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EDITORIAL

# New findings showing how DNA methylation influences diseases

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## Abstract

In 1975, Holliday and Pugh as well as Riggs independently hypothesized that DNA methylation in eukaryotes could act as a hereditary regulation mechanism that influences gene expression and cell differentiation. Interest in the study of epigenetic processes has been inspired by their reversibility as well as their potentially preventable or treatable consequences. Recently, we have begun to understand that the features of DNA methylation are not the same for all cells. Major differences have been found between differentiated cells and stem cells. Methylation influences various pathologies, and it is very important to improve the understanding of the pathogenic mechanisms. Epigenetic modifications may take place throughout life and have been related to cancer, brain aging, memory disturbances, changes in synaptic plasticity, and neurodegenerative diseases, such as Parkinson's disease and Huntington's disease. DNA methylation also has a very important role in tumor biology. Many oncogenes are activated by mutations in carcinogenesis. However, many genes with tumor-suppressor functions are "silenced" by the methylation of CpG sites in some of their regions. Moreover, the role of epigenetic alterations has been demonstrated in neurological diseases. In neuronal precursors, many genes associated with development and differentiation are silenced by CpG methylation. In addition, recent studies show that DNA methylation can also influence diseases that do not appear to be related to the environment, such as IgA nephropathy, thus affecting the expression of some genes involved in the T-cell receptor signaling. In conclusion, DNA methylation provides a whole series of fundamental information for the cell to regulate gene expression, including how and when the genes are read, and it does not depend on the DNA sequence.

**Key words:** DNA methylation; Stem cells; Enhancer; IgA nephropathy; Gene regulation

**Core tip:** DNA methylation in eukaryotes acts as a hereditary regulation mechanism that influences gene expression and cell differentiation. Recently, we have begun to understand that the features of DNA methylation are not the same for all the cells. Major differences have been found between differentiated cells and stem cells. However, epigenetic modifications may take place throughout life and influence various diseases, and they are very important for improving the understanding of pathogenic mechanisms. New studies show that DNA methylation can also influence diseases that do not appear to be related to the environment.

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## INTRODUCTION

In 1975, Holliday and Pugh as well as Riggs independently hypothesized for the first time that DNA methylation in eukaryotes could act as a hereditary regulation mechanism to influence gene expression and cell differentiation. Epigenetics was born as the study of inheritable changes in the genome that occur without modification of the DNA sequence and affect its functionality. DNA methylation is an extremely important mechanism. Together with covalent modifications of histones (proteins that compact the DNA), methylation modifies the chromatin structure and the accessibility of DNA to the regulation factors of gene expression.

DNA methylation density strongly varies with each chromosome. The sub-telomeric regions near the extremities often show a high methylation rate, which is important for controlling telomere length and recombination. In general, a lower methylation density is observed at the level of "CpG islands" and transcription initiation sites[1]. In 2008, a definition of epigenetic characteristics was formulated during the Cold Spring Harbor Meeting as a "stably inheritable phenotype deriving from a chromosomal alteration not connected to DNA variations"[2].

DNA methylation is maintained throughout the life of the affected cells and can be transmitted to subsequent cellular generations without modification of the DNA sequence. The processes responsible for epigenetic changes can take place both before and after transcription. In the first case, they are mainly represented by DNA methylation, which is bound by a covalent bond (and therefore reversible) to a methyl group coming from the universal methyl donor S-adenosylmethionine in position 5 of the cytosine residue of the CpG dinucleotide (Cytosine-phosphate-Guanidine). The phenomenon is due to the intervention of the specific enzyme DNA methyltransferase (DNMT).

The hypermethylation of DNA determines the "silencing" of the gene of interest, while hypomethylation causes activation[3]. Anomalous chromatin states that lead to abnormal gene expression patterns have been defined as epimutations, which have been detected in numerous diseases, including cancer. Epimutations can affect one or both alleles of a gene. Epimutations in cancer usually occur in somatic cells and cause cancer progression[4-8].

## FEATURES OF DNA METHYLATION ARE NOT THE SAME FOR ALL CELLS

Recently, we have begun to understand that the features of DNA methylation are not the same for all cells. Major differences have been found between differentiated cells and stem cells. For example, 99.98% of the methylated DNA regions in normal cells, such as fibroblasts, are rich in C and G, whereas the methylation in stem cells is concentrated in sequences that are rich in A and T. Moreover, embryonic cells that are induced to differentiate lose the methylation at the level of non-CG sequences, while they maintain the methylation in the sequences rich in C and G. This indicates that widespread methylation at the non-CG level is lost during differentiation.

Previous studies have hypothesized the existence of methylation almost exclusively in C and G-rich sequences in mammals, but observations led to the supposition that

non-CG methylation is a general feature, at least for human embryonic stem cells. The absence of non-CG methylation in fibroblasts coincides with a reduced presence of *de novo* DNMTs (DNMT3A, DNMT3B, and DNMT3L)-that is, enzymes that catalyze the addition of methyl groups in previously unmethylated residues. In contrast, "maintenance" methyltransferases reproduce the methylation pattern in a DNA strand based on what is present in the other filament[1].

A positive correlation between gene expression and methylation density is also observed at the level of non-CG sequences. The most expressed genes contain three times higher methylation density than unexpressed genes. However, no correlation has been detected between CpG methylation density and gene expression in stem cells. A particularly high methylation density has been observed for genes involved in RNA processes, such as splicing and RNA metabolic processes. Unexpectedly, an enrichment of non-CG methylation was found at the level of the antisense strand of the coding regions of genes, but the potential roles of this methylation are currently unknown.

Numerous studies have documented a correlation between DNA methylation and the ability of some proteins to interact with their target sequences. A decrease in DNA methylation density has been noted in correspondence with the protein interaction sites.

Another very important modality of gene expression regulation involves the "enhancer" regions, which are short DNA sequences that can to bind activating proteins, which in turn facilitate the recruitment of RNA polymerase and thus the transcription of the regulated gene. A decrease in methylation has been observed at the level of enhancers specific for fibroblasts. Conversely, at the level of specific stem enhancers, the methylation density does not change in either the embryonic stem cells themselves or in fibroblasts. This indicates the maintenance of these elements in an unmethylated state, thus preventing interference in the protein-DNA interaction process. The specific type of de-methylation (non-CG in stem cells and CG in fibroblasts) could indicate the use of different types of methylation specific to each cell type. Another paradigm of DNA methylation is that it controls aspects of cell differentiation. Obviously, this implies that methylation patterns vary in different cell types, as documented in several studies[9,10].

## METHYLATION INFLUENCES VARIOUS PATHOLOGIES

Interest in the study of epigenetic processes has been inspired by their reversibility and their potentially preventable or treatable consequences[11,12]. It is easy to understand how methylation influences various pathologies and the importance that it covers to understand better pathogenic mechanisms. In recent years, several studies have shown that DNA methylation influences many diseases. Cancer has been the most studied among the numerous diseases in which epigenetic modifications are the object of greater attention. DNA methylation also has a very important role in tumor biology.

Cancer is considered an essentially genetic disease, in which mutations alter the functioning of genes, causing the cell to proliferate in an uncontrolled manner. In recent decades, however, numerous indications have led to the suspicion that epigenetic factors-particularly DNA methylation-may be involved in the genesis of a tumor. In carcinogenesis, many oncogenes are activated by mutations[13-16]. However, many genes with tumor suppressor function are "silenced" by the methylation of CpG sites present in some regions[17].

We have known that epigenetic changes are associated with cancer, but until a few years ago, we did not know if they were the cause or a consequence of the disease. Recently, the development the new "epigenetic engineering" approach in some studies has allowed us to verify that even changes in DNA methylation alone can induce cancer. In fact, in new research, Yu *et al*[18] created a line of genetically modified mice where a small fragment of DNA adjacent to the p16 gene behaved like a magnet that attracted DNA methyltransferases and methyl groups, which hypermethylated the gene promoter and blocked the possibility of transcription. p16 has the function of blocking the cell cycle and preventing mitosis when necessary. For this reason, the p16 gene is considered a tumor-suppressor gene.

The results of the study showed that in the population of transgenic adult mice, the gene encoding p16 is more substantially activated during aging. The incidence of spontaneous tumors was higher than that in the control population of normal mice, in which the tumor suppressor gene continued to act regularly. Obviously, this kind of regulation can be extended to several other oncogenes in several other diseases. This result has profound implications for future studies because epigenetic changes are

potentially reversible. Therefore, new epigenetic therapies may be very effective for both tumors and other diseases, such as neurodevelopmental diseases, obesity, and diabetes.

The role of epigenetic alterations has also been demonstrated in neurological diseases. In neuronal precursors, many genes associated with development and differentiations are silenced by CpG methylation. The regulation of the proteins that bind to the methylated CpG is subject to mutations, duplications, and insertions. One example of a condition that depends on these processes is Rett's syndrome, which involves severe mental retardation linked to the X chromosome. Studies carried out with animal models of this disease report very interesting results in that the modifications of the CpG are at least partially reversible[19]. Epigenetic modifications may take place throughout life and have been related to brain aging, memory disturbances, and changes in synaptic plasticity[20]. The resulting alterations are increased over the years[21] and become significant in various neurodegenerative diseases, such as Parkinson's disease and Huntington's disease[14,22].

Various observations have led to the suspicion of the existence of epigenetic mechanisms in the pathogenesis of asthma, which is present in both twin homozygotes in as much as half of the cases[23]. There are complex interactions between genetics and the environment, which could lead to epigenetic modifications of the genome. One example is the demonstrated interaction between maternal smoking during pregnancy and the activity of the interleukin-1 receptor antagonist in newborns, which is associated with a significant increase in the risk of asthma[24]. In contrast, exposure to some endotoxins *in utero* appears to have a protective effect[25]. Moreover, many studies on the genetics of asthma have shown the existence of gene de-regulations that can be explained with only epigenetic alterations and DNA hypomethylation of 14 CpG sites that are gained after birth and linked with childhood asthma[26,27].

Recent studies show that DNA methylation can also influence diseases that do not appear to be related to the environment, such as IgA nephropathy (IgAN). This condition is the most common form of primary glomerulonephritis worldwide and has a strong genetic component. DNA methylation in the CD4+ T cells of IgAN patients influences the expression of some genes involved in the T-cell receptor signaling, which is the pathway that transfers the signal for the presence of antigens and activates the T-cells[28]. In particular, TRIM27 and DUSP3 genes were found to be hypomethylated in correspondence to the site that modulates their transcription, and these genes are upregulated in the CD4 T cells of IgAN patients.

The DNA region encoding vault RNA 2-1 (VTRNA2-1) non-coding RNA was also found to be hypermethylated, leading to its down-regulation. In turn, following CD3/CD28 T-cell receptor (TCR) stimulation, the lower levels of VTRNA2-1 cause a decrease in the proliferation of CD4+ T-cells, which plausibly occurs through the activation of the interferon-inducible kinase protein kinase R. Lower VTRNA2-1 levels also increase transforming growth factor beta expression. Together with DUSP3 and TRIM27, the increased transforming growth factor beta expression impairs the proliferation and activation of CD4+ T-cells, thus reducing the effect of the CD3/CD28 activation[28]. This deregulation causes reduced TCR strength and a T-cell anergy-like status. The lower activation of CD4+ T-cells and the lower TCR strength can determine Th1 polarization with higher interleukin 2 production in some biological settings. The aberrantly methylated DNA regions of CD4+ T-cells in IgAN patients thus offers a way to improve the understanding of the molecular mechanisms implicated in this disease. They could also lead to a new point of view for new therapeutic targets for the treatment of the IgAN.

In addition to these DNA methylation processes, we should also consider that various other inheritable mechanisms of post-transcriptional gene regulation can be used by cells, particularly the synthesis of non-coding microRNA, which binds to the corresponding messenger RNA and causes degradation or inhibition[29]. Recent studies also reveal an interesting interaction between these two kinds of epigenetic regulating systems. Gene expression can be affected by DNA methylation operating at a distance through the methylation or demethylation of the regulatory regions of miRNAs. The diversity of miRNA targets may produce the concurrent regulation of numerous biological pathways, such as apoptosis, cell proliferation, and migration.

Many *in vitro* and *in vivo* studies have shown that even epigenetic modifications of microRNAs can intervene in the pathogenesis of atherosclerotic lesions. For example, miR-33 inhibits the genes involved in the ability to expel cell cholesterol, the metabolism of high-density lipoproteins, lipid oxidation, and glucose metabolism. In mice, miR-33 deficiency is associated with a reduction of induced atherosclerotic lesions[30]. In neuroblastoma, a cluster of aberrantly methylated miRNA genes could lead to impaired regulation of the cell cycle, apoptosis, and the control of V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog expression[31].

These data show that a complex arrangement of connections between epigenetically managed miRNAs and target genes may affect the control of cell homeostasis at different levels.

## CONCLUSION

The knowledge is changing in regard to the regulation of pathways and biological processes in living organisms, including humans. DNA methylation provides a whole series of fundamental information for cells to regulate their gene expression, including how and when the genes are read, and it does not depend on the DNA sequence. As discussed, these reversible DNA modifications are influenced by the surrounding context and can heavily influence diseases.

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MINIREVIEWS

# Autism and carnitine: A possible link

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## Abstract

Patients with autism spectrum disorders (ASD) present deficits in social interactions and communication, they also show limited and stereotypical patterns of behaviors and interests. The pathophysiological bases of ASD have not been defined yet. Many factors seem to be involved in the onset of this disorder. These include genetic and environmental factors, but autism is not linked to a single origin, only. Autism onset can be connected with various factors such as metabolic disorders: including carnitine deficiency. Carnitine is a derivative of two amino acid lysine and methionine. Carnitine is a cofactor for a large family of enzymes: the carnitine acyltransferases. Through their action these enzymes (and L-carnitine) are involved in energy production and metabolic homeostasis. Some people with autism (less than 20%) seem to have L-carnitine metabolism disorders and for these patients, a dietary supplementation with L-carnitine is beneficial. This review summarizes the available information on this topic.

**Key words:** Autism; Carnitine; Neurodevelopmental; Metabolism; Pathophysiological bases



**Core tip:** Autism spectrum disorder is characterized by impaired communication, altered social skills, stereotypical behaviors and limited interests. The pathophysiological bases of autism have not been defined yet. Several publications have pointed a possible connection between autism and carnitine deficiency. Carnitine is a cofactor for a large family of enzymes: the carnitine acyl transferases. Through their action these enzymes are involved in energy production and metabolic homeostasis. Low plasma carnitine were reported in autism patients and for some of them, defects in L-carnitine metabolism have been reported. This review summarizes the available information on the possible link between autism and carnitine.

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## AUTISM

### Description

Autism is a heterogeneous neurodevelopmental disorder actually known as Autism spectrum disorders (ASD). It is characterized by a dyad of symptoms: impaired communication and altered social skills[1]. ASD symptoms include (1) persistent deficits in initiating and sustaining social interaction and social communication; and (2) limited, repetitive and stereotypical patterns of behaviors and interests.

The impact of ASD on people can be very dissimilar: some people with ASD can live independently, others require life-long care and support. The onset of this disease occurs, typically, in early childhood usually before the age of three. For some patients, the symptoms will become apparent later when daily autonomy requirements will not be handled by the patient. According to the Diagnostic and Statistical Manual of mental disorders (DSM-5®), the symptoms must appear during the childhood to be considered as autism[1].

Most people affected by autism are healthy otherwise but for some of them, autism is associated with other health problems. Among those pathologies associated with autism, one can find metabolic disorders such as phenylketonuria, chromosomal abnormalities, infectious diseases such as rubella or neurocutaneous disorders.

In most cases, deficits are severe enough to affect the personal life: the different aspects of social, educational, occupational life are generally affected in people affected by ASD. The spectrum is large and among patients, a full range of mental abilities and communication skills can be observed[2].

In the literature, some theories explain autism by cognitive deficit. Baron-Cohen studied the hypothesis of a deficit of theory of mind and showed that patients with ASD have difficulties in imputing mental states (beliefs, desires, intentions, emotions, *etc*.) to others[3]. It has been proposed that people with ASD have a deficit of executive functions. Evaluations of cognitive functions objectivize deficits in inhibition, cognitive flexibility and working memory. Happé and Frith[4] developed another theory indicating that a person with ASD has a weak central coherence; this theory suggested that a person with autism tends to be more focused on details: the perception of a person with ASD can be defined as fragmented. There are other theories that have been developed to explain what happens in ASD patients: like the perception and sensory theory[5].

### Evolution

When ASD is diagnosed in childhood, the symptoms tend to persist during adolescence and adulthood.

With appropriate interventions, the autistic spectrum can be improved: behavioral treatment can improve communication and social behavior, usually associated with a positive impact on wellbeing for people with ASD and their family.

### Data and statistics

In the 60$^{th}$, the prevalence of ASD was estimated to be around 4-5 in 10000, today this number is around 100 (or even more) in 10000 people[6,7] and some authors, even reported a prevalence of 3.6%[7]. This increase may not find its origin in genetic and thus, environmental factors may play a role in the onset of ASD[8]. This rise may also be due to a more efficient diagnosis and a better detection of the disease. For ASD, a male-to female ratio of 3.75:1 has been found[9].

ASD can be diagnosed as early as 2 years old, but most children are not diagnosed with ASD until the age of 4. Usually, the age of diagnosis depends of the severity of ASD. The DSM-5 defined 3 severity levels which depend on the requiring support. A schematic representation of the major features found in ASD patients is summarized in Figure 1.

Parents who have a child with ASD have a 2%-18% risk of having a second child who is also affected[10]. This data can be linked to the genetic aspects of ASD.

ASD is found in every country and in every ethnic group and in both sexes. Reports indicate that prevalence might be different according to the ethnical origin[11] even if

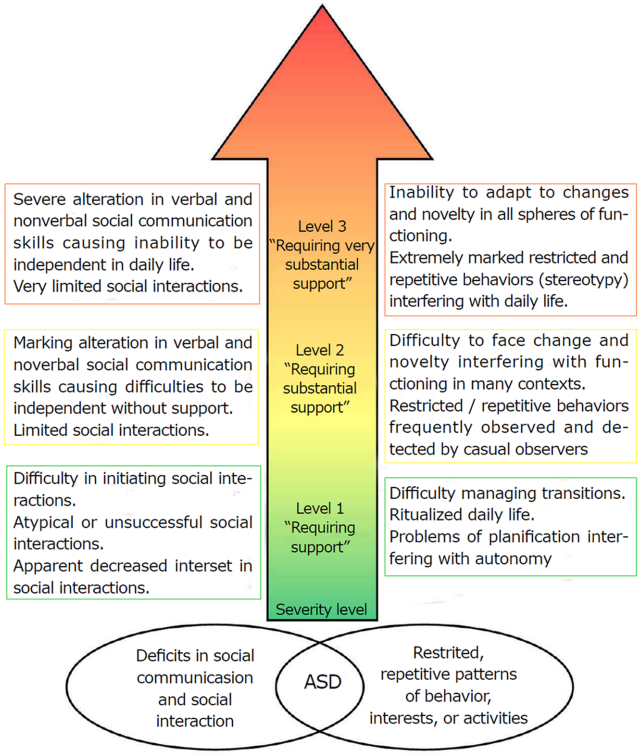


**Figure 1 Schematic representation of the major symptoms observed in autism spectrum disorders according to the degree of severity of the disease.** ASD: Autism spectrum disorders.

contradictory data suggest that more standardized protocol for diagnosing are required. The differences among these numbers in various countries may also be due to the lack of homogeneity that exists all around the world for diagnosing autism. Professionals agreed to homogenize diagnostic criteria. The main diagnostic guides *i.e.*, the DSM-5 edited by the American Psychological Association (APA) and the International Classification of Diseases 11th Revision (ICD-11) published by the World Health Organization (WHO) are considering this problem.

For several years, ASD is recognized as a public health problem, some people consider ASD as becoming epidemic and as a target for health policy. The support of people with ASD is costly; in fact, people with ASD need more medical examinations and drug prescriptions than most healthy people. An important proportion of children with ASD requires special educational services and some stay in health institution at adulthood. In 2014, in the United States, the total cost for children with ASD was estimated between 11.5 billion to 60.9 billion United States dollars per year[12].

### *Pathophysiology - Causes*

The pathophysiological origin of ASD has not been defined yet. Many factors have been suggested including genetic and environmental factors. These aspects are extensively detailed in a recent review[13]. What seems clear today is the fact that autism is not linked to a single origin. Autism can be associated with many factors, and among those, metabolic disorders can possibly increase the risk of the development of autism.

Some people with ASD also have other health problems, including anxiety and depression, epilepsy, attention deficit hyperactivity disorder (ADHD). In people with ASD, the intellectual level is extremely variable, ranging from profound impairment to higher levels.

In most cases, the etiology of ASD is not known, but a genetic factor, involving possibly 15 or more loci, is widely has been proposed for contributing to the development of ASD[14]. ASD traits are also found in patients affected by several genetic diseases such as Rett syndrome or Angelman syndrome[15].

The strong heterogeneity among individuals with ASD has limited the pure genetic implication[16].

Among potential environmental factors the role of perinatal factors was studied by Gardener *et al*[17], they performed a meta-analysis of the association between perinatal and neonatal factors and the risk of autism. In this study they described associations

between more than 60 potential perinatal risk factors and ASD[17].

Durkin *et al*[18] showed that the age of the parents is associated with the risk of ASD for the children. They noticed that the risk is even more pronounced for the elder children. Premature birth has been identified as a risk factor[19]. Prematurity (infants born at < 37 wk of gestation) and low birth weight (< 2500 g) have also been examined as a risk factor for the development of ASD, among several other potential risk factors[20]. Recently a few studies have associated ASD and pesticides: pesticide exposures during pregnancy is a risk factor for ASD[21].

Some people with ASD have metabolic disorders and/or health problems such as mitochondrial dysfunction and gastrointestinal abnormalities. More than thirty years ago, Coleman and Blass[22] suggested an abnormality in carbohydrate metabolism in individuals with ASD, several years later, it was proposed that ASD may be a disorder associated with an impairment in mitochondrial function[23]. Recently a meta-analysis examined the possible link between mitochondria and autism, the conclusions of this study are that it is not clear if mitochondrial dysfunction contributes to the development or pathogenesis of ASD or if mitochondrial dysfunction is just an epiphenomenon of ASD[14].

## CARNITINE

Carnitine occurs in two racemic forms: L- and D-carnitine. In the human body, only the L-isomer is present. L-carnitine is an amino acid derivative found in almost any cell in the body. When discovered, a century ago, L-carnitine was considered as a vitamin as it was shown that the development of a worm *(Tenebrio Molitor*) was dependent on L-carnitine. Several decades later, it was shown that mammals are able to synthesize L-carnitine and subsequently, L-carnitine was not considered as a vitamin anymore.

L-carnitine is mainly found in muscles where it plays a major role in the use of fatty acid for energy production and carnitine found in the human body can either come from an endogenous synthesis or from the foodstuffs.

### *L-carnitine biosynthesis*

L-carnitine biosynthesis is performed with 2 ultimate precursors: lysine and methionine and the enzymatic reactions involved in this synthesis requires several cofactors: vitamin C, iron, vitamin B6 and niacin (Figure 2).

The first step corresponds to the methylation of lysyl residues included in various proteins such as histones, cytochrome c or calmodulin. This reaction is catalyzed by enzymes known as protein lysyl methyltransferases. The product of this reaction is trimethyllysyl residues which are released from proteins by protein hydrolysis as free trimethyllysine (TML).

Subsequently, TML enters the mitochondria and interacts with the trimethyllysine hydroxylase (TMLD, encoded by the trimethyllysine hydroxylase epsilon gene: TMLHE) which converts TML into 3-hydroxy-N-trimethyllysine[24]. 3-hydroxy-N-trimethyllysine is then cleaved into gamma-trimethylaminobutyraldehyde, a reaction catalyzed by hydroxyl N-trimethyllysine aldolase (HTMLA)[25].

Gamma-trimethylaminobutyraldehyde is then dehydrogenated and forms gamma-butyrobetaine a reaction catalyzed by the 4-trimethylammoniobutyraldehyde dehydrogenase. Finally, L-carnitine is formed by the hydroxylation of gamma-butyrobetaine a reaction catalyzed by the gamma-butyrobetaine hydroxylase (BBOX1).

L-carnitine biosynthesis involves different organelles (the nucleus, the mitochondria, the peroxisome and the cytosol) in various tissues and organs: kidney, liver, brain, *etc*.[26]. Between 1 and 2 μmol of carnitine are synthesized/kg b.w. per day in a human body.

### *Dietary origin of carnitine*

L-carnitine is mainly present in meat and meat products, dairy and fishes provide also a significant amount of carnitine. Most fruits and vegetables are not riche in L-carnitine. An omnivorous diet brings about 50 to 100 mg of carnitine per day, 80% coming from meat while a vegetarian diet brings around 10 mg of carnitine/day.

For regular foods, L-carnitine bioavailability varies from 54% to 87%[27] and for dietary supplements, the bioavailability is only around 15%.

In Human, L-carnitine concentration in muscles is around 3 and 6 μmol of per gram making muscle the major reservoir for L-carnitine in the body, however, it has been shown that L-carnitine present in the muscle does exchange easily with the plasma and muscle is unable to synthesize carnitine and relies on L-carnitine synthesized elsewhere in the body or from the dietary carnitine. In contrast, L-carnitine level in the

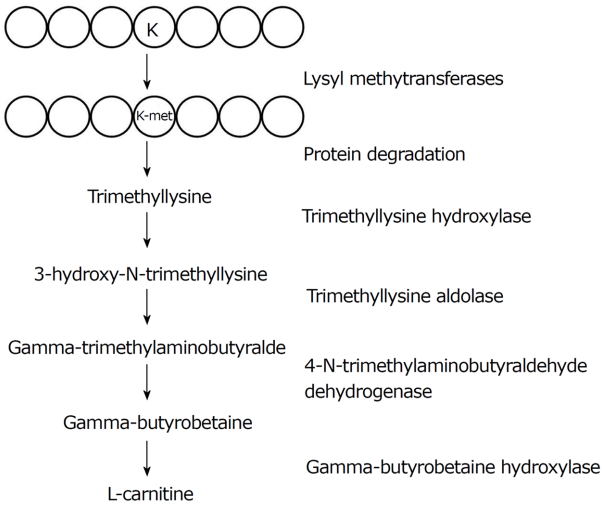


**Figure 2 Summarized biosynthetic pathway for L-carnitine.** The names of the enzymes are on the right part of the figure.

liver is much less than in the muscle (around 0.5 to 1 μmol of L-carnitine per g of tissue) but the hepatic carnitine can be quickly released in the plasma. High levels of L-carnitine are also found in the testes and the brain.

There is no evidence for a catabolism of L-carnitine in mammalian cells and L-carnitine is eliminated as it from the body in the urine.

### *Deficiency in human*

L-carnitine can be synthesized in several organs (liver, kidney, testis and brain), and can be provided by the foodstuffs. In the human body, carnitine is mainly found in muscles and as muscles are unable to synthesize carnitine they rely on an active transport across the sarcolemma to provide L-carnitine to muscle cells. L-carnitine transport across membrane is done by transporters. The major transporters for L-carnitine belongs to the organic cation transporter (OCTN) family. L-carnitine transport is done through the activity of three transporters: OCTN1 (SLC22A4) and OCTN2 (SLC22A5) in humans and animals and Octn3 (Slc22a21) in mice. A defect in OCTN2 is known to induce primary systemic carnitine deficiency (SCD) a defect that leads to alteration in beta-oxidation of long-chain fatty acids, causing various symptoms, such as myopathy, cardiomyopathy, fatty liver and male infertility[28].

Secondary carnitine deficiency is due to defects in other metabolic pathways or to drugs that impair intestinal or renal absorption of L-carnitine. The consequences of this deficiency are basically the same than those observed in primary deficiency.

### *Biochemical functions*

The major actions of carnitine are due to the fact that carnitine acts as a cofactor for a large family of enzymes: the carnitine acyltransferases. These enzymes are responsible for the esterification of carnitine with acyl groups, allowing the formation of acyl-carnitines. The carnitine acyltransferases are widely distributed in the cell. Carnitine acetyltransferase, is an enzyme that catalyzes the esterification of carnitine into short chain acyl-coenzyme A (acyl-CoA); this activity was described in different organelles: the cytosol, the mitochondrion, the peroxisome and the endoplasmic reticulum in various tissues: the heart, the brain, the kidney, the sperm cells and the liver[29]. Carnitine octanoyltransferase (CrOT) is an enzyme required for the peroxisomal metablism of very long-chain fatty acids and branched-chain fatty acids[30]. Carnitine palmitoyltransferase 1 (CPT 1) is an enzyme located on the outer mitochondrial membrane: it catalyzes the esterification of long chain acyl-coenzyme A to acyl-carnitine. Carnitine palmitoyltransferase 2 (CPT 2) is present in the inner mitochondrial membrane; it catalyzes the conversion of acyl-carnitine back to acyl-coenzyme A and together with CPT 1 and a transporter the carnitine acyl-carnitine translocase allows for the transport of acyl-Co across mitochondrial membranes. Once in the mitochondrial matrix, acyl-CoA can be used in several metabolic pathways: primarily the beta-oxidation.

**Mitochondrial metabolism of long chain fatty acids:** The mitochondrial metabolism of long chain fatty acids requires several steps: one of them is the entry of fatty acids inside the mitochondria. This transport is done through a system known as the

carnitine system. This 3-protein complex is composed of 2 enzymes: the carnitine palmitoyl transferases 1 and 2 and a transporter: the carnitine acyl-carnitine translocase (CACT). Together these 3 proteins allow the transfer of activated fatty acids from the cytosol to the mitochondrial membrane. In this process, L-carnitine is a key compound that is involved in all the steps of this pathway. Once in the mitochondria matrix, activated fatty acids can enter the beta oxidation pathway and generate energy[31].

**Mitochondrial acyl-CoA/free CoA ratio control:** Coenzyme A is one of the key compounds in cell physiology and many pathways use this cofactor. Coenzyme A may be present in the cell either as a free compound or bound to various molecules (*e.g*. acyls). Inside the cell, a stable equilibrium between free and acylated CoA exists but this equilibrium may be destabilized. To restore this equilibrium, several process can be activated, one involves L-carnitine. This process involves (1) an increase in acyl-carnitine synthesis which leads to (2) an increase in the mitochondrial beta oxidation. Together these two events induce an increase in the level of free coenzyme and restore the free/acylated coenzyme A ratio[32].

**Peroxisomal beta oxidation:** In the peroxisome; the β-oxidation of very long chain fatty acids leads to the formation of medium chain-acyl CoAs and acetyl CoA, and is sometimes considered as incomplete contrarily to the mitochondrial beta oxidation which leads to the formation of acetyl-coA only[31]. In the peroxisome, two enzymes dependent on L-carnitine are involve in the beta oxidation of fatty acids. These enzymes are the carnitine acetyltransferase and the carnitine octanoyltransferase. These enzymes seem to be necessary for the exit of medium-chain and short-chain acyl-CoAs from the peroxisomal matrix to the cytosol[30] but the precise role of these enzymes is not clearly defined yet.

**Acetylation of histones:** Acetylation and deacetylation of histones are crucial mechanisms for the regulation of the transcription. Acetylation requires available acetyl-CoA and it has been suggested that acetyl-carnitine formed in mitochondria[32] can enter the nucleus and be converted into acetyl-CoA and then be used for histone acetylation[33].

**Free radical production:** Free radicals may interact with various molecular species: lipids, nucleic acids or proteins leading to altered cell function. Several studies have proposed that a dietary supplementation in L-carnitine may exert a protective effect against the deleterious effects of free radicals[34], however, the mechanisms involved in this potential protective effect remain unclear[35].

## LINKS BETWEEN AUTISM AND CARNITINE

Although the spectrum of autism is thought to be highly heritable, and may result from multigene susceptibility interactions, no single gene has been identified that can adequately explain the disorder's complex heterogeneity and alarmingly increasing prevalence.

Researches carried out on this topic have pinpointed many genetic variations, including genes involved in carnitine biosynthesis and glutamatergic transmission, which may augment susceptibility to neurodevelopmental disorders like ASD.

The links between L-carnitine and autism rely on 3 major observations (1) the alteration of mitochondrial function occurring in patients with ASD. This aspect has been several times reviewed in the literature and will not be detailed in the present paper (for more information: see ref. [36]); (2) the relationships between L-carnitine levels and the severity of autism and (3) the genetic aspects of autism associated with L-carnitine metabolism. These two aspects are detailed in the present review.

### *L-carnitine level in ASD patients*

A few publications have looked at carnitine levels in patients with ASD and one single publication has studied the effect of a L-carnitine supplementation on ASD patients.

The study of Filipek *et al*[37] measured the level of total and free carnitine in control and ASD patients. They observed that total and free carnitine values were significantly reduced in the autistic individuals with a $P < 0.001$ and that more than 80% of ASD patients have total and free carnitine levels below the reference value. This information was also reported by another group in 2005[38]. Mostafa *et al*[38] also reported a decrease in L-carnitine levels in ASD patients. They described a decrease of almost 50% in L-carnitine levels in ASD individuals. They also reported decreases in lactate levels and poly unsaturated fatty acid levels. This study was done on a

significant number of individuals (30 control *vs* 30 ASD).

Besides this purely quantitative aspect, Frye *et al*[39] analyzed the acyl-carnitine profile of ASD patients, in a large cohort ($n$ = 213), these authors reported that 17% of children with ASD exhibited an elevation in short-chain and long-chain, but not medium-chain, acyl-carnitines. These authors indicated that this pattern of acyl-carnitine abnormalities is similar to what is observed in the brain of propionic acid rodent, model of ASD. This defect in L-carnitine levels is associated with mitochondrial dysfunction.

The acyl-carnitine composition was studied in a population of Chinese children with ASD. The goal of this publication was to identify a possible relation between the acyl-carnitine profile and the intelligence level. This study was carried out on 90 children: 60 with ASD and 30 control. The intelligence level was assessed using the Chinese Wechsler Young Children Scale of Intelligence (C-WYCSI). Blood analysis for L-carnitine derivatives showed that glutaryl carnitine and carnosyl carnitine were significantly decreased in ASD group and the authors of this publication suggested that those 2 compounds may be potential biomarkers for diagnosis of ASD. For these authors, these alterations indicate a potential mitochondrial dysfunction leading to an abnormal fatty acid metabolism in children with ASD[40].

L-carnitine was used as a potential treatment for patients diagnosed with autism. In the study published by Fahmy, 30 children diagnosed with autism (median age 69 mo, ranging from 29 mo to 103 mo) were randomly allotted into either the placebo ($n$ = 14) group or the group receiving 100 mg/kg bodyweight per day of liquid L-carnitine ($n$ = 16) for 6 mo. Several parameters were analyzed in these children: parameters associated with autism such as the childhood autism rating scale (CARS) form and parameters related to carnitine such as free and total carnitine levels. The results presented by the authors revealed a significant improvement in CARS scores in patients receiving L-carnitine, this improvement was associated with an increase in total and free carnitine levels. The authors concluded about (1) the good tolerance of the treatment; and (2) the improvement of autism severity in patient treated for 6 mo with L-carnitine, they also concluded that subsequent studies will be welcome[41].

In conclusion, the analysis of studies carried out on patients with autism for parameters related to L-carnitine suggest that L-carnitine may be lowered in patients with ASD. Furthermore, a L-carnitine supplementation seems to improve the symptoms of autism in patients. However, the relatively low number of publications describing these parameters implies some moderation.

### *Carnitine synthesis defect and autism*

With the overall goal to identify exonic copy number variants in the genome of ASD patients, Celestino-Soper and coauthors[42] analyzed 3743 samples for detecting disease-causing copy number variants (CNVs) that are not detected by most techniques used in conventional research and clinical diagnosis laboratories. This was done on 297 samples from 99 trios (probands with ASD, mother, father). Fifty-five potentially pathogenic CNVs were identified and validated and in a male proband, an exonic deletion of the TMLHE.

Using genome-wide chromosomal analysis methods, Celestino-Soper and coauthors[43] identified 55 potentially pathogenic copy number variants, among those and in the male samples, a deletion of exon 2 of the TMLHE gene (trimethyllysinehydroxylase epsilon) was found, this gene encodes the first enzyme in the biosynthesis of carnitine which is located in mitochondria. The lack of this enzyme leads to a decrease in plasmatic levels of 3-hydroxy-6-N-trimethyllysine and γ-butyrobetaine and in an increase in 6-N-trimethyllysine concentration in the plasma.

In the same time, the entire chromosome X exome was analyzed by next-generation sequencing in 12 unrelated families with ASD affected males. Thirty-six possibly deleterious variants were found located in 33 candidate genes. Among those a mutation in TMLHE, was identified in two brothers with autism. The screening of the TMLHE coding sequence in 501 male patients with ASD allowed the identification of 2 additional missense substitutions. These mutations were shown to induce a loss-of-function and led to an increase in trimethyllysine in the plasma of patients[44].

Based on these observations, a 4-year-old male with a mutation in the TMLHE gene and developing an autism spectrum disorder was supplemented with L-carnitine (200 mg/kg per day). In this young patient, the levels of carnitine were very low. The authors reported that two weeks after the initiation of the treatment, his family reported "noticeable increases in language, non-verbal expression, and engagement with others"[45]. The evolution was positive as the patient's regression ended and even started progressing. In the same time, the levels of carnitine in the plasma increased. Under such circumstances (with a TMLHE deficit) a supplementation in L-carnitine seemed efficient.

More recently, another potential mechanism involving the role of carnitine in the

onset of autism has been propose, it implies a defect in the transport of L-carnitine into the cells. The amino acid transporter SLC7A5, also able to transport carnitine, has been recently shown to be associated with the onset of autism[46]. The same transporter seems also to be implicated in the metabolism of drugs such as Risperidone given for limiting the symptoms in ASD patients. Depending on the polymorphism of the gene, the drug can be differently catabolized[47]. One might notice that the transporter SLC7A5 is not purely a transporter of carnitine but a protein able to transport various amino acids.

## CONCLUSION

A few aspects in the relation between carnitine and autism should be highlighted: (1) Low plasma carnitine is reported in autism, but not systematically. Furthermore, it seems that if some affected infants have low plasma carnitine during the early childhood, their plasma carnitine return to normal when measured a few years later[48]; (2) Typically, L-carnitine levels are measured in the plasma, but it is very likely that the important levels of L-carnitine for brain development should be measured in the brain. And little to no information are available on these values; and (3) In any cases, not all patients with ASD have altered levels of carnitine.

In conclusion, some patients with ASD might have L-carnitine synthesis disorders (around 10%-20%) and for these patients, a supplementation with L-carnitine is beneficial. It is still remaining 80%-90% of the patients who have no L-carnitine synthesis or transport defects and for whom the origin of the disease should be find elsewhere.

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*MINIREVIEWS*

# Last decade update for three-finger toxins: Newly emerging structures and biological activities

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## Abstract

Three-finger toxins (TFTs) comprise one of largest families of snake venom toxins. While they are principal to and the most toxic components of the venoms of the *Elapidae* snake family, their presence has also been detected in the venoms of snakes from other families. The first TFT, α-bungarotoxin, was discovered almost 50 years ago and has since been used widely as a specific marker of the α7 and muscle-type nicotinic acetylcholine receptors. To date, the number of TFT amino acid sequences deposited in the UniProt Knowledgebase free-access database is more than 700, and new members are being added constantly. Although structural variations among the TFTs are not numerous, several new structures have been discovered recently; these include the disulfide-bound dimers of TFTs and toxins with nonstandard pairing of disulfide bonds. New types of biological activities have also been demonstrated for the well-known TFTs, and research on this topic has become a hot topic of TFT studies. The classic TFTs α-bungarotoxin and α-cobratoxin, for example, have now been shown to inhibit ionotropic receptors of γ-aminobutyric acid, and some muscarinic toxins have been shown to interact with adrenoceptors. New, unexpected activities have been demonstrated for some TFTs as well, such as toxin interaction with interleukin or insulin receptors and even TFT-activated motility of sperm. This minireview provides a summarization of the data that has emerged in the last decade on the TFTs and their activities.

**Key words:** Three-finger toxins; Snake; Venom; Structure; Biological activity



**Core tip:** The three-finger toxins (TFTs) of snake venoms are principal to and the most toxic components of elapid venoms. Over 700 TFT amino acid sequences are listed in the UniProt Knowledgebase currently, with new members added constantly. The past decade has also seen multitudinous new discoveries, including structural variations in

TFTs (*i.e.* disulfide-bound dimers), new types of biological activities for the well-known TFTs (*e.g.*, α-bungarotoxin's inhibition of ionotropic receptors of γ-aminobutyric acid), and other new, unexpected activities for the TFTs (*i.e.* interaction with interleukin or insulin receptors and activation of sperm motility). This minireview provides an up-to-date overview of these data.

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## INTRODUCTION

Three-finger toxins (TFTs) form an abundant family of nonenzymatic proteins found in snake venoms[1]. The TFTs were so-named according to their characteristic spatial structure, in which three loops (fingers) protrude from the central core, stabilized by four conserved disulfide bonds. The TFTs contain from 57 to 82 amino acid residues, with some toxin types having an extra fifth disulfide bond, located in either their central loop II or N-terminal loop I. The position of the bond affects the toxin's biological activity.

The TFTs manifest a wide array of biological activities, ranging from selective interaction with certain receptor types to nonselective cell lysis[2]. Typically, the TFTs represent the main components of elapid venoms[3]. Thus, in the venom of the desert coral snake *Micrurus tschudii*, 95% of toxins are represented by TFTs[4]. Their presence has also been detected in the venoms of snakes from other families. For example, TFTs were found in different snake genera from colubrid family[5,6]. The TFTs also represent one of the largest families of snake toxins, having more than 700 TFT amino acid sequences deposited in the UniProt Knowledgebase free-access database. Intriguingly, nontoxic structural counterparts of the TFTs have been found in animal organisms, namely the lymphocyte antigen 6 (Ly6) proteins. Based on their commonality of three-finger folding, the TFTs and Ly6 proteins are combined into one Ly6/neurotoxin family[7].

The first TFT discovered, α-bungarotoxin (α-Bgt), was published almost 50 years ago[8]. Since then, α-Bgt has become a widely used specific marker of the α7 and muscle-type nicotinic acetylcholine receptors (nAChRs). In addition, a tremendous number of other TFTs have been discovered, and new members of this family possessing original structures and biological activities are emerging constantly. Moreover, new activities have been recognized for the well-known TFTs, and the discovery of new activities for both the new and the well-known TFTs may be regarded as a recent trend in toxinology.

This minireview briefly summarizes the data obtained for TFTs during the last decade (Table 1). The toxins with new structural features appearing in the recent literature are considered herein; those that have garnered the most interest is the covalently-bound TFT dimers. In addition, the recently discovered uncommon biological activities of some TFTs are discussed; these include the so-called mambalgins that exert a potent analgesic effect upon central and peripheral injection and represent the most remarkable discovery of late.

## RECENTLTY-DISCOVERED TFTS WITH NEW STRUCTURAL FEATURES

Ten years ago, the first data revealing covalently-bound TFT dimers were published[9]. Disulfide-bound dimers of TFTs, including heterodimers formed by α-cobratoxin (α-CTX; a long-chain α-neurotoxin) with different cytotoxins and the homodimer of α-CTX were isolated from the *Naja kaouthia* cobra venom. Determination of the homodimer crystal structure allowed identification of the intermolecular disulfides formed by Cys3 in the protomer one and Cys20 in the protomer two, and other way round (Figure 1A). All other disulfides in protomers had the same pairing as in natural α-CTX[10].

The dimerization itself strongly affected the biological activity of the original

**Table 1 Novel three-finger toxin biological effects and their potential applications**

| | Toxin | Effect/Target | Potential application |
|---|---|---|---|
| Impact on signal transduction | Mambalgins | Inhibitors of ASICs | Analgesics |
| | Micrurotoxins | Modulators of GABAA receptor | Biochemical instruments for the GABAA receptor study |
| | α-Neurotoxins | Inhibitors of GABAA receptor | |
| | Muscarinic toxin MTα | Antagonist of α2B-adrenoceptor | Treatment of blood pressure disorders |
| | Toxin CM-3 | Interaction with α1A-adrenoceptor | |
| | Toxin AdTx1 (ρ-Da1a) | Specific and selective inhibitor for the α1A-adrenoceptor | |
| | Toxin ρ-Da1b | Antagonist of α2A-adrenoceptors | |
| | Toxin Tx7335 | Potassium channel activator | Biochemical instrument for the study of potassium channels |
| | Calliotoxin (δ-elapitoxin-Cb1a) | Activator of voltage-gated sodium channel | Biochemical instrument for the study of sodium channels |
| Impact on blood coagulation | Toxin KT-6.9 | Inhibitor of platelet aggregation | Treatment of blood coagulation disorders |
| | Ringhalexin | Inhibitor of FX activation | |
| | Exactin | Inhibitor of FX activation | |
| Insulinotropic activity | Cardiotoxin-I | Induction of insulin secretion from β-cells | Treatment of type 2 diabetes |
| Impact on sperm motility | Actiflagelin | Activator of sperm motility in vitro | Infertility treatment |

ASIC: Acid sensing ion channel; GABAA: Type A receptor of gamma-amino butyric acid; FX: Factor X.

toxins, with the cytotoxic activity of cytotoxins within dimers being completely abolished. However, the dimers were found to retain most of the α-CTX capacity to interact with *Torpedo* and α7 nAChRs as well as with the *Lymnea stagnalis* acetylcholine-binding protein. Moreover, in contrast to the α-CTX monomer, the α-CTX dimer acquired the capacity to interact with α3β2 nAChR, similar to that seen with κ-bungarotoxin (κ-Bgt), a dimer with no disulfides between its monomers (Figure 1D). Collectively, these data show that dimerization of three-fingered neurotoxins is essential for binding to heteromeric α3β2 nAChRs.

In 2009, from venom of the brown cat snake *Boiga irregularis* a new heterodimeric TFT, irditoxin, was isolated[11]. Irditoxin spatial structure determined by X-ray analysis displayed two subunits possessing a three-finger fold, characteristic for nonconventional toxins (Figure 1B). The subunits in the irditoxin dimer are connected by an interchain disulfide bond, formed by extra cysteine residues present in each subunit. In contrast to the dimeric toxins discussed above, irditoxin does not inhibit mouse neuronal α3β2 and α7 nAChRs. However, irditoxin is a bird- and reptile-specific postsynaptic neurotoxin which inhibits the chick muscle nAChR three orders of magnitude more efficiently than the mouse receptor. *In vivo*, it was lethal to birds and lizards and was nontoxic toward mice[11].

Covalently-bound dimers are undoubtedly the most interesting TFT posttranslational modification recently found. A new TFT forming a noncovalent dimer was discovered recently as well; this neurotoxin, haditoxin, was isolated from the venom of king cobra *Ophiophagus hannah*[12]. The high-resolution X-ray analysis revealed that haditoxin is a homodimer (Figure 1C), with a structure very similar to that of κ-Bgt (Figure 1D). However, in contrast to κ-Bgt, the amino acid sequences of the monomeric subunits of haditoxin correspond to those of α-neurotoxins of the short-chain type. It should be noted that κ-Bgt targets the neuronal α3β2 and α4β2 nAChRs and α-neurotoxins of the short-chain type block the muscle-type nAChRs only, while haditoxin demonstrated new pharmacological features, being antagonist toward muscle (αβγδ) and neuronal (α7, α3β2, and α4β2) nAChRs, and having the highest affinity ($IC_{50}$ 180 nmol/L) for α7 nAChRs[12].

The above data indicate that TFT dimerization may underlie the capacity for interaction with neuronal nAChRs.

The disulfide bonds play an essential role in maintaining the spatial structure of TFTs, and their pairing pattern is largely conserved. However, an unusual disulfide bond scaffold was found in a TFT isolated from the venom of eastern green mamba *Dendroaspis angusticeps*[13]. This toxin, named Tx7335, has the highest amino acid sequence similarity to the TFTs of nonconventional type, but differs from them in the number and positions of cysteines (Figure 2). Similar to α-neurotoxins of the short-chain type, Tx7335 has only eight cysteines, while nonconventional toxins have ten

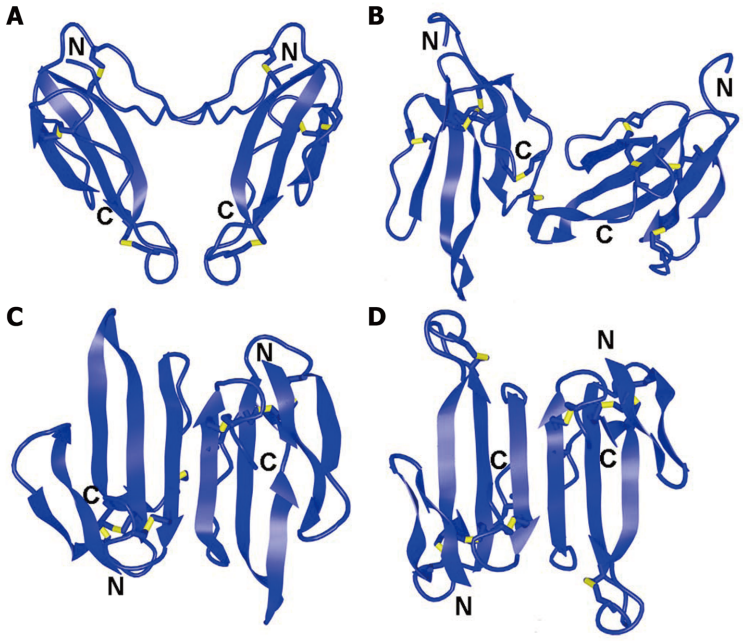


**Figure 1 Spatial structures of dimeric three-finger toxins.** A: Homodimer of α-cobratoxin, Protein Data Bank Identification code (PDB ID): 4AEA; B: Irditoxin, PDB ID: 2H7Z; C: Haditoxin, PDB ID: 3HH7; D: κ-bungarotoxin, PDB ID: 1KBA. Disulfide bonds are shown in yellow. N and C indicate N- and C-terminus, respectively.

cysteines. Furthermore, Tx7335 contains a tyrosine residue at position 43, which is occupied by a cysteine in all TFTs, while a cysteine is found at position 25. In most other TFTs this position is occupied by a tyrosine (Figure 2). According to structure modeling, Cys55 may make a disulfide bond with Cys25; due to the spatial proximity of these residues no major distortion in the three-finger structure occurs. This novel arrangement of disulfides may explain the unique functional effects observed for Tx7335. Indeed, it has been shown to activate the bacterial pH-gated potassium channel KcsA by a dose-dependent increase in both mean open time and open probability. Moreover, Tx7335 binds at the KcsA extracellular domain at a site probably different from that of channel inhibitors[11].

Several new TFTs retaining the classical arrangement of disulfide bonds but possessing novel structural features have been identified recently. So, from the venom of black mamba *Dendroaspis polylepis polylepis*, a non-typical long-chain TFT, α-elapitoxin-Dpp2d (α-EPTX-Dpp2d), was isolated[14]. α-EPTX-Dpp2d contains an amidated C-terminal arginine, a posttranslational modification that had not been observed before in snake TFTs. Biological activity studies showed that, at a 1 μmol/L concentration, the α-EPTX-Dpp2d potently inhibited neuronal α7 ($IC_{50}$ 58 nmol/L) and muscle-type nAChRs ($IC_{50}$ 114 nmol/L) however showed no effect on α3β2 and α3β4 receptors. Therefore, this amidation was deemed to have no significant effect on the toxin's selectivity, as the activity profile is fairly similar to that of the classic long-chain TFTs with a free carboxyl termini[14].

It was shown that the earlier characterized TFT Oh9-1[15] from *Ophiophagus hannah* venom may represent a new group of competitive nAChR antagonists, known as the Ω-neurotoxins[16]. Electrophysiology experiments on *Xenopus* oocytes showed that Oh9-1 inhibited rat muscle-type α1β1εδ (adult, $IC_{50}$ 3.1 μmol/L) andα1β1γδ (fetal, $IC_{50}$ 5.6 μmol/L) and rat neuronal α3β2 nAChRs ($IC_{50}$ 50.2 μmol/L), but manifested low or no affinity for other human and rat neuronal subtypes. Interestingly, Oh9-1 potentiated the human glycine receptor (homopentamer composed of α1 subunits), with activity increase by about 2-fold. Alanine-scan mutagenesis showed a novel mode of interaction with the ACh binding pocket of nAChRs *via* a set of functional amino acid residues that are different from those in the classical α-neurotoxins. Herewith, the central loop of Oh9-1 interacts with α1β1εδ nAChR by both sides of the β-strand, while only one side of the β-strand interacts with the α3β2 receptor[16].

The taxon-specific dimeric TFT irditoxin, isolated from a rear-fanged snake, was discussed above. Another taxon-specific TFT, fulgimotoxin, was isolated from the venom of the rear-fanged green vine snake *Oxybelis fulgidus*[17]. This toxin is a monomer and contains five disulfides, typical for the nonconventional TFTs. It is highly neurotoxic to lizards; however, mice are unaffected. Similar to other colubrid TFTs, fulgimotoxin has an extended N-terminal amino acid sequence and a


**Figure 2 Alignment of amino acid sequence of toxin Tx7335 with those of nonconventional toxins.** Cysteine residues are marked in yellow. Black lines indicate the locations of the typical disulfide bond in nonconventional toxins; red line indicates the unusual disulfide bond 25-55 in Tx7335. 3NOJ_DENAN: Toxin Tx7335 from *Dendroaspis angusticeps* (Eastern green mamba); 3NOJ_BUNCA: Bucandin from *Bungarus candidus* (Malayan krait); 3NOJ6_DENJA: Toxin S6C6 from *Dendroaspis jamesoni kaimosae* (Eastern Jameson's mamba); 3NOJ_WALAE: Actiflagelin from *Walterinnesia aegyptia* (desert black snake).

pyroglutamic acid at the N-terminus.

The longest TFT, the nonconventional toxin BMLCL, consisting of 82 amino acid residues and five disulfide bridges, was identified in *Bungarus multicinctus* venom[18]. Earlier studies of biological activity revealed no interaction with the muscarinic acetylcholine receptors (mAChRs) M1 and M2 nor with the muscle-type nAChR[19]. However, recent studies showed that BMLCL interacted efficiently with both α7 ($IC_{50}$ 43 nmol/L) and muscle-type nAChR ($IC_{50}$ 31 nmol/L)[19]. Thus, the longest TFT functions as an antagonist of nAChRs.

It should be noted that so far, no TFTs have been found in the venoms of snakes from the *Viperidae* family; however, transcripts encoding these toxins were identified in venom gland transcriptomes of several *Viperidae* species. To address the question of biological activity of *Viperidae* TFTs, two toxins were obtained by heterologous expression in *Escherichia coli*. Based on the nucleotide sequences of cDNA encoding TFTs in the venom glands of vipers *Azemiops feae* and *Vipera nikolskii*, the corresponding genes optimized for bacterial expression were synthesized[20]. Expressed *A. feae* TFT (TFT-AF) and *V. nikolskii* TFT (VN-TFT), both of the nonconventional type, were refolded under the conditions elaborated on earlier for cobra TFTs. The biological activity of the toxins obtained was studied by electrophysiological techniques, calcium imaging, and radioligand analysis. Both toxins inhibited neuronal α3-containing and muscle-type nAChRs in the micromolar concentration range, but they were each very weak antagonists of neuronal α7 nAChRs. Thus, viper TFTs can function as antagonists of nAChRs of neuronal and muscle-type[20].

## RECENTLY DISCOVERED TFTS WITH NEW BIOLOGICAL ACTIVITIES

### *Novel TFTs affecting signal transduction*

The most fascinating biological activity of TFTs discovered during the last decade is their capacity to interact with acid-sensing ion channels (ASICs). ASICs are proton activated and $Na^+$-selective ion channels, widely distributed throughout the peripheral and central nervous systems (CNS) in vertebrates. ASICs take part in an array of physiological processes, from synaptic plasticity and neurodegeneration to pain sensation. Therefore, the finding of new regulatory modes for these proteins opens up a new avenue of research for pain management, as well as for addiction or fear.

The new mambalgins class of TFTs has been characterized as potent, rapid and reversible inhibitors of ASICs, based on studies with the protein from African black mamba (*Dendroaspis polylepis*) venom[21]. The mambalgins are composed of 57 amino acids and eight cysteine residues, and have about 50% amino acid sequence identity to other snake TFTs. While mambalgins were found to be nontoxic in mice, they were found to exert a potent analgesic effect, as strong as that of morphine but causing much less tolerance than morphine and no respiratory distress. Pharmacological studies showed that mambalgins produce their analgesic effect through the blockade of heteromeric channels containing ASIC1a and ASIC2a subunits in CNS and of channels including ASIC1b subunit in nociceptors. Mambalgins were also shown to inhibit heteromeric channels including ASIC1a and ASIC1b subunits, homomeric rodent and human ASIC1a channels and homomeric rodent ASIC1b channels, the $IC_{50}$s being in the range from 11 nmol/L to 252 nmol/L[22,23].

The structure of an ASIC1a–mambalgin-1 complex was determined by cryoelectron microscopy at a resolution of 5.4 Å[24]. The data obtained showed that mambalgin-1

binds precisely to the thumb domain of ASIC1a but not to the acid-sensing pocket, as suggested earlier[25]. However, mambalgin-1 binding induced conformational changes in the thumb domain of the channel, which may disturb the sensing of an acidity in ASIC1a[24]. The structural data obtained might provide a structural basis for further development of ASIC modulators.

No less significant than the discovery of mambalgins was the finding of TFTs that interact with ionotropic GABA receptors ($GABA_A$). Almost simultaneously, three research groups found that snake TFTs were able to bind $GABA_A$ receptors[26-28]. Thus, two TFTs, called micrurotoxin 1 (MmTX1) and 2 (MmTX2), were isolated from Costa Rican coral snake (*Micrurus mipartitus*) venom and sequenced[26]. It was shown that at subnanomolar concentrations MmTX1 and MmTX2 increased receptor affinity for the agonist by binding to allosteric site, and thus potentiated opening and macroscopic desensitization of the receptor. The authors suggested that at the molecular level, the α+/β− subunit interface might be involved in toxin action. When injected into mouse brain, both toxins evoked seizures against the background of reduced basal activity[26]. The discovery of toxins enhancing $GABA_A$ receptor sensitivity to agonist established a new class of ligands for this receptor family.

In 2006, it was shown that α-Bgt, a classical blocker of α7 and muscle-type nAChRs, binds to and blocks $GABA_A$ receptors containing the interface of β3/β3 subunit[29]. No effects were observed for α-Bgt on heterooligomeric $GABA_A$ receptors which contain α-, β- and γ-subunits or α-, β- and δ-subunits. However, recently, two research groups independently showed that α-Bgt and some other TFTs could bind to recombinant and native $GABA_A$ receptors[27,28]. Both electrophysiology experiments and fluorescent measurements with α-Bgt coupled to Alexa-Fluor 555 revealed the highest toxin affinity to α2β2γ2 receptor subtype[27]. GABA reduced fluorescent labeling by α-Bgt, suggesting that the α-Bgt binding site overlaps the GABA binding site at the interface of β/α subunits[27].

Binding at the β/α subunit interface was demonstrated for the long-chain α-neurotoxin α-CTX[28], and this toxin interacted more efficiently with the $GABA_A$ receptor than α-Bgt. Electrophysiology experiments showed mixed competitive and noncompetitive α-CTX action, with highest affinity of this toxin being to the α1β3γ2 receptor ($IC_{50}$ 236 nmol/L). Other receptor subtypes were inhibited less potently, as follows: α1β2γ2 ≈ α2β2γ2 > α5β2γ2 > α2β3γ2 and α1β3δ. Among the several TFTs studied, the long α-neurotoxins Ls III (*Laticauda semifasciata*) and neurotoxin I (*Naja oxiana*) as well as the nonconventional toxin WTX (*Naja kaouthia*) interacted with the $GABA_A$ receptor. These data demonstrate that $GABA_A$ receptors are a target for diverse TFTs, including the very well-studied α-Bgt and α-CTX.

Among the vast variety of TFTs there is a class of toxins that interact with mAChRs, which are G-protein coupled receptors (GPCRs)[30]. For many years, the mAChRs were thought to be the only GPCRs affected by TFTs; however, over the last decade, several TFTs capable of interacting with other GPCRs, namely adrenoreceptors of different types, were reported.

It was shown that muscarinic toxin α (MTα) was a more potent antagonist for the $\alpha_{2B}$-adrenoceptor than for mAChR[31]. MTα inhibited the $\alpha_{2B}$-adrenoceptor, but did not affect the $\alpha_{2A}$-, $\alpha_{2C}$-, $\alpha_{1A}$- or $\alpha_{1B}$-adrenoceptors. In ligand binding experiments, MTα superseded the radioligand efficiently ($IC_{50}$ 3.2 nmol/L) and decreased the maximum binding without any influence on the radioligand affinity, demonstrating a noncompetitive inhibition mode[31]. The study of another MT, MTβ, showed nonselective low affinity interaction with the five muscarinic receptor subtypes[32]. Study of the toxin CM-3 (having undefined biological function to date) and MTβ demonstrated high efficacy for α-adrenoceptors and particularly a subnanomolar affinity for the receptor of $\alpha_{1A}$-subtype[32]. Both toxins were isolated more than 20 years ago from the venom of the African mamba *Dendroaspis polylepis*[33,34]. No or very weak affinity of these toxins were found for muscarinic receptors in the work of Blanchet *et al*[32].

Targeted searches for toxins interacting with α-adrenoceptors have yielded novel information in the last decade. The interactions of fractions obtained from green mamba (*Dendroaspis angusticeps)* venom with $\alpha_1$-adrenoceptors were tested in binding experiments using $^3$H-prazosin as a radioligand[35]. A new TFT inhibitor, AdTx1 (renamed later as ρ-Da1a[36]), comprising 65 amino acid residues with four disulfide bridges, was found. ρ-Da1a showed subnanomolar affinity with $K_i$ of 0.35 nmol/L and demonstrated high specificity for the human adrenoceptor of $\alpha_{1A}$-subtype. Interestingly, the biological activity profile of ρ-Da1a appeared very similar to those of MTβ and CM-3; however, these latter two toxins interacted more potently than ρ-Da1a with $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptor subtypes[32]. ρ-Da1a was, thus, characterized as a specific and selective peptide inhibitor for the $\alpha_{1A}$-adrenoceptor, acting as a potent relaxant of smooth muscle[35].

Using a similar targeted screening approach, but with application of $^3$H-

rauwolscine as a radioligand, the effects of venom fractions obtained from green mamba on $\alpha_2$-adrenoceptors from rat brain synaptosomes were studied[37]. A novel TFT, ρ-Da1b, comprising 66 amino acid residues with four disulfide bridges was isolated. It inhibited binding of $^3$H-rauwolscine to the three $\alpha_2$-adrenoceptor subtypes by 80% with affinity in the range of 14-73 nmol/L and with Hill coefficient of about unity. Furthermore, calcium imaging experiments on human $\alpha_{2A}$-adrenoceptors expressed in mammalian cells showed that ρ-Da1b was an antagonist of this adrenoceptor type[37].

The structural scaffold of aminergic TFTs that are known to interact with various α-adrenergic, muscarinic and dopaminergic receptors was used to generate experimental toxins with new functions. Specifically, the ancestral protein resurrection methodology was applied to identify the functional substitutions that might happen during evolution, and then utilize them for molecular design[38]. Six variants of ancestral toxin (AncTx, 1-6) were generated, and their biological activity was studied. AncTx1 was found to be the toxin possessing to date the highest selectivity to $\alpha_{1A}$-adrenoceptor. AncTx5 was the strongest inhibitor for $\alpha_2$-adrenoceptor of the three subtypes. The toxin ρ-Da1a affinities for the $\alpha_1$- and $\alpha_{2C}$-adrenoceptor subtypes were modulated most strongly by amino acids at positions 28, 38 and 43 in the evolutionary pathway[38]. Thus, this molecular engineering study represents the first successful attempt to engineer more potent aminergic TFTs.

Among the snake venoms, the mamba ones are unique in their variety of toxins affecting signal transductions[39]. A multitude of toxins capable of disturbing the different stages of cholinergic and adrenergic (see above paragraphs) transmission have been isolated from these venoms. Several toxins affecting voltage-gated ion channels have been isolated as well. The very recently discovered TFT Tx7335, in eastern green mamba *Dendroaspis angusticeps* venom, interacts with the KcsA potassium channel[13]. The unusual structure of this toxin was discussed above. Interestingly, Tx7335 is a channel activator but not an inhibitor, as evidenced by its ability to increase in a dose-dependent mode both mean open times and open probabilities of KcsA incorporated in artificial bilayers; yet, the Tx7335 binding site on KcsA is distinct from that of the canonical pore-blocker toxins. The authors of this study suggested that the toxin allosterically reduced inactivation of KcsA that results in increase of potassium flow through the channel[13].

Blue coral snake *Calliophis bivirgatus* belong to the Elapidae family of snakes the neurotoxic venoms of which typically produce the flaccid paralysis. However it was shown that the *C. bivirgatus* venom uniquely produced spastic paralysis[40]. The toxin producing this paralysis was isolated and called calliotoxin (protein name: δ-elapitoxin-Cb1a). Although calliotoxin is a TFT, it has low amino acid sequence similarity to the other known toxins. It comprises 57 amino acid residues with four disulfide bridges in the classical scaffold. Biological activity studies using HEK293 cells heterologously expressing $Na_V1.4$ showed that the voltage-dependence of channel activation was shifted to more hyperpolarized potentials by calliotoxin. It inhibited inactivation and produced significant ramp currents. These data conformed with profound effects of calliotoxin on contractile force in preparation of isolated skeletal muscle. Thus, calliotoxin represents a functionally novel class of TFTs and is the first activator of voltage-gated sodium channel purified from snake venoms[40].

### Novel TFTs affecting blood coagulation

TFTs affecting blood coagulation are not so numerous as those affecting signal transduction. Nevertheless, a new member of the TFT family that is capable of influencing different stages of blood coagulation appeared recently. TFTs inhibiting both primary and secondary hemostasis have been reported.

Primary hemostasis involves platelets, which immediately form a plug at the site of injury. A novel TFT which inhibits the human platelet aggregation process in a dose-dependent manner was purified from cobra *Naja kaouthia* venom and named KT-6.9[41]. KT-6.9 was shown to inhibit platelet aggregation induced by adenosine diphosphate (ADP), thrombin and arachidonic acid but not by collagen and ristocetin. It was 25-times more active than the antiplatelet drug clopidogrel. Based on the data showing significant inhibition (70%) of the platelet aggregation induced by ADP, the authors suggested toxin binding to ADP receptors located on the platelet surface[41].

As for secondary hemostasis, two TFTs capable of inhibiting the extrinsic tenase complex (ETC) were purified from the venom of African ringhals cobra *Hemachatus haemachatus*[42,43]. ETC activates conversion of factor X (FX) to factor Xa (FXa) and represents an important target for the development of novel anticoagulants. A novel TFT anticoagulant, ringhalexin (the ringhals extrinsic tenase complex inhibitor) was shown to inhibit FX activation with an $IC_{50}$ of 123.8 nmol/L[42]. As an inhibitor of mixed type, on chick biventer cervicis muscle preparations ringhalexin manifested an irreversible weak neurotoxicity. The amino acid sequence of ringhalexin is 94%

identical to that of NTL2, an uncharacterized neurotoxin-like protein from *Naja atra*. X-ray crystallography of ringhalexin revealed a typical three-finger structure stabilized by four conserved disulfide bridges[42].

Another novel anticoagulant TFT from *Hemachatus haemachatus* venom, called exactin, can specifically and potently inhibit the activation of FX by ETC ($IC_{50}$ 116.49 nmol/L), similar to ringhalexin[43]. It is also a mixed-type inhibitor of ETC and weakly inhibits FX activation by intrinsic tenase complex ($IC_{50}$ 4.05 μmol/L) and prothrombin activation by prothrombinase complex ($IC_{50}$ 17.66 μmol/L). In contrast to other TFT anticoagulants that are structurally similar to snake cytotoxins, exactin manifests structural similarity to postsynaptic neurotoxins. It also has 82% identity to the weak toxin CM1b from *H. haemachatus* venom and 58% identity to a number of *Ophiophagus hannah* neurotoxins, including the Ω-neurotoxin Oh9-1 discussed above.

### *Novel TFTs with unexpected biological activities*

The last decade has also seen the discovery of several new TFTs possessing quite unusual biological activities.

TFTs, being structurally well defined, thermally stable and resistant to proteolysis, are very good subjects for directed evolution. When a randomization scheme was applied to α-neurotoxin amino acid residues in the loops involved in binding with nAChRs, followed by the cDNA display screening method, new modulators of the interleukin-6 receptor (IL-6R) were obtained[44]. The proteins obtained possessed nanomolar affinity and high specificity for IL-6R. The IL-6-dependent cell proliferation assay revealed both antagonists and agonists in the protein pool. Application of the size minimization procedure resulted in proteins with the molecular mass of about one-third of the original toxin; no significant loss of activities was observed. Moreover, the loops important for function were identified[44]. In another work by the same group, directed evolution was applied to produce a trypsin inhibitor based on the TFT scaffold[45]. The DNA sequences converged after seven rounds of selection. The recombinant proteins obtained were good inhibitors s of trypsin ($K_i$ of 33-450 nmol/L). Three groups of proteins had $K_i$ values close to those of soybean trypsin inhibitor and bovine pancreatic trypsin inhibitor. Two proteins inhibited chymotrypsin and kallikrein as well. The authors suggested that the technique developed may be widely applied for the targeted generation of different regulatory molecules based on the TFT motif[45].

Studies of a TFT cardiotoxin showed a quite unexpected effect on insulin secretion. The fractions of cobra *Naja kaouthia* venom obtained by combination of ultrafiltration and reversed-phase high-performance liquid chromatography were screened for insulinotropic activity using the rat INS-1E β-cell line[46]. Only one fraction of the total 22 obtained induced secretion of insulin from the INS-1E cells with no influence on cell integrity and viability. Liquid chromatography-tandem mass spectrometry analysis revealed that this fraction represented the cardiotoxin-I (CTX-I) isolated earlier from *Naja kaouthia* venom. Analysis of the isolated CTX-I toxin in INS-1E cells showed that its insulin stimulation ability persisted even in the absence of glucose. In contrast to typical cobra cardiotoxin, CTX-I did not induce direct hemolysis of human erythrocytes and showed no potent vasoconstriction capability. Based upon this toxin, a truncated analogue [Lys(52)CTX-I(41-60)] was obtained by structure-guided modification[47]. This analogue showed insulinotropic activity similar to CTX-I and appeared to exert its action through $K_v$ channels[47]. As such, it may serve as a basis for the design of new therapeutic agents for the treatment of type 2 diabetes (Table 1).

A new paradoxical TFT, nakoroxin, was isolated from the cobra *Naja kaouthia* venom[48]. Nakoroxin belongs to the group of orphan TFTs (group "XX"), the biological activities of which are practically unknown. Nakoroxin was not cytotoxic to rat pheochromocytoma PC12 cells nor to human lung carcinoma HT1080 cells. It did not inhibit the binding of α-Bgt to α7 or muscle-type nAChRs, but potentiated the binding of α-Bgt to the acetylcholine-binding protein from *Lymnaea stagnalis*. The reason for this unusual property of nakoroxin is not clear.

Another quite interesting TFT, actiflagelin, was isolated from cobra *Walterinnesia aegyptia* venom by combination of reverse-phase and ion-exchange chromatography[49]. Actiflagelin activated *in vitro* motility of sperm from OF1 male mice. The amino acid sequence established by Edman sequencing combined with tandem mass spectrometry analyses showed that the protein comprised 63 amino acid residues with five disulfide bonds, the pattern of which corresponded to that of nonconventional toxins. Actiflagelin had a noticeable homology to bucandin, a nonconventional toxin from *Bungarus candidus* venom[49]. The authors suggested that the protein found may have therapeutic potential for cases of infertility when the problem is related to the sperm motility.

## CONCLUSION

TFTs were among the first toxins isolated from snake venoms. At present, they form one of the largest toxin families and their number is increasing constantly. Several TFTs are used as sophisticated pharmacological tools to study the function and structure of their molecular targets. The new TFTs that have emerged recently possess both novel structural and functional characteristics, expanding the possibilities for their future applications. The TFTs discovered during the last decade have good prospects to be transformed into novel drugs. For example, mambalgins are perfect candidates for the design of powerful analgesics, and on the basis of CTX-I possessing insulinotropic activity, new therapeutics for the treatment of diabetes may be created. The data presented in this minireview (Table 1) show that TFTs continue to be important and promising for both basic science and medicine.

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REVIEW

# Fasciculation and elongation zeta proteins 1 and 2: From structural flexibility to functional diversity

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## Abstract

Fasciculation and elongation zeta/zygin (FEZ) proteins are a family of hub proteins and share many characteristics like high connectivity in interaction networks, they are involved in several cellular processes, evolve slowly and in general have intrinsically disordered regions. In 1985, *unc-76* gene was firstly described and involved in axonal growth in *C. elegans*, and in 1997 Bloom and Horvitz enrolled also the human homologues genes, *FEZ1* and *FEZ2*, in this process. While nematodes possess one gene (*unc-76*), mammalians have one more copy (*FEZ1* and *FEZ2*). Several animal models have been used to study FEZ family functions like: *C. elegans*, *D. melanogaster*, *R. novergicus* and human cells. Complementation assays were performed and demonstrated the function conservation between paralogues. Human FEZ1 protein is more studied followed by UNC-76 and FEZ2 proteins, respectively. While FEZ1 and UNC-76 shared interaction partners, FEZ2 evolved and increased the number of protein-protein interactions (PPI) with cytoplasmatic partners. FEZ proteins are implicated in intracellular transport, acting as bivalent cargo transport adaptors in kinesin-mediated movement. Especially in light of this cellular function, this family of proteins has been involved in several processes like neuronal development, neurological disorders, viral infection and autophagy. However, nuclear functions of FEZ proteins have been explored as well, due to high content of PPI with nuclear proteins, correlating FEZ1 expression to *Sox2* and *Hoxb4* gene regulation and retinoic acid signaling. These recent findings open new avenue to study FEZ proteins functions and its involvement in already described processes. This review intends to reunite aspects of evolution, structure, interaction partners and function of FEZ proteins and correlate them to physiological and pathological processes.

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**Core tip:** Fasciculation and elongation zeta/zygin (FEZ) proteins are intrinsically disordered and hub proteins involved in many cellular functions, acting as a bivalent adaptor of kinesin-based movement. These proteins are associated to several processes like neuronal development, neurological disorders, viral infection and autophagy. However, novel nuclear functions are being described, shedding more light to their role. This review intends to reunite aspects of evolution, structure, interaction partners and function of FEZ proteins and correlate them to physiological and pathological processes.

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## INTRODUCTION

The fasciculation and elongation zeta/zygin (FEZ) protein family was described by Bloom and Horvitz[1] in 1997 as two human homologs of UNC-76 protein (Table 1). In attempt to characterize the *unc-76* gene, which in *Caenorhabditis elegans* mutants caused locomotory defects (uncoordinated), they found that these mutants presented axonal abnormalities: axons in fascicles did not reach their full lengths, and also failed to bundle tightly together. In addition, human *FEZ1* gene (protein code Q99689) was capable to partially restore *unc-76* mutant locomotion defects and axonal fasciculation, thus suggesting that FEZ family members share conserved evolutionary function and structure from *C. elegans* to *Homo sapiens*[1].

The worm has one copy of *unc-76* gene, while humans have two copies, FEZ1 and FEZ2 (protein code Q9UHY8). It has been later proposed that *unc-76* gene duplication occurred after divergence in the amphioxus branch, concomitant with chordates origin[2]. Synteny analysis evidences two rounds of genomic duplication in the chordate branch, after cephalochordate divergence but before the division of teleost and tetrapod[3]. Probably, the *unc-76* gene duplication has occurred during these rounds of genomic duplication.

Bloom and Horvitz[1] in 1997 also gave some insights into FEZ1/UNC-76 structure, function and expression pattern, which during more than 20 years of research were - and still are - the main subjects of study from different groups around the world[1]. Further in this paper we will discuss these topics in details.

## EXPRESSION PATTERNS IN TISSUES

As previously stated, Bloom and Horvitz[1] in 1997 briefly reported the expression patterns regarding FEZ1 and FEZ2, with the former being present in the brain while the latter also in non-neuronal tissues. Later, Honda *et al*[4] in 2004 characterized the expression of FEZ1 in the developing rat brain by *in situ* hybridization. It was shown that FEZ1 mRNA in adult rat brain was more expressed in olfactory bulb and cortical and hippocampal neurons, while the signal in cerebellum was weak.

Regarding the expression levels during development in rat, FEZ1 mRNA expression was low in the hippocampus by E16 and E18 prenatal development stages, by E20 there was a signal in pyramidal cells, and by P0 there was an intense signal in both pyramidal cells of the CA1-3 regions and granule cells of the dentate gyrus. The highest signal of FEZ1 mRNA was detected at P7 and in adult rats the expression decreased[4].

Another study compared the mRNA expression levels of FEZ1 and FEZ2 in rat tissue and mouse embryos. FEZ1 mRNA was observed almost exclusively in the brain, while FEZ2 mRNA was ubiquitously present in all tissues, although weaker when compared to FEZ1. In mouse developing embryos, FEZ1 mRNA was greatly

**Table 1 Identities and similarities between human proteins FEZ1, FEZ2 and UNC-76 from *C. elegans***

| | UNC-76 identity (%) | UNC-76 similarity (%) | FEZ1 identity (%) | FEZ1 similarity (%) |
|---|---|---|---|---|
| FEZ1 | 35 | 46 | - | - |
| FEZ2 | 34 | 45 | 49 | 56 |

Alignment of 376 and 385 amino acid UNC-76, 251 amino acid FEZ2, and 392 amino acid FEZ1. It is noteworthy that the N-terminal region shows to be more divergent, but yet with substantial similarity. The C-terminal region presented three particular regions with more similarity (amino acids 179-197, 251-307, and 354-381 – numbered according to the *C. elegans* protein)[1]. FEZ: Fasciculation and elongation zeta/zygin; UNC: Uncoordinated.

increased around 11 dpc (days post-coitum) and gradually faded as development continued. FEZ2 mRNA, otherwise, showed to be constantly expressed from 7 to 17 dpc[5]. Figure 1 presents a schematic view of FEZ1 expression.

Northern blot analysis with RNA from adult human tissues showed weak presence of FEZ1 RNA in prostate, testis, ovary, small intestine, colon, liver, especially when compared with very high expression of FEZ1 RNA in the brain[6].

Moreover, a gene array analysis of rat type-1 astrocytes (T1As) and T2As has also shown the expression of FEZ1 mRNA. At both mRNA and protein levels, T2As expressed more FEZ1 than T1As. Immunofluorescent staining with specific antibody showed the presence of FEZ1 in the cytoplasm of both astrocytes and also neurons[7]. More recently, the same group reported a new study using a rat model for Parkinson's disease (PD) by injecting 6-Hydroxydopamine Hydrobromide in the medial forebrain bundle. The results showed an increase of FEZ1 mRNA and protein in both striatum and substantia nigra of PD rats, peaking at 2 and 3 wk respectively after injury, and then decreasing to control levels for striatum and also decreasing for substantia nigra but to levels that were still higher when compared to control rats. In addition, after performing immunostaining of brain tissue after 2 wk of injection, the authors observed a change of FEZ1 expression from dopamine neurons in control group to a significantly increase of its expression in astrocytes from PD rats, suggesting an association between FEZ1 expression and astrocyte activation after injury[8].

More recently, it has been reported that FEZ1 is expressed in oligodendroglia (OL) lineage cells originated from mouse, rat, and human, through immunofluorescence. In addition, the authors also found that protein and mRNA levels of FEZ1 were increased by day 4 of differentiation process in primary cultured oligodendroglia progenitor cells (OPCs) in comparison to undifferentiated proliferating OPCs. Moreover, upregulation of FEZ1 protein and mRNA was detected in optic nerves during intense myelinogenesis between postnatal days 14 and 30[9].

## PROPOSED STRUCTURE

Human FEZ1 possess 392 amino acids and through different software predictions it was shown that its whole sequence, especially N-terminal and central regions, exhibited disordered structure. The C-terminal region was predicted to possess higher probability of structure. Indeed after performing circular dichroism spectro-polarimetry with purified protein, the authors confirmed the prediction of a largely disordered structure for FEZ1 protein and also suggested that it could gain structure upon binding to interacting partners[10].

It was also *in silico* predicted for FEZ1 to present one region with high probability to form coiled-coils in the regions between amino acid residues 230-265 (approximately 96% probability) and 278-306 (63%) (Figure 2). At the N-terminal three regions with low probability were also predicted[10]. Similar structure organization is found at homologues FEZ2 (human) and UNC-76 (*C. elegans*) (Figure 2).

Due to its unstructured characteristic, small angle X-ray scattering (SAXS) experiments were performed to determine low 3D resolution conformational model of FEZ1. Besides showing that FEZ1 has a long, extended and flexible conformation, the dimeric state of the protein in solution was also demonstrated. The authors compared the theoretical value of molecular mass for full length FEZ1 (48.6 kDa) with the one of the dimer (approximately 95 kDa), confirming that the latter was twice the former[10]. It is also important to highlight, especially for didactic purpose that due to the high content of charged amino acids and intrinsically disordered structure, FEZ1 shows an

| | Expression of FEZ1 | | |
|---|---|---|---|
| Developing rat brain (hippocampus) | E16 and E18 | E20: Pyramidal cells | P0: Pyramidal cells in CA1-3 and granular cells of dentate gyrus<br>P7: Highest signal |
| Adult rat brain | Cerebellum: Granular cells Purkinje cells, brainstem neurons | Olfactory bulb: Mitral and granular cells<br>Hippocampus: Pyramidal cells in CA1-3 | Hippocampus CA1-3 and granular cells of dentate gyrus |
| Mouse embryo | | | 11 d post-coitum |

**Figure 1 Schematic representation demonstrating FEZ1 expression in the developing rat brain and adult, and also in the mouse embryo[4,5].**

anomalous mobility in SDS-PAGE.

A second publication about FEZ1 dimerization deepened our understanding on this matter. After also performing SAXS experiments, the authors confirmed that the N-terminal region (protein fragment 92-194 amino acids, contain coiled-coil motif) was able to dimerize in solution without a disulfide bond reducing agent, but become a monomer after its addition. Moreover, through mass spectroscopy in full length FEZ1, it was shown that the dimeric peptide was formed by a Cys133–Cys133 disulfide bond. The authors also confirmed the presence of endogenous FEZ1 as a dimer in HEK293 cell lysate. Regarding FEZ2 (N-terminal fragment 106-189 amino acids, with coiled-coil), due to low concentration expression, the dimer state was only analyzed by SDS-PAGE followed by Western blot. Two bands were shown: monomeric band of 20 kDa upon reduction and dimer band of 40 kDa under nonreducing condition[11].

Finally, the structure model proposed (Figure 3) by all these authors determines that FEZ1 forms a dimer at the N-terminal region through a disulfide bond and that the outwards pointing C-terminal regions from each monomer can freely interact with the many partner proteins of FEZ1 interactome (discussed below). Hence, the N-terminal serves as a platform for dimerization while the C-terminal works as a protein-protein docking domain.

## INTERACTION PARTNERS AND FUNCTIONS

FEZ1 and FEZ2 are considered hub proteins, meaning that they interact with a great number of other proteins, which possess different functions (Figure 2). Makino *et al*[12] suggested that the evolution rate of a protein with many interaction partners is slower than those with few of them. After gene duplication, three functional pathways are possible. First, one copy can be silenced while the other copy remains with the original function. Second, while one copy remains with original function, the other copy can acquire new functions by accumulation of mutations that are positively selected. Third, both gene copies gain additional new functions, but the original function is cooperatively retained[12]. FEZ interaction partners indicate that the second option is currently happening with *Fez* genes.

Two independent yeast two-hybrid studies were performed to identify interaction partners (see details in: Assmann *et al*[13], 2006; Alborghetti *et al*[2], 2011). Here we summarized the main functions and interactors in Figure 4.

Alborghetti *et al*[2] in 2011 demonstrated that UNC-76 interacted with practically all FEZ1 interaction partners. FEZ2 was also able to interact with all FEZ1 partners and acquired new exclusive partners. The authors found that 83.3% of nuclear proteins interacting with FEZ2 also interacted with FEZ1. Proteins involved with translation seemed to be the novelty specific to FEZ2, and 56% of cytoplasmic proteins found interacting with FEZ2 were specific and did not interact with FEZ1[2].

This interaction pattern and the previously proposed history of *unc-76* gene duplication are in accordance to phenotypes displayed in mutation, knockout and complementation studies in *C. elegans*, *Drosophila melanogaster* and *Mus musculus*. Defects in axonal development were observed in gene mutations in invertebrates and human FEZ1 was able to complement them in *C. elegans*. However, FEZ1 knockout mice did not show any impairment in axonal development, although these animals were more responsive to methamphetamine[1,14-16]. Since FEZ2 is able to interact with all FEZ1 interaction partners, it is possible, though still speculative, that FEZ2 can

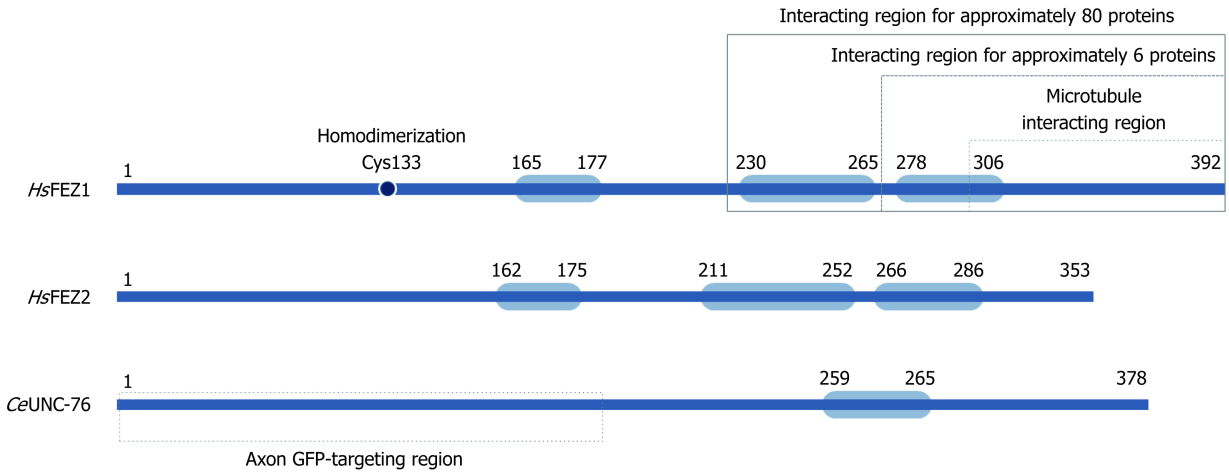


**Figure 2 Schematic structure for FEZ proteins.** Blue boxes indicate predicted coiled-coil regions involved in homodimerization or protein-protein interaction.

complement FEZ1 function in its absence.

### *Phosphorylation and degradation*

The first interaction partner reported for FEZ1 was the protein kinase C zeta (PKCζ), which is an atypical isoform of the PKC family. Kuroda *et al*[17] in 1999 performed a yeast two-hybrid screening using a rat brain cDNA library and found that the regulatory domain of rat PKC zeta interacted with FEZ1, most likely in its C-terminal. The interaction was confirmed by immunoprecipitation in COS-7 cells overexpressing both FEZ1 and PKC zeta, and the authors found that the complex could be phosphorylated after performing a phosphorylation assay. One interesting aspect discovered was that transfected FEZ1 seemed to translocate from the plasma membrane and cytoplasm periphery to uniform cytoplasm localization when there was a change in overexpressing PKC zeta to a constitutively active form of PKC (caPKCζ). Moreover, it was also shown that PC12 cells' rate of differentiation into neurons was higher (approximately 48%) in the presence of transfected FEZ1 and caPKCζ than when only caPKCζ was transfected (approximately 18%)[17]. A subsequent study showed that FEZ2 and PKCζ were immunoprecipitated and that PC12 could differentiate more in the presence of both FEZ2 and caPKCζ[5].

Still regarding the matter of phosphorylation, it was later reported that UNC-76 interacted and was phosphorylated by UNC-51, a Ser/Thr kinase, in *Droshophila*. The authors performed experiments *in vitro*, in HEK293T cells and in extracts from wild-type larvae in order to address the phosphorylation status. Mass spectrometry analysis revealed that UNC-76 Ser143 was a phosphorylation site. In addition, phosphorylated UNC-76 was detected in wild-type and absent in the *unc-51* fly mutant. Also, a mutated version of phospho-UNC76 (S143A) could not rescue the defects present in *unc-76* mutants, in opposition to the rescued effect caused by UNC-76 wild-type and a phosphomimetic UNC-76. Those defects were related to axonal transport and synaptic vesicles and kinesin heavy chain (KHC) aggregation. Moreover, co-expression in HEK293T cells followed by immunoprecipitation and FRET analysis in COS-7 cells, showed the interaction between UNC-76 and Synaptotagmin-1, a protein of synaptic vesicles, and that this interaction was dependent of UNC-76 phosphorylation by UNC-51. *Drosophila unc-51* mutants produced a phenotype where axonal cargoes transport was affected, especially the one of synaptic vesicles and partially the mitochondria[18].

Using liquid chromatography coupled with tandem mass spectrometry, another work identified four FEZ1 phospho-serine sites (S58, S134, S301, S316) from HEK293 cells lysates expressing FEZ1-GFP[19]. The phosphorylation of the S58 site of FEZ1 by microtubule-affinity regulating kinases (MARKs) was confirmed *in vitro* and in HEK293 cells. Specific antibody raised against the residue S58 tested in HeLa cells confirmed that, in the presence of MARK, phospho-FEZ1 was detected[20].

Regarding the degradation of FEZ1, some studies shed light on how this could happen. Starting with a yeast two-hybrid assay using a human brain cDNA library, it was showed that FEZ1 interacted with the U-box-type ubiquitin ligase E4B. The interaction was confirmed in HEK293T and PC12 cells. The authors reported that the interaction was enhanced in the presence of caPKCζ. In addition, they found that co-expression of E4B with FEZ1 and caPKCζ increased the proportion of PC12 cells

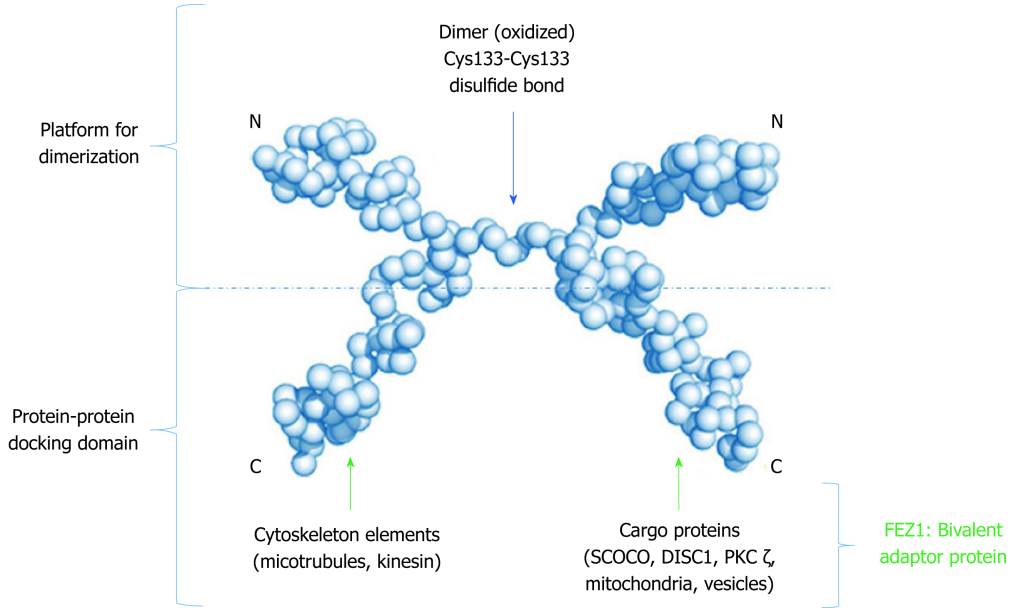


**Figure 3 Low resolution model proposed to the dimeric structure of FEZ1.** Monomers connect through a cysteine present in the N-terminal. The C-terminal of each monomer is free to perform protein-protein interactions. Adapted from the model proposed by Alborghetti *et al*[11], 2010. SCOCO: Short-coiled coil protein; DISC1: Disrupted-In-Schizophrenia 1; PKC: Protein kinase C.

differentiated into neurons. Although there was some polyubiquitylation of FEZ1 by E4B, the authors could not directly affirm that this modification causes FEZ1 degradation by the proteasome[21]. Nonetheless, it was shown that the presence of the proteasome inhibitor MG132 prevented the degradation of FEZ1[22,23], indicating that FEZ1 is likely to be degraded by the 26S proteasome.

A more recent study showed that FEZ1 interacted with cell-division cycle 20/anaphase-promoting complex (Cdc20/APC), which is an ubiquitin ligase (E3) and modulates dendrite development. Experiments carried *in vitro*, in HEK293T cells and in hippocampal lysates confirmed the interaction. The authors reported that over-expression of Cdc20 increased the polyubiquitination of FEZ1 and that the suppression of Cdc20 increased the levels of FEZ1, suggesting that the degradation of FEZ1 is regulated by Cdc20 in dendrite development. In addition, the authors also reported that the Cdc20/APC complex is regulated by Hdac11[23]. A subsequent new study with *Hdac11* knockout mice showed decrease of *Fez1* in the hippocampus of adult mice[24].

### *Intracellular transport*

It was first reported by Gindhart *et al*[14] in 2003 that UNC-76 was found as an interaction partner of KHC tail domain after performing a two-hybrid assay using a *Drosophila* embryonic cDNA library. The interaction was further confirmed *in vitro*. A general scheme of FEZ proteins acting on cytoplasmatic intracellular transport is depicted on Figure 5. The authors generated *unc-76* fly mutants and performed detailed characterization. They found that UNC-76 was essential for *Drosophila* development and the phenotypes were similar to the mutants for kinesin. The function of UNC-76 as a membrane cargo transporter in axons was not observed since there was no co-localization with axon clogs, which are related to vesicle transport[14].

Nonetheless, in line with this first study, subsequent work provided more evidence about FEZ1 as an important component of the kinesin transport pathway. *In vitro* experiments showed the interaction between the C-terminal of FEZ1-tubulin and FEZ1-kinesin, and the complex FEZ1-kinesin (KIF5)-β tubulin was immuno-precipitated from PC12 cells differentiated into neurons. The authors also showed the co-localization of endogenous FEZ1 and mitochondria in PC12 cells, both before and after differentiation into neurons upon treatment with nerve growth factor (NGF)[25]. They also confirmed another work[26] showing that mitochondria anterograde movement was reduced when FEZ1 was repressed by interference RNA, both in hippocampal neurons isolated from rat embryonic brain and PC12 cells treated with NGF[25]. Therefore, it was shown that FEZ1 is important in mitochondrial anterograde transport, morphology and neuronal polarity and this transport along microtubules was mediated by FEZ1-Kinesin interaction. More recently, it has been reported that UNC-76 regulated mitochondrial density in anterior *C. elegans* touch receptor

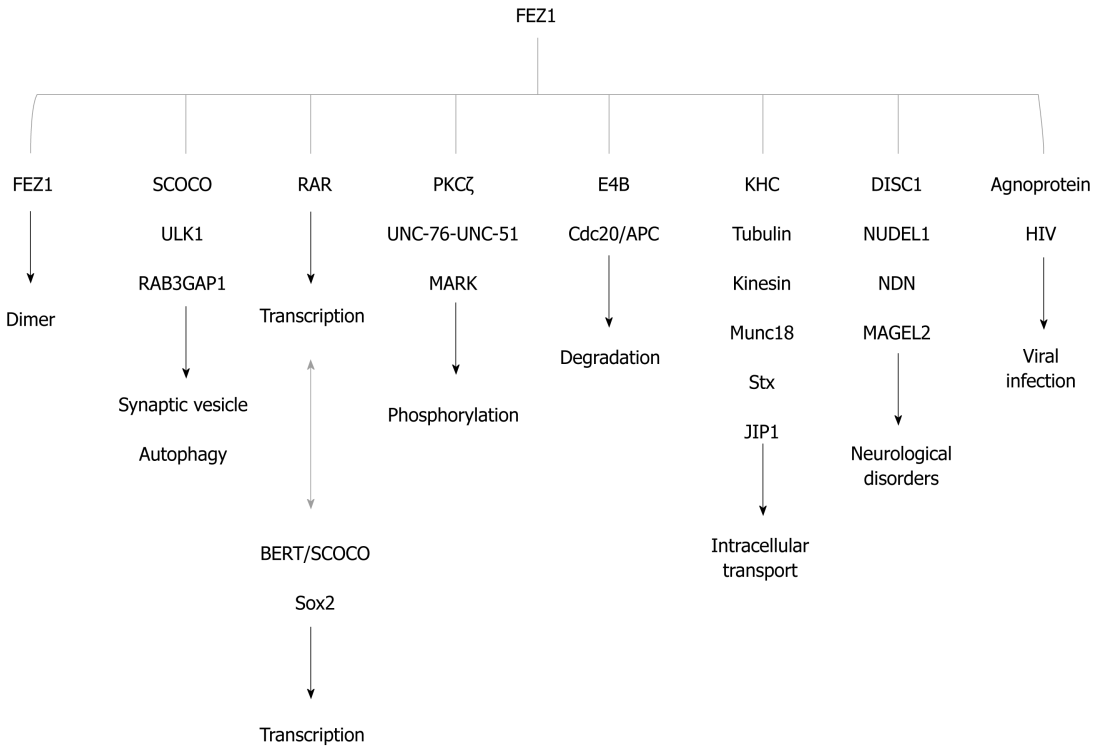


**Figure 4 FEZ1 and proteins reported to associate either by direct binding or by the presence in a protein complex.** The function of the association is also depicted. BERT/SCOCO was reported to regulate the expression of Sox2 gene. SCOCO: Short-coiled coil protein; DISC1: Disrupted-In-Schizophrenia 1; PKC: Protein kinase C; KHC: Kinesin heavy chain; RAR: Retinoic acid receptor; HIV: Human immunodeficiency virus; NUDEL: Nuclear distribution element-like; NDN: Necdin; ULK1: UNC-51-like kinase; JIP1: c-Jun N-terminal kinase-interacting protein 1; Stx: Syntaxin 1a; MARK: Microtubule-affinity regulating kinase.

neuron[27].

Another study identified a transport complex involving Kinesin-1 (KIF5C), FEZ1, Munc18 and Syntaxin 1a (Stx). Immunoprecipitation in HEK293 and also neurons from postnuclear supernatants from rat brain confirmed the function of FEZ1 as an adaptor that connected Munc18 and Stx (cargoes) to the kinesin-1 (motor). Phosphatase treatment ceased the interaction of FEZ1 with Munc18 and KIF5C. Stx and Munc18 colocalized with FEZ1 in growth cones of primary hippocampal neurons. *C. elegans* mutants for UNC-76 and UNC-116 (kinesin) presented aggregation of UNC-64 (Stx) in cells bodies and axons[19].

Using label-free quantitative mass spectrometry in immunoisolated FEZ1 and Kinesin-1 samples of the cytosolic fraction containing vesicles from the rat brain, it was found that over one thousand proteins were enriched in the samples. More surprisingly, more than 90% of these proteins were common for both FEZ1 and Kinesin-1. The authors also generated transgenic *C. elegans* and found that the disruption of Par-1 (the only MARKs in worms) altered the phenotype of FEZ1 and Stx1 with the appearance of aggregates in axons and cells bodies, probably due to impairment of axonal transport[20].

In addition, another component of the complex was found to be the protein c-Jun N-terminal kinase-interacting protein 1 (JIP1). After confirming the interaction between kinesin-1 (kinesin heavy and light chain) with FEZ1 and JIP1 in COS cells by co-immunoprecipitation (co-IP), the authors performed a microtubule binding assay showing that the presence of both FEZ1 and JIP1 was necessary for the activation of kinesin-1 and the microtubule motility. The presence of only one of the proteins could not activate kinesin-1 and, in the absence of kinesin-1, there was no interaction between FEZ1 and JIP1[28].

### *Neurological disorders*

One of the genes related to schizophrenia encodes the protein Disrupted-In-Schizophrenia 1 (DISC1) and it was identified to interact with FEZ1 through a two-hybrid assay. More specifically, the C-terminal of both proteins was required for this interaction. In addition, the interaction was further confirmed by experiments in mammalian cells. The authors also demonstrated that during neuron differentiation in PC12 cells, the interaction FEZ1-DISC1 was crucial for neurite outgrowth[29]. Another study showed that the knockdown of FEZ1 in cultured adult mouse neural progenitors derived from hippocampus caused increased soma size of neurons and

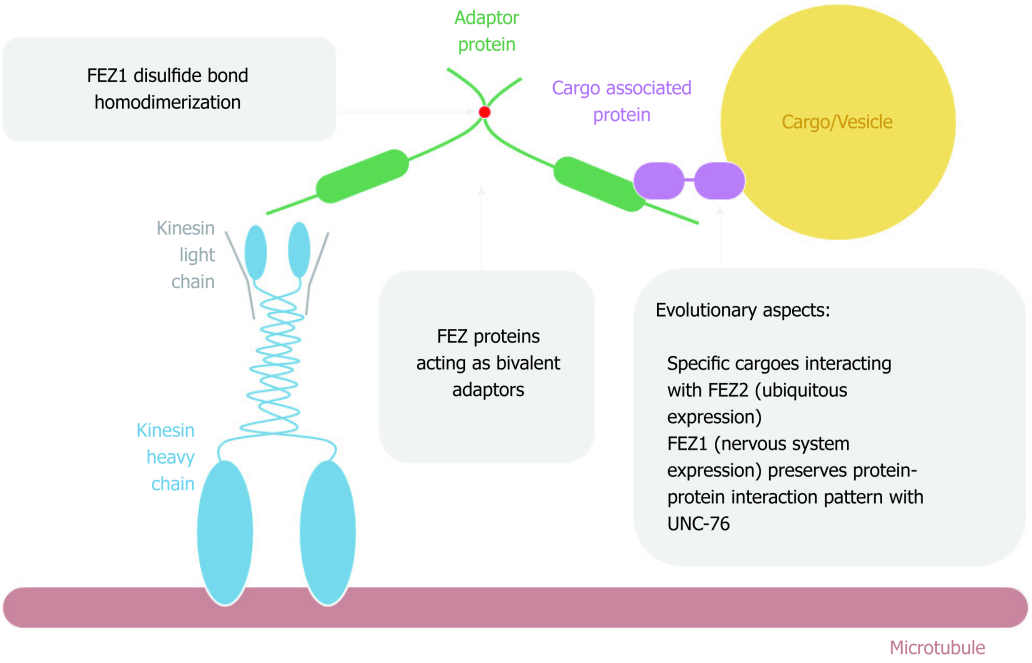


**Figure 5 FEZ proteins cytoplasmic function as transport bivalent adaptors.**

accelerated dendritic development, being both phenotypes also present in DISC1 knockdown neurons. Moreover, double knockdown experiment for both of these proteins confirmed an enhancement of the dendritic growth phenotype when compared to FEZ1 knockdown alone, suggesting a synergistic functional role of DISC1-FEZ1 interaction in regulating dendritic development[30].

In accordance, the work from Honda *et al*[4] in 2004 has previously reported similarities regarding the expression patterns of DISC1 and FEZ1 in rat brain during prenatal stages and at birth, suggesting a role for this interaction during the nervous system development in mammals. Moreover, it is noteworthy that the region containing the translocation breakpoint responsible for disrupting DISC1 in schizophrenia was critical for the interaction with FEZ1[29]. These findings motivated some interesting reports where genotyping of different patient populations was performed in order to associate *Fez1* gene with schizophrenia. The study with a Japanese population showed this association for a specific group of patients carrying rare homozygosis[31]. A more robust study was further performed in the Japanese population and the former could not be replicated[32]. In accordance with the latter, a study conducted in Caucasian and African American populations could not associate any of the nine single nucleotide polymorphisms tested for *Fez1* with schizophrenia[33].

Nevertheless, studies have continued to be performed in attempt to correlate FEZ1 with schizophrenia, especially in association with other genes. One work conducted with postmortem hippocampal and dorsolateral prefrontal cortex from normal controls and patients with schizophrenia revealed a significant decrease in FEZ1 mRNA expression in the test group[34]. More recently, another study has also shown the same down-regulation of FEZ1 mRNA levels in peripheral blood samples from patients with schizophrenia when compared to healthy volunteers[35]. In addition, FEZ1 mRNA showed to be a significant diagnosis predictor. The mRNA from nuclear distribution element-like (NUDEL) was also significantly decreased in hippocampus and proved to be useful as a diagnosis predictor[34]. Interestingly, it was later described a significant positive correlation between FEZ1 and NUDEL regarding their expression patterns in RNA samples obtained from postmortem human frontal cortex[36]. At protein level, co-IP experiments with adult mouse brain tissue confirmed the interaction between FEZ1/NUDEL1 and DISC-1/NUDEL1. Functionally, double knockdown of FEZ1 and NUDEL1 showed additive phenotype described for each individual protein knockdown, demonstrating different roles in regulation of neurogenesis in adult mouse brain[30].

Another aspect of FEZ's association with neurological diseases is its interaction with proteins necdin (NDN) and MAGEL2, both inactivated in Prader-Willi syndrome and predicted to relate with hypothalamic dysfunction. The authors reported the interaction of FEZ2 with NDN by yeast two-hybrid Ras rescue system and also the interaction of both FEZ1 and FEZ2 with NDN by co-IP in HEK293 cells transiently

transfected. There was also interaction of FEZ1 with MAGEL2 when performing co-IP. Immunofluorescence performed in HEK293 cells overexpressing NDN and FEZ1 showed co-localization of these proteins in juxtanuclear bodies as well as the presence of FEZ1 surrounding and partially overlapping the gamma-tubulin at the centrosomes. Co-IP of FEZ1, NDN and gamma-tubulin showed that these proteins were all present in the immuno complex. Together with other results, the authors proposed that the NDN-FEZ1 interaction in centrosomal structure could cause cytoskeletal rearrangements that interfere in axonal outgrowth[22].

### *Viral infection*

The JC virus (JCV) agnoprotein is related to viral growth and JCV infection. Using a human brain cDNA library and the full-length agnoprotein, Suzuki *et al*[37] in 2005 performed a two-hybrid yeast assay and found FEZ1 as a binding partner of agnoprotein. The FEZ1 region retrieved contained the coiled-coil domain. Deletion mutants of FEZ1 and purified protein precipitation assays further confirmed the direct interaction of agnoprotein and the coiled-coil at C-terminal of FEZ1. Moreover, a stable cell line expressing JCV agnoprotein derived from HEK293 cells was transfected with FEZ1 and the interaction was also confirmed by immuno-precipitation. Confocal microscopy showed agnoprotein in the perinuclear region and also extended to the cytoplasm, while FEZ1 was seen throughout the cytoplasm and colocalized with agnoprotein only in the perinuclear region[37].

Functionally, it was found that agnoprotein significantly inhibited neurite extension in transfected PC12 cells stably expressing FEZ-GFP with DsRed-agnoprotein. Additionally, a human glial cells (SVG-A) stably expressing FEZ1 was inoculated with JCV and the expression of agnoprotein and VP1 (protein from the viral capsid) was reduced in the cells overexpressing FEZ1 when compared to control. Conversely, the same proteins showed increased expression in cells transfected with FEZ1 siRNA. Thus, FEZ1 suppressed the expression of JCV proteins in SVG-A cells. It was also shown by immunocytofluorescence in the same cell line that overexpression of FEZ1 suppressed the propagation of VP1-positive cells after 7 d of infection and that this viral protein was restricted to the nucleus in the presence of FEZ1, suggesting a role as an inhibitor of nuclear to cytoplasm translocation[37].

Interestingly both FEZ1 and agnoprotein were also shown to interact with microtubules through microtubule cosedimentation assays. The binding of FEZ1 and microtubules occurred at the C-terminal of FEZ1 (residues 297-392), which does not contain the coiled-coil region, meaning that binding regions of agnoprotein and microtubules with FEZ1 were different from each other. More importantly it was reported that agnoprotein and FEZ1 seemed to compete for microtubule: while more expression level of agnoprotein was detected, less amount of FEZ1 cosedimented with microtubules and vice-versa. The authors suggested that there might be some conformational change in the C-terminal of FEZ1 when there is an interaction with agnoprotein, resulting in the disruption of FEZ1-microtubule binding. This disruption could also explain the reason why the presence of agnoprotein inhibited the neurite extension in PC12 cells, since FEZ1 and its association with microtubule could lead to neurite outgrowth[37].

Another study with a gene array performed in virus-resistant cell mutant isolated from Rat2 cell, a parental fibroblast line, named R3-2 was conducted in order to investigate the resistant phenotype of an apparent constrain of the viral DNA to the cytoplasm. After confirming the results from microarray through quantitative RT-PCR, FEZ1 gene was up-regulated > 30-fold in the R3-2 cell line. Stable Rat2 cell line over-expressing myc-His-FEZ1 and controls were infected with retroviruses (MLV: Moloney murine leukemia, and HIV-1: human immunodeficiency virus type 1). The cells over-expressing FEZ1 and infected with both viruses showed significant resistance when compared to control cells. This was later confirmed by knockdown of FEZ1 in mutant and wild-type cells. In addition, human cells 293T stably overexpressing FEZ1 and infected with HIV-1 were also capable of blocking infection when compared to control[38].

To assess the time point of this mechanism, the course of viral DNA was analyzed by checking the amount of viral DNA from different intermediates throughout the infection (total viral DNA, earlier steps in reverse transcription and nuclear viral DNA). When compared to R3-2 and controls, cells over-expressing FEZ1 showed similar phenotype: no defect in overall synthesis of viral DNA and similar levels of DNA at early step in reverse transcription. The difference observed was related to the levels of circular viral DNA: Control cell lines showed high levels of it, while low levels were seen in over-expressed FEZ1 and mutant R3-2 cell lines, suggesting that the viral block occurred after reverse transcription but before nuclear entry[38].

Another study further investigated the resistance of neurons expressing high levels of FEZ1 to HIV-1 infection. Also this work interestingly investigated, especially for

didactic purposes, the endogenous expression levels of FEZ1, both as mRNA and protein, in different human brain cell lines. Derived neuronal cells (SH-SY5Y) exhibited the highest levels of endogenous expression, while astrocytes (1321N1) and microglia (CHME3) derived cell lines showed significantly lower levels[39].

Similarly to the experiments previously described with R3-2 cells, the authors infected SH-SY5Y, 1321N1 and CHME3 cell lines with HIV-1 and assessed the susceptibility and the intermediates of infection. They found that SH-SY5Y presented the highest resistance to infection. Regarding the total viral DNA, all the three cell lines presented similar levels, while the levels of circular DNA was significantly lower in SH-SY5Y. These experiments were conversely confirmed by RNA interference specific for FEZ1. Both results showed that there was a robust block of HIV-1 infection in the neuronal cell line highly expressing endogenous FEZ1, especially regarding nuclear trafficking of viral DNA[39].

Additional experiments with microglia cell line CHME3 transiently and stably overexpressing FEZ1 were also performed and again showed that the presence of FEZ1 made cells more resistant to viral DNA before nuclear entry. It is noteworthy to mention that FEZ1 expression was not induced in CHME3 by interferon treatment, and as suggested by the authors, the blocking role of FEZ1 may come from its natural expression as a neuron-specific determinant of retroviral infectivity, instead of part of a wider antiviral response[39].

One more study associating FEZ1 with retroviral infection shed light on how this protein affects the delivery of viral DNA to the nucleus. Firstly it was shown that different cells lines presented a potent reduction in HIV-1 infection in the presence of FEZ1 siRNA. Besides CHME3, the cells tested this time were primary normal human dermal fibroblasts (NHDFs) and macrophages (differentiated Thp-1). Conversely, NHDFs cells overexpressing FEZ were able to restore the infection. These results may present a contradiction, however the authors pointed that the effect of FEZ1 as a positive regulator of HIV-1 infection began to decline in cells expressing very high level of this protein, suggesting that indeed the antiviral effect of FEZ1 previously reported may be related to the excessively high expression level of FEZ1 in neuronal cells lines[40].

More strikingly, this work showed that cell extracts containing FEZ1-FLAG could interact to *in vitro* assembled HIV-1 capsid-nucleocapsid (CA-NC). Moreover, using FEZ1 siRNA and infecting NHDFs with HIV-1 wild-type as well as two HIV-1 mutated capsids (N74D and P90A), both related to entry of the virus into the nucleus, it was reported that the block of infection was similar to all forms of HIV-1. This suggested that FEZ1 was not directly participating in the nuclear entry process but instead could affect the movement of the virus to the nucleus[40].

Since FEZ1 was already reported as a kinesin-1 adaptor, the authors assessed the role of kinesin-1 regarding HIV-1 infection and trafficking. They confirmed that viral particles in knockdown cells for kinesin-1 entered the cytosol and remained largely at the cell periphery, failing to move towards the nucleus. Also using stable NHDFs expressing FEZ1-FLAG and FEZ1-S58A-FLAG (mutant that fails to bind kinesin-1) with further infection with HIV-1, it was reported that only FEZ1-FLAG increased infection, while the mutant did not when compared to control. Therefore it was proposed that FEZ1 binds to HIV-1 and that the virus exploits the role of FEZ1 as a kinesin-1 adaptor, regulating the trafficking of viral particles to the nucleus. Additionally, it was pointed out that while high levels of FEZ1 in neurons may attribute a role as an antiviral factor, in other cells FEZ1 may actually work as a positive host cofactor that facilitates infection by the stated mechanism[40].

The same authors (Malikov and Naghavi[41] in 2017) further provided more details about the role of phosphorylated FEZ1 regarding transport and uncoating of HIV-1. In addition to the association of FEZ1 and Kinesin-1 heavy chain (Kif5B) to regulate virus trafficking, it was also reported that both FEZ1 and Kif5B were required to regulate uncoating in infected siRNA microglia (CHME3) by *in situ* fluorescence microscopy. In Jurkat cells, FEZ1 also needed to bind Kif5B to promote HIV-1 infection and uncoating[41].

The strategy of HIV-1 to exploit microtubules for intracellular movement was reported to be mediated by FEZ1, since FEZ1 efficiently interacted with HIV-1 core, and no direct interaction between HIV-1 core and Kif5B has been identified. Conversely, the authors reported that Kif5B was expressed in the FEZ1-S58A mutant cells that exhibited defects in transport and uncoating, therefore FEZ1 was suggested to be the bridging factor that enables kinesins to control HIV-1 motility and capsid disassembly[41].

Finally, in line with previous reported work about FEZ1 phosphorylation[20], the authors also demonstrated that in 293T cells over-expressing GFP-MARK2 and FEZ1-FLAG, there was an increase in FEZ1 S58 phosphorylation. In addition, the amounts of HIV-1 CA-NC bound to phosphorylated FEZ1 were also increased. Moreover, the

depletion of FEZ1 abrogated the ability of MARK2 expression to enhance infection, thus suggested by the authors that MARK2 effects occurred through its substrate, FEZ1. Therefore, they proposed that HIV-1 binds MARK2 to regulate FEZ1 phosphorylation on HIV-1 cores[41].

### Special partner short-coiled coil protein, and autophagy

The UNC-69 protein in *C. elegans* is the homolog of mammalian short-coiled coil protein (SCOCO). SCOCO presents a coiled-coil region conserved among species with the N-terminus showed to be more divergent. Conserved function was seen by restoration of locomotion in *unc-69* mutants in the presence of human SCOCO. In addition, *unc-69* fly mutants showed defective axonal outgrowth and guidance. Northern blot analysis in human fetal tissue showed expression in lung, liver, kidney, and it was enriched in the fetal brain[42].

The interaction with UNC-76 was first identified by yeast two-hybrid screen and subsequently confirmed *in vitro* by pull-down assay, showing that the coiled-coil of UNC-76 was important for interaction. Moreover, UNC-76 and UNC-69 co-localized in round, perinuclear dots in the soma of *C. elegans*. This co-localization was disrupted in *unc-116* (KHC) mutants, although axonal transport was still occurring. The authors proposed that UNC-69 and UNC-76 could act together in order to promote extension and regulation of synaptic vesicles in axons[42].

Another aspect of SCOCO-FEZ1 interaction is the role in autophagy. Autophagy is a catabolic process and it is initiate by autophagosome formation by activation of UNC-51-like kinase (ULK1)[43,44]. The process requires transport of organelles and macromolecules to the lysosomes for degradation[45-47].

It was previously reported that UNC-51 binds to UNC-76[18]. The mammalian ortholog of UNC-51 is the ULK1 protein and its activation is the initial step of autophagy. Co-IP experiments revealed that ULK1 interacts with FEZ1 and mutations in FEZ1 that disrupted the interaction with SCOCO did not disturbed ULK1-FEZ1 interaction. There was no interaction between ULK1 and SCOCO and siRNA depletion of SCOCO affected FEZ1 and ULK1 interaction. The authors performed a screening in HEK293 cells and found that SCOCO was required for starvation-induced autophagy. They also found that both SCOCO and FEZ1 interacted with UV radiation resistance associated gene (*UVRAG*), another protein that is also a member of autophagy complexes, by co-IP, and that SCOCO-UVRAG was regulated by FEZ1 and sensitive to starvation[43].

A subsequent study showed that FEZ1 co-localized with RAB3GAP1, which is part of a complex that modulates autophagy[48]. It had been previously reported by yeast two-hybrid screen that these proteins interacted[13]. Spang *et al*[48] in 2014 showed that the knockdown of FEZ1 and FEZ2 increased autophagic activity, while deficiency of RAB3GAP1/2 decreased this activity.

The autophagy scenario is complex, highly regulated, and the previously related function of FEZ1 as an adaptor of cargo transport - and probably scaffolds protein - places FEZ proteins as emergent targets in this process. Indeed, thirteen FEZ interaction partners are involved in autophagy (Table 2). As already described, the complex of FEZ1 with ULK1, SCOC, RAB3GAP1 or RAB3GAP2 is already correlated to autophagy. However, there is no work demonstrating the involvement of FEZ1 complex with TBC1D25, HAP1, HTT, TLK2, NBR1, PTPRS or TTR in this process.

TBC1D25, also known as OATL1, is a putative Rab guanosine triphosphatase-activating protein (GAP) that interacts with Atg8 homologues and participates in the fusion of lysosomes and autophagosomes[49]. In 2014, it was demonstrated in neurons that HTT and HAP1 are regulators of autophagosome retrograde transport. HAP1 interacts with HTT and control dynein and kinesin motors during the processive transport[50]. Moreover, HTT is able to bind ULK1, releasing ULK1 from negative regulation by mTOR[51]. TLK2, a nuclear kinase, was reported as a negative regulator of autophagy, however the mechanism remains elusive[43]. Several targets are ubiquitinated for degradation and NBR1 is proposed to act as receptor for these targets at autophagosome[52]. PTPRS is a protein tyrosine phosphatase involved in several cell functions and its loss causes autophagy induction[53]. Finally, a mutation in TTR (Y114C) has impaired autophagy[54]. Further studies about FEZ proteins' involvement in autophagy, if it acts as a protein adaptor or scaffold for proteins above described, can shed light in neurological disorders and cancer[55-57]. It is also important to mention that two studies provided insights on SCOCO's structure and its binding to FEZ1[58,59].

### Nuclear role and flower-like phenotype

Another important aspect of FEZ function lies around its transcriptional regulators identified as interactors (Table 3). A general scheme of FEZ protein and its nuclear function is depicted on Figure 6.

**Table 2 FEZ1 interaction partners[1] involved in autophagy**

| Gene | Protein | FEZ1 interaction reference | Autophagy reference |
|---|---|---|---|
| *RAB3GAP2* | Rab3 GTPase-activating protein non-catalytic subunit | [13] | [48] |
| *RAB3GAP1* | Rab3 GTPase-activating protein catalytic subunit | [13] | [48] |
| *FEZ1* | Fasciculation and elongation protein zeta-1 | [13] | [43] |
| *FEZ2* | Fasciculation and elongation protein zeta-2 | [13] | [43] |
| *TBC1D25* | TBC1 domain family member 25 | [13] | [49] |
| *HAP1* | Huntingtin-associated protein 1 | [57] | [50] |
| *HTT* | Huntingtin | [57] | [51] |
| *SCOCO* | Short coiled-coil protein | [13] | [43] |
| *TLK2* | Serine/threonine-protein kinase tousled-like 2 | [13] | [43] |
| *NBR1* | Next to *BRCA1* gene 1 protein | [6] | [52] |
| *PTPRS* | PTPRS protein | [55] | [53] |
| *TTR* | Transthyretin | [55] | [54] |
| *ULK1* | UNC-51 like kinase 1 | [43] | [56] |

[1]Interactions described to *Homo sapiens* sequence. Rab: Ras superfamily of small GTP-binding proteins; TBC: Tre-2/Bub2/Cdc16; BRCA: Breast cancer gene; PTPRS: Protein tyrosine phosphatase, receptor type S; UNC: Uncoordinated, serine/threonine protein kinase.

In addition, and still in reference to the permanent SCOCO partner aforementioned, Papanayotou *et al*[60] in 2008 showed that BERT (*Gallus gallus* SCOCO), which is equivalent to human SCOCO, participates in the regulation of *Sox2* gene expression in the neural plate. This regulation is performed by the binding of BERT to both Geminin and ERNI. Upon BERT binding, the interaction between Geminin-ERNI is disrupted, releasing HP1γ-ERNI-BERT and leaving BERT-Geminin-Brahma in the N2 enhancer and causing the induction of *Sox2*[60].

In this context, it was recently reported that *Sox2* gene was induced when FEZ1 was over-expressed in U87 cells and this was confirmed when FEZ1 was depleted and there was no *Sox2* activation, supporting the hypothesis that the presence of FEZ1 could be part of a regulatory complex responsible for *Sox2* activation. Moreover, the authors also reported a dramatic induction of *Hoxb4* gene in the context of FEZ1 over-expression and the interaction with the retinoic acid receptor (RAR), in the presence of the ligand retinoic acid[61]. *Hoxb4* is known to be related to development[62] and experiments showed *Fez1* expression by *in situ* hybridization in chicken embryos during neurulation and somitogenesis (Kobarg *et al*, non-published observation).

More surprisingly, HOXB4 was reported to correlate with acute myeloid leukemia[63], which is very consistent with our findings regarding increased expression of FEZ1 in this disease by immunohistochemistry experiments with tissues from patients (Kobarg *et al*, not published). The interesting link between FEZ1 and HOXB4 differential expression in acute myeloid leukemia could lie in the discovery that FEZ1 over-expression caused the phenotype of multi lobulated nuclei (also known as flower-like nuclei) in mammalian cell line[64]. This nuclear phenotype has already been reported to be a marker of myeloid leukemia of M4/5 subtype[65,66].

It is important to mention that another unrelated protein had been synonymously called "*FEZ1*"/LZTS1 (Q9Y250, 596 amino acids length), in the past. This transcription factor and tumor suppressor protein has been reported also as "*FEZ1*" ("F37 /Esophageal cancer-related gene-coding leucine Zipper motif 1")[67] but today has been renamed to: LZTS1 ("Leucine zipper putative Tumor Suppressor 1"), and is no longer called *FEZ1*[68].

## CONCLUSION

FEZ proteins are implicated in neuronal development and viral infection and their role as kinesin adaptor has been studied in these context. However, FEZ proteins are hubs and novel nuclear functions that could be involved in these processes have been described. The structural, evolutionary and functional data available for this family of proteins provides tools to understand the role of hub proteins in physiological and pathological states.

**Table 3 FEZ interaction protein partners related to nuclear function and transcription**

| Protein | Description | Reference |
|---|---|---|
| DRAP1 (NC2α) | DR1 associated protein 1 (negative co-factor 2 alpha) | [13] |
| BAF60a (SMARCD1) | Component of SWI/SNF chromatin remodeling complex | [13] |
| SAP30L | Sin3A-associated protein, 30 kDa-like | [2,13] |
| BRD1 | Bromodomain containing protein 1 | [13] |
| TLK2 | Tousled like kinase 2 | [13] |
| ZNF251 | Zinc finger protein 251 | [13] |
| RARA | Retinoic acid receptor alpha | [2,61] |
| MED7 | Mediator complex subunit 7 | [2] |
| MLF1IP | MLF1 interacting protein | [2] |
| SAP30 | Sin3A-associated protein, 30kDa | [2] |
| SFRS8 | Splicing factor, arginine/serine-rich 8 | [2] |

DR1: Down-regulator of transcription 1; SMARCD1: SWI/SNF-Related Matrix-Associated Actin-Dependent Regulator of Chromatin, Subfamily D, Member 1; BAF60a: Brg1/Brm associated factor, 60kDa, subunit A (Brm: brahma, Brg1: brahma-related gene 1); SWI/SNF: Switching defective/sucrose nonfermenting; MLF1: Myeloid leukemia factor 1.

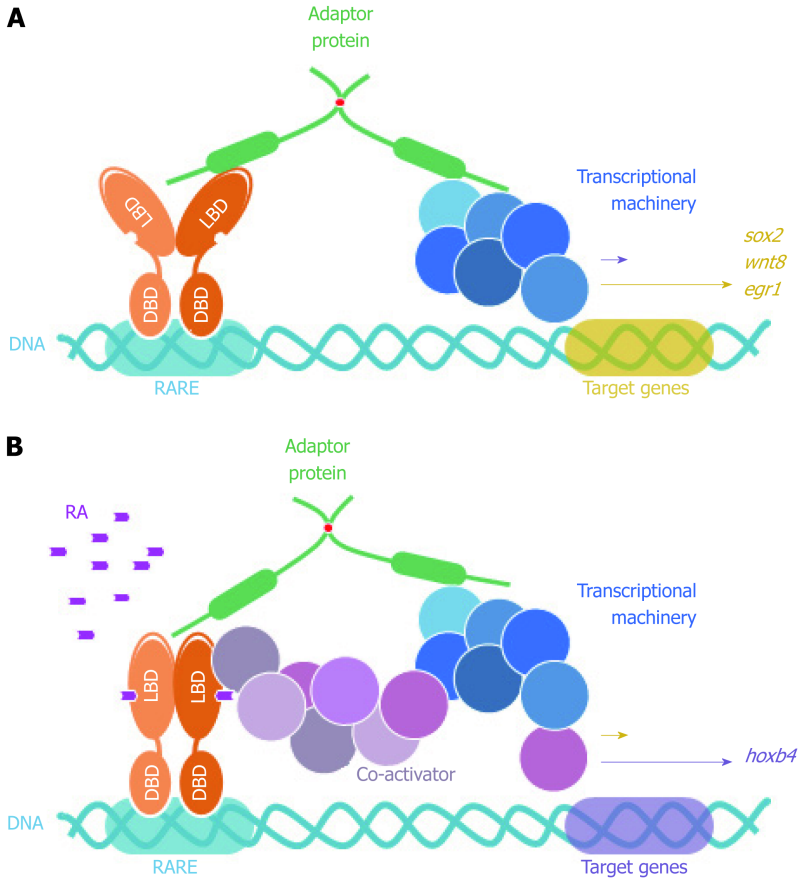


**Figure 6 FEZ proteins nuclear function as scaffold between nuclear receptors (retinoic acid receptor) and transcriptional machinery.** DBD: DNA binding domain; LBD: Ligand binding domain; RARE: Retinoic acid response element; RA: Retinoic acid.

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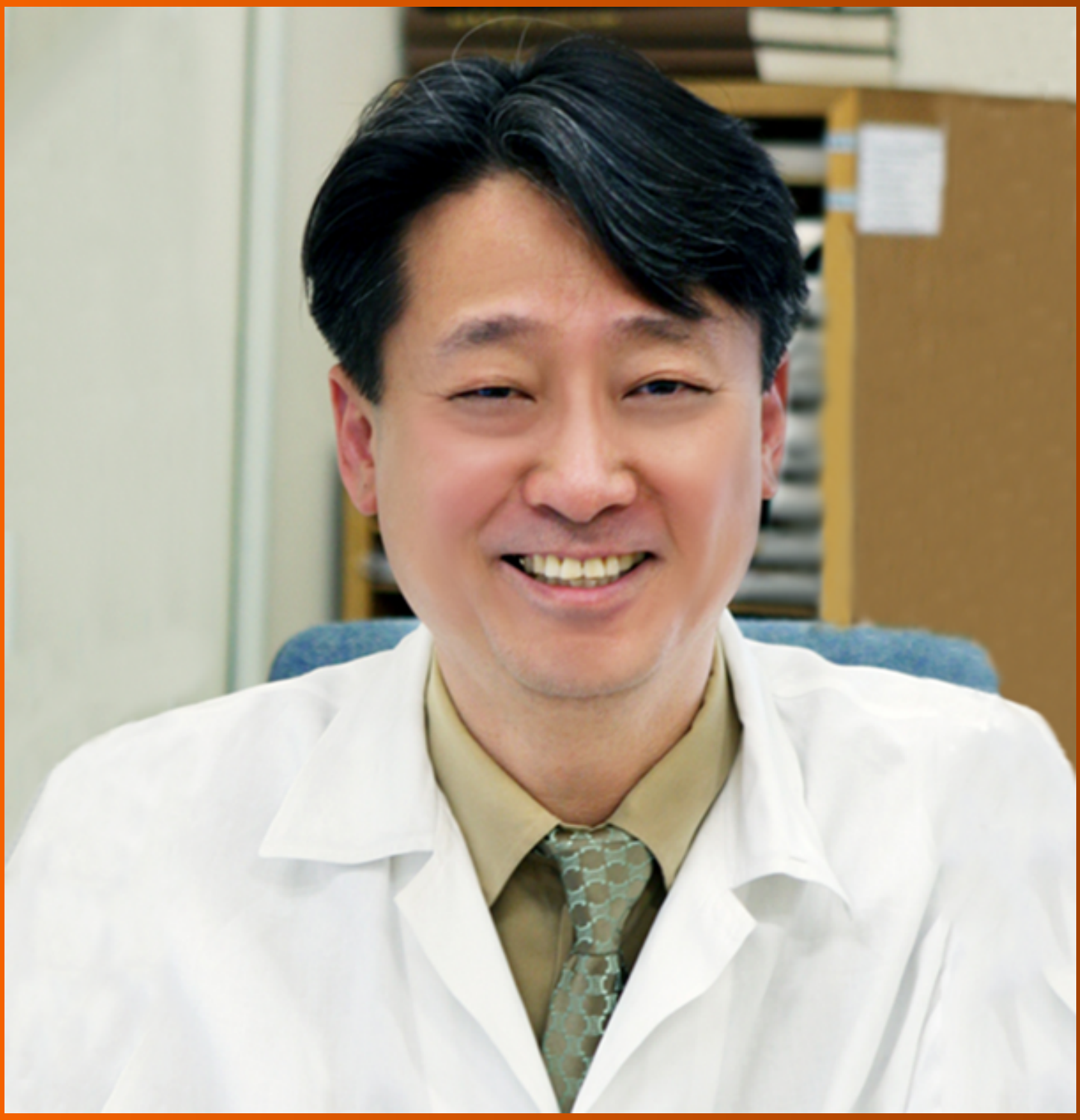

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REVIEW

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REVIEW

# Complex interactomes and post-translational modifications of the regulatory proteins HABP4 and SERBP1 suggest pleiotropic cellular functions

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**Author contributions:** Colleti C, Melo-Hanchuk TD, da Silva FRM, Saito Â and Kobarg J performed the literature search, analyzed and interpreted the data, generated the figures, contributed different sections of the manuscript and collated the final version of the text together. All authors read, revised and approved the final version.

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## Abstract

The 57 kDa antigen recognized by the Ki-1 antibody, is also known as intracellular hyaluronic acid binding protein 4 and shares 40.7% identity and 67.4% similarity with serpin mRNA binding protein 1, which is also named CGI-55, or plasminogen activator inhibitor type-1-RNA binding protein-1, indicating that they might be paralog proteins, possibly with similar or redundant functions in human cells. Through the identification of their protein interactomes, both regulatory proteins have been functionally implicated in transcriptional regulation, mRNA metabolism, specifically RNA splicing, the regulation of mRNA stability, especially, in the context of the progesterone hormone response, and the DNA damage response. Both proteins also show a complex pattern of post-translational modifications, involving Ser/Thr phosphorylation, mainly through protein kinase C, arginine methylation and SUMOylation, suggesting that their functions and locations are highly regulated. Furthermore, they show a highly dynamic cellular localization pattern with localizations in both the cytoplasm and nucleus as well as punctuated localizations in both granular cytoplasmic protein bodies, upon stress, and nuclear splicing speckles. Several reports in the literature show altered expressions of both regulatory proteins in a series of cancers as well as mutations in their genes that may contribute to tumorigenesis. This review highlights important aspects of the structure, interactome, post-translational modifications, sub-cellular localization and function of both regulatory proteins and further discusses their possible functions and their potential as tumor markers in different cancer settings.

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**Core tip:** Intracellular hyaluronic acid binding protein 4 and serpin mRNA binding protein 1 are paralog human regulatory proteins that share 41% amino acid sequence identity. The characterization of their protein interactomes suggested their functional association with transcriptional regulation, mRNA metabolism and in the cell's DNA damage and stress responses. Their complex post-translation modifications, involving phosphorylation, arginine methylation and SUMOylation, as well as their finely regulated sub-cellular localization in the nucleus and cytoplasm as well as in several cytoplasmic and nuclear granules suggest extensive functional regulation. This review discusses the functional and structural aspects and emerging roles of these regulatory proteins in human cancer.

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## INTRODUCTION

Ki-1 was the first monoclonal antibody specific for Hodgkin and Stenberg-Reed cells in Hodgkin's lymphoma[1]. Ki-1 recognizes CD30, a glycoprotein of 120 kDa found on the surface of Hodgkin's cells, and cross-reacts with an intracellular antigen of 57 kDa, named Ki-1/57, which was functionally and structurally uncharacterized at that time[2,3]. Ki-1/57 was also named intracellular hyaluronic acid binding protein 4 (IHABP4; GeneBank: AF241831) as it bound to hyaluronic acid *in vitro*[4]. Huang and co-workers also observed that IHABP4 binds to others negatively charged molecules such as glycosaminoglycans, e.g. chondroitin sulfate and heparin sulfate, and to RNA. However, the functional role of these interactions is not completely understood. The recommended name is hyaluronic acid binding protein 4 (HABP4).

HABP4 shares 40.7% identity and 67.4% similarity with serpin mRNA binding protein 1 (SERBP1), indicating that they might be paralogs, possibly with similar or redundant functions in human cells[5]. The name of its putative paralog was originally CGI-55, derived from: "Comparative Gene Identification", a method used to search for related genes. In the year 2000, Lin' s group obtained 150 potential full-length novel human genes through CGI, identified from the *Caenorhabditis elegans* proteome[6], with the number 55 being one of the SERBP1 transcript variants (GenBank: AF151813). Independently, CGI-55 was identified as an interactor of plasminogen activator inhibitor type-1 (PAI-1) RNA; therefore, it was also called PAI-1 RNA-binding protein or PAI-RBP1[7]. Moreover, other names such as HABP4L and SERPINE 1 were also used. As SERBP1 is most widely used it will be adopted in this review.

Since the identification of these two proteins, several studies have addressed their structure and function. Here, we present a detailed report on the current knowledge on the HABP4 and SERBP1 proteins.

## HABP4 AND SERBP1 STRUCTURE

Structurally, HABP4's amino acid sequence, has a high level of disorder-promoting amino acids (Alanine, Arginine, Glycine, Glutamine, Serine, Proline, Glutamic acid, Lysine), a high net charge and a low mean hydropathy value in its amino acid composition[8]. These features are observed for most intrinsically unstructured proteins (IUP) and inhibit the formation of a hydrophobic core or a regular secondary structure[9,10]. Bressan *et al*[8] demonstrated using size exclusion chromatography (SEC), analytical ultracentrifugation and small angle X-ray scattering (SAXS) studies on the HABP4 C-terminal region ($HABP4_{122-413}$), that it is an elongated monomer in solution,

without a well-defined core. Thus, the HABP4 C-terminal has been shown to be a pre-molten globule of 37 kDa.

A proteinase K sensitivity assay showed that $HABP4_{122-413}$ was readily degraded, confirming its flexibility and absence of a stable hydrophobic core. Additionally, the spectrum obtained by circular dichroism (CD) experiments was typical of a random coil or denatured proteins, indicating the absence of a regular secondary structure[8]. However, after the addition of 2,2,2-trifluoroethanol (TFE), the CD pattern for HABP4 shifted, showing an increase in secondary structure. TFE is an alcohol used to promote increased hydrogen bonding, and thus increases propagation of the secondary structures in polypeptides[11,12]. The appearance of secondary structure is commonly seen for IUPs when they associate with their interactors[13]. The gain of the structure may be explained by the existence of secondary structural elements in the protein sequence[14].

Despite the lack of structural studies for SERBP1, its high level of similarity and identity to HABP4 on the amino acid sequence level, allowed comparative bioinformatics analyses, which suggested that SERBP1 may also be an IUP. The protein secondary structure prediction (PSIPRED) analysis of both proteins illustrated that both HABP4 and SERBP1, have similar contents of predicted secondary structure and random coil. This may lead to the conclusion that that both HABP4 and SERBP1, are unstructured proteins (Figure 1).

Proteins belonging to the IUP family are associated with a plethora of cellular processes, such as translation, RNA recognition, transcriptional regulation, cell cycle control, membrane fusion and transport, protein phosphorylation, storage of small molecules and the regulation and assembly of protein complexes[13,15]. All these biological processes are in accordance with the present knowledge on the interaction network of HABP4 and SERBP1 (see the Functional aspects of HABP4 and SERBP1 in the following sections for more details).

## POST-TRANSLATIONAL MODIFICATIONS

Post-translational modifications (PTMs) control protein functions by covalently attaching molecules to specific amino acid residues. The types of modifications exceed 200, such as phosphorylation, glycosylation, methylation, acetylation, ubiquitinylation, and SUMOylation among others, with phosphorylation being the most widely studied[16-19].

The PTMs of HABP4 and SERBP1 have been discovered over the years mainly due to the identification of their interaction with modifying proteins. Until now, HABP4 and SERBP1 were predicted to have phosphorylation, methylation and SUMOylation sites, as shown in Figure 2[20-22]. These PTMs and their impact on the functions of HABP4 and SERBP1 are described below.

### *Phosphorylation*

Initially, immunoprecipitates of HABP4 from three different tumor cell lines (L540, U266/B1 and Raji Burkitt), using the Ki-1 antibody, revealed an associated serine/threonine protein kinase activity[23]. Based on this it was also hypothesized that HABP4 enzymatic activity could be regulated by self-phosphorylation. This hypothesis was ruled out by Nery and co-workers[20], who demonstrated that full-length recombinant HABP4 did neither exert kinase activity itself nor towards other proteins. These results are in agreement with the sequence analyses that also do not show any kinase domain features in the HABP4 amino acid sequence (Figure 2).

Yeast two-hybrid (Y2H) screens identified the Receptor of ACtivated Kinase 1 (RACK1), a protein kinase C (PKC) adaptor protein, as a HABP4 interactor and it was hypothesized that HABP4 could be a substrate for phosphorylation by PKC, explaining the earlier findings of the co-precipitated kinase activity[23].

Experiments with L540 cells showed that HABP4 is indeed phosphorylated by PKC and that its phosphorylation level is increased when cells are activated by the addition of phorbol 12-myristate 13-acetate (PMA)[20]. Furthermore, HABP4 can be phosphorylated by different PKC isoforms, such as PKCαβ, $PKC_{\delta}$, PKCλ/ζ and more strongly by $PKC_{\theta}$. $PKC_{\mu}$ was the only member of the PKC family that did not phosphorylate HABP4 *in vitro*[20].

The phosphorylation of HABP4 does not seem to be affected by the presence or absence of RACK1, which binds to HABP4 C-terminal domain. On the other hand, the interactions between HABP4 and nuclear proteins, including RACK1 and CHD3[5,20], were down-regulated in response to phosphorylation. This modulation of interaction in response to phosphorylation shows that HABP4, at some level, may regulate the functions of the adaptor protein RACK1 or other protein interactors.

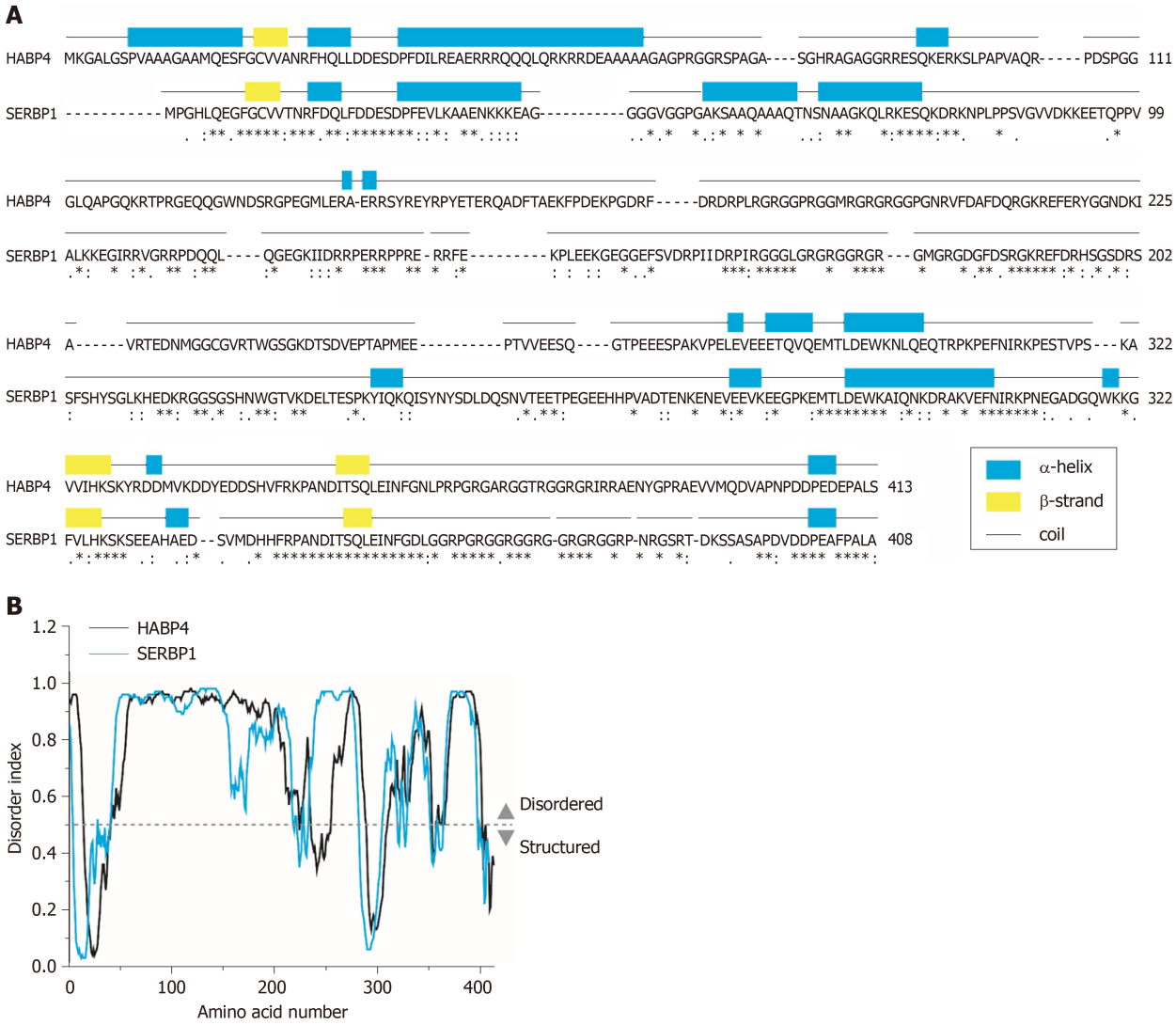


**Figure 1 Bioinformatics analysis of hyaluronic acid binding protein 4 and serpin mRNA binding protein 1 amino acid sequences.** A: Alignment between hyaluronic acid binding protein 4 (HABP4) and serpin mRNA binding protein 1 (SERBP1) and their predicted secondary structure content obtained by Clustal Omega and PSIPRED 4.0, respectively; B: Predictable disorder of HABP4 and SERBP1 structure obtained by DISOPRED 3. Below amino acids: Asterisk: Identical amino acid residues; colon: Strong similar properties; period: Weak similar properties. HABP4: Hyaluronic acid binding protein 4; SERBP1: Serpin mRNA binding protein 1.

PKC phosphorylates HABP4 only in its C-terminal domain, which contains 15 potential Ser/Thr residues that could be targets of phosphorylation. Interestingly, only two of the threonine residues (T354 and T375) were indeed phosphorylated *in vitro* by commercial PKC-Pan, showing that PKC activity on HABP4 is highly specific[20] (Figures 2A and 3A).

More recently, SERBP1 was also established as a RACK1 interactor through Y2H screens[24]. Since RACK1 can recruit PKC, we can predict that SERBP1 is also likely to be modified by phosphorylation, since several of the Ser/Thr residues are conserved in both amino acid sequences (Figure 2).

### Methylation

Many of the cellular processes that are mediated by specific interactions between proteins and other proteins or nucleic acids, are regulated by arginine methylation. The RGG/RXR box, where X is any amino acid, is the main target of arginine methyltransferases, such as PRMT1. In general, these motifs are found in proteins related to transcriptional regulation and RNA processing. HABP4 and SERBP1 present conserved RGG/RXR boxes in their sequences, localized mostly in their C-terminal regions (Figure 2). Both, HABP4 and SERBP1, were methylated by PRMT1 *in vitro*[26-28]. *In vivo* assays with L540 cells showed that the levels of HABP4 decrease in the cytoplasm in response to the methylation inhibitor Adox (adenosine-2',3'-dialdehyde) treatment, indicating that the methylation status of HABP4 can affect its cellular distribution. Additionally, nuclear HABP4 was stronger methylated than that localized in the cytoplasm. Interestingly, the paralog SERBP1 behaved otherwise. In

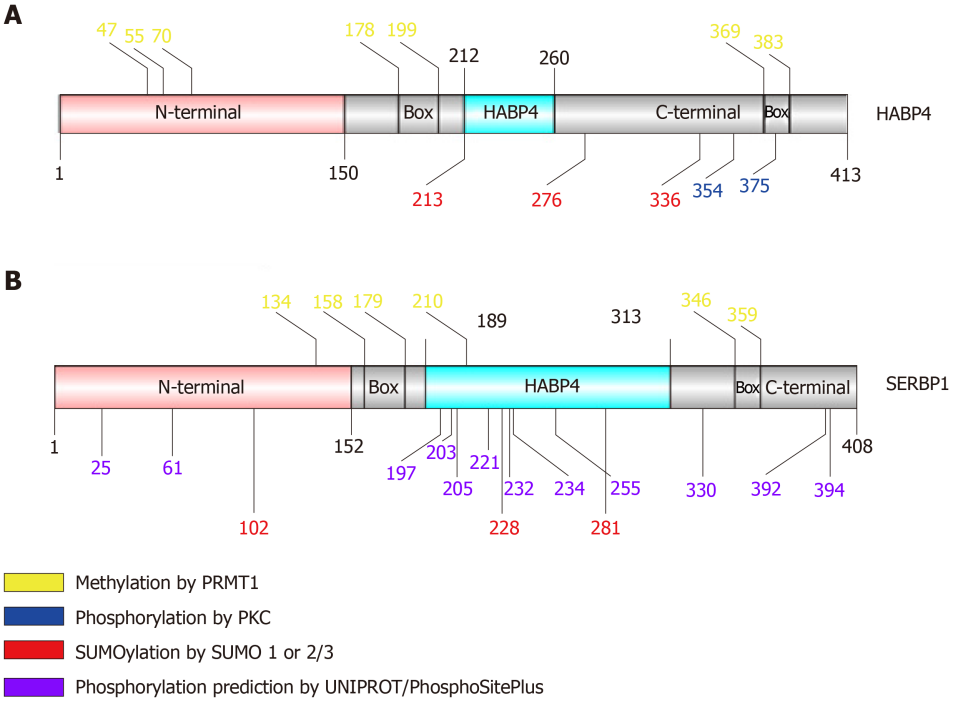


**Figure 2 Schematic view of hyaluronic acid binding protein 4 and serpin mRNA binding protein 1 primary structure identifying residues that exhibit post-translational modification.** The pink region corresponds to the N-terminal domain; gray corresponds to the C-terminal; light blue corresponds to the HABP4 domain. "Box" indicates RGG/RXR boxes.

untreated cells, SERBP1 in mostly found in the nucleus and upon Adox treatment more in the cytoplasm[26] (Figure 3C).

The interaction between HABP4 and RACK1 did not influence the methylation pattern. Although RACK1 interacts with the C-terminal domain of HABP4, methylation of the RGG/RXR box cluster 369-383 continued to occur. However, it was reported that significant inhibition of methylation in the C-terminal domains of HABP4 and SERBP1 occurred, when they were previously phosphorylated[26-28].

### *SUMOylation*

SUMOylation is the attachment of Small Ubiquitin-like Modifier (SUMO) proteins to a lysine residue in the specific target protein. This PTM regulates proliferation[29], transcription[30], mRNA processing and metabolism[31], among many other processes. The insight that HABP4 and its paralog SERBP1 could be SUMOylated was derived by the finding that both interact with proteins related to the SUMOylation machinery, including: UBC9, PIAS3 and TOPORS interacting with HABP4 and UBA2, PIAS-1/ -3 /-y and TOPORS all interacting with SERBP1[32,33].

HABP4 has seven predicted sites for SUMOylation, of which three are highly likely: Lysine 213, 276 and 336 (Figure 2A). Likewise, SERBP1 exhibited fifteen potential SUMOylation sites, of which six had a higher probability of being conjugated with SUMO (Figure 2B). *In vitro* assays showed that HABP4 is indeed SUMOylated by SUMO-2/3 at the three main targets[22]. *In vivo* experiments revealed that wild-type HABP4, but not the SUMOylation-defective mutant HABP4$^{K213R/K276R/K336R}$, co-immunoprecipitated with anti-SUMO-1 and anti-SUMO-2 antibodies.

The same approach was used for SERBP1, and mutations in the three lysine residues with the highest score (K102R/K228R/K281R), resulted in SERBP1 being unavailable for modification by SUMO conjugation that was observed in the wild-type protein[22].

SUMOylation by SUMO-1 or SUMO-2 does not affect the profile of SERBP1's interaction partners. The analysis of the partners identified by immunoprecipitation followed by tandem mass spectrometry (IP-MS/MS) with or without SUMO-1 or SUMO-2 transfection, resulted in the identification of proteins related to gene expression regulation. Specifically proteins involved in transcriptional control, RNA splicing and translation, ribosome biogenesis, apoptosis or mitosis, but no significant differences were observed between SUMO-1 and SUMO-2 co-transfection[22].

On the other hand, HABP4 displayed functional differences when co-expressed with SUMO-1 or SUMO-2. HABP4 co-immunoprecipitated 68 proteins when co-expressed with SUMO-1, whereas only 29 proteins were detected when HABP4 was

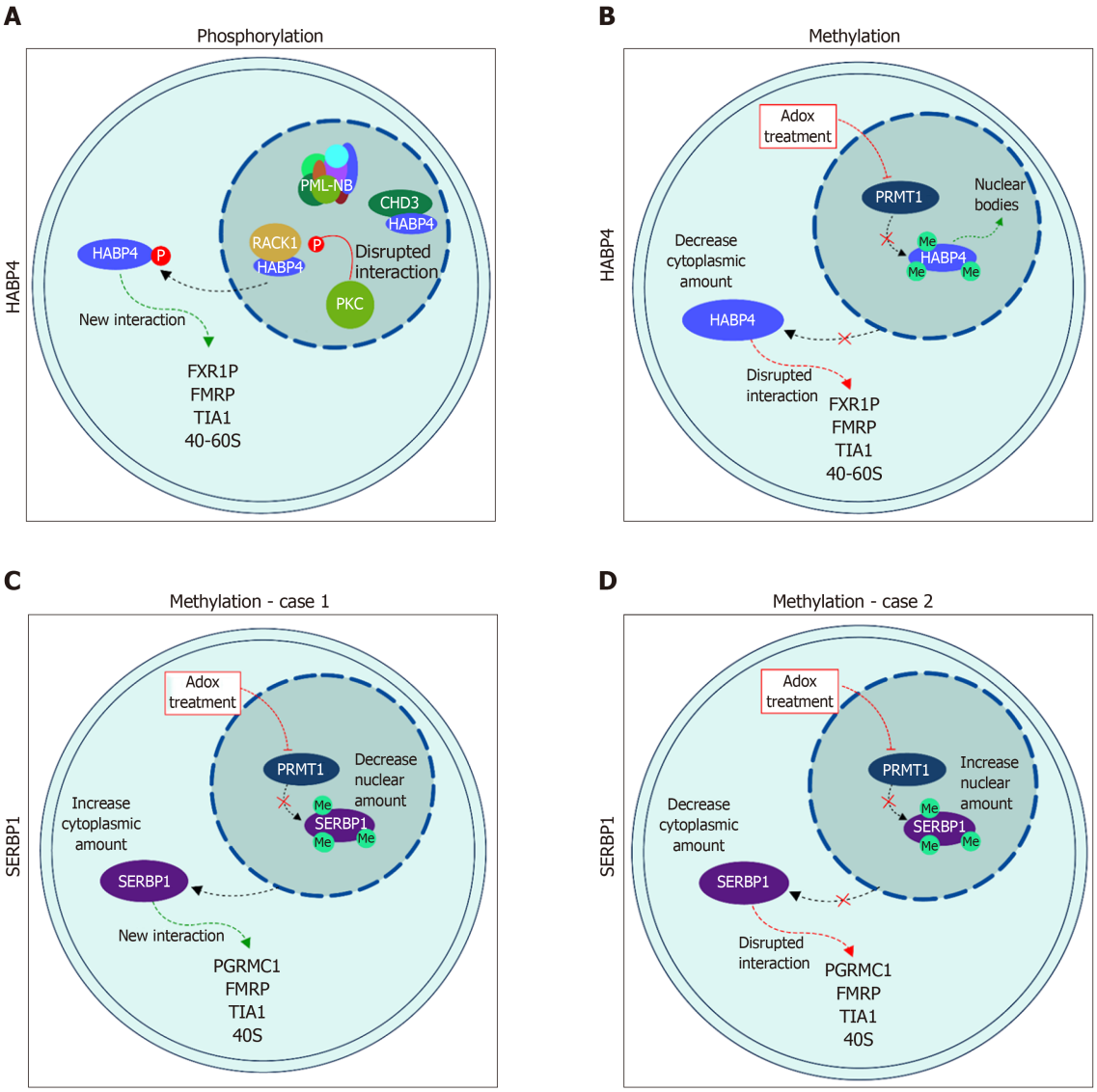


**Figure 3 Cellular localization of hyaluronic acid binding protein 4 and serpin mRNA binding protein 1 in response to post-translational modification.** A: Localization of hyaluronic acid binding protein 4 (HABP4), in light blue, after phosphorylation; and B: after methylation; C and D: Localization of serpin mRNA binding protein 1 (SERBP1), in purple, after methylation. SERBP1 methylation is still controversial and both possibilities are presented as related in the literature: C[26] and D[28]. Please see text for details. HABP4: Hyaluronic acid binding protein 4; SERBP1: Serpin mRNA binding protein 1.

co-expressed with SUMO-2. The enrichment of biological processes also presented some differences: HABP4 co-expressed with SUMO-1 was found to be involved in the regulation of transcription, RNA splicing, translation, ribosome biogenesis, mitotic cell cycle, the apoptotic process, and DNA repair. However, when HABP4 was co-expressed with SUMO-2, much fewer biological processes were observed, such as gene expression regulation (transcription, RNA splicing and translation) and telomere maintenance[22]. In summary, these data showed that HABP4 and SERBP1, despite having similar modification sites, respond in different ways to these PTMs in the context of their protein interactomes.

### *Cellular localization of HABP4 and SERBP1*

More than two decades ago, Rhode and co-workers showed that HABP4 has both cytoplasmic and nuclear localizations. In the nucleus it is often associated with heterochromatin, euchromatin, and the nucleolus[3]. The paralog SERBP1 also exhibited shuttling between cytoplasm and nuclei[5]. Since then, numerous data on these protein localizations have been reported. For example, Nery and co-workers[20] showed that the phosphorylation of HABP4 by PKC affects its cellular localization. Upon PMA-stimulus, HABP4 was no longer found in the nucleus, whereas its cytoplasmic level increased (Figure 3A).

Methylation also influences HABP4 and SERBP1 localization patterns. The methylation inhibition by Adox treatment leads to a decrease in HABP4 cytoplasmic

staining, but shuttling from the nucleus to the cytoplasm of SERBP1[26]. The localization of SERBP1 is still controversial. Lee and co-workers[27,28], showed that SERBP1 nuclear staining is stronger than cytoplasmic staining after methylation inhibition (Figure 3D). Altogether, these data suggest that HABP4 and SERBP1 are involved in nuclear functions, this possibly depends on the phase of the cell cycle, or on specific cell growth conditions.

Once in the nucleus, HABP4 was detected at sub-structures such as nucleoli, where ribosome biogenesis and maturation occur[34]. Both, SERBP1 and HABP4, were found to co-localize with p80-coilin, a marker for Cajal bodies, and HABP4 has also been observed to co-localize with GEMS (Gemini of coiled bodies) in cells treated with Adox[33,34]. Cajal bodies and GEMS are both considered nuclear compartments involved in small nuclear ribonucleoproteins snRNP storage or the assembly of pre-mRNA splicing complexes[35,36]. Upon Adox treatment, HABP4 has also been shown to co-localize with SC-35, a marker protein for splicing speckles[34]. These sub-compartments are known to store pre-mRNA splicing complexes, and HABP4 interacts with SFRRS1/9 and hnRNPQ, both known as splicing regulatory proteins[34]. In summary, these data suggest that methylation not only promotes nuclear import but also directs HABP4 more specifically to selected nuclear bodies.

The fact that HABP4 and SERBP1 interacted with several proteins related to promyelocytic leukemia nuclear bodies (PML-NBs), raised interest to determine if both proteins would play a role in the formation and distribution of these bodies. PML-NBs are related to protein modification, transcriptional regulation, DNA-damage response, DNA repair, cell proliferation and apoptosis[37]. The relationship of HABP4 and SERBP1 with PML-NBs was explored by Saito and co-workers, who showed that the number of PML-NBs decreases in response to HABP4 or SERBP1 over-expression. After treatment with arsenic trioxide ($As_2O_3$), which under normal conditions increased PML-NBs formation, their abundance was significantly lower in cells over-expressing HABP4 and SERBP1. Interestingly, SERBP1 and HABP4 SUMOylation-defective mutants revealed distinct behaviors in relation to PML-NBs. While the SERBP1 SUMOylation-defective mutant displayed an effect similar to the wild-type protein, the HABP4 SUMOylation-defective mutant did not have any impact on the number of PML-NBs. In addition, the presence of HABP4 or SERBP1 seems to affect the diffuse distribution of the PML protein[22].

The regulation of HABP4´s localization to PML-NBs by SUMOylation is exactly the opposite of what was observed for PML protein. PML only co-localizes with nuclear bodies when it is SUMOylated[38]. Although HABP4 and SERBP1 influenced the formation and distribution of PML-NBs, the PML protein, the main component of the PML-NBs, did not interact with HABP4 and SERBP1 in IP-MS/MS experiments. It is known that changes in the formation and distribution of PML-NBs can also occur in response to UV-C light exposure in a p53-dependent manner[39]. It has also been well established that HABP4 interacts with p53, reducing its transcriptional activity[32]. It could be hypothesized that the role of HABP4 in PML-NBs formation is mediated by regulation through p53, which on the other hand is part of PML-NBs.

Outside the nucleus, HABP4 interacts with the Fragile X-Related Protein 1 (FXR1P) and the Fragile X Mental Retardation Protein (FMRP), both of which are related to translational regulation[40,41]. Furthermore, it co-localizes with TIA1 upon arsenite challenge, which is a marker protein for stress granules[40,42].

In ribosomal fractions, HABP4 was detected in fractions containing the 40 and 60S ribosomal subunits and in small amounts in the polysome fraction, thereby pointing to possible additional roles of HABP4 in the context of translation[40]. Furthermore, SERBP1 also co-localizes with TIA1, after arsenite treatment. This migration of SERBP1 to the stress granules is affected by its methylation. Cells pre-treated with Adox, before the arsenite challenge, showed a decrease in SERBP1 recruitment to stress granules, but later also showed high retention of it in the granules[27].

## FUNCTIONAL ASPECTS OF HABP4 AND SERBP1

### *HABP4 and SERBP1: Interaction partners and functions*

Frequently, HABP4 and SERBP1 are described as hubs in signaling networks[13], as they can bind to their targets through multiple sites, facilitating the dynamic assembly of complexes. These multiple sites also allow for allosteric responses in biological signaling[43,44]. Besides the ability to have multiple interaction partners, IUPs are targets of PTMs, often having clusters of modifications[45].

The Y2H system is a powerful tool used to access the pairwise interaction of proteins[5,26,33,34,46]. Such assays with SERBP1 as bait identified several nuclear proteins as interactors and many of them are related to the transcriptional control of gene

expression, such as CHD3, DAXX, TOPORS, PIAS1, and PIAS3 (Figure 4)[5,33].

By using the N- and C- terminal domains of HABP4 as bait, a protein-protein interaction network revealed the pleiotropic functions of this protein. Many of its partners act at different levels of signaling processes (RACK1, PRMT1, EB-1, RIL, ALEX 2, CTGF and EPS8, APLP1, Myosin IXA). Other interactors include DNA binding factors and transcription regulators (such as CHD3, TOPORS, ZFP106, ZFP189, TIP60, BTBD2, YB-1/NSEP1, GADD34, DAXX, PIAS, p100 and HMG). A third category includes RNA metabolism-associated proteins (CIRBP, YB-1 /NSEP1, SFRS9 and SF2 / p32, FXRP). All this evidence seems to suggest that HABP4 has functions in all of the above related biological processes (Figure 4).

Interestingly, at the time of the initial discovery of the Ki-1/57 antigen in 1992[3] a series of cellular localization studies, using electron microscopy, with the original Ki-1 antibody, were performed using mostly the L540 Hodgkin analogous cells. Part of the data were published, but some figures were criticized as potentially nuclear "artifacts" (Figure 5). With today's knowledge of Ki-1/57/ HABP4 interaction with a number of transcriptional regulators (p53, p100, HMG, Topors, Daxx) and chromatin remodeling machines (CHD3), the images obtained may be interpreted, although in a speculative way, in a different light. Is it possible that the Ki-1 gold-labeling observed represents a chromosome, or chromatin? Is it transcriptionally active or in a repressed state? Future studies should address which parts of the genome are regulated by HABP4.

Based on the protein interaction profile and other assays, SERBP1 and HABP4 were functionally related to the regulation of gene expression[5,26,34,36,46]. To identify the candidate genes regulated by SERBP1 and HABP4 over-expression, a global gene expression (DNA microarray, Affymetrix) analysis was performed and revealed that most of the affected genes are related to apoptosis, proliferation, the cell cycle and mRNA metabolism[47]. After over-expression of both SERBP1 or HABP4 around 90% of the genes were down-regulated and these target genes were related to mRNA metabolism and transcription, suggesting that SERBP1/HABP4 may act mainly as a gene expression repressor[47].

### *The role of SERBP1 in the progesterone response*

The plasminogen activator inhibitor type-1 (PAI-1) is the major physiological inhibitor of fibrinolysis and plays important roles in cell adhesion, migration, and invasion[48]. PAI-1 has been related to tumor vascularization and metastasis and some inhibitors are currently being evaluated in cancer therapy[49].

As mentioned before, SERBP1 was previously called PAI-1 mRNA binding protein 1, because it binds to PAI-1 mRNA, and regulates its stability, thereby causing a decrease in overall PAI-1 protein levels in the cell[7]. SERBP1 binds to an AU-rich element located in the 3′-untranslated region of the PAI-1 mRNA. AU-rich elements have been determined as RNA instability promoting sequence motifs. The SERBP1/AU-rich element interaction may be regulated by Sphingosine 1-phosphate (S1P), a sphingolipid metabolite[50].

PAI-1 and SERBP1 mRNA are both over-expressed in ovarian tumors and the expression is higher in more advanced diseases[51]. In fact, the ovarian hormones progesterone (P4) and 17β-estradiol (E2), both regulate SERBP1 mRNA levels, especially in brain regions. These regions are important for the neuroendocrine control of female reproduction[52]. Additionally, the expression of SERBP1 mRNA was also increased in the hypothalamus, which is also important for female reproduction. The expression of SERBP1 mRNA in the brain correlates with the expression of its target mRNA: Progesterone receptor membrane component-1 (PGRMC1) mRNA[52,53]. PGRMC1 mediates the anti-apoptotic action of P4. The over-expression of SERBP1 increased the anti-apoptotic effects of P4 by 10-fold in spontaneously immortalized granulosa cells[54]. However, its effects seem not to be mediated by direct interaction of SERBP1 with the P4 hormone, as this protein possesses only the hyaluronan-binding region (Figure 2). SERBP1 seems to have allosteric effects on PGRMC1 by binding to its C-terminus, which is distant to the putative P4 binding site but could mediate the actions of P4 by serving as a scaffolding protein or co-activator/regulator of PGRMC1[55]. Besides the well-characterized interaction between SERBP1 and PGRMC1, other partners of SERBP1 and HABP4 are also related to P4- and estrogen-related processes, some of which are listed in Table 1.

### *HABP4 and SERBP1 in the context of the DNA damage response*

DNA damage is a constant event in cells due to exposure to exogenous (ultraviolet light, ionizing radiation, chemotherapy, radiotherapy) or endogenous agents (reactive oxygen species, oxidation of bases, formation of adducts)[56-60]. Each type of damage is able to activate different cellular responses, including DNA damage repair, changes in the transcriptional response, triggering of apoptosis, senescence, or activation

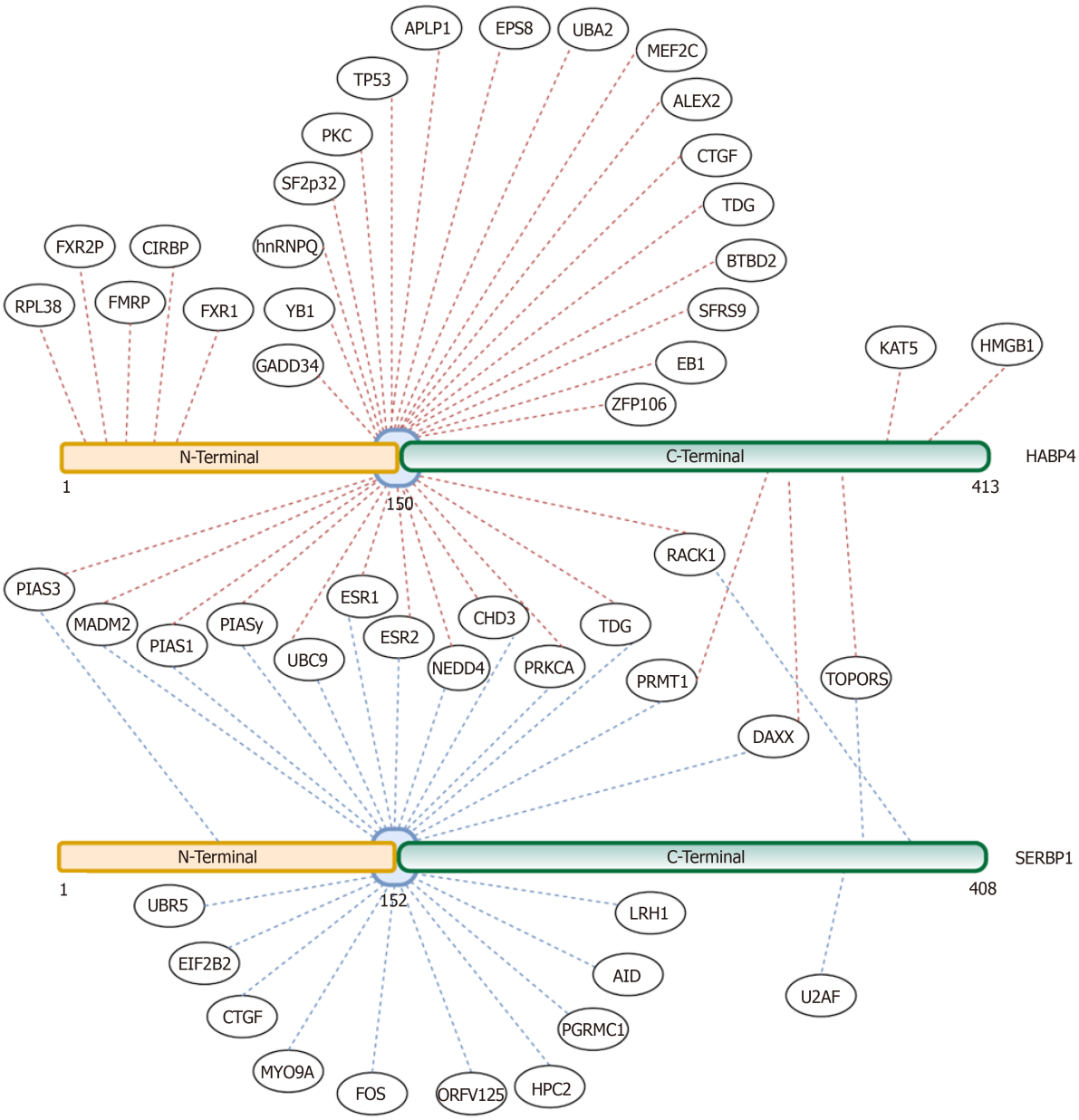


**Figure 4 Global protein interaction network of hyaluronic acid binding protein 4 and serpin mRNA binding protein 1.** The published interactors of hyaluronic acid binding protein 4 (HABP4) (top) and serpin mRNA binding protein 1 (SERBP1) (below) were linked to the specific domains of interaction. Proteins that interact with both N- and C-terminal domains, or the site of interaction is unknown, were linked to the blue center. Common interactors between HABP4 and SERBP1 are connected to both proteins and are located between both proteins. Red dotted lines are published interactors of HABP4, and blue dotted lines are interactors of SERBP1. HABP4: Hyaluronic acid binding protein 4; SERBP1: Serpin mRNA binding protein 1.

/blockage of the cell cycle checkpoints[61].

Both HABP4 and SERBP1 interacted with proteins related to the DNA damage response. In response to DNA double-strand breaks, SERBP1-depleted cells showed defects in the activation by phosphorylation of CHK1 and RPA2[62]. The effect of SERBP1 in the Homologous Repair pathway is partially explained by the regulation of CtIP (C-terminal binding protein interacting protein) translation in the S phase, once SERBP1 targets CtIP mRNA, thereby controlling its expression levels[62]. CtIP is important for end resection-mediated double-strand break repair *via* both the Homology Repair (HR) and Non Homology End Joining (NHEJ) pathways[63].

SERBP1 and HABP4 interact further with UBC9, DAXX and PIAS, all of which are related to DNA damage/repair pathways[33,34,46]. HABP4 also interacted in the Y2H system with GADD34, p53, and YB-1[34,46]. The YB-1 protein under normal conditions is mainly located in the cytoplasm, but after genotoxic stress, either by UV-radiation or by treatment with cisplatin or mitomycin C, the protein is translocated to the nucleus, where it has a high affinity for damaged DNA sites[64,65]. In addition, YB-1

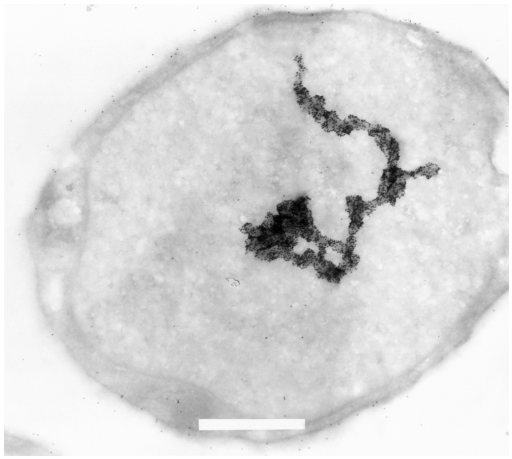

**Figure 5 Electron microscopic image of a L540 Hodgkin-derived cell labeled with 15 nm gold-particle-coupled Ki-1 antibody (× 29.000 fold magnification).** Some labeling on the cell surface is due to Ki-1 binding to the CD30 cell surface receptor. Strong hyaluronic acid binding protein 4 (HABP4) labeling can be detected in the nucleus and the format of the large macrostructure seems to represent part of a chromosome or also (transcriptionally active?) chromatin. A part of the upper end of the macrostructure seems to follow a "corkscrew"-like pattern. Each small black dot is a single Ki-1 gold labeled antibody. Please see[3] for more experimental details. In brief, cells were pelleted by centrifugation. The cell pellet was solidified on ice and the solid cell block was prepared in slices of 2 mm that were dehydrated by incubation in a solution containing stepwise increasing sucrose concentration up to 70%. After fixation in frozen nitrogen, ultrathin cryosections were prepared with an ultracut E, FC-4D and collected on nickel grids. After incubation with Ki-1 antibody, washing, and incubation with secondary reagent *Staphylococcal aureus* protein A-gold labeled, and further washing, the sections were fixed and contrasted with 4% uranyl acetate to be subsequently analyzed in a Siemens Elmiskop 101, Electron Microscope. We would like to thank Prof. Dr. Hilmar Lemke (Kiel, Germany) for generously providing the electron micrography.

interacts with key proteins of the base excision repair (BER) and nucleotide excision repair (NER) pathways, such as APE1, DNA polymerase β and DNA glycosylases[66-68].

The Δ113 isoform of p53, another HABP4 partner, antagonizes the effects of p53 towards apoptotic activity and its expression is increased after irradiation, promoting the repair of the DNA double-strand breaks through the HR and NHEJ pathways. Δ113p53/Δ133p53 promotes repair by regulating the expression of RAD51, LIG4 and RAD52[69]. p53 levels are also regulated by the ubiquitin ligase MDM2, which in turn is regulated by the DAXX protein, that also interacts with HABP4. After treatment with etoposide, ATM phosphorylates DAXX and the complex DAXX-MDM2 is broken down, thereby promoting the activation of p53[70].

Other HABP4 interactors also have functions related to DNA damage. PRMT1, for example, has a function related to the DNA damage response, once it accumulates in cytoplasmic bodies responsive to DNA damage[71]. The protein inhibitor of activated STAT (PIAS) also interacts with HABP4 and when over-expressed in HeLa cells promoted higher resistance to ionizing radiation through its involvement in the repair pathways HR and NHEJ[72]. Additionally, PIAS3 acts as E3 ligase and mediates the required BRCA1 SUMOylation, which in turn is essential for the ubiquitin ligase activity of BRCA1 itself[73].

### *The emerging regulation of SERBP1 through miRNAs*

The microRNAs (miRNAs) are small non-coding RNAs that modulate gene expression by binding to target mRNA[74]. By binding in the 3' -untranslated region of target mRNAs they inhibit translation or promote degradation of the mRNA. As a consequence the protein expression or non-coding RNAs may be regulated[75]. miRNAs have been explored as biomarkers and therapeutic targets for many pathological conditions including cancer[76], diabetes[77], viral infections[78], cardiovascular disease[79], and neurodegenerative diseases[74], among others.

The study of the regulation of SERPB1 by miRNA has emerged in the past few years. The analysis of miRNA expression in peripheral blood of patients with osteonecrosis of the femoral head revealed that many miRNAs target SERBP1 and p53[80]. In hepatocellular carcinoma (HCC) SERBP1 is regulated by miR-218[81]. miR-218 plays a role as a tumor suppressor in certain types of human cancers and is involved in biological processes such as tumor initiation, progression and metastasis[82]. Despite the negative correlation between the expression of miR-218 and SERBP1 in HCC tissues, when both are co-transfected, miR-218´s ability to inhibit metastasis and reverse Epithelial Mesenchymal Transition, was abolished, suggesting that the

**Table 1 Table summarizing the interactions of hyaluronic acid binding protein 4 and serpin mRNA binding protein 1 with proteins related to the hormones progesterone and estradiol**

| Gene | Protein | Biological process reference | Interaction reference |
|---|---|---|---|
| UBR5 | E3 ubiquitin-protein ligase UBR5 | Progesterone receptor signaling pathway[158] | SERBP1[22] |
| EIF2B2 | Translation initiation factor eIF-2B subunit beta | Ovarian follicle development[159] | SERBP1[22] |
| FOS | Proto-oncogene c-Fos | Response to progesterone stimulus[160] | SERBP1[22] |
| PGRMC1 | Membrane-associated progesterone receptor component 1 | Progesterone receptor signaling pathway[161,162] | SERBP1[149] |
| ESR2 | Estrogen receptor beta | Cellular response to estradiol stimulus[163] | SERBP1, HABP4[22] |
| MDM2 | E3 ubiquitin-protein ligase Mdm2 | Cellular response to progesterone stimulus[164] | SERBP1, HABP4[165] |
| NEDD4 | E3 ubiquitin-protein ligase NEDD4 | Progesterone receptor signaling pathway[166] | SERBP1, HABP4[22] |
| ESR1 | Estrogen receptor | Cellular response to estradiol stimulus[163] | SERBP1, HABP4 [22] |
| PKC | Protein Kinase C | Cellular response to estradiol stimulus[167] | HABP4[20] |
| p53 | Cellular tumor antigen p53 | Cellular response to estradiol stimulus[168] | HABP4[21] |

function of miR-218 is dependent on SERBP1[81]. In pancreatic cancer cells, miRNAs also act on SERPB1, mainly through miR-448, in a pathway dependent on the long non-coding RNA-PVT1, which regulates proliferation and migration[83].

## HABP4 AND SERBP1 INVOLVEMENT IN CANCER

### *HABP4 and SERBP1 in cancer*

Based on the broad clinical spectrum and the underlying, associated molecular behavior, cancer is an extremely complex disease. Tumor initiation and progression result from inherited or acquired genomic alterations within the cells[84], resulting in the acquisition of advantageous features that lead to uncontrolled growth and proliferation[85]. For each type of cancer, specific proteins are frequently altered during cancer initiation and progression, disrupting or promoting protein-protein interactions[86], or causing loss- or gain of function protein variants. The interaction profiles of HABP4 and SERBP1 suggest that they are involved in important cellular events which are related to tumorigenesis[47]. This includes the regulation of gene transcription, translation, RNA splicing, mRNA metabolism, mitotic cell cycle, and apoptosis, as described before[5,22,46,47].

The over-expression of HABP4 in HEK293 cells followed by microarray analysis, showed alterations in gene expression related to proliferation, including those coding for the proteins PLAU, PDXK, NRG1 and α-taxilin (TXLNA). Repression of the TXLNA protein correlates with the proliferative activity of HCC and the metastatic and invasive potential of renal cell carcinoma[87,88]. The over-expression of SERBP1 also resulted in reduced expression of genes related to proliferation, such as GNB1 and CCL14. Based on these findings, both HABP4 and SERBP1 are involved in repressive control mechanisms of proliferation[47]. The proliferation status of cells with HABP4 and SERBP1 over-expression was also evaluated by MTS and EdU incorporation and a lower rate of proliferation was observed, relative to non-transfected cells.

In the same study, HABP4 over-expression led to down-regulation of negative apoptosis regulators, including MAP2K5, ADNP, ANXA4, as well as the positive regulator BCLAF1[47]. In addition, under treatment with the ER-stress inducer thapsigargin, it was found that HABP4 over-expression increased the mRNA level of HSP90B1, an endoplasmic reticulum chaperone that protects against apoptosis[89]. In cells with SERBP1 over-expression, down-regulation of negative apoptotic regulators MAP2K5, PAK7 and FOXO1[47], was observed. The expression of some genes related to the cell cycle or cell division control, such as cyclin-dependent kinase 15 (CDK15), MAPK12[90,91], and the nuclear distribution genes NDEL1 and GORASP1[92,93], was also down-regulated by HABP4 and SERBP1 over-expression, respectively[47].

Taken together, these data suggest that HABP4 and SERBP1 have similar functions, mainly as repressors of genes involved in proliferation, cell cycle and apoptosis regulation, cellular processes that are frequently de-regulated in cancer[47]. However, the target genes can be different for both proteins and consequently the outcome. Several studies suggest important functions of the HABP4 and SERBP1 proteins in human cells that can contribute to a better understanding of the tumorigenic process[47].

### *HABP4 and SERBP1 are related to epigenetic modifications*

Epigenetic modifications, such as alterations in chromatin structure, represent critical regulatory events for cellular proliferation and tumor formation. Many tissue types have altered epigenetic profiles, which contribute to cancer development[94,95]. Chromatin remodeling is one of the epigenetic alterations that is important for the maintenance of chromatin structure and genomic stability[95]. It can change the gene expression patterns and plays important roles in tumor growth, coordinating the transcription factors and protein complexes during the regulation of gene expression[96]. HABP4 and SERBP1 interact with the chromatin remodeling protein "Chromo-Helicase DNA-binding domain-3" (CHD-3), which regulates gene transcription[5,33]. Although the exact mechanisms by which the complexes HABP4- and SERBP1-CHD-3 act, are still unknown, they might influence gene expression regulation of genes related to tumorigenesis. These mechanisms require further analysis. However, the altered transcriptional regulation through pathways targeting chromatin-remodeling has already been reported in tumors[95,97].

The histone modification patterns are crucial for the organization and maintenance of chromatin and as a result, transcriptional regulation. These processes are fundamental in understanding epigenetic mechanisms and their involvement in cancer development, as transcriptional control is essential for appropriate cell proliferation and differentiation[98,99].

Furthermore, HABP4 over-expression in HEK293 cells revealed an increase in the expression in histone genes[47]. These histones could be involved in chromatin compaction during the gene repression process caused by HABP4 over-expression. The above-mentioned repressed genes may also be associated to other, already mentioned, biological processes, such as cellular proliferation, apoptosis and the cell cycle[47,100].

HABP4 may regulate gene expression in response to stress[47]. Some of the histones triggered by HABP4 over-expression are related to the stress response. For example, H2AX, which is responsible for recruiting multiple proteins to chromatin, during the DNA damage/repair response[101]. Additionally, HABP4 and SERBP1 over-expression also influence some histone gene clusters that are preferentially associated with PML-NBs, which respond to basic physiological processes and several forms of stress[102]. Interestingly, PML-NBs also play a role in chromatin regulation and contain histone-modifying enzymes and transcription factors within them[102].

For example, DAXX, a histone component of PML-NB that interacts with HABP4 and SERBP1, possibly coordinates chromatin dynamics by binding to histone deacetylases and chromatin remodeling proteins[103]. Also, HABP4 over-expression reduces histone-lysine N-methyltransferase (SETMAR) expression, which is responsible for the methylation of lysine residues in histone and heterochromatin formation[104].

The histone methyltransferases (HMTs) transfer methyl groups from S-adenosyl methionine (AdoMet) to the lysine and arginine residues of target substrates, which may affect gene transcription, chromatin compaction and effector protein binding[105-107]. HMTs have been found to play fundamental roles in cell differentiation, gene regulation, DNA recombination and DNA damage repair[108-110]. The over-expression of different HMTs and their interaction with oncogenes is associated with the cancer phenotype[111]. Interestingly, HABP4 and SERBP1 not only interact with HMTs but are also methylated by the protein arginine methyltransferase PRMT1[26]. A misregulation of methyltransferases modifies the balance of transcription and leads to changes in cell destination, which in turn may result in tumor development[107]. Further studies are required to better understand the roles of HABP4 and SERBP1 proteins in tumorigenesis events mediated through methyltransferases such as PRMT1 and SETMAR.

### *HABP4 and cancer*

Kobarg and co-workers[112] showed, for the first time, the expression of HABP4 in some tumor cell lines and in activated leukocytes. The HABP4 gene was mapped in the human chromosome 9, bands 9q22.3-31, an area associated with secondary chromosomal aberrations in acute myeloid leukemia and in colon neoplasia[113]. Although previous experiments with HABP4 protein were performed in the Hodgkin's lymphoma analogous cell line L540[5,112], the relationship between HABP4 and Hodgkin's disease is still not clear[47].

However, in the colon, the 9q22.2-31.2 region, which contains the HABP4 gene, was found to be in linkage disequilibrium with SNP haplotypes found in families with a certain colon neoplasia risk[113]. Preliminary studies have revealed that HABP4 has characteristics also very common in several onco-proteins, such as PTMs, shuttling between the cytoplasm and nucleus, and transcriptional regulation activity[5,40,112,114]. Additionally, HABP4 interacted with PKC[3,21,112], which is considered a tumor suppressor, and loss of function mutations were linked to breast, bladder, skin, and

other forms of cancer[115]. However, the exact role of HABP4 in human cells and cancer remains unknown[46].

Another correlation of HABP4 protein with cancer is based on the fact that it belongs to the class of IUPs[8]. Members of the IUPs have received considerable attention lately, due to some of them being involved in the development of several pathologies[46,116]. Indeed, many proteins or domains that are functionally associated with cancer and other human diseases have long disordered regions[116-118]. Additionally, HABP4 plays a role in important regulatory mechanisms such as transcription, translation, cell-cycle checkpoints, and signal transduction, through its interacting proteins and *via* DNA/RNA either directly or mediated by other proteins[8]. This suggests its functional plasticity and ability to bind to various partners involved in the tumorigenic process[8,116].

The p53 is a nuclear transcription factor that regulates numerous target genes involved in important cellular processes, such as cell cycle arrest and monitoring of the G1 checkpoint, apoptosis, senescence, repair of damaged DNA, as well as metabolic regulation, playing a central role in human cancer as a tumor suppressor[119,120]. When DNA is damaged, free p53 is induced to accumulate in the cell nucleus, mediated through PTMs such as phosphorylation and acetylation[121]. Some studies have shown that the p53 gene is mutated in over 50% of human cancers. The tumor suppressor p53 has also been associated with PML-NBs[32,122], which play significant roles in genome maintenance[37]. Y2H system assays have shown that HABP4 interacts with p53 and with other p53 interacting proteins, as well as with several nuclear proteins involved in the regulation of transcription in the human Hodgkin's disease analogous cell line L540[32]. HABP4 can negatively influence p53-dependent transcription by blocking its DNA binding. The p53/HABP4 interaction was reported to be inhibited by *in vitro* phosphorylation of p53[32].

### *HABP4 and mRNA splicing in cancer*

Pre-mRNA splicing is a post-transcriptional process of the eukaryotic gene expression machinery and consists of the removal of introns and the junction of exons in gene transcripts, leading to mature RNAs[123,124]. Deregulation of the splicing process has been discovered to be a critical contributor to the genesis and development of different types of cancers[125,126]. Mutations were found in diverse types of cancers and are linked to alterations in splicing, regulation of specific transcripts and control of spliceosomal activity[123,127].

Several HABP4 interaction partners are involved in gene expression regulation, at the transcriptional level or pre-mRNA splicing[26,32,40,128]. In this way, HABP4 is a potential candidate that could affect cellular fate and function in cancer[127]. HABP4 can also play an important role in tumorigenic events through pre-mRNA splicing alterations[128]. Following this argument, further studies are required to identify the expression of some specific subsets of mRNA that may be regulated by HABP4 in cancer, considering that altered proteins originating from alternative RNA splicing are promising candidates for the diagnosis and even targets for novel therapeutic strategies[123].

It is worth noting that two protein partners were identified for HABP4 that are involved in the regulation of pre-mRNA splicing and cancer. They are serine/arginine-rich splicing factor 9 (SFRS9), a member of the serine/arginine-rich (SR) protein family and heterogeneous nuclear ribonucleoproteins (such as hnRNPQ)[128-130]. In immunoprecipitation and pull-down assays of HeLa cell extracts, both proteins were pulled down by HABP4, suggesting that these splicing proteins interact specifically with HABP4, probably forming functional complexes *in vivo*[128].

In addition, these proteins have some similar characteristics with HABP4. SFRS9 protein, for example, is a target of arginine methylation, which is required for its localization and trafficking to mammalian cell nuclei[128]. hnRNP proteins act on pre-mRNA splicing through site-specific binding (Arg/Gly-rich clusters) within the target RNA[131], and HABP4 has several RGG-box (Figure 2A) that are important for the interaction with many RNA-binding proteins that mediate splicing decisions[128,132].

Interestingly, SRSF9 has been considered a proto-oncogene by promoting cell proliferation *via* β-catenin (key effector of the Wnt signaling pathway). β-catenin accumulation in the cytosol and nucleus was found in colon cancer cell lines, and led to increasing colony formation in SRSF9 over-expressing NIH3T3 cells. Furthermore, when these cells were implanted in nude mice they generated tumors of increased sizes compared to control cells, that do not over-express SRSF9[129].

Moreover, elevated levels of SRSF9 expression were found in glioblastoma, colon adenocarcinoma, squamous cell lung carcinoma and malignant melanoma[129], in cancer tissue arrays, when compared to normal tissues. In addition, SRSF9 is implicated in the proliferation of a bladder cancer cell line *via* an unknown mechanism and SRSF9 over-expression was found in a clinical bladder cancer

sample[133].

The hnRNPQ splicing protein has also been reported to be a proto-oncogene[134]. It can promote cell proliferation and translationally regulates cell cycle-related genes in SW480 colon cancer cells, by translation control. In this way, it may increase cell growth ability during tumor formation. Moreover, both its mRNA and protein levels were found to be elevated in colon tumor tissue, possibly, involving transcriptional or post-transcriptional regulation mechanisms.

The contribution of hnRNPs to the control of splicing site selection has been found for apoptotic genes. It was also reported that there was tight control of the balance between the activities of pro- and anti-apoptotic variants produced by apoptotic peptidase activating factor (APAF-1)[135], Bcl-x[136], Fas[137] and caspases[138]. hnRNPs also have a suppressive effect on DNA damage repair[139]. In general, studies have documented that several hnRNPs are involved in human malignancies and metastasis, being promising biomarkers of lung, head and neck, colon, breast, and pancreatic cancers and acute myeloid leukemia[140-142].

### *SERBP1 and cancer*

The over-expression of SERBP1 was reported in epithelial ovarian cancer, in breast-, colon-, prostate- and lung cancer as well as in glioblastoma[51,143,144-146]. In ovarian carcinoma, over-expression of SERBP1 was associated with higher tumor grades (Grade III *vs* Grades II and I tumors)[143,147]. Indeed, SERBP1 has prognostic value in ovarian cancer and other solid tumors[51]. Although its exact mechanism of action is not well known, SERBP1 was implicated in tumorigenicity and resistance to anti-cancer drugs[51,62].

In human breast cancer, SERBP1 over-expression was classified as a new prognostic marker[144]. A correlation between the expression of SERBP1 and nuclear P4 receptors was observed in malignant breast epithelial cells[27,144,148]. This relation is very important, as P4 receptors are ligand-activated transcription factors, playing a crucial role in the regulation of growth, survival, and differentiation of normal and malignant breast epithelial cells[144,148]. Furthermore, as pointed out above, SERBP1 interacts with PGRMC1, which is involved in mediating anti-apoptotic actions through the P4 receptor[55,144,149].

Interestingly, abundant SERBP1 expression in human breast cancer was associated with low PAI-1 protein levels in Western blot analysis[144], showing that SERBP1 can not only stabilize but can also destabilize PAI-1 mRNA, depending on the cellular context[7]. Heaton and co-workers documented an inverse relationship between SERBP1 and PAI in rat hepatoma, reporting that high SERBP1 protein levels, lead to increased degradation of PAI-1 mRNA and consecutively to low PAI-1 protein levels in rat hepatoma[150].

Other studies showed over-expression and high protein levels of PAI-1 in breast cancer, which was associated with poor prognosis. PAI-1 is considered a valuable factor in clinical practice[51,144,151]. In ovarian cancer, the over-expression of PAI-1 was also detected and related to advanced tumor stages, and poor prognosis in ovarian cancer patients[51,152-154]. Moreover, studies have reported an association between the high expression of PAI-1 in ovarian cancer and its histological grade[153,155], tumor stage[156], tumor recurrence[153] and residual tumor[157] (Table 2).

## CONCLUSION

HABP4 and SERBP1 share high levels of amino acid sequence identity and similarity and seem to have overlapping functions in the cell, related to transcription regulation, mRNA metabolism and DNA damage and stress responses. However, they have also exclusive interacting partners and might be differentially regulated. Thus, HABP4 and SERBP1 may be required in different situations to exert unique functions specific to each paralog protein. In this review, we presented the emerging role of HABP4 and SERBP1 in the cancer field, and the need for further studies to understand more deeply the cellular functions of both proteins.

**Table 2 Hyaluronic acid binding protein 4 and serpin mRNA binding protein 1 expression in different types of cancer**

| Protein | Expression/overexpression | Cancer types | Ref. |
|---|---|---|---|
| HABP4 | Expression | Hodgkin's lymphoma | Kobarg *et al*[112] |
| HABP4 | Expression | B-cell lymphatic leukemia | Kobarg *et al*[112] |
| HABP4 | Expression | non-Hodgkin-T-cell lymphoma | Kobarg *et al*[112] |
| HABP4 | Expression | Bladder | Kobarg *et al*[112] |
| SERBP1 | Overexpression | Ovarian | Koensgen *et al*[51] |
| SERBP1 | Overexpression | Breast | Serce *et al*[144] |
| SERBP1 | Overexpression | Colon | Wang *et al*[81] |
| SERBP1 | Overexpression | Prostate | Guo *et al*[145] |
| SERBP1 | Overexpression | Lung | Sun *et al*[147] |
| SERBP1 | Overexpression | Glioblastoma | Hlavaty *et al*[146] |

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