;  $\alpha$ -KG:  $\alpha$ -ketoglutarate;  $\alpha$ -KIC:  $\alpha$ -ketosiocaproate; Trp: Tryptophan; Arg: Arginine; Gln: Glutamine; Glu: Glutamate; BCAAs: Branched chain amino acids; Ser: Serine; Gly: Glycine; mTORC1: Complex 1 of the mammalian target of rapamycin; IDO: Indoleamine-2,3-dioxygenase; TDO: Tryptophan-2,3-dioxygenase; TPH1: Tryptophan hydroxylase-1; BCATc: Cytosolic branched chain aminotransferase; ASS1: Argininosuccinate synthetase 1; ASL: Argininosuccinate lyase; GLS2: Glutaminase 2; PHGDH: Phosphoglycerate dehydrogenase; SHMT1: Serine hydroxymethyl transferase 1.

proliferation to support their increased biosynthetic and energy demands<sup>[1,2]</sup>. To meet these demands, cancer cells need a continuous supply of nutrients that maintain abnormal growth and rapid division during cancer progression. Activated T cells also rely on a continuous nutrient supply to ensure proper differentiation and performance during cell-mediated immunity against pathogen attacks or while fighting cancer<sup>[3]</sup>. In areas with poor nutrient and oxygen access, such as the tumor microenvironment<sup>[4]</sup> and inflammatory sites<sup>[5]</sup>, cellular metabolism must adapt to promote continued survival and function of the cells residing in and/or migrating to these areas. Not surprisingly, both cancer and T cells rely on high rates of glycolysis while retaining mitochondrial respiration, a phenomenon known as the Warburg effect<sup>[6,7]</sup>. Ever since Otto Warburg observed that cancer cells produced lactate from glucose even under non hypoxic conditions<sup>[7]</sup>, subsequent reports demonstrated that high rates of glycolysis are beneficial for cell survival, providing energy and biosynthetic material for the synthesis of new proteins, lipids, and nucleic acids in proliferating cells<sup>[8-13]</sup>. Recent advances in cancer biology and cellular immunity reveal that not only glucose but also amino acids are essential to support the high metabolic demands of tumor and/or immune cells and different strategies for targeting amino acid metabolism and corresponding enzymes are now a focus of innovative treatment approaches<sup>[14-17]</sup>. The purpose of

this review is to highlight the importance of amino acid metabolism in the tumor microenvironment and use this knowledge to generate more effective immunotherapies while keeping in mind that both cancer and immune cells have similar metabolic requirements for growth and function and therefore may compete for common nutrients.

## ARGININE AND TRYPTOPHAN METABOLISM IN THE TUMOR MICROENVIRONMENT

The amino acid arginine has considerable nutritional and physiological significance as it is recognized as an important precursor for the synthesis of proteins, urea, and creatine as well as for the synthesis of signaling molecules such as glutamate, nitric oxide, and agmatine<sup>[18]</sup> (Figure 1). Although arginine is a dispensable (nonessential) amino acid for healthy humans, it is conditionally essential under certain physiological conditions or disease state<sup>[10,19,20]</sup>. For example, many tumors are dependent on exogenous arginine for growth as they lack the enzyme argininosuccinate synthetase 1 (ASS1)<sup>[21]</sup>. ASS1 catalyzes the conversion of citrulline into argininosuccinate in an ATP-dependent manner, completing one of the last steps in the arginine biosynthetic pathway<sup>[18]</sup> (Figure 1). Loss of ASS1 prevents the production or arginine and may lead

to arginine depletion. Osteosarcoma and bladder cancer cell lines expressing low levels of ASS1 failed to grow in an arginine-free medium, indicating that ASS1 behaves as a tumor suppressor<sup>[22,23]</sup>. However, arginine depletion in ASS1-negative tumor cells is usually associated with an aggressive phenotype and negative prognostic impact<sup>[22]</sup>. Kobayashi et al<sup>[22]</sup>, showed that ASS1 deficiency in osteosarcoma patients correlated with the development of pulmonary metastasis in patients with osteosarcoma and can be used as a predictive biomarker for unfavorable prognosis. The aggressiveness of ASS1-negative tumors can be explained partially by the ability of these tumors to utilize more efficiently exogenous arginine coming from the tumor microenvironment. It is hypothesized that tumor associated myeloid cells (TAMCs), that consist of macrophages, monocytes, myeloid suppressor cells, and neutrophils<sup>[24]</sup>, form metabolic relationship with the tumor cells in the tumor microenvironment. They provide arginine to help the tumor cells by-pass the effect of arginine deprivation<sup>[25]</sup>. In addition, TAMCs express high levels of another enzyme in the arginine metabolism, arginase 1, that hydrolyses arginine into urea and ornithine and sustains tumor growth by providing precursors for polyamine synthesis<sup>[26,27]</sup>. By producing high levels of arginase 1 in the tumor microenvironment, TAMCs reduce arginine availability for other immune cells such as T cells. However, TAMCs can arrest cytotoxic T cell proliferation and induce T cell dysfunction by more than one mechanism, including generation of nitric oxide from arginine by nitric oxide synthase<sup>[28]</sup>. Additionally, these cells can induce regulatory T cell differentiation<sup>[29]</sup> potentially promoting immune tolerance in the tumor microenvironment. Thus, TAMCs have the ability to suppress the protective anti-tumor immune response by targeting arginine metabolism and helping tumors escape immune destruction. While immunotherapeutic targeting of arginine metabolism in the tumor microenvironment is still in its infancy, several decades of substantial in vitro and in vivo studies on arginine metabolism led to the development of arginine deprivation therapy, which is currently the subject of ongoing clinical trials with several arginine depleters, such as pegylated arginine deiminase (ADI-PEG20, Polaris group) and bioengineered forms of human arginase<sup>[25,30,31]</sup> (Table 1). A small study group of patients with hepatocellular carcinoma revealed response rates to ADI-PEG20 between 25%-47% and this compound holds great potential in the arginine depravation therapy<sup>[30]</sup>. ADI-PEG20 is not limited to cancer therapy as there is ADI-PEG20 with anti-viral activity designed for treatment of hepatitis C by Polaris group.

Tryptophan is another amino acid linked to the regulation of immune tolerance and anti-tumor immune responses<sup>[32-35]</sup>. Tryptophan degradation occurs *via* the kynurenine pathway where two different enzymes, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3dioxygenase (TDO), catalyze the conversion of tryptophan into kynurenine, while tryptophan hydroxylase-1 (TPH-1) converts tryptophan to 5-hydroxytryptophan and provides precursors for serotonin biosynthesis<sup>[32,36]</sup> (Figure 1). IDO is the most studied enzyme in tryptophan metabolism expressed by both immune cells (dendritic cells, macrophages) and tumor cells<sup>[33,37-39]</sup>. Due to the fact that tryptophan readily crosses the plasma membrane, dendritic cells expressing IDO are capable of depleting tryptophan in the extracellular space, limiting tryptophan supply to surrounding T cells. T cell activation is sensitive to local tryptophan concentrations, and lack of tryptophan blocks their proliferation<sup>[40]</sup>. By degrading tryptophan, IDO inhibits T cell proliferation and plays a role in autoimmunity and anti-inflammatory responses<sup>[41-43]</sup>. For example, Apoe<sup>-/-</sup> mice treated with the IDO inhibitor, 1-methyl-Trp, showed a significant increase in atherosclerotic lesions in the aortic arch and root of their hearts along with enhanced vascular inflammation<sup>[41]</sup>. Additionally, IDO-expressing dendritic cells suppressed the allograft rejection and increased the survival time of small bowel transplanted mice<sup>[44]</sup>. In the tumor microenvironment, however, IDO limits the T cell response to tumor growth<sup>[45-47]</sup>. By using an IDO-negative subline of the mouse tumor model P815B, Uyttenhove et al<sup>[45]</sup> elegantly showed that mice transfected with this subline completely rejected the tumor challenge while mice transfected with IDO-expressing cells developed progressive tumors and died. These results suggest that effector T cells that infiltrate the tumor microenvironment are more susceptible to the effects of IDO than are tumor cells. Indeed, immunohistochemical staining for IDO expression in endometrial cancer tissues revealed significant correlation between high IDO expression and low numbers of CD3<sup>+</sup>, CD8<sup>+</sup>, and CD57<sup>+</sup> immune cells, which possibly contributed to disease progression and impaired clinical outcome for patients with endometrial cancer<sup>[48]</sup>. In addition to IDO, tumors use other enzymes in tryptophan metabolism to resist immune destruction<sup>[36,49]</sup>. TDO, a well described liver enzyme, is another immunosuppressive enzyme found in bladder carcinomas, melanomas, and hepatocarcinomas<sup>[50]</sup>. In a preclinical model, Pilotte et al[50] demonstrated that systemic treatment of immunized mice with a TDO inhibitor prevented the growth of TDO-expressing tumor cells. Lastly, TPH-1 expressed by mast cells is also necessary for long-term graft tolerance and a suppressive antitumor microenvironment<sup>[51]</sup>. Therefore, more than one tryptophan catabolic enzyme contribute to the establishment of immune tolerance and could be suitable for combinatorial therapies. Thus far, IDO inhibitors, specifically designed for cancer immunotherapy, have been broadly used in preclinical and clinical trials alone or in combination with T cell checkpoint inhibitors<sup>[52-54]</sup>, while systemic inhibition of TDO, although promising, may raise safety concerns<sup>[54]</sup> (Table 1). Another limitation of cancer trials targeting tryptophan metabolism is that they do not select cancer patients based on assessment of systemic IDO/TDO activities or by analysis of their metabolites in patients' serum<sup>[54]</sup>. Nevertheless, combinatorial approaches targeting tryptophan metabolism will continue to deliver novel therapeutic avenues in cancer therapy.

#### Ananieva E. Amino acid metabolism and cancer

| Amino acid<br>metabolism          | Targeted<br>enzyme                            | Drug design                | Drug toxicity, adverse events                                                                                 | Cancer type                                                                                       | Response rate                   | Clinical<br>studies    | Ref.       |
|-----------------------------------|-----------------------------------------------|----------------------------|---------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|---------------------------------|------------------------|------------|
| Arginine                          | ASS1,<br>Arginine<br>deaminase                | ADI-PEG20                  | <sup>1</sup> Grade 3-4: Fatigue, hyperuricemia, anemia                                                        | HCC (nonresectable and metastatic)                                                                | 31%-47%                         | Phase I / II           | [30,94,95] |
|                                   |                                               | ADI-PEG20                  | <sup>1</sup> Grade 3-4: Pain in extremity,<br>arthralgia, pruritus, lymphedema,<br>seizure                    | Melanoma (stage III and IV)                                                                       | 25%                             | Phase I / II           | [96,97]    |
|                                   | Arginase 1                                    | rhArgIpeg5000              | <sup>1</sup> Grade 3-4: Elevated ALT, AST,<br>bilirubin and GGT                                               | HCC (advanced)                                                                                    | 26.70%                          | Phase I                | [31]       |
|                                   |                                               | Co-hArgI                   | Nude BALBc mouse model: Weight<br>loss, hunch posture, lethargy, bone<br>marrow injuries (> 15 mg/kg)         | HCC (HepG2),<br>pancreatic cancer<br>(Panc-1); tumor site (right<br>flank)                        | Smaller<br>tumors               | Preclinical<br>studies | [98]       |
| Tryptophan                        | IDO                                           | Indoximod and<br>docetaxel | Grade 1: Anemia, fatigue,<br>hyperglycemia<br>Grade 3-4: Headache, hypotension,<br>infection                  | Various metastatic solid<br>tumors                                                                | 41% PD                          | Phase I                | [99]       |
|                                   |                                               | 1-D-MT                     | Grade 1: Fatigue; Grade 2:<br>Hypophysitis                                                                    | Various metastatic solid<br>tumors                                                                | 4P (SD), 3P<br>(PD)             | Phase I                | [100]      |
|                                   |                                               | Indoximod and anti-CTLA4   | C57BL/6 mouse model: No weight<br>loss or acute/delayed toxicity<br>observed                                  | B16 F10 melanoma cell<br>line, tumor site -flank                                                  | Delayed<br>tumor growth         | Preclinical<br>studies | [101]      |
| Tryptophan                        | TDO                                           | Indole LM10                | DBA/2 mouse model: No liver toxicity observed for 3 mo                                                        | P815B tumor cell line,<br>tumor site –peritoneal<br>cavity                                        | Delayed<br>tumor<br>progression | Preclinical<br>studies | [50]       |
| Serine                            | PHGDH                                         | NA                         | NA                                                                                                            | Melanoma and breast<br>cancer cell lines<br>(SkBr3, MCF7)                                         | NA                              | Preclinical<br>studies | [15]       |
|                                   |                                               | NA                         | NA                                                                                                            | Murine mammary fat<br>pad tumors with MDA-<br>MB-468 cells expressing<br>PHGDH-shRNA              | Reduced<br>tumor growth         | Preclinical<br>studies | [63]       |
| Glycine                           | SHMT1                                         | NA                         | $\lambda$ -Myc <i>Shmt1-/-</i> transgenic mice: no toxicity observed                                          | Accelerated<br>lymphomagenesis                                                                    | NA                              | Preclinical<br>studies | [102]      |
| Glutamine                         | Glutamine<br>-dependent<br>enzymatic<br>steps |                            | None reported                                                                                                 | Various animal and<br>human xenografted<br>tumors                                                 | Tumor<br>growth<br>inhibition   | Preclinical<br>studies | [80]       |
|                                   |                                               | Acivicin <sup>2</sup>      | Grade1-3: neurological toxicity<br>Grade 2: vomiting, infections                                              | High grade astrocytoma                                                                            | Median 128<br>day survival      | Phase II               | [103]      |
| Leucine,<br>Isoleucine,<br>Valine | BCATc                                         | NA                         | CD-1 nude mouse model bearing<br>tumors with BCATc knockdown:<br>lethargy and uncoordinated motor<br>activity | U-87MG glioblastoma<br>cells with BCATc-<br>shRNA; tumor<br>site-intracerebral<br>transplantation | Smaller<br>tumors               | Preclinical<br>studies | [16]       |
|                                   |                                               | NA                         | NA                                                                                                            | Nasopharyngeal<br>carcinoma (5-8F, 6-10B),<br>colorectal cancer                                   | Induced cell proliferation      | Preclinical studies    | [92,93]    |

<sup>1</sup>The highest grade is shown (Grade 3-4) and is summarized for either one or more studies; <sup>2</sup>Discontinued. ADI-PEG20: Pegylated arginine deiminase; rhArgIpeg5000: Pegylated recombinant human arginase I; HCC: Hepatocellular carcinoma; Co-hArgI: Co<sup>2+</sup> substitution of the Mn<sup>2+</sup> metal cofactor in human arginase I; Indoximod (NLG8189, 1-D-MT1): 1-methyl-D-tryptophar; Indole LM10, tetrazolyl-vinyl substituted (fluoro)indole; L-DON: 6-diazo-5oxo-L-norleucine; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma glutamyltransferase; PD: Progressed disease; SD: Stable disease; P: Patient; NA: Not available.

### SERINE AND GLYCINE METABOLISM IN TUMORIGENESIS

Serine and glycine metabolism are interconnected *via* the glycine cleavage system, a major metabolic pathway in one-carbon metabolism that provides cofactors for purine and pyrimidine nucleotide biosynthesis for proliferating lymphocytes, cancer cells, and/or fetal tissues<sup>[55-57]</sup>

(Figure 1). Studies from the late 1980s to more recent years, strongly suggest that cancer cells have an increased capacity for *de novo* serine synthesis *via* the phosphoglycerate dehydrogenase (PHGDH) pathway. PHGDH oxidases around 10% of 3-phosphogycerate produced during glycolysis by converting it to 3-phosphohydroxypyruvate<sup>[58-61]</sup>. This compound is then transaminated, forming 3-phosphoserine, and dephos-

phorylated to yield serine (Figure 1). PHGDH along with other enzymes in the serine biosynthetic pathway were upregulated in highly metastatic breast cancer, a finding associated with overall poor patient survival<sup>[62]</sup>. Two independent studies published in 2011 reported that the gene encoding PHGDH was recurrently amplified in melanoma and breast cancers and this amplification was not associated with oncogene regulation<sup>[15,63]</sup>. Apart from PHGDH, serine hydroxymethyl transferase (SHMT), that converts serine to glycine, has also been implicated in tumorigenesis. Two isoforms of SHMT (cytoplasmic SHMT1 and mitochondrial SHMT2) were both described as targets of c-myc oncogene<sup>[64]</sup>, a transcriptional factor abnormally expressed in many tumors that controls transcription of up to 15% of the genes in human cells<sup>[65]</sup>. Both SHMT1 and SHMT2 were described as downstream effectors of c-myc function and rescued the growth defects of c-myc-null cells<sup>[64]</sup>. At first glance, the role of serine and glycine metabolism in tumorigenesis may appear disadvantageous as PHGDH diverts metabolites from glycolysis to de novo synthesis of serine followed by conversion to glycine by SHMT (Figure 1). However, both serine and glycine are major sources of methyl groups for the one carbon pool required for a variety of biosynthetic pathways and/or DNA methylation that tumor cells use. Similar to cancer cells, immune cells are also shown to use glycolytic intermediates for serine/glycine biosynthesis with the ultimate goal of synthesizing building materials for cell growth and proliferation<sup>[66]</sup>. Preclinical studies are underway to test the efficacy of many one carbon metabolic enzymes, including PHGDH<sup>[67]</sup>, as anti-tumor targets (Table 1). Dietary intervention is another strategy to target cancer metabolism and although preclinical studies restricting serine and glycine metabolism showed promising results<sup>[68,69]</sup>, global or systematic interventions need to be carefully examined in the context of the immune system that rely on the same metabolic pathways for proper function.

### GLUTAMINE METABOLISM, A HALLMARK OF PROLIFERATING CELLS

As early as 1951, Mider<sup>[70]</sup> described that tumors behaved as "nitrogen traps" where glutamine was the preferred nitrogen donor<sup>[70,71]</sup>. More reports form the 1980's, demonstrated that not only cancer cells but also rapidly dividing cells such as lymphocytes, thymocytes, and colonocytes had high glutamine consumption rates<sup>[72-74]</sup>. Despite the fact that glutamine is a nonessential amino acid, proliferating cells display addiction to glutamine implying that glutamine plays more roles in cell metabolism than simply being a nitrogen donor. As such, glutamine is a conditionally essential amino acid for the proliferating cells as well as critically ill humans<sup>[75]</sup>. Glutamine provides intermediates for the TCA cycle, restores glutathione to its reduced form suppressing oxidative stress, and maintains mitochondrial membrane integrity thus contributing to the survival of proliferating cells (reviewed by Wise and Thompson)<sup>[76]</sup>. Tumor suppressors (p53) and oncogenes (c-myc) were shown to regulate glutamine metabolism. Hu et al<sup>[77]</sup> demonstrated that p53 targeted the mitochondrial isoform of glutaminase [glutaminase 2 (GLS2)], that converts glutamine to glutamate (Figure 1). p53 increased GLS2 expression and led to enhanced mitochondrial respiration and generation of ATP along with reduction in reactive oxygen species due to increased glutathione levels<sup>[77]</sup>. Likewise, introduction of an inducible c-myc transgene in mouse embryonic fibroblasts led to induction of glutaminase 1 along with lactate dehydrogenase and glutamine transporters, suggesting that c-myc is required to support cellular dependence on glutamine<sup>[78]</sup>. Cancer "addiction" to glutamine had been explored in cancer therapeutics and three compounds, 6-diazo-5-oxo-Lnorleucine (L-DON), azaserine, and acivicin, showed significant activity as glutamine analogs<sup>[79]</sup>. In early preclinical and clinical studies, these compounds showed promising results against different tumor types by inhibiting ribonucleotide biosynthesis, glutamine oxidation and reducing cell viability<sup>[80,81]</sup> (Table 1). However, due to the important role of glutamine metabolism for normal tissue physiology, these compounds were discontinued while better tumor-targeting options with less general toxicity are currently considered<sup>[76,82]</sup>.

## EMERGING ROLE OF BRANCHED CHAIN AMINO ACID METABOLISM IN CANCER THERAPY

Branched chain amino acids (BCAAs, leucine, isoleucine, and valine) constitute about 40% of the essential amino acid requirements of healthy individuals<sup>[83]</sup>. They play an important role in protein synthesis and serve as major nitrogen donors for alanine and glutamine synthesis<sup>[84]</sup>. Increasing evidence shows that BCAAs, especially leucine, are not merely building blocks necessary to support biosynthetic demands but also nutrient signals regulating the mammalian target of rapamycin (mTOR) pathway<sup>[85,86]</sup>. By controlling protein translation, cell growth, proliferation, and autophagy, the mTOR pathway is recognized as a critical regulator of cellular function<sup>[87]</sup>. Immune cells are particularly sensitive to mTOR regulation as mTOR pathway responds to environmental cues and coordinates immune cell differentiation and function, accordingly<sup>[88]</sup>. For example, inhibition of mTOR pathway in T cells promoted T cell tolerance and the mechanism responsible for the maintenance of tolerance was failure of T cells to upregulate mTOR activity in the presence of metabolic inhibitors including leucine antagonists<sup>[89]</sup>. In this regard, leucine appears to be an important nutrient signal that is sensed by the immune cells via mTOR pathway and is critical for their proliferation. Leucine supply to mTOR pathway is regulated through BCAA metabolism as shown recently in T cells<sup>[90]</sup>. The cytoplasmic branched chain aminotransferase (BCATc), that catalyzes leucine transamination, was induced in activated T cells, where



it regulated leucine supply to complex 1 of the mTOR pathway. Loss of BCATc expression eliminated cytosolic leucine catabolism leading to upregulation of complex 1 of the mTOR pathway and increased glycolysis<sup>[90]</sup>. While mTOR pathway is upregulated in many cancer types and mTOR-targeted cancer therapy has been a part of clinical research<sup>[91]</sup>, a direct link between mTOR pathway and leucine/BCAA metabolism in the tumor microenvironment awaits to be explored. Similar to the tryptophan degrading enzymes, BCATc may play an immunosuppressive role in the tumor microenvironment possibly contributing to tumor escape mechanisms. Another mechanism of BCATc function in cancer was explored in glioblastomas<sup>[16]</sup>. Tönjes et al<sup>[16]</sup> demonstrated that BCATc and BCAA metabolism are attractive targets for the development of therapeutic approaches to treat glioma patients. The majority of gliomas show mutations in their isocitrate dehydrogenase enzyme 1 (IDH1<sup>mut</sup>) and although IDH1 mutation status is a powerful prognostic factor, it was insufficient to induce tumors in mice alone. BCATc was overexpressed in normal (wild type) IDH1<sup>wt</sup> gliomas but not in gliomas with mutated IDH1, demonstrating a connection between IDH1 mutation and BCATc<sup>[16]</sup>. Additionally, BCATc was identified as a c-myc target in nasopharyngeal carcinoma and BCATc overexpression induced cancer cell proliferation and migration<sup>[92]</sup>. Although the majority of these studies imply that cancer cells require BCAA metabolism to sustain growth, and overexpression of BCATc results in increased cell proliferation<sup>[92,93]</sup>, the mechanism through which changes in BCAA metabolism affect cancer growth is currently unknown. It is possible that T cells and cancer cells share similar requirements for BCAA catabolism, where mTOR pathway is dependent on leucine regulation. Thus, future use of leucine antagonists or specific inhibitors aimed at BCATc may be suitable for targeted cancer therapies.

#### CONCLUSION

Research on amino acid metabolism in cancer cells in the last decades has provided valuable insights on the potential impact of metabolic control and regulation in the tumor microenvironment. Amino acids are no longer regarded solely as building materials but also as nutrient signals that regulate important signaling pathways. A number of amino acid metabolic enzymes are regulated by oncogenes and tumor suppressors and have been explored as targets for cancer therapies. Design and use of inhibitors targeting tryptophan, arginine and/or glutamine metabolism either alone or in combination with anti-tumor drugs has been introduced in clinical trials. However, cancer and immune cells share similar requirements for amino acid metabolic enzymes and often compete for the same nutrients. Therefore, therapeutic interventions in the tumor microenvironment must be cautiously explored to eliminate potential negative impacts on the anti-tumor immunity. Understanding the underlying mechanisms of metabolic interplay between tumor and immune cells will provide new directions to manipulate the tumor

microenvironment and unleash the anti-tumor immune response.

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REVIEW

## Regulation of *MYC* gene expression by aberrant Wnt/ $\beta$ -catenin signaling in colorectal cancer

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#### Abstract

The Wnt/ $\beta$ -catenin signaling pathway controls intestinal homeostasis and mutations in components of this pathway are prevalent in human colorectal cancers (CRCs).

These mutations lead to inappropriate expression of genes controlled by Wnt responsive DNA elements (WREs). T-cell factor/Lymphoid enhancer factor transcription factors bind WREs and recruit the  $\beta$ -catenin transcriptional co-activator to activate target gene expression. Deregulated expression of the *c-MYC* proto-oncogene (MYC) by aberrant Wnt/ β-catenin signaling drives colorectal carcinogenesis. In this review, we discuss the current literature pertaining to the identification and characterization of WREs that control oncogenic MYC expression in CRCs. A common theme has emerged whereby these WREs often map distally to the MYC genomic locus and control MYC gene expression through long-range chromatin loops with the MYC proximal promoter. We propose that by determining which of these WREs is critical for CRC pathogenesis, novel strategies can be developed to treat individuals suffering from this disease.

Key words: Wnt;  $\beta$ -catenin; Chromatin looping; Wnt responsive DNA element; MYC

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Core tip: In colon cancer, mutations in components of the Wnt/ $\beta$ -catenin signaling pathway result in inappropriate *c-MYC* proto-oncogene (*MYC*) expression. To understand colorectal carcinogenesis requires the identification of Wnt responsive DNA elements (WREs) that control MYC expression in colorectal cancer (CRC). Through efforts to characterize MYC WREs, a model has emerged where several of these WREs appear largely dispensable for intestinal homeostasis, but are instead "hijacked" by oncogenic Wnt/ $\beta$ -catenin signaling to drive CRC. These findings raise the intriguing possibility that these WREs may be targeted therapeutically as an alternative approach to treat individuals afflicted by CRC. In this review, we summarize the literature describing the identification of MYC WREs and discuss how those involved in colorectal carcinogenesis may be targeted to limit progression of CRC.



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#### INTRODUCTION

The canonical Wnt/ $\beta$ -catenin signaling pathway regulates stem cell self-renewal, cell proliferation, and cell-fate decisions in the intestinal crypt microenvironment<sup>[1]</sup>. The β-catenin transcriptional co-activator governs the Wnt response, therefore, its distribution and levels within a cell are tightly regulated<sup>[2]</sup>. When cells, such as the differentiated cells in the apical regions of the intestinal crypts, are not exposed to Wnt, cytoplasmic  $\beta$ -catenin associates with a multi-protein "destruction complex"<sup>[3]</sup>. This complex contains adenomatous polyposis coli (APC), the axis inhibition proteins 1 and/or 2 (AXIN1/2), casein kinase 1 (CK1), and glycogen synthase kinase three beta (GSK3<sub>β</sub>). Here, APC and AXIN1/2 function as scaffolds to position  $\beta$ -catenin in proximity of CK1 and GSK3 $\beta$ . Phosphorylation of β-catenin by CK1 and GSK3β prime it for ubiquitination by the  $\beta$ -transducin-repeat-containing protein ( $\beta$ -TrCP) and subsequent degradation *via* the proteasome<sup>[4]</sup>. In the absence of Wnt, members of the T-cell factor/Lymphoid enhancer factor (TCF/Lef) family of sequence-specific transcription factors bound to Wnt responsive DNA elements (WREs) recruit transcriptional corepressor complexes. These complexes include transducin-like enhancer (TLE) and C-terminal binding protein, which associate with histone deacetylases to repress target gene expression (Figure 1A)<sup>[5]</sup>.

The basal regions of the intestinal crypts contain stem cells and transit-amplifying progenitor cells<sup>[6]</sup>. These cells are exposed to Wnt ligand, secreted by the surrounding mesenchyme and differentiated Paneth cells, which binds frizzled (FZD)/low-density lipoprotein receptor-related protein 5 or 6 receptor complexes expressed on the cell surface<sup>[3]</sup>. This binding results in the subsequent recruitment of AXIN1/2 to the plasma membrane via interaction with dishevelled proteins (DVLs) and inactivation of the destruction complex. β-Catenin then escapes proteasomal degradation, accumulates in the cytoplasm, and translocates into the nucleus where it displaces TLE from TCF/Lef bound WREs<sup>[1]</sup>. β-Catenin/ TCF/Lef complexes in turn recruit histone-modifying complexes, such as CBP/p300 protein acetyltransferases and MLL/Set methyltransferases, and chromatin remodeling complexes, including SWI/SNF, to induce expression of Wnt/ $\beta$ -catenin target genes and drive cellular proliferation (Figure 1B)<sup>[7]</sup>.

Colorectal cancer (CRC) is the third leading cause of cancer related deaths in the United States. In 2015, 140000 individuals are predicted to be diagnosed with CRC and over 50000 patients are predicted to succumb to the disease<sup>[8]</sup>. Approximately 90% of sporadic CRCs contain mutations in components of the Wnt/β-catenin signaling pathway<sup>[9]</sup>. These mutations are found in the earliest neoplasms suggesting that this pathway serves as a critical gatekeeper to prevent colorectal carcinogenesis<sup>[10]</sup>. Most tumors contain a mutation in a single component of the pathway, although recent data from the cancer genome atlas consortium indicates that mutations in multiple components can cooccur<sup>[11]</sup>. The majority of mutations, some 85%, map to "hotspots" within APC and often lead to expression of a truncated APC protein from this allele<sup>[12]</sup>. This truncated protein is incapable of incorporating into a functional  $\beta$ -catenin destruction complex. Thus, when the wildtype APC is inactivated by mutation or lost through loss of heterozygosity,  $\beta$ -catenin levels inappropriately accumulate in the cell, leading to aberrant expression of Wnt/β-catenin target genes and the development of benign adenomas (Figure 1C)<sup>[1]</sup>. As these adenomas accumulate additional mutations in other signaling pathways, they transition into carcinomas<sup>[13]</sup>. Therefore, CRC is a disease of uncontrollable Wnt/β-catenin signaling where  $\beta$ -catenin/TCF complexes bound to WREs drive pathogenic expression of downstream target genes. To understand CRC initiation and progression requires identification of these genes and the WREs that control their expression.

The *c*-MYC proto-oncogene (MYC) was identified as a Wnt/ $\beta$ -catenin target gene using a differential RNA expression screen conducted in the human HT29 CRC cell line harboring mutant *APC* alleles<sup>[14]</sup>. MYC is a basic helix-loop-helix zipper (bHLHZ) transcription factor that heterodimerizes with bHLHZ factor MAX<sup>[15]</sup>. MYC: MAX heterodimers bind E-box sequence motifs to predominantly activate transcription of genes by recruiting histone modifying and chromatin remodeling complexes<sup>[16,17]</sup>. MYC: MAX regulates expression of thousands of target genes whose products control a widerange of cellular processes including metabolism, ribosome biogenesis, and protein synthesis<sup>[18]</sup>. As such, MYC promotes cellular proliferation and cell growth<sup>[19]</sup>.

MYC expression is deregulated in 50% of all cancers, including CRC<sup>[20]</sup>. In fact, Myc is required for tumorigenesis in mouse models of CRCs<sup>[21-25]</sup>. Given that deregulated MYC expression by oncogenic Wnt/β-catenin signaling is critical for colorectal carcinogenesis, several groups have sought to identify and characterize WREs that control its expression in human CRC cell lines over the years (Table 1). In this review, we summarize the literature pertaining to WREs that regulate MYC in CRC cells. We define a MYC WRE as a region of DNA that is: (1) bound by  $\beta$ -catenin/TCF complexes; (2) associated with histone modifications that demarcate enhancer elements, such as monomethylated lysine 4 on histone H3 (H3K4me1) and acetylated lysine 27 on histone H3 (H3K27Ac); (3) responsive to  $\beta$ -catenin/ TCF complexes in luciferase reporter assays; and/or (4) juxtaposed to the MYC promoter region through chromatin loops, if it maps distal to the MYC gene. We then discuss

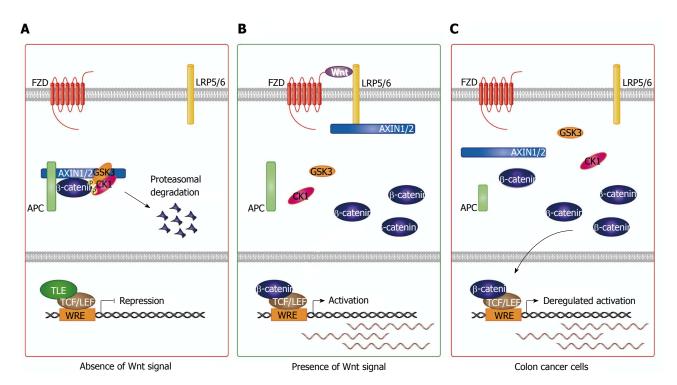


Figure 1 The Wnt/ $\beta$ -catenin signaling pathway. A: In the absence of a Wnt ligand, cytoplasmic  $\beta$ -catenin is targeted for proteasomal degradation by a multiprotein "destruction complex". Within the nucleus, TCF/Lef at WREs associates with the TLE co-repressor to repress Wnt/ $\beta$ -catenin target gene expression; B: Upon Wnt ligand binding to the FZD and LRP5/6 co-receptor complex, AXIN1/2 is recruited to the plasma membrane and the destruction complex is inactivated.  $\beta$ -Catenin subsequently translocates to the nucleus where it binds TCF/Lef assembled at WREs and recruits co-activator complexes to activate Wnt/ $\beta$ -catenin target gene expression; C: In CRCs, truncations of the APC protein prevent efficient targeting of  $\beta$ -catenin for proteasomal degradation. Therefore, nuclear  $\beta$ -catenin levels are elevated and aberrantly associate with TCF/Lef at WREs to drive deregulated expression of Wnt/ $\beta$ -catenin target genes. WRE: Wnt responsive DNA element; TCF/ Lef: T-cell factor/Lymphoid enhancer factor; TLE: Transducin-like enhancer; FZD: Frizzled; LRP5/6: Lipoprotein receptor-related protein 5 or 6; AXIN1/2: Axis inhibition proteins 1 and/or 2; CRC: Colorectal cancer; APC: Adenomatous polyposis coli; GSK3: Glycogen synthase kinase three; CK1: Casein kinase 1.

| MYC WRE            | Chromosomal position | Distance to MYC TSS | Ref.          |
|--------------------|----------------------|---------------------|---------------|
| MYC 5' WREs        | chr8: 128747425      | 889-bp upstream     | [14,28]       |
| MYC 3' WRE         | chr8: 128755419      | 7105-bp downstream  | [26,27,31]    |
| Myc 3' WRE         | chr15: 61992066      | 6725-bp downstream  | [32,33]       |
| MYC -335 WRE       | chr8: 128413279      | 335036-bp upstream  | [46-51,55,56] |
| Myc -335 WRE       | chr15: 61450712      | 534630-bp upstream  | [52]          |
| MYC -520 WRE       | chr8: 128227619      | 520696-bp upstream  | [59,61]       |
| MYC -517 WRE       | chr8: 128231269      | 517046-bp upstream  | [59]          |
| MYC -513 WRE       | chr8: 128235209      | 513106-bp upstream  | [59,61]       |
| MYC -488 WRE       | chr8: 128259779      | 488536-bp upstream  | [59,61]       |
| MYC -470 WRE       | chr8: 128278129      | 470186-bp upstream  | [59,61]       |
| MYC super-enhancer | chr8: 128275000      | 473314-bp upstream  | [61,69]       |

TSS: Transcription start site; WRE: Wnt responsive DNA element.

future avenues of research aimed at determining which of these are the critical drivers for oncogenic *MYC* expression and how these results may be leveraged to develop new therapeutic options to manage individuals afflicted by CRC.

#### PROMOTER PROXIMAL MYC WREs

In the aforementioned study identifying *MYC* as a target of oncogenic Wnt/ $\beta$ -catenin signaling in human CRC cells, He *et al*<sup>[14]</sup> localized a  $\beta$ -catenin-responsive region within the *MYC* proximal promoter (Figure 2). This region

contained two TCF binding elements, TBE1 and TBE2, which mediated  $\beta$ -catenin/TCF-responsiveness of this element in luciferase reporter assays. While expression of a dominant negative TCF4 protein lacking its aminoterminal  $\beta$ -catenin interaction domain decreased *MYC* expression in CRC cells, it was not evaluated at that time whether  $\beta$ -catenin/TCF complexes occupied this region at the endogenous *MYC* locus. However, this study identified the first *MYC* WRE and we have referred to it as the *MYC* 5' WRE in our studies of WREs that control *MYC* expression in human CRC lines<sup>[26,27]</sup>.

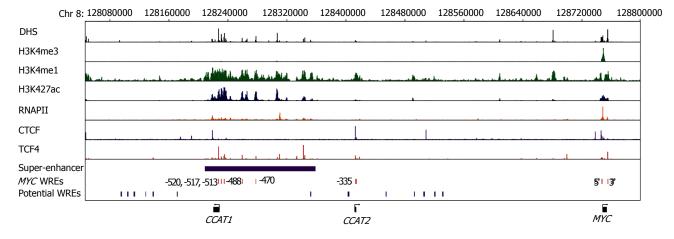


Figure 2 The MYC genomic locus in colorectal cancer. ChIP-Seq and DHS data in the HCT116 CRC cell line were downloaded from the WashU Epigenome Browser (http://epigenomegateway.wustl.edu/). The MYC distal super-enhancer is denoted as a purple rectangle, the MYC WREs discussed in this review are depicted as red lines, and potential WREs are denoted as blue lines<sup>[50,73]</sup>. WRE: Wnt responsive DNA element; CTCF: CCCTC-binding factor; H3K4me1: Monomethylated lysine 4 on histone H3; H3K27Ac: Acetylated lysine 27 on histone H3; RNAP: RNA Polymerase II; TCF: T-cell factor; CCAT: Colon-cancer associated transcript; DHS: DNase hypersensitivity.

Sierra *et al*<sup>[28]</sup> characterized a region mapping approximately 1200-bp upstream from the *MYC* transcriptional start site (TSS), and slightly upstream from *MYC* 5' WRE, that harbors a third TBE, TBE3. In an extensive set of chromatin immunoprecipitation (ChIP) analyses, they found that  $\beta$ -catenin/TCF complex binding to this region correlated with *MYC* transcription in CRC cells. They also demonstrated that when Wnt signaling was shut-off, APC removed  $\beta$ -catenin and transcriptional co-activators from this site and replaced them with transcriptional corepressors to repress *MYC* expression. Interestingly, unlike wild-type APC, truncated APC found in CRC cells was unable to mediate this corepressor exchange, providing one mechanism underlying how APC mutations lead to aberrant *MYC* expression.

The identification and characterization of *MYC* promoter proximal WREs was an important step forward in our understanding of how  $\beta$ -catenin/TCF complexes drive *MYC* expression. However, very low levels of *MYC* transcripts are produced from a transgene in mice containing the human *MYC* genomic locus, and regions 2.3-kb upstream and 0.4-kb downstream, suggesting that additional WREs may contribute to regulating *MYC* expression<sup>[29]</sup>.

# THE DOWNSTREAM *MYC* PROXIMAL WRE

To identify regulatory elements that control *MYC* expression, Mautner *et al*<sup>(30]</sup> mapped DNaseI hypersensitivity sites in proximity to the *MYC* gene in the Colo320 CRC cell line. This strategy mapped a strong hypersensitivity site 1.5-kb downstream from the *MYC* transcription stop site, although it was not determined at that time whether this region demarcated a WRE. In a subsequent study, some 13 years later, a strong  $\beta$ -catenin binding region that overlapped this hypersensitive site was identified in the HCT116 human CRC cell line<sup>[31]</sup>. Using a combination of ChIP assays, luciferase reporter assays, and MYC transcript analysis, it was determined that this  $\beta$ -catenin/TCF binding element demarcated a WRE termed the MYC 3' WRE (Figure 2)<sup>[26]</sup>. Using chromosome conformation capture (3C), it was demonstrated that  $\beta$ -catenin/TCF4 complexes coordinated a chromatin loop that integrated MYC 5' and 3' WREs in CRC cells<sup>[27]</sup>. This chromatin conformation was absent in guiescent CRC cells when MYC expression is silenced, but was induced by serum mitogens as MYC levels increased, indicating that loop formation accompanied MYC transcriptional activation. Interestingly, in HEK293 cells that contain an intact Wnt/β-catenin signaling pathway, either treatment with Wnt3A ligand or activation of the pathway with the GSK3 inhibitor lithium chloride (LiCl), failed to induce the chromatin loop even though MYC expression was increased. However, over-expression of oncogenic  $\beta$ -catenin (S45F) in these cells induced the MYC 5'3' loop. Thus, the MYC 5'3' chromatin conformation required elevated levels of nuclear  $\beta$ -catenin that typifies CRC.

In the colonic crypts of mice harboring a germ-line deletion of the Myc 3' WRE, there was a 2-fold increase in Myc transcript levels and a 2.5-fold increase in MYC protein levels relative to levels seen in the colons of wild-type littermates<sup>[32]</sup>. This increase in MYC correlated with an increase in the number of proliferative cells and a decrease in the number of differentiated cells comprising the colonic crypt epithelium. Two approaches were taken to ascertain the role of this WRE in colorectal carcinogenesis<sup>[33]</sup>. First, Myc 3' WRE<sup>-/-</sup> mice were bred to Apc<sup>Min/+</sup> mice that harbor a mutation in one Apc allele and spontaneously form intestinal tumors as the mice age and the second wild-type Apc allele is lost<sup>[34,35]</sup>. In comparison to Apc<sup>Min/+</sup>, Apc<sup>Min/+</sup> Myc 3' WRE<sup>-/-</sup> mice contained 4-fold more colonic tumors. Second, Myc 3' WRE<sup>-/-</sup> mice and wild-type littermates were subjected to the azoxymethane/dextran sodium sulfate model of colitis-associated colorectal cancer (CAC). *Myc* 3' WRE<sup>/-</sup> mice subjected to this protocol contained an elevated number of tumors along the entire colonic tract, most notably in the ceca, which presented 20-fold more tumors. Thus, the primary function of this WRE is to repress *Myc* in the colonic epithelium.

#### DISTAL MYC WREs

#### The MYC -335 WRE

The MYC locus maps within a gene-poor region on chromosome 8q24<sup>[36]</sup>. Upstream of MYC, results from genome-wide association studies identified regions that harbor single nucleotide polymorphisms that are associated with an increased risk for the development of colon, breast, and prostate cancers<sup>[37-44]</sup>. One SNP in particular, rs6983267, has received interest in the field, with the G allele associated with increased risk of CRC over the T allele<sup>[40,42,44]</sup>. Despite this relationship, it was unknown how this SNP contributed to CRC, as MYC, the nearest protein-coding region, resides 335-kb downstream<sup>[45]</sup>. Using the enhancer element locator computational method, Tuupanen et al<sup>[46]</sup> found that a region encompassing rs6983267 was predicted to harbor a strong enhancer. Importantly, rs6983267 is adjacent to a TCF4 binding motif and was predicted to influence the binding of this factor. Indeed, the G allele conferred greater Wnt-responsiveness through this site that correlated with enhanced TCF4 binding. In an accompanying manuscript, Pomerantz et al[47] shed light on the mechanism for how the rs6983267 risk variant might influence MYC expression. They found that this MYC -335 WRE was physically juxtaposed to the MYC promoter region through a long-range chromatin loop. This result was confirmed by both Ahmadiyeh et al<sup>[48]</sup> and Jäger et al<sup>[49]</sup> in studies to identify regions of 8q24 that interact with the MYC -335 WRE by 3C and capture Hi-C, respectively. Furthermore, Sotelo et al<sup>(50)</sup> also reported evidence for this long-range interaction and found that overexpression of  $\beta$ -catenin and TCF4 increased the frequency of the association. However, a subsequent study found that while the SNP had no effect on the efficiency of chromatin looping, the risk-associated allele increased expression of the linked MYC allele<sup>[51]</sup>. Although the precise mechanistic details remain to be fully defined, these findings offer a potential explanation for how transcription factors bound to a distal regulatory element can drive oncogenic MYC expression in CRC.

In 2012, Sur *et al*<sup>(52)</sup> reported findings from a mouse model containing a germ-line deletion in the *Myc* -335 WRE. As was the case in *Myc* 3' WRE<sup>-/-</sup> intestines, deletion of the *Myc* -335 WRE did not cause gross phenotypic alterations of the intestinal architecture. Moreover, deletion of the *Myc* -335 WRE did not alter the cellular composition of the intestinal epithelium or influence expression of *Myc* in the duodenum. However, *Myc* expression in the colons of *Myc* -335 WRE<sup>-/-</sup> was decreased relative to levels seen in the colons of wildtype littermates. Importantly, when these mice were bred to  $Apc^{Min/+}$  mice, deletion of the Myc -335 WRE led to a reduced number of tumors that formed in both the small intestines and colons. Thus, this element is a critical regulator of intestinal tumors that arise from pathogenic Wnt/ $\beta$ -catenin signaling.

As the Apc<sup>Min/+</sup> mouse is an important model for CRC, it is somewhat limited by the fact that tumors preferentially arise in the small intestine and not the colon<sup>[53]</sup>. A recent study suggests that this phenotype may be due to the fact that stem cells in the small intestine divide more rapidly than those in the colons and hence there is an increased chance of accumulating mutations in the small intestine<sup>[54]</sup>. In addition, tumors that arise in  $Apc^{Min/+}$  mice are primarily adenomas, which do not progress to carcinomas because the mice become moribund<sup>[53]</sup>. Therefore, to determine whether the MYC -335 WRE is required in colorectal carcinomas requires deleting this regulatory element in a human CRC cell line. As part of an elegant study to functionally annotate genes whose expression is influenced by colon cancer risk SNPs, Yao et al<sup>[55]</sup> used clustered regulatory interspaced short palindromic repeats/Cas9 (CRISPR/ Cas9) to delete the MYC -335 WRE in HCT116 human CRC cells. While MYC -335 WRE<sup>-/-</sup> cells expressed less MYC relative to parental cells, it was not reported whether this influenced the oncogenic properties of these cells.

In an intriguing study, Ling et al<sup>[56]</sup> offered an additional explanation for how the MYC -335 WRE influences MYC-driven colorectal carcinogenesis. They found that a long non-coding RNA, which they termed colon-cancer associated transcript two (CCAT2), is expressed from this region and contains the rs6983267 SNP. CCAT2 expression is elevated in colon tumors relative to levels detected in adjacent and uninvolved colonic mucosa. Overexpression of CCAT2 in HCT116 cells promoted tumorigenesis when these cells were implanted into immunocompromised mice, whereas CCAT2 knockdown diminished the invasive capacity of the KM12SM CRC cell line. Moreover, CCAT2 overexpression increased MYC expression in HCT116 cells, whereas CCAT2 knockdown decreased MYC expression in these cells. CCAT2 itself is a Wnt/β-catenin target gene, and it interacts directly with TCF4 to augment TCF4-dependent expression of Wnt/ $\beta$ -catenin target genes, including *MYC*. Further studies are necessary to fully understand how the rs6983267 SNP impinges upon CCAT2 function.

In an effort to understand the function of rs6983267 in colorectal carcinogenesis, Kim *et al*<sup>[57]</sup> doned seven lncRNAs derived from the 8q24.21 gene desert region. It was found that one of these lncRNAs, termed cancer-associated region long non-coding RNA number 5 (*CARLo5*), played an important role in driving CRC. *CARLo5* expression was elevated in CRC relative to expression in normal adjacent tissues, and knocking down *CARLo5* expression diminished CRC cell proliferation and growth of these cells as tumors in athymic nude mice. Interestingly, a region containing the

*MYC*-335 WRE was juxtaposed to the *CARLo5* promoter region to drive its expression in CRC cells.

#### The MYC distal super-enhancer

In 2010, Bottomly et al<sup>[58]</sup> reported results from a ChIP-Seq screen to localize  $\beta$ -catenin binding sites throughout the genome in the HCT116 human CRC cell line. This screen confirmed that  $\beta$ -catenin bound the MYC 5' WRE, 3' WRE, and -335 WRE in these cells. In addition, they noted that a cluster of  $\beta$ -catenin binding sites mapped to a region approximately 400-500-kb upstream from the MYC TSS. In a follow-up study, it was found that these regions bound TCF4,  $\beta$ -catenin, and RNA Polymerase II (RNAP) and are demarcated by histones with modifications that typify enhancer regions including H3K4me1 and H3KAc<sup>[59,60]</sup>. Four of five of these distal β-catenin-bound regions formed longrange chromatin loops with the MYC proximal promoter region. Interestingly, these conformations were not only present in HCT116 cells, but also the non-CRC cell lines HEK293 and TIG-1, suggesting that  $\beta$ -catenin might use these pre-existing chromatin loops to activate MYC gene expression. Moreover, the interaction frequencies between two of these regions and the MYC proximal promoter were induced upon stimulation of the cells with serum mitogens.

Several years later, Hnisz et al<sup>[61]</sup> found that this distal cluster of MYC WREs overlapped with a super-enhancer. According to Whyte *et al*<sup>[62]</sup>, super-enhancers are "...large clusters of transcriptional enhancers - formed by binding of high levels of master TFs (transcription factors) and Mediator coactivator - that drive expression of genes that define cell identity". These super-enhancers also bind high levels of the chromatin-associated protein bromodomain containing protein 4 (BRD4)<sup>[63]</sup>. Through its bromodomain, BRD4 associates with acetylated histones and recruits the positive transcription elongation factor (PTEF-b) to promote transcriptional elongation by RNAP<sup>[64,65]</sup>. BRD4 also interacts directly with the Mediator complex, and Mediator has been shown to play a role in chromatin looping<sup>[64-66]</sup>. JQ1 is a selective inhibitor of BRD4 activity that competes with BRD4 binding to acetylated substrates<sup>[67]</sup>. Interestingly, treatment of multiple myeloma cells with JQ1 led to a preferential loss of BRD4 occupancy at super-enhancers and repression of oncogene expression, including  $MYC^{[63,68]}$ . A comparison of H3K27Ac patterns obtained from ChIP-Seg analysis in normal colonic mucosa and the HCT116 CRC cell line revealed that high levels of nucleosomes containing this modification map to the distal MYC super-enhancer in CRC cells<sup>[61]</sup>. By overlaying the ChIP-Seq profile for TCF4 binding in these cells, Hnisz et al<sup>[61]</sup> identified four putative WREs embedded within the distal superenhancer that corresponded to four of the five MYC WREs identified previously in this region by Yochum<sup>[59]</sup>. Indeed, luciferase assays conducted with reporters containing these elements confirmed that they functioned as WREs<sup>[61]</sup>.

As is the case for the MYC -335 WRE, a IncRNA temed colon cancer-associated transcript one (CCAT1), localizes within the *MYC* super-enhancer<sup>[69]</sup>. Two isoforms of CCAT1 are expressed; a long form, CCAT1-L, which is 5200-bp in length, and a short form, CCAT1-S, which is 2600-bp in length. Both isoforms are expressed at higher levels in CRCs relative to levels in patient matched uninvolved colonic mucosa<sup>[69-71]</sup>. In contrast to CCAT1-S, which localizes to the cytoplasm, CCAT1-L localizes to the nucleus. CCAT1-L knockdown in CRC cells reduces MYC expression, while increased expression of CCAT1-L from its chromosomal locus enhances MYC expression. It was demonstrated that CCAT1-L is an important regulator of chromatin looping between MYC WREs and the MYC promoter. Interestingly, this IncRNA not only promoted chromatin looping between the MYC super-enhancer and the MYC promoter, but also between the MYC superenhancer and the MYC -335 WRE. In addition, the CCCTC-binding factor (CTCF) was shown to play a role in mediating the interactions between distal MYC WREs and the MYC promoter. CCAT1-L interacted directly with CTCF and stabilized CTCF binding to distal WREs, providing a mechanism for CCAT1-L-mediated regulation of MYC.

## ACTIVATION OF *MYC* EXPRESSION BY ONCOGENIC WNT/β-CATENIN SIGNALING

To summarize, mutations in components of the Wnt/  $\beta$ -catenin signaling pathway lead to aberrant MYC expression in CRCs through  $\beta$ -catenin/TCF transcription complexes bound to WREs. The MYC 3' WRE, MYC -335 WRE, and distal super-enhancer are juxtaposed to the MYC 5' WRE within the proximal promoter region through long-range chromatin loops (Figure 3)<sup>[27,47-51,59,69]</sup>. We therefore propose a model in which "hijacked" distal WREs align to the MYC proximal promoter and increase the local concentration of  $\beta$ -catenin/TCF transcription complexes to drive oncogenic MYC expression in CRC. Thus, the MYC proximal promoter serves as a "landing pad" to coordinate chromatin conformations at MYC. As the 3C technique represents an average interaction frequency across a population of cells, it is unknown whether these chromatin loops occur simultaneously at a single MYC allele<sup>[72]</sup>. It is also important to note that in several cases, these chromatin loops are not restricted to CRC cells and their formation does not depend on Wnt/ $\beta$ -catenin signaling<sup>[48,51,59]</sup>. Thus, the conformation itself, and not its formation, may poise the MYC locus to receive oncogenic signals. Finally, it is probable that additional WREs and other enhancer elements contribute to deregulated MYC expression in CRC cells<sup>[50,73]</sup>.

#### CONCLUSION

Despite the identification of MYC over 30 years ago



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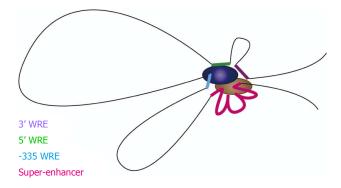


Figure 3 Model for the chromatin interaction network at the MYC gene locus in colorectal cancer cells with elevated nuclear  $\beta$ -catenin.  $\beta$ -Catenin "hijacks" MYC WREs in CRC cells, therefore, driving or stabilizing a distinct promoter-enhancer interaction network that is required for deregulated MYC gene expression. TCF/Lef and  $\beta$ -catenin are depicted as brown and purple ovals, respectively. WRE: Wnt responsive DNA element; CRC: Colorectal cancer; TCF/Lef: T-cell factor/Lymphoid enhancer factor.

and numerous reports linking deregulated MYC gene expression to tumorigenesis, there is still no clinically available drug that targets MYC in cancer cells<sup>[74-76]</sup>. This is in part due to the fact that, unlike other proteins, MYC is very rarely mutated in cancers<sup>[76]</sup>. Instead, MYC is overexpressed due to aberrant activation of upstream signaling pathways or due to events that trigger amplification of the MYC gene locus or insertions of activating sequences<sup>[77]</sup>. It is, therefore, difficult to target MYC in cancer cells vs normal, healthy cells where MYC is required for cellular proliferation<sup>[76]</sup>. Despite these difficulties, recent approaches to inhibit the BRD4 chromatin reader have been demonstrated to specifically and profoundly diminish MYC gene expression in several cancer cells<sup>[63,68,78-80]</sup>. These studies indicate that targeting MYC at the transcriptional level is an effective therapeutic strategy for treating cancers with deregulated MYC gene expression. Therefore, further characterization of the MYC WREs discussed in this review will likely provide new avenues for targeting MYC gene expression in CRC cells.

Recent reports have indicated that active DNA enhancer elements express short, bidirectional RNAs termed enhancer RNAs (eRNAs)<sup>[81-84]</sup>. Expression level changes observed for eRNAs correspond to changes in mRNA levels of the target gene, but the mechanism through which eRNAs activate target gene expression appears to vary based on cellular context. eRNAs have been found to stabilize enhancer-promoter interactions and also to relieve transcriptional pausing by inactivating the negative elongation factor complex<sup>[85,86]</sup>. It is unknown whether eRNAs are transcribed from the MYC WREs described in this report. It is feasible that eRNA transcripts derived from these MYC WREs facilitate MYC gene expression by stabilizing MYC WRE interactions with the MYC promoter. Additional factors could also be functioning to stabilize chromatin loops and activate *MYC* expression.  $\beta$ -Catenin was recently demonstrated to recruit cohesin and direct enhancerpromoter interactions in human embryonic stem cells<sup>[87]</sup>. If β-catenin also recruits cohesin at MYC WREs in CRC cells, it could explain how "hijacked" *MYC* WREs interact with the *MYC* promoter or suggest that  $\beta$ -catenin stabilizes pre-existing chromatin loops. CTCF could also be a critical factor for maintaining the genomic architecture at the *MYC* gene locus, as the interaction frequency of the *MYC* super-enhancer with the *MYC* promoter is diminished after CTCF knockdown<sup>[69]</sup>. To provide a better understanding of how  $\beta$ -catenin drives deregulated *MYC* gene expression in CRC cells, future work is needed to define the factors critical for *MYC* chromatin conformations and to determine the role of these conformations in activating *MYC* expression in CRCs.

Before candidate WREs can be considered as therapeutic targets, proof-of-principle experiments are required. Namely, whether these elements are dispensable in the normal colonic epithelium, but essential for colon carcinogenesis, which would suggest that a subset of WREs may be "hijacked" by oncogenic Wnt/β-catenin signaling. Indeed the mouse studies described by Sur et al<sup>[52]</sup> indicated that the Myc -335 WRE did not play a role in intestinal homeostasis, but was required for intestinal tumorigenesis caused by mutations in Apc. To determine whether this element was required in HCT116 human CRC cells, Yao et al<sup>[55]</sup> deleted it using CRISPR/Cas9 gene editing. While this deletion reduced MYC expression in these cells, it was not reported whether it altered the chromatin conformation at the MYC locus or reduced the oncogenic potential of these cells. Interestingly, the MYC distal super-enhancer is preferentially activated in CRC and not uninvolved colonic mucosa<sup>[61,88]</sup>. However, due to its size and complexity, it may be difficult to target using gene editing strategies. Although we reported that the Myc 3' WRE suppressed colonic tumorigenesis in mice, we noted that deletion of this element decreased tumorigenesis in the small intestines of  $Apc^{Min/+}$  mice<sup>[33]</sup>. Therefore, we used CRISPR/Cas9 to target the MYC 3' WRE in HCT116 cells (SAR, GSY, unpublished). Using this approach, we generated clonal cell lines that harbored homozygous deletions in one of two TBEs within this element. These cells contain reduced MYC expression at the transcript and protein levels and also display reduced oncogenic properties.

Further investigation of the factors required for WREmediated transcriptional regulation of *MYC* will provide a more detailed model of how *MYC* gene expression becomes deregulated in CRCs. This model can then be applied to investigate deregulated *MYC* expression as a result of constitutive upstream signaling pathway activation in other cancers and also to identify potential therapeutic targets.

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REVIEW

## Crosstalk between mitochondria and peroxisomes

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#### Abstract

Mitochondria and peroxisomes are small ubiquitous organelles. They both play major roles in cell metabolism, especially in terms of fatty acid metabolism, reactive oxygen species (ROS) production, and ROS scavenging, and it is now clear that they metabolically interact with each other. These two organelles share some properties, such as great plasticity and high potency to adapt their form and number according to cell requirements. Their functions are connected, and any alteration in the function of mitochondria may induce changes in peroxisomal physiology. The objective of this paper was to highlight the interconnection and the crosstalk existing between mitochondria and peroxisomes. Special emphasis was placed on the best known connections between these organelles: origin, structure, and metabolic interconnections.

Key words: Peroxisome; Mitochondrion; Beta-oxidation; Reactive oxygen species; Dynamic; Fatty acids

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**Core tip:** The goal of this review was to highlight the links between mitochondria and peroxisomes in terms of dynamic and metabolism. This review of the literature shows that these two organelles, even if they derive from distinct ancestors, share several common functions and coordinate their activities. The division of peroxisomes and mitochondria uses similar mechanisms, and autophagic processes are used to limit the number of both organelles. The metabolic implication of mitochondria and peroxisomes in fatty acid metabolism is remarkable, as these organelles use closely-related pathways for oxidizing fatty acid, but with different metabolic goals. All together, the available data suggest a major interconnection between these organelles.

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#### INTRODUCTION

Mitochondria and peroxisomes are small organelles present in almost every cell of higher organisms. They share some properties (such as size and function), but differ in terms of origin, structure, and physiological roles. With the current knowledge of the subject, it



seems clear that these two organelles communicate in the cell, and in some cases participate at the same biosynthetic pathways. They share many enzymes and mitochondrial function has been shown to regulate peroxisomal activity.

Peroxisomes and mitochondria share a common range size of 0.1 to 1  $\mu$ m. However, they differ in terms of structure: Mitochondria are surrounded by a double membrane, while peroxisomes are bordered by a single membrane system. In the cell, the number of peroxisomes and mitochondria varies according to the cell type (*e.g.*, mitochondria are very abundant in brown adipose tissue, but relatively scarce in white adipocytes). Peroxisomal and mitochondrial abundance is regulated by several cellular parameters: (1) organelle formation; (2) organelle dynamic; and (3) organelle death.

### ARE MITOCHONDRIA AND PEROXISOME DERIVED FROM A COMMON ANCESTOR?

#### Origin

Both, peroxisomes and mitochondria are very dynamic organelles. They show high plasticity and are able to adopt various shapes depending on the cell's requirements. Their number and morphology can change according to the metabolic needs of the cell and/or the physiopathological environment.

**Peroxisomal origin:** The origin of peroxisomes is still not fully understood. From the early days of peroxisome discovery, peroxisomes were supposedly of endosymbiotic origin<sup>[1]</sup>. However, this theory is no longer considered, as many reports have suggested that peroxisomes are instead derived from the endoplasmic reticulum<sup>[2]</sup>. This has been especially shown in experiments in which cells with no peroxisomes were able to engender new peroxisomes<sup>[3]</sup>. In fact, several studies have established that peroxisomes can be formed from pre-existing peroxisomes, as well as *de novo* from the endoplasmic reticulum (ER) under certain circumstances<sup>[4,5]</sup>.

When looking at the origin of proteins present in peroxisomes, two categories of proteins can be described: those of a prokaryotic origin and those of an eukaryotic origin<sup>[6]</sup>. Eukaryotic originating proteins are essentially involved in peroxisomal biogenesis, while peroxisomal proteins originating from bacteria appear to be initially targeted to mitochondria. In accordance with the finding that peroxisomes are derived from the ER, many conserved proteins involved in peroxisome biogenesis and repairs are homologous to proteins present in the ER<sup>[7]</sup>. About 25% of proteins in peroxisomes have an origin that is difficult to precisely define. This suggested that some peroxisomal proteins have evolved from mitochondrial proteins. Many proteins are present in both organelles, which suggests that these enzymes may be retargeted from mitochondria to peroxisomes<sup>[8]</sup>.

This would indicate that peroxisome formation is influenced by mitochondria.

However, if peroxisomes are derived from ER, why are mitochondrial proteins included in the organelle and why are mitochondria not assuming these functions? What are the links between ER and mitochondria/peroxisomes? These questions remain as yet unanswered.

**The origin of mitochondria:** The origin of mitochondria is more likely to be endosymbiotic. The endosymbiotic theory indicates that mitochondria were initially free-living prokaryotes that entered eukaryotic cells and became organelles. However, this theory has not always been accepted by the scientific community; it was first proposed for plastids at the beginning of the 20<sup>th</sup> century, but was only accepted for mitochondria for a few years before being rejected by cell biologists. It was not until the 1960's that the theory was reconsidered and more widely accepted<sup>[9]</sup>.

This theory is based on several facts: (1) endosymbiotic organelles retain a small genome encoding several dozen proteins. Regardless of this genome reduction, mitochondria harbor at least 2000 proteins<sup>[10]</sup> involved in many biochemical pathways and, in particular, in energy production. The difference between the number of proteins encoded by the mitochondrial genome and the number of proteins located in this organelle is usually explained by another aspect of endosymbiotic mechanisms, *i.e.*, and (2) the endosymbiotic gene transfer. This process of gene transfer occurs during the evolution of the organism and, in time, will become a protein import mechanism, such as the mitochondrial import system for proteins. This closes the endosymbiotic process circle; the organelle carries out functions that the eukaryotic cell is unable to realize, while the eukaryotic cell provides extra proteins for the organelle. This process gives evolutionary advantages to both the eukaryotic cell and the "new" organelle<sup>[11]</sup>.

Even if they share a few similarities, mitochondria and peroxisomes do not derive from the same evolutionary process, with mitochondria deriving from an endosymbiotic process and peroxisomes from an intracellular maturation process.

#### Protein import

Protein import in peroxisomes: Proteins present in the peroxisome are strictly dependent on nuclear genes and to an import system. Peroxisomes do not possess any DNA sequences and are not able to synthesize any proteins. They rely on cytosolic synthesis from nuclear genes and on the import of these proteins into the peroxisome. This protein import relies on the presence of a peroxisomal targeting signal in the protein sequence: either PTS1 or PTS2. The import of proteins into the peroxisomal matrix is a coordinated process involving the intervention of many proteins known as peroxins (Pex)<sup>[12]</sup>. Pex proteins control peroxisome structure, division, and inheritance. Over a dozen peroxins have

been described<sup>[13]</sup>.

PTS1 is composed of the consensual sequence SKL (or their conserved variants) and is located at the C terminal domain of the protein. It is mostly used by proteins located in the peroxisomal matrix<sup>[14]</sup>. PTS1-containing proteins are recognized by Pex5 protein, whose C-terminal domain (and in rare cases its N-terminal domain) interacts with the PTS1 sequence<sup>[15,16]</sup>. This is a method of peroxisomal import unique to several species, such as the nematode *Caenorhabditis elegans*<sup>[17]</sup>. In mammals, several proteins contain another type of PTS: Type 2 PTS. This motif is recognized by the Pex7 protein, and proteins carrying this PTS2 signal are transported into the peroxisome with the same mechanisms<sup>[18]</sup>.

**Protein import into mitochondria:** Mammalian mitochondria possess their own genome, which is a circular DNA chain of about 16000 base pairs. In animal mitochondria, the genetic code is slightly different from the "universal" code<sup>[19]</sup>. The structure of this genome is simple: there are virtually no non-coding regions and the genes are mostly adjacent to each other. The mitochondrial genome encodes for 2 rRNA, 22 tRNA, and 13 polypeptides involved in mitochondrial respiration<sup>[20]</sup>.

All proteins synthesized from the mitochondrial genome participate in the respiratory chain (complex I, III, IV, and V) and are located in the inner membrane of the mitochondria<sup>[21]</sup>. Among these 13 proteins, 7 are present in complex I (NADH: Ubiquinone oxidoreductase), 1 is part of complex III (ubiquinone: Cytochrome c oxidoreductase), 3 belong to complex IV (cytochrome c: Oxygen oxidoreductase), and 2 are part of complex V (ATP synthase).

This means that the other proteins present in the mitochondria (i.e., around 2000 proteins) are the products of nuclear genes. As for any nuclear genes, the corresponding proteins are synthesized in the cytosol, although mitochondrial proteins are subsequently imported into the mitochondria. The import of these proteins requires that they find their way to the mitochondria. The journey of these precursors throughout the cytosol is supported by mitochondrial targeting elements involved in the transport of the precursors to specific receptors on the mitochondrial surface. This mechanism also depends on cytosolic factors<sup>[22]</sup>. The most common mitochondrial targeting signal is a positively-charged sequence, known as the presequence, which is located at the N-terminus of the protein<sup>[23]</sup>. The presequence addresses proteins to the mitochondrial matrix, the inner membrane, or the mitochondrial intermembrane space. This process is universal as an important part of proteins located in the mitochondrial outer membrane, although many proteins of the inner membrane and the intermembrane space do not possess the classical presequence, and instead enclose internal cryptic targeting sequences in their amino acid sequence.

Once onto the mitochondrial outer membrane,

mitochondrial protein precursors go through the lipid of this membrane with the intervention of the TOM complex (mitochondrial outer membrane preprotein translocase). The TOM complex is made of seven subunits and forms a channel that allows for the crossing of the outer membrane<sup>[24]</sup>.

#### Dynamic

Contrarily to the nucleus that is present as a single organelle in almost all cells, numerous peroxisomes and mitochondria are present, with the actual number depending on the metabolic needs of the cells. The shape and the interconnection among and between these organelles also change depending on the metabolic environment<sup>[25]</sup>.

**Peroxisomal dynamic:** The peroxisomes show high plasticity and a high capacity of adaptation in response to developmental, metabolic, and environmental alterations. Their number, protein content, and shape can be modulated. Peroxisome number can increase either by division of pre-existing organelles or, at least under certain circumstances, from *de novo* biosynthesis from the ER<sup>[26]</sup>. While most of the biochemical processes are involved in this dynamic process, the basic mechanisms and nature of the control of these processes are still poorly understood<sup>[27]</sup>.

**Mitochondrial dynamic:** Mitochondria are also dynamic organelles that permanently change their morphology, size, and number. This dynamic is associated with the processes of fusion/fission that permit the fusion of two mitochondria or the division of a mitochondrion to give rise to two mitochondria, respectively<sup>[28]</sup>.

In the cell, mitochondrial fusion and fission participate in maintaining an adequate mitochondrial number. The fusion process allows mitochondria to combine their whole content. This process participates in mtDNA repair, complementation of proteins, and in the balance of metabolites. Fission also participates in the dynamic of mitochondria, as this process participates in mtDNA segregation. It may also participate in the removal of altered mitochondria through the process of mitophagy. Additionally, these mechanisms of fission/fusion participate in the positive segregation of mitochondria.

The key enzyme for fission of mitochondria is Drp1. This enzyme has a GTPase activity that promotes the fission of mitochondrial lipid membrane<sup>[29]</sup>. Drp1 is the ortholog of Dnm1, a yeast enzyme. The action of Drp1 requires the translocation of this protein onto specific sites located in the outer mitochondrial membrane. Although initially only two proteins were described as docking proteins for Drp1 (Fis1 and Mff), another two (MiD49 and MiD51) were later discovered<sup>[30,31]</sup>.

Drp1 is a very controlled enzyme. Its intracellular level and activity are regulated through various mechanisms, including SUMOylation and phosphorylation<sup>[32,33]</sup>. A recent review has listed and analyzed all of the factors that participate in the regulation of mitochondrial fission<sup>[34]</sup>. The authors emphasized the regulatory role played by Bcl2 family proteins and the regulatory role of TNF-alpha and PKA.

Mitochondrial fusion involves Mfn1 and Mfn2 proteins<sup>[35]</sup>. These mitofusins are large proteins located in the mitochondrial outer membrane. They exhibit GTPase activity and allow the fusion of two outer mitochondrial membranes coming from two distinct mitochondria<sup>[35]</sup>. OPA1, on the other hand, is used in the fusion of mitochondrial inner membranes, and is located in the outer side of the inner mitochondrial membrane<sup>[36,37]</sup>. This process of fusion is highly regulated. To identify the physiological roles of these proteins, mouse mutants have been realized. Mice carrying mutations in the OPA1 gene were created, with the resulting homozygous mice dying during gestation<sup>[38]</sup>. Mfn1- and Mfn2-deficient mice were made, which die during midgestation. Similarly, animal models carrying a deletion of the Drp1 gene also died at the embryonic stage in mice<sup>[39]</sup>.

**Comparison:** While it seems clear that mitochondria and peroxisomes do not derive from a common ancestor, it may be surprising that the fission machinery is in a good part conserved between these organelles. The genetic deletion of Drp1 leads to peroxisomes and mitochondria with an altered structure<sup>[39,40]</sup>. The docking proteins Mff and Fis1 are also important for both organelles<sup>[41]</sup>. This suggests that whatever the origin of these organelles, they are able to interact with each other.

#### Organelle degradation

Autophagy is a genetically programmed process that degrades and removes proteins present in the cell, as well as participating in the removal of damaged or excessive organelles through the initial formation of a structure known as the autophagosome; these structures will then fuse with lysosomes and have their content degraded<sup>[42]</sup>.

The process of autophagy can be divided into five major steps: (1) Development of the isolation membrane; (2) Elongation of this membrane; (3) Closure of the isolation membrane with the formation of the autophagosome; (4) Fusion between the autophagosome and lysosomes; and (5) Degradation of the autophagosome content<sup>[43]</sup>. The overall mechanism is similar in yeast and mammalian cells. In the yeast *Saccharomyces cerevisiae*, nearly 30 autophagy-related proteins have been identified<sup>[44,45]</sup>.

**Pexophagy:** An autophagic process called pexophagy is responsible for regulating the number of peroxisomes in the cell<sup>[46]</sup>. While, in mammalian cells, all aspects of this programmed peroxisome death are not yet fully characterized, some aspects of this process have been described. It has been shown that Pex11p, Pex25p, and Pex27p positively control this mechanism. It was also reported that Drp1 was directly involved in peroxisome division. The implication of Drp1a and Pex11 is not clear

yet. Drp1 and Pex11-beta are overexpressed during this process, but they did not seem to act directly upon  $it^{[47]}$ .

The mechanisms involved have been extensively studied in yeast and have been recently reviewed<sup>[48]</sup>.

**Mitophagy:** Mitophagy is a biological process that allows the elimination of mitochondria using an autophagic process<sup>[49]</sup>. During mitophagy, mitochondria are initially integrated into an autophagosome, which subsequently fuses with lysosomes and leads to the degradation of its content. This mechanism was initially described in cells undergoing starvation, but mitophagy also participates in the regulation of mitochondrion numbers<sup>[50]</sup>.

#### METABOLIC CROSSTALK

#### Reactive oxygen species production and scavenging

Intense oxidation activities occur in both mitochondria and peroxisomes. These organelles are also directly implicated in the production of reactive oxygen species (ROS) through physiological and extraphysiological processes. ROS are able to activate the inflammasome system, which is a multiprotein system coupled with the caspase and interleukin activating systems. The activation of inflammasome leads to a programmed cell death process, and its dysregulation may play a significant role in various diseases<sup>[51]</sup>.

On the other hand, both mitochondria and peroxisomes possess biological tools that allow for the scavenging of damaging reactive oxygen species<sup>[52]</sup>. It is important to note that ROS scavenging is crucial in limiting the cellular damage that these compounds may induce. It should also be noticed that ROS physiologically contribute to various pathways, including those involved in metabolism and signaling.

ROS production in peroxisomes: Many oxidases that produce several kinds of ROS (including nitric oxide, superoxide radicals, hydroxyl radicals, and hydrogen peroxide) are present in peroxisomes. H<sub>2</sub>O<sub>2</sub> is mainly produced by oxidases that use many different types of substrates, such as lactate, urate, or oxalate<sup>[53]</sup>. Peroxisomes are also potential sources of O2• and NO•, via the enzymatic activity of xanthine oxidase and nitric oxide synthase<sup>[54]</sup>. Xanthine oxidase also produces H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>• as byproducts<sup>[54]</sup>. Nitric oxide synthases (NOS) are a broad family of enzymatic proteins, with the inducible form of NOS catalyzing the oxidation of L-arginine to NO• and citrulline in response to induction by cytokines or endotoxins. This reaction requires O<sub>2</sub>, FAD, tetrahydrobiopterin, NADPH, and FMN and, in the absence of adequate amounts of substrates, the enzyme can also produce important amounts of  $O_2 \bullet^{-[55]}$ . Mammalian peroxisomes do not seem to contain enzymes that directly produce •OH or ONOO<sup>-[54]</sup>. However, these ROS might be able to produce them as secondary products from H2O2, O2•, and NO•.

**ROS production in mitochondria:** The mitochondrial electron transport chain is also an important source of ROS. At the complex 1 level, superoxide ions are produced; these ions can be subsequently transformed into more potent ROS.

ROS can induce a vicious cycle that may lead to cell death. Excessive production of ROS is able to damage many macromolecules. Lipids, proteins, and DNA can become targets for ROS, and an inadequate production of ROS will damage mitochondrial enzymes and mitochondrial DNA. Subsequently, these initial damages will induce an altered functioning of the electron transport system and ultimately increase the production of ROS. In addition, overproduction of ROS in mitochondria may lead to the release of cytochrome c and the triggering of apoptosis<sup>[56]</sup>.

**Scavenging of ROS in peroxisomes:** Several antioxidant systems are present in peroxisomes: catalase, Mn-SOD, Cu/Zn-SOD, peroxiredoxin I , epoxide hydrolase, peroxisomal membrane protein 20, and glutathione peroxidase are present in the peroxisomal matrix and contribute to the defense against excessive ROS.

Catalase is the "historic" marker of peroxisomal activity and has a crucial protective function against the peroxides generated in peroxisomes and their toxicity<sup>[57]</sup>. This common marker for peroxisomes metabolizes both  $H_2O_2$  (catalytic function) and many other substrates, such as ethanol, methanol, phenol, and nitrites, through its peroxidatic activity<sup>[58]</sup>. Catalase is targeted to peroxisomes, as it possesses modified PTS1.

The relationship between oxidative stress and the peroxisomal ROS scavenging system has been studied. It has been shown that an increase in oxygen concentration induced a moderate increase in the activity of enzymes involved in the scavenging of ROS<sup>[59]</sup>. On the other hand, low levels of enzymes involved in ROS scavenging, such as catalase, glutathione peroxidase, and Mn-SOD, are commonly observed in malignant cells. In cultured cells, it has been observed that oxidative stress (i.e., UV irradiation or exposure to H2O2) induces pronounced elongation of peroxisomes  $^{\scriptscriptstyle \left[ 60\right] }$  , with antioxidant treatment blocking this elongation process. This elongation step seems a prerequisite for peroxisome division<sup>[61]</sup>. This suggests that peroxisomes can be activated when oxidative stress occurs, indicating that peroxisomes may actively participate in the control of ROS accumulation in the cell.

**Scavenging of ROS in mitochondria:** Mammalian mitochondria possess enzymes and non-enzymatic antioxidants systems for ROS scavenging<sup>[62]</sup>. Enzymes with antioxidant activities such as MnSOD, glutathione reductase, glutathione-S-transferase, and molecules with anti-oxidant properties such as thioredoxin, glutaredoxin, peroxiredoxins, cytochrome c, glutathione, and NADH are present in the mitochondria and participate in limiting oxidative damage; these aspects have been extensively

reviewed by Andreyev *et al*<sup>[62]</sup>. Exposure of cells to a overproduction of ROS induces an increase in activity of the mitochondrial defense system<sup>[62]</sup>.

Scavenging of ROS mobilizes both mitochondria and peroxisomes, as both organelles possess similar systems for counteracting excessive production of ROS. However, the underlying mechanisms involved in the recruitment of either system remain unknown.

#### Fatty acid metabolism in peroxisome and mitochondria

One of the most remarkable common features between mitochondria and peroxisome is the cooperative function for fatty acid oxidation. Fatty acids play many important role in energy production, inflammation and its resolution<sup>[63]</sup>, etc. In mammalian cells, both peroxisomes and mitochondria contain a beta-oxidative pathway. Beta-oxidation is a key pathway for the breakthrough of fatty acids. In yeasts and plants, fatty acid oxidation occurs uniquely in peroxisomes<sup>[64]</sup>, as mitochondria are not able to catabolize fatty acids. In mammalian cells, both peroxisomes and mitochondria can beta-oxidize fatty acids. These two pathways share many similarities, especially in terms of enzymatic reactions, but they differ in terms of substrates and enzymatic reactions. Furthermore, the metabolic implication and final products are not the same in mitochondria and peroxisomes<sup>[65]</sup> (Table 1).

The overall pathways are essentially the same in both mitochondria and peroxisomes: Fatty acids are first activated as acyl-CoA and then the activated fatty acid (acyl-CoA) is dehydrogenated. This represents the first step of beta-oxidation. Hydration of the double bound then occurs, and is followed by dehydrogenation and cleavage. This allows for the removal of 2 C from C: n acyl-CoA, leading to the formation of C:n-2 acyl-CoA.

Peroxisomal fatty acid oxidation: In peroxisomes, the very first step of fatty acid oxidation is the conversion reaction of fatty acid into acyl-CoA. The thus-formed acyl-CoA can be directed to the peroxisomal matrix after crossing the peroxisomal membrane, with the intervention of an ABC transporter (ABCD1) being implied<sup>[66]</sup>. Once in the peroxisomal matrix, betaoxidation starts via a reaction catalyzed by acyl-CoA oxidase (ACOX), an enzyme that is often considered a key element of the process. During the reaction catalyzed by ACOX, electrons provided by FAD are transferred to oxygen, thereby leading to the formation of H<sub>2</sub>O<sub>2</sub>. In the mitochondrial pathway, the reaction is essentially the same, with the exception that the electrons are transferred to the respiratory chain instead. ACOX isoforms have been described in mammals and are all dimeric proteins. The next reaction is catalyzed by an enzyme known as the multifunctional enzyme (MFE), which realizes both the second and the third reactions of peroxisomal beta-oxidation. In the peroxisomal matrix, two MFE are present: MFE-1 [L-bifunctional protein (LBP)] and MFE-2 [D-bifunctional protein (DBP)]; both

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| Table 1 Comparison between mitochondrial and peroxisomal beta-oxidation |                                                  |                                                  |  |  |
|-------------------------------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--|--|
|                                                                         | Mitochondrial                                    | Peroxisomal                                      |  |  |
| Substrates                                                              | LCFA                                             | VLCFA, branched FA, leukotrienes                 |  |  |
| Entry system                                                            | Carnitine system (including CPT1, CACT and CPT2) | ABC transporters                                 |  |  |
| End products                                                            | Acetyl-CoA (and subsequently ATP)                | Acetyl-CoA and MCFA (and subsequently H202)      |  |  |
| Physiological implications                                              | Energy production                                | Biosynthesis of specific fatty acids (e.g., DHA) |  |  |

LCFA: Long-chain fatty acids; VLCFA: Very long chain fatty acids; FA: Fatty acids; CPT: Carnitine palmitoyltransferase; CACT: Carnitine-acylcarnitine translocase; ABC: ATP-binding cassette; MCFA: Medium-chain fatty acids; DHA: Docosahexaenoic acid.

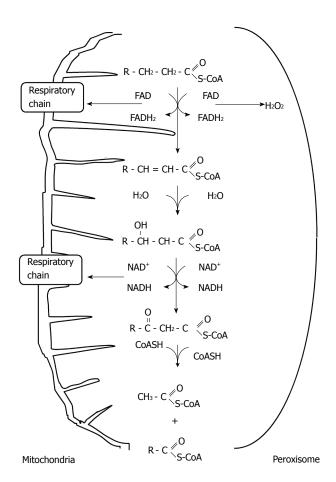


Figure 1 Mitochondrial and peroxisomal beta-oxidations. The reactions involved in mitochondria are presented on the left side of the figure, while the reactions occurring in peroxisome are on the right side of the figure.

catalyze the formation of 3-ketoacyl-CoA intermediates from substrate mirror image stereochemistry. Although the two enzymes catalyze the same reaction, they do not show any similarities in terms of structure<sup>[67]</sup>. The final reaction in this pathway is catalyzed by thiolase. This enzyme catalyzes the thiolytic cleavage of 3-ketoacyl-CoA to acetyl-CoA and Cn-2-acyl-CoA<sup>[68]</sup>. The peroxisomal beta-oxidation is incomplete, as it climaxes with shortened acyl-CoA (medium chain, with one of the common being octanoyl-CoA). These compounds are converted into acylcarnitine by carnitine octanoyl transferase, and can then leave the peroxisome<sup>[69]</sup>.

Peroxisomal beta-oxidation mainly concerns very long chain fatty acids (> C22) and branched fatty acids, as well as some prostaglandins and leukotrienes. While mitochondrial beta-oxidation relies on the energy needs of the cells, peroxisomal beta-oxidation does not. Peroxisomal beta-oxidation is essentially involved in biosynthesis pathways, while the mitochondrial pathway is related to catabolism and energy production. The end product of peroxisomal oxidation of fatty acid is H<sub>2</sub>O<sub>2</sub>, while mitochondrial beta-oxidation is coupled with the production of ATP (Figure 1).

**Mitochondrial fatty acid oxidation:** Mitochondrial beta-oxidation mainly involves long chain fatty acids provided by foodstuff. This pathway supplies the acetyl-CoA used, at least in part, for ATP synthesis (Figure 1).

As for the peroxisomal pathway, mitochondrial fatty acid beta-oxidation requires the initial esterification of fatty acids into acyl-CoA and then the entry of acyl-CoA into the mitochondrial matrix. The activation of fatty acid into acyl-CoA is catalyzed by acyl-CoA synthases, which are ATP-dependent enzymes located in the cytosol. According to the size of the fatty acid, several acyl-CoA synthases with various affinities for different types of substrates carry out these reactions. The following are present in the cytosol: short-chain acyl-CoA, medium-chain acyl-CoA, and long-chain acyl-CoA. Once converted into acyl-CoA, these compounds can cross the mitochondrial membranes; short and mediumchain acyl-CoAs seem to be able to freely cross the mitochondrial membrane while, for long chain acyls, the crossing of the mitochondrial double membrane system requires the intervention of the carnitine system. This system consists of two acyl-transferases (carnitine palmitoyltransferase 1 and 2) and the transporter carnitine acylcarnitine translocase. The presence of L-carnitine is also required as an essential part of this system<sup>[70]</sup>.

Four enzymatic reactions compose the mitochondrial beta-oxidation. The initial reaction is catalyzed by acyl-CoA dehydrogenase, while the subsequent steps are catalyzed by 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-oxoacyl-CoA thiolase, successively. The overall goal of mitochondrial beta-oxidation is the production of energy, mainly as ATP. The beta-oxidation itself consists of successive cycles (consisting of the four aforementioned enzymatic reactions) leading to the removal of 2C from C: n acyl-CoA, thereby generating C: n-2 acyl-CoA. The end-product of this pathway is acetyl-CoA, while mitochondrial beta-oxidation is metabolically coupled with the respiratory chain and tricarboxylic acid

cycle<sup>[71]</sup>.

#### CONCLUSION

While peroxisomes and mitochondria do not derive from a common ancestor (the origin of mitochondria is endosymbiotic, while peroxisomes derive from the endoplasmic reticulum), several proteins are common among these organelles and they share not only a few enzymes, but also full metabolic pathways. Their divisions are closely related and use identical factors and enzymes. This suggests efficient crosstalk between peroxisomes and mitochondria.

However, the physiological function of the two organelles are different. In terms of fatty acid metabolism, mitochondria degrade the majority of long-chain fatty acids to supply acetyl-CoA for the production of ATP and for anabolic reactions, while peroxisomal beta-oxidation is more involved in anabolic processes. However, the two organelles work together for the metabolism of fatty acids. Peroxisomes and mitochondria are independent organelles but their interaction is necessary for optimal function of the cell.

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REVIEW

# Modulation of the matrix redox signaling by mitochondrial Ca<sup>2+</sup>

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#### Abstract

Mitochondria sense, shape and integrate signals, and thus function as central players in cellular signal transduction. Ca<sup>2+</sup> waves and redox reactions are two such intracellular signals modulated by mitochondria. Mitochondrial Ca<sup>2+</sup> transport is of utmost physio-pathological relevance with a strong impact on metabolism and cell fate. Despite its importance, the molecular nature of the proteins involved

in mitochondrial Ca<sup>2+</sup> transport has been revealed only recently. Mitochondrial Ca<sup>2+</sup> promotes energy metabolism through the activation of matrix dehydrogenases and downstream stimulation of the respiratory chain. These changes also alter the mitochondrial NAD(P)H/NAD(P)<sup>+</sup> ratio, but at the same time will increase reactive oxygen species (ROS) production. Reducing equivalents and ROS are having opposite effects on the mitochondrial redox state, which are hard to dissect. With the recent development of genetically encoded mitochondrial-targeted redoxsensitive sensors, real-time monitoring of matrix thiol redox dynamics has become possible. The discoveries of the molecular nature of mitochondrial transporters of Ca<sup>2+</sup> combined with the utilization of the novel redox sensors is shedding light on the complex relation between mitochondrial Ca<sup>2+</sup> and redox signals and their impact on cell function. In this review, we describe mitochondrial Ca<sup>2+</sup> handling, focusing on a number of newly identified proteins involved in mitochondrial Ca<sup>2+</sup> uptake and release. We further discuss our recent findings, revealing how mitochondrial Ca<sup>2+</sup> influences the matrix redox state. As a result, mitochondrial Ca2+ is able to modulate the many mitochondrial redox-regulated processes linked to normal physiology and disease.

Key words: Calcium transport; Signal transduction; Redox regulation; Mitochondria; Oxidation-reduction; Mitochondrial membrane transport proteins; Mitochondrial Ca<sup>2+</sup> uniporter; Mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger

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**Core tip:** Deregulated redox signaling in mitochondria leads to mitochondrial dysfunction, associated with several disorders and disease states. Matrix Ca<sup>2+</sup> rising can be linked through multiple pathways to the mitochondrial redox state. Here we describe recent progress in the field of mitochondrial Ca<sup>2+</sup> handling. We further summarize how mitochondrial Ca<sup>2+</sup> signals are influencing the mitochondrial redox state. This link between Ca<sup>2+</sup> and redox signals is likely



of central importance in the regulation of mitochondrial function in health and disease.

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#### INTRODUCTION

Mitochondria are versatile multifunctional organelles best known for their contribution to cellular energy homeostasis. Mitochondria are also central regulators of cell fate. The involvement of mitochondria in a large number of biological processes is dependent on two unique characteristics. First, mitochondria are organelles able to sense and influence a number of intracellular signals (ions and small molecules, such as Ca<sup>2+</sup>, ATP, pH and the redox potential). Second, these organelles are very dynamic. Mitochondria undergo morphological changes (they fragment and fuse)<sup>[1,2]</sup>, and they increase or decrease in the mass due to mitochondrial biogenesis<sup>[3]</sup> and mitophagy<sup>[4]</sup>. Finally, they can move within the cell<sup>[5]</sup>. Mitochondrial dynamics combined with their ability to control the fluxes of ions and small molecules makes this organelle a central player in signal transduction. Two of the signals strongly affected by mitochondria are Ca<sup>2+</sup> and redox state.

Ca<sup>2+</sup> is a key intracellular messenger that coordinates a vast repertoire of cellular functions, ranging from contraction, secretion and fertilization to the control of transcription, proliferation, several aspects of development as well as learning and memory<sup>[6-8]</sup>. Cells express a large number of proteins for the precise spatial and temporal control of Ca<sup>2+</sup> rising<sup>[6]</sup>. Mitochondria efficiently contribute to the shaping of Ca<sup>2+</sup> signals through Ca<sup>2+</sup> uptake and release<sup>[9-12]</sup>. The associated matrix Ca<sup>2+</sup> rises (transient or prolonged) act as a signal per se that can modulate energy metabolism and cell fate<sup>[12-16]</sup>. Although the basic properties of mitochondrial Ca<sup>2+</sup> handling have been established several decades ago, the molecular identities of the mitochondrial Ca<sup>2+</sup> transport systems have only been revealed over the last 6 years (see section on "Molecular identification of mitochondrial Ca<sup>2+</sup> transporters", below). The identification and study of these transporters has improved our understanding of the physio-pathological role of mitochondrial Ca<sup>2+</sup> transport and provided researchers with new opportunities for molecular intervention.

Signals other than Ca<sup>2+</sup> are generated/integrated in the mitochondrial matrix, notably redox reactions linked to the production of reactive oxygen species (ROS), and changes in the oxidation state of thiol groups in proteins (thiol switches). Redox reactions and associated changes can serve as cellular signals. Metabolites and proteins can activate specific cellular signaling pathways in a redox state-dependent manner<sup>[17,18]</sup>. Thiol switches in proteins are controlled by the balance of two opposite influences, oxidizing and reducing. ROS are able to shift the equilibrium to a more oxidized state. This is particularly relevant in mitochondria, which are a major source of ROS<sup>[19,20]</sup>. Such oxidation in mitochondria is counteracted by reducing systems that depend on the availability of NAD(P)H, which is generated by mitochondrial metabolism<sup>[18]</sup>. The oxidation/reduction of thiol groups in mitochondrial target proteins can modulate their activity, localization and stability. Such changes can regulate mitochondrial functions, including nutrient oxidation, oxidative phosphorylation, ROS production, mitochondrial permeability transition, cell death and mitochondrial morphology<sup>[21]</sup>. The study of redox switches in vivo has been a challenge<sup>[22]</sup>. However, the recent development of fluorescent protein redox sensors has revolutionized the study of redox processes in living cells. They allow real-time compartment-specific monitoring of thiol redox dynamics<sup>[22-25]</sup>.

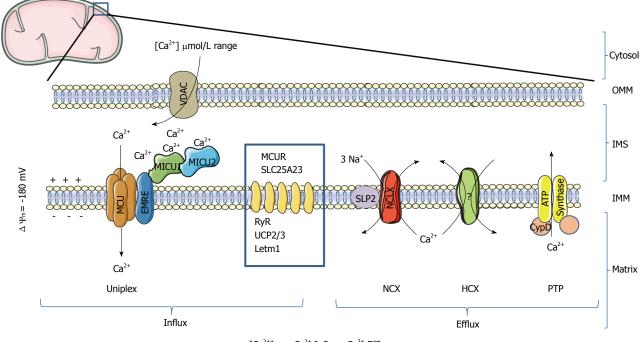
Excellent reviews have covered the mechanisms of ROS production<sup>[19]</sup>, the importance of redox signals in the modulation of mitochondrial function, and how deregulation of these signals can lead to the development of various disease states<sup>[21,26,27]</sup>. The effect of Ca<sup>2+</sup> on ROS generation and in the regulation of cellular energetics has also been reviewed recently<sup>[13,28]</sup>. Here we will review the transporters mediating and regulating the entry and extrusion of calcium across the inner membrane. We will further discuss the evidences linking mitochondrial Ca<sup>2+</sup> rises to the modulation of the matrix redox signals.

#### MOLECULAR IDENTIFICATION OF MITOCHONDRIAL Ca<sup>2+</sup> TRANSPORTERS

Mitochondria are equipped with sophisticated machinery mediating  $Ca^{2+}$  fluxes across the inner mitochondrial membrane (Figure 1). This system is composed of channels, exchangers, regulatory proteins and a poorly characterized matrix  $Ca^{2+}$  buffer system. In this section, we will focus on the recently identified  $Ca^{2+}$  channels and exchangers.

#### Mitochondrial Ca<sup>2+</sup> uptake mechanisms

**Mitochondrial Ca<sup>2+</sup> uniporter:** The mitochondrial Ca<sup>2+</sup> uniporter (MCU) is the principal mediator of Ca<sup>2+</sup> transport into the mitochondrial matrix. The MCU catalyzes the passive and unidirectional transport of Ca<sup>2+</sup> across the inner mitochondrial membrane, a process that is driven by the electrochemical gradient ( $\Delta \psi$ mt) in energized mitochondria. The inside negative potential of -180 mV is generated as electrons transferred stepwise along the respiratory chain from electron donors inside the matrix to the end-acceptor molecular oxygen. Given the large electrical gradient across the inner mitochondrial membrane, the organelle has



 $[Ca^{2+}]_{mit} = Ca^{2+}$  Influx - Ca<sup>2+</sup> Efflux

**Figure 1 Ca<sup>2+</sup> transport proteins of mitochondria.** In mammalian mitochondria, the uptake of Ca<sup>2+</sup> is mediated by the Ca<sup>2+</sup>-selective channel MCU, which is part of a high molecular weight protein complex called Uniplex. At least 4 additional proteins (MCUb, MICU1, MICU2 and EMRE) regulate MCU activity. Ca<sup>2+</sup> is then extruded by a sodium/calcium exchange (NCX) or proton/calcium exchange (HCX). If the protein NCLX has been confirmed to be the mitochondrial NCX, which is down-regulated by the protein SLP-2, the molecular nature of the mitochondrial HCX is still debated. Dimers of mitochondrial ATP synthase have been proposed to form the PTP, a mitochondrial channel regulated by CypD, that facilitates PTP opening by desensitizing PTP to Ca<sup>2+</sup>. Besides being activated by Ca<sup>2+</sup>, PTP has also been proposed to act as a reversible fast Ca<sup>2+</sup> release channel. Other non-MCU mitochondrial proteins with an indirect or debated effect on Ca<sup>2+</sup> transport are represented in the blue square (MCUR; SLC25A23; ryanodine receptor, RyR; UCP2; UCP3; LETM1). OMM: Outer mitochondrial membrane; IMS: Inter-membrane space; IMM: Inner mitochondrial membrane; VDAC: Mitochondrial porin; PTP: Permeability transition pore; CypD: Cyclophilin D; MCU: Mitochondrial Ca<sup>2+</sup> uptake protein 1; MICU2: Mitochondrial Ca<sup>2+</sup> uptake protein 2; EMRE: Essential MCU regulator.

the potential to import large amounts of Ca<sup>2+</sup>. At a concentration of 100 nmol/L cytosolic Ca2+ in a resting cells and a mitochondrial potential of -180mV, the Nernst equation would predict that the equilibrium will only be reached at 100 mmol/L mitochondrial [Ca<sup>2+</sup>]. The result would be a gradient of six orders of magnitude between matrix and cytosolic calcium concentration. However, this matrix Ca<sup>2+</sup> concentration is never reached under physiological conditions for three reasons: (1) the mitochondrial Ca<sup>2+</sup> uptake mechanism displays low Ca<sup>2+</sup> affinity, and the K<sub>d</sub> has been estimated close to 10  $\mu$ mol/L. Therefore efficient mitochondrial Ca<sup>2+</sup> uptake only occurs in the  $\mu$ mol/L cytosolic [Ca<sup>2+</sup>] range, protecting the mitochondria from  $Ca^{2+}$  overload in resting cells; (2) mitochondria activate  $Ca^{2+}$  extrusion as soon as [Ca<sup>2+</sup>] rises in the mitochondrial matrix, following cell stimulation; and (3) the mitochondrial matrix contains a poorly defined high capacity Ca<sup>2+</sup> buffer system, composed of PO<sub>4</sub><sup>-</sup>, Ca<sup>2+</sup> binding proteins and metabolites that significantly reduce free mitochondrial  $[Ca^{2+}]$ .

Electrophysiological recordings in mitoplasts have revealed the existence of an inward rectifying highly Ca<sup>2+</sup>-selective current across the inner mitochondrial membrane  $(I_{MCU})^{[29]}$ . This current was shown to be reflecting mitochondrial Ca<sup>2+</sup> uniport activity as it was blocked by two well-characterized pharmacological inhibitors of MCU, ruthenium red and Ru360<sup>[29]</sup>. This study was the first to define the electrophysiological properties of the MCU.

Following a long-lasting search for the proteins responsible for mitochondrial calcium uniport, two groups independently identified the essential component of the MCU in 2011<sup>[30,31]</sup>. The Mitocarta, the most complete compendium of mitochondrial proteins, was used as a starting point for the identification of MCU1 (mitochondrial Ca<sup>2+</sup> uptake protein 1). Baughman *et al*<sup>[30]</sup> identified MCU1 on the basis of an integrative genomic approach combining whole-genome phylogenetic profiling, genomewide co-expression analysis and organelle-wide protein co-expression analysis to predict proteins functionally related with MICU1. MICU1 had previously been identified as essential for mitochondrial Ca<sup>2+</sup> uptake<sup>[32]</sup>. De Stefani et al<sup>[31]</sup> uncovered MCU1 by analyzing well-known and predicted characteristics of the mitochondrial uptake mechanism: the ubiquitous expression in mammalian cells, its absence in yeast (which lacks a ruthenium

red-sensitive  $Ca^{2+}$  uptake mechanism), its presence in kinetoplastids (which express a ruthenium red-sensitive  $Ca^{2+}$  uptake mechanism), and the presence of two or more predicted transmembrane domains.

The MCU is part of a high molecular weight protein complex called Uniplex (Uniporter Complex, Figure 1). This complex is comprised of at least 5 different proteins:  $MCU1^{[30,31]}$ ,  $MCUb^{[33]}$ ,  $MICU1^{[32]}$ ,  $MICU2^{[34,35]}$  (mitochondrial Ca<sup>2+</sup> uptake protein 2) and essential MCU regulator (EMRE)<sup>[36]</sup>. Less well established is the interaction with and functional relevance of 2 additional mitochondrial proteins: MCUR and SLC25A23. MCU1 constitutes the pore-forming subunit. This essential component of the uniporter is sufficient for uniporter activity<sup>[37]</sup>. MCU1 contains two a-helix trans-membrane domains connected by a loop in the inter-membrane space<sup>[30]</sup>. Biochemical evidence and computational modeling predict MCU1 forming a tetramer. The trans-membrane domains build the pore of the channel. The loops, facing the intermembrane space, constitute the mouth of the channel, which also confers Ca<sup>2+</sup> selectivity. Sequence analysis of MCU led to the identification of a new MCU1 paralogue, named MCUb. This protein can replace MCU1 subunits resulting in different MCU1/MCUb ratios as observed in different tissues. MCUb can be considered a dominant negative pore-forming version of MCU1. Its presence adds a regulatory mechanism that modulates the properties of the channel<sup>[33]</sup>. MICU1 and MICU2 are Ca<sup>2+</sup>-sensitive subunits of the complex<sup>[32,38]</sup>. Both carry EF-hand (helixloop-helix) Ca<sup>2+</sup> binding domains facing the intermembrane space (Figure 1). Indirectly, these EF-hands sense cytosolic signals. MICU1 and MICU2 are likely to work as a gatekeeper defining the activation threshold of the channel, preventing the activity at resting  $Ca^{2+}$ levels (100 nmol/L) and triggering MCU activity when Ca<sup>2+</sup> microdomains (several µmol/L) are generated close to the channel<sup>[39,40]</sup>. MICU1 has been shown to control cooperativity of Ca<sup>2+</sup> uptake, a well-defined characteristic of the MCU. This regulatory mechanism favors the active state of the channel at high cytosolic [Ca<sup>2+</sup>]<sup>[39]</sup>. The Ca<sup>2+</sup> binding affinity of each active helix-loop-helix domain on MICU1 and MICU2 has been estimated to be in the range of 15-21  $\mu$ mol/L. These values are consistent with the necessity to reach high Ca<sup>2+</sup> microdomains as an essential requirement for full MCU activation<sup>[41]</sup>. The recently obtained crystal structure of MICU1 suggests that in the absence of Ca<sup>2+</sup>, the protein forms hexamers that inhibit MCU1. Conversely, in the presence of Ca<sup>2+</sup>, MICU1 undergoes a conformational change, forming multiple oligomers that activate MCU1<sup>[41]</sup>. Biochemical evidence suggests that MICU2 physically interacts with MICU1, which in turn interacts with MCU1. Functional interaction studies between MCU1, MICU1 and MICU2 suggest that both regulatory subunits contribute to MCU activation as a function of the amount of cytosolic [Ca<sup>2+</sup>]. At high cytosolic [Ca<sup>2+</sup>], the stimulatory effect of MICU1 drives the rapid response of mitochondria to cytosolic [Ca<sup>2+</sup>] rises. Conversely, at low cytosolic [Ca<sup>2+</sup>], MICU2 is required to prevent mitochondrial Ca<sup>2+</sup> uptake<sup>[34,35]</sup>. EMRE<sup>[36]</sup> was discovered as part of the mitochondrial calcium uptake machinery by quantitative mass spectrometry, and was shown to mediate MCU1-MICU1 physical and functional interaction. When EMRE was missing, the interaction between MCU1 and MICU1/2 was disrupted, despite intact MCU1 oligomers and preserved MICU1-2 interactions<sup>[36]</sup>. EMRE knock-out and knock-down models display a strong decrease in the ability to take up Ca<sup>2+</sup> in permeabilized mitochondria. Furthermore, *Imcu* Ca<sup>2+</sup> current is virtually absent in mitoplasts from EMRE knocked-out cells. These results are in conflict with earlier results suggesting that MCU1 alone is sufficient for MCU activity<sup>[37]</sup>.

A large variety of mitochondrial  $Ca^{2+}$  currents has been observed across different tissues and cell types<sup>[42]</sup>. Based on the current knowledge of the function of MCU components, it will be possible to study how their stoichiometry influences the variability of mitochondrial  $Ca^{2+}$  currents in different cell types.

A number of additional proteins have been shown to be important for mitochondrial Ca<sup>2+</sup> uptake<sup>[43-47]</sup>. These proteins are not likely part of the Uniplex, but rather may be involved in alternative mechanisms of mitochondrial Ca<sup>2+</sup> uptake. Therefore, this group of proteins is discussed separately. A genome-wide siRNA screen, designed to detect new proteins involved in mitochondrial Ca<sup>2+</sup> uptake<sup>[43,44]</sup>, has identified leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) as a molecule able to mediate high affinity (200 nmol/L) mitochondrial Ca<sup>2+</sup> uptake in exchange for H<sup>+</sup>. Several laboratories have been able to reproduce the mitochondrial Ca<sup>2+</sup> uptake defect in LETM1 knock-down cells<sup>[48,49]</sup>. Furthermore, when reconstituted in liposomes, LETM1 mainly promotes electro-neutral Ca<sup>2+</sup>/2H<sup>+</sup> exchange<sup>[50]</sup>. However, LETM1 was previously proposed to exchange  $K^+$  against  $H^{+[51,52]}$ . Also, thermodynamic considerations would argue against electro-neutral Ca<sup>2+</sup>/2H<sup>+</sup> exchange, as this should promote Ca<sup>2+</sup> extrusion rather than uptake given the proton gradient (0.8 pH units more alkaline in the matrix) across the inner mitochondrial membrane<sup>[53]</sup>. Knockout of LETM1 causes mitochondrial dysfunction, swelling and depolarization, thus reducing the driving force for mitochondrial Ca<sup>2+</sup> uptake. Depolarization of the inner mitochondrial membrane as a secondary consequence of LETM1 disruption may explain impaired MCU-mediated Ca2+ transport<sup>[51,52,54]</sup>. The role of LETM1 in mitochondrial ion homeostasis remains controversial. MCUR1 was originally reported to be a component of the Uniplex, contributing to ruthenium red-sensitive mitochondrial Ca<sup>2+</sup> uptake<sup>[45]</sup>. However, MCUR1 was not identified in mass spectrometry experiments of the purified Uniplex, arguing against a direct role in mitochondrial Ca<sup>2+</sup> uptake<sup>[36]</sup>. In the absence of MCUR1 expression, oxidative phosphorylation is impaired and cellular ATP levels lower, leading to activation of AMP kinase. Recent evidence suggests that MCUR1 may instead work as a cytochrome-c oxidase assembly factor<sup>[55]</sup>. The evidence suggests that loss of MCUR1 impairs respiratory function, leading to diminished  $\Delta \psi_{mit}$  and

thereby a reduction of mitochondrial Ca<sup>2+</sup> uptake. Recently, the protein SLC25A23, a member of the mitochondrial Ca<sup>2+</sup>-dependent solute carrier family, previously considered to be an ATP-Mg/PO4 carrier, has been shown to physically interact and positively modulate MCU activity. Lack of SLC25A23, as well as overexpression of the SLC25A23 EF-hand mutants, was shown to reduce MCU activity<sup>[46]</sup>. SLC25A23 may therefore be a regulatory subunit of the Uniplex. UCP2 and UCP3 have also been proposed to be essential for mitochondrial Ca<sup>2+</sup> uptake<sup>[47]</sup>. This is unlikely, since mitochondria isolated from tissues of the UCP2 and UCP3 knock-out mice displayed unaltered Ca<sup>2+</sup> uptake<sup>[56]</sup>. Recently, impaired mitochondrial Ca<sup>2+</sup> uptake has been confirmed in UCP3 knock-out cells<sup>[57]</sup>. Even though MCU activity did not rely on the UCP3 in intact cells, the lack of UCP3 decreased cytosolic ATP available for SERCA pumps. As a result, IP3-driven cytosolic and mitochondrial Ca<sup>2+</sup> rises were reduced<sup>[57]</sup>. The contribution of UCP2 and UCP3 to mitochondrial calcium uptake is therefore via their impact on Ca<sup>2+</sup> handling in the ER.

Studies in cardiac cells indicate that ryanodine receptors, one of the main endoplasmic reticulum Ca<sup>2+</sup> release channels, contribute to an alternative mitochondrial Ca<sup>2+</sup> uptake mechanism<sup>[58,59]</sup>. Consistent with this, localization of ryanodine receptors to mitochondria was demonstrated using electron microscopy and Western-blotting<sup>[58,59]</sup>. Pharmacological inhibition with ryanodine diminished inward Ca<sup>2+</sup> current in mitoplasts insensitive to the MCU blocker Ru360<sup>[60]</sup>. Along the same line of evidence, single channel recordings in mitoplasts from HeLa cells after knock-down of MCU1 revealed a 2.5-fold increase in the occurrence of the extra-large conductance Ca<sup>2+</sup> current<sup>[61]</sup>. These observations are consistent with an alternative and compensatory molecular mechanism for mitochondrial Ca<sup>2+</sup> uptake.

#### Mitochondrial Ca<sup>2+</sup> release mechanisms

Two main mechanisms have been proposed to account for mitochondrial  $Ca^{2+}$  release<sup>[62]</sup>: (1) Na<sup>+</sup>-dependent, mediated by a recently identified mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger named NCLX; and (2) Na<sup>+</sup>-independent, probably mediated by a H<sup>+</sup>/Ca<sup>2+</sup> exchanger. These two mechanisms operate to extrude Ca<sup>2+</sup> during physiological mitochondrial [Ca<sup>2+</sup>] transients. A third mechanism, called permeability transition pore (PTP, see below) opening gets activated under specific physiopathological conditions when mitochondria experience Ca<sup>2+</sup> overload for extended periods of time<sup>[62]</sup>.

The mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchange discovered by Carafoli *et al*<sup>[63]</sup> constitutes the main pathway for mitochondrial Ca<sup>2+</sup> extrusion<sup>[64,65]</sup>. The stoichiometry of ion exchange has been estimated to be  $3Na^+/Ca^{2+}$ . This electrogenic export mode favors Ca<sup>2+</sup> extrusion in energized mitochondria<sup>[66]</sup>. Like MCU, NCLX is highly selective for Ca<sup>2+</sup> when compared with other divalent ions, but less selective for Na<sup>+</sup> that can be replaced by Li<sup>+[63,67]</sup>. Although NCLX has recently been thought to localize to the plasma membrane, electron microscopy and cell fractionation experiments clearly showed that NCLX is targeted to the mitochondrial inner membrane. Na<sup>+</sup>-dependent Ca<sup>2+</sup> release was strongly reduced in NCLX knock-down cells, whereas NCLX overexpression enhanced it<sup>[68]</sup>. NCLX-driven Ca<sup>2+</sup> extrusion is inhibited by 7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157), the most selective inhibitor of mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchange. Taken together, the evidence led to the conclusion that NCLX encodes the so-called mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger<sup>[68]</sup>. Several studies suggest the existence of regulatory mechanisms controlling mitochondrial Ca<sup>2+</sup> export kinetics. The protein kinases PKC<sup>[69]</sup> and PINK1<sup>[70]</sup> were reported to modulate the activity of this ion exchanger. Stomatin-like protein 2 (SLP-2), which localizes to the inner mitochondrial membrane, was also shown to inhibit mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchange<sup>[71]</sup>. CGP-37157, an inhibitor of the mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, was used to demonstrate that this exchange mechanism contributes to shape cytosolic [Ca<sup>2+</sup>] transients<sup>[72]</sup>, to mediate the Ca<sup>2+</sup> transfer from the extracellular medium to the ER during IP<sub>3</sub>-driven Ca<sup>2+</sup> signaling<sup>[73]</sup>, and modulates the NAD(P)H redox state<sup>[74]</sup> and ATP production<sup>[72]</sup>.

Mitochondria suspended in buffers devoid of Na<sup>+</sup> retain their capacity to extrude Ca<sup>2+</sup>, pointing to a Na<sup>+</sup>-independent mechanism. This Ca<sup>2+</sup> efflux pathway is catalyzed by a to date non-identified H<sup>+</sup>/Ca<sup>2+</sup> exchanger (reviewed in Bernardi)<sup>[62]</sup>. LETM1 has been proposed recently to exchange Ca<sup>2+</sup> against H<sup>+</sup>. LETM1 may therefore drive extrusion of Ca<sup>2+</sup> from energized mitochondria<sup>[43,44]</sup>. In conflict with this interpretation, LETM1 expression in HeLa cells did not alter Ca<sup>2+</sup> efflux rates, regardless of the amplitude of Ca<sup>2+</sup> elevation reached during agonist stimulation<sup>[74]</sup>. These findings cast doubt on the Ca<sup>2+</sup> exchanger function of LETM1.

**Permeability transition pore:** The PTP is a Ca<sup>2+</sup> and ROS-activated, voltage-dependent and cyclosporine A-sensitive channel located in the inner mitochondrial membrane. Opening of the permeability transition pore causes a sudden increase in the mitochondrial inner membrane permeability to solutes with molecular masses up to 1500 Dalton<sup>[75-77]</sup>. Opening of PTP leads to mitochondrial permeability transition, which plays an important role in intracellular death signaling and in events ranging from tissue damage upon infarction to muscle wasting in some forms of dystrophy<sup>[75]</sup>. Given that PTP activation occurs under several pathological conditions<sup>[14,75]</sup>, the channel has been extensively characterized as a pharmacological target. The proteins forming the PTP channel have been recently rediscovered. The classical model envisioned a supramolecular complex spanning the double membrane system of mitochondria including the protein voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocator in the inner membrane, cyclophilin D in the mitochondrial matrix, and also including additional

proteins such as Bax. However, genetics studies have demonstrated that permeability transition and/or even single channel activity can be observed in mitochondria devoid of the proposed components of the  $PTP^{[78-83]}$ . Recent evidence from the Bernardi laboratory has revolutionized our view of the PTP. They propose that the pore of the channel is formed by dimers of the ATP synthase<sup>[84]</sup> (Figure 1). To be activated by Ca<sup>2+</sup>, PTP has also been proposed to act as a reversible fast Ca<sup>2+</sup> release channel<sup>[85]</sup>. Such transient opening of the PTP may be induced by physiological stimuli for fast mitochondrial Ca<sup>2+</sup> release, preventing Ca<sup>2+</sup> overload<sup>[86]</sup>.

# Reevaluation of the physiological role of mitochondrial $Ca^{2+}$

Mitochondrial Ca<sup>2+</sup> uptake has been related with a plethora of cell functions, including exocytosis, gene transcription, cell cycle regulation, respiration and cell death<sup>[12,14]</sup>. Much of the evidence linked to the function of mitochondrial Ca2+ was obtained over the last few decades using pharmacological tools. The identification of the molecular machinery governing mitochondrial Ca<sup>2+</sup> fluxes has led to a large number of genetic studies that re-evaluate the role of mitochondrial Ca<sup>2+</sup> in cell physiology/pathology. Furthermore, they have allowed for the first time their role to be studied in the context of the whole organism. Recently, an MCU1 knock-out mouse has been generated. Analysis of the mouse phenotype has led to new questions regarding the importance of mitochondrial Ca<sup>2+</sup> uptake. Unexpectedly, the mouse is viable. It only displays limited impairment of muscle function, even during exercise. Contrary to expectations, MCU KO hearts were also not protected from the damage induced by ischemia/reperfusion<sup>[87,88]</sup>. It is worth mentioning that the mice analyzed were obtained from a mixed genetic population (CD1) background, because disruption of the MCU1 gene in pure C57/BL/6 inbred mice led to embryonic lethality<sup>[89]</sup>. Developmental defects were also observed in a Zebra fish knock-down model that showed defects in gastrula morphogenesis<sup>[90]</sup>. The results with MCU1 KO mice on a mixed genetic background suggest that in these animals, alternative mechanisms are able to compensate for the absence of MCU function. It has been postulated that alternative Ca<sup>2+</sup> uptake mechanisms may be responsible for this compensation. This hypothesis is hard to reconcile with the absence of regulated Ca<sup>2+</sup> uptake in mitochondria isolated from MCU1 KO mice<sup>[87]</sup>. Despite the negative results obtained in KO mice on a mixed genetic background, MCU activity seems to play an important role in muscle physiology. Manipulation of MCU1 expression in skeletal muscle cells in vivo revealed that MCU levels modulate muscle size. The phenomenon is linked to the PGC1a4 and on IGF1-AKT/PKB signaling pathways<sup>[91]</sup>. MCU-dominant negative transgenic mice showed similar heart rates compared to the wild type animals under resting conditions, but failed to increase the beating frequency upon physiological

adrenergic stimulation<sup>[92]</sup>. Detailed assessment of hearts from mice lacking MCU revealed markedly impaired mitochondrial Ca<sup>2+</sup> uptake. Surprisingly, the hearts of these animals appear to function relatively normally, even during stress<sup>[93]</sup>. MICU1 loss of function mutations in human fibroblasts led to a defect in mitochondrial Ca<sup>2+</sup> homeostasis<sup>[94]</sup>. Patients carrying such mutations displayed neurological disorders and muscle disease.

### MATRIX REDOX SIGNALING MODULATION BY MITOCHONDRIAL Ca<sup>2+</sup> EXTRUSION

Energy metabolism and redox balance are regulated by mitochondrial Ca<sup>2+</sup>. There is a complex relationship between Ca<sup>2+</sup> and redox signaling, as Ca<sup>2+</sup> promotes both oxidizing and reducing biological processes. Matrix Ca<sup>2+</sup> rises stimulate respiratory chain activity<sup>[95,96]</sup>, which as a side-product also produces ROS<sup>[97]</sup>. ROS production occurs at complexes I ,  ${\rm I\hspace{-0.1em}I}$  and  ${\rm I\hspace{-0.1em}I}$  of the respiratory chain and several flavoproteins in different cellular compartments<sup>[18]</sup>. In fact, it was shown that mitochondria are able to produce H<sub>2</sub>O<sub>2</sub><sup>[98,99]</sup>. The peroxide is formed from dismutation of superoxide (O2.-), which is generated within mitochondria<sup>[100,101]</sup>. By promoting ROS formation, matrix Ca<sup>2+</sup> causes net oxidation of the mitochondrial redox state. On the other hand, several Ca<sup>2+</sup>-activated dehydrogenases of the mitochondrial matrix form reducing equivalents, therefore favoring reduction of mitochondrial redox couples. Notably, studies in Bristol in the 1960s and 1970s led to the recognition that mitochondrial Ca<sup>2+</sup> promotes the supply of reducing equivalents in the form of NADH or FADH2<sup>[102-104]</sup>. Four Ca<sup>2+</sup>-activated mitochondrial dehydrogenases were described: FAD-glycerol phosphate dehydrogenase (located on the outer surface of the inner mitochondrial membrane; influenced by changes in cytoplasmic Ca<sup>2+</sup> concentration), pyruvate dehydrogenase, NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase (the latter three located within mitochondria and regulated by changes in mitochondrial matrix  $Ca^{2+}$  concentration). Following early studies with isolated mitochondria, the results on Ca<sup>2+</sup> regulation of mitochondrial metabolism were confirmed in situ<sup>[13]</sup>. Recent evidence on insulinsecreting INS-1E-cells demonstrates that the interplay between mitochondrial Ca2+ and matrix production of reducing equivalents may be even more complex<sup>[105]</sup>. Matrix calcium signals accelerate respiration and increase cytosolic ATP levels<sup>[16]</sup>. Under the same conditions, NAD(P)H levels increased rapidly to reach a new steadystate in both INS-1E cells and human pancreatic islets<sup>[105]</sup>. Surprisingly, this substrate-dependent increase of NAD(P)H was also observed when calcium signaling was prevented. The data is consistent with Ca<sup>2+</sup>-dependent control both at the level of dehydrogenases and the respiratory chain. The accelerated formation of reducing equivalents by dehydrogenases is balanced by enhanced

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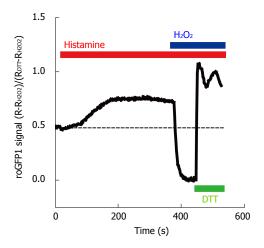


Figure 2 Ratiometric fluorescence intensity response of roGFP1 to histamine stimulation and exogenous H<sub>2</sub>O<sub>2</sub> and DL-dithiothreitol in living HeLa cells. HeLa cells were transfected with the mitochondrial targeted redox sensor roGFP1. Cells were excited at 410 and 480 nm and emission was collected at 535 nm. The 410/480 fluorescence ratio (R) was normalized to the minimum of fluorescence (obtained after the addition of 1 mmol/L H<sub>2</sub>O<sub>2</sub>) and the maximum (after the addition of 10 mmol/L dithiothreitol). DL-dithiothreitol). The effect of intracellular Ca<sup>2+</sup> was assessed by stimulating the cell with 100  $\mu$ mol/L histamine. DTT: DL-Dithiothreitol.

oxidation of NADH and FADH<sub>2</sub> by the respiratory chain. Such coordinated activation of oxidative metabolism and respiratory chain activity allows the respiratory rate to change several fold with only small or no alterations of the NAD(P)H/NAD(P)<sup>+</sup> ratio<sup>[105]</sup>. These results underline the complex connection between matrix Ca<sup>2+</sup> and the control of mitochondrial redox signaling.

The recently developed fluorescent green fluorescence protein (GFP)-based redox sensors can be used to further clarify the interplay between Ca<sup>2+</sup> and redox regulations (see for a recent review describing in detail novel sensor variants and their utilization to understand redox biology in living cells)<sup>[22]</sup>. These roGFP sensors equilibrate predominantly with the glutathione redox couple (GSSG/2GSH)<sup>[22]</sup>. This redox balance depends on glutaredoxins, which catalyze thiol-disulfide exchange between the glutathione pool and the redoxsensitive protein<sup>[106]</sup>. By coupling human glutaredoxin to an roGFP, new redox sensors have been developed that can be used even in compartments lacking glutaredoxin activity<sup>[107]</sup>. Glutathione can be oxidized by superoxide radical, hydrogen peroxide and other oxidizing agents<sup>[108,109]</sup>, therefore mitochondrial targeted roGFP1 was demonstrated to be in dynamic equilibrium with the mitochondrial redox status and to respond to membrane-permeant reductants and oxidants<sup>[24]</sup> (Figure 2).

We have recently used the mitochondrially-targeted roGFP probe to study the link between mitochondrial  $Ca^{2+}$  signals and matrix redox state. The kinetics of mitochondrial  $Ca^{2+}$  transients were analyzed by focusing on the rate of mitochondrial  $Ca^{2+}$  extrusion<sup>[74]</sup>. Following agonist-induced  $Ca^{2+}$  mobilization, maximal mitochondrial  $Ca^{2+}$  efflux rates were calculated as a function of the signal amplitude. A large heterogeneity of matrix Ca2+ extrusion rates was observed. Thus, only single-cell analysis is able to capture the complexity of this biological process. Manipulation of the mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCLX)<sup>[68]</sup> expression has a strong impact on agonist-induced matrix Ca<sup>2+</sup> transients. These experimental conditions were also used to assess the effect of mitochondrial Ca2+ signals on the matrix redox state (Figure 2). During HeLa cell stimulation with the agonist histamine, calcium is mobilized from the ER, leading to a mitochondrial calcium rise. Concomitant with the Ca<sup>2+</sup> rise, the mitochondrial redox state was increasingly reduced as measured with a mitochondriallytargeted roGFP1. These changes likely reflect a shift of the mitochondrial glutathione pool towards the reduced form. By promoting mitochondrial Ca<sup>2+</sup> extrusion in cells overexpressing NCLX, histamine-induced redox changes were completely prevented. This effect was reverted by blocking NCLX activity using CGP-37157<sup>[68,74]</sup>. Consistent with these results, NCLX expression was able to limit histamine-induced mitochondrial NAD(P)H production in HeLa cells, and this response was fully restored by CGP-37157. We conclude that the calcium rises induced by histamine stimulate matrix dehydrogenases, as reflected by the increased NAD(P)H/NAD(P)<sup>+</sup> ratio. These changes favor the formation of reduced glutathione measured by an increase of the roGFP1 signal. Mitochondrial Ca<sup>2+</sup> is also a powerful activator of respiratory chain complexes. The associated acceleration of ROS production should have a net oxidizing effect on the mitochondria. Our findings demonstrate that the reducing effects are dominant during physiological calcium mobilization. Furthermore, our data establish a causal relationship between NCLX activity and matrix redox state (Figure 3).

#### REDOX REGULATION OF MITOCHONDRIAL FUNCTION

Redox control of mitochondrial proteins is an important topic in cell physiology and pathology because many mitochondrial functions are linked to redox reactions<sup>[21]</sup>. An increasing number of publications demonstrate a role for redox signals in the control of mitochondrial functions, including nutrient oxidation, oxidative phosphorylation, ROS production, mitochondrial permeability transition, mitochondrial morphology and cell death (reviewed in<sup>[21]</sup>). A number of reviews have covered the role of redox switches in the control of specific mitochondrial functions<sup>[21,110-113]</sup>. They highlight the fact that mitochondria harbor a unique environment that promotes thiol modifications and redox signaling. The mitochondrial proteome is very rich in protein thiols. The total concentration of such thiol groups was estimated to be in the range of 60-90 mmol/L<sup>[114]</sup>. In addition, as previously mentioned, mitochondria are a very important source for ROS (notably superoxide anion radical and hydrogen peroxide), reduced glutathione and NAD(P)H, which are required for oxidation/reduction reactions. Importantly, redox potentials are strongly

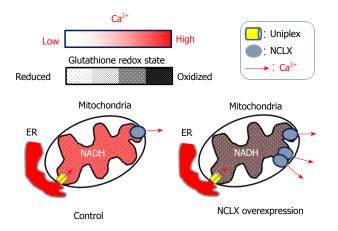


Figure 3 Proposed model for the modulation of the matrix redox signaling by mitochondrial Ca<sup>2+</sup>. After cell stimulation, Ca<sup>2+</sup> released from the endoplasmic reticulum (ER) enters in mitochondria by the mitochondrial uniplex (left), stimulating matrix Ca<sup>2+</sup>-dependent dehydrogenases, which increase NADH levels and promoting a reduction of the mitochondrial glutathione pool. In cells over-expressing the mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCLX, right) Ca<sup>2+</sup> extrusion is more efficient and therefore Ca<sup>2+</sup>-dependent dehydrogenases less activated. Such lowering the mitochondrial Ca<sup>2+</sup> response blunts NADH formation and prevents matrix redox changes of the mitochondrial glutathione pool. NADH: Reduced form of nicotinamide-adenine dinucleotide; NCLX: Mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger.

influenced by pH, and mitochondria are able to dynamically regulate mitochondrial proton gradient during cytosolic and mitochondrial Ca<sup>2+</sup> elevations<sup>[115]</sup>. Changes in mitochondrial pH seem to play an important role in physiological and pathological situations such as apoptosis, neurotransmission, pancreatic beta cells activation and insulin secretion<sup>[116,117]</sup>. Interestingly, spontaneous flashes of alkalinization have been reported in the mitochondrial matrix of living cells<sup>[118]</sup>; they can even spread between contiguous mitochondria, but their potential impact on redox potential has not yet been studied. Since the redox potential of any redox reaction involving H<sup>+</sup> is pHdependent, it is likely that matrix pH influences redox reactions. By extension, pH flashes could promote localized matrix redox reactions. Given the described properties, mitochondria represent a perfect microenvironment to promote redox signaling via cysteine oxidation reactions.

Several types of redox modifications have been observed in mitochondria. These modifications include S-oxidation (sulfenylation and sulfinylation), S-glutathionylation and S-nitrosylation<sup>[21]</sup>. A proteomic method has been recently developed to profile quantitatively free cysteine thiol groups based on their intrinsic reactivity in situ<sup>[119]</sup>. It is noteworthy that among the 50 most reactive cellular cysteine residues listed, 19 were found in mitochondrial proteins. The most reactive mitochondrial cysteines were found in aldehyde dehydrogenases. Other examples of enzymes that undergo physiologically-relevant thiol switches have been reviewed elsewhere<sup>[18]</sup>. They include mitochondrial thiolases, creatine kinase, aconitase, homoaconitase and branched chain aminotransferase. The importance of mitochondrial thiol switches and the role of "physiological" ROS production to trigger those switches has also been highlighted by Riemer *et al*<sup>[18]</sup>. For instance,</sup>

the phenotypes of knock-out mice for several mitochondrial redox-regulating enzymes (superoxide dismutases 1 and 2; glutaredoxins 1 and 2; glutathione peroxidases 1 and 4; thioredoxin 2; thioredoxin reductases 1 and 2; peroxiredoxin 3) were reviewed. The observed phenotypes in these animals range from embryonic lethality, developmental aberrations and neurodegeneration to impaired signal transduction.

Mitochondrial proteins involved in oxidative metabolism and energy production are primary targets regulated by reactive cysteines. A subunit of pyruvate dehydrogenase, which links glycolysis to the citric acid cycle, is reversibly inactivated by hydrogen peroxide<sup>[120]</sup>. The activity of several tricarboxylic acid cycle enzymes (aconitase, isocitrate dehydrogenase, ketoglutarate dehydrogenase and succinyl-CoA synthetase) is modulated by redox reactions as well<sup>[121-124]</sup>. In addition, mitochondrial respiratory chain complexes are a target of thiol-base redox regulation<sup>[125]</sup>.

Glutathionylation of uncoupling protein 2 (UCP2) and UCP3, two mitochondrial protein paralogues of UCP1, has been proposed to modulate proton leak<sup>[126-128]</sup>. Interestingly, UCP2 and UCP3 modulate the activity of sarco/endoplasmic reticulum Ca<sup>2+</sup> ATPases by decreasing mitochondrial ATP production<sup>[57]</sup>, revealing an additional link between Ca<sup>2+</sup> and redox signals.

The matrix redox state also influences mitochondrial function through sirtuins, a class of NAD<sup>+</sup>-dependent deacetylases having beneficial health effects<sup>[129]</sup>. Among the seven members of this family, SIRT3, SIRT4 and SIRT5 are found in the mitochondria, where they govern mitochondrial processes<sup>[130]</sup>. Also, some mitochondrial transport systems have been reported to be regulated by the redox potential. For example, the carnitine/acylcarnitine carrier, required for the transport of fatty acid into mitochondria, is regulated by glutathionylation<sup>[131]</sup>.

Redox regulation of mitochondrial proteins plays a crucial role also in pathology, as ROS promote the opening of the mitochondrial permeability transition pore<sup>[14,75,132]</sup>. The role of thiol oxidation during mitochondrial permeability transition has been carefully characterized<sup>[133,134]</sup>. For example, redox-active compounds belonging to the polyphenol family are able to modulate both PTP singlechannel activity and PTP-dependent colloidosmotic swelling of isolated mitochondria<sup>[135]</sup>. Mitochondrial morphology has a strong impact on metabolism and cell death decisions, and this process is also modulated by redox reactions. Chronic ROS exposure promotes mitochondrial fragmentation<sup>[136]</sup>. Treatment with sublethal amounts of H2O2 or other acute stresses induces hyperfusion and can be prevented using antioxidants<sup>[137]</sup>. A recent genomewide screen using RNA interference has identified ROMO1 as an essential redox-regulator, which is required for mitochondrial fusion and normal cristae morphology<sup>[138]</sup>.

Deregulated mitochondrial redox signaling is associated with several diseases and condition<sup>[21]</sup>. The involvement of mitochondrial thiol oxidation has been reported in cardiovascular diseases<sup>[139]</sup>. Redox proteomics



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of hearts subjected to ischemia/reperfusion indicates major changes in the redox state of thiol groups in mitochondrial proteins, including components of electron transport complexes and enzymes involved in lipid metabolism<sup>[139]</sup>. Moreover, mitochondrial PTP opening has been demonstrated to be a causative event in reperfusion damage of the heart  $^{\scriptscriptstyle [140]}$  . Mitochondrial redox signals have been implicated also in neurodegenerative disorders, and deregulation of glutaredoxin-1 and thioredoxin-1 have been proposed to be important events in Alzheimer's disease pathogenesis<sup>[141]</sup>. A wellknown feature of Parkinson's diseases is an imbalanced redox state<sup>[142]</sup>. Acting on mitochondrial redox signals has been suggest as an approach to attenuate oxidative stress in dopaminergic neurons of the substantia nigra in individuals with Parkinson's disease. ROS production and associated mitochondrial dysfunction may also play an important role during progression of type 2 diabetes<sup>[143]</sup>. The relevance of redox signaling in the development of type 2 diabetes has been highlighted recently<sup>[144]</sup>. In pancreatic beta cells, mitochondria are particularly important as they link nutrient metabolism to down-stream signals essential for insulin secretion. In this cell type the identification of mitochondrial proteins controlled by redox state may lead to the identification of novel signaling pathways modulating insulin secretion. Finally aging and age-related diseases in general are influenced by intracellular free radicals<sup>[145]</sup>. Disruption of mitochondrial redox signals seems to contribute to ageing<sup>[146,147]</sup> and the redox state of protein thiol group has been proposed to play key role in this process<sup>[148]</sup>.

#### CONCLUSION

Several disorders and disease states are associated with deregulated redox signaling, including cardiovascular and neurodegenerative diseases, insulin resistance, obesity, diabetes and aging (discussed in<sup>[21]</sup>). Novel approaches are needed to rescue cellular function due to deregulated redox signaling. Polyphenols<sup>[149]</sup> are a good example as they have anti-oxidant properties and should prevent free radical damage, and thereby potentially normalize redox signaling. However, as discussed by Visioli et al<sup>[149]</sup>, "basic and clinical science is showing that the reality is much more complex than this and that several issues, notably content in foodstuff, bioavailability, or in vivo antioxidant activity are yet to be resolved". Mitochondria constitute an optimal target to face those issues because they drive/modulate their functions by redox reactions. The ability of mitochondrial Ca<sup>2+</sup> to modulate matrix redox state offers potential novel strategies for the manipulation of the mitochondrial redox state. Several natural compounds are known to modulate mitochondrial Ca<sup>2+</sup> transport<sup>[150]</sup>. Such compounds affecting mitochondrial Ca<sup>2+</sup> handling may have beneficial health effects by rescuing mitochondrial redox-related functions.

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REVIEW

# Signal transducer and activator of transcription 3 regulation by novel binding partners

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#### Abstract

Signal transducers and activators of transcription (STATs) mediate essential signals for various biological processes,

including immune responses, hematopoiesis, and neurogenesis. STAT3, for example, is involved in the pathogenesis of various human diseases, including cancers, autoimmune and inflammatory disorders. STAT3 activation is therefore tightly regulated at multiple levels to prevent these pathological conditions. A number of proteins have been reported to associate with STAT3 and regulate its activity. These STAT3-interacting proteins function to modulate STAT3-mediated signaling at various steps and mediate the crosstalk of STAT3 with other cellular signaling pathways. This article reviews the roles of novel STAT3 binding partners such as DAXX, zipperinteracting protein kinase, Krüppel-associated box-associated protein 1, Y14, PDZ and LIM domain 2 and signal transducing adaptor protein-2, in the regulation of STAT3mediated signaling.

Key words: Janus kinase/signal transducer and activator of transcription; Signal transduction; Signal transducer and activator of transcription 3; DAXX; Zipper-interacting protein kinase; Krüppel-associated box-associated protein 1; Y14; PDZ and LIM domain 2; Signal transducing adaptor protein-2; Nuclear factor-κB

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**Core tip:** Signal transducer and activator of transcription 3 (STAT3) has been proposed its physiological and pathological significance in malignant and inflammatory diseases; therefore, the targeting of the STAT3 pathways is likely to be suitable for clinical application. In this review, we introduced novel regulatory molecules of STAT3 binding partners, such as DAXX, zipper-interacting protein kinase, Krüppel-associated box-associated protein 1, Y14, PDZ and LIM domain 2 and signal transducing adaptor protein-2. These proteins positively or negatively regulate critical steps of STAT3-mediated signals *via* individually unique mechanism. We hope that the information described here will help to develop a new strategy to clinically control the STAT3 activities.



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#### INTRODUCTION

Cytokines selectively activate Janus kinases (JAKs), which in turn activate one or more signal transducers and activators of transcription (STATs) via their tyrosine phosphorylation<sup>[1-3]</sup>. STATs have cytoplasmic signaling regions, such as a Src-homology 2 (SH2) domain and tyrosine phosphorylation sites. Upon cytokine stimulation, STATs are phosphorylated and dimerize via their SH2 domains and then move into the nucleus<sup>[4]</sup>. STAT3 is a central member of the STAT protein family, and is activated by various cytokine signals, such as interleukin 6 (IL-6)<sup>[1,5-7]</sup>, which plays a role in immune regulation, hematopoiesis, inflammation and oncogenesis<sup>[6,7]</sup> (Figure 1). The majority of IL-6 functions are in turn mediated by STAT3<sup>[8,9]</sup>. Of importance, abnormal expression of STAT3 has been reported in several cancer cells as well as autoimmune diseases, suggesting the involvement of STATs in a wide range of diseases<sup>[5,10-13]</sup>. Because of important physical roles, STAT3 activity is strictly regulated by multiple molecular mechanisms. For example, the protein inhibitor of activated STAT suppresses transcriptional activities of STAT3 by interfering STAT3 from DNA binding in the nucleus<sup>[14]</sup>. Suppressor of cytokine signaling (SOCS), which is induced by STAT3, participates in the negative feedback of STAT3 activities<sup>[15,16]</sup>. Cytoplasmic tyrosine phosphatases, such as SH2-containing phosphatase 1 (SHP1), SHP2 and protein-tyrosine phosphatase 1B, function to stop STAT activities<sup>[14,15]</sup>. Nuclear tyrosine phosphatases, such as TC45, also dephosphorylate nuclear STAT3, resulting in their translocation from the nucleus to the cytoplasm<sup>[14,17]</sup>. We have identified some STAT3-interacting molecules, including DAXX<sup>[18,19]</sup>, zipper-interacting protein kinase (ZIPK)<sup>[20,21]</sup>, Krüppel-associated box-associated protein 1 (KAP1)<sup>[22]</sup>, Y14<sup>[23,24]</sup>, PDZ and LIM domain 2 (PDLIM2)<sup>[25]</sup> and signal transducing adaptor protein-2 (STAP-2)<sup>[26-28]</sup>. Here, we describe functions of each of these molecules in the STAT3-mediated signaling pathway. DAXX negatively regulates STAT3-mediated transactivation and cell proliferation through the IL-6 signal transducer gp130<sup>[18,19]</sup>. ZIPK positively regulates STAT3 transactivation through STAT3 Ser727 phosphorylation<sup>[20,21]</sup>. KAP1 negatively regulates STAT3 Ser727 phosphorylation and transactivation by interacting with HDAC3 within the nucleus<sup>[22]</sup>. Y14 is a novel type of STAT3 binding partner and influences IL-6-induced STAT3 transactivation through altering its tyrosine-phosphorylation state<sup>[23,24]</sup>. PDLIM2 acts as a nuclear E3 ligase for STAT3 and terminates STAT3mediated signaling<sup>[25]</sup>. STAP-2 is a novel adaptor protein, composed of pleckstrin homology (PH) and SH2-like domains, and a STAT3-binding (YXXQ) motif<sup>[26-28]</sup>. Taken together, STAT3 activity is positively and negatively regulated at multiple steps.

#### NOVEL STAT3 BINDING PARTNERS

The STAT3 activities are strictly regulated, and recent reports have suggested several novel STAT3 regulators, whose characters are summarized in Table  $1^{[25,26,29-33]}$ .

# A nuclear STAT3 repressor, Death domain-associated protein (DAXX)

DAXX, which mainly located in the nucleus, has an ability to modulate transcription as well as cell death<sup>[34]</sup>. DAXX interacts with a number of transcription factors, including ETS1<sup>[35]</sup>, PAX5<sup>[36]</sup>, Glucocorticoid receptor<sup>[37]</sup>, RelA<sup>[38]</sup>, RelB<sup>[39]</sup>, TCF4<sup>[40]</sup>, SMAD4<sup>[41]</sup>, C/ EBP<sup>[42]</sup> and AIRE<sup>[43]</sup>, and regulates their transcriptional activities. Because DAXX is also known to bind to histone deacetylases<sup>[44]</sup>, DNA methyltransferases and their associated proteins<sup>[29,45,46]</sup>, and the chromatin-modifying a-thalassemia syndrome protein<sup>[47,48]</sup>, DAXX is likely to regulate cellular processes by regulating the transcription of specific genes via epigenetic modification. We found that DAXX regulates STAT3-transcriptional activity and that IFN-induced DAXX functionally links to IL-6/LIF/ STAT3-mediated signaling<sup>[18]</sup>. Pretreatment of HeLa and Hep3B cells with IFN caused a decrease of IL-6-induced STAT3 transcriptional activities. Importantly, DAXX directly interacts with STAT3 in the nucleus, leading to the decreased STAT3-transcriptional activities. Indeed, knockdown of DAXX significantly enhanced STAT3 activation and gene expression after IL-6-stimulation.

The IL-6 family cytokines recognize gp130 membrane protein as a signal-transducing receptor component<sup>[8,9]</sup>. Dimerization of gp130 activates JAK family proteins (JAK1, JAK2 and TYK2), which then phosphorylate and activate STAT3. In lymphocytes, STAT3 is involved in IL-6- and/or IL-27-dependent cell growth<sup>[49,50]</sup>. In addition, STAT3 is also required for pro-B cell survival as well as efficient B lymphocyte production<sup>[51]</sup>. DAXX was reported to play a role in STAT3-mediated growth signals through gp130<sup>[19]</sup>. DAXX constitutively interacts with STAT3, leading to the impairment of STAT3 binding to the consensus DNA sequences of its target genes. In this regard, DAXX preferentially suppresses gp130-mediated Bcl-2 expression, which control cell survival. During lymphocyte apoptosis, an inverse correlation between DAXX and Bcl-2 expression levels is often observed. When progenitor B lymphocytes were treated with IFN-β, DAXX expression and nuclear localization were enhanced in parallel to Bcl-2 down-regulation<sup>[52]</sup>.

Therefore, DAXX has an important function to control STAT3 activity and Bcl-2 expression during cytokine

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#### Matsuda T et al. Novel STAT3-interacting proteins

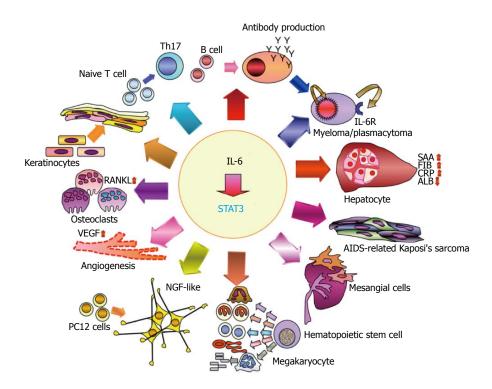


Figure 1 Interleukin 6 modulates a variety of physiological events, such as cell proliferation, differentiation, survival, and apoptosis, through signal transducer and activator of transcription 3. IL-6-STAT3 axis plays roles in the immune, the endocrine, the nervous and the hematopoietic systems, and on bone metabolism. IL-6 has been implicated in the pathology of different diseases including multiple myeloma, rheumatoid arthritis, Castleman's disease, AIDS, mesangial proliferative glomerulonephritis, psoriasis, Kaposi's sarcoma, sepsis and osteoporosis. SAA: Serum amyloid A; FIB: Fibrinogen; ALB: Albumin; CRP: C-reactive protein; NGF: Nerve growth factor; VEGF: Vascular endothelial growth factor; NF-k: Nuclear factor-k:B; RANKL: Receptor activator of NF-kB ligand; Th17: T helper type 17; IL-6: Interleukin 6; STAT3: Signal transducer and activator of transcription 3; AIDS: Acquired immunodeficiency syndrome.

stimulation.

#### A STAT3 Ser727 kinase, ZIPK

Tyrosine and/or serine residues of STATs are phosphorylated in response to ligand stimulation<sup>[53,54]</sup>. In the case of STAT3, a single serine phosphorylation (serine residue at the position of amino acid 727; Ser727) in the transcriptional activation domain is needed for its maximal transcriptional activity. A mutant form of S727A of STAT3, in which serine 727 was replaced by alanine, was estimated to have approximately 50% of transcriptional activity when compared with wild type<sup>[54]</sup>. To analyze the meaning of Ser727 phosphorylation in vivo, SA mutant mice whose STAT3 Ser727 was substituted to alanine, were produced<sup>[55]</sup>. Embryonic fibroblasts from SA/SA homozygous mice displayed approximately 50% of the transcriptional cellular responses when compared with wild-type mice; therefore, Ser727 phosphorylation is important for maximal transcriptional activities of STAT3 even in vivo. Serine phosphorylation increases STAT3 activity via the association with some cofactors, such as p300<sup>[56]</sup>. Several kinases were implicated in serine phosphorylation of STAT3, and interactions between STAT signaling and serine kinase signaling pathways have been proposed<sup>[53]</sup>.

With a yeast two-hybrid screen using the C-terminal region of STAT3 as bait, we identified ZIPK as a new STAT3-binding protein<sup>[20]</sup>. ZIPK selectively bound to STAT3, but not other STAT proteins, in mammalian cells.

Furthermore, the kinase domain of ZIPK interacted with the DNA binding and C-terminal domains of STAT3 although ZIPK kinase activities were not essential for their binding. Of importance, ZIPK phosphorylates STAT3 Ser727 in the nucleus, and functionally enhances STAT3-mediated transcription after IL-6- or LIFstimulation. siRNA-mediated knock down of endogenous ZIPK expression also proposed participation of ZIPK in STAT3-mediated transcriptional activation and target gene expression after LIF-stimulation. ZIPK, a serine/ threonine-specific protein kinase, binds to ATF4, which belongs to the activating transcription factor/cyclic AMPresponsive element binding protein family<sup>[57]</sup>. ZIPK aggregates via its leucine zipper domain to become an active enzyme form. Over-expression of wild type ZIPK, but not the kinase-inactive mutant ZIPK K42A, induces apoptosis in NIH 3T3 cells, indicating that ZIPK stimulates the apoptotic process via its catalytic activity<sup>[57]</sup>. The kinase domain of ZIPK shows high sequence homology to that of death-associated protein kinase (DAPK), and these proteins establish a family with DAPK2/DRP-1, DRAK1 and DRAK2, all of which are related to apoptosis<sup>[58-60]</sup>. In collaboration with DAXX and Par-4, ZIPK induces apoptosis by way of nuclear PML oncogenic domains (PODs)<sup>[61]</sup>. We previously reported that activated STAT3 enhanced ZIPK activity after IL-6or LIF-stimulation<sup>[20,21]</sup>. In this regard, IL-6/LIF/STAT3 signaling is likely to mediate apoptotic activity via inducing the translocation of ZIPK into PODs, together

| Protein | STAT3-binding site       | Binding site in STAT3                                            | Function             | Reported KO mice phenotype                                                                                                                                                                 | Ref. |
|---------|--------------------------|------------------------------------------------------------------|----------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| DAXX    | N-terminal (1-240)       | DNA-BD (320-493)                                                 | STAT3<br>suppression | Extensive apoptosis and embryonic lethality                                                                                                                                                | [29] |
| ZIPK    | Kinase domain (1-275)    | DNA-BD (320-493) and SH2-TAD<br>(494-750)                        | STAT3<br>activation  | Not yet reported                                                                                                                                                                           |      |
| KAP1    | Not determined           | Coiled-coil (138-319) and DNA-<br>BD (320-493)                   | STAT3<br>suppression | Severe hypoproliferative anemia (hematopoietic-restricted deletion of KAP1)                                                                                                                | [30] |
|         |                          |                                                                  |                      | Significant expansion of immature thymocytes, imbalances<br>in $CD4^{+}/CD8^{+}$ cell ratios, and altered responses to TCR<br>and TGF- $\beta$ stimulation (T-cell-specific Kap1-deletion) | [31] |
|         |                          |                                                                  |                      | Male-predominant hepatosteatosis and development of<br>liver adenoma (Liver-specific ablation of KAP1)                                                                                     | [32] |
|         |                          |                                                                  |                      | Heightened levels of anxiety-like and exploratory activity<br>and stress-induced alterations in spatial learning and<br>memory (Conditional Deletion of KAP1 in the Forebrain)             | [33] |
| Y14     | Not determined           | DNA-BD (320-493) and SH2-TAD<br>(494-750)                        | STAT3<br>activation  | Not yet reported                                                                                                                                                                           |      |
| PDLIM2  | LIM domain               | N-terminal (1-137), DNA-BD<br>(320-493) and SH2-TAD (494-750)    | STAT3<br>suppression | Enhanced Th17-cell dependent responses                                                                                                                                                     | [25] |
| STAP-2  | SH2 domain<br>YXXQ motif | Coiled-coil (138-319), DNA-BD<br>(320-493) and SH2-TAD (494-750) | STAT3<br>activation  | Reduction in LPS-induced acute phase response                                                                                                                                              | [26] |

STAT3: Signal transducer and activator of transcription 3; KO: Knock out; DNA-BD: DNA binding domain; SH2: Src homology 2 domain; TAD: Transactivation domain; TCR: T cell receptor; TGF: Transforming growth factor; Th17: T helper type 17; LPS: Lipopolysaccharide.

with PML and DAXX. Conversely, ZIPK induces STAT3 Ser727 phosphorylation, and enhances STAT3-mediated transcription. However, ZIPK K42A expression decreased STAT3 Ser727 phosphorylation in early but not late phase of IL-6-stimulation, suggesting that other kinases may be involved in the late phase of STAT3 Ser727 activation after IL-6-stimulation. Phosphorylation of Ser727 can increase STAT3 activity *via* associations with some coactivators, such as p300<sup>[56]</sup>. Of importance, ZIPK also interacts with p300 and forms a complex with STAT3.

Therefore, the binding of STAT3 to ZIPK in the nucleus may contribute to the stabilization of coactivator-transcription factor complexes.

#### A nuclear STAT3 binder, KAP1

KAP1, also known as transcriptional intermediary factor 1 $\beta$  and Tripartite motif-containing 28 (TRIM28), is a corepressor of Krüppel-associated box-domain-containing zinc finger proteins<sup>[62-64]</sup>. KAP1 has an ability to coordinate various components involving in gene silencing; therefore, it can control the histone deacetylase (HDAC) complex<sup>[65-67]</sup> and a histone methyltransferase<sup>[68]</sup>. In other words, KAP1 inhibits the transcription of its target genes *via* orchestrating functions of the co-repressor complexes.

We isolated KAP1 as a STAT3-interacting protein using a yeast two-hybrid screening of a mouse embryo cDNA library<sup>[22]</sup>. Co-immunoprecipitation experiments confirmed that KAP1 binds to STAT3 in Hep3B cells. Endogenous KAP1 was present within the nucleus even in the absence of stimulation. After IL-6 stimulation, STAT3 was predominantly found in the nucleus, where it overlapped with KAP1, demonstrating that activated STAT3 translocates into the nucleus and interacts with KAP1. In Hep3B cells, KAP1 knockdown by specific siRNA significantly enhanced STAT3 activation as well as mRNA expression of SOCS3 and C/EBP $\delta$  in response to IL-6. Thus, KAP1 negatively regulates STAT3-mediated transcriptional activation and gene expression after IL-6-stimulation. Importantly, phosphorylation of STAT3 Ser727, but not STAT3 Tyr705, increased in parallel to reduction of KAP1 expression. Coincident with these data, reduction of KAP1 expression showed enhanced nuclear accumulation of STAT3 phosphorylated at Ser727. This may be in part related to the association with some cofactors, such as p300. Therefore, KAP1 is likely to recruit protein phosphatases to dephosphorylate STAT3 Ser727 in the nucleus. Alternatively, the direct interaction of KAP1 with HDACs may also be another mechanism for KAP1-mediated transcriptional repression because STAT3 has an ability to associate with HDAC3<sup>[69]</sup>.

Therefore, KAP1 has a potential to suppress transcriptional activities of STAT3 in multiple ways.

#### A novel type of STAT3 binder, Y14

We identified Y14 as a novel associating protein with STAT3<sup>[23,24]</sup>. Y14, an RNA-binding protein, forms an exonjunction complex (EJC) with MAGOH. This complex selectively recognizes spliced forms of mRNAs immediately upstream of exon-exon junctions, and the binding is kept even after nuclear export<sup>[70,71]</sup>. In general, mRNAs produced by splicing are translated more efficiently than those from similar intronless precursors<sup>[72,73]</sup>. The EJC is in part involved in this translational enhancement because both Y14 and MAGOH recognize spliced form of mRNAs in the cytoplasm until mRNAs are translated. Human Y14 is known to shuttle mRNAs to interact with MAGOH<sup>[74]</sup>. However, only limited information is available regarding an mRNA shuttling protein involved in the regulation of transcription factors, such as STAT3.

We found that endogenous Y14 directly binds to STAT3 in Hep3B cells and affects STAT3 transactivation activity at several steps of IL-6-mediated signaling, including the tyrosine-phosphorylation, the nuclear accumulation and the DNA-binding of STAT3<sup>[23,24]</sup>. Furthermore, MAGOH inhibits complex formation between STAT3 and Y14, and MAGOH knockdown by specific siRNA enhances IL-6-induced gene expression.

Therefore, Y14 positively regulates IL-6-induced STAT3 activation, and MAGOH interferes with this effect by displacing Y14 from STAT3.

#### PDLIM2, a nuclear E3 ligase for STAT3

STAT3 activation is tightly regulated at multiple levels, including the ubiquitin/proteasome-dependent degradation of STAT3<sup>[75,76]</sup>. We found that a nuclear ubiquitin E3 ligase, PDLIM2 (also known as SLIM or mystique) binds to and degrades STAT3. PDLIM2 is a nuclear protein, composed of PDZ (postsynaptic density 65-discs large-zonula occludens 1) and LIM (abnormal cell lineage 11-isket 1-mechanosensory abnormal 3) domains<sup>[77,78]</sup>. PDLIM2 promoted to polyubiquitinate and degrade STAT3 in a proteasome-dependent manner by means of its LIM domain<sup>[25]</sup>. Consistently, PDLIM2-deficiency, as well as targeted gene disruption or knockdown of PDLIM2, caused insufficient STAT3 degradation, leading to nuclear accumulation of STAT3 and enhanced STAT3-mediated gene expression.

The LIM domain of PDLIM2 is needed for the recognition of STAT3. Ubiquitination reactions require three types of enzymes: An ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). RING-type E3 ligases provide the polyubiquitin chain from E2 to their substrate by binding to E2 via their RING-finger domain, as well as by interacting with substrate proteins via the other domain<sup>[79]</sup>. The LIM domain forms a zinc finger structure and PHD domains. Proteins containing these domains generally possess ubiguitin E3 ligase activity and polyubiguitinate their target proteins<sup>[80]</sup>. Thus, the LIM domain of PDLIM2 is thought to be enough to bind to both E2 and its substrate STAT3. This possibility was consistent with the finding that the LIM domain of PDLIM2 could individually polyubiquitinate STAT3 in vitro. Interestingly, PDLIM2 binds to both phosphorylated and unphosphorylated STAT3, but PDLIM2 was shown to bind to phosphorylated but not unphosphorylated STAT4.

Therefore, PDLIM2 may regulate STAT3 activation *via* a different mechanism from that used on STAT4.

#### STAP-2 as a novel adaptor protein for STAT3

STAP-2, which we isolated as a *c-fms*-interacting protein, is composed of an N-terminal PH domain and an SH2-like domain<sup>[26]</sup>. A proline-rich region as well as a STAT3binding YXXQ motif are also present in its C-terminal region<sup>[26]</sup>. STAP-2 is a murine homologue of an adaptor molecule BKS, which is a substrate of BRK tyrosine kinase<sup>[81]</sup>. Upon stimulation with epidermal growth factor, STAP-2 is tyrosine-phosphorylated and moves to the plasma membrane in STAP-2-overexpressing fibroblasts. IL-6 strongly induced STAP-2 mRNA in cultured hepatocytes; in addition, lipopolysaccharide-injection also induced STAP-2 mRNA in mice liver. In STAP-2deficient hepatocytes, mRNA expression of acute-phase proteins and the tyrosine-phosphorylation of STAT3 are specifically impaired at the late phase of IL-6 stimulation. Thus, STAP-2 regulates IL-6/STAT3-mediated acutephase protein responses during systemic inflammation. Furthermore, transient overexpression of STAP-2 mutant constructs revealed that STAP-2 enhances STAT3 activation through the YXXQ motif<sup>[26]</sup>. STAP-2 tyrosine-250 (Tyr250), a major tyrosine phosphorylation site by v-Src, JAK2 and LIF, is also required for the enhancement of STAT3 activity<sup>[27]</sup>. Indeed, the Y250F mutant, in which Tyr250 is substituted with phenylalanine, does not enhance STAT3 transcriptional activity.

As announced first, STAP-2 is a substrate of BRK<sup>[81]</sup>. BRK, also known as PTK6, is a non-receptor tyrosine kinase, composed of an SH3 domain, an SH2 domain and a tyrosine kinase catalytic domain, lacking an N-terminal myristoylation site for membrane targeting<sup>[82]</sup>. BRK is expressed by several malignant cells, such as metastatic melanomas and colon and prostate tumors as well as breast cancers<sup>[83-87]</sup>. In mammary gland, a large proportion of breast cancer cells express BRK, while normal mammary cells do not<sup>[88]</sup>. Notably, growth of breast cancer cells was impaired by siRNA-mediated down-regulation of BRK expression<sup>[89]</sup>. Our manipulation of STAP-2 expression indicates that STAP-2 plays an essential role in STAT3 activation by BRK. Indeed, STAP-2 bound to both BRK and STAT3, and STAP-2 knockdown by specific siRNA greatly decreased STAT3 activation induced by BRK in a breast cancer line T47D. Notably, an artificial STAP-2-BRK fusion protein had robust kinase activity and strongly induced activation and tyrosine phosphorylation of STAT3<sup>[28]</sup>.

Therefore, STAP-2 is involved in BRK-mediated STAT3 activation and tumor cell growth.

#### Possible clinical utility of targeting STAT3-related molecules in future

Of note, most of these STAT3 binding proteins also directly interact with nuclear factor- ${}_{\kappa}B$  (NF- ${}_{\kappa}B$ ) (p65/ RelA) or NF- ${}_{\kappa}B$  signaling molecules<sup>[38,78,90-92]</sup>. NF- ${}_{\kappa}B$ , as well as STAT3, is a central signaling hub in inflammation and oncogenesis. NF- ${}_{\kappa}B$  is also a transcription factor, which regulates gene expression of antiapoptosis as well as proinflammatory cytokines and chemokines<sup>[93,94]</sup>. Like STAT3, constitutively active NF- ${}_{\kappa}B$  is found in many types of cancers<sup>[95]</sup>. Both STAT3 and NF- ${}_{\kappa}B$  are also involved in the expression of target genes relating to tumor cell growth, migration and invasion<sup>[93,94,96]</sup>. Furthermore, target genes regulated by positive or negative crosstalk between STAT3 and NF- ${}_{\kappa}B$  are gradually increasing<sup>[96,97]</sup>.

In normal immune cells, activated STAT3 promotes serine-phosphorylation and subsequent proteasomemediated degradation of  $I\kappa B\alpha$ , resulting in the activation of I<sub>K</sub>B kinase<sup>[98]</sup>. In cardiomyocytes as well as non-small cell lung cancer cells, the activation of NF-KB up-regulates STAT3 expression<sup>[99,100]</sup>. Importantly, STAT3 is known to directly bind to the transactivation domain of NF- $\kappa B$ through its DNA-binding domain<sup>[101,102]</sup>. Furthermore, it has been shown that, besides nuclear translocation after cytokine-stimulation, STAT3 continuously shuttles between the cytoplasm and the nucleus, independently of its tyrosine phosphorylation. Unphosphorylated STAT3 can interact with transcription factors, such as NF-KB, bind to DNA and drive gene expression in a distinct manner from phosphorylated STAT3<sup>[103]</sup>. Thus, direct interactions between STAT3 and NF-KB can regulate gene expression in several forms of NF-kB-dependent transcription. Therefore, STAT3-regulating molecules as well as STAT3 are likely to be key players during oncogenesis or inflammation, proposing that STAT3 could be a suitable target for malignant and/or inflammatory diseases. Although many manuscripts have showed that STAT3 has physiological and/or pathological significance, clinical meanings of the interactions with STAT3 and its binding partners should be clarified in future. STAT3 binding proteins described here are likely to have a potential to regulate STAT3 activity under some malignant or inflammatory circumstance; therefore, further experiments, including the establishment of low molecular compounds to inhibit their interaction with STAT3 could help for us to gather information about their clinical utility as well as physiological and/or pathological significance. Because STAT3 deficient mice are embryonic lethal<sup>[104]</sup>, the targeting of STAT3 binding proteins may have fewer adverse effects than that of STAT 3 itself.

#### CONCLUSION

In this review, we summarized the functions of newly identified STAT3-interacting proteins. DAXX negatively regulates STAT3-mediated transactivation and cell proliferation through an IL-6 signal transducer, gp130<sup>[18,19]</sup>. ZIPK positively regulates STAT3 transactivation through STAT3 Ser727 phosphorylation<sup>[20,21]</sup>. KAP1 negatively regulates STAT3 Ser727 phosphorylation and transactivation by interacting with HDAC3 inside the nucleus<sup>[22]</sup>. Y14 regulates STAT3 transactivation via influencing tyrosinephosphorylation after IL-6-stimulation<sup>[23,24]</sup>. PDLIM2 acts as a nuclear E3 ligase for STAT3 and terminates STAT3signals<sup>[25]</sup>. STAP-2, a new adaptor protein, recognizes STAT3 through its YXXQ motif and stimulates STAT3 transactivation<sup>[26]</sup>. Although constitutive STAT3 activation is frequently observed in malignancies, few mutations in the STAT3 gene have yet been described. Therefore, it is very informative to clarify the mechanism how STAT3 is activated in malignant cells. Although direct proof is lacking, STAT3-associated proteins described here may be involved in this malignant process.

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REVIEW

# Global histone post-translational modifications and cancer: Biomarkers for diagnosis, prognosis and treatment?

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#### Abstract

Global alterations in epigenetic landscape are now recognized as a hallmark of cancer. Epigenetic mechanisms such as DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs are proven to have strong association with cancer. In particular, covalent post-translational modifications of histone proteins are known to play an important role in chromatin remodeling and thereby in regulation of gene expression. Further, histone modifications have also been associated with different aspects of carcinogenesis and have been studied for their role in the better management of cancer patients. In this review, we will explore and discuss how histone modifications are involved in cancer diagnosis, prognosis and treatment.

Key words: Epigenetics; Cancer; Diagnosis; Prognosis; Histone post-translational modifications; Treatment

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**Core tip:** The purpose of the review is to describe the potential of histone post-translational modifications in the field of cancer.

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#### INTRODUCTION

Cancer is a manifestation of both genetic and epigenetic alterations leading to the genomic instability and thus affecting several classes of genes, such as oncogenes, tumor suppressor genes, apoptotic genes and DNA repair genes. The field of cancer genetics which include the study of point mutation, deletion, insertion, gene amplification, chromosomal deletion/inversion/translocation, and allelic



loss/gain has got the attention of most cancer researchers in the last few decades. However, the appreciation of cancer epigenetics is more recent as several studies have now shown that in addition to numerous genetic alterations human cancers also harbor global epigenetic abnormalities<sup>[1,2]</sup>.</sup>

Epigenetics, was initially defined by C. H. Waddington as "the causal interactions between genes and their products, which bring the phenotype into being"<sup>[3]</sup>. With time, the definition of epigenetics has evolved and is implicated in a wide variety of biological processes. The current definition is "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence". Epigenetic mechanisms include DNA methylation<sup>[4]</sup>, noncoding RNA<sup>[5,6]</sup>, histone variants<sup>[7]</sup> and histone post translational modifications (PTMs). These mechanisms together alter the local structural dynamics of chromatin to regulate the functioning of the genome, mostly by regulating its accessibility and compactness. All together, these mechanisms govern the chromatin architecture and gene function in various cell types, developmental and disease states<sup>[2,8-12]</sup>. Disruption in the proper maintenance of these heritable epigenetic mechanisms can result in activation or inhibition of various critical cell signaling pathways thus leading to disease states such as cancer<sup>[1,13]</sup>. Epigenetic mechanisms also cooperate with genetic alteration and work together at all stages of cancer development from initiation to progression<sup>[14]</sup>. Unlike genetic alterations, epigenetic changes are reversible in nature and can be potentially restored back to their original state by epigenetic therapy. These findings have inspired many studies aimed to understand the role of epigenetics in tumorigenesis and further explore its utility in cancer diagnosis, prognosis and therapy<sup>[15]</sup>. In recent years, research focus has been shifted to understand various post translational modifications for gaining deeper insights in to the functioning of histone/chromatin associated proteins. Information about the PTMs and the related modifying enzymes is available in the database HIstome: The Histone Infobase (http://www.actrec.gov.in/ histome/)<sup>[16]</sup>. This review will discuss the role of histone post-translational modifications and its utility in cancer diagnosis, prognosis and treatment.

#### **HISTONE PTMS: A DYNAMIC PROCESS**

Histones are highly conserved and basic proteins with a globular C-terminal domain and an unstructured N-terminal tail<sup>[17]</sup>. They are also the most important proteins for converting a linear naked genome in to physiologically sensible architecture, chromatin. Nucleosomes are fundamental units of chromatin, consisting an octamer of H2A, H2B, H3 and H4 (two each) around which 146 base pairs of DNA is wrapped-. There are sequence variants of these histones which are expressed and incorporated into chromatin in a context dependent manner in normal and disease related processes. In cancer, histone H2A variants, H2A.1, H2A. Z and macroH2A have also been reported to express aberrantly<sup>[18-20]</sup>. Also, histones proteins can undergo a variety of PTMs some of which are methylation (me), acetylation (ac), ubiquitylation (ub), sumoylation (su) and phosphorylation (ph) on specific amino acid (Figure 1)<sup>[10]</sup>. Apart from these modifications, histones are also known to undergo homocysteinylation, crotonylation and glucosylation amongst others<sup>[21]</sup>. These histone modifications occur at several degrees, for example, methylation can be of monomethyl (me), dimethyl (me2) and trimethyl (me3).

Histone PTMs are added and removed from histones by enzymes called "writers" and "erasers" respectively. Histone acetyltransferases (HATs), histone methyltransferases (HMTs) and histone kinases are the examples of "writers" which add acetyl, methyl and phosphoryl groups, whereas histone deacetylases (HDACs), histone demethylases (HDMs) and histone phosphatases are examples of "erasers" which remove acetyl, methyl and phosphoryl groups, respectively (Figure 2)<sup>[22-24]</sup>. Histone-modifying enzymes are also known to interact with each other as well as other chromatin related proteins thus influencing key cellular processes such as transcription, replication and repair<sup>[10]</sup>.

The mechanism behind the regulation of key cellular processes by histone post-translational modifications is not fully understood; however, it can be generalized into two categories. First, the addition of any PTM on histone protein affects inter/intra-nucleosomal interactions and their binding to DNA by steric hindrance or charge interactions. Second, addition of these PTMs to histone proteins inhibits or facilitates the binding of various proteins to chromatin<sup>[10]</sup>. These mechanisms allow a vast range of flexibility in regulating chromatin dynamics and signaling transmission and thereby regulating the gene expression. As an example of first mechanism, histone acetylation is proposed to be associated with chromatin relaxation and transcription activation, H4K16ac inhibits the formation of compact 30 nm fibers and higher order chromatin structures<sup>[25,26]</sup>. As an example of second mechanism, evolutionarily conserved specialized proteins, termed "histone readers," possess the ability to specifically bind certain histone modifications and affects a defined nuclear process such as transcription, DNA repair and replication, etc. (Figure 2). For example, through its evolutionary conserved chromodomain heterochromatin protein 1 recognize and gets recruited to H3K9me3 and leads to the formation of compact chromatin which in turn inhibits the access of the transcriptional machinery<sup>[27,28]</sup>. Moreover, the fact that there are different variants of each histone protein differing from few to many amino acids adds another level of complexity in functional aspects of histone PTMs. Such complicated and multilayered regulatory mechanisms of cellular processes through histone modifications have led to the hypothesis of "histone code" where a set of histone variants and modifications together perform a specific function<sup>[29]</sup>. However, due to its complexity histone code is still not fully understood<sup>[30]</sup>. Further, the status of one histone modification also regulates that of another by



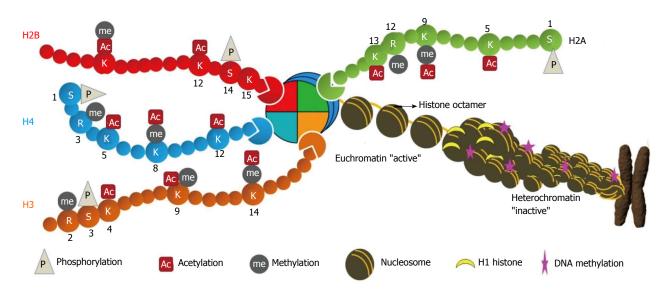


Figure 1 Chromatin architecture. The DNA is wrapped in two turns around histone octamers (nucleosomes) at intervals of about 200 bp along the DNA. Histones within the nucleosome (two each of H2A, H2B, H3 and H4) undergo numerous post-translational modifications at their N-terminal tail which protrudes from the nucleosome. Further folding of nucleosome with linker histone H1 creates a spiral structure, the heterochromatin leading to metaphase chromosome. These modifications directly regulate the chromatin structure and thus DNA-mediated cellular processes. The diagram indicates some modifications at specific residues: M: Methylation; A: Acetylation; P: Phosphorylation.

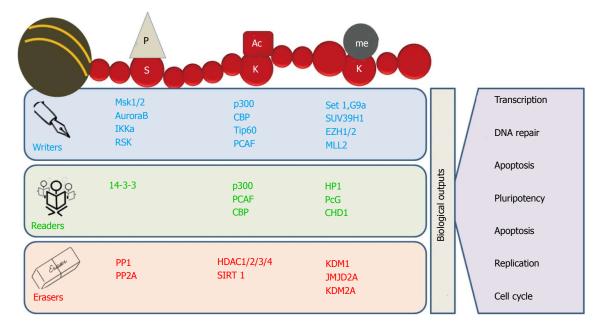


Figure 2 Readers, writers and erasers of chromatin marks. Histone modifications are highly dynamic in nature. The "writers" like histone acetyltransferases (HATs), histone methyltransferases (HMTs) and kinases add specific marks on specific amino acid residues on histone tails. These marks are identified by various proteins containing specific domains such as bromodomains, chromodomains and Tudor domain containing proteins called "readers". The written marks are removed by "erasers" like histone deacetylases (HDACs), lysine demethylases (KDMs) and phosphatases. In addition, removal and identification of these post-translational modifications on histone tails regulate various biological processes, including transcription, DNA replication and DNA repair.

cross-talk and affects chromatin remodeling and gene expression. Cross-talk between H3S10ph and H3K14ac, H2Bub and H3K4me and H3K4ac and H3K4me3 and H3K14ac are few prominent examples regulating gene expression<sup>[31]</sup>. For example, acetylation of H3K18 and H3K23 by CBP (CREB binding protein) can promote the methylation of H3R17 by Coactivator-Associated Arginine Methyltransferase 1 (CARM1), resulting in activation of estrogene-responsive genes<sup>[32]</sup>.

#### **HISTONE PTMS IN CANCER**

In cancer, several histone PTMs have been reported to be misregulated; however, their involvement in cancer pathophysiological characteristics like cellular transformation, angiogenesis and metastasis *etc.*, is not well understood. Moreover, there are very few studies commenting on the cancer specific regulatory mechanism behind the alteration of histone PTMs. It has been a

decade when global loss of H4K16ac and H4K20me3 was reported for their association with cancer and considered as a common hallmark of tumor cells<sup>[33]</sup>. However, still there are no reports of their direct involvement in cellular transformation or any other cancer characteristics. Despite of the awareness of hMOF (human Male absent Of First) and HDAC4 as writer and eraser of H4K16ac, it is a recent development that low expression of hMOF has been implicated for its loss in gastric cancer<sup>[34]</sup>. Moving on to histone methylation, Lin et al<sup>[35]</sup> showed histone lysine demethylase KDM1A mediated loss of H3K4me2 is associated with epithelial to mesenchymal transition (EMT) in human breast cancer cells. Loss of H3ac, H3K9me3 and H3S10ph is observed at the promoters of Sfrp2, Sfrp5 and Wnt5a during genistein induced development of colon cancer in the rat model system<sup>[36]</sup>. Alterations in methylation patterns of H3K9 and H3K27 are related to aberrant gene silencing in many cancers<sup>[37,38]</sup>. Tissue microarrays done to compare the levels of H2B ub1 levels in normal mammary epithelial tissue as well as benign, malignant, and metastatic breast cancer samples have clearly shown a sequential decrease in H2B monoubiquitination with breast cancer progression and metastasis in comparision with normal epithelia<sup>[39]</sup>. A very important discovery has been made in term of phosphorylation of H3S10 as the only histone marks directly associated with cellular transformation. The knockdown and mutant (S10A) of histone H3 suppressed LMP1-induced proliferation of nasopharyngeal carcinoma cell line CNE1<sup>[40]</sup>. H3S10P has been reported to increase and has been established as indispensable for cellular transformation<sup>[41,42]</sup>. Cellular transformation by v-src constitutively activated phosphorylation of histone H3 at Ser10 in a transformation-specific manner; while, non-transforming mutant of v-src did not activate H3 phosphorylation<sup>[43]</sup>. Further, Mitogen- and stress-activated kinase 1 (MSK1) has been shown to phosphorylate H3S10 in TPA and EGF mediated cellular transformation<sup>[44]</sup>. Unpublished data from our lab has also shown increase in H3S10ph in gastric cancer, which is regulated by p38-MAPK/MSK1 pathway.

It has now been clear that acetylation, methylation and phosphorylation of histones are the most studied histone marks. In cancer, most of the studies have been done for these modifications with respect to the identification of their enzymes, regulation, effect on cellular physiology and as well as molecular biological markers for the disease management. The National Institute of Health defines a biological marker (biomarker) as a biological molecule found in blood, other body fluids, or tissues that are an objective indicator of normal or abnormal process, or of a condition or disease<sup>[45]</sup>. From the next part of the review we will see how histone acetylation, methylation and phosphorylation can be exploited as biomarkers for cancer diagnosis, prognosis and treatment.

# **HISTONE PTMS IN CANCER DIAGNOSIS**

Diagnosis of a disease majorly depends on the analysis

of physical symptoms, body fluids and fecal samples. A sensitive and specific diagnostic marker is not only useful in early diagnosis, but also helps in assessing the risk of developing the disease. Advances in the technology have enabled investigators to isolate metabolites, proteins and DNA from body fluids and fecal material and correlate them with pathophysiological symptoms of diseases including cancer.

Decades of research have discovered a battery of markers for cancer diagnosis; however, only few could reach to clinics because of issues of sensitivity and specificity. Therefore, at one side there is a need to improve techniques and on the other hand discovery of new markers is of immense importance. The discovery of the presence of DNA in fecal and urine samples<sup>[46]</sup> and circulating nucleosomes in serum<sup>[47,48]</sup> has led to the foundation of identifying epigenetic markers such as DNA methylation and histone posttranslational modification for cancer diagnosis. Ahlquist et al<sup>[49]</sup> demonstrated the recovery of DNA from frozen fecal samples of colorectal cancer patients which was followed by other investigators showing matching DNA methylation patterns between DNA from tissue and fecal samples of gastric and colorectal cancer patients<sup>[50-52]</sup>. Methylation pattern of DNA isolated from urine samples was also used to diagnose bladder and prostate cancer<sup>[53-57]</sup>. All these methylation studies have successfully detected global hypomethylation and gene specific hypermethylation of DNA, as established from tissue based studies.

Presence of histone proteins is not known in fecal and urine samples; therefore, histone posttranslational modifications have been utilized as cancer diagnostic markers using circulating nucleosomes (cNUCs) in serum samples. Two histone methylation marks, H3K9me3 and H4K20me3, the hallmarks of pericentric heterochromatin<sup>[58]</sup>, were investigated in circulating nucleosomes by subsequent studies. Gezer et al<sup>[59]</sup> investigated the correlation between the H3K9me3 and H4K20me3 of cNUCs in healthy subjects and patients with colorectal cancer (CRC) and multiple myeloma and found low levels of these PTMs in cancer. Sera of patients with malignant tumors including colorectal, lung, breast, ovarian, renal, prostate cancer, and lymphoma showed high level of nucleosome concentration compared with those of healthy persons and patients with benign diseases<sup>[60]</sup>. Further, the same group showed high level ALU115 DNA sequence associated H3K9Me in multiple myeloma patients compared to healthy individuals<sup>[61]</sup>. ChIP based analysis of circulating nucleosomes in serum samples by Gloria et al reported a low level of H3K9me3 and H4K20me3 in patients with colorectal, pancreatic, breast and lung cancer compared to healthy control<sup>[62,63]</sup>. Moreover, H3K9me3 and H4K20me3 have been found to be lower at the pericentromeric satellite II repeat in patients with CRC when compared with healthy controls or patients with multiple myeloma. In summary, identification of histone PTMs from serum isolated circulating nucleosomes have open the doors of immense possibility that blood samples collected by

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| Histone PTM | Writer                 | Eraser                | Function       | Cancer Diagnosis/ Prognosis/ Treatment                       |
|-------------|------------------------|-----------------------|----------------|--------------------------------------------------------------|
| -13K9ac     | GCN-5                  | SIRT-1; SIRT-6        | Transcription  | Diagnosis: ?                                                 |
|             |                        |                       | initiation     | Prognosis: Lung, breast, ovarian                             |
|             |                        |                       |                | Treatment: ?                                                 |
| H3K18ac     | CBP/p300               | ?                     | Transcription  | Diagnosis: ?                                                 |
|             |                        |                       | initiation and | Prognosis: Lung, prostate, breast, esophagus                 |
|             |                        |                       | repression     | Treatment: ?                                                 |
| H4K5ac      | CBP/P300; HAT1; TIP60; | ?                     | Transcription  | Diagnosis: ?                                                 |
|             | HB01                   |                       | activation     | Prognosis: Lung                                              |
|             |                        |                       |                | Treatment: ?                                                 |
| H4K8ac      | TIP60; HB01            | ?                     | Transcription  | Diagnosis: ?                                                 |
|             |                        |                       | activation     | Prognosis: Lung,                                             |
|             |                        |                       |                | Treatment: ?                                                 |
| H4K16ac     | TIP60; hMOF            | SIRT-1; SIRT-2        | Transcription  | Diagnosis: Colorectal                                        |
|             |                        |                       | activation     | Prognosis: Lung, breast                                      |
|             |                        |                       |                | Treatment: ?                                                 |
| H3K4me      | SETD1A; SETD1B; ASH1L; | KDM1A; KDM1B; KDM5B;  | Transcription  | Diagnosis: ?                                                 |
|             | MLL; MLL2; MLL3: MLL4; | NO66                  | activation     | Prognosis: Prostate, kidney                                  |
|             | SETD7                  |                       |                | Treatment: ?                                                 |
| H3K4me2     | SETD1A; SETD1B; MLL;   | KDM1A; KDM1B; KDM5A;  | Transcription  | Diagnosis: ?                                                 |
|             | MLL2; MLL3; MLL4;      | KDM5B; KDM5C; KDM5D;  | activation     | Prognosis: Prostate, lung, kidney, breast, pancreatic, liver |
|             | SMYD3                  | NO66                  |                | Treatment: ?                                                 |
| H3K4me3     | SETD1A; SETD1B; ASH1L; | KDM2B; KDM5A; KDM5B;  | Transcription  | Diagnosis: ?                                                 |
|             | MLL; MLL2; MLL3; MLL4; | KDM5C; KDM5D; NO66    | elongation     | Prognosis: Kidney, liver, prostate                           |
|             | SMYD3; PRMD9           |                       |                | Treatment: ?                                                 |
| H3K9me      | SETDB1; G9a; EHMT1;    | KDM3A; KDM3B§; PHF8;  | Transcription  | Diagnosis: Myeloma                                           |
|             | PRDM2                  | JHDM1D                | initiation     | Prognosis: Kidney, pancreas, prostate                        |
|             |                        |                       |                | Treatment: ?                                                 |
| H3K9me2     | SUV39H1; SUV39H2;      | KDM3A; KDM3B§; KDM4A; | Transcription  | Diagnosis: ?                                                 |
|             | SETDB1; G9a; EHMT1;    | KDM4B; KDM4C; KDM4D;  | initiation and | Prognosis: Prostate, pancreas                                |
|             | PRDM2                  | PHF8; KDM1A; JHDM1D   | repression     | Treatment: ?                                                 |
| H3K9me3     | SUV39H1; SUV39H2;      | KDM3B§; KDM4A; KDM4B; | Transcription  | Diagnosis: Colorectal, myeloma, prostate, breast and lun     |
|             | SETDB1; PRDM2          | KDM4C; KDM4D          | initiation and | Prognosis: Lung, prostate, breast, leukemia, stomach         |
|             |                        |                       | repression     | Treatment: ?                                                 |
| H3K27me     | EZH2; EZH1             | JHDM1D                | Transcription  | Diagnosis: ?                                                 |
|             |                        |                       | activation     | Prognosis: Kidney                                            |
|             |                        |                       |                | Treatment: ?                                                 |
| H3K27me3    | EZH2; EZH1             | KDM6A; KDM6B;         | Transcription  | Diagnosis: ?                                                 |
|             |                        |                       | repression     | Prognosis: Breast, pancreatic, ovarian, prostate, stomach,   |
|             |                        |                       |                | Esophagus, Liver                                             |
|             |                        |                       |                | Treatment: ?                                                 |
| H4K20me3    | SUV420H1; SUV420H2     | ?                     | Transcription  | Diagnosis: Colorectal, myeloma, prostate, breast and lung    |
|             |                        |                       | repression     | Prognosis: Breast, lymphoma, colon, ovarian                  |
|             |                        |                       | 1              | Treatment: ?                                                 |

#### Table 1 Global post-translational modifications of histones in cancer

PTM: Post translational modification.

cancer patients can also be used for histone PTM based cancer diagnosis.

### **HISTONE PTMS IN CANCER PROGNOSIS**

In cancer, to date, histones PTMs have been mostly studied for their potential as prognostic marker (Table 1). The first report in this area strongly suggested the utility of histone PTMs in cancer diagnosis and showed loss of H4K16ac and H4K20me3 in several cancers and establish these two marks as a hallmark of tumor and establishes the correlation of H4K16ac with tumor progression<sup>[33]</sup>. Further, loss of H4K20me3 is as also detected in various cancer animal models<sup>[64,65]</sup>. A study on prostate cancer showed a positive correlation of H3K18ac, H4K12ac and H4R3me2 with increasing tumor grade<sup>[66]</sup>. Another

study on prostate cancer showed independently of other clinical and pathologic parameters, high rate of tumor recurrence in low-grade prostate carcinoma patients with low level of H3K4me2<sup>[66]</sup>. Loss of H3K4me2/me3 is reported in various neoplastic tissues such as non-small cell lung cancer, breast cancer, renal cell carcinoma and pancreatic adenocarcinoma serving as a predictor of clinical outcomes<sup>[67-72]</sup>.

Acetylation of histone H3K9 has shown ambiguous results with the increase in some and decrease in other cancers. Decrease of H3K9ac has been linked with tumor progression, histological grading and clinical stage in prostate and ovarian tumors, hence is coupled with a poor prognosis for these patients<sup>[66,73-75]</sup>. Patients with non-small cell lung adenocarcinoma exhibited better prognosis on the reduction of the H3K9ac expression



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level<sup>[68,76]</sup>. In contrast, increase in H3K9ac levels was reported in liver cancer<sup>[73]</sup>. Methylation of the same residue K9 of histone H3 requires loss of H3K9ac and is also linked to number of cancers. An association with the increase in methylation of H3K9 and aberrant gene silencing, has been found in many cancers<sup>[37,77]</sup> and its high level is associated with poor prognosis in gastric adenocarcinoma patients<sup>[77]</sup>. However, in patients with acute myeloid leukemia decrease in H3K9me3 has been found to be associated with better prognosis<sup>[78]</sup>. Decrease in H3K18ac is correlated with poor prognosis in prostate, pancreatic, lung, breast and kidney cancers<sup>[66,69,71]</sup>. It has also shown a strong correlation with tumor grade, signifying its importance in tumor progression<sup>[69]</sup>. In this regard, Kurdistani laboratory has confirmed that oncogenic transformation by the adenovirus protein E1a is associated with drastic changes in the global H3K18 acetylation pattern<sup>[79,80]</sup>. In addition, H3K18 hypoacetylation has been associated with an high risk of tumor recurrence in lowgrade prostate cancer patients<sup>[66]</sup>. However, in contrast to this, low expression of H3K18ac has been correlated with a better prognosis for esophageal squamous cell carcinoma and glioblastoma patients<sup>[76,81]</sup>. This suggests that a single histone modification could predict differential prognosis in different cancers depending on it tissue specificity.

Another histone mark, H3K27me3 has been evaluated as a prognostic factor in patients with prostate, breast, ovarian, pancreatic and esophageal cancer<sup>[81-84]</sup>, however, some of the results are perplexing and need further investigation. High level of H3K27me3 correlates with poor prognosis in esophageal cancers<sup>[81,84]</sup>. On the other hand H3K27me3 showed a negative correlation with overall survival time in breast, prostate, ovarian and pancreatic cancer patients<sup>[83]</sup>. Zhang et al<sup>[85]</sup> have identified many genes like oncogenes, tumor suppressor genes, cell cycle regulators, and genes involved in cell adhesion with significant differences in H3K27me3 pattern in gastric cancer samples in comparison to adjacent non-neoplastic gastric tissues. Further they were able to correlate changes in H3K27me3 to gene expression pattern of MMP15, UNC5B, and SHH. In non-small cell lung cancer enhanced H3K27me3 was correlated with longer overall survival (OS) and better prognosis. Moreover, both univariate and multivariate analyses indicated that H3K27me3 level was a significant and independent predictor of better survival<sup>[86]</sup>. Recently, a study showed K27M mutations of histone H3.3 variants in 31% pediatric glioblastoma tumors suggesting another level of complexity in alteration of histone PTMs in cancer which is independent of histone modifying enzymes<sup>[87]</sup>. Mass spectrometry based analysis showed high level of H3K27ac in colorectal cancer than the corresponding normal mucosa<sup>[88]</sup>. Immunohistochemical analysis on metachronous liver metastasis of colorectal carcinomas by Tamagawa et al<sup>[89]</sup> has correlated H3K4me2 and H3K9ac with the tumor histological type. In addition, lower levels of H3K4me2 correlated with a poor survival rate and also found to be an independent prognostic factor.

Recently DNA damage mark yH2AX also have shown its prognostic value. In triple negative breast tumors, high level of  $\gamma$ H2AX was associated poor overall survival<sup>[90]</sup> and which was further found to be associated with shorter telomere length<sup>[91]</sup>. In colorectal cancer a high yH2AX expression in CRC tissues was associated with tumor stage and perineurial invasion. Furthermore, a high yH2AX expression was associated with poor distant metastasisfree survival (DMFS) and OS. Cox regression analysis also revealed that yH2AX was an independent predictor of DMFS and OS. A high yH2AX expression in CRC tissues is associated with a more malignant cancer behavior, as well as poor patient survival<sup>[92]</sup>. ELISA based analysis in glioblastoma multiformes tumors showed the high level of H3T6ph,H3S10p and H3Y41ph as signatures associated with a poor overall survival<sup>[93]</sup>. Increase in H3S10ph has been associated with poor prognosis in several cancers including glioblastoma multiformes<sup>[93]</sup>, cutaneous nodular melanoma<sup>[94]</sup>, cutaneous melanoma<sup>[95]</sup>, breast cancer<sup>[96,97]</sup>, esophageal squamous cell carcinoma<sup>[98]</sup>, gastric cancer<sup>[99,100]</sup>, melanoma<sup>[101]</sup> and nasopharyngeal carcinoma<sup>[40]</sup>.

## **HISTONE PTM'S IN CANCER TREATMENT**

Reversible nature of epigenetic changes or mechanisms has drawn major attention of scientific community to study the molecular mechanism regulating the alteration in epigenetic marks, specifically the histone post-translational modifications. Such efforts have led to the discovery of several histone modifying enzymes<sup>[102]</sup> and their chemical inhibitors<sup>[103]</sup> which has emerged as an attractive strategy in cancer treatment. Targeting these enzymes can reactivate epigenetically silenced tumor-suppressor genes by modulating the levels of histone posttranslational modifications<sup>[104]</sup>. Further, these drugs have also given additional advantage in the area of combinatorial chemotherapy<sup>[105,106]</sup>.

# Histone acetyl-transferases and histone deacetylases as the targets

Loss of histone acetylation has a strong correlation with aberrant gene silencing in cancer. Treatment with HDAC inhibitors reactivate silenced tumor suppressor genes by increasing histone acetylation levels and act as antitumorigenic agent by promoting growth arrest, apoptosis and cell differentiation<sup>[107]</sup>. Additionally, HDACi have shown their potential in reversing chemoresistance and induce antiproliferative effects on a number of cancer cell lines<sup>[108-113]</sup>. However, the question still remains whether the promise shown in the above studies by HDAC inhibitors are mainly due to their potency to alter epigenetic mechanisms or mere its effect on key cellular growth regulatory pathways.

Initial results upon treatment with HDACi like valproic acid and phenylbutyrate, as a single agent against hematologic malignancies were not encouraging<sup>[81]</sup>. However, the field showed much promise with the development of more



potent HDACi such as the class-specific inhibitors (entinostat and romidepsin) and the pan HDAC inhibitors (vorinostat, belinostat and panobinostat). The field however gained boost when in a landmark Phase IIb multicenter trial, Yu et  $al^{(82)}$  have shown vorinostat as effective treatment modality for refractory cutaneous T-cell lymphoma. Further, in Phase II multi-institutional trial, romidepsin has also been shown to have significant and durable efficacy against cutaneous T-cell lymphoma<sup>[83]</sup>. Due to their great successes in many studies, HDACi romidepsin and vorinostat have been approved by FDA as the treatment regime of cutaneous T-cell lymphoma, and romidepsin also for the treatment of relapsed peripheral T-cell lymphoma<sup>[84]</sup>. Since then many other HDACi have been under study of phase I and/or  ${\rm II}$ trials as monotherapy, including belinostat, panobinostat, entinostat, chidamide, SB939 and LAQ824 in various cancers like ovarian, lung, soft tissue carcinoma, nonsmall-cell lung and breast<sup>[114-121]</sup>. However, unlike that of earlier success in treatment of lymphomas the majority of the results among solid tumor patients have been disappointing. In spite of achieving only intermittent anecdotal clinical responses, HDACi been related with severe toxicities.

Interactions between different epigenetic mechanisms have led to the foundation of research on combinatorial approach of cancer treatment using epigenetic drugs. Indeed, combinations of DNA methyltransferase and histone deacetylase inhibitors appear to synergize effectively in the reactivation of epigenetically silenced genes<sup>[107,122-124]</sup>. Such combinatorial approaches of cancer treatment have been found to be more effective than treatment with a single therapeutic agent. For example, treatment with 5-Aza-CdR and trichostatin-A in combination led to the derepression of certain putative tumor suppressor genes unlike individual treatments<sup>[107]</sup>. Pre-treatment of HDAC inhibitor SAHA relaxes the chromatin sensitizes cells to DNA damage induced by Topoisomerase II inhibitor<sup>[125]</sup>. Similarly pretreatment of valproic acid act in synergy with epirubicine and reduces the tumor volume in breast cancer mouse model<sup>[126]</sup>.

Furthermore, synergistic activity of decitabine and HDACi sodium phenylbutyrate was shown to decrease the lung cancer formation by more than 50% in comparison with decitabine alone in a murine model based study by Belinsky *et al*<sup>[124]</sup>. The same group also reported that the combination of HDACi entinostat with the DNMTi azacitidine was able to decrease tumor size and reduce the growth of K-ras/p53 mutant lung adenocarcinomas orthotopic engrafted in immunocompromised nude rats<sup>[127]</sup>. In another case HDACi sodium butyrate reduces the cell proliferation of MCF-7 cell when combine with vitamin-A<sup>[128]</sup>.

# Histone methyl-transferases and histone demethylases as the targets

Studies on histone methylation and their modifiers have been slow. Only few histone methylases (HMT) and demethylases (HDM) and their inhibitors have been discovered. However, studies on histone methylation could be more fruitful for their therapeutic potential because the less redundancy in HMTs and HDM compared to HATs and HDACs in targeting specific amino acid residue of histone<sup>[129]</sup>. This property of HMTs and HDMs provides exciting opportunities with more tailored treatment, while potentially minimizing side effects.

LSD1/KDM1 was among the first identified histone demethylases selectively targeting H3K4me1 and H3K4me2<sup>[130]</sup> and mediate gene repression. LSD1 has been reported to be overexpressed in many cancers like brain, breast, and prostate, thus thought to be a promising target for drug therapy<sup>[130-132]</sup>. Small molecules such as SL11144 and tranylcypromine have been developed to inhibit LSD1<sup>[133,134]</sup>, Since then have shown to restore expression many silenced tumor suppressors like secreted frizzled-related protein and GATA transcription factors in many cancer cell lines. They have also been shown to possess antitumor activity in a study involving neuroblastoma xenografts model<sup>[132]</sup>. However, similar to HDACi, HDM and HMT inhibitors also have off-target effects on H3K9me2 and DNMT1 thus limiting their  $\mathsf{use}^{\scriptscriptstyle[135]}$  and further in-depth studies are required. EZH2 is another methyltransferase responsible for H3K27me3 leads to gene silencing by promoting DNA methylation<sup>[136]</sup>. EZH2 is overexpressed in head and neck, breast, and prostate cancers<sup>[137]</sup> and can be targeted by a hydrolase inhibitor called 3-deazaneplanocin A (DZNep). It induces differentiation as well as apoptosis in cancer cell lines and xenografts by countering EZH2 and inhibiting H3K27 trimethylation<sup>[138,139]</sup>, while sparing normal cells.

#### Histone kinases and phosphatases as the targets

Compared to histone acetylation and methylation, the effort of regulating histone phosphorylation by targeting kinases and phosphatases for therapeutic uses is new. High level of several histone H3 phosphorylations such as H3S10ph, H3T6ph has been reported in a number of cancers. Unpublished data from our lab shows increase of H3S10ph in cisplatin resistance gastric cancer cell lines AGS and KATOIII. Our observation further supported the finding that p38 MAPK pathway mediated increase in H3S10ph in response to cisplatin treatment<sup>[140]</sup> in HeLa and MCF7 cells. Pacaud et al<sup>[93]</sup> recently reported that the kinase inhibitors like Enzastaurin (PKC-beta inhibitor), AZD1152 (Aurora-B inhibitor) and AZD1480 (Jak2 inhibitor) increases the cell death of TMZ-Irrad resistant GBM and decreases H3T3ph, H3S10ph and H3Y41ph respectively. Further, H89 (MSK1 inhibitor) treatment reduces the TPA and EGF mediated cellular transformation and by decreasing H3S10ph<sup>[44]</sup>. All these studies represent the potential of regulating histone phosphorylation for therapeutic use in cancer; however, these observations need to be further explored.

Despite of all this progress in the utilization of histone PTMs in chemotherapeutic interventions, a very little is known about their utility in monitoring the response to chemotherapy. For this purpose, levels of cNUCs and their modifications can be utilized. Because, circulating nucleosomes in serum are a result of apoptosis of



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actively dividing cells; therefore, after chemotherapy/ radiotherapy increase in the circulating nucleosomes correlates with progressive disease and decrease was associated with disease regression. Increase in the concentration of serum nucleosomes has been shown at 24-72 h after the first application of chemotherapy and 6-24 h after the start of radiotherapy<sup>[60]</sup>. Thus, the concentration of nucleosomes in serum might be a useful tool for monitoring the biochemical responses during antitumor therapy, particularly for the early estimation of therapeutic efficacy. Histone modifications such as H4K16ac for example, can be utilized in this regard as its loss has been reported in several cancers and also chemosensitize cancer cells<sup>[33,69,141]</sup>. Histone modifications like H3K27me3 have indeed showed perplexing results when analyzed with respect to various cancers. This can be attributed to tissue type, and indeed histone PTMs are known to be showing their abundance in a tissue specific manner<sup>[142]</sup>. This might be as because many writers and erasers utilize co-factors or substrates like acetyl CoA, SAM, NAD<sup>+</sup>, FAD<sup>+</sup> or ATP which are crucial metabolites in core pathways of intermediary metabolism<sup>[143]</sup>. The cellular concentrations of these metabolites fluctuate with the metabolic status of the cells and thus, the activity of these enzymes gets affected thus the histone PTMs.

# **CONCLUSION AND FUTURE DIRECTIONS**

The role of histone modifications in governing cellular functions has been not yet fully understood. However, with increased research over the past decade, all the organisms studied so far (from yeast to man) have bought to light the importance of chromatin environment especially histone PTMs in development and disease. These observations have revolutionized the field of epigenetics and have challenged the old hypothesis of the genetic code being the sole determinant of the pathophysiology of any disease. In cancer, especially this is further established with the discovery of small molecule inhibitors targeting histone modifying enzymes, which can restore the expression of various genes to normal and can induce apoptosis of transformed cells. The best studied examples of these drugs are HDACi, which have proven to be highly effective anticancer drugs, thus are in clinics. Although the exact nature of the mechanism by which these drugs act is not understood yet, still these drugs are faring better against cancer. Future studies need to be directed more towards understanding these mechanisms and increasing the potency of these drugs. Though many histone PTMs are known to change during cancer, less is understood regarding the significance and mechanistic details of the change observed. Much of the work done in this direction has been hindered due to technical limitations. However with the advent of new technologies, and also decrease in the cost of high throughput technologies like ChIP-seq and TMA amongst other global approaches, it is a matter of time we have more knowledge of these mechanisms. Also, new targets for development of more potent drugs need

to be explored by careful understanding of an already existing chromatin atlas of various cancer cell lines and tissues. Further work in the next decade may gain deeper understanding of the global patterns of histone posttranslational modifications and their corresponding changes which will hopefully reveal many molecular targets that can be employed as new weapons in long fought battle against cancer.

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MINIREVIEWS

# Cyclin A2: At the crossroads of cell cycle and cell invasion

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# Abstract

Cyclin A2 is an essential regulator of the cell division cycle through the activation of kinases that participate to the regulation of S phase as well as the mitotic entry. However, whereas its degradation by the proteasome in mid mitosis was thought to be essential for mitosis to proceed, recent observations show that a small fraction of cyclin A2 persists beyond metaphase and is degraded by autophagy. Its implication in the control of cytoskeletal dynamics and cell movement has unveiled its role in the modulation of RhoA activity. Since this GTPase is involved in both cell rounding early in mitosis and later, in the formation of the cleavage furrow, this suggests that cyclin A2 is a novel actor in cytokinesis. Taken together, these data point to this cyclin as a potential mediator of cell-niche interactions whose dysregulation could be taken as a hallmark of metastasis.

Key words: Cyclin; Mitosis; Mesenchymal transition; Metastasis; Autophagy; RhoA; Phospholipase C

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**Core tip:** Cyclin A2, as an essential regulator of the cell division cycle, is commonly associated to dividing cells and, like Ki67, is usually taken as a marker of cell proliferation. However, the level of this cyclin does not always correlate with the aggressiveness of the tumor, more particularly with respect to its invasiveness. Surprisingly, recent data suggest that it plays with RhoA



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also a role in the late phase of mitosis during which it is degraded by autophagy. Moreover, its dysregulation appears to be associated with the epithelial to mesenchymal transition.

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### INTRODUCTION

The cell division cycle is controlled by the coordinated expression of regulatory proteins whose degradation is orchestrated by specific and timely phosphorylation/ dephosphorylation events. Cyclins, as binding and activating partners of cyclin-dependent kinases (CDK) constitute a key subset of the regulatory components of the cell cycle engine. In animal cells, cyclins A and B play a central role in the control of mitosis, with cyclin A being degraded before cyclin B by the proteasome, just after the nuclear envelope breakdown. In mammalian cells there are two A-type cyclins: Cyclin A1 that is specifically expressed in the testis, and cyclin A2, that is ubiquitously expressed. Accordingly, cyclin A2 is usually linked to cell proliferation and as such is often found expressed at a high level in human cancers<sup>[1]</sup>. However, the level of this cyclin does not always correlate with the aggressiveness of the tumor, more particularly with respect to its invasiveness<sup>[2]</sup>.

A wealth of information has accumulated suggesting that some cell cycle regulators play a more general role in cellular transactions<sup>[3,4]</sup>, and along these lines, cyclin A2 has been shown to participate in the control of cytoskeleton dynamics and cell motility<sup>[5]</sup>.

# CYCLIN A2: A LATE ACTOR OF CYTOKINESIS?

Using cyclin A2-EGFP to study its ubiquitylation by FRET, we recently demonstrated that it starts in foci in prometaphase and then spreads throughout the cell in metaphase<sup>[6]</sup>. Indeed, endogenous cyclin A2 colocalized within these structures with Cdc20, a key regulator of its ubiquitylation, as well as with active proteasome, as detected with DQ-ovalbumin<sup>[6]</sup>. This unprecedented observation revealed two unexpected aspects of cyclin A2 expression: (1) not all foci colocalized with Cdc20 or DQ-ovalbumin; and (2) cyclin A2 foci persisted until late mitosis, *i.e.*, when its proteasomal degradation is thought to be complete. Since this cyclin is absent at the beginning of the G1 phase of the next round of cell division, this led us to see whether autophagy, another major intracellular proteolysis pathway, could also be involved in its degradation.

Autophagy is a degradation pathway that eliminates misfolded, damaged, or superfluous cell components, whether individual molecules or organelles, into recycled pools of biomolecules. The operating structures of autophagic degradation are the lysosomes that contain many hydrolases that break down proteins, lipids as well as nucleic acids<sup>[7]</sup>. Interestingly, some endogenous cyclin A2 foci colocalized with light chain 3-B protein (LC3-B), a marker of autophagosomes, in metaphase as well as with p62<sup>[6]</sup>, a receptor for ubiquitylated proteins necessary for their degradation by selective autophagy<sup>[8]</sup>. Furthermore, our data suggest that, in prometaphase, cyclin A2 foci colocalized mainly with Cdc20 or activated proteasome, while in metaphase, they colocalized mainly with LC3-B, p62, or lysosomes<sup>[6]</sup>.

Characterized first as a general recycling process for defective structures, autophagy is now proposed to participate in regular cellular regulatory pathways. Accordingly, our observation showing that it plays a complementary role in cyclin A2 degradation to prevent its accumulation at the end of mitosis, points to a potential novel and unexpected function for this cyclin late in cytokinesis. Indeed, formation of the mitotic cleavage furrow depends upon the activity of RhoA via its exchange factors such as Ect2<sup>[9]</sup> and GEF-H1 that localizes to the mitotic apparatus<sup>[10,11]</sup>. Our previous observation unveiled the potentiating effect of cyclin A2 on RhoA GTP loading by its exchange factors<sup>[5]</sup>. Thus, formation of the contractile ring, which is dependent upon local activation of the GTPase, would appear to result from its concomitant interaction with Ect2 and/or microtubule-activated GEF-H1 and cortical cyclin A2.

Finally, autophagy has already been shown to participate in abscission of the cleavage furrow after telophase and to promote the degradation of active RhoA<sup>[12]</sup>. This suggests that cyclin A2 may be degraded by autophagy with the GTPase (in the same complex?) when localized to cortical membranes, whereas degradation of its soluble fraction would occur mainly through proteasomal activity (Figure 1).

# EPITHELIAL TO MESENCHYMAL TRANSITION AND CYCLIN A2 DYSREGULATION

The epithelial to mesenchymal transition (EMT) is a normal biological process that is important in organogenesis during early development and in wound healing. It has recently become a leading concept to explain metastasis<sup>[13]</sup>. This process entails the trans differentiation of epithelial cells to mesenchymal cells, with an associated increase in invasive properties. Cyclin A2 depletion in fibroblasts leads to an increase in cell motility and cooperates with oncogenic transformation to increase invasiveness in collagen matrices<sup>[5,14]</sup>, a phenomenon that is associated with its cytoplasmic localization and independent of its association with the CDKs. When performed in epithelial cells, cyclin

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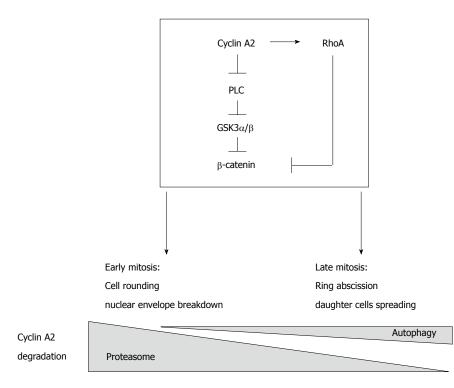


Figure 1 Presumptive regulatory network involving cyclin A2 and RhoA in the control of mitosis and epithelial to mesenchymal transition. Cyclin A2 degradation by the proteasome in mid mitosis is essential for mitosis to proceed. However, recent observations show that a small fraction of cyclin A2 persists beyond metaphase and is degraded by autophagy. Cyclin A2 participates in the modulation of RhoA activity; since this GTPase is involved in both cell rounding early in mitosis and later, in the formation of the cleavage furrow, this suggests that cyclin A2 plays also a role in cytokinesis. Taken together, these data point to this cyclin as a potential mediator of cell-niche interactions, its down regulation leading to EMT *via* PLC and RhoA converging pathways. EMT: Epithelial to mesenchymal transition; PLC: Phospholipase C.

A2 knockdown induces an EMT in RasV12-transformed mammary epithelial cells, and increases their invasiveness *in vitro* as well as in chicken embryos<sup>[14]</sup> (discussed in 15). Interestingly, these cells form tumorospheres and are more resistant to anoikis under non-adherent conditions. Whereas the Rho GTPases, RhoA and RhoC, were shown to be responsible for the invasion phenotype, with RhoA involved in the maintenance of cell-cell junctions, they do not fully account for the observed EMT phenotype<sup>[15]</sup>.

Oncogenic EMT, which is thought to arise during tumorigenesis, is generally characterized by a decrease and/or a delocalization of epithelial markers (E-cadherin, occludin), upregulation of mesenchymal markers (N-cadherin, vimentin) and transcriptional factors (Zeb1/2, Slug, Snail, Twist1/2, *etc.*), as well as functional attributes such as increase in migration, invasion, cell scattering and resistance to anoikis<sup>[16]</sup>.

Multiple signalling pathways are known to be dysregulated during tumorigenesis and EMT. Among them, the WNT pathway<sup>[17]</sup> has been shown to play a major role in metastasis and promotion of EMT in breast cancer, in which expression of WNT ligands or suppression of their inhibitors induces EMT and metastasis<sup>[18]</sup>. The canonical WNT signalling pathway is initiated by binding of a WNT ligand to a frizzled (FZD) receptor and its associated co-receptors, low-density lipoprotein receptor–related proteins 5/6. This inactivates glycogen synthase kinase- $3\beta$  (GSK- $3\beta$ ) *via* phosphorylation, followed by the release, dephosphorylation, and nuclear translocation of  $\beta$ -catenin, where it binds to T-cell factor/lymphocyte enhancer factor components and activate transcription of target genes, such as the mesenchymal marker, fibronectin<sup>[19]</sup>.

β-catenin is also known to be regulated independently of the WNT pathway, through inactivation of GSK-3 $\beta$ and/or delocalization of E-cadherin, which ultimately liberates  $\beta$ -catenin to undergo dephosphorylation and nuclear translocation. Some reports have shown that WNT-independent  $\beta$ -catenin regulation can occur through transduction components such as extracellular signalregulated kinases 1 and 2, protein kinase A, protein kinase B (AKT1), as well as phospholipases A and C isoforms. Recently, phospholipase C (PLC) isoforms have been shown to be major players in EMT, mainly in metastatic breast cancer cells, due to their roles in regulating cytoskeletal organization, differentiation and other signal transduction pathways<sup>[20]</sup>. Interestingly, isoforms of PLC are known to be activated by Rho GTPases<sup>[21,22]</sup>, and PLC- $\gamma$ is also regulated by the E-cadherin/<sub>β</sub>-catenin complex at the plasma membrane<sup>[23]</sup>.

Our recent observations suggest that inhibition of the  $\beta$ -catenin pathway and/or PLC might lead to a reversion of EMT induced by cyclin A2 depletion in Ras-transformed cells, with a concomitant increase in E-cadherin expression and localization to cell membrane, as well as decrease in mesenchymal traits in the same cellular context<sup>[24]</sup>. Thus, EMT induced by cyclin A2 depletion could well be dependent on PLC and  $\beta$ -catenin activation.

Interestingly, a previous report showed that the



integrity of adherens junctions results from the balance of the antagonistic activities of RhoA and RhoC<sup>[25]</sup>, a low level of cyclin A2 displacing the equilibrium toward the escape of  $\beta$ -catenin from the junction. That the two processes are linked or belong to synergistic pathways remains to be established. In any case, a down regulation of cyclin A2 expression appears to promote EMT and invasive properties. Accordingly, cyclin A2 protein levels have been shown to be much lower in human samples obtained from metastatic sites in comparison to matched primary colon adenocarcinomas, which suggest that a decreased expression level of cyclin A2 could be linked to cancer metastasis<sup>[25]</sup>.

## CONCLUSION

Metastasis relies on the acquisition by the candidate cell of invasive properties based on morphological changes that allow it to modify its interactions with its neighbours and, more generally, to reinterpret cues emanating from its surrounding niche. Mitosis offers such an opportunity to a cell embedded within an epithelial structure to change its fate. Indeed, while it is associated to a drastic change in cell shape, it also entails dramatic alterations of intracellular structures such as chromosome condensation and nuclear envelope breakdown. It is thus not surprising to find that dysregulation of cyclin A2 and RhoA, two major actors of its control, involved in both its early step, cell rounding, and then later, the spreading of the two daughter cells, be instrumental in giving rise to cells that escape niche controls. Interestingly, recent studies on circulating tumor cells have revealed that a large number of them harboured a phenotype that supports the idea that they were generated through an EMT-like mechanism<sup>[26]</sup>. Thus, promoting a mechanism such as EMT is nothing else but another example of how a cancer cell co-opts fundamental cellular mechanisms that are important in the early development or in wound healing.

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MINIREVIEWS

# Klotho in cardiovascular disease: Current and future perspectives

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# Abstract

Protein Klotho, beyond its role as a regulator of the phosphatemia, is also involved in the maintaining of the cardiovascular health, being associated its alterations with the development of cardiovascular damage and increased morbi-mortality. For all this, nowadays Klotho is the subject of a thorough research which is focused on uncover its intimate mechanisms of action, and in analyzing the utility of its modulation as a potential strategy with clinical applicability. Molecular mechanisms of Klotho are not well understood but an emerging research area links Klotho deficiency with vascular pathology. Changes in this protein have been associated with cardiovascular-related complications like inflammation, vascular calcification, and endothelial dysfunction. All this is particularly relevant if considering the recent discovery of Klotho expression in vascular tissue.

Key words: Klotho; Cardiovascular disease; Chronic kidney disease; Mechanism of action; Therapeutics

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**Core tip:** Protein Klotho, beyond its role as a regulator of the phosphatemia, has also been involved in the maintaining of the cardiovascular health. For all this, is the subject of a thorough research which focused on uncover its intimate mechanisms of action, and in analyzing the utility of its modulation as a potential strategy for clinical applicability. Emerging research links Klotho deficiency with vascular pathology. Changes in this protein have been associated, among others cardiovascular-related



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complications, with inflammation, vascular calcification, and endothelial dysfunction.

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## INTRODUCTION

The protein Klotho (formerly called  $\alpha$ -Klotho to distinguish it of a homologue subsequently discovered and called β-Klotho) has opened an extraordinarily wide research area because of its implication in diverse biological processes, many of them related to human longevity. Although the molecular mechanisms of action of Klotho are not well understood, but current knowledge points to the high pleiotropy of this protein with diverse biological functions and downstream targets. Functions of Klotho include regulation of energy metabolism, antiinflammatory and anti-oxidative effects, modulation of ion transport, and regulation of mineral metabolism<sup>[1]</sup>. Many of these effects have been related to the maintaining of the vascular health and the mismatch of Klotho levels has been related with the onset of cardiovascular disease (CVD).

In the organism, protein Klotho is present as a transmembrane form, which participates in the signal transduction of the phosphatonin fibroblast growth factor (FGF) 23, and as an endocrine soluble factor, detectable in blood, urine, and cerebrospinal fluid<sup>[2]</sup>. Soluble form predominates in humans<sup>[2]</sup>, declines with age<sup>[3]</sup>, and it can be generated through different ways including an alternative RNA splicing<sup>[2,4]</sup>, which generates a short soluble form of 65 kDa, and a proteolytic cleavage by membrane-anchored A Disintegrin and Metalloproteinases (ADAM)-17 and ADAM-10 and by the beta-site amyloid precursor protein-cleaving enzyme 1<sup>[5,6]</sup> which generates a longer soluble form of 130 kDa. This longer form predominates in humans.

Klotho protein deficiency was first related to CVD in the Klotho-deficient mice aging-model, which manifests many human progeroid symptoms including accelerated arteriosclerosis, associated with extensive medial calcification of the aorta, and both medial calcification and intimal thickening of medium-sized muscular arteries<sup>[7]</sup>. In addition, they exhibit impaired angiogenesis<sup>[8]</sup> and endothelial dysfunction<sup>[9]</sup> which can be ameliorated by *in vivo* gene delivery of the *Klotho* gene or by parabiosis with the Klotho wild-type specimen<sup>[10,11]</sup>.

Most of the research in Klotho has been focused in its role as renal cofactor for the binding of FGF23. The presence of Klotho in the kidneys elicits the phosphaturic effect of FGF23 and the inhibition of the synthesis of the active form of the vitamin  $D^{[12,13]}$ . However, the

existence of a soluble form of Klotho<sup>[4,5]</sup> and, importantly, its recently discovered expression in vascular tissue<sup>[14,15]</sup> and in blood<sup>[16]</sup>, has allowed to consider this molecule as a novel factor able to exert important effects in multiple organs including the cardiovascular (CV) system.

# **KLOTHO AND CVD**

The kidneys are probably the main source of soluble Klotho and are also the target of multiple mineral metabolismrelated effects of Klotho. Interestingly, both genetic and chronic kidney disease (CKD) Klotho deficiencies generate very similar systemic manifestations including CV lesions. This has prompted the recent appearance of numerous investigations focused on the association of Klotho deficiency with the extremely high CV morbidity and mortality in the renal patient<sup>[17,18]</sup>. This is a particularly relevant research area if we consider that mortality from CVD disease in a 20-year-old dialysis patient is the same as in a 80-year-old person not on dialysis<sup>[19]</sup>, and that the traditional risk factors, highly prevalent in CKD, are not enough to account for this incidence. In any case, studies focused on studying the relationship of Klotho with CVD should consider the presence of many confusing factors including age, kidney function, active vitamin D, FGF23, parathyroid hormone (PTH), medications, and Ca<sup>2+</sup> and Pi levels which affect blood soluble Klotho levels in these patients.

CVD is the leading cause of mortality in CKD patients which universally suffer from vascular calcification, inflammation, endothelial dysfunction and oxidative stress<sup>[20,21]</sup>. Reduced Klotho, in addition to traditional and CKD-related risk factors, has been proposed as a novel contributor to the appearing of these complications<sup>[22-24]</sup>.

The absence of Klotho in murine models causes accelerated aging syndrome and atherosclerosis, vascular calcifications<sup>[15]</sup>, and defects in angiogenesis<sup>[8]</sup>, and endothelial dysfunction<sup>[23]</sup>, which is reversed by administering the Klotho gene or by parabiosis with the wild-type. More recent studies have confirmed the protective effects of Klotho on the vascular system, including its participation in the maintenance of endothelial homeostasis and vascular functionality<sup>[9,23]</sup>, correlating their absence with the appearance of endothelial dysfunction and vascular calcifications<sup>[23,24]</sup>. Clinical studies have shown that low serum Klotho is associated with arterial stiffness in CKD patients<sup>[25]</sup> and independently associated with severity of coronary artery disease (CAD) in patients with normal kidney function<sup>[26]</sup>. In addition, genetic variation studies have demonstrated that Klotho gene polymorphisms might be also associated with longevity and CAD<sup>[27-29]</sup>. In particular, the KL-VS allele, characterized by six SNPs in a region of 800 bp in exon 2 and flanking sequence, is prevalent in the population and is associated with a reduced longevity<sup>[27]</sup>. In a study where two different groups of healthy siblings were tested, Arking *et al*<sup>[28]</sup> found that this functional variant of Klotho gene is an independent risk factor for CAD. This risk is modulated by



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modifiable risk factors, such as hypertension, increased high-density lipoprotein cholesterol levels or smoking<sup>[28]</sup>. Likewise, in an Ashkenazi Jew group it was found that homozygous KL-VS individuals were at higher risk of stroke than wild-type subjects<sup>[30]</sup>. In the case of G-395A polymorphism, the A allele has been found to be an independent predictor of atherosclerotic CAD but not of vasospastic angina in Japanese population<sup>[29]</sup>. This polymorphism affects the promoter of the Klotho gene, so that the  $G \rightarrow A$  substitution impairs protein binding to the region and consequently affects gene expression<sup>[31]</sup> and soluble Klotho levels. Similarly, Jo et al[32] observed an association of the G-395A allele with CAD but not with coronary artery calcification in Korean patients. Besides, subjects with the T allele for the C1818T polymorphism (located in exon 4) have lower prevalence of CAD than those with CC genotype<sup>[33]</sup>.

CAD is mainly caused by established coronary arteriosclerosis derived from endothelial dysfunction which could be developed by low Klotho levels. Stimulation of nitric oxide (NO) synthesis by Klotho, verified in experimental models<sup>[34]</sup>, could be essential for this endothelial protective effect. Consistent with this, the deficiency in Klotho expression in rats reduces the ability of arterial vasodilatation, with a decrease in the excretion of NO urinary metabolites<sup>[11]</sup>. Furthermore, this situation can be reversed by parabiosis with wild-type specimens, and also through administration of the *Klotho* gene<sup>[9,11]</sup>. Moreover, *Klotho*-deficient mice exhibit impaired angiogenesis which depends on endothelium-derived NO<sup>[35]</sup>.The mechanism by which Klotho regulates NO synthesis remains to be determined.

Klotho protein also appears to be associated with the inflammatory process. Thus, the proinflammatory cytokines tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and TNF- $\alpha$  cause the NF $\kappa$ Bdependent decrease in the expression of Klotho, both *in vitro* and *in vivo*<sup>[36]</sup>, while in human umbilical endothelial cells, the addition of Klotho is able to suppress the expression of adhesion molecules in the endothelium induced by TNF- $\alpha$ <sup>[37]</sup>. Moreover, the soluble form of Klotho is capable of inhibiting the Wnt signaling<sup>[38]</sup>. Although this signal is essential to ensure the proliferation and survival of stem cells, an excess exposure to it can contribute to depletion and accelerated senescence<sup>[39]</sup>. Therefore, the attenuation of the Wnt signal by Klotho contributes to the anti-aging properties of this protein.

Ectopic calcifications in soft tissues, including cardiac valves and aorta, have been also related to low Klotho soluble levels<sup>[7,24,40]</sup>. Vascular calcification is prominent in the Klotho mutant mice, similarly to CKD subjects, and can be reversed by Klotho overexpression through adenoviral delivery of *Klotho* gene<sup>[10]</sup>. Albeit in CKD the release of Ca<sup>2+</sup> and Pi, by disturbed PTH, low calcitriol and soluble Klotho and high FGF23, may trigger or accelerate vascular calcification<sup>[41]</sup>, the existence of direct effects of Klotho and also of FGF23 is the subject of extensive research at present.

# PLAUSIBLE MECHANISMS OF ACTION OF KLOTHO IN THE VASCULATURE

Many vascular molecular actions are proposed to explain Klotho vascular-protective effects. Klotho is expressed in the vasculature<sup>[14,15]</sup> and is probably that the formation of both, the membrane and the soluble form, coexists in the vascular beds. Since membrane Klotho protein has an extremely short intracellular domain, it is unlikely that displays a signal transduction by itself. More probably, membrane Klotho solely serves as a correceptor for the binding to FGF receptors (FGFRs) and as a source of a soluble form<sup>[42]</sup>. However, it is still unknown what is the contribution of the vascular bed to the blood levels, and which are the regulatory mechanisms of Klotho shedding. Serum Klotho levels determined by ELISA varies between 200 and 740 pg/mL<sup>[43]</sup>.

Similarly, the mechanisms of action of soluble Klotho remain unknown and the existence of a receptor for this protein is a matter of debate. Because FGFRs are quite ubiquitous, the binding of soluble Klotho to these receptors has been proposed<sup>[44]</sup> as an explanation to the pleiotropic effects of Klotho. Theoretically, soluble Klotho can bind to this FGFRs but there is no evidence that this activates signalling events<sup>[44]</sup>. Another possibility is that soluble Klotho does not act as an hormone sensu stricto, since it lacks of a specific receptor. In this scenario, the hypothetical functions of soluble Klotho would be based on its putative enzymatic abilities. Different works point to the glycan-modifying activity of circulating Klotho. Thus, a putative activity as sialidase which removes terminal sialic acids from N-linked glycans of glycoproteins in the cell surface has been proposed to explain the effect of circulating Klotho in preventing the endocytosis of the transient receptor potential cation channel, subfamily V, member 5 (TRPV5) and the renal outer medullary potassium channel 1 in the kidneys<sup>[45,46]</sup>, thereby increasing renal reabsorption of Ca<sup>2+</sup> and excretion of K<sup>+</sup>, respectively. This enzymatic activity is also responsible of promoting the endocytosis of the NaPi-2a cotransporter in the renal proximal tubule<sup>[47]</sup>. But circulating Klotho acting as a glycan-modifying enzyme could also exert a broad range of effects beyond the kidneys; the addition of recombinant soluble Klotho protein to rat vascular smooth muscle cells (VSMCs) cultures is able to decrease high Pi-induced calcification by diminishing expression and activity of Na/Pi cotransporters type 3 (Na/Pi-3, also known as Pit 1 and Pit 2)<sup>[24,47]</sup>. Na/Pi-3 cotransporters are widely expressed in tissues such as intestinal epithelium, liver, lung, heart and VSMCs<sup>[48,49]</sup>, but to date there is no evidence for an effect of Klotho on Pit-1 and 2 in the vascular beds. Finally, the aminoacidic sequence of Kotho shares homology with a  $\beta$ -glucosidase. However, this glucosidase activity has not been confirmed<sup>[7,50]</sup>.

Another possible explanation for some of the CV disorders observed in situations of low levels of Klotho comes from FGF23. In this sense, vascular calcification might be an off-target effect of FGF23 levels. Lim *et* 

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 $al^{(15)}$  proposed that vascular Klotho acts not only as an endogenous inhibitor of vascular calcification in VSMCs but also, as a cofactor required for vascular FGF23 signaling. In this study, restoration of Klotho expression in knock-down cells unmasked FGF23 anticalcific effects<sup>[15]</sup>. Finally, a second effect of FGF23 on CVD derives from the extremely high levels of this hormone observed in primary (mainly genetic) and secondary (commonly in CKD) Klotho deficiency states<sup>[7,24,51-55]</sup>. In CKD, uremic cardiomyopathy, characterized by cardiac hypertrophy and fibrosis, is a principal cardiovascular pathological feature and includes left ventricular hypertrophy (LVH). Novel risk factors for LVH in uremic cardiomyopathy are low levels of soluble Klotho and high levels of FGF23<sup>[55,56]</sup>. In fact, FGF23 causes LVH after intramyocardial and intravenous administration in wild-type mice<sup>[55]</sup>. Since Klotho is not normally expressed in the ventricles, the effects caused by Klotho deficiency might be due to other parameters. One of the parameters considered is FGF23. Some in vivo experiments show that Klotho influences FGF23 production<sup>[57]</sup>, however, there are no *in vitro* data supporting this effect.

Klotho is also involved in the modulation of inflammation. The mechanism of regulation of inflammation by Klotho may be based in the inhibition of NF- $\kappa$ B activity in the endothelium<sup>[37]</sup> by unknown mechanisms. Similarly, secreted Klotho protein is also able to suppress the Wnt biological activity<sup>[38,58]</sup>. In this case the binding of Klotho to various Wnt family members results in the signal inhibition.

Finally, Klotho has been related to the regulation of the intrinsic generation of reactive oxygen species (ROS) which play a role in aging and longevity determination. Yamamoto et al<sup>[59]</sup> determined that cell surface-bound Klotho inhibited FOXO3a phosphorylation and promoted its nuclear translocation. FOXO3a is a member of the O subclass of the forkhead family of transcription factors which are characterized by a fork head DNA binding domain. Nuclear translocated FOXO3a then became bound to the MnSOD promoter and upregulating its expression which results in suppressing ROS formation. Moreover, Ohta et al<sup>[60]</sup> investigated the effect of Klotho gene delivery on blood pressure and oxidative stress in vivo finding an upregulation of MnSOD expression and total SOD activity in the aorta of mice, enhanced NO production, and downregulated lipid peroxide concentration in serum of mice. It was concluded that Klotho gene infusion into the tail vein of mice and rats suppressed ROS formation in animals.

# ABOUT THE THERAPEUTIC UTILITY OF KLOTHO

Injection of soluble Klotho produces biological effects in experimental animal models with kidney injury<sup>[24,61,62]</sup>, which is a state of Klotho deficiency, and in intact animals<sup>[44,47]</sup>. This supports the idea of a therapeutic approach based on the administration of soluble Klotho. In CKD, one of the

earliest events is Klotho deficiency, and establishing whether Klotho has therapeutic potential treating this disease and its complications is a critical step. However, no study has documented the therapeutic effect of Klotho in humans with renal disease.

Another approximation is based on the stimulation of Klotho expression. Experimental studies show that calcitriol administration promotes expression of Klotho *via* the activation of vitamin D receptor<sup>[63,64]</sup>. A recent work has demonstrated that administration of alfacalcidol, a vitamin D receptor activator, promoted an up regulation of *Klotho* gene expression in the kidney of nephrectomized spontaneously hypertensive rats<sup>[65]</sup>. According this, Klotho variants associated with lower *Klotho* gene expression have been associated with a decrease in survival of dialysis patients, more pronounced among patients not treated with active forms of vitamin D<sup>[66]</sup>.

Strategies aimed to increase extrarenal Klotho production might be of particular importance in end-stage renal disease patients with a significant loss of functional renal tissue. In these patients, administration or stimulation of synthesis of Klotho may potentially reverse or retard the disease progression. Therapeutic effects of Klotho may result from the modulation of circulating hormones, regulation of mineral parameters or from direct effects on target organs, leading to induction of phosphaturia, anticalcifying, antifibrotic, and antioxidative effects, and antagonism of angiotensin II effects.

Another therapeutic approach to increase Klotho levels could be based on the recently examined relationship between inflammation and Klotho expression, showing that the inflammatory cytokines TWEAK and TNF $\alpha$  promote the NF $\kappa$ B dependent downregulation of Klotho expression both *in vitro* and *in vivo*<sup>[36]</sup>. Activation of these inflammatory cytokines and the subsequent reduction of Klotho expression could contribute to organ damage. In this sense, regulation of Klotho expression by anti-inflammatory therapeutic treatments, including statins, could suppose a new approach to delay the progression of renal damage.

More specifically, Klotho expression in the vasculature may also become an important option in therapeutics. If resident vascular Klotho protects the vasculature against vascular disorders, either as a transmembrane or as a soluble form in paracrine and/or autocrine manner, upregulation of vascular synthesis could be reflected in the prevention of many vascular disorders such as endothelial dysfunction, arterial stiffness and vascular calcification.

# CONCLUSION

Nowadays, the major interest to the clinical application of Klotho research is related to CKD, where FGF23 and Klotho have been linked with CV morbidity and mortality independently of phosphatemia<sup>[67]</sup>. Importantly, these associations remain in population without impaired kidney function. Thus, Klotho has been proposed as a



potential therapeutic option. Another open question is the elucidation of the contribution of soluble vascular Klotho in advanced stages of CKD, where low serum Klotho levels are frequent. Thus far, measurements of vascular Klotho mRNA and protein in humans have been limited. It is unknown if vascular expression is also lowered in advanced CKD stages or remains at physiologic levels. Anyway, it is probably that vascular derived Klotho plays a more important role in these patients since renal contribution is dramatically diminished. What we know is that vascular expression of Klotho, along with blood soluble levels, are decreased in CAD patients without renal disease<sup>[26]</sup>.

Although depicted results clearly point to Klotho as a potential therapeutic agent in mineral-cardiovascular disorders, further studies are needed to evaluate the reliability and practical utility of this protein. Despite the controversies regarding Klotho expression and its effects, current data point to a pathogenic role of Klotho deficiency the CVD. Overall, the recent discovery of FGF23 and Klotho, and the elucidation of their regulatory effects on the phosphate homeostasis, has been accompanied by significant contributions to the CVD researching field. These discoveries will revert in the development of new targets and therapies with potential clinical applications.

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MINIREVIEWS

# Caldecrin: A pancreas-derived hypocalcemic factor, regulates osteoclast formation and function

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# Abstract

Caldecrin was originally isolated from the pancreas as a

factor that reduced serum calcium levels. This secreted serine protease has chymotrypsin-like activity and is also known as chymotrypsin C; it belongs to the elastase family. Although intravenous administration of caldecrin decreases the serum calcium concentration even when its protease activity is blocked, this effect does require cleavage of caldecrin's pro-peptide by trypsin, converting it to the mature enzyme. Ectopic intramuscular expression of caldecrin prevented bone resorption in ovariectomized mice. Caldecrin inhibited parathyroid hormone-stimulated calcium release from fetal mouse long bone organ cultures. Furthermore, caldecrin suppressed the formation of osteoclasts from bone marrow cells by inhibiting the receptor activator of nuclear factor-k B ligand (RANKL)stimulated phospholipase Cy-calcium oscillation-calcineurinnuclear factor of activated T-cells, cytoplasmic 1 pathway. Caldecrin also suppressed the bone resorption activity of mature osteoclasts by preventing RANKL-stimulated Src activation, calcium entry, and actin ring formation. In vivo and in vitro studies have indicated that caldecrin is a unique multifunctional protease with anti-osteoclastogenic activities that are distinct from its protease activity. Caldecrin might be a potential therapeutic target for the treatment of osteolytic diseases such as osteoporosis and osteoarthritis. This mini-review describes caldecrin's historical background and its mechanisms of action.

Key words: Serine protease; Osteoclasts; Hypocalcemia; Chymotrypsin; Bone resorption; Calcium signaling

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**Core tip:** Caldecrin (also known as chymotrypsin C) reduces serum calcium levels. This activity is distinct from its protease activity but also requires trypsin-mediated cleavage of the pro-peptide, converting caldecrin to its active form. Ectopic intramuscular expression of caldecrin prevented bone resorption in ovariectomized mice. Caldecrin inhibited parathyroid hormone-stimulated calcium release from fetal mouse long bones. Furthermore, caldecrin suppressed receptor activator of nuclear factor-



kappa B ligand-induced activation of intracellular calcium signaling, thereby reducing osteoclast formation and bone resorption. Caldecrin is a unique multifunctional protease that possesses anti-osteoclastogenic activity, resulting in reduced serum calcium levels.

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## INTRODUCTION

Calcium homeostasis is controlled by intestinal calcium absorption and calcium resorption in the kidney, as well as by bone formation and resorption. Clinical and experimental observations have also linked the pancreas to calcium homeostasis. Pancreas-derived glucagon<sup>[1,2]</sup>, amylin<sup>[3,4]</sup>, and calcitonin gene-related peptide<sup>[5,6]</sup> have been shown to regulate calcium homeostasis, while acute and chronic pancreatitis have been shown to associate with hypocalcemia<sup>[7]</sup>.

In the 1960's, the pioneering work of Takaoka et  $al^{[8,9]}$  demonstrated that a porcine pancreatic extract had hypocalcemic activity. In 1992, we first successfully purified a hypocalcemic factor named caldecrin from a pancreatic extract using chromatographic separation techniques including ion exchange, gel filtration chromatography, and high-performance liquid chromatography<sup>[10]</sup>. To identify caldecrin, each fraction was intravenously administered to overnight-fasted mice and serum calcium concentrations were measured 4 h postinjection. In addition, the samples were assayed for their inhibition of parathyroid hormone-stimulated calcium release from fetal mouse long bone organ cultures. Caldecrin is an anionic protein (pI: 4.5) with a molecular weight of about 28 kDa; it was found to be a serine protease with chymotryptic activity.

In 1995, we isolated rat caldecrin cDNA from pancreatic cDNA expression library by immunoscreening with an anti-caldecrin antibody<sup>[11]</sup>. A partial amino acid sequence of caldecrin purified from rat pancreas was completely matched with that encoded by the cDNA. The nucleotide sequence was almost identical (except for three nucleotides) to that of a PCR clone referred as elastase IV (ELA4)<sup>[12]</sup>. Comparison of the amino acid sequences encoded by these two cDNAs indicated that the central region of caldecrin differed from that of ELA4 due to a frame shift caused by this minor nucleotide change (Figure 1). The amino acid sequences of the purified caldecrin fragments, including the central region, were consistent with the deduced amino acid sequence of caldecrin but not with that of ELA4. Over-expression of the ELA4 PCR clone in Sf9 cells caused a complete loss of secretion, low expression levels, and much lower protease activity<sup>[13]</sup>. Furthermore, the rat genomic DNA

| Species | Pancreatic protease | Identity (%) | Similarity (%) |
|---------|---------------------|--------------|----------------|
| Rat     | Caldecrin           | 100          | 100            |
|         | Chymotrypsin B      | 41           | 55             |
|         | Elastase I          | 51           | 67             |
|         | Elastase II A       | 59           | 72             |
|         | Elastase III B      | 57           | 71             |
| Human   | Caldecrin           | 78           | 88             |
|         | Chymotrypsin B      | 41           | 56             |
|         | Elastase II A       | 61           | 74             |
|         | Elastase II B       | 56           | 70             |
|         | Elastase III A      | 57           | 70             |
|         | Elastase ∭B         | 55           | 69             |
| Cow     | Chymotrypsin A      | 39           | 57             |

Sequence identity: Percent of same amino residues in a sequence alignment between 2 sequences; Sequence similarity: Percent amino acid sequence identity and percent positive substitutions between 2 sequences.

sequence matched that of the caldecrin cDNA, but not that of the ELA4 clone<sup>[13]</sup>. Therefore, the ELA4 PCR clone may be a cloning artifact or represent a mutant caldecrin gene. In 1995, the crystalline structure of bovine chymotrypsinogen C was reported<sup>[14-16]</sup> and its amino acid sequence was very close to that of rat caldecrin, thereby suggesting a similarity between caldecrin and chymotrypsin C (CTRC). It is now known that CTRC, caldecrin, and ELA4 are the same protein, which is encoded by the CTRC gene and known officially as CTRC (caldecrin), according to the HUGO Gene Nomenclature Committee. Table 1 compares the amino acid sequence of rat caldecrin with that of other members of the rat and human pancreatic chymotrypsin and elastase families. Caldecrin shows a greater similarity with elastase than with chymotrypsin. In addition, expressed recombinant human caldecrin also showed serum calcium-decreasing activity, even following phenylmethylsulfonyl fluoride treatment to abolish its protease activity<sup>[17]</sup>.

In 1996, another research group purified a calcium metabolism-regulating factor from the porcine pancreas by determining its stimulatory effects on proliferation of the osteosarcoma MG-63 cell line and its inhibition of 1, 25 vitamin D<sub>3</sub>-stimulated calcium release in organ cultures<sup>[18]</sup>. The terminal sequence of the 28-kDa protein that was isolated corresponded to that of human elastase III B. Recombinant elastase III B decreased interleukin-1-induced hypercalcemia and this effect was dependent on its protease activity. Although both have been isolated from the pancreas, caldecrin and elastase III B were found to be different molecules that exerted their hypocalcemic effects *via* different mechanisms of action.

# PROTEIN STRUCTURE AND PROTEASE ACTIVITY OF CALDECRIN

The human *CTRC* gene maps to chromosome 1p36.21. The homologous mouse and rat genes are located on chromosomes 4E1 and 5q36, respectively. The *CTRC* genes consist of 8 exons in these species. Northern blot



|       |                             |                       |                       |                       |                       |                       |                      |                        |                       |                     |                        |                        |                       |                       |                 | ł                     | ,                     |                       |                       |                        |                   |
|-------|-----------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|----------------------|------------------------|-----------------------|---------------------|------------------------|------------------------|-----------------------|-----------------------|-----------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------------|-------------------|
| rCal: | ATG<br>M                    | TTG<br>L              | GGA<br>G              | ATT<br>I              | ACG<br>T              | GTC<br>V              | CTC<br>L             | GCT<br>A               | GCC<br>A              | ATC<br>I            | CTG<br>L               | GCC<br>A               | TGC<br>C              | GCC<br>A              | TCT<br>S        | TGC<br>C              | TGC<br>C              | GGG<br>G              | AAC<br>N              | CCC<br>P               | 60<br>4           |
| Ela4: | · · ·<br>*                  | <br>*                 | *                     | ••••                  | ••••<br>•             | *                     | ••••<br>*            | ••••                   | ••••                  | *                   | *                      | ••••                   | ••••                  | <br>*                 | ••••            | *                     | ••••                  | • • •                 | ••••                  | *                      | 60<br>4           |
| rCal: | GCC                         | TTC                   | CCA                   | CCT                   | AAC                   | CTG                   | TCA                  | ACC                    | AGA                   |                     | GTA                    | GGA                    | GGA                   | GAG                   | GAT             | GCT                   | GTC                   | CCC                   | AAC                   | AGC                    | 120               |
| Ela4: | A<br><br>*                  | <br>*                 | <br>*                 | <br>*                 | N<br><br>*            | L<br><br>*            | <br>*                | т<br><br>*             | R<br><br>*            | <br>*               | <br>*                  | G<br><br>*             | G<br><br>*            | E<br><br>*            | D<br><br>*      | A<br><br>*            | <br>*                 | Р<br><br>*            | N<br><br>*            | <br>*                  | 24<br>120<br>24   |
| rCal: |                             | ССТ                   |                       | CAG                   |                       | TCT                   | CTC                  | CAG                    | TAC                   |                     | AAG                    |                        | GAC                   | ACA                   |                 | AGG                   |                       | ACC                   | TGT                   | GGG                    | 180               |
| Ela4: | <br>*                       | Р<br>G・・<br>А         | . W<br><br>*          | .Q<br>*               | <br>*                 | <br>*                 | ц<br><br>*           | .Q<br>*                | . Y<br><br>*          | <br>*               | к<br><br>*             | D<br><br>*             | D<br><br>*            | т<br><br>*            | <br>*           | R<br><br>*            | н<br><br>*            | т<br><br>*            | с<br><br>*            | G<br><br>*             | 44<br>180<br>44   |
| rCal: |                             | AGT<br>S              | CTC                   | ATC                   | ACC<br>T              | ACC<br>T              | AGC<br>S             | CAC                    | GTC                   | CTC                 | ACT                    | GCC                    | GCC                   | CAC                   | TGC<br>C        | ATC                   |                       | AAA                   | GAC                   | TTC                    | 240               |
| Ela4: | G<br><br>*                  | <br>*                 | <br>*                 | <br>*                 | <br>*                 | <br>*                 | ъ<br><br>*           | . H<br><br>*           | <br>*                 | <br>*               | т<br><br>*             | A<br><br>*             | <br>*                 | ( <u>H</u> ).<br>*    | <br>*           | п<br><br>*            | N<br><br>*            | К<br><br>*            | D<br><br>*            | F<br><br>*             | 64<br>240<br>64   |
| rCal: | ACT                         | TAC<br>Y              | CGT                   | GTG<br>V              | GGC                   | CTG                   |                      | AAG<br>K               | TAT                   | AAT                 | CTG                    | ACA<br>T               | GTG                   | GAG<br>E              | GAT             | GAG                   | GAA<br>E              | GGC                   | TCC                   | GTG<br>V               | 300<br>84         |
| Ela4: | т<br><br>*                  | <br>*                 | R<br><br>*            | <br>*                 | <br>*                 | <br>*                 | G<br><br>*           | <br>*                  | . Y<br><br>*          | N<br><br>*          | ц<br><br>*             |                        | <br>*                 |                       | D<br><br>*      | ·C ·<br>A             |                       | G<br>G<br>A           | CCC<br>P              |                        | 300<br>84         |
| rCal: |                             |                       | GAG                   | GTG                   |                       |                       | ATC                  | TAC                    | GTC                   | CAT                 | GAG                    |                        |                       | AAC                   | CGA             |                       | TTC                   | CTG                   |                       | AAC                    | 360               |
| Ela4: | Y<br>AC <mark>Z</mark><br>T | A<br>CTO<br>L         | E<br>G AGO<br>R       | V<br>G TGO<br>W       | D<br>G ACI<br>T       | T<br>A CCZ<br>P       | I<br>A TCI<br>S      | Y<br>F ACO<br>T        | V<br>G TCO<br>S       | H<br>CATO<br>M      | E<br>G AGZ<br>R        | K<br>A AGI<br>S        | W<br>GG2<br>G         | N<br>A ACO<br>T       | R<br>C GAC<br>D | L<br>TCI<br>S         | F<br>TCC<br>S         | L<br>TGI<br>C         | W<br>GGZ<br>G         | A ACC<br>T             | 104<br>360<br>104 |
| rCal: | GAC<br>(D)                  | ATC<br>I              | GCT<br>A              | ATC<br>I              | ATT<br>I              | AAG<br>K              | TTG<br>L             | GCT<br>A               | GAG<br>E              | CCT<br>P            | GTG<br>V               | GAA<br>E               | CTG<br>L              | AGC<br>S              | AAC<br>N        | ACC<br>T              | ATC<br>I              | CAG<br>Q              | GTG<br>V              | GCC<br>A               | 420<br>124        |
| Ela4: |                             | . <del>.</del> .<br>* |                       | . <del>.</del> .<br>* | . <del>.</del> .<br>* | *                     | . <del></del> .<br>∗ | . <del>**</del> .<br>* | . <del></del> .<br>.★ | . <del>.</del><br>* | <br>*                  | . <del>.</del> .<br>*  | . <del></del> .<br>★  | .~.<br>*              | .".<br>*        | . <del>.</del> .<br>* | . <del>.</del> .<br>* | .~.<br>*              | . <b>.</b> .<br>*     | . <del>**</del> .<br>* | 420<br>124        |
| rCal: | TGC<br>C                    | ATC<br>I              | CCA<br>P              | GAG<br>E              | GAA<br>E              | GGT<br>G              | TCC<br>S             | CTG<br>L               | CTG<br>L              | CCT<br>P            | CAG<br>Q               | GAC<br>D               | TAT<br>Y              | CCC<br>P              | TGC<br>C        | TAT<br>Y              | GTC<br>V              | ACG<br>T              | GGC<br>G              | TGG<br>W               | 480<br>144        |
| Ela4: | · · ·<br>*                  | . <del>.</del> .<br>* | <br>*                 | . <del>.</del> .<br>* | . <del>.</del> .<br>* | <br>*                 | <br>*                | . <del>.</del> .<br>*  |                       | <br>*               | <br>*                  |                        |                       | . <sup>-</sup><br>*   | <br>*           |                       | <br>*                 | . <del>.</del> .<br>* |                       | · · · ·<br>*           | 480<br>144        |
| rCal: | GGT<br>G                    | CGC<br>R              | CTC<br>L              | TGG<br>W              | ACC<br>T              | AAT<br>N              | GGT<br>G             | CCC<br>P               | ATC<br>I              | GCT<br>A            | GAA<br>E               | GTG<br>V               | CTC<br>L              | CAG<br>Q              | CAG<br>Q        | GGC<br>G              | CTG<br>L              | CAG<br>Q              | CCC<br>P              | ATC<br>I               | 540<br>164        |
| Ela4: | · · ·<br>*                  |                       | . <del>.</del> .<br>* | <br>*                 |                       | *                     | <br>*                | . <del>.</del> .<br>*  | . <del>.</del> .<br>* |                     | . <del>.</del> .<br>*  | <br>*                  | . <del>.</del> .<br>* | .~.<br>*              | .~.<br>*        | <br>*                 | . <del>.</del> .<br>* | .~.<br>*              | . <del>.</del> .<br>* | . <del>.</del> .<br>*  | 540<br>164        |
| rCal: | GTG<br>V                    | AGC<br>S              | CAT<br>H              | GCC<br>A              | ACG<br>T              | TGC<br>C              | TCC<br>S             | AGG<br>R               | TTG<br>L              | GAC<br>D            | TGG<br>W               | TGG<br>W               | TTC<br>F              | ATC<br>I              | AAG<br>K        | GTC<br>V              | CGG<br>R              | AAG<br>K              | ACG<br>T              | ATG<br>M               | 600<br>184        |
| Ela4: | · · · ·<br>*                | <br>*                 | . <del>.</del> .<br>* | <br>*                 | . <del>.</del> .<br>* | <br>*                 | <br>*                | <br>*                  | . <del>.</del> .<br>* |                     | <br>*                  | ."<br>*                | . <sup>-</sup><br>*   | . <del>.</del> .<br>* |                 | <br>*                 |                       |                       |                       | · · · ·<br>*           | 600<br>184        |
| rCal: | GTG<br>V                    | TGC<br>C              | GCT<br>A              | GGG<br>G              | GGT<br>G              | GAT<br>D              | GGC<br>G             | GTC<br>V               | ATC<br>I              | TCT<br>S            | GCC<br>A               | TGT<br>C               | AAC<br>N              | GGA<br>G              | GAT<br>D        | TCT                   | GGC<br>G              | GGC<br>G              | CCA<br>P              | CTG<br>L               | 660<br>204        |
| Ela4: | · · ·<br>*                  | <br>*                 |                       | <br>*                 | <br>*                 |                       | <br>*                | <br>*                  | . <del>.</del> .<br>* | <br>*               | . <sup>11</sup> .<br>* | <br>*                  |                       | <br>*                 |                 | *                     | <br>*                 | <br>*                 | . <sup>-</sup><br>*   | . <del>.</del> .<br>*  | 660<br>204        |
| rCal: | AAC<br>N                    | TGC<br>C              | CAA<br>Q              | GCA<br>A              | GAA<br>E              | GAC<br>D              | GGC<br>G             | TCA<br>S               | TGG<br>W              | CAG<br>Q            | GTG<br>V               | CAC<br>H               | GGC<br>G              | ATC<br>I              | GTG<br>V        | AGC<br>S              | TTC<br>F              | GGT<br>G              | TCC<br>S              | AGT<br>S               | 720<br>224        |
| Ela4: | · · · ·<br>*                | <br>*                 | ·~·<br>*              |                       |                       | <br>*                 | <br>*                | <br>*                  | ."<br>*               | .*.<br>*            | <br>*                  | . <sup>11</sup> .<br>* | <br>*                 | .†<br>*               | <br>*           |                       | . <sup>-</sup><br>*   | <br>*                 |                       | · · ·<br>*             | 720<br>244        |
| rCal: | AGC<br>S                    | GGC<br>G              | TGC<br>C              | AAC<br>N              | GTA<br>V              | CAC<br>H              | AAG<br>K             | AAA<br>K               | CCG<br>P              | GTA<br>V            | GTC<br>V               | TTC<br>F               | ACC<br>T              | CGA<br>R              | GTG<br>V        | TCT<br>S              | GCC<br>A              | TAC<br>Y              | AAT<br>N              | GAC<br>D               | 780<br>244        |
| Ela4: | <br>*                       | <br>*                 | ••••<br>*             | <br>*                 | ••••<br>•             | <br>*                 | <br>*                | <br>*                  |                       | <br>*               | ••••<br>•              |                        | . <del>.</del> .<br>* | <br>*                 | <br>*           | <br>*                 |                       | . <del>.</del> .<br>* | <br>*                 | <br>*                  | 244<br>780<br>244 |
| rCal: | TGG<br>W                    | ATC<br>I              | AAC<br>N              | GAG<br>E              | AAA<br>K              | ATA<br>I              | CAA<br>Q             | CTG<br>L               |                       |                     |                        |                        |                       |                       |                 |                       |                       |                       |                       |                        |                   |
| Ela4: | <br>*                       | .†<br>*               | <br>*                 |                       | <br>*                 | . <del>.</del> .<br>* |                      |                        |                       |                     |                        |                        |                       |                       |                 |                       |                       |                       |                       |                        |                   |

Figure 1 Nucleotide and deduced amino acid sequences of rat caldecrin (rCal) and elastase IV (Ela4). The nucleotide (upper row) and amino acid (lower row) sequences of the indicated molecules are shown. The dots and asterisks indicate nucleotides and amino acid residues, respectively, that are conserved between rCal and Ela4. Circle: Charge-relay system; Vertical arrowhead: Proteolytic cleavage site.

analysis has indicated that caldecrin is mainly expressed in the pancreas (Figure 2A).

CTRC (caldecrin) is a single protein consisting of 268 amino acids, with a signal peptide (16 amino acids), propeptide (13 amino acids), and the mature protein (239 amino acids; Figure 2B). The three-dimensional structure demonstrated that five disulfide bridges were formed at Cys1-Cys125 (according to the chymotrypsin numbering),

Cys43-Cys59, Cys139-Cys206, Cys170-Cys186, and Cys196-Cys227 (Figure 2B). CTRC (caldecrin) was shown to have a two-barrel structure, each composed of 6-7  $\beta$ -sheets and a C-terminal  $\alpha$ -helix long tail<sup>[14-16]</sup> (Figure 2C). Following tryptic cleavage at Arg13-Val14, the caldecrin pro-peptide remains associated with the mature enzyme *via* the Cys1-Cys125 disulfide bridge; this generates a structure resembling those of chymotrypsin A and B, as

#### Tomomura M et al. Caldecrin is an anti-osteoclastogenic factor

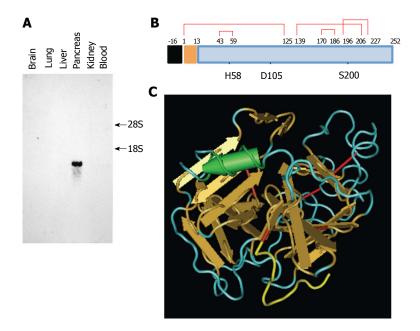


Figure 2 Caldecrin expression and protein structure. A: Caldecrin expression was analyzed by Northern blot. 18S, 28S: 18S, 28S ribosomal RNA; B: Domain structures of caldecrin. Black box: signal peptide; orange box: pro-peptide; blue box: mature protein; red line: disulfide bridges with cysteine number; the H (histidine), D (aspartic acid), S (serine) catalytic triad; C: Ribbon diagram of the crystal structure of human caldecrin (adapted from PDB ID: 4H4F, prepared from [16]). Red line: Disulfide bridge; Yellow line: Pro-peptide; Arrow:  $\beta$ -sheet structure; Cylinder:  $\alpha$ -helix structure.

well as elastase II A, but not those of elastase I , III A, and III B, where the pro-peptide is removed from the mature enzyme after tryptic activation<sup>[11,14-16]</sup>.

CTRC (caldecrin) is a serine protease with the characteristic charge-relayed catalytic triad (His58, Asp105, and Ser200), located in the active site cleft between the barrel structures<sup>[14-16]</sup>. After tryptic activation, caldecrin changes its structure to a substrate-accessible catalytic cleft form. Active caldecrin hydrolyzes the leucyl bond (e.g., in the N-Succinyl-Ala-Ala-Pro-Leu-p-nitroanilide substrate) more efficiently than chymotrypsin A and B; Caldecrin also cleaves the phenylalanyl bond (e.g., in the N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide substrate) and the tyrosyl bond (e.g., in the N-Succinyl-Leu-Leu-Val-Tyr-p-nitroanilide substrate)<sup>[10,19-21]</sup>. The protease activity of caldecrin is inhibited by serine protease inhibitors (phenylmethylsulfonyl fluoride or diisopropyl fluorophosphate), chymotrypsin inhibitor (chymostatin), and the Bowman-Birk trypsin and chymotrypsin inhibitor. The amino acid sequence and protease activity of caldecrin indicate that it is a hybrid of chymotrypsin and elastase.

### CALDECRIN AND BONE METABOLISM

Caldecrin produces dose-dependent decreases in serum calcium concentrations<sup>[10]</sup>. The administration of purified porcine and rat caldecrin *via* the tail vein of mice decreased their serum calcium concentration dose-dependently and the maximum effect was attained 2-4 h post-injection with 20-100  $\mu$ g (about 0.7-3.6 nmol)/kg body weight. The hypocalcemic potency of caldecrin was almost equivalent to that of porcine calcitonin (1 nmol/kg body weight, Tomomura *et al*<sup>[10]</sup> data not shown). The caldecrin proform (pro calderon), purified from the porcine pancreas in the presence of diisopropyl fluorophosphate, appeared to show time- and concentration-dependent chymotryptic activity following cleavage by trypsin. Administration of activated caldecrin reduced the serum calcium level

in mice, even after treatment with the serine protease inhibitor, phenylmethylsulfonyl fluoride, which abolished the chymotryptic activity. However, administration of procaldecrin did not decrease serum calcium levels<sup>[22]</sup>. Recombinant rat<sup>[11]</sup> and human<sup>[17]</sup> caldecrin also decreased serum calcium levels. In addition, rat protease activitydeficient caldecrin mutants (with His58Ala or Ser200Ala substitutions) decreased the levels of serum calcium. Therefore, the effect of caldecrin on serum calcium levels *in vivo* requires its activation by trypsin cleavage. An intramolecular responsive region required for this calcium decreasing activity may therefore be exposed by trypsin activation.

The caldecrin-induced serum calcium decrease occurred concomitantly with a decrease in the serum concentration of hydroxyproline, which is a marker of bone resorption. This observation suggested that this serum calcium decrease may be due to the suppression of bone resorption<sup>[10]</sup>. The effects of caldecrin on osteoclast function have also been investigated; recombinant wild-type and protease activity-deficient mutant caldecrin produced concentration-dependent suppression of bone resorption in isolated rabbit mature osteoclasts<sup>[23]</sup>.

Osteoclasts execute bone resorption, which is modulated by macrophage colony-stimulating factor and receptor activator of nuclear factor-kappa B (NF- $\kappa$ B) ligand (RANKL), produced by osteoblasts and osteocytes. An imbalance between bone formation and resorption leads to bone diseases, including osteoporosis. Osteoclast differentiation and maturation involves the following three steps: (1) Osteoclast precursor cells are generated from bone marrow cells in response to macrophage colonystimulating factor; (2) osteoclasts begin to differentiate from the precursor cells following stimulation by RANKL; and (3) at the later stage of differentiation, osteoclasts fuse to become multinucleated giant cells, leading to the cytoskeletal actin ring formation required for bone resorption. These processes are tightly regulated to

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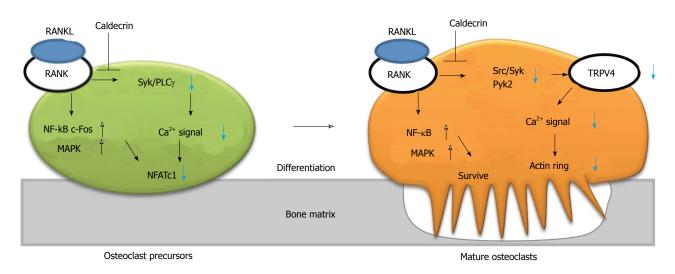


Figure 3 Caldecrin suppresses RANKL-induced osteoclast differentiation and bone resorption. RANKL binds to its receptor (RANK) on the osteoclast precursor, leading to simultaneous activation of two pathways: the NF- $\kappa$ B/ MAPK/c-Fos axis and the Syk/PLC $\gamma$ -calcium oscillation- NFATc1 axis. In mature osteoclasts, RANKL also activates the NF- $\kappa$ B/MAPK and Src/Syk/Pyk2-TRPV4 channel–calcium entry–actin ring formation axes. Caldecrin inhibits the latter pathways (but not the NF- $\kappa$ B/MAPK pathway) in the precursor and mature osteoclasts. TRPV4: Transient receptor potential vanilloid channel 4; MAPK: Mitogen-activated protein kinase; RANKL: RANK ligand; PLC $\gamma$ : Phospholipase C $\gamma$ ; Pyk2: Proline-rich tyrosine kinase 2; NFATc1: Nuclear factor of activated T-cells cytoplasmic 1; NF- $\kappa$ B: Nuclear factor-kappa B; Syk: Spleen tyrosine kinase.

maintain bone homeostasis, and many molecules are involved in osteoclast differentiation<sup>[24-26]</sup>. The key molecule involved in osteoclastogenesis is RANKL, which is a member of the tumor necrosis factor superfamily that is expressed by osteoblasts and osteocytes in membranebound and secreted forms<sup>[27-33]</sup>. RANKL induces osteoclast differentiation by activating two signaling pathways: the mitogen-activated protein kinase (MAPK), NF- $\kappa$ B, and c-Fos activation axis and the phospholipase C  $\gamma$  (PLC $\gamma$ )mediated calcium oscillation-calcineurin-nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) axis. Caldecrin did not inhibit macrophage colony-stimulating factorinduced osteoclast progenitor formation from bone marrow cells but did inhibit RANKL-induced osteoclast differentiation, even in the absence of protease activity<sup>[34]</sup>. Caldecrin inhibited the RANKL-stimulated spleen tyrosine kinase- and PLC<sub>7</sub>-induced calcium oscillation, leading to an inhibition of calcineurin and NFATc1 activity (Figure 3). Caldecrin also inhibited the RANKL-mediated actin ring formation in mature osteoclasts, which is associated with RANKL-evoked calcium entry via the transient receptor potential vanilloid channel 4<sup>[35]</sup>. Caldecrin significantly inhibited RANKL-stimulated phosphorylation of c-Src in association with spleen tyrosine kinase, which is upstream of transient receptor potential vanilloid channel 4 and actin ring formation. On the other hand, caldecrin did not inhibit RANKL-mediated stimulation of MAPK, NF-<sub>K</sub>B, and c-Fos activation in osteoclast precursors or mature osteoclasts<sup>[34,35]</sup>. Therefore, caldecrin antagonized the RANKL-stimulated calcium signaling pathway involved in both osteoclast differentiation and activation.

Caldecrin is a therapeutic target in osteoporosis. The ovariectomized mouse provides a model of postmenopausal osteoporosis and exhibits an increased serum calcium level due to elevated bone resorption. This is evidenced by an increase in the bone surface to bone volume ratio, increased trabecular separation, decreased bone volume density, and decreased trabecular thickness and number. Expression of the caldecrin plasmid vector, which harbors the wild-type rat caldecrin cDNA, in the femoral muscle of this mouse model reversed this increase in serum calcium levels and restored bone resorption parameters to normal levels<sup>[36]</sup>.

An important, but unaddressed, question relates to how the caldecrin released from the pancreas targets the bone. Recently, osteocalcin, which is osteoblastderived, stored in the bone matrix, and then released by osteoclastic bone resorption, was shown to increase insulin secretion from pancreatic islets<sup>[37,38]</sup>. This activity appears to provide a physiological link between bone and pancreas, in relation to the regulation of energy metabolism. It is possible that some of the caldecrin derived from the pancreas enters the circulation and then inhibits osteoclasts, in order to regulate calcium homeostasis. The physiological activation and functions of caldecrin are not defined; however, considering its obvious effects on serum calcium levels and osteogenesis, caldecrin might be an intrinsic calcium regulating factor. The expression and distribution of caldecrin, peptide fragments of caldecrin, and its binding proteins should be explored in order to determine their physiological roles in bone metabolism.

# **OTHER BIOLOGICAL ASPECTS**

The *CTRC* gene modulates risk for pancreatitis. Rosendahl *et al*<sup>[39]</sup> reported that *CTRC* gene mutations were significantly associated with hereditary chronic pancreatitis. Masson *et al*<sup>[40]</sup> also identified a *CTRC* mutation in patients with idiopathic chronic pancreatitis. CTRC hydrolyzes the pro-peptide and calcium-binding

loop of the trypsinogens, enhancing their activation and degradation, respectively<sup>[41-43]</sup>. Loss-of-function *CTRC* variants increase the risk for chronic pancreatitis. *CTRC* is also a susceptibility gene for tropical calcific pancreatitis, which is a juvenile form of chronic nonalcoholic pancreatitis that occurs in Asians and Africans and is associated with nearly 90% pancreatic calcium deposition<sup>[44]</sup>.

It is of clinical interest that five decades ago, Takaoka et al<sup>[8,9,45]</sup> administered pancreatic extract to patients diagnosed with myasthenia gravis and muscular dystrophy. The symptoms of the patients treated with the extract improved progressively, suggesting that the hypocalcemic effect of the extract could have contributed to protecting them against the development of muscular dystrophy. The effect of caldecrin was also investigated in the dy/dy muscular dystrophic mouse model<sup>[46]</sup>. These mice genetically lack M-laminin and exhibit defective muscle basement membranes. Peritoneal administration of caldecrin protein or intramuscular expression of a caldecrin vector inhibited muscular destruction in the dy/dy mice. This indicated that caldecrin was responsible for the effects of the pancreatic extract on muscular dystrophy.

In 2011, Lacruz *et al*<sup>[47]</sup> found that CTRC (caldecrin) was expressed by ameloblasts and was up-regulated during enamel maturation, suggesting that caldecrin might be involved in tooth development.

CTRC (caldecrin) has been reported to be associated with pancreatic cancer, where its expression is drastically reduced. Individuals with chronic pancreatitis who show low or no activity of caldecrin show an increased risk for pancreatic cancer<sup>[48]</sup>. Furthermore, Wang *et al*<sup>[49]</sup> demonstrated that overexpression of CTRC (caldecrin) downregulated the migration of human pancreatic adenocarcinoma Aspc-1 cells, whereas the knockdown of CTRC (caldecrin) increased cell migration. It would be interesting to explore the potential use of caldecrin in pancreatic cancer diagnosis and treatment. In addition, breast cancer is highly associated with osteolytic metastatic disease. RANKL is important in mammary gland development and also in the progression of metastatic breast cancer cells<sup>[50,51]</sup>. RANKL may partly contribute to the activation of metastatic breast cancer via the calcineurin/NFAT pathway<sup>[52]</sup>, which is modulated by caldecrin. It would therefore be interesting to investigate whether caldecrin suppresses RANKLdependent tumor metastases.

### CONCLUSION

The serum calcium-decreasing factor, caldecrin, was discovered in the pancreas. Caldecrin inhibits osteoclast differentiation and bone resorption in mature osteoclasts *via* inhibition of RANKL-induced intracellular calcium signaling. This effect occurs independently of its inherent protease activity. Therefore, caldecrin might be a potential therapeutic target for the treatment of osteolytic diseases such as osteoporosis and osteoarthritis.

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ORIGINAL ARTICLE

#### **Basic Study**

# Disruption of NAD<sup>+</sup> binding site in glyceraldehyde 3-phosphate dehydrogenase affects its intranuclear interactions

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# Abstract

**AIM:** To characterize phosphorylation of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and mobility of GAPDH in cancer cells treated with chemotherapeutic agents.

**METHODS:** We used proteomics analysis to detect and characterize phosphorylation sites within human GAPDH. Site-specific mutagenesis and alanine scanning was then performed to evaluate functional significance of phosphorylation sites in the GAPDH polypeptide chain. Enzymatic properties of mutated GAPDH variants were assessed using kinetic studies. Intranuclear dynamics parameters (diffusion coefficient and the immobile fraction) were estimated using fluorescence recovery after photobleaching (FRAP) experiments and confocal microscopy. Molecular modeling experiments were performed to estimate the effects of mutations on NAD<sup>+</sup> cofactor binding.

**RESULTS:** Using MALDI-TOF analysis, we identified novel phosphorylation sites within the NAD<sup>+</sup> binding center of GAPDH at Y94, S98, and T99. Using polyclonal antibody specific to phospho-T99-containing peptide within GAPDH, we demonstrated accumulation of phospho-T99-GAPDH in



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the nuclear fractions of A549, HCT116, and SW48 cancer cells after cytotoxic stress. We performed site-mutagenesis, and estimated enzymatic properties, intranuclear distribution, and intranuclear mobility of GAPDH mutated variants. Site-mutagenesis at positions S98 and T99 in the NAD<sup>+</sup> binding center reduced enzymatic activity of GAPDH due to decreased affinity to NAD<sup>+</sup> (Km = 741  $\pm$  257  $\mu$ mol/L in T99I  $\nu s$  57  $\pm$  11.1  $\mu$ mol/L in wild type GAPDH. Molecular modeling experiments revealed the effect of mutations on NAD<sup>+</sup> binding with GAPDH. FRAP (fluorescence recovery after photo bleaching) analysis showed that mutations in NAD<sup>+</sup> binding center of GAPDH abrogated its intranuclear interactions.

**CONCLUSION:** Our results suggest an important functional role of phosphorylated amino acids in the NAD<sup>+</sup> binding center in GAPDH interactions with its intranuclear partners.

Key words: NAD<sup>+</sup>; Binding site; Fluorescence recovery after photobleaching; Nuclear proteins; Mutation; Glyceraldehyde 3-phosphate dehydrogenase; Anticancer agents

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**Core tip:** We detected the phosphorylated amino acid residues Y94, S98, T99 within the NAD<sup>+</sup> binding center of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Substitution of these amino acids with non-phosphorylated alanine residues did not abrogate intranuclear localization of GAPDH. Instead, such mutations altered the molecular dynamics parameters of intranuclear GAPDH probably by hindering its interactions with yet to be identified nuclear biomolecules. Our molecular modeling experiments invoke an important structural feature -T99-E97 H-bond likely involved in stabilization of NAD<sup>+</sup> binding center.

Phadke M, Krynetskaia N, Mishra A, Barrero C, Merali S, Gothe SA, Krynetskiy E. Disruption of NAD<sup>+</sup> binding site in glyceraldehyde 3-phosphate dehydrogenase affects its intranuclear interactions. *World J Biol Chem* 2015; 6(4): 366-378 Available from: URL: http://www.wjgnet.com/1949-8454/full/v6/i4/366.htm DOI: http://dx.doi.org/10.4331/wjbc.v6.i4.366

### INTRODUCTION

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an intriguing example of moonlighting proteins, which performs multiple functions in several unrelated cellular pathways<sup>[1]</sup>. Participation of GAPDH in diverse biochemical pathways inside distinct cellular compartments poses a problem from evolutionary point of view. Because energy metabolism of cancer cells relies mainly on glucose utilization *via* the glycolytic pathway, rather than on oxidative phosphorylation (the Warburg effect), GAPDH is important for glycolysis-dependent energy supply. Depletion of GAPDH arrests cell proliferation, and induces accelerated senescence in cancer cells<sup>[2-4]</sup>.

In response to stress, GAPDH acts as a signaling molecule linking stress factors and cellular apoptotic machinery<sup>[5-7]</sup>. S-nitrosylation of Cys149 in the GAPDH catalytic center induces its intranuclear translocation and activation of apoptosis-related signaling cascade<sup>[5,8]</sup>.

An additional level of complexity in regulating intracellular functions of GAPDH comes from the fact that multiple GAPDH isoforms are detected in cytosolic and nuclear fractions. Seo *et al*<sup>[9]</sup> identified multiple posttranslational modifications of GAPDH (19 types of modification on 42 sites), whose physiological role still remains to be elucidated.

Details of GAPDH-mediated intranuclear functions are incompletely known. Several lines of evidence suggest involvement of GAPDH in chemotherapy-induced DNA damage response. First, GAPDH demonstrates uracil-DNA-glycosylase activity contributing to DNA repair activity<sup>[10]</sup>. Viability of human leukemia cells treated with 6-mercaptopurine correlates with intranuclear level of GAPDH; the higher level of intranuclear GAPDH was detected in cells with lower viability<sup>[11]</sup>. Next, GAPDH was demonstrated to be a component of DNA-protein complexes formed on short DNA duplexes containing inserted modified nucleosides cytarabine, fluorouridine, and thioguanosine<sup>[12]</sup>. Finally, GAPDH binds DNA covalently linked to saframycin, a natural product with potent antiproliferative effect<sup>[13]</sup>. DNA alkylation with an antitumor drug S23906-1 results in chromatin binding with GAPDH and its protein partner HMGB1<sup>[14]</sup>.

In the present study, we detected post-synthetic modifications in three amino acid residues encompassing the NAD<sup>+</sup> binding center in GAPDH. In order to elucidate the functional significance of these modifications, we prepared the mutant forms of GAPDH, and assessed enzyme properties, intracellular localization, and intranuclear interactions of the mutated proteins. Our results indicate that NAD<sup>+</sup> binding center in GAPDH is an important structural element which regulates GAPDH interactions with other nuclear components.

### MATERIALS AND METHODS

#### Cell cultures, drug treatment and plasmids

Lung carcinoma cells A549 were obtained from the ATCC collection (ATCC, Manassas, VA). HCT116-1640 ( $p53^{+/+}$ ) and SW48-297 ( $p53^{+/+}$ ) cells were a generous gift from Dr. Vogelstein (John Hopkins University, Baltimore, MR). Cells were maintained in RPMI1640 medium (A549 cells; ATCC, Manassas, VA) or in McCoy's A5 (HCT116 and SW48 cells; Lonza, Allendale, NJ) at 40%-80% confluence. Cells were treated with araC {cytarabine, cytosine arabinoside, 4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl) oxolan-2-yl] pyrimidin-2-one} dissolved in water as 500-1000 × stock solutions; drug concentrations were determined spectrophotometrically (araC,  $\epsilon_{272}$  = 9259; The Merck Index, 2001). Human GAPDH cDNA was inserted in frame with enhanced green fluorescent protein (EGFP) into pcDNA3.1 as described



#### Phadke M et al. NAD+ binding site in intranuclear GAPDH

| Table 1         Mutations introduced to glyceraldehyde 3-phosphate dehydrogenase cDNA ORF |                                               |                   |              |  |  |  |  |
|-------------------------------------------------------------------------------------------|-----------------------------------------------|-------------------|--------------|--|--|--|--|
| GAPDH variant                                                                             | Mutagenic primers <sup>1</sup>                | Nucleotide change | Codon change |  |  |  |  |
| Y94A                                                                                      | CGATGCTGGCGCTGAG <b>GC</b> CGTCGTGGAGTCCACTGG | 280-281           | UAC->GCC     |  |  |  |  |
|                                                                                           | CCAGTGGACTCCACGACGGCCTCAGCGCCAGCATCG          |                   |              |  |  |  |  |
| S98A                                                                                      | GAGTACGTCGTGGAGGCCACTGGCGTCTTCACC             | 290               | UCC- > GCC   |  |  |  |  |
|                                                                                           | GGTGAAGACGCCAGTGGCCTCCACGACGTACTC             |                   |              |  |  |  |  |
| T99A                                                                                      | AGTACGTCGTGGAGTCCGCTGGCGTCTTCACCACC           | 295               | ACU- > GCU   |  |  |  |  |
|                                                                                           | GGTGGTGAAGACGCCAGCGGACTCCACGACGTACT           |                   |              |  |  |  |  |
| T99I                                                                                      | GAGTACGTCGTGGAGTCCATTGGCGTCTTCA               | 296               | ACU- > AUU   |  |  |  |  |
|                                                                                           | TGAAGACGCCAATGGACTCCACGACGTACTC               |                   |              |  |  |  |  |

<sup>1</sup>Mutated nucleotides are indicated in bold font; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

earlier<sup>[3]</sup> and verified by sequencing. Site-mutagenesis of GAPDH ORF was performed using QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene, CA), per manufacturer's instructions. Oligonucleotides used for mutagenesis are listed in Table 1. Wild type and mutated GAPDH cDNA were inserted into pET28a plasmid, expressed in BL21 (DE3) Escherichia coli (*E. coli*) expression system (Novagen, WI) and purified using Ni-NTA chromatography.

#### Two dimensional gel electrophoresis

Separation of cytosolic and nuclear fractions of cells was performed with NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Rockford, IL), per manufacturer's instructions as described earlier<sup>[3]</sup>. The extracts (cytoplasmic fraction and nuclear extract) were snap-frozen on dry ice and stored at -80  $^{\circ}$ C.

Protein extracts were precipitated by acetone, and re-dissolved in 2D buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L Tris, 60 mmol/L DTT, and 1 × protease inhibitor cocktail (Roche Applied Science). Further procedures for sample preparation, the first and the second dimension separations were performed according to the published protocol<sup>[15]</sup>. Briefly, samples were diluted to 120 µL with rehydration buffer, applied on Immobiline Drystrip (7 cm) with 3-11 or 6-11 pH range (Amersham Biosciences) overnight. Isoelectric focusing was performed by applying a voltage gradient as follows: 30 min to 250 V, 1 h to 1500 V, 1 h to 3000 V and then 3 h to 8000 V. After IEF, the strips were incubated for 30 min in equilibration buffer and treated with iodoacetamide. The second dimension (SDS-PAGE) was performed on 12% Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> Precast Gels (456-1041, Biorad). Separated proteins were stained with SYPRO® Ruby protein gel stain (Life Technologies) or transferred onto the nitrocellulose membrane by electroblotting procedure<sup>[16]</sup>. Membranes were developed with mouse monoclonal anti-GAPDH antibody at 1:10000 dilution (Millipore, CA) or phospho-T99-GAPDH antibody (Rockland Immunochemicals, PA). Polyclonal rabbit anti-pT99 antibody was raised by immunization with KLH-conjugated phosphopeptide AEYVVES [pT] GVFT corresponding to the sequence 92-103 in human GAPDH. Antibody was further purified by affinity chromatography, and used at a 1:10000

dilution. Bands were visualized by treatment with secondary antibody - IRDye 800CW donkey anti-rabbit antibody or IRDye 680 goat anti-mouse antibody (Rockland, Gilbertsville, PA) at 1:10000 dilution, and quantified by Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE) using two-color fluorescence detection at 700 and 800 nm.

#### **Proteomics protocols**

**Preparation of protein samples and in-gel trypsin digestion:** Protein spots were processed for gel electrophoresis-liquid chromatography-mass spectroscopy (GeLC-MS/MS) proteomics analysis. After visualization with SYPRO staining, gel images were acquired in an image reader FLA-5000 (FujiFILM) and analyzed densitometrically using the multi gauge v3.0 (FujiFILM).

The GAPDH spots were extracted and diced into - 1 mm × 1 mm. After destaining with 50% v/v Acetonitrile (ACN) in 25 mmol/L ammonium bicarbonate buffer (bicarbonate buffer), proteins within gel pieces were reduced with 10 mmol/L DTT in bicarbonate buffer, and alkylated by incubation with 50 mmol/L iodoacetamide. After gel dehydration with 100% ACN, the gel pieces were covered with approximately 40  $\mu$ L of 12.5  $\mu$ g/mL trypsin in bicarbonate buffer. In-gel digestion was performed at 37 °C for 12 h, trypsin was inactivated with formic acid at 2% final volume, and peptides were extracted and cleaned-up using C18 Tip column (ZipTips<sup>®</sup>) as previously described<sup>[17]</sup>.

**GeLC-MS/MS proteomics analysis:** Peptides were dried in a vacuum centrifuge, then resuspended in 30  $\mu$ L of 0.1% v/v TFA/H<sub>2</sub>O. Peptide samples were loaded onto 2  $\mu$ g capacity peptide traps (CapTrap; Michrom Bio-resources) and separated using a C18 capillary column (15 cm 75 mm, Agilent) with an Agilent 1100 LC pump delivering mobile phase at 300 nL/min. Gradient elution using mobile phases A (1% ACN/0.1% formic acid, balance H<sub>2</sub>O) and B (80% ACN/0.1% formic acid, balance H<sub>2</sub>O) was as follows (percentages for B, balance A): Linear from 0 to 15% at 10 min, linear to 60% at 60 min, linear to 100% at 65 min. The nano ESI MS/ MS (electrospray ionization mass spectrometry) was performed using a HCT Ultra ion trap mass spectrometer (Bruker). ESI was delivered using distal-coating spray Silica tip (id 20  $\mu$ m, tip inner id 10  $\mu$ m, New Objective, Ringoes, NJ). Mass spectra were acquired in positive ion mode, capillary voltage at -1200 V and active ion charge control trap scanning from 300 to 1500 m/z. Using an automatic switching between MS and MS/MS modes, MS/MS fragmentation was performed on the two most abundant ions on each spectrum using collision-induced dissociation with active exclusion (excluded after two spectra, and released after 2 min). The complete system was fully controlled by HyStar 3.2 software.

### MS data analysis and post-translational modif-

**ications:** Mass spectra processing was performed using BrukerDaltonics esquire 6.1 Data-Analysis (Version 3.4). The generated de-isotoped peak list was submitted to an in-house Mascot server 2.2.07 for searching against the Swiss-Prot database (Release 2011-06) (version 56.6, 536029 sequences). Mascot search parameters were set as follows: species, Homo sapiens (20413 sequences); enzyme, trypsin with maximum 2 missed cleavage sites. Post-translational modifications were analyzed specifically for GAPDH: Fixed modification, cysteine carboxymethylation; variable modifications: Methionine oxidation, phosphorylation of serine, threonine and tyrosine. All peptides matches were filtered using an ion score cutoff of 30.

# Transfection with pEGFP-GAPDH and fluorescence recovery after photobleaching experiments

About 25000 cells/dish were seeded in 35 mm glass bottom Petri dishes (MatTek, MA) and transfected with pEGFP (Clontech, Palo Alto, CA) or pEGFP-GAPDH plasmid using FuGene6 transfection reagent (Roche, NJ). Alternatively, HCT116-4016 cells were transiently transfected by electroporation (Neon Transfection System, Invitrogen, CA). The next day, cells were treated with 1-10  $\mu$ mol/L araC and incubated for 24 h.

After incubation, fluorescence recovery after photobleaching (FRAP) experiments were performed on a Leica TCS SP2 AOBS confocal microscope equipped with a 63 imes 1.4 N.A. oil immersion objective at 37  $^\circ\!\!\mathbb{C}$ , as described earlier<sup>[3]</sup>. Briefly, pre-bleaching plateau was defined by acquiring 20 single section images with  $6 \times zoom$  on an area 7  $\times$  7  $\mu$ m, with acquisition speed 287 msec/frame. Bleaching was performed with 3 pulses using the 458, 476, and 488 nm lines of Ar laser. Fluorescence recovery was monitored collecting 40 single-section images at 287 msec intervals with low laser intensity (5% of the bleach intensity with the single 488 laser line, detection 495-600 nm). Quantitative analysis was performed after background subtraction, correction for laser fluctuations and acquisition photobleaching, and normalization as described by Rabut and Ellenberg<sup>[18]</sup>. 5-10 cells were analyzed on each dish; all experiments were repeated 4-5 times. Diffusion coefficient D value was calculated using the equation  $D = 0.88 \text{ w}^2/(4t_{1/2})$  where w is a radius of bleached area, with the assumptions that the bleached area is a disc and that diffusion occurs only laterally<sup>[19,20]</sup>.

The immobile fraction was calculated after correction for loss of signal due to photobleaching<sup>[18]</sup>.

#### Glycolytic assay

GAPDH glycolytic activity was measured by spectrophotometric assay at 340 nm as described earlier<sup>[21]</sup>. Briefly, the assay was carried out in 0.015 mol/L sodium pyrophosphate, 0.03 mol/L sodium arsenate (Sigma), pH 8.5, in the presence of 3.5 mmol/L DTT, 0.26 mmol/L NAD<sup>+</sup> and 0-2 mmol/L glyceraldehyde-3-phosphate (Sigma), or 0.51 mmol/L glyceraldehyde-3-phosphate, and 0-2 mmol/L NAD<sup>+</sup> catalyzed by wild type or mutated GAPDH expressed in *E. coli*. Human GAPDH (Sigma) was used as control.

# Molecular modeling of the effects of mutation on NAD<sup>+</sup> cofactor binding

Molecular modeling experiments were performed utilizing the Sybyl molecular modeling environment. The X-ray crystal structure of human GAPDH at 1.75 Angstrom resolution (1U8F.pdb) was obtained from the Protein Data Bank and preprocessed for virtual mutagenesis studies by initial extraction of the crystallographic water molecules from the tetramer, selection and extraction of the P chain monomer with its corresponding NAD<sup>+</sup> cofactor, and final extraction of the cofactor as separate files. The resulting monomer was then subjected to a localized energy minimization (annealing) routine involving the region around the amino acid of interest (T99). The subset of residues within 8 Å of T99 were considered to be the "hot" region, while a radius of 14 Å comprised the "interesting" region for the annealing process, and the region was minimized to a gradient of 0.05 utilizing the AMBER7FF99 force field and charges. The resulting structure was saved, and T99 was mutated to Ala and to Ile, the resulting structures then undergoing the same local annealing process after substitution. The structures for the native T99, and the mutant forms T99A and T99I were prepared for submission to a canonical (NVT) molecular dynamics calculation utilizing the same force field and charges as above.

#### Statistical analysis

The statistical analyses were carried out using Student's *t* test with Statistica software program (StatSoft, OK), and non-linear regression analysis with GraphPad Prizm 4.0 software (GraphPad software, CA). A *P* value < 0.05 was considered statistically significant. Data are presented as the mean  $\pm$  SE.

### RESULTS

# Proteomics analysis of intranuclear GAPDH after genotoxic stress

Nuclear and the cytosolic fractions were extracted from either untreated A549 cells, or cells treated 1  $\mu$ mol/L araC for 24 h. Nuclear and cytosolic GAPDH isoforms were detected by one- or two-dimensional Western blot with



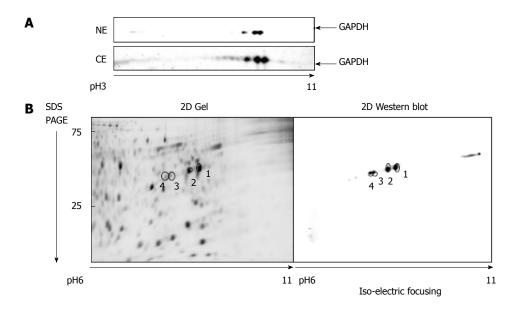


Figure 1 Glyceraldehyde 3-phosphate dehydrogenase isoforms in A549 cells. A: 2D gel separation (pH range 3-11) of proteins from the nuclear (NE) and the cytosolic (CE) fraction of A549 cells after 1 µmol/L araC treatment for 24 h. The membrane was developed with anti-GAPDH antibody as described in "Materials and Methods"; B: Proteins from the cytosolic fraction were separated by 2D gel (pH range 6-11). Separated proteins were stained with SYPRO<sup>®</sup> Ruby (left panel), or developed with anti-GAPDH antibody (right panel). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; 2D: Two dimensional.

anti-GAPDH antibodies. Because intranuclear GAPDH in untreated cells was below detection level<sup>[3]</sup>, we analyzed the nuclear fraction of A549 cells after treatment with araC. Figure 1A demonstrates 2D gel separation (pH range 3-11) of the nuclear (upper part) and the cytosolic (bottom part) fractions of A549 cells treated with araC. Western blot analysis of cytosol revealed the presence of multiple GAPDH isoforms in cytosolic fraction. Only basic isoform accumulated in the nuclear fraction of treated A549 cells (Figure 1A). 2D separation (pH range 6-11) of the cytosolic fraction from untreated A549 cells is shown on (Figure 1B). The left panel shows the gel stained with SYPRO® Ruby, and the right image depicts proteins detected with anti-GAPDH antibody. Proteomics analysis of spot 1 and spot 2 on Figure 1B identified a peptide WGDAGAEYVVESTGVFTTMEK corresponding to the amino acid sequence 87-107 of human GAPDH. Further analysis of this peptide resulted in identification of phosphorylated amino acids at position 94, 98 and 99 as revealed by MS detection of the precursor ion 850.51 m/z (MS1) (Figure 2A), and the subsequent fragmentation ions (MS2) (Figure 2B).

The presence of phosphorylated T99-GAPDH in the cytosolic and nuclear fractions of treated A549 cells was confirmed with anti-phospho-T99-GAPDH antibody (Figure 3). In cytosol of araC- treated A549 cells, we detected the basic forms of GAPDH with phosphorylated T99 (Figure 3A). We also observed accumulation of basic isoforms in the nuclear fraction of treated A549 cells (Figure 3B upper image). Consecutive immuno staining of 2D gel with anti-GAPDH antibody and anti-phospho-T99-GAPDH antibody revealed phosphorylated T99 in the spots co-localized with the basic GAPDH isoforms in the nuclear fraction (Figure 3B merged image).

# Site-specific mutagenesis of NAD<sup>\*</sup> binding domain in GAPDH polypeptide

Phosphorylated amino acid residues identified in our proteomics experiments are localized within the NAD<sup>+</sup> binding center of GAPDH, as demonstrated by high-resolution structural analysis of human GAPDH<sup>[22]</sup>. To elucidate the functional role of newly found phosphorylated amino acids in NAD<sup>+</sup> binding domain of GAPDH in chemotherapy-induced stress response, we prepared plasmids coding for wild type- and mutated forms of GAPDH polypeptide with mutations that prevented phosphorylation at position 94, 98, and 99 of GAPDH.

Plasmids coding for (His)-tagged GAPDH variants Y94A, S98A, T99A, and T99I were used for heterologous expression of mutated GAPDH polypeptides in a bacterial expression system. Enzymatic activity of purified GAPDH proteins was assayed *in vitro*. Constructs coding for GAPDH variants Y94A, S98A, T99A, and T99I in-frame with EGFP cDNA under CMV promoter were generated from pEGFP-GAPDH as described earlier<sup>[3]</sup>, and these constructs were used in transfection experiments with human lung carcinoma cells A549, human colorectal carcinoma cells HCT 116-4016, and SW48-297.

# *Mutations in NAD<sup>+</sup> binding domain of GAPDH inhibit glycolytic activity*

Enzymatic properties of wild type and mutated forms of GAPDH were assessed spectrophotometrically using the varying concentrations of glyceraldehyde-3-phosphate or NAD<sup>+[21]</sup>. Results of these experiments are shown on Figure 4, and summarized in Table 2. All GAPDH mutant proteins had affinity for glyceraldehyde-3-phosphate close to that of wild type enzyme. In contrast, mutated polypeptides manifested decreased affinity to NAD<sup>+</sup>

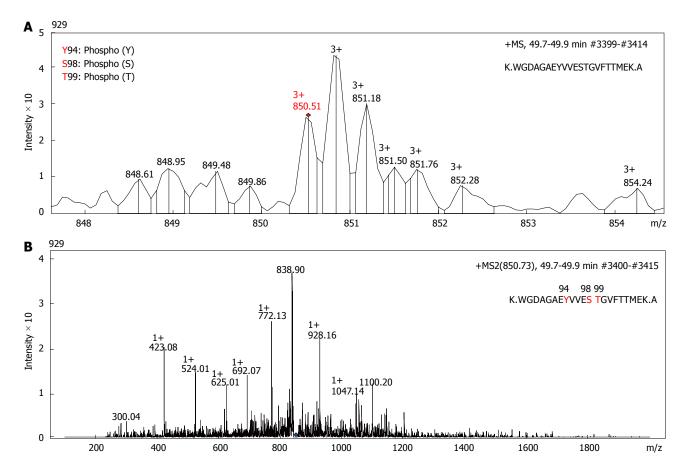


Figure 2 The tandem mass spectrometry (MS/MS) profile of glyceraldehyde 3-phosphate dehydrogenase peptides. Proteomics characterization of a basic GAPDH isoform (spot 1 from Figure 1B) from the cytosolic fraction of A549 cells by mass spectroscopy. A: Spectrum profile of the precursor ion 850.51 m/z (MS 1) selected for further fragmentation; B: Spectrum profile of the fragmentation ions (MS 2) of the peak 850.51 m/z from panel A. Retention time and number of scans where the peptide was detected are shown in the right top part of each panel. GAPDH: Glyceraldehyde 3-phosphate. dehydrogenase.

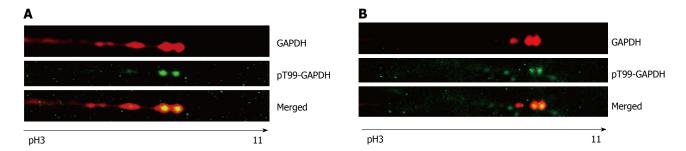


Figure 3 Phospho-T99-glyceraldehyde 3-phosphate dehydrogenase was detected in nuclei of A549 cells after genotoxic stress. A: 2D gel analysis of the cytosolic fraction extracted from A549 cells. Only a section of the membrane containing GAPDH isoforms is shown. Membranes were developed with anti-GAPDH antibody or anti-phospho-T99-GAPDH antibody in untreated and araC-treated cells. pH gradient is indicated under the bottom image; B: The nuclear fraction from A549 cells after treatment with araC. Only a section of the membrane containing GAPDH isoforms is shown. Membranes were developed with anti-GAPDH antibody (top image) or anti-phosphoT99-GAPDH antibody (bottom image). pH gradient is indicated under the bottom image. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; 2D: Two dimensional.

compared with wild type protein (Table 2). T99I variant had the lowest affinity to NAD<sup>+</sup>, its K<sub>m</sub> (NAD<sup>+</sup>) was increased by more than an order of magnitude compared with wild type GAPDH (741 ± 257 *vs* 57 ± 11.1 µmol/L). This low binding of NAD<sup>+</sup> explains why T99I variant has low saturation under conditions of experiment (*i.e.*, at NAD<sup>+</sup> concentration 260 µmol/L), as depicted on Figure 4A. Extremely low binding of T99I-GAPDH to NAD<sup>+</sup> coenzyme accounts for decreased velocity of glycolytic

reaction catalyzed by this mutated enzyme (Figure 4).

# Mutations in NAD<sup>+</sup> binding domain do not affect nuclear accumulation of EGFP-GAPDH in response to genotoxic stress

As we demonstrated earlier, endogenous GAPDH or EGFP-GAPDH fusion polypeptides localized predominantly in cytoplasm of untreated A549, SW620, and DLD1 cultured cells<sup>[3,21]</sup>. To assess the effect of mutation in NAD<sup>+</sup> binding

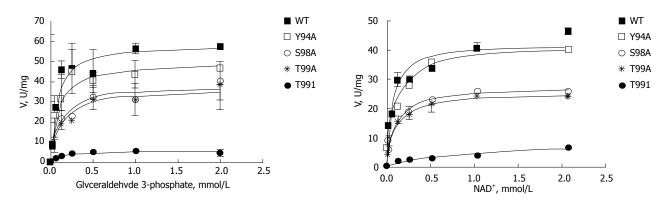


Figure 4 Kinetic analysis of wild type and mutated forms of Hisx6-tagged glyceraldehyde 3-phosphate dehydrogenase prepared in BL21 (DE3) *Escherichia coli* expression system. Conditions for the glycolytic assay are indicated in "Materials and Methods". A: Reaction was performed with 0.26 mmol/L NAD<sup>+</sup> and varying concentrations of D-glyceraldehyde-3-phosphate (0-2 mmol/L); B: Reaction was performed with 0.51 mmol/L D-glyceraldehyde-3-phosphate and varying concentrations of NAD<sup>+</sup> (0-2 mmol/L). Vmax and Km were calculated by non-linear regression analysis with GraphPad Prizm 4.0 as described in Materials and Methods. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

| Table 2         Kinetic parameters of wild type and mutated glyceraldehyde 3-phosphate dehydrogenase variants |                          |                                 |  |  |  |
|---------------------------------------------------------------------------------------------------------------|--------------------------|---------------------------------|--|--|--|
| GAPDH variant                                                                                                 | <b>Km (G3P) (μmol/L)</b> | Km (NAD <sup>+</sup> ) (μmol/L) |  |  |  |
| Wild type                                                                                                     | 77 ± 25.9                | 57 ± 11.1                       |  |  |  |
| V04 A                                                                                                         | 87 ± 22 1                | $07.4 \pm 11$                   |  |  |  |

| Y94A         87±33.1         97.4±11           S98A         100±36.7         101±7.2           T99A         112±44.7         105±17.7 | Wild type | 77 ± 25.9      | 57 ± 11.1      |
|---------------------------------------------------------------------------------------------------------------------------------------|-----------|----------------|----------------|
| T99A         112 ± 44.7         105 ± 17.7                                                                                            | Y94A      | 87 ± 33.1      | $97.4 \pm 11$  |
|                                                                                                                                       | S98A      | $100 \pm 36.7$ | $101 \pm 7.2$  |
|                                                                                                                                       | T99A      | $112 \pm 44.7$ | $105 \pm 17.7$ |
| 1991 $106 \pm 38.3$ $741 \pm 257$                                                                                                     | T99I      | $106 \pm 38.3$ | $741 \pm 257$  |

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

center on intracellular localization of GAPDH, we used confocal microscopy and image analysis to compare GAPDH distribution between cytoplasmic and nuclear compartments in HCT116-4016 and SW48-297 cells expressing EGFP-GAPDH (wild type), or mutated fusion proteins. After genotoxic stress caused by araC treatment, wild type EGFP-GAPDH as well as all mutated fusion proteins accumulated in the nuclei. Figure 5A shows the distribution of EGFP-GAPDH wild type, T99A and T99I mutated forms in HCT116-4016 cells before and after treatment. The basal (i.e., before the genotoxic stress) intranuclear level of mutant forms was higher compared to wild type form (up to 15% vs 2.3%). Neither mutation at positions 94, 98, or 99 prevented GAPDH nuclear accumulation after genotoxic stress. In HCT116-4016 cells, T99I demonstrated the most prominent nuclear accumulation (45% of total EGFP-GAPDH, Figure 5B). Similar results were received in p53-proficient SW48-297 cells (not shown).

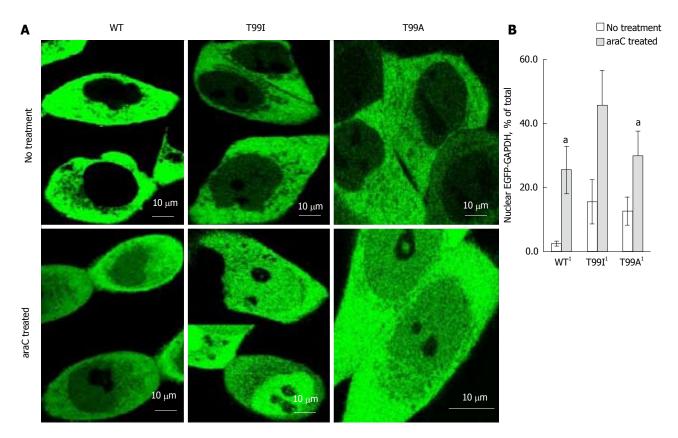
# Mutations within the NAD<sup>\*</sup> binding domain alter intranuclear mobility of GAPDH

FRAP technique provides important information about dynamic properties of fluorescent proteins in the cytoplasmic and nuclear compartments of the live cells<sup>[3,23]</sup>. After short-term photo bleaching of the preselected spots inside the cytoplasmic or nuclear compartments of the cells, recovery of fluorescence intensity in the cytosolic and nuclear compartments was measured as described in "Materials and Methods". Recovery of fluorescence in the nuclear and cytosolic fractions of EGFP-GAPDH in HCT116-4016, and SW48-297 occurred differently depending on the nature of mutation in the NAD<sup>+</sup> binding center. As shown on Figure 6, recovery of the fluorescent signal in cytosol for all EGFP-GAPDH fusion proteins was fast and reached plateau after approximately 7.5 msec (Figure 6B, open symbols). The rate of fluorescence recovery in the nuclei of cells expressing mutant EGFP-GAPDH fusion proteins was similar to fluorescence recovery in cytosol (Figure 6B, closed symbols). In contrast, recovery of wild type EGFP-GAPDH was slow, and didn't reach plateau even after 12.5 msec (Figure 6B). Importantly, only wild type nuclear EGFP-GAPDH demonstrated about 10 times lower D value and about 3 time higher immobile fraction (1-M<sub>f</sub>) compared with cytosolic EGFP-GAPDH (Table 3).

# Molecular modeling of the effects of mutation on NAD<sup>+</sup> cofactor binding

To probe the impact of site-directed mutagenesis on the affinity of GAPDH for NAD<sup>+</sup>, we performed molecular modeling experiments. The X-ray crystal structure of human GAPDH at 1.75 Angstrom resolution (1U8F,pdb) was obtained from the Protein Data Bank (Figure 7A)<sup>[22]</sup>. Because of the high degree of conserved secondary structure near the region of interest, only the loop containing amino acid at position 99 was subjected to the dynamics calculation, the rest of the protein being held fixed as an aggregate. We determined the loop region to extend from E97 (end of the beta sheet) through T104 (first residue in the attached alpha helix). The in vacuo dynamics calculation was then submitted starting with a random initial velocity for a total duration of 100 ps at 300 K for the native and each mutant construct.

The results of the trajectory calculations are shown in Figure 7B, with the average structure for each simulation being shown overlaid in green (T99), yellow (T99A), and red (T99I), along with the cofactor as initially bound in the active cleft. In the native T99, an intramolecular H-bond between T99 and E97 is likely to exist, and this



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Figure 5 Nuclear accumulation of wild type and mutated enhanced green fluorescent protein-glyceraldehyde 3-phosphate dehydrogenase in live HCT116-4016 cells after transient transfections with plasmids coding for enhanced green fluorescent protein fusions with WT-glyceraldehyde 3-phosphate dehydrogenase, T99I-glyceraldehyde 3-phosphate dehydrogenase, and T99A-glyceraldehyde 3-phosphate dehydrogenase polypeptides. A: Distribution of EGFP-GAPDH variants in HCT116-4016 cells before (upper images) and after (lower images) treatment with 1  $\mu$ mol/L araC for 24 h; B: Quantitative analysis of images shown in Panel A was performed using ImageJ 1.48 v software (NIH, United States). For statistical evaluation, pixel analysis of 20-25 cells was performed for each experiment (mean  $\pm$  SE). <sup>1</sup>Significant (P < 0.03) alteration in nuclear GFP-GAPDH between cells treated with araC and untreated cells. EGFP: Enhanced green fluorescent protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GFP: Green fluorescent protein.

# Table 3 Fluorescence recovery after photobleaching analysis of dynamic properties of wild type and mutated enhanced green fluorescent protein-glyceraldehyde 3-phosphate dehydrogenase

| Protein         | Compartment | (1-Mf)           | <i>t</i> 1/2, s  | <b>D</b> , μ <b>m</b> <sup>2</sup> /s |  |
|-----------------|-------------|------------------|------------------|---------------------------------------|--|
| EGFP-GAPDH-WT   | cyto        | $0.18\pm0.038$   | $0.19 \pm 0.055$ | $27.17 \pm 1.632$                     |  |
| EGFP-GAPDH-Y94A | cyto        | $0.17 \pm 0.129$ | $0.09 \pm 0.014$ | $43.76 \pm 7.158$                     |  |
| EGFP-GAPDH-S98A | cyto        | $0.20 \pm 0.009$ | $0.12 \pm 0.016$ | $29.15 \pm 3.885$                     |  |
| EGFP-GAPDH-T99A | cyto        | $0.11 \pm 0.062$ | $0.11 \pm 0.023$ | $35.52 \pm 8.133$                     |  |
| EGFP-GAPDH-T99I | cyto        | $0.17 \pm 0.06$  | $0.22 \pm 0.041$ | $17.46 \pm 1.733$                     |  |
| EGFP-GAPDH-WT   | nuclei      | $0.62 \pm 0.048$ | $1.41 \pm 0.033$ | $2.91 \pm 0.331$                      |  |
| EGFP-GAPDH-Y94A | nuclei      | $0.23 \pm 0.127$ | $0.09 \pm 0.034$ | $39.05 \pm 6.291$                     |  |
| EGFP-GAPDH-S98A | nuclei      | $0.35 \pm 0.136$ | $0.13 \pm 0.018$ | $26.57 \pm 3.747$                     |  |
| EGFP-GAPDH-T99A | nuclei      | $0.38 \pm 0.120$ | $0.11 \pm 0.027$ | $33.01 \pm 9.209$                     |  |
| EGFP-GAPDH-T99I | nuclei      | $0.29\pm0.028$   | $0.21 \pm 0.059$ | $14.97 \pm 1.855$                     |  |

Cyto: Cytoplasmic compartment; nuclei: Nuclear compartment; EGFP: Enhanced green fluorescent protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; FRAP: Fluorescence recovery after photobleaching.

H-bond was maintained throughout the simulation. This H bond has a stabilizing effect holding the loop in a restricted conformation, thereby permitting the cofactor to fully occupy the upper region of the cleft as illustrated by Figure 7C. Mutation of T99 to A99 removes the stabilizing H-bond, and the resultant loop is able to expand somewhat into the cleft and potentially affect the binding affinity of the cofactor to GAPDH. This hypothesis is most clearly demonstrated in the mutation to I99, where the average structure places the terminus of the sidechain well within the Van der Waals radii of the adenine ring of NAD<sup>+</sup>, thereby displacing or blocking the cofactor from its initial association with GAPDH, as illustrated by the surfaces shown in Figure 7D.

#### Phadke M et al. NAD+ binding site in intranuclear GAPDH

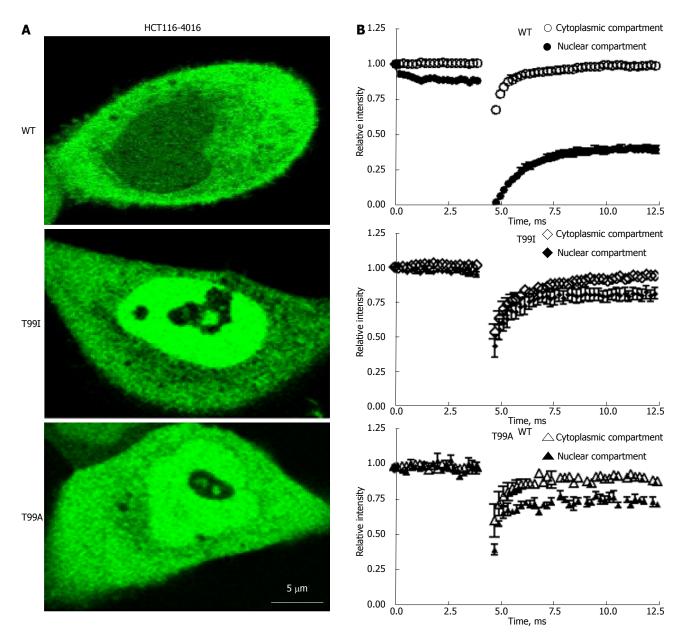


Figure 6 Intranuclear mobility of wild type and mutated variants of enhanced green fluorescent protein-glyceraldehyde 3-phosphate dehydrogenase in HCT116-4016 cells after genotoxic stress. A: FRAP analysis of fluorescent fusion protein EGFP-GAPDH in subcellular compartments of HCT116-4016 cells. Confocal images were collected from transfected cells expressing WT-EGFP-GAPDH (WT), EGFP-GAPDH-T99I (T99I), and EGFP-GAPDH-T99A (T99A) after treatment with 1 µmol/L araC for 24 h; B: Recovery rate of wild type and mutant EGFP-GAPDH fusion proteins after photo bleaching. Open symbols, cytoplasmic compartment, and closed symbols, nuclear compartment. The dynamic parameters mobile fraction (1-Mf), half-time of equilibration *t*(1/2) (s), and diffusion coefficients D were calculated from FRAP experiments, as described in "Materials and Methods" and summarized in Table 3. Where not seen, the error bars are smaller than the data point symbols. EGFP: Enhanced green fluorescent protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GFP: Green fluorescent protein; FRAP: Fluorescence recovery after photobleaching.

## DISCUSSION

Multiple GAPDH isoforms characterized by different isoelectric points have long been known to exist in human and non-human tissues, as summarized in<sup>[24]</sup>. Because no kinetic differences were detected among the GAPDH isozymes, their role remains poorly understood. Nuclear isoforms of GAPDH are differentially regulated during apoptosis indicating important, yet to be characterized intranuclear functions<sup>[25]</sup>. In line with these data, our observations (Figure 1) also indicated the presence of post-translational modifications in GAPDH polypeptide<sup>[9]</sup>.

Proteolytic digestion and subsequent MS analysis of the basic GAPDH forms with pI 8.3 revealed phospho amino acids within peptide 87-107, which were further identified as Y94, S98, and T99<sup>[26]</sup>. While multiple phosphorylation sites in GAPDH have been predicted or experimentally demonstrated, their functional role still remains obscure.

Western blot analysis with anti-GAPDH or antiphosphoT99 antibody revealed two basic phosphorylated GAPDH isoforms in cytosol of the cells (Figure 3A). Consistently, GAPDH was excluded from the nuclei of untreated cells, but after treatment with araC, we found GAPDH phosphorylated at position T99 in the nuclei

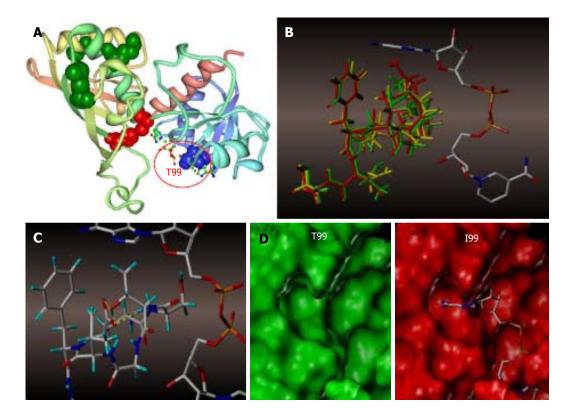


Figure 7 Molecular modeling of NAD<sup>\*</sup> binding site in human glyceraldehyde 3-phosphate dehydrogenase. A: NAD<sup>\*</sup> binding site within a GAPDH polypeptide (circled). Only one of four GAPDH subunits is shown. T99 is shown as a space-filled amino acid; B: The results of the trajectory calculations, with the average structure for each simulation shown overlaid for T99 (green), A99 (yellow), and I99 (red), along with the NAD<sup>\*</sup> molecule bound in the active cleft. Isoleucine side chain (red) of the T99I polypeptide acquires the closest position relative to the purine ring; C: In the native T99, an intramolecular H-bond between T99 and E97 (highlighted yellow) is likely to exist which holds the loop in a restricted conformation. This hydrogen bond could be a stabilizing factor permitting NAD<sup>\*</sup> to fully occupy the upper region of the cleft; D: Side-by-side comparison of T99 (green) and I99 (red) NAD<sup>\*</sup> binding site surfaces. In I99, the average structure places the terminus of the side chain well within the Van der Waals radii of the adenine ring of NAD<sup>\*</sup>, thereby displacing or blocking the cofactor from its initial association with GAPDH. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

#### (Figure 3B).

Peptide 87-107 is a part of NAD<sup>+</sup> binding center, as demonstrated by X-ray analysis<sup>[22]</sup>. Specifically, T99 is involved in non-polar interactions with the adenine residue of NAD<sup>+</sup>, while S98 forms electrostatic interactions with 5'-0-phosphate adjacent to nicotinamide nucleoside. The same NAD<sup>+</sup> binding center is also involved in binding of GAPDH with RNA<sup>[27]</sup>.

In order to characterize the role of phosphorylated amino acids in NAD<sup>+</sup> binding center, we performed alanine scanning by consecutive substituting Y94, S98, and T99 with Ala. Additionally, we prepared T99I mutated GAPDH. T99I mutation results from a single nucleotide variation rs11549329 found in NCBI dbSNP database, which is likely a rare mutation.

Kinetic parameters of wild type and four mutated GAPDH variants were quite different depending on the nature of mutation. The most dramatic changes were detected in T99I variant where  $K_m$  for NAD<sup>+</sup> was increased by more than an order of magnitude compared with wild type GAPDH. In line with these findings, the rate of the reaction catalyzed by T99I was very low (Figure 4). Mutation T99A had less effect on GAPDH parameters, with Km (NAD<sup>+</sup>) about 2 times higher, and Km (G3P) about 1.5 times higher compared with wild type GAPDH.

Mutation S98A at position adjacent to T99 showed an effect similar to that of T99A. Interestingly, Y94A mutation only slightly affected kinetics of the enzyme, while its effect on molecular dynamics parameters of nuclear GAPDH was quite prominent (Tables 2 and 3). The results of the molecular modeling experiments were in good agreement with the observed changes in enzyme kinetics of the mutated polypeptides (Figure 7).

Intracellular distribution of wild type and mutant GAPDH fused to EGFP was assessed in p53-proficient HCT116-4016 and SW48-297 cells using confocal microscopy and image analysis. Unexpectedly, mutations in the NAD<sup>+</sup> binding center changed distribution of fused EGFP-GAPDH proteins between cytoplasmic and nuclear compartments. Consistent with our previous results, wild type EGFP-GAPDH was excluded from nuclei of untreated cells, and showed intranuclear accumulation following genotoxic stress. In contrast, T99A and T99I mutant proteins were already notable in the nuclei of untreated cells. Upon incubation with araC, significantly higher levels of intranuclear T99A, and T99I GAPDH were observed far exceeding the level of wild type intranuclear GAPDH under stress conditions (Figure 5B). Both mutated proteins contained non-phosphorylated amino acids in the NAD<sup>+</sup> binding center, and manifested decreased affinity to NAD<sup>+</sup>.

It remains to be elucidated what molecular events alter the intracellular distribution of the fusion proteins. Earlier, we demonstrated that GAPDH export from the nucleus was facilitated by CRM1<sup>[21]</sup>. We hypothesize that the redistribution of mutated GAPDH between the cytoplasmic and nuclear compartments could result from less effective interaction of mutated GAPDH polypeptides with the CRM1-mediated nuclear export system.

In our previous studies, we estimated the diffusion coefficient and the immobile fraction of GAPDH inside the living A549 cells<sup>[3]</sup>. In the present study, we performed FRAP analysis of live HCT116-4016 and SW48-297 cells treated with araC. The results of these experiments in HCT116-4016 are shown on Figure 6, and summarized in Table 3. Similarly to our experiments on intracellular distribution of wild type and mutated polypeptides, we detected profound difference in molecular dynamics characteristics of GAPDH variants. After photo bleaching, the wild type GAPDH fusion protein demonstrated ten-fold lower mobility in the nuclear compartment of HCT116-4016 cells under genotoxic stress, compared to cytoplasm. The fraction of immobile wild type EGFP-GAPDH was  $0.62 \pm 0.048$  in the nuclei of araC-treated cells vs 0.18 ± 0.055 in cytoplasmic compartment. This observation parallels our findings for wild type EGFP-GAPDH in the A549 cell line<sup>[3]</sup>. Our results indicate that, inside the nucleus of the stressed cells, GAPDH loses it mobility, and significant fraction of polypeptides remains immobile presumably due to strong binding to as yet unidentified macromolecule or macrostructure. In parallel to our findings, intranuclear GAPDH in dexamethasonetreated S49 cells was found resistant to extraction with high salt or DNase treatment, consistent with our FRAP experiments<sup>[28]</sup>. David-Cordonnier's group reported binding of GAPDH with chromatin following DNA alkylation with antitumor drug S23906-1<sup>[14]</sup>.

The immobile fraction was notably lower in Y94A, S98A, T99A, and T99I (Table 3). All four mutated variants had similar diffusion coefficients in cytoplasmic and nuclear compartments, in contrast to wild type nuclear GAPDH which had lower D and higher (1-Mf) values. These results indicate that mutated polypeptides are involved in less tight intranuclear interactions than their wild type counterpart, with as yet unknown nuclear components (Table 3). We conclude that the phosphorylated amino acids or functionality of NAD<sup>+</sup> binding center is important for GAPDH intranuclear immobilization.

Tight interaction of intranuclear GAPDH with nuclear component(s) is an intriguing observation, considering the fact that multiple stress factors promote intranuclear accumulation of this protein. At least two groups of intranuclear proteins, sirtuins and ADP-ribosyl transferases, which control stress response and DNA repair, are NAD<sup>+</sup>-dependent. Both groups are present in the nucleus, and require NAD<sup>+</sup> for their activity. Importantly, GAPDH was demonstrated to directly interact with NAD<sup>+</sup>-dependent Sir2 histone deacetylase, and poly (ADP-ribose) polymerase- $1^{[29,30]}$ . Therefore, we speculate that GAPDH

could provide a nuclear NAD<sup>+</sup> reservoir, and deliver it to these proteins following the stress stimulus.

T99I variant is especially interesting as it is not an entirely artificial construct. The cDNA clone containing missense rs11549329 leading to T99I mutation was prepared from melanotic melanoma cells (GenBank: BM904848.1). This mutated variant manifests very low affinity to NAD<sup>+</sup> (Figure 4 and Table 2), high level of intranuclear accumulation (Figure 5), and a modest immobile fraction after genotoxic stress (Figure 6). Treatment with genotoxic drug does not alter mobility (diffusion coefficient) of this mutated form inside the nucleus or cytosol (Table 3).

Molecular modeling of NAD<sup>+</sup> binding site in human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed differential effects of amino acid changes at position 99 (Figure 7A). Interestingly, our molecular modeling experiment suggested the existence of a hydrogen bond between T99 and E97. Such intramolecular H-bond is likely to stabilize the loop extending from E97 (end of the beta sheet) through T104 (first residue in the attached alpha helix). This H-bond suffices to hold the loop in a restricted conformation, thereby permitting the cofactor to fully occupy the upper region of the cleft (Figure 7C).

Though T99A mutation does not prevent NAD<sup>+</sup> binding, decreased T99A GAPDH catalytic activity is best explained by the disruption of such T99-E97 intramolecular H-bond. Mutation of T99A removes the stabilizing H-bond, and the resultant loop is able to expand somewhat into the cleft and potentially affect the binding affinity of the cofactor to GAPDH. This hypothesis has been supported by our kinetic experiments with mutated GAPDH proteins in vitro, as Km of the mutant forms did decrease by 2-10 fold (Table Further confirmation for T99-E97 interaction requires additional testing, for example by mutational scanning at E97. Finally, analysis of the average potential energies of the dynamics trajectories also indicates that the system exhibits decreased stabilization (higher average potential energy and total energy) upon mutation and concomitant expansion of the loop into the cleft (Table 4).

Our model predicts T99I to have the strongest effect on NAD<sup>+</sup> binding and catalytic activity of GAPDH. Indeed, insertion of Ile at position 99 brings its side chain in close proximity with adenine of the cofactor (Figure 7). Modeling of the van der Waals surfaces inside the NAD<sup>+</sup> binding center shows sterically unacceptable interaction so that such interaction either displaces, or blocks cofactor binding by T99I GAPDH.

In conclusion, we detected the phosphorylated amino acid residues Y94, S98, T99 within the NAD<sup>+</sup> binding center of GAPDH. Substitution of these amino acids with non-phosphorylated alanine residues did not abrogate intranuclear localization of GAPDH. Instead, such mutations altered the molecular dynamics parameters of intranuclear GAPDH probably by hindering its interactions with yet to be identified nuclear biomolecules. Our

| Table 4 Average potential energy, kinetic energy, total energy and temperature calculated from dynamic simulations |                                |                                  |         |                                |  |
|--------------------------------------------------------------------------------------------------------------------|--------------------------------|----------------------------------|---------|--------------------------------|--|
| Polypeptide<br>variant                                                                                             | Average<br>potential<br>(kcal) | Average kinetic<br>energy (kcal) | •       | Average<br>temperature<br>(°C) |  |
| T99                                                                                                                | -5021.8                        | 97.1                             | -4924.7 | 293.4                          |  |
| A99                                                                                                                | -5007.3                        | 93.7                             | -4913.6 | 293.7                          |  |
| 199                                                                                                                | -4997.8                        | 101.4                            | -4896.4 | 293.3                          |  |

molecular modeling experiments invoke an important structural feature -T99-E97 H-bond likely involved in stabilization of NAD<sup>+</sup> binding center.

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# COMMENTS

#### Background

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an important enzyme of the glycolytic pathway, and an intriguing moonlighting protein involved in multiple, seemingly unrelated biochemical processes. It is known to exist in multiple isoforms, biological role and physiological significance of which remain enigmatic. Distribution of GAPDH between cellular compartments reveals complex patterns, and is regulated by partially characterized mechanisms.

## **Research frontiers**

Among multiple functions ascribed to GAPDH, its contribution to the nuclear processes remains obscure. The role of intranuclear GAPDH isoforms in maintaining the integrity of genome is a new direction of study.

## Innovations and breakthrough

This is the first study in which the role of phosphorylation sites in a NAD<sup>+</sup> binding center of GAPDH has been explored by site-mutagenesis, and the dynamics parameters of nuclear GAPDH have been directly measured in fluorescence recovery after photobleaching experiments.

## Applications

These results indicate an unexpected, important role phosphorylated amino acids within the NAD<sup>\*</sup> binding center play in intranuclear functions of GAPDH, and suggest a single mechanism by which GAPDH contributes to formation and functioning of its multiple binding partners.

## Terminology

Fluorescence recovery after photobleaching is an optical technique for quantifying the diffusion of the fluorescently labeled biological molecules inside the living cells.

#### Peer-review

In this study, Phadke *et al* investigate the phosphorylated amino acid residues Y94, S98, T99 within the NAD\* binding center of glyceraldehyde 3-phosphate dehydrogenase. Substitution of these amino acids with non-phosphorylated

alanine residues did not abrogate intranuclear localization of GAPDH. Instead, such mutations altered the molecular dynamics parameters of intranuclear GAPDH probably by hindering its interactions with yet to be identified nuclear biomolecules. Molecular modeling experiments suggest an important structural feature -T99-E97 H-bond likely involved in stabilization of NAD\* binding center.

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ORIGINAL ARTICLE

#### **Basic Study**

# Connective tissue growth factor differentially binds to members of the cystine knot superfamily and potentiates platelet-derived growth factor-B signaling in rabbit corneal fibroblast cells

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Author contributions: Pi L and Chung PY are co-first authors and share equal contribution; Pi L performed yeast two-hybrid analysis; Chung PY carried SPR experiments; Rahman MM contributed to SPR analysis; Sriram S established rabbit corneal fibroblast cell culture; Pi L, Chung PY, Scott EW, Petersen BE, and Schultz GS substantially contributed to the design of the study, acquisition, analysis and interpretation of data; Pi L and Chung PY wrote the paper; all authors made critical comments related to the intellectual content of the manuscript, and approved the final version of the article to be published.

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and dataset available from the corresponding author at lpi@peds. ufl.edu.

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# Abstract

**AIM:** To study the binding of connective tissue growth factor (CTGF) to cystine knot-containing ligands and how this impacts platelet-derived growth factor (PDGF)-B signaling.

**METHODS:** The binding strengths of CTGF to cystine knot-containing growth factors including vascular en-



dothelial growth factor (VEGF)-A, PDGF-B, bone morphogenetic protein (BMP)-4, and transforming growth factor (TGF)- $\beta$ 1 were compared using the LexAbased yeast two-hybrid system. EYG48 reporter strain that carried a wild-type LEU2 gene under the control of LexA operators and a lacZ reporter plasmid (p80placZ) containing eight high affinity LexA binding sites were used in the yeast two-hybrid analysis. Interactions between CTGF and the tested growth factors were evaluated based on growth of transformed yeast cells on selective media and colorimetric detection in a liquid  $\beta$ -galactosidase activity assay. Dissociation constants of CTGF to VEGF-A isoform 165 or PDGF-BB homo-dimer were measured in surface plasma resonance (SPR) analysis. CTGF regulation in PDGF-B presentation to the PDGF receptor  $\beta$  (PDGFR $\beta$ ) was also quantitatively assessed by the SPR analysis. Combinational effects of CTGF protein and PDGF-BB on activation of PDGFR<sub>β</sub> and downstream signaling molecules ERK1/2 and AKT were assessed in rabbit corneal fibroblast cells by Western analysis.

**RESULTS:** In the LexA-based yeast two-hybrid system, cystine knot motifs of tested growth factors were fused to the activation domain of the transcriptional factor GAL4 while CTGF was fused to the DNA binding domain of the bacterial repressor protein LexA. Yeast cotransformants containing corresponding fusion proteins for CTGF and all four tested cystine knot motifs survived on selective medium containing galactose and raffinose but lacking histidine, tryptophan, and uracil. In liquid  $\beta$ -galactosidase assays, CTGF expressing cells that were co-transformed with the cystine knot of VEGF-A had the highest activity, at 29.88  $\pm$  0.91 fold above controls (P < 0.01). Cells containing the cystine knot of BMP-4 expressed the second most activity, with a 24.77  $\pm$  0.47 fold increase (P < 0.01). Cells that contained the cystine knot of TGF- $\beta$ 1 had a 3.80 ± 0.66 fold increase (P < 0.05) and the ones with the cystine knot of PDGF-B had a 2.64  $\pm$  0.33 fold increase of  $\beta$ -galactosidase activity (P < 0.01). Further SPR analysis showed that the association rate between VEGF-A 165 and CTGF was faster than PDGF-BB and CTGF. The calculated dissociation constant (KD) of CTGF to VEGF165 and PDGF-BB was 1.8 and 43 nmol/L respectively. PDGF-BB ligand and PDGFR $\beta$  receptor formed a stable complex with a low dissociation constant 1.4 nmol/L. Increasing the concentration of CTGF up to 263.2 nmol/L significantly the ligand/receptor binding. In addition, CTGF potentiated phosphorylation of PDGFR<sub>B</sub> and AKT in rabbit corneal fibroblast cells stimulated by PDGF-BB in tissue culture condition. In contrast, CTGF did not affect PDGF-B induced phosphorylation of ERK1/2.

CONCLUSION: CTGF has a differential binding affinity to VEGF-A, PDGF-B, BMP-4, and TGF- $\beta$ . Its weak association with PDGF-B may represent a novel mechanism to enhance PDGF-B signaling.

Key words: Connective tissue growth factor; Vascular endothelial growth factor A; Platelet derived growth factor

B; Transforming growth factor- $\beta$ ; Bone morphogenetic protein-4; Yeast two-hybrid analysis; Surface plasma resonance; Rabbit corneal fibroblast cells; Platelet-derived growth factor receptor  $\beta$ ; Platelet-derived growth factor-B signaling

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**Core tip:** The relative binding strength of connective tissue growth factor (CTGF) was vascular endothelial growth factor-A > bone morphogenetic protein-4 > transforming growth factor- $\beta$ 1 > platelet-derived growth factor (PDGF)-B. CTGF binding could potentiate PDGF-B signaling as evidenced by enhanced phosphorylation of PDGF receptor  $\beta$  and downstream AKT molecules in rabbit corneal fibroblast cells. Our findings provide deep insight into CTGF action in fine-tuned regulation of extracellular signaling mediated by different cysteine knot containing growth factors.

Pi L, Chung PY, Sriram S, Rahman MM, Song WY, Scott EW, Petersen BE, Schultz GS. Connective tissue growth factor differentially binds to members of the cystine knot superfamily and potentiates platelet-derived growth factor-B signaling in rabbit corneal fibroblast cells. *World J Biol Chem* 2015; 6(4): 379-388 Available from: URL: http://www.wjgnet.com/1949-8454/full/v6/ i4/379.htm DOI: http://dx.doi.org/10.4331/wjbc.v6.i4.379

# INTRODUCTION

Connective tissue growth factor (CTGF), also termed as CCN2, is a secreted cysteine-rich protein in the CCN (Cyr61/CTGF/Nov) family involved in a diverse variety of biological processes ranging from angiogenesis, chondrogenesis, osteogenesis, and tissue repair, to cancer development. CTGF was originally isolated from human umbilical vein endothelial cells as a 349-amino acid mitogen with biological activities similar to plateletderived growth factor (PDGF)-B<sup>[1]</sup>. CTGF protein contains a modular architecture starting with an N-terminal signal peptide followed by an insulin-like growth factor-binding (IGFBP) domain, a von Willebrand factor type C domain, a thrombospondin type I homology domain, and a C terminal cystine knot motif<sup>[2]</sup>. These domains enable CTGF interaction with a broad repertoire of proteins including cell surface proteins, extracellular matrix proteins, and growth factors. Through these interactions, CTGF functions as an extracellular signaling modulator and regulates cell adhesion, migration, activation and differentiation<sup>[3]</sup>.

The cystine-knot motif is structurally conserved in various kinds of growth factors, ligands, and mucins<sup>[4,5]</sup>. Consensus sequences of this structure can be identified by a pattern of six cysteine amino acids with a defined space consisting of three intertwined disulfide bridges. Two of the disulfide bonds and their adjacent amino acids



form a ring that is passed by the third disulfide. This structure forces exposure of many hydrophobic residues in monomers and favor the formation of homo- or hetero-dimers to decrease hydrophobicity in an aqueous environment. These dimers represent active states of the cystine knot-bearing growth factors able to elicit signal transduction via their cognate receptors. Interestingly, many members of this superfamily including transforming growth factor (TGF)- $\beta$ , bone morphogenetic protein (BMP)-4, BMP-2, vascular endothelial growth factor (VEGF)-A, PDGF-B, von Willebrand factor (vWF), the neuronal guidance cue Slit3, the growth and differentiation factor 5 (GDF5), and GDF15 are able to interact with CTGF<sup>[6-11]</sup>. It has been shown that CTGF binding can sequester VEGF-A from VEGF receptor 1 and inhibit VEGF-A stimulated angiogenesis<sup>[6]</sup>. Moreover, the high affinity of CTGF inhibits the presentation of BMP-4 to BMP receptor type Ia and the phosphorylation of this receptor and downstream molecule Smad1, whereas its low affinity potentiates TGF- $\beta$ 1 binding to  $T\beta R II$  receptor and enhances phosphorylation of Smad2<sup>[7]</sup>. So far, little has been determined concerning the effect of CTGF on PDGF-B activity and signaling. In this study, we compared the binding strengths of CTGF to VEGF-A, PDGF-B, TGF-B1 and BMP-4 using the LexA-based yeast two-hybrid system. The effect of CTGF on PDGF-B presentation to PDGFRβ receptor was determined in surface plasma resonance (SPR) analysis. The modulation of CTGF on PDGF-B signaling was investigated using rabbit corneal fibroblast cells.

# MATERIALS AND METHODS

## Yeast two-hybrid analysis

Yeast two-hybrid assays were performed according to the Clontech yeast protocol handbook. Synthetic dropout minimal medium (SD) and supplements used in this study were purchased from Clontech (Mountain View, CA). Dropout supplements containing nucleotides and amino acid residues as well as additional supplements of glucose, galactose (Gal), and raffinose (Raf) were prepared according to the manufacturer's instructions. CTGF, cystine knot motifs in VEGF-A and PDGF-B were described previously<sup>[9]</sup>. The cystine knot motif of TGF-B1 was amplified using primers 5' AGGTCGACCTCCTGGCGATACCTCAGCAACGC 3' (sense) and 5' TTGCGGCCGCTGCACTTGCAGGAGCGCAC 3' (antisense) from a human cDNA clone (Genebank Accession # NM 000660.5). The cystine knot motif of BMP-4 was amplified using primers 5' AGGTCGACC GGACACCTCATCACACGACTA 3' (sense) and 5' TTGCG-GCCGCGGCATCCACACCCCTCTAC 3' (antisense) from a mouse cDNA clone (Genebank Accession #NM\_007554.2). All the constructs contained restriction enzyme sites Sal I and Not I that facilitated their cloning into the prey pB42AD vector carrying an activation domain (AD) of VP16 or the bait pLexA vector containing a LexA-DNA binding domain (BD).

In yeast two-hybrid analysis, *Saccharomyces cerevisiae* EGY48 was used as the reporter strain, which carried a

wild-type LEU2 gene under the control of LexA operators and a *lacZ* reporter plasmid p80p-*lacZ* containing eight high affinity LexA binding sites. SD medium that lacked uracil (Ura) was used for the maintenance of p8oplacZ plasmids. BD or AD plasmids containing CTGF and cystine knot motifs of tested growth factors were cotransformed into EGY48(p8op-lacZ) and selected using histidine (His; a selection marker for pLexA plasmid) and tryptophan (Trp; for selection of pB42-AD plasmids). Co-transformants were initially selected on SD medium lacking Ura, His, and Trp but supplemented with glucose, which repressed fusion protein expression. Then these co-transformed cells were grown overnight prior to dilution into SD media that contained Gal and Raf but lacked Trip, His, and Ura. Gal and Raf supplements were added to induce fusion gene expression. The diluted cells were grown to mid-log phase with optical densities of 0.5-0.8 units. One-half of each cell culture was used to determine activation of the Leu2 reporter gene by growth assay based on survival in SD medium contained Gal and Raf but lacked Trp, His, Ura, and leucine (Leu). The remaining mid-log-phased cells was pelleted, washed and resuspended in Z buffer (60 mmol/L Na2HPO4, 40 mmol/L NaH2PO4, 10 mmol/L KCl, 10 mmol/L MgSO4, pH = 7.0).  $\beta$ -galactosidase activity was measured by O-nitrophenyl β-D-galactopyranoside (ONPG) cleavage in a liquid assay (Pierce Biotechnology, Rockford, IL). The results were obtained by reading absorbance at 420 nm and normalized against cell density (A600) and reaction time. Values are the average folds of activation over pB42 vector alone (arbitrarily set to 1) from three independent experiments. Statistical analysis was carried out using Student t test in Microsoft Excel software.

# SPR analysis

Biacore 2000 and CM4 dextran sensor chips (GE healthcare, Piscataway, NJ) were used to characterize proteinprotein interactions based on SPR technology. Filtered and degassed HBS-EP buffer (pH = 7.4) containing 0.01 mol/L HEPES, 0.15 mol/L NaCl, 3 mmol/L EDTA, and 0.005% Tween 20 was used as running buffer. Prior to the kinetic measurements, the sample channel was immobilized with carrier free recombinant human CTGF protein (BioVendor, Asheville, NC), and the reference channel was blocked with ethanolamine. Then, serial concentrations of recombinant human homodimeric PDGF-BB protein (Shenandoah, Warwick, PA) and recombinant human VEGF-A variant 165 (VEGF<sub>165</sub>) (Abcam, Cambridge, MA) in HBS-EP buffer were injected over the sampling and reference channels simultaneously at 30 uL/min for 3 min. The sensorgrams acquired from the reference channels were deducted from those of CTGFimmobilized channels to eliminate the responses caused by the refractive index changes. The dissociation was then followed by injecting HBS-EP buffer for 10 min. The difference in SPR response unit (RU) between the baseline and the signal after the dissociation reflects the amount of bound analytes. Before starting the next injection, the chip surface was regenerated by exposing



the chip surface with 10 mmol/L of cysteine-HCl buffer pH 2.0 at 30  $\mu\text{L/min}$  for 30 s.

To determine the effect of CTGF on the binding between PDGF-BB and PDGFR $\beta$ , a mixture of PDGF-BB (2 nmol/L) and varied concentrations of CTGF (0 to 263.2 nmol/L) was injected over the PDGFR<sub>β</sub>-immobilized channel and reference channel. Amine coupling strategy was used to immobilize the recombinant human PDGFR $\beta$ Fc chimera protein (R&D Systems, Minneapolis, MN) on the sampling channel. Briefly, a mixture of 0.4 mol/L 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 0.1 mol/L N-hydroxysuccinimide in equal proportion was used to activate the surface of a CM4 dextran sensor chip at a flow rate of 10  $\mu$ L/min for 7 min. Then, PDGFR $\beta$ was injected over separate sampling channels for 7 min to covalently immobilize on the surfaces. Remaining active groups were blocked with 1 mol/L ethanolamine-HCl at pH 8.5 on both sampling and reference channels. The sensorgrams acquired from the reference channels were deducted from those of the PDGFR $\beta$ -immobilized channels to eliminate the responses caused by the refractive index changes. All data were analyzed with the BIAevaluation software for calculating dissociation constants.

# Determination phosphorylation of PDGFR $\beta$ , ERK1/2 and AKT in Western analysis

Primary rabbit corneal fibroblast cells were isolated as described previously<sup>[12]</sup>. Cells were cultured to 100% confluence and serum-starved for 20 h in DMEM with 0.1% FBS. PDGF-B (1.6 nmol/L), CTGF (6.6 nmol/L), or a combination was added to the prepared cells. At 10 min and again at 1 h after stimulation, cells were collected and lysed in RIPA buffer (150 mmol/L NaCl, 1.0% IGEPAL<sup>®</sup> CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris, pH = 8.0) containing proteinase inhibitor (Sigma, St Louis, MI). Total protein lysates (20  $\mu$ g) were separated on 10% SDS-PAGE gels followed by transferring to polyvinylidene difluoride membranes. The primary antibodies for anti-phospho-PDGFR<sub>β</sub>, anti-PDGFR<sub>β</sub>, anti-phospho-ERK1/2 (Thr202/ Tyr204), anti-ERK1/2, anti-phospho-AKT, and anti-AKT were purchased from Cell Signaling (Danvers, MA). HRP conjugated rabbit or mouse secondary antibodies were used followed by detection with the ECL kit (GE Health Biosciences).

# RESULTS

# Differential binding ability of CTGF to VEGF-A, PDGF-B, TGF- $\beta$ , and BMP-4 in the yeast two-hybrid LexA system

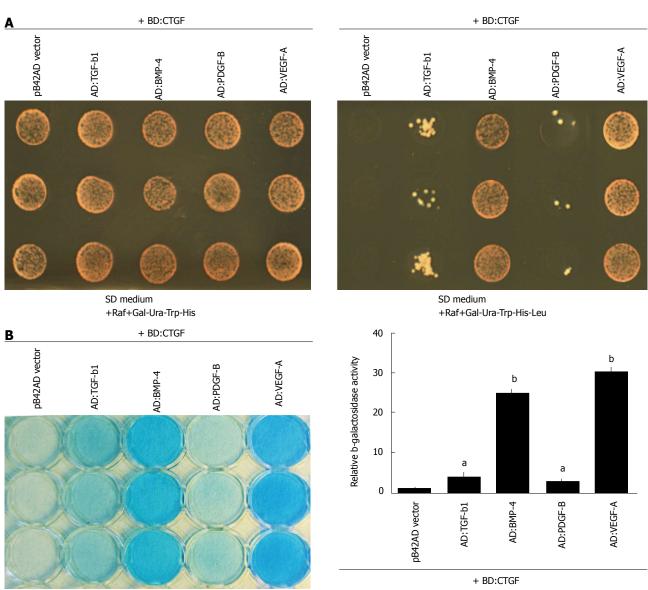
To compare the relative binding activity of CTGF to VEGF-A, PDGF-B, TGF- $\beta$ 1, and BMP-4, we took the yeast two-hybrid approach and expressed the cystine knot motifs of these specific growth factors as AD fusion proteins under the control of the GAL1 promoter in the pB42-AD plasmids. In addition, full-length CTGF cDNA was expressed in fusion with LexA BD under the control

of an alcohol dehydrogenase promoter in pLexA plasmids. These plasmids and their corresponding empty vectors were transformed into the EGY48 (p8op-lacZ) reporter strain (Figure 1A). CTGF itself was not able to selfactivate in this LexA system since yeast cells that were co-transformed with the BD:CTGF plasmid and pB42 vector did not grow without Leucine supplementation and did not turn blue in  $\beta$ -galactosidase assays. Nor were cystine knot motifs of tested growth factors because they also failed to trigger Leu2 and LacZ reporter genes when co-transformed with pB42-AD empty vector. In contrast, co-transformation with CTGF allowed survival of cells that expressed cystine knot motifs of all four growth factors on selective medium lacking leucine, supporting our previous observations that CTGF has broad binding capabilities to members of cystine knot superfamily<sup>[9]</sup>. In addition, we carried out an ONPG-based assay and assessed relative β-galactosidase activity in comparison to control cells that carried plasmids for CTGF and pLexA vector. As shown in Figure 1B, CTGF expressing cells that were co-transformed with the cystine knot of VEGF-A had the highest  $\beta$ -galactosidase activity, at 29.88 ± 0.91 fold above controls. Cells containing the cystine knot of BMP-4 expressed the second most  $\beta$ -galactosidase activity, with a 24.77  $\pm$  0.47 fold increase. Cells that contained the cystine knot of TGF- $\beta$ 1 had a 3.80 ± 0.66 fold increase and the ones with the cystine knot of PDGF-B had a 2.64 ± 0.33 fold increase. These observations indicated that VEGF-A and BMP-4 were strong interactors whereas PDGF-B and TGF- $\beta$  were weak binding proteins for CTGF.

## Binding kinetics of CTGF to VEGF-A and PDGF-B in SPR analysis

SPR analysis was performed to characterize the affinity of CTGF to VEGF165 and PDGF-BB. Initial experiments indicated that injecting homodimeric PDGF-BB over a CM5 dextran sensor chip caused a significant non-specific response on the reference channels, which may be due to the electrostatic interaction between the cationic growth factors and negatively charged carboxymethyl dextran covered on the chip. Therefore, we chose a CM4 dextran sensor chip with a lower degree of carboxymethylation to avoid the non-specific interaction. The kinetic analyses between PDGF-BB/CTGF and VEGF165/CTGF complexes were carried out by injecting different concentrations of PDGF-BB (19.3 to 385 nmol/L) or VEGF165 (3.879 to 52.267 nmol/L) over the CTGF-immobilized channel and reference channel simultaneously at 30  $\mu$ L/min for 3 min. The dissociation process was followed by injecting HBS-EP buffer at 30  $\mu$ L/min for 10 min. The higher flow rate was required to reduce the mass transfer effect and obtain kinetic parameters accurately. As shown in Figure 2A-C, the association rate between VEGF165 and CTGF was faster than PDGF-BB and CTGF. The calculated dissociation constant (KD) of CTGF to VEGF165 and PDGF-BB was 1.8 and 43 nmol/L respectively. These results indicated that CTGF had higher affinity to VEGF165 than PDGF-BB.





# Pi L et al. CTGF binding to cystine knot containing growth factors

Figure 1 Determination of connective tissue growth factor binding to cystine knot motifs of vascular endothelial growth factor-A, platelet-derived growth factor-B, transforming growth factor- $\beta$ 1, and bone morphogenetic protein-4 in yeast two-hybrid LexA system. A: CTGF binding to cystine knot motifs of tested growth factors was assessed using growth assay on selective SD medium lacking leucine (lower panel); B: The liquid  $\beta$ -galactosidase assay was also carried out determining the binding strength of CTGF to cystine knot motifs of tested growth factors. The relative  $\beta$ -galactosidase activity was quantified in graph on the right. <sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01. Values are the average -folds of activation over pB42-AD vector alone (arbitrarily set to 1) from three independent experiments performed in triplicate  $\pm$  SD. CTGF: Connective tissue growth factor; VEGF: Vascular endothelial growth factor; BMP: Bone morphogenetic protein-4; TGF- $\beta$ 1: Transforming growth factor- $\beta$ 1; PDGF-B: Platelet-derived growth factor-B.

# CTGF promotes the binding of PDGF-B to PDGFR $\beta$ determined by SPR analysis

PDGF-BB ligand and PDGFR $\beta$  receptor formed a stable complex indicated by a low dissociation constant 1.4 nmol/L in our SPR analysis (Figure 3A). To determine whether CTGF protein influenced the formation of this ligand/receptor complex, we injected a mixture of CTGF and PDGF-BB over the PDGFR $\beta$ -immobilized channel and measured the SPR response unit. As shown in Figures 3B and 4, the presence of CTGF was able to potentiate the binding of PDGF-BB to its receptor. Increasing the amount of CTGF further promoted this effect, especially when the concentration of CTGF was more than 3.3 nmol/L. Specifically, two-fold more ligand/receptor binding was observed when 26.3 nmol/L CTGF was mixed with 2 nmol/L PDGF-BB in comparison to the same amount of PDGF-BB alone. Moreover, three and a half-fold more ligand/receptor binding was seen when 263.2 nmol/L CTGF was mixed with 2 nmol/L PDGF-BB. In other words, seven-fold more ligand/receptor binding response was seen when CTGF to PDGF-BB ratio in molar concentration was 131.6 in comparison to PDGF-BB alone. To validate if the binding was indeed attributable to PDGF-BB, we also injected pure 263.2 nmol/L CTGF over PDGFR $\beta$ -immobilized channels as a control. We found no binding interaction (Figure 5).

# CTGF potentiates activation of PDGFR $\beta$ and downstream signaling molecule AKT

 $\text{PDGFR}_\beta$  is recognized by PDGF-B ligand and deletion



#### Pi L et al. CTGF binding to cystine knot containing growth factors

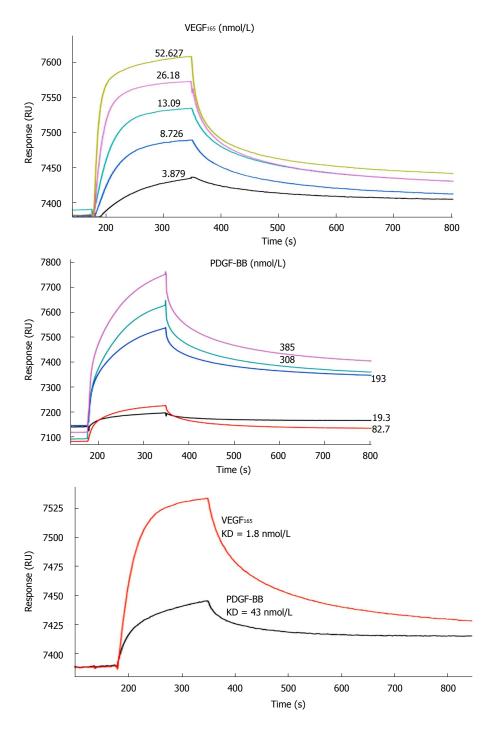


Figure 2 Quantitative measurement of connective tissue growth factor binding to vascular endothelial growth factor 165 and platelet-derived growth factor-BB in surface plasma resonance analysis. The binding kinetics of CTGF to VEGF165 (A) or to PDGF-BB (B) were determined by injecting VEGF165 ranging from 3.879 nmol/L to 52.267 nmol/L or PDGF-BB ranging 19.3 nmol/L to 385 nmol/L over CTGF immobilized channel and reference channel at 30  $\mu$ L/min for 3 min simultaneously. The reference sensorgram has already deducted to eliminate the signal from a bulk refractive index shift; C: Sensorgrams of SPR analysis showed that the equilibrium dissociation constant for CTGF binding to PDGF-BB or VEGF165 was 43 nmol/L or 1.8 nmol/L respectively. CTGF: Connective tissue growth factor; VEGF: Vascular endothelial growth factor; PDGF-B: Platelet-derived growth factor-B; SPR: Surface plasma resonance.

of this receptor gene affects key fibroblast functions in wound healing<sup>[13,14]</sup>. PDGF-BB binding to the extracellular region of PDGFR $\beta$  induces dimerization and activation of intracellular kinase domain of this receptor. As a result, PDGFR $\beta$  receptors undergo auto-phosphorylation in critical tyrosine residues and gain full enzymatic activity to phosphorylate substrates. These phosphorylation regions can function as docking sites and recruit signal molecules containing SH2 and SH3 domains. For instances, Tyr751 in the kinase-insert region of PDGFR $\beta$  is the docking site for PI3 kinase, which in turn activates downstream molecules including the serine/threonine kinase AKT<sup>[15]</sup>. We used cultured rabbit corneal fibroblast cells and detected that PDGF-BB (1.6 nmol/L) quickly

stimulated PDGFR $\beta$  phosphorylation at Tyr751 within minutes (Figure 6). This phosphorylated PDGFR $\beta$ gradually decreased probably due to a rapid turnover of this receptor in cultured rabbit corneal fibroblast cells. This pattern was correlated with phosphorylation of AKT and ERK1/2 in PDGF-BB stimulated cells. The phosphorylation of both PDGFR $\beta$  and AKT induced by 1.6 nmol/L PDGF-BB was enhanced by the addition of CTGF (6.6 nmol/L) to rabbit corneal fibroblast cells. In contrast, CTGF alone was unable to cause significant changes of PDGFR $\beta$  receptor and AKT levels in the cultured cells. Moreover, phosphorylation of ERK1/2 stimulated by PDGF-BB was not influenced by presence of CTGF. These results indicated that CTGF could potentiate some PDGF-

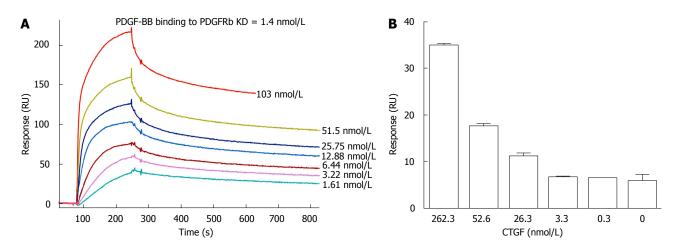


Figure 3 Connective tissue growth factor is able to enhance the binding of platelet-derived growth factor-BB to platelet-derived growth factor receptor $\beta$ . SPR assay was carried out to quantitatively measure the binding of PDGF-BB to PDGFR $\beta$ . A: Sensorgrams of SPR analyses showing the binding dissociation equilibrium constant of PDGF-BB and PDGFR $\beta$ ; B: Measurement of SPR response unit showed that CTGF promoted PDGF-BB/PDGFR $\beta$  interaction. A mixture of PDGF-BB (2 nmol/L) with different concentrations of CTGF (0-263.2 nmol/L) was run over PDGFR $\beta$  sensor chips. CTGF: Connective tissue growth factor; PDGF-B: Platelet-derived growth factor-B; SPR: Surface plasma resonance; PDGFR $\beta$ : Platelet-derived growth factor receptor  $\beta$ .

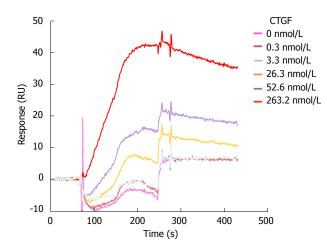


Figure 4 Connective tissue growth factor enhances platelet-derived growth factor-BB binding to platelet-derived growth factor receptor  $\beta$  in surface plasma resonance analysis. PDGF-BB (2 nmol/L) was mixed with different concentrations of CTGF (0-263.2 nmol/L) and run over PDGFR $\beta$  sensor chips. SPR response units were measured and used for quantitative comparisons of interaction between PDGF-BB and PDGFR $\beta$  in presence of different concentrations of CTGF proteins in Figure 3B. Representative sensorgrams from two independent trials are shown. CTGF: Connective tissue growth factor; PDGF-B: Platelet-derived growth factor-B; PDGFR $\beta$ : Platelet-derived growth factor receptor  $\beta$ .

BB mediated signaling including activation of  $PDGFR\beta$  and AKT in rabbit corneal fibroblast cells.

## DISCUSSION

The yeast two-hybrid system is designed to measure protein-protein interaction by reconstitution of a functional transcription factor that activates receptor genes. Previously, we used a GAL4 system and revealed CTGF's broad ability to bind with multiple cystine knot-bearing factors using *His3* reporter<sup>[9]</sup>. This is a qualitative assay with potential variation dependent upon the expression levels of the fusion proteins under investigation. In this study, we took

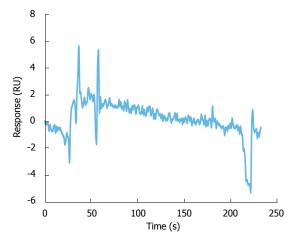


Figure 5 Connective tissue growth factor is not associated with plateletderived growth factor receptor  $\beta$  in surface plasma resonance analysis. CTGF protein (263.2 nmol/L) was run over PDGFR $\beta$  sensor chips and the SPR response unit was measured. Sensorgrams showed little binding response between CTGF and PDGFR $\beta$  proteins. CTGF: Connective tissue growth factor; PDGF-B: Plateletderived growth factor-B; SPR: Surface plasma resonance; PDGFR $\beta$ : Plateletderived growth factor receptor  $\beta$ .

advantage of LexA-responsive *LEU2* and *lacZ* reporter genes, and carried out quantitative assessments according to both colony survival and  $\beta$ -galactosidase activity to discriminate high- and low-affinity interactions. Our results suggested that CTGF strongly bound to VEGF-A and BMP-4 but loosely interacted with PDGF-B and TGF- $\beta$ 1. These observations were consistent with published results about measurement of dissociation constants for BMP-4, TGF- $\beta$ 1, PDGF-BB, and VEGF-A using SPR or I<sup>125</sup> labeled solid phase protein binding assays<sup>[7,10]</sup>. This study further supported a previous report that the degree of protein interaction determined by yeast two-hybrid approaches correlates well with those examined in biochemical analysis<sup>[16]</sup>.

The cystine-knot superfamily is composed of



#### Pi L et al. CTGF binding to cystine knot containing growth factors

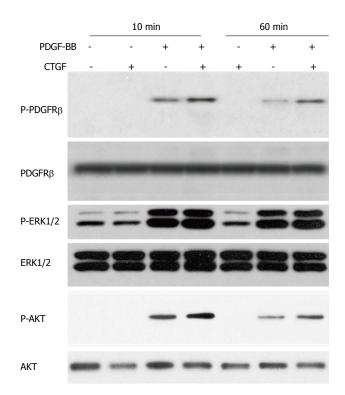


Figure 6 Connective tissue growth factor potentiates platelet-derived growth factor-BB stimulated activation of platelet-derived growth factor receptor $\beta$  and downstream signal molecule Akt. Western analysis was carried out to determine phosphorylated or total PDGFR $\beta$ , ERK1/2 and AKT in rabbit corneal fibroblast cells stimulated by CTGF (6.6 nmol/L), PDGF-B (1.6 nmol/L) or combination. CTGF: Connective tissue growth factor; PDGF-B: Platelet-derived growth factor-B; PDGFR $\beta$ : Platelet-derived growth factor receptor  $\beta$ .

several subgroups of proteins involved in a diverse variety of pathophysiological functions including cell growth, organogenesis, embryonic development, cell-to-cell communication, differentiation, tissue repair and cancer progression. In mammals, the PDGF subfamily has at least 10 members (i.e., VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E from the VEGF family; PDGF-A, PDGF-B, PDGF-C, and PDGF-D from the PDGF family; and placenta growth factors PIGF-1 and PIGF-2)<sup>[14]</sup>. The TGF- $\beta$  subfamily has about 37 members (BMP2-7, BMP8A, BMP8B, BMP10, BMP15, GDF1-3, GDF5-11, GDF15, Activin  $\beta$ E, Inhibin  $\alpha$ , Inhibin βA, Inhibin βB, Inhibin βC, TGF-β1-3, Nodal, Anti-Mullerian hormone, LEFTY-A, LEFTY-B, GDNF, Neurturin, Persephin, and Artemin-21)<sup>[17]</sup>. The Slit subfamily has three members (Slit1-3). The mucin-like subgroup include nine BMP antagonists (Follistatin, FSTL-3, Cerberus, Chordin, Gremlin, Noggin, SOST/Sclerostin, TSG, and USAG-1)<sup>[18]</sup>, five mucin related protein (Norrin, mucin-2, MUC5AC protein, secretory mucin), and vWF<sup>[4]</sup>. So far, CTGF is found to interact with at least one member in each subgroup of the cystine knot superfamily. This broad binding spectrum of CTGF may be due to unique structure organization of members of the CCN family. Based on small angle X-ray scattering data in CCN3 and CCN5, CCNs seem to form long extended structures able to move and flex to allow multiple domains to bind a ligand<sup>[19]</sup>. It is conceivable that the four modular structures of CTGF protein form long extended and flexible scaffolds that "glue" the cystine knot proteins from destruction by cleavage in extracellular space.

Differential binding activity of CTGF to cystine knot-bearing ligands appears to give rise to disparate regulatory mechanisms for the ligand presentation to their receptors. One mechanism is through a high affinity/interaction with CTGF. Examples are VEGF-A, BMP-4, and BMP-2, which are strong interactors for CTGF. Our study, together with others<sup>[10]</sup> show that VEGF-A has a dissociation constant for CTGF in SPR assays around 1.8-4.8 nmol/L. BMP-4 and BMP-2 have dissociation constants of 5 and 0.77 nmol/L with CTGF respectively<sup>[7,10]</sup>. CTGF binding sequesters VEGF-A from its receptor and inhibits downstream signaling leading to anti-angiogenesis<sup>[6]</sup>. Similarly, CTGF binding inhibits BMP-4 from BMPRIa receptor and downstream signaling leading to altered development of *Xenopus* embryo<sup>[/]</sup>. BMP-2 activity on the proliferation of chondrocytes is also inhibited by CTGF<sup>[12]</sup>. Hence, we speculate that strong interaction with CTGF causes sequestration of the bound ligands from their receptors thereby preventing signal transduction.

In contrast, other cystine knot-bearing growth factors such as TGF- $\beta$ 1 and PDGF-B are weak interactors and their signal transduction pathways are not inhibited by CTGF<sup>[7]</sup>. The dissociation constant for CTGF binding to TGF- $\beta$ 1 is 30 or 64.7 nmol/L measured by two different groups<sup>[7,10]</sup>. We found that CTGF bound to PDGF-BB with a dissociation constant 43 nmol/L. Much lower binding kinetics between CTGF and PDGF-BB (K<sub>D</sub> = 230 nmol/L) is observed by Khattab et al<sup>[10]</sup>. Variable factors including purity of tested recombinant proteins and variation of Biacore SPR systems may contribute to these discrepancies. Nevertheless, the measured dissociation constants for these weak interactors are often more than 10<sup>-10</sup> mol/L. Despite of this low affinity, CTGF seems to increase the activity of TGF- $\beta^{[7]}$ . We found that CTGF was able to potentiate PDGF-BB binding to PDGFR $\beta$  and enhance activation of PDGFR $\beta$  and downstream AKT. Given that CTGF is a matricellular protein and can trigger adhesive signaling through association with cell surface proteins such as integrins<sup>[3]</sup>, its binding to TGF- $\beta$ 1 or PDGF-B may be part of crosstalk for integration of these different signal pathways. Lack of overlapping in some downstream signaling between CTGF and PDGF-BB may explain little enhancement of ERK1/2 activation in cultured rabbit corneal fibroblast cells.

TGF- $\beta$ 1, PDGF-B and CTGF are known profibrotic partners for fibroblast activation<sup>[20]</sup>. Low affinity of CTGF may account for its synergistic effect on PDGF-BB and TGF- $\beta$  in promoting chronic fibrosis. In particular, TGF- $\beta$ 1 can transcriptionally activate CTGF expression through SMAD responsive elements in the CTGF promoter<sup>[21]</sup>. In mice, co-injection of CTGF and TGF- $\beta$  results in sustained, persistent fibrosis, whereas subcutaneous injection of just TGF- $\beta$  causes a transient fibrotic response<sup>[22]</sup>. In addition, PDGF is a major regulator for vascular cells and its deficiency causes vascular abnormalities including



capillary dilation and microaneurysms during mouse embryonic development<sup>[23]</sup>. VEGF-A is critical for the proliferation and survival of vascular endothelial cells. Loss of VEGF-A expression results in severe abnormalities in the vasculature and causes embryonic death in heterozygous mice<sup>[24]</sup>. Given the importance of PDGF-B and VEGF-A in the assembly of blood vessels, the inherent stickiness of CTGF to PDGF-B and VEGF-A may represent a fine-tuned regulation of vascular smooth muscle cells, pericytes, and vascular endothelial cells during angiogenesis. Future studies about the nature of CTGF binding to VEGF-A, PDGF-B, and TGF- $\beta$  may help the development of therapeutic strategies for pathological processes involving angiogenesis and fibrosis.

# ACKNOWLEDGMENTS

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# COMMENTS

#### Background

Connective tissue growth factor (CTGF), also termed CCN2, is one prototype member in the Cyr62/CTGF/Nov family of matricellular proteins. It contains a four conserved modular structure and regulates cell proliferation, migration, differentiation, apoptosis, and survival through interaction with extracellular matrix protein, growth factors, and cell surface proteins. CTGF exhibits a broad binding ability to a structurally conserved motif characteristic of the cystine knot superfamily. However, molecular action of CTGF in modulation of the cystine knot-containing growth factors has not been fully understood.

#### **Research frontiers**

CTGF binding can sequester some of the cystine knot-containing growth factors such as vascular endothelial growth factor (VEGF)-A and bone morphogenetic protein (BMP)-4 from their cognate receptors x and inhibit their signaling. For other growth factors such as transforming growth factor (TGF)- $\beta$ 1, CTGF has distinct effects and potentiates TGF- $\beta$ 1 mediated downstream signal transduction pathway. Correlation between the binding strength of CTGF and its action on the majority of cystine knot-containing growth factors has not been investigated.

#### Innovations and breakthroughs

This paper combined LexA-based yeast two-hybrid system with surface plasma resonance (SPR) analysis and quantitatively assesses the binding strengths of CTGF to VEGF-A, platelet-derived growth factor (PDGF)-B, BMP-4, and TGF- $\beta$ 1. CTGF had high affinities with VEGF-A and BMP-4 but only weakly interacted with TGF- $\beta$ 1 and PDGF-B. These binding strengths were correlated with inhibition or potentiation by CTGF on signal transduction pathways mediated by these cystine knot-containing growth factors, implicating that stickiness to CTGF results in sequestration of the ligands from their cognate receptors while weak association facilitates ligand/receptor presentation. Our findings support that CTGF is a critical modulator of extracellular signaling and functions to integrate different pathways into signaling networks resulting in tight regulation and fine-tuning of cellular responses during diverse physiological and pathological processes.

## Applications

Many cystine knot-containing growth factors are key regulators in angiogenesis and tissue repair. Understanding of the binding of CTGF to these growth factors may hold promise as a strategy of rational therapeutic design for the prevention or treatment of pathological processes in cancer, vascular and fibrotic disorders of many organs.

#### Terminology

Cystine knots refer to a common overall topology that was initially discovered in structure of nerve growth factor solved by Tome Blundell using X-ray crystallography in 1991. This tertiary structure favors dimer formation and is unique in extracellular signaling molecules of multicellular organisms since it is not found in the unicellular yeast genome. All cysteine knot growth factors are believed to be homo- or hetero- dimers and initiate signal transduction through dimerization of their cognate receptors.

#### Peer-review

CTGF is known as an important factor in regulating diverse biological functions, including cell adhesion, migration, tissue wound repair, fibrotic diseases, and cancers. In this paper, the author demonstrated that CTGF had different binding strengths to VEGF-A, PDGF-B, BMP-4, and TGF- $\beta$ , which regulated these growth factors and triggered downstream signaling pathways. The topic of this work is of high interest. The authors approach to the study of the interactions between CTGF and a number of its interactors using the LexA-based yeast two-hybrid system and SPR analysis, and tried to explain the results using some functional aspects.

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ORIGINAL ARTICLE

#### **Basic Study**

# DNA microarray unravels rapid changes in transcriptome of MK-801 treated rat brain

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Data sharing statement: The obtained gene expression data deposited at the NCBI, Gene Expression Omnibus (GEO) site (accession number GSE63639) is freely available to the scientific community (http://www.ncbi.nlm.nih.gov/geo/).

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# Abstract

**AIM:** To investigate the impact of MK-801 on gene expression patterns genome wide in rat brain regions.

**METHODS:** Rats were treated with an intraperitoneal injection of MK-801 [0.08 (low-dose) and 0.16 (high-dose) mg/kg] or NaCl (vehicle control). In a first series of experiment, the frontoparietal electrocorticogram was recorded 15 min before and 60 min after injection. In a second series of experiments, the whole brain of each animal was rapidly removed at 40 min post-injection, and different regions were separated: amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum on ice followed by DNA microarray (4 × 44 K whole rat genome chip) analysis.

**RESULTS:** Spectral analysis revealed that a single systemic injection of MK-801 significantly and selectively augmented the power of baseline gamma frequency (30-80 Hz) oscillations in the frontoparietal electroencephalogram. DNA microarray analysis showed the largest number (up- and down- regulations) of gene expressions in the cerebral cortex (378), midbrain (376), hippocampus (375), ventral striatum (353), amygdala (301), and hypothalamus (201) under low-dose (0.08 mg/kg) of MK-801. Under high-dose (0.16 mg/kg), ventral striatum (811) showed the largest number of gene expression changes. Gene expression changes were functionally categorized to reveal expression of genes and function varies with each brain region.

**CONCLUSION:** Acute MK-801 treatment increases synchrony of baseline gamma oscillations, and causes very early changes in gene expressions in six individual rat brain regions, a first report.

**Key words:** Dizocilpine; Dye-swap; Gene expression; Microarray; MK801; N-Methyl-D-aspartate receptors

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Core tip: N-Methyl-D-aspartate receptors (NMDAr) are involved in multiple physiological functions and neuropsychiatric disorders. Dizocilpine (commonly referred to as MK-801) is a well-known non-competitive NMDAr antagonist with psychotomimetic properties. A combination of electrophysiological and molecular analyses reveals not only the increased synchrony of baseline cortical gamma oscillations by MK-801, but also more importantly new insight into differential gene expressions in the cerebral cortex, midbrain, hippocampus, ventral striatum, amygdala, and hypothalamus regions after acute low-dose (0.08 mg/kg) MK-801 treatment; only the ventral striatum showed increased gene expression at a high dose (0.16 mg/kg) of MK-801. We believe that our present study will contribute in the understanding of the pathogenic mechanisms of neuropsychiatric disorders.

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# INTRODUCTION

N-Methyl-D-aspartate receptor (NMDAr) is an ionotropic subtype of glutamate receptors, one of the excitatory amino acids, forming high calcium (Ca<sup>2+</sup>)-permeable cation channels that specifically accept NMDA as a selective agonist<sup>[1]</sup>. The NMDAr is a tetramer, composed of assemblies of NMDAR1 (NR1) and NMDAR2 (NR2) subunits<sup>[2]</sup>. NMDAr integrates chemical and electrical stimuli into a Ca<sup>2+</sup> signal, and plays crucial roles in synaptic plasticity, which may be involved in learning, memory, and motion. Dysfunction of NMDAr has been suggested to be involved in stroke, Parkinson's disease, and schizophrenia<sup>[3-6]</sup>. Decreased Ca<sup>2+</sup> influx in the response to glutamate activation leads to the impairment of NMDAr function, disrupting intra- and extracellular communication. Impaired NMDAr function in the prefrontal cortex causes injury to learning and memory formation, probably causing impulsivity, hyperactivity, and attention deficit, seen in attention-deficit hyperactivity disorder (ADHD)<sup>[7]</sup>. The state of NMDAr inhibition is also similar to the schizophrenia model<sup>[8,9]</sup>.

It has been demonstrated that NMDAr play potential roles in nociceptive (the neural processes of encoding and processing noxious stimuli) transmission, particularly in the spinal cord<sup>[10]</sup>. NMDAr antagonists reduced the successive increase in response to repetitive stimuli, namely windup, in dorsal and ventral horn neurons<sup>[11]</sup>. Because of the psychotomimetic action of NMDAr antagonists, it was suggested that human psychosis and NMDAr blockade are correlated<sup>[12]</sup>. The drugs that non-competitively block NMDAr simulate schizophrenic psychopathology in healthy humans<sup>[13]</sup>. Of importance, using fMRI it has recently been demonstrated that acute ketamine administration in healthy subjects increases global brain functional connectivity<sup>[14]</sup>, creating a state resembling that recorded in patients during the early stages of schizophrenia but not that recorded in patients with chronic (since several years) schizophrenia<sup>[15]</sup>. The acute ketamine effects are quick and reversible. These findings are consistent with preclinical studies demonstrating that in rodent's ketamine or MK-801 transiently increases the power of baseline gamma oscillations in cortical and subcortical structures<sup>[16,17]</sup>. Furthermore, there is proof that NMDAr antagonists induce a broad range of symptoms, behaviors, and cognitive deficits that resemble aspects of endogenous psychoses, particularly schizophrenia and dissociative states<sup>[9]</sup>.

Phencyclidine (PCP), a well-known NMDAr antagonist, is one of the most effective psychotropic drugs. The PCP-elicited psychosis symptoms closely resemble schizophrenia such as positive/negative symptoms and cognitive deficits<sup>[8,18,19]</sup>. It should be noted that the glutamate hypothesis is one of few hypotheses of etiology and/or pathophysiology of



schizophrenia. Javitt hypothesized that hypofunction of NMDAr is involved in the pathogenic mechanism of schizophrenia<sup>[4]</sup>. As part of that evidence, it was reported that there is a (1) decrease in the phosphorylation level of NR1 subunit in postmortem brains of schizophrenia patients<sup>[20]</sup>; and (2) occurrence of abnormal behavior similar to schizophrenia symptom in the NR2A subunit (GRIN2A) lacking mice<sup>[21,22]</sup>. Other than PCP, dizocilpine (commonly referred to as MK-801) is another wellknown non-competitive NMDAr antagonist. These molecules have psychotomimetic properties. Intraperitoneal (ip) injection of MK-801 induces hyperlocomotion, ataxia, abducted hindlimbs, flat body posture, and stereotyped behavior such as head weaving in rat<sup>[23]</sup>. It should be emphasized that these conditions are also included in the ADHD symptoms. It has been proposed that ADHD-like symptoms can be produced by stopping development of the dopaminergic neuron<sup>[24]</sup>. Moreover, it has been demonstrated that NMDAr expression was enhanced in the striatum in ADHD model animals, where activities of the dopaminergic neurons were inhibited<sup>[25]</sup>.

MK-801 has been used in studies of NMDAr in schizophrenia and psychosis<sup>[26-28]</sup>. Systemic administration of MK-801 causes dysfunction of learning and memory<sup>[29]</sup>. Acute ip injection of MK-801-induced transient behavior, and which correlated with schizophreniform psychosis in the rat. MK-801-treated animals progressed to an acute episode involving abnormal behavior reminiscent of symptoms of schizophrenia<sup>[16,17,30]</sup>. Furthermore, significant reproducible behavior was found, such as increased locomotion, stereotyped sniffing and ataxia<sup>[31,32]</sup>. These behavioral changes are dose-dependent, varied with age and sex, and represents a rat EAA hypofunction model of psychosis<sup>[33]</sup>.

The overall goal of the present study was to identify gene expression patterns along rat chromosomes in different brain regions after a single injection of MK-801, which exerts a longer acute effect than ketamine on ongoing brain activities<sup>[16]</sup>. The brain regions (amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum) of MK-801-treated rats were subjected to a genome-wide transcriptome mapping analysis (4 × 44 K). The present study should contribute in the understanding of the pathogenic mechanisms of neuropsychiatric disorders. To note, a previous study in 2004, was the first study on uncovering gene expressions using rat model to investigate the effects of both memantine and MK-801 in adult rat brain<sup>[34]</sup>. In that study, a cDNA-based microarray chip carrying 1090 well-characterized transcripts was used to profile gene expression changes in the posterior cingulate and anterior retrosplenial cortices of the rat brain following 5 to 50 mg/kg memantine and 1 mg/kg MK-801 brain<sup>[34]</sup>, but which is different from our study in that six brain regions were sampled following low-dose (0.08 mg/kg) and high-dose (0.16 mg/kg) single injection of MK-801.

# MATERIALS AND METHODS

#### Ethics

In this study, all animal care procedures were achieved in accordance with European Union Guidelines (Directive 2010/63/EU) and with CREMEAS, the National and Regional Ethics Committee. The second part of the study (for genome-wide analysis experiment) was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at the National Institute of Advanced Industrial Science and Technology (AIST), Japan.

#### Animals, MK-801 injection, and electrophysiology

Four adult (3-6 mo old) male Wistar rats (280-380 g body weight, BW) were used for the electrophysiology experiment performed in the French laboratory. All animal care procedures were achieved in accordance with European Union Guidelines (Directive 2010/63/EU) and with CREMEAS, the National and Regional Ethics Committee. Rats were implanted under deep anesthesia in a stereotaxic frame. For bilateral recordings two stainless steel screws were implanted extradurally over the left and right frontoparietal cortices (from bregma: 1 mm posterior and 2 mm lateral). Two other screws were fixed: in the frontal bone for ground connection and in the bone covering the cerebellum for reference. The screws were connected to a subminiature connector fixed to the skull with dental acrylic. Following the surgery, the rats were housed in separate cages with food and water ad libitum. Recording sessions began after 1 wk of recovery. Every rat had a 15 min habituation period before the recording session. It was gently stimulated to ensure it did not fall asleep. During this habituation period, each rat was injected with vehicle (NaCl, 0.9%, 1 mL/kg) and MK-801 (0.08 mg/kg, intraperitoneal, ip; low-dose) and the recordings were carried out about 60 min after injection<sup>[16]</sup>. The electrocorticogram (ECoG) signals were processed with a bandpass of 0.1-800 Hz and digitized at 10 kHz. Spectral analysis of ongoing spontaneously occurring activity was done with Fast Fourier Transformation (FFT). The total power was the sum of all FFT values computed between 30 and 80 Hz (resolution 2.4 Hz).

# Animals, treatment with MK-801, dissection of brain, and preparation of fine brain tissue sample powders

This part of the experiment was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at the National Institute of AIST, Japan. Nine male 10-wk-old Wistar rats (300-350 g BW) were housed in acrylic cages (3 per cage) at 24 °C and given access to tap water and laboratory chow ad libitum. The rats were divided into two groups, and each group rats received ip injection of 0.08 (low-dose) and 0.16 (high-dose) mg/kg of MK-801, respectively. Three rats were treated with saline as sham (vehicle control group) using the same method (see the experimental strategy in Figure 1). After 40 min post-injection, the whole brain of each animal was rapidly removed and put on ice, and brain regions were separated according to the method of Glowinski and Iversen (1996)<sup>[35]</sup>, with minor modifications<sup>[36,37]</sup>. Each brain region was placed in a sterile 2 mL Eppendorf microtube, quickly immersed in liquid nitrogen before being stored in -80  $^\circ C$  prior to further analysis. The



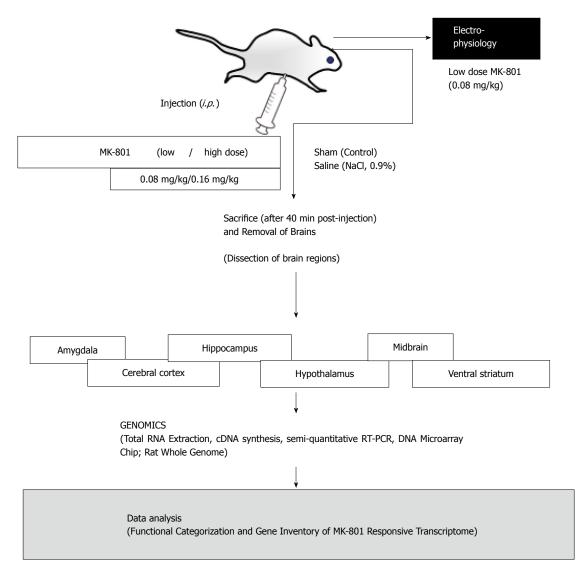


Figure 1 Experimental strategy and design to investigate the MK-801 effects on rat brain. Details are mentioned in the Materials and Methods section.

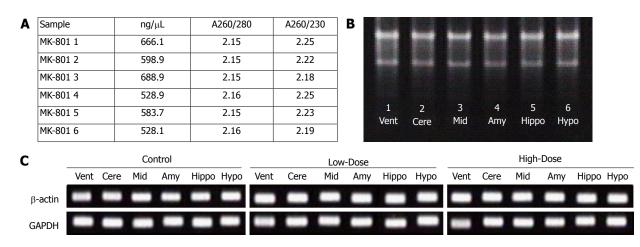
deep frozen brain regions (amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum) were transferred to a pre-chilled (in liquid nitrogen) mortar and pestle and ground to a very fine powder (for details see protocol by Masuo *et al*<sup>(38,39]</sup>). The powdered samples (aliquots of 70 mg) were transferred to sterile 2 mL Eppendorf microtubes (pre-chilled in liquid nitrogen) and stored at -80 °C till used for extraction of total RNA.

# Total RNA extraction, cDNA synthesis, and reverse transcription-polymerase chain reaction

Total RNA was extracted from about 70 mg sample powder using the QIAGEN RNeasy Mini Kit (QIAGEN, Maryland, United States). To verify the quality of this RNA, the yield and purity were determined spectrophotometrically (NanoDrop, Wilmington, DE, United States) and visually confirmed using formaldehyde-agarose gel electrophoresis. To validate the total RNA quality and subsequently synthesized cDNA, reverse transcription-polymerase chain reaction (RT-PCR) was carried out using two commonly used house-keeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and  $\beta$ -actin as positive controls  $^{[40,41]}$ . The 3'-UTR gene-specific primers were designed in-house and are listed in Table 1.

Briefly, total RNA samples were first DNase-treated with RNase-free DNase (Stratagene, La Jolla, CA, United States). First-strand cDNA was then synthesized in a 20 µL reaction mixture with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Agilent Technologies, La Jolla, United States) according to the protocol provided by the manufacturer, using 1  $\mu$ g total RNA isolated from each control and treated samples. The reaction conditions were: 25 °C for 5 min, 42 °C for 5 min, 55 °C for 40 min and 95  $^\circ\!\!\mathbb{C}$  for 5 min. The synthesized cDNA was mixed up to a volume of 50  $\mu$ L with sterile water supplied in the kit. The reaction mixture contained 0.6 µL of the firststrand cDNA, 7 pmols of each primer set and 6.0  $\mu$ L of the Emerald Amp PCR Master Mix ( $2 \times \text{premix}$ ) (TaKaRa Shuzo, Shiga, Japan) in a total volume of 12  $\mu$ L with sterile water supplied in the kit. Thermal-cycling (S1000 Thermal Cycler, Bio-Rad, Tokyo, Japan) parameters

| Table 1         Primer design for reverse transcription-polymerase chain reaction validation experiment |                |                                             |                                   |                                    |                      |  |
|---------------------------------------------------------------------------------------------------------|----------------|---------------------------------------------|-----------------------------------|------------------------------------|----------------------|--|
| Accession<br>(Gene)                                                                                     | Gene<br>symbol | Description                                 | Nucleotide sequence (5'-3'): Left | Nucleotide sequence (5'-3'): Right | Product<br>size (bp) |  |
| X02231 X00972                                                                                           | Gapdh          | Glyceraldehyde-3-phosphate<br>dehydrogenase | TCCCTCAAGATTGTCAGCAA              | AGATCCACAACGGATACATT               | 308                  |  |
| NM031144                                                                                                | $\beta$ -actin | β-actin                                     | CCTGTATGCCTCTGGTCGTA              | CCATCTCTTGCTCGAAGTCT               | 260                  |  |
| NM_031327                                                                                               | Cyr61          | Cysteine rich protein 61                    | GTCCTTGTGGACAACCAGTGTA            | CCTTTAGTCCCTGAACTTGTGG             | 341                  |  |
| NM_001010970                                                                                            | Amy1a          | Amylase, alpha 1A (salivary)                | CTTCTGACAGAGCCCTTGTCTT            | AATGGTCACTTCTTTGGTTGCT             | 254                  |  |



Gene-specific (rat) primers are designed in-house and original to our group.

Figure 2 Total RNA quality and quantity check, and reverse transcription-polymerase chain reaction. Total RNA quality was confirmed spectrophotometrically (A) and by formaldehyde agarose-gel electrophoresis (B). Stable expression levels of GAPDH and  $\beta$ -actin genes are shown (C). For further details, see the Materials and Methods section. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

were as follows: after an initial denaturation at 97  $^\circ\!\!\mathbb{C}$  for 5 m, samples were subjected to a cycling regime of 20 to 40 cycles at 95  $^\circ\!\!\mathbb{C}$  for 45 s, 55  $^\circ\!\!\mathbb{C}$  for 45 s, and 72  $^\circ\!\!\mathbb{C}$ for 1 min. At the end of the final cycle, an additional extension step was carried out for 10 min at 72 °C. After completion of the PCR the total reaction mixture was spin down and mixed (3 µL) was loaded into wells of a 1.8% agarose [Agarose (fine powder) Cat no. 02468-95, Nacalai Tesque, Kyoto, Japan] gel. Electrophoresis was then performed for about 22 min at 100 Volts in 1  $\times$ TAE buffer using a Mupid-ex electrophoresis system (ADVANCE, Tokyo, Japan). The gels were stained (8 µL of 10 mg/mL ethidium bromide (EtBr) in 200 mL 1 imesTAE buffer) for about 7 min and the stained bands were visualized and quantified using an UV-transilluminator (ATTO, Tokyo, Japan). Each gene expression analysis was performed at least twice as independent PCR reactions and electrophoresis on gel, and one of the images was presented as a representative data for each gene in the respective figures (Figures 2 and 3) for no change or upand down-regulated expressions.

#### Rat whole genome DNA microarray analysis

A rat 4  $\times$  44K whole genome oligo DNA microarray chip (G4131F, Agilent Technologies, Palo Alto, CA, United States) was used for global gene expression analysis. Total RNA (800 ng) was labeled with either Cy3 or Cy5 dye using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescently labeled targets of control as well as treated samples were hybridized to the same microarray slide with 60-mer probes. A flip labeling (dye-swap or reverse labeling with Cy3 and Cy5 dyes) procedure was followed to nullify the dye bias associated with unequal incorporation of the two Cy dyes into cRNA<sup>[41-45]</sup>. Briefly, the same total RNA (800 ng) samples were labeled twice with Cy3 or Cy5: A Cy5-labeled treatment  $(T^{Cy5})$  and a Cy3-labeled control  $(C^{Cy3})$  were hybridized on a slide and then a Cy3-labeled treatment (T<sup>Cy3</sup>) and a Cy5-labeled control (C<sup>Cy5</sup>) were reversely hybridized on another slide. Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray scanner G2565BA. For the detection of significantly differentially expressed genes between control and treated samples each slide image was processed by Agilent Feature Extraction software (version 9.5.3.1). This program measures Cy3 and Cy5 signal intensities of whole probes. Dye-bias tends to be signal intensity dependent therefore, the software selected probes using a set by rank consistency filter for dye-normalization. Said normalization was performed by LOWESS (locally weighted linear regression) which calculates the log ratio of dye-normalized Cy3- and Cy5-signals, as well as the final error of log ratio. The significance (P) value based on the propagate error and universal error models (Agilent). In this analysis, the

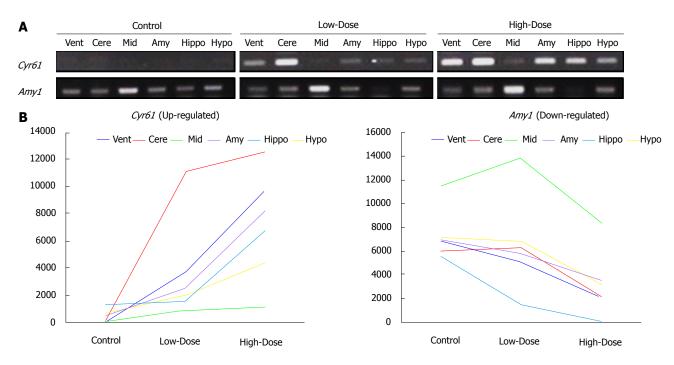


Figure 3 Reverse transcription-polymerase chain reaction analyses of Cyr61 and Amy1 genes that are differentially expressed in the six (6) brain regions by MK-801 treatment. For the stable expression levels of GAPDH and  $\beta$ -actin genes, please see Figure 2C. For further details, see the Methods and Materials section. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

threshold of significant differentially expressed genes was < 0.01 (for the confidence that the feature was not differentially expressed). In addition, erroneous data generated due to artifacts were eliminated before data analysis using the software. The differentially expressed gene lists (up- and down-regulated genes) were generated and annotated using the GeneSpring version GX 10 (Agilent). The outputs of microarray analysis used in this study are available under the series number GSE63639, at the NCBI Gene Expression Omnibus (GEO) public functional genomics data repository (http://www. ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63639).

To validate the microarray data, RT-PCR analysis was performed on two selected up- and down-regulated genes using 3'-UTR specific gene primers (Table 1).

# RESULTS

# MK-801 increases the power of spontaneously-occurring cortical gamma oscillations

To examine the neurophysiological impact of MK-801, we conducted bilateral high-resolution ECoG recordings in free-moving awake rats during the resting state. Spectral analysis revealed that a single subcutaneous administration of MK-801 at a low (0.08 mg/kg) or higher (0.16 mg/kg) dose significantly and dose-dependently augmented the power of baseline gamma oscillations (Figure 4). The MK-801 effects lasted more than one hour and were transient<sup>[16]</sup>. It is worth mentioning that a single injection of a psychotomimetic dose of a non-competitive NMDAr antagonist (MK-801 or ketamine) transiently induces persistent aberrant gamma oscillations in multiple cortical and subcortical structures, including

the prefrontal cortex, accumbens, amygdala, basalis, hippocampus, striatum and thalamus<sup>[17]</sup>.

# Quality of total RNA and expression level of GAPDH and $\beta$ -actin genes in the brain regions

To investigate global changes in gene expression in the brain regions, the quantity and quality of the total RNA is a critical factor in further downstream analyses, and was confirmed as described in Materials and Methods section. The quantity and quality of total RNA was shown in Figure 2. This RNA was then used for synthesizing cDNA. Prior to DNA microarray analysis, we examined the expression of two commonly used house-keeping genes, namely GAPDH and  $\beta$ -actin in all the six brain regions. We used these two genes as positive controls rather than simply loading or using internal controls<sup>[40]</sup>. This simple test of gene expression showed that the mRNAs for GAPDH and  $\beta$ -actin were expressed almost uniformly across regions and conditions (Figure 2C). Following this preliminary but extremely important analysis of sample quantity and quality, we proceeded to conduct a DNA microarray analysis.

## Genome-wide transcriptome analysis reveals numerous and early changes in gene expression

The differentially expressed genes in each brain region, amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain, and ventral striatum were analyzed based on their up- or down-regulations. Genes were up-regulated if they displayed a fold-change ratio greater than or equal to 1.5, whereas genes were down-regulated if they showed a fold-change ratio less than or equal to 0.75 in both the chips carrying different Cy3 and Cy5



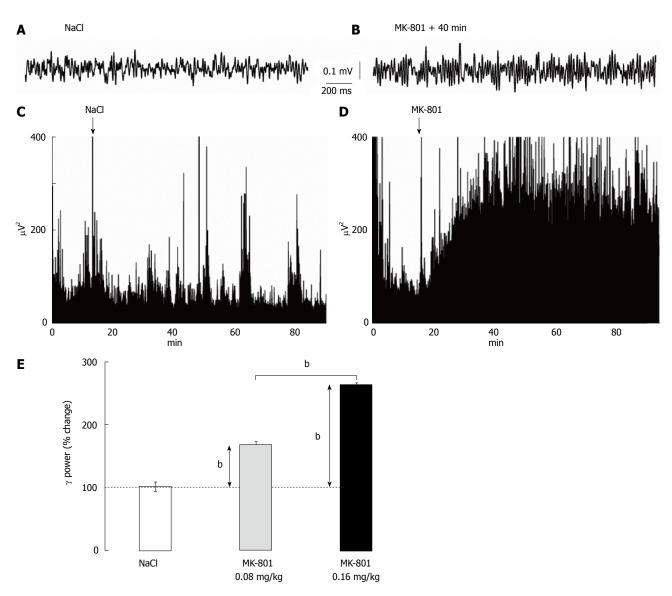


Figure 4 MK-801 increases the power of spontaneously-occurring gamma (30-80 Hz) oscillations. A-B: Typical 2-s bouts of frontoparietal electrocorticogram (bandpass: 20-80 Hz) recorded in a free-moving awake rat under control (vehicle: NaCl, 0.9%, 1 mL/kg, A) and MK-801 (about 40 min after injection, 0.08 mg/kg, B) conditions; C-D: Each chart shows the total power (resolution: 2.4 Hz; average of 8 x 2.5-s epochs) of oscillations during a 90-min recording session, during which the rat received an injection [NaCl (C) or MK-801 (D)] at 15 min (arrow). Note that MK-801 induces a dramatic increase in power, which is still present at the end of the recording session; E: The histogram shows that MK-801 significantly (*t*-test,  ${}^{b}P < 0.001$ ) and in a dose-dependent manner increases the power during the 30 to 40 min post-injection period. All values are means  $\pm$  SEM (5 rats > 100 values/rat, spectral analysis 2.5 s epochs).

labels; *i.e.*, dye-swap experiment<sup>[41-45]</sup>. Each brain region showed different patterns and numbers of changed gene expressions, reflecting the different responses of the brain regions to MK-801 treatment. Moreover, differential gene expression was also observed with the dose of applied MK-801, suggesting a clear dose-dependent effect of the antagonist. In Figure 5A and B, the numbers of changed genes in each region under low- and highdoses of MK-801, respectively, are shown. Results show that under low-dose of MK-801, the largest number (upand down-regulations) of gene expressions was affected in the cerebral cortex (378) followed by midbrain (376), hippocampus (375), ventral striatum (353), amygdala (301) and hypothalamus (201) (Figure 5A). Results showed that under high-dose of MK-801, the largest number (up- and down-regulations) of gene expressions

were affected in the ventral striatum (811) followed by midbrain (689), hippocampus (443), cerebral cortex (341), hypothalamus (325) and amygdala (269) (Figure 5B). The common genes between regions were numbered and indicated over the lined arrows (Figure 5A and B).

As the only other study available to date was the 2004 report by Marvanová *et al*<sup>[34]</sup> who showed gene expression changes post-MK-801 injection in adult rat brain, we compared those genes (34) with our gene inventory for all the six brain regions. Two genes were found to be in common, namely *C-fos* or *Fos* (NM\_022197, FBJ osteosarcoma oncogene; an immediate early gene encoding a nuclear protein involved in signal transduction: http://www.ncbi.nlm.nih.gov/nuccore/NM\_022197), and *RL/IF-1* or *Nfkbia* (NM\_001105720, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,

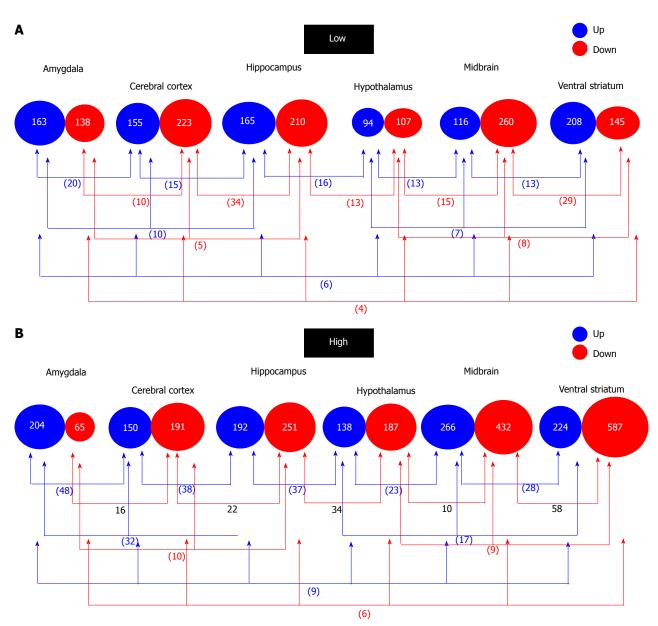


Figure 5 Number of changed genes in each brain region and the number of common genes among each region. A: Low-dose of MK-801; B: High-dose of MK-801.

alpha; inhibitor of NF-kappa-B/binds NF kappa B and retains it in the cytoplasm: http://www.ncbi.nlm.nih.gov/ nuccore/NM\_001105720). The Fos gene was found to be up-regulated in the cerebral cortex (1.7 fold), amygdala (1.87 fold), and hippocampus (1.55 fold) whereas Nfkbia gene was up-regulated in midbrain (1.79 fold), and only under high dose (0.16 mg/kg) MK-801. Although the Nfkbia gene expression may be a generalized response to the MK-801 injection as also suggested by Marvanová et  $al^{(34)}$ , the up-expression of the Fos gene may have some meaning in the brain, as it is also a marker of neuronal activation. A recently published study showed significant increase in the c-Fos protein in amygdala, hippocampus and thalamus, after local injection of MK-801 leading those authors to suggest that local blockade of NMDA receptors in the medial prefrontal cortex could lead to the activation of diverse downstream structure<sup>[46]</sup>. These

low commonalities of gene expressions between the two studies are ascribed to: (1) use of posterior cingulate and anterior retrosplenial cortices of the rat brain over six distinct regions used in our study; (2) 1090 cDNA probes *vs* 41090 gene probes used here; and (3) 1 mg/kg MK-801 dose administration compared to low-dose (0.08 mg/kg) and high-dose (0.16 mg/kg) MK-801 used in our study. Nevertheless, the 2004 study contributed to the identification of 34 (and 28 following memantine injection) well characterized transcripts to delineate molecular pharmacologic effects of NMDA/glutamate receptor antagonists in the rat brain<sup>[34]</sup>.

## Confirmatory RT-PCR on two candidate genes

To confirm alterations in gene expression observed by DNA microarray, we selected two genes expressed differentially among brain regions and doses, namely

the up-regulated gene (*Cyr61*) and the down-regulated gene (*Amy1*) (Table 1 lists the gene-specific primers used for these two genes). The results of the RT-PCR analysis of these two genes, *Cyr61* and *Amy1* are presented in Figure 3 as both EtBr-stained PCR gene products and as a graph for clarity. Results from RT-PCR revealed that the intensity of bands were higher than control in all regions under both low- and high- dose, especially under high-dose for the *Cyr61* gene. For *Amy1*, the intensity of bands was lower than control in all regions under both sere results imply that the microarray data can be re-confirmed using appropriate primer design followed by RT-PCR. The possible function of these two genes in context of MK-801 action is discussed below under up- and down-regulated genes.

# Functional categorization of differential gene expressions

As a next step, all the genes in each region (low- and high- doses) were functionally characterized based on the Gene Ontology (GO), and are presented in Figures 6 to 11. These genes were divided into 38 functional categories in total. We selected some remarkably differentially expressed genes showing up-/down-regulations in all regions examined in this study, along with some genes with very high-/low-fold values and those with having characteristic functional annotations related to this study. The functional categories were annotated by NCBI database or Rat Genome Database (RGD, http://rgd.mcw.edu/). These genes are discussed below in up-regulated and down-regulated categories, respectively.

## **Up-regulated genes**

Cyr61: Cysteine-rich, angiogenic inducer 61; an extracellular heparin binding protein involved in supporting smooth muscle cell (SMC) adhesion, promoting cell migration and enhancing growth factor-stimulated mitogenesis; NM 031327. The expression of this gene was found to be up-regulated in all regions [amygdala (30.92 fold), cerebral cortex (22.85 fold), ventral striatum (20.89 fold), hippocampus (13.41 fold), hypothalamus (4.61 fold) and midbrain (2.04 fold)] with high-dose of MK-801. Under low-dose, the expression was up-regulated in five regions [cerebral cortex (16.84 fold), ventral striatum (7.98 fold), amygdala (4.78 fold), hippocampus (2.88 fold) and hypothalamus (1.85 fold)]. Cyr61 is prominent in extracellular matrix binding (molecular function, MF), cell adhesion (biological process, BP) and extracellular region (cellular component, CC) categories. This gene induced angiogenesis and vascular SMC chemotaxis; increased protein levels were detected after balloon angioplasty<sup>[47]</sup>. Ito et  $a^{48}$  confirm that subcutaneously (s.c.) injection of PCP induced remarkable up-regulation of this gene expression after 60 min in the neocortex of the rats at post-natal day 56. MK-801 also caused a prominent up-regulation of neocortical expression of this gene in adult rats. It has been suggested that this gene or protein could be implicated in a molecular cascade associated with the age-dependent onset of schizophrenia. Our data indicate that up-regulation of *Cyr61* expression by the injection of NMDA receptor antagonist is induced rapidly in the adult rat brain. This gene may act as a chemotactic factor that influences neurite outgrowth in response to a stimulating acetylcholine signal<sup>[49]</sup>. Thus, the up-regulated *Cyr61* expression might be related to neurodegenerative diseases.

Verge: The synonym of Apold1, apolipoprotein L domain containing 1; may regulate endothelial cell differentiation, activation and signaling; NM 001003403. The expression of this gene was found to be up-regulated in all regions [cerebral cortex (10.47 fold), hippocampus (6.63 fold), amygdala (6.31 fold), ventral striatum (5.11 fold), hypothalamus (2.90 fold) and midbrain (2.35 fold)] with high-dose of MK-801. Under low-dose, the expression was up-regulated in four regions [cerebral cortex (6.59 fold), ventral striatum (2.45 fold), amygdala (2.34 fold) and hippocampus (2.11 fold)]. This gene mainly belongs to lipid binding (MF), angiogenesis (BP) and extracellular region (CC) categories. The Verge mRNA and protein are induced selectively in the endothelium of adult vasculature by chemical seizures. Further, this gene may function as a dynamic regulator of endothelial cell signaling and vascular function<sup>[50]</sup>. Thus, MK-801 treatment may have an effect on the signaling related to angiogenesis.

Ccl2: C-C motif (chemokine) ligand 2; a chemokine involved in leukocyte taxis and inflammation; associated with many diseases; NM\_031530. The expression of this gene was found to be up-regulated in the cerebral cortex (3.80/7.06 fold), with both low- and high-dose of MK-801. Under high-dose, the expression was up-regulated in four regions [amygdala (4.28 fold), ventral striatum (4.10 fold), hippocampus (3.94 fold) and midbrain (2.02 fold)]. Ccl2 mainly functions in CCR2 chemokine receptor binding, G-protein-coupled receptor binding, chemokine activity, immune response, inflammatory response (BP), cell soma, cytoplasm, extracellular region and extracellular space (CC) and brain injuries, brain ischemia, and bipolar disorder (pathway) categories. Drexhage et al<sup>[51]</sup> reported that increased serum Ccl2 levels in the schizophrenia patients are significantly higher than in healthy adult human. Independent of the use of antipsychotic medication, both regarding the class of drug and the individual drug Ccl2 is associated with divergent diseases, including schizophrenia. In the brain, the change of Ccl2 expression in the cerebral cortex during early stage of schizophrenia was seen. Application of Ccl2 on dopaminergic neurons increases their excitability, dopamine release and related locomotor activity<sup>[52]</sup>. Therefore, characteristic function of this gene may be related to increased locomotor activity which is included in the positive symptoms in schizophrenia.

KIF4: Kruppel-like factor 4 (gut); a transcription factor



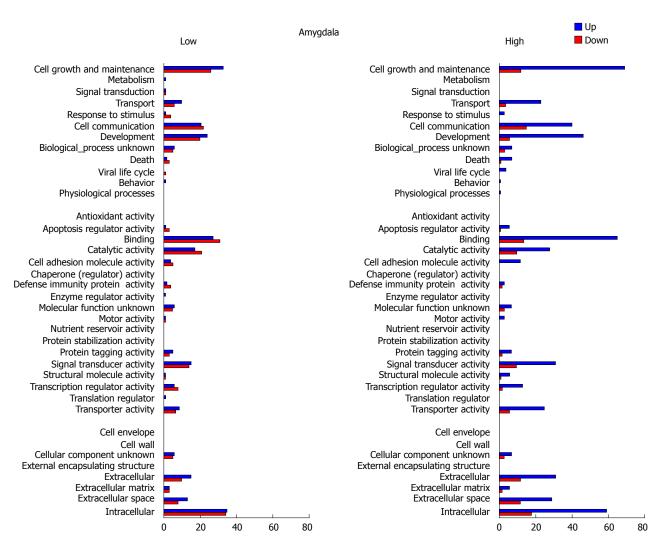


Figure 6 Functional categorization of the differentially expressed amygdala genes based on Gene Ontology. Changed genes divided into 38 function categories in the amygdala.

that works with Sp1 to activate the Laminin gamma1 chain gene; NM 053713. The expression of this gene was found to be up-regulated in the cerebral cortex (3.09/4.22 fold), with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under lowdose in the ventral striatum (1.93 fold), under high-dose in four regions [amygdala (3.11 fold), ventral striatum (3.03 fold), hippocampus (2.82 fold) and hypothalamus (1.87 fold)]. This gene belongs to RNA polymerase II transcription factor activity (MF), cell differentiation (BP) and chromatin (CC) categories. Zhu et al<sup>[53]</sup> suggested that this gene can be up-regulated by activation of NMDA receptors and such up-regulation is dependent on calcium. MK-801 completely abolished NMDA-induced Klf4 expression in their experiment. From these results, it is implied that NMDA-induced KIf4 mRNA expression is dependent on Ca<sup>2+</sup> influx, and is completely blocked by MK-801. Thus, this gene can be suggested to be related to NMDA receptor function in the episode of schizophrenia. Coming back to the study of Zhu et al<sup>[53]</sup>, it is difficult to compare those data obtained from cultured neurons and brain slices, with our present study, and also whether the MK-801 concentration used (20 µmol/L) is equivalent to the amount of MK-801 available in brain tissue following a low- of high-dose of MK-801 administered ip. However, in the study of Zhu *et al*<sup>[53]</sup> it seems that the concentration of MK-801 is appropriate for measuring glutamate excitotoxicity with excessive Ca2+ influx. The up-regulation of the Klf4 found in the present study might reflect a hyperglutamatergic state that would be induced following GABAergic disinhibition (at least of pyramidal neurons) subsequent to NMDAr blockade on GABAergic interneurons<sup>[54,55]</sup>. On the basis of an *in vitro* investigation and biophysical stimulation of a hippocampal disynaptic recurrent inhibitory circuit, it is generally thought that the blockade or hypofunction of NMDAr by ketamine or MK-801 or PCP would initially attenuate the excitation of PV+GABAergic interneurons, which are strongly excited by NMDAr activation<sup>[56]</sup> and which are more sensitive to NMDAr antagonists than glutamatergic neurons<sup>[57]</sup>. This difference might be due to a difference in NMDAr subunit assembly between local GABAergic interneurons and projection glutamatergic neurons.

**Adamts1:** ADAM metallopeptidase with thrombospondin type 1 motif, 1; disintegrin and metalloprotease that



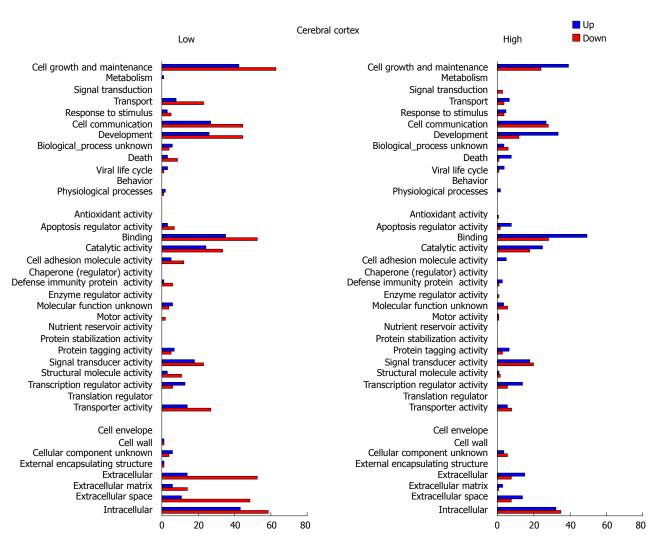


Figure 7 Functional categorization of the differentially expressed cerebral cortex genes based on Gene Ontology. Changed genes divided into 38 function categories in the cerebral cortex.

may be necessary for normal kidney morphology and function; NM\_024400. The expression of this gene was found to be up-regulated in three regions [cerebral cortex (5.26/3.53 fold), amygdala (3.92/1.81 fold) and ventral striatum (3.33/1.58 fold)] with both low- and high-dose of MK-801. Under high-dose, the expression was upregulated in two regions [hippocampus (2.21 fold) and hypothalamus (1.55 fold)]. The functional categories are heparin binding, metal ion binding, metalloendopeptidase activity and peptidase activity (MF), proteolysis (BP) and extracellular matrix and extracellular region (CC). It is reported that the expression of Adamts1 gene increases during a physical or toxic injury<sup>[58,59]</sup> and in neurological diseases, such as Alzheimer's disease and Parkinson disease<sup>[60]</sup>. It seems that the changed gene expressions associated with models of neurodegenerative disorders may be a sensitive molecular response to the pathological circumstances of the brain areas<sup>[61]</sup>. In that study, Adamts1 expression was up-regulated dosedependently in three regions. These results suggest that Adamts1 gene expression might be modulated in response to cranial nerve diseases.

Zfp36: Zinc finger protein 36; acts as a transcriptional activator; NM\_133290. The expression of this gene was found to be up-regulated in the cerebral cortex (2.35/3.50 fold), with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under highdose in the amygdala (1.52 fold), under low-dose in the ventral striatum (2.09 fold). The functional categories are AU-rich element binding (MF), mRNA catabolic process (BP) and cytoplasm, cytosol and nucleus (CC). Itokawa et al<sup>[21]</sup> determined that a microsatellite repeat in the promoter region of the GRIN2A gene suppresses transcriptional activity and correlates with the symptom severity in chronic schizophrenia patients. Furthermore, mice lacking the GRIN2A are known to show abnormal behavior similar to symptoms in schizophrenia. A 2001 study on schizophrenia drug-gene interactions revealed some bridge genes that included GRIN2A, GRIN3B, GRIN2C, and GRIN2B<sup>[62]</sup>. Therefore, *Zfp36* may act as a transcriptional activator in the schizophrenia patients. Of note, in our study, we also identified a Grin3b gene that was slightly down-regulated in the hippocampus and only under low-dose MK-801 treatment.

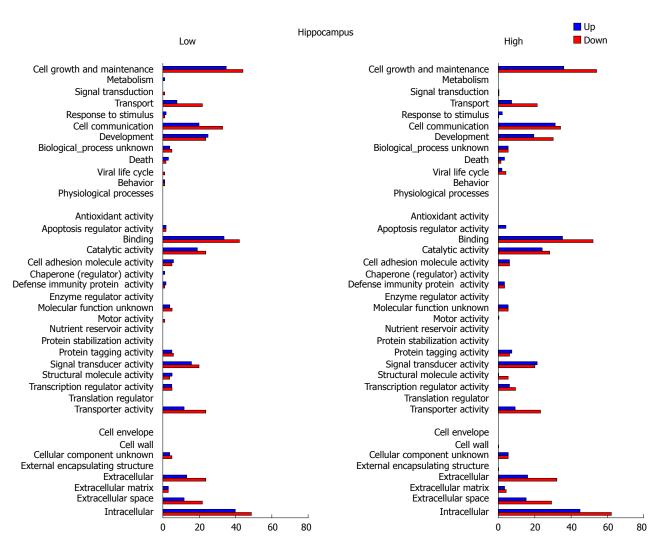


Figure 8 Functional categorization of the differentially expressed hippocampus genes based on Gene Ontology. Changed genes divided into 38 function categories in the hippocampus.

**LOC685106:** Similar to ribosomal protein L6; XM\_001062312. The expression of this gene was found to be up-regulated in all regions [hypothalamus (13.25 fold), hippocampus (12.90 fold), amygdala (10.82 fold), cerebral cortex (9.40 fold), ventral striatum (8.54 fold) and midbrain (7.67 fold)] with low-dose of MK-801. Under high-dose, the expression was not changed. It functions as a structural constituent of ribosome (FC), translation (BP) and ribosome (CC), and is therefore related to protein synthesis. Thus, MK-801 treatment might cause enhanced protein synthesis due to increased gene expression.

## Down-regulated genes

**Amy1:** The synonym of Amy1a, amylase, alpha 1A (salivary); key enzyme in the digestion of starches and glycogen; NM\_00101970. The expression of this gene was found to be down-regulated in all regions [ventral striatum (0.12 fold), cerebral cortex (0.28 fold), midbrain (0.48 fold), amygdala (0.53 fold), hippocampus (0.63 fold) and hypothalamus (0.65 fold)] with high-dose of MK-801. Under low-dose, the expression was down-regulated in the cerebral cortex (0.56 fold). This gene shows alpha-

amylase activity (MF), carbohydrate metabolic process (BP), extracellular space (CC) and starch and sucrose metabolic pathway (pathway) functions. The *Amy1* gene function in the brain remains unknown, but it may be involved in glycogen degradation under normal conditions, and therefore affected by treatment with MK-801.

**Avp:** Arginine vasopressin; a neuropeptide hormone involved in the regulation of natriuresis, vasoconstriction, cell growth and proliferation, and various behaviors; associated with hypertension, diabetes, and epilepsy; NM\_016992. The expression of this gene was found to be down-regulated in the amygdala (0.13/0.19 fold) with both low- and high-dose of MK-801. In the ventral striatum, the expression was up-regulated under high-dose (1.80 fold). *Avp* shows V1A vasopressin receptor binding, V1B vasopressin receptor binding and V2 vasopressin receptor binding (MF), G-protein coupled receptor protein signaling pathway (BP), dendrite, extracellular region, extracellular space and secretory granule (CC), cerebrovascular accident, dehydration, dementia (disease) and abnormal anxiety-related response, abnormal coping response,

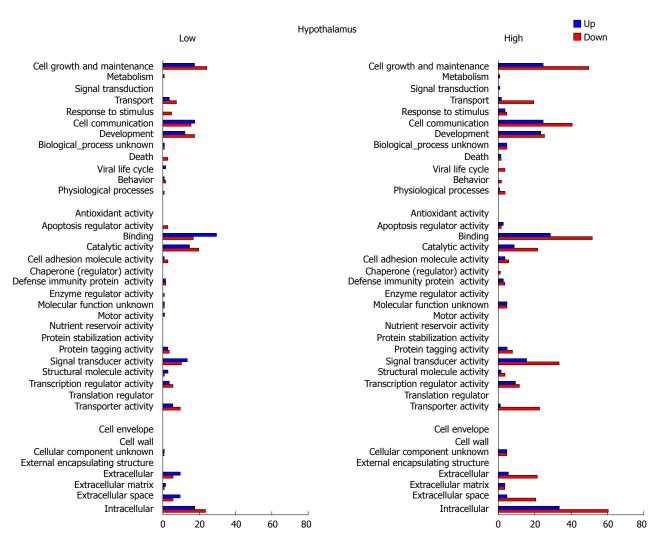


Figure 9 Functional categorization of the differentially expressed hypothalamus genes based on Gene Ontology. Changed genes divided into 38 function categories in the hypothalamus.

abnormal depression-related behavior, abnormal emotion/affect behavior, hyperactivity (phenotype) and vasopressin signaling pathway (pathway) functions. The Avp was shown to be involved in the regulation of brain water content and cerebral edema<sup>[63]</sup>. The Avp protein is synthesized in and secreted by the suprachiasmatic nucleus (SCN) in a circadian pattern and is expressed in the inner medulla<sup>[64]</sup>. The Avp gene expression in the SCN is mediated by MAPK signaling pathway<sup>[64]</sup>. Further, Avp activated by the protein kinase C activator, phorbol 12-myristate 13-acetate, and sodium channel blocker of Avp, tetrodotoxin (TTX), greatly decreases heteronuclear RNA levels and suppresses rhythmicity. Matsuoka et al<sup>65</sup> reported that Avp expression was down-regulated in the amygdala of MK-801 treated rats. Their results match our present data obtained from DNA microarray analysis. Furthermore, it was also proposed that Avp is related to water intoxication which is a symptom in schizophrenia patients<sup>[66]</sup>. It has been suggested that Avp receptors, V1a and V1b, may be implicated in the psychiatric disorders associated with dysfunction of social behavior such as schizophrenia and autism<sup>[67]</sup>. The result of this study was consistent with the previous reports. We believe that the *Avp* expression is rapidly affected in the amygdala by MK-801, and therefore might be an important factor in neurodegenerative disorders such as schizophrenia and autism.

Sostdc1: Sclerostin domain containing 1; may be involved in the onset of endometrial receptivity for implantation/ sensitization; NM\_153737. The expression of this gene was found to be down-regulated in the hippocampus (0.20/0.19 fold) with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under both low- and high-dose in the amygdala (1.50/3.18 fold), and down-regulated under high-dose in the hypothalamus (0.40 fold). The major functional categories for Sostdc1 are protein binding (MF), Wnt receptor signaling pathway and negative regulation of bone morphogenetic protein (BMP) signaling pathway (BP) and extracellular region and extracellular space (CC). Sostdc1 belongs to a family of BMP antagonists and encodes a secretory protein called uterine sensitization-associated gene-1 or ectodin<sup>[68]</sup>. Interestingly, a recent study on altered brain gene

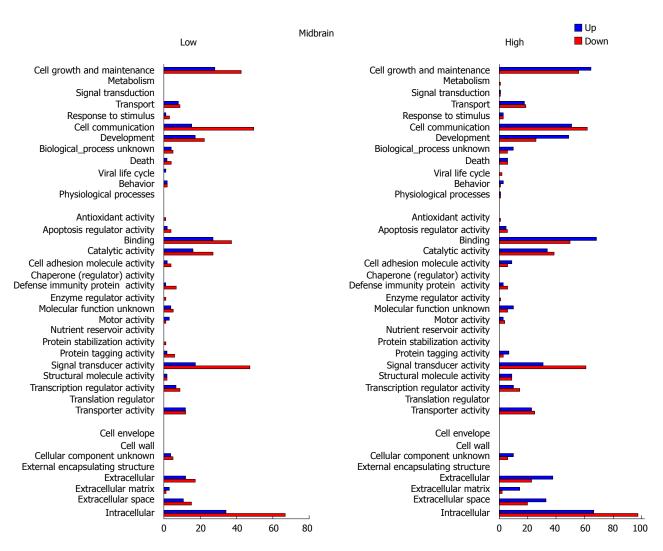


Figure 10 Functional categorization of the differentially expressed midbrain genes based on Gene Ontology. Changed genes divided into 38 function categories in the midbrain

expression profiles associated with the pathogenesis of phenylketonuria (PKU) in a mouse model revealed its upregulation in the PKU mouse than in the wild-type<sup>[69]</sup>. It was proposed that the further study of this gene may provide new insights into the mechanisms involved in neurological damage in the PKU brain. The function of this gene in respect to MK-801 treatment remains unknown, but will also benefit from further studies.

**Kcnj13:** Potassium inwardly-rectifying channel, subfamily J, member 13; inwardly rectifying K+ for the Na+,K(+)-ATPase; NM\_053608. The expression of this gene was found to be down-regulated in the hippocampus (0.08/0.15 fold) with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under high-dose in two regions [midbrain (2.21 fold) and amygdala (1.86 fold)], and down-regulated under low-dose in three regions [cerebral cortex (0.32 fold), midbrain (0.55 fold) and vent (0.74 fold)], under high-dose in the hypothalamus (0.25 fold). It mainly shows inward rectifier potassium channel activity, potassium ion binding and voltage-gated ion channel activity (MF), ion transport,

potassium ion transport (BP) and integral to membrane and membrane (CC) functions. The *Kcnj13* was found to be expressed in thyroid, intestine and choroid plexus<sup>[70]</sup>. MK-801 is an open channel blocker, which inhibits only the ion channel site which is opened by ligand binding. Thus this gene with function of voltage-gated ion channel activity, also responded to MK-801 in this study.

**Ttr:** Transthyretin; binds thyroxine (T4) and 3,5,3' triiodothyronine (T3); plays a role in thyroid hormone transport in serum; NM\_012681. The expression of this gene was found to be down-regulated in the hippocampus (0.18/0.24 fold) with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under high-dose in two regions [amygdala (2.79 fold) and ventral striatum (2.31 fold)], and down-regulated under high-dose in the hypothalamus (0.28 fold), under low-dose in the midbrain (0.46 fold). The functional categories are hormone activity, hormone binding and thyroid hormone transmembrane transporter activity (MF), thyroid hormone metabolic process and transport (BP), extracellular region and extracellular space (CC)

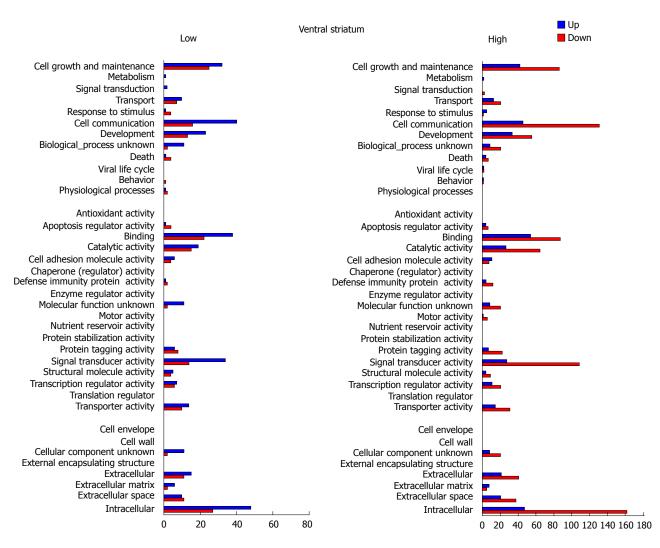


Figure 11 Functional categorization of the differentially expressed ventral striatum genes based on Gene Ontology. Changed genes divided into 38 function categories in the ventral striatum.

and Alzheimer's disease, amyloid neuropathies and brain ischemia (disease). It has been reported that Ttr protein expression in cerebrospinal fluid (CSF) of schizophrenia patients was down-regulated in contrast to that in plasma. Ttr tetramer is a retinoid transporter, and the dysfunction of retinoid may be involved in pathology of schizophrenia<sup>[71]</sup>. It was confirmed that *Ttr* expression, which is specifically expressed in the choroid plexus in that study, was completely decreased in the brain of adult rat treated with maternal separation during the neonatal period used as a model of depression<sup>[72]</sup>. Tsai *et al*<sup>[73]</sup> also suggested that the change in the expression level of this gene is correlated with the pathogenesis of the Alzheimer's disease. Thus, it is suggested that this gene may related to the psychiatric disorders. In this study, Ttr gene expression was also down-regulated in hippocampus, hypothalamus (high-dose) and midbrain (low-dose). Taken together with previous reports, Ttr may be involved in the etiology of schizophrenia in the intracerebral substances. To note, in the present study we find the Ttr gene down-regulation after acute MK-801 treatment, which is a model of first stages of schizophrenia, in contrast to its up-regulation after chronic MK-801 treatment, which is a model from chronic schizophrenia<sup>[65]</sup>. Interestingly, a recent study on altered brain gene expression profiles associated with the pathogenesis of PKU in a mouse model revealed up-regulation of *Ttr* in the PKU mouse than in the wild-type<sup>[69]</sup>. The authors suggested that this might be due to dopamine deficiency.

**Pmch:** Pro-melanin-concentrating hormone; cyclic neuropeptide; induces hippocampal synaptic transmission; NM\_012625. The expression of this gene was found to be down-regulated in the hypothalamus (0.12/0.28 fold) with both low- and high-dose of MK-801. Major functional categories are melanin-concentrating hormone activity and type 1 melanin-concentrating hormone receptor binding (MF), regulation of neuronal synaptic plasticity (BP) and extracellular region and extracellular space, nucleus (CC). The *Pmch* regulates appetite, and it activates Y1 receptor which in turn mediates Npy<sup>[74]</sup>. *Pmch* may play a role in retrograde facilitation on the inhibitory avoidance<sup>[75]</sup>. It plays a role in regulation of feeding behavior<sup>[76]</sup>. The *Pmch* gene is expressed in the lateral hypothalamus and

zona incerta of the central nervous system<sup>[76]</sup> and is found in the brain lateral hypothalamic area<sup>[74]</sup>. In schizophrenia patients treated with antipsychotic agents, the symptom weight gain is observed. It is suggested that increased *Pmch* is associated with weight gain<sup>[77-79]</sup>. However, in this study, *Pmch* expression was down-regulated in the hypothalamus. The *Pmch* expression may be downregulated in schizophrenia patients without treatment, whereas it may be up-regulated by treatment with antipsychotic agents, where it is involved in weight gain.

SIc6a3: Solute carrier family 6 (neurotransmitter transporter, dopamine), member3; acts as a sodium-dependent dopamine transporter; may play a role in regulation of dopamine metabolism and signaling; NM\_012694. The expression of this gene was found to be down-regulated in the hypothalamus (0.01/0.09 fold) with both low- and high-dose of MK-801. It belongs to multiple functional categories of dopamine binding, dopamine transmembrane transporter activity and dopamine: sodium symporter activity (MF), dopamine biosynthetic and catabolic processes (BP), integral to plasma membrane, plasma membrane and synaptosome (CC), Parkinsonian disorders (disease), abnormal movement/ locomotion, abnormal spatial learning, hyperactivity (phenotype), ADHD (behavior) and dopamine signaling pathway (pathway). Slc6a3 is sodium-dependent dopamine transporter<sup>[80-82]</sup>. Slc6a3 is expressed in substantia nigra and ventral tegmental area<sup>[80]</sup>. Sáiz et al<sup>[83]</sup> reported that their study provides evidence that possible interaction between dopamine-3 receptor (DRD3) and Slc6a3 genes are associated with schizophrenia. It was also reported previously that the variations at Slc6a3 are important determinants of schizophrenia susceptibility, with additional risk due to some variant genes. These genes encode proteins that regulate synaptic dopamine concentrations<sup>[84]</sup>. It has been proposed that the hyperenhancement of the dopamine function is involved in the positive symptom of schizophrenia. Adriani et al<sup>[85]</sup> found that the dopamine transporter overexpressing rats showed increased impulsivity in the absence of general locomotor effects. In this study, the expression of Slc6a3, one of the genes encode proteins that regulate synaptic dopamine concentrations, was down-regulated in the hypothalamus by the injection of MK-801. Therefore, the dopamine function might have been hyper-enhanced. Thereby, dopamine would modulate the excitability of neurons, for instance via its action on GABAergic neurons<sup>[86,87]</sup>. This dopaminergic action would interfere with MK-801 action since GABAergic neurons are thought to be the first target of NMDAr antagonists<sup>[88]</sup>.

**Lect1:** Leukocyte cell derived chemotaxin 1; cartilagespecific angiostatic factor; stimulates growth of chondrocytes and inhibits tube formation of endothelial cells; NM\_030854. The expression of this gene was found to be highly down-regulated in the midbrain (0.09 fold) with low-dose of MK-801. Under high-dose, the expression was up-regulated in the hypothalamus (2.26 fold). The functional categories are cartilage development, cell differentiation, multicellular organismal development, negative regulation of angiogenesis and negative regulation of vascular endothelial growth factor receptor signaling pathway (BP) and extracellular region and integral to membrane and membrane (CC). In this study, the expression of genes having the function of positive regulation of angiogenesis, *Cyr61* and *Verge*, were upregulated. While this gene (*Lect1*), having the function of negative regulation of angiogenesis, was found to be down-regulated. Therefore, it can be suggested that MK-801 treatment leads to a signaling related to angiogenesis (see *Verge*).

# DISSCUSSION

Our study provides an exhaustive inventory of gene expressions, and shows that MK-801 is highly effective in causing large number of gene expressions as early as 40 min after treatment in rat brain. Using a highthroughput DNA microarray screening approach, in the core of this study we compare and provide, for the first time, transcriptome profiles in six brain regions, namely amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum, after MK-801 treatment using rat model. It should be mentioned that one of the goals of the study was also to identify potential biomarkers closely associated with neurological damage in rat brain after MK-801 treatment. Our investigation has provided not only new insight into the differential genes expressed upon MK-801 treatment but also has identified several genes which are related to psychiatric disorders, such as schizophrenia or depression. This is interesting since the NMDAr antagonist ketamine has not only psychotomimetic but also antidepressant effects<sup>[89]</sup>. Moreover, as we pooled brain samples to decrease inter-animal variation during DNA microarray analysis, this may have decreased the specificity of the potential biomarkers. However, the approach used here has allowed us to present these candidate biomarkers to the scientific community, and undoubtedly, further functional analysis will be needed to determine their importance. Finally, detailed bioinformatics analysis, such as utilizing the Ingenuity Pathway Analysis (IPA, Ingenuity<sup>®</sup> Systems, www.ingenuity.com) tool, will be essential to reveal predominant pathways and networks of genes affected by MK-801 providing new meaning to this vast gene resource presented in this study.

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# COMMENTS

#### Background

*N*-Methyl-D-aspartate receptor (NMDAr) hypofunction is thought to be involved in various neuropsychiatric disorders, including schizophrenia. Non-competitive NMDAr antagonists, like phencyclidine, ketamine and MK-801 (or dizocilpine), are suitable pharmacological tools used to understand the pathogenesis of psychosis, as they disturb - in both humans and rodents - behavior, brain connectivity and oscillations, cognition and sensory-perceptual processes which, together, resemble aspects of schizophreniform psychoses.

#### **Research frontiers**

Combining pharmacological, electrophysiological and DNA microarray approaches is a great challenge to understand the initial genetic-to-molecular mechanisms and gene-gene interactions responsible for the NMDAr hypofunction thought to underlie the pathogenesis of neurobiological disorders.

#### Innovations and breakthroughs

The present study used two low doses (0.08 and 0.16 mg/kg) of MK-801, which increases in a dose-dependent manner the power of spontaneously occurring gamma (30-80 Hz) and higher frequency oscillations in the adult rat cortex and subcortical structures. Using genome DNA chip (4 × 44 K), it provides, from MK-801-treated rats, a detailed gene inventory and resource from six brain regions (amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum). The present study reveals several genes identified as being potential biomarker candidates for schizophrenia and depression.

#### Applications

As this study primarily provides a gene resource, the outputs of microarray analysis are freely available to the public scientific community under the series number GSE63639, at the NCBI Gene Expression Omnibus public functional genomics data repository (http://www.ncbi.nlm.nih.gov/geo/query/acc. cgi?acc=GSE63639). These data can be downloaded and analysed for further bioinformatics and targeted functional analysis.

#### Terminology

Genome-wide transcriptome analysis is the analysis of the total set of transcripts present in a cell, tissue or organism, usually by DNA microarray technique. The mRNA abundance or expression of genes could be unraveled in the brain regions examined at the set point of 40 min post-injection MK-801.

#### Peer-review

This study and analysis of genes genome wide, in particular could help in the potential identification of biomarkers associated with neurological and neuropsychiatric illnesses.

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ORIGINAL ARTICLE

#### **Basic Study**

# JAK3 inhibitor VI is a mutant specific inhibitor for epidermal growth factor receptor with the gatekeeper mutation T790M

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## Abstract

**AIM:** To identify non-quinazoline kinase inhibitors effective against drug resistant mutants of epidermal growth factor receptor (EGFR).

METHODS: A kinase inhibitor library was subjected to screening for specific inhibition pertaining to the in vitro kinase activation of EGFR with the gatekeeper mutation T790M, which is resistant to small molecular weight tyrosine kinase inhibitors (TKIs) for EGFR in nonsmall cell lung cancers (NSCLCs). This inhibitory effect was confirmed by measuring autophosphorylation of EGFR T790M/L858R in NCI-H1975 cells, an NSCLC cell line harboring the gatekeeper mutation. The effects of a candidate compound, Janus kinase 3 (JAK3) inhibitor VI, on cell proliferation were evaluated using the MTT assay and were compared between T790M-positive and -negative lung cancer cell lines. JAK3 inhibitor VI was modeled into the ATP-binding pocket of EGFR T790M/L858R. Potential physical interactions between the compound and kinase domains of wild-type (WT) or mutant EGFRs or JAK3 were estimated by calculating binding energy. The gatekeeper residues of EGFRs and JAKs were aligned to discuss the similarities among EGFR T790M and JAKs.

**RESULTS:** We found that JAK3 inhibitor VI, a known inhibitor for JAK3 tyrosine kinase, selectively inhibits EGFR T790M/L858R, but has weaker inhibitory effects on the WT EGFR *in vitro*. JAK3 inhibitor VI also specifically reduced autophosphorylation of EGFR T790M/L858R in NCI-H1975 cells upon EGF stimulation, but did not show the inhibitory effect on WT EGFR in A431 cells. Furthermore, JAK3 inhibitor VI suppressed the proliferation



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of NCI-H1975 cells, but showed limited inhibitory effects on the WT EGFR-expressing cell lines A431 and A549. A docking simulation between JAK3 inhibitor VI and the ATP-binding pocket of EGFR T790M/L858R predicted a potential binding status with hydrogen bonds. Estimated binding energy of JAK3 inhibitor VI to EGFR T790M/ L858R was more stable than its binding energy to the WT EGFR. Amino acid sequence alignments revealed that the gatekeeper residues of JAK family kinases are methionine in WT, similar to EGFR T790M, suggesting that TKIs for JAKs may also be effective for EGFR T790M.

**CONCLUSION:** Our findings demonstrate that JAK3 inhibitor VI is a gatekeeper mutant selective TKI and offer a strategy to search for new EGFR T790M inhibitors.

Key words: Epidermal growth factor receptor; Tyrosine kinase inhibitor; Gatekeeper mutation; Non-small cell lung cancers

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Core tip: Non-small cell lung cancers caused by mutations in the epidermal growth factor receptor (EGFR) initially respond to tyrosine kinase inhibitors (TKIs). However, the therapeutic efficacy of EGFR-TKIs is limited by drugresistant mutations such as the gatekeeper mutation T790M. Our present study rediscovered JAK3 inhibitor VI, a known TKI for Janus kinases (JAKs), as a selective EGFR T790M inhibitor. Our structural analysis revealed similarities among EGFR T790M and JAKs, offering a possible strategy to search for EGFR T790M inhibitors from known kinase inhibitors. Repositioning of the existing therapeutics may facilitate solving clinical problems such as drug resistance and toxicity.

Nishiya N, Sakamoto Y, Oku Y, Nonaka T, Uehara Y. JAK3 inhibitor VI is a mutant specific inhibitor for epidermal growth factor receptor with the gatekeeper mutation T790M. *World J Biol Chem* 2015; 6(4): 409-418 Available from: URL: http:// www.wjgnet.com/1949-8454/full/v6/i4/409.htm DOI: http:// dx.doi.org/10.4331/wjbc.v6.i4.409

## INTRODUCTION

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib are clinical therapeutics effective on non-small cell lung cancers (NSCLCs) with activating mutation in EGFR<sup>[1,2]</sup>. Most frequent activating mutations in EGFR, a point mutation L858R and an exon19 deletion (delE746-A750), increase affinity for EGFR-TKIs relative to that for wild type (WT)<sup>[3,4]</sup>. However, the development of drug-resistant mutations such as the gatekeeper mutation T790M limits clinical efficacies of the EGFR-TKIs and is the major cause of acquired resistance in NSCLC patients<sup>[5,6]</sup>.

An irreversible EGFR-TKI with a reactive acceptor

group covalently binds to a conserved cysteine residue in the EGFR kinase domain (Cys 797) *via* a Michael addition reaction. The covalent bond formation increases stability of the complex formation between a TKI and the ATP-binding site of the kinase domain in comparison with reversible inhibitors. Therefore, irreversible EGFR-TKIs provide the ability to inhibit EGFR T790M<sup>[7-12]</sup>. However, EGFR T790M inhibition by the quinazolinebased irreversible TKIs causes simultaneous inhibition of the WT EGFR because of similar ATP affinity between WT and the T790M mutant, resulting in adverse effects such as skin rash and diarrhea. Therefore, the use of irreversible EGFR-TKIs has been limited in gefitinibor erlotinib-resistant NSCLCs and by the dose-limiting toxicity<sup>[13,14]</sup>.

Here we report that Janus kinase 3 (JAK3) inhibitor  $VI^{[15]}$ , a known TKI for a tyrosine kinase JAK3, selectively inhibits EGFR T790M/L858R, but has less inhibitory effect on the WT EGFR *in vitro*. JAK3 inhibitor VI also suppresses the proliferation of NCI-H1975 cells, an NSCLC cell line harboring EGFR T790M/L858R. This inhibitor is modeled into the ATP-binding pocket of EGFR T790M/L858R and forms potential hydrogen bonds with the kinase domain of EGFR T790M/L858R. The gatekeeper residue of JAK3 is methionine in WT, and the overall structure of the catalytic domain of JAK3 is closely related to that of EGFR. Our findings indicate that a JAK3 inhibitor is a mutant-selective reversible TKI for EGFR T790M.

## MATERIALS AND METHODS

#### **Reagents and cell lines**

JAK3 inhibitor VI and JAK inhibitor I were purchased from Calbiochem (San Diego, CA). Gefitinib was purchased from JS Research Chemical Trading (Wedel, Germany). SCADS inhibitor kit 3 was provided by Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan. Antibodies against EGFR, pEGFR (Y1045), pEGFR (Y1068), and STAT3 were purchased from Cell Signaling Technology (Beverly, MA). AntipSTAT3 (Y705) was purchased from BD (Franklin Lakes, NJ). HRP-conjugated anti-phosphotyrosine antibodies PY20 and 4G10 were purchased from BD and Millipore (Billerica, MA). NCI-H1975 cell line was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 2 mmol/L L-glutamine and 10% Fetal Bovine Serum at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

In vitro kinase assay and screening for kinase inhibitors One hundred nanograms of recombinant cytoplasmic domains (amino acid residues 696 to the C-terminus) of the WT EGFR, L858R, or T790M/L858R (Cell Signaling Technology) were preincubated with kinase inhibitors in 25  $\mu$ L of kinase reaction buffer (120 mmol/L HEPES, pH 7.5; 10 mmol/L MqCl<sub>2</sub>; 10 mmol/L MnCl<sub>2</sub>; 6  $\mu$ mol/ L Na<sub>3</sub>VO<sub>4</sub>; and 2.5 mmol/L DTT) at 25  $^{\circ}$ C for 30 min. Subsequently, 25  $\mu$ L of ATP/substrate solution containing 2 or 6  $\mu$ mol/L ATP and 6  $\mu$ mol/L poly (Glu-Tyr) biotinylated peptide (Cell Signaling Technology) was added to the preincubation mixture. The kinase reaction was performed at 25  $^{\circ}$ C for 30 min and terminated by adding 50  $\mu$ L of stop buffer (50 mmol/L EDTA, pH 8.0). Kinase activity was estimated using ELISA with avidin-coated 96-well plates and anti-phosphotyrosine antibodies (PY20 and 4G10).

## Phosphorylation analysis

NCI-H1975 or A431 cells were pretreated using JAK3 inhibitor VI for 1 h in 0.2% serum conditions and were stimulated using 200 ng/mL EGF for 15 min. Alternatively, NCI-H1975 cells were treated with kinase inhibitors at the indicated concentration for 16 h. Cells were lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 1 mmol/L NaF; 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>; and protease inhibitors) and cleared by centrifugation. SDS sample buffer was added to the supernatants. Protein samples were separated using SDS-PAGE and analyzed using Western blotting with antibodies against phospho-specific or total proteins.

#### **Proliferation assay**

Cells were cultivated in a flat-bottomed 96-well plate at  $1 \times 10^4$  cells per well in 150  $_{\mu}L$  media containing various concentrations of kinase inhibitors for 72 h. The inhibitor-treated cells were incubated in the presence of 0.5 mg/mL 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in a CO<sub>2</sub> incubator for 4 h. One hundred microliters of 20% SDS was added to each well for dissolving the insoluble purple formazan product into a colored solution. The absorption at 570 nm was measured using a spectrophotometer. The means of data values from at least three independent experiments were calculated.

## Docking model analysis

All starting templates, except the WT EGFR, were prepared by superimposing the crystal structure of the WT EGFR (PDB ID: 4WKQ) onto crystal structures of EGFR-LRTM (PDB ID: 4I22) and JAK3 (PDB ID: 3LXL) without ligands. Docking studies were performed to analyze interactions of some inhibitors in the binding site of the WT EGFR, EGFR-TMLR, and JAK3 through PyRx<sup>[16]</sup> using AutoDock 4.2.6<sup>[17]</sup>. The adopted standard protein preparation protocol involves the addition of missing hydrogen atoms to the starting template and the assignment of an ionizable state to each charged group. The docking grid of 11 Å × 12 Å × 14 Å was generated based on the observed binding site of the WT EGFR using gefitinib (PDB ID: 4WKQ). Schematic representations were generated using the UCSF Chimera package<sup>[18]</sup>.

#### Statistical analysis

All data are presented as the mean ± SE. The Mann-

Whitney *U*-test was used to determine statistically significant differences.

## RESULTS

To profile sensitivities of EGFR mutants against different classes of kinase inhibitors, we assayed in vitro recombinant cytoplasmic domains of the WT EGFR, EGFR L858R, or EGFR T790M/L858R for 95 kinase inhibitors (Table 1). Several compounds such as Ellagic acid, Damnacanthal, and RAF1 kinase inhibitor I inhibited all three EGFR kinases activity, but other kinase inhibitors showed selectivity in their inhibitory activity against WT or mutant kinases. Among inhibitors with selectivity, JAK3 inhibitor VI<sup>[15]</sup> and Gö7874<sup>[19]</sup> reduced kinase activity of EGFR T790M/L858R. Gö7874 is a known PKC inhibitor and has an indolocarbazole structure that is also seen in staurosporine, a classical kinase inhibitor (Figure 1B). Because it has been reported that indolocarbazole compounds selectively inhibit EGFR T790M but show limited effects on the WT EGFR<sup>[20]</sup>, JAK3 inhibitor VI was used in further studies. JAK3 inhibitor VI, a 3'-pyridyl oxindole compound (Figure 1A), inhibited EGFR T790M/ L858R (IC<sub>50</sub> = 1.43  $\mu$ mol/L) mutant, but weaker effects were observed on the L858R mutant (IC<sub>50</sub> > 10  $\mu$ mol/L) and WT EGFR (IC<sub>50</sub> > 10  $\mu$ mol/L) (Figure 1C).

Next, we tested whether JAK3 inhibitor VI decreased EGFR signaling in the EGFR T790M/L858R-positive NCI-H1975 cells. EGF-induced EGFR autophosphorylation at Y1045 was significantly reduced in 10 or 5  $\mu$ mol/L JAK3 inhibitor VI-treated NCI-H1975 cells (Figure 2A). In contrast, JAK3 inhibitor VI had no effect on the WT EGFR Y1045 phosphorylation in A431 cells (Figure 2B). These data confirm the mutant EGFR-specific blockage by JAK3 inhibitor VI in the T790M/L858R positive NCI-H1975 cells.

Although JAK 3 inhibitor VI specifically inhibited EGFR T790M/L858R in cells, it was not clear whether the T790M/L858R inhibition was a direct effect on the EGFR kinase or an indirect effect via JAK3 inhibition. To clarify this point, we analyzed phosphorylation levels of STAT3, a direct JAK3 substrate. The STAT3 phosphorylation level was unchanged even after stimulation using 200 ng/mL EGF, which efficiently elevated EGFR Y1045 phosphorylation (Figure 2A and C). In addition to the effects of JAK3 inhibitor VI on the EGF-stimulated phosphorylation of EGFR T790M/L858R, those on the basal phosphorylation of EGFR T790M/L858R and STAT3 were analyzed pertaining to NCI-H1975 cells in a 10% serum culture condition. In contrast to a decrease in the phosphorylation level of EGFR T790M/L858R at 5 µmol/L JAK3 inhibitor VI, STAT3 Y705 phosphorylation was apparent at 5  $\mu$ mol/L and began to decrease from a higher concentration, 10 µmol/L (Figure 2D). Another JAK inhibitor, JAK inhibitor I, significantly reduced STAT3 Y705 phosphorylation at 2.5  $\mu$ mol/L, but did not affect EGFR Y1068 phosphorylation at higher concentrations such as 5 or 10  $\mu$ mol/L (Figure 2D). These data suggest that JAK3 inhibitor VI reduces the phosphorylation level

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| Target        | Compound name                                                                                                        |  |  |
|---------------|----------------------------------------------------------------------------------------------------------------------|--|--|
| AK            | ABT-702                                                                                                              |  |  |
| AKT (PKB)     | Akt Inhibitor IV, Akt Inhibitor VII, Akt Inhibitor XI                                                                |  |  |
| AMPK          | compound C                                                                                                           |  |  |
| ATM           | ATM/ATR kinase inhibitor, ATM kinase inhibitor                                                                       |  |  |
| Aurora kinase | Aurora kinase/CDK inhibitor, aurora kinase inhibitor II, Aurora kinase inhibitor II                                  |  |  |
| Bcr-abl       | AG957                                                                                                                |  |  |
| BTK           | LFM-A13, terreic acid                                                                                                |  |  |
| CAMKII        | KN-93, KN-62, Lavendustin C                                                                                          |  |  |
| CDK           | Kenpaullone, purvalanol A, Olomoucine, Alsterpaullone, Cdk1/2 inhibitor Ⅲ, Cdk2/9 inhibitor, NU6102, Cdk4 inhibitor, |  |  |
| CDR           | NSC625987                                                                                                            |  |  |
| Chk           | SB218078, Isogranulatimide, Chk2 inhibitor, Chk2 inhibitor II                                                        |  |  |
| CK            |                                                                                                                      |  |  |
| Clk           | Ellagic acid (dihydrate), TBB, DMAT, D4476                                                                           |  |  |
|               | TG003<br>Disadaharan Ibiana indikitan H                                                                              |  |  |
| DGK           | Diacylglycerol kinase inhibitor II                                                                                   |  |  |
| DNA-PK        | IC60211                                                                                                              |  |  |
| eEF2 kinase   | TX-1918                                                                                                              |  |  |
| EGFR          | BPIQ-II, AG1478, AG490                                                                                               |  |  |
| FGFR          | SU4984, SU5402                                                                                                       |  |  |
| Flt-3         | Flt-3 Inhibitor                                                                                                      |  |  |
| Fms           | cFMS Receptor Inhibitor                                                                                              |  |  |
| Fyn           | SU6656                                                                                                               |  |  |
| GSK           | GSK-3 inhibitor IX, 1-Azakenpaullone, Indirubin-3'-monoxime                                                          |  |  |
| HER2          | AG825                                                                                                                |  |  |
| IGF-IR        | AG1024, AGL 2263                                                                                                     |  |  |
| IKK           | BMS-345541, IKK-2 inhibitor VI                                                                                       |  |  |
| IRAK          | IRAK-1/4 inhibitor                                                                                                   |  |  |
| JAK           | JAK Inhibitor I, JAK3 Inhibitor VI                                                                                   |  |  |
| JNK           | SP600125, JNK inhibitor Ⅷ                                                                                            |  |  |
| Lck           | Damnacanthal, PP2                                                                                                    |  |  |
| MAPK          | ERK inhibitor II                                                                                                     |  |  |
| MEK           | PD98059, U-0126, MEK inhibitor I                                                                                     |  |  |
| Met           | SU11274                                                                                                              |  |  |
| MLCK          | ML-7                                                                                                                 |  |  |
| p38 MAPK      | SB202190, SB239063                                                                                                   |  |  |
| PDGFR         | AG1296, SU11652, PDGF receptor inhibitor V, PDGF receptor inhibitor IV                                               |  |  |
| PI3K          | LY-294002, Wortmannin                                                                                                |  |  |
| PKA           | H-89, 4-cyano-3-methylisoquinoline                                                                                   |  |  |
| PKC           | Bisindolymaleimide I, Go7874                                                                                         |  |  |
| PKG           | Rp-8-CPT-cGMPS, KT5823                                                                                               |  |  |
|               | PKR inhibitor                                                                                                        |  |  |
| PKR           |                                                                                                                      |  |  |
| Raf<br>ROCK   | RAF1 kinase inhibitor I, ZM 336372                                                                                   |  |  |
|               | H-1152, Y-27632                                                                                                      |  |  |
| Hsp90         | radicicol                                                                                                            |  |  |
| Src           | PP1 analog                                                                                                           |  |  |
| Syk           | Syk inhibitor                                                                                                        |  |  |
| TGF-bRI       | SB431542, TGF-beta RI kinase inhibitor II                                                                            |  |  |
| Tpl2          | Tpl2 kinase inhibitor                                                                                                |  |  |
| TrKA          | TrkA inhibitor                                                                                                       |  |  |
| VEGFR         | VEGFR receptor inhibitor II, VEGF recptor 2 inhibitor I, SU1498                                                      |  |  |

AK: Adenosine kinase; AKT(PKB): Protein kinase B; AMPK: 5'-adenosine monophosphate-activated protein kinase; ATM: Ataxia telangiectasia mutated; Aurora: Aurora kinase; Bcr-abl: Breakpoint cluster region-Abelson fusion kinase; BTK: Bruton's tyrosine kinase; CAMKII: Calcium/calmodulin-dependent protein kinase II; CDK: Cyclin-dependent kinase; Chk: Checkpoint kinase; CK: Casein kinase; Clk: Cdc2-like kinase; DGK: Diacylglycerol kinase; DNA-PK: DNA-dependent protein kinase; eEF2 kinase: Eukaryotic elongation factor 2 kinase; EGFR: Epidermal growth factor receptor; FGFR: Fibroblast growth factor receptor; Flt-3: Fms-related tyrosine kinase 3; Fms: Feline McDonough sarcoma (Colony-stimulating factor-1 receptor); GSK: Glycogen synthase kinase; HER2: Human epidermal growth factor receptor 2; IGF-IR: Insulin-like growth factor I receptor; IKK: IkB kinase; IRAK: Interleukin-1 receptorassociated kinase; JAK: Janus kinase; JNK: Jun N-terminal kinase; Lck: Lymphocyte-specific protein tyrosine kinase; MAPK: P38 mitogen-activated protein kinase; PDGFR: Platelet-derived growth factor receptor; PI3K: Phosphatidylinositol-4-Kinase; PKA: Protein kinase A; PKC: Protein kinase C; PKG: Protein kinase G; PKR: Double-stranded RNA-activated protein kinase; Raf: Rapidly accelerated fibrosarcoma; ROCK: Rho-associated protein kinase; Hsp90: Heat shock protein; Syk: Spleen tyrosine kinase; TGF-bRI: Transforming growth factor-beta type I receptor; Tpl2: Tumor progression locus 2; TrKA: Tyrosine kinase receptor A (tyrosine kinase receptor for nerve growth factor); VEGFR: Vascular endothelial growth factor receptor.

of EGFR T790M/L858R without JAK3 inhibition, and that JAKs are not major contributors to EGFR phosphorylation

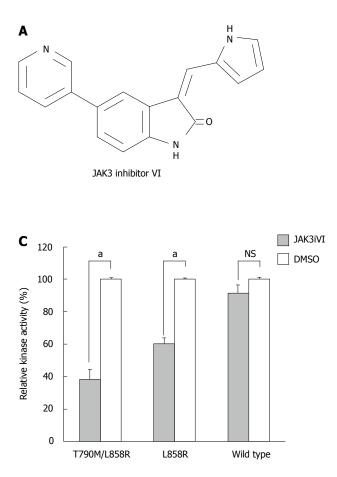
#### in NCI-H1975 cells.

Next, we performed MTT assays to test the effects



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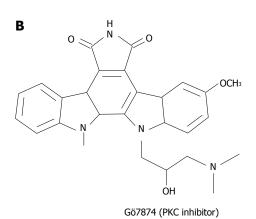


Figure 1 Janus kinase 3 inhibitor VI selectively inhibits kinase activity of the epidermal growth factor receptor gatekeeper mutant T790M/L858R *in vitro*. A, B: Chemical structures of JAK3 inhibitor VI (A) and Gö7874 (B) are shown; C: Effects of JAK3 inhibitor VI on kinase activities of WT, L858R, and T790M/L858R EGFRs were compared. Recombinant EGFR kinase domains were treated using JAK3 inhibitor VI at 10  $\mu$ mol/L, and phosphorylation was initiated by adding ATP in the presence of an artificial substrate peptide. Phosphorylation levels were measured by ELISA using an anti-phosphotyrosine antibody. Error bars indicate SEM (*n* = 4); <sup>a</sup>*P* < 0.05. NS: Not significant; EGFR: Epidermal growth factor receptor; JAK3: Janus kinase 3.

of JAK3 inhibitor VI on proliferation of EGFR T790M/ L858R positive NCI-H1975 cells. NCI-H1975 cells were cultured in 96-well plates for 3 d with or without different concentrations of compounds. JAK3 inhibitor VI showed growth suppression at 10 and 30  $\mu$ mol/L, but gefitinib or JAK inhibitor I did not (Figure 3A). The effects of JAK3 inhibitor VI on proliferation of the WT EGFR expressing A431 (Figure 3B) and A549 (Figure 3C) cells were also analyzed. These cells were less sensitive to JAK3 inhibitor VI than NCI-H1975 cells.

To obtain structural insight, docking simulations between JAK3 inhibitor VI and kinases were performed. In the model structure, JAK3 inhibitor VI bound to the ATP-binding pocket of EGFR T790M/L858R and formed hydrogen bonds with M793 and T854 residues (Figure 4A). Estimated binding to EGFR T790M/L858R was more stable than that to the WT EGFR and was equivalent with that to JAK3 (EGFR T790M/L858R: -7.38 kcal/mol; JAK3: -7.59 kcal/mol; WT EGFR: -6.23 kcal/mol). Amino acid sequence alignments of the EGFR T790 flanking regions and the corresponding regions of JAKs revealed that the gatekeeper residues of WT JAKs are methionine, similar to those of EGFR T790M (Figure 4B).

## DISCUSSION

EGFR T790M limits the clinical efficacy of EGFR-TKIs. Irreversible TKIs for EGFR T790M are associated with toxic effects due to simultaneous inhibition of the WT EGFR. In the present study, we found that JAK3 inhibitor VI selectively inhibits EGFR T790M/L858R and had a lesser inhibitory effect on the WT EGFR *in vitro*. JAK3 inhibitor VI also suppressed the proliferation of NCI-H1975 cells harboring EGFR T790M/L858R. Thus, our findings demonstrate that a JAK3 inhibitor is a mutant selective TKI for EGFR T790M/L858R and offer a potential strategy to develop EGFR T790M-specific inhibitors by applying known inhibitors for JAK family kinases closely related to the ErbB family.

JAK3 inhibitor VI selectively inhibits the gefitinibresistant EGFR T790ML858R. JAK3 inhibitor VI (10  $\mu$ mol/L) inhibited kinase activity of EGFR T790M/L858R; however, it was ineffective on the activity of the WT EGFR *in vitro* kinase assay (Figure 1C). Furthermore, JAK3 inhibitor VI efficiently reduced autophosphorylation of EGFR T790M/L858R in gefitinib-resistant lung cancer cells, NCI-H1975 (Figure 2A), in contrast to resistance of the WT EGFR in A431 cells (Figure 2B). Dosage restriction



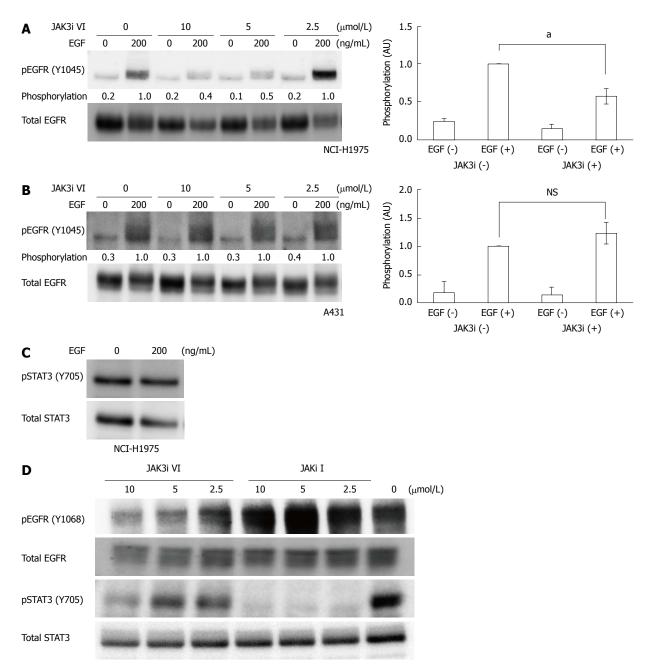


Figure 2 Janus kinase 3 inhibitor VI reduces epidermal growth factor receptor autophosphorylation in NCI-H1975 cells harboring epidermal growth factor receptor T790M/L858R. A, B: NCI-H1975 (A) or A431 (B) cells were treated with the indicated concentration of JAK3 inhibitor VI and were stimulated using 200 ng/mL EGF. Phosphorylation levels of EGFR Y1045 were analyzed using Western blotting. Phosphorylation levels of EGFR were normalized against total EGFR levels. Relative phosphorylation levels to the sample treated with DMSO and 200 ng/mL EGF are indicated. Right panels show the averages of relative phosphorylation levels from three independent experiments; C: NCI-H1975 cells were stimulated using 200 ng/mL EGF. Phosphorylation levels of STAT3 Y705 were analyzed using Western blotting; D: NCI-H1975 cells were treated using JAK3 inhibitor VI or JAK inhibitor I, as indicated. Phosphorylation levels of EGFR Y1068 and STAT3 Y705 were analyzed using Western blotting with phospho-specific antibodies. Error bars indicate SEM (n = 3); <sup>a</sup>P < 0.05. NS: Not significant; EGF (-): 0 ng/mL EGF; EGF (+): 200 ng/mL EGF; JAK3i (-): 0  $\mu$ mol/L JAK3 inhibitor VI; JAK3i (+): 10  $\mu$ mol/L JAK3 inhibitor VI; EGFR: Epidermal growth factor receptor; JAK3: Janus kinase 3.

of quinazoline-based EGFR-TKI treatments is attributable to the toxicity derived from simultaneous inhibition of the WT EGFR. As a possible solution for the issue, Zhou *et al*<sup>(21)</sup> reported that compounds with a non-quinazoline structure, pyrimidine, were specifically effective on mutant EGFRs. Staurosporine-related indolocarbazole compounds such as Gö6976 and PKC412 are also more selective for EGFR T790M than quinazoline-based EGFR inhibitors<sup>[19]</sup>. Therefore, applications of non-quinazoline compounds can confer T790M mutant specific inhibition without affecting the WT EGFR. Our data indicate that JAK3 inhibitor VI, an indol-based compound, provides an additional example of EGFR T790M-specific structures.

JAK3 inhibitor VI suppresses proliferation of NSCLCs harboring the gatekeeper mutation EGFR T790M. EGFR T790M/L858R-expressing NCI-H1975 cells showed reduced proliferation in the presence of JAK3 inhibitor VI, but not in the presence of gefitinib or JAK inhibitor

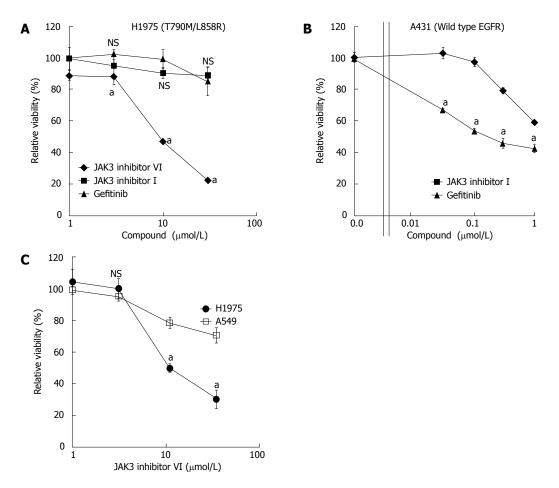


Figure 3 Janus kinase 3 inhibitor VI suppresses proliferation of NCI-H1975 cells. A-B: NCI-H1975 (A) or A431 (B) cells were treated with JAK3 inhibitor VI, JAK inhibitor I, or gefitinib. Effects of indicated concentration of kinase inhibitors on cell proliferation were analyzed using the MTT assay; C: Effects of JAK3 inhibitor VI on proliferation were compared between NCI-H1975 and A549 cells using the MTT assay. Error bars indicate S.E.M. (*n* = at least 3); <sup>a</sup>*P* < 0.05. NS: Not significant; JAK3: Janus kinase 3; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.

I (Figure 3A). Furthermore, WT EGFR-expressing cell lines, A431 and A549, were less sensitive to JAK3 inhibitor VI in comparison with NCI-H1975 cells (Figures 3B and C), suggesting specific inhibition of EGFR T790M by JAK3 inhibitor VI. Because JAK3 inhibitors block JAK3 that may phosphorylate EGFR, indirect effects via JAK3 inhibition may cause a reduction in autophosphorylation of EGFR T790M/L858R in NCI-H1975 cells. However, EGFR T790M/L858R inhibition was observed in lower concentrations of JAK3 inhibitor VI than those of JAK3 inhibition. Another JAK inhibitor, JAK inhibitor I, did not show EGFR T790M/L858R inhibition. Furthermore, T790M kinase inhibition was observed in an in vitro assay using recombinant kinases. Therefore, the decrease in autophosphorylation of EGFR T790M/L858R seems to be caused by direct inhibition in EGFR T790M/ L858R rather than by JAK3 inhibition-mediated indirect effects.

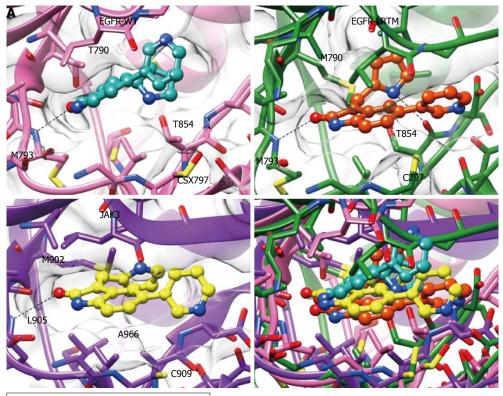
Structural characteristics of EGFR T790M mutants may be analogous to those of JAK3. In the docking study, JAK3 inhibitor VI bound to the ATP-binding pocket of EGFR T790M/L858R with an additional hydrogen bond (Figure 4A). Binding energy calculations predicted that JAK3 inhibitor VI would more stably bind to EGFR T790M/L858R than to the WT EGFR with an equivalent

stability to the simulated binding to JAK3 (Figure 4A). Furthermore, EGFR and JAK family kinases are located in neighboring clans in the human kinome<sup>[22]</sup>, indicating that overall structures of the catalytic domains of JAKs are closely related to those of EGFRs. The gatekeeper residues of WT JAKs are methionines, which are identical to the gatekeeper mutant EGFR T790M (Figure 4B). Sequence alignments of the gatekeeper flanking regions between BCR-ABLs and Aurora kinases indicated that the corresponding residues in Aurora kinases are isoleucines, similar to the gatekeeper mutation T315I. These findings suggest a hypothesis in which inhibitor sensitive kinases such as EGFR L858R, exon19-deleted EGFR, and BCR-ABL obtained structural similarities to other kinases during acquisition of drug resistance. In fact, Aurora kinase inhibitors are effective on BCR-ABL T315I<sup>[23,24]</sup> in addition to our findings of JAK3 inhibitor VI as an EGFR T790M inhibitor. Therefore, it may be a rational strategy to screen compounds effective on different kinases structurally similar to gatekeeper mutants in the fight against drug resistant kinases.

## ACKNOWLEDGMENTS

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| В |                                                                                              | Gatekeeper                                                                                                             |
|---|----------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|
|   | EGFR WT<br>EGFR T790M<br>JAK1<br>JAK2                                                        | 786 VQLITQLMP 794<br>786 VQLIMQLMP 794<br>952 IKLIMEFLP 960<br>925 LKLIMEYLP 933                                       |
|   | JAK3<br>BCR-ABL WT<br>BCR-ABL T315I<br>Aurora kinase A<br>Aurora kinase B<br>Aurora kinase C | 898 LRLVMEYLP 906<br>311 FYIITEFMT<br>311 FYIIIEFMT 319<br>205 RVYLILEYA 213<br>149 RIYLILEYA 157<br>115 RVYLILEYA 123 |

Figure 4 Structural analysis of janus kinase 3 inhibitor VI binding to the epidermal growth factor receptor T790M. A: JAK3 inhibitor VI (cyan in EGFR WT: top left, red in EGFR T790M/L858R: top right, or yellow in JAK3: bottom left) was modeled into EGFR WT, EGFR T790M/L858R (LRTM), or JAK3. A superimposed image is also shown (bottom right). The dotted lines indicate putative hydrogen bonds; B: Amino acid sequence alignments of EGFRs and JAKs, and BCR-ABL and Aurora kinases. Asterisk indicates the gatekeeper residues. EGFR: Epidermal growth factor receptor; JAK: Janus kinase; WT: Wild type.

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## COMMENTS

#### Background

Tyrosine kinase inhibitors against the epidermal growth factor receptor (EGFR-TKIs) are initially effective on non-small cell lung cancers (NACLCs) caused by EGFR mutations. However, drug-resistant mutations such as the gatekeeper mutation T790M limit the clinical efficacy of EGFR-TKIs.

## **Research frontiers**

Irreversible EGFR-TKIs that covalently bind to the EGFR kinase domain have provided a way to inhibit the gatekeeper mutant EGFR T790M.

## Innovations and breakthroughs

EGFR T790M inhibition by the quinazoline-based irreversible TKIs causes simultaneous inhibition of the WT EGFR, resulting in adverse effects such as skin rash and diarrhea. The present study rediscovered Janus kinases 3 (JAK3) inhibitor VI as a reversible EGFR-TKI selective for EGFR T790M.

#### Applications

The authors' structural analysis revealed similarities among EGFR T790M and Janus kinases (JAKs). This may offer a new strategy to screen for EGFR T790M inhibitors from known kinase inhibitors.

#### Terminology

JAKs are protein tyrosine kinases, whose catalytic domain structures are closely related to those of EGFRs.

#### Peer-review

In current manuscript entitled "JAK3 inhibitor VI is a mutant specific inhibitor for EGFR with gatekeeper mutation T790M", the authors demonstrated that JAK3



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inhibitor  $\rm VI$  could specifically inhibit EGFR gatekeeper mutation (T790M) in non-small cell lung cancers.

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