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Diverse roles of the nucleic acid binding protein KHSRP in cell differentiation and disease

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Abstract

The single-stranded nucleic acid binding protein KHSRP (KH-Type Splicing Regulatory Protein) modulates RNA life and gene expression at various levels. KHSRP controls important cellular functions as different as proliferation, differentiation, metabolism and response to infectious agents. We summarize and discuss experimental evidence providing a potential link between changes in KHSRP expression/function and human diseases including neuromuscular disorders, obesity, type II diabetes, and cancer.

Keywords

KHSRP; RNA-binding protein; neuromuscular disorders; inflammation; obesity; cancer

KHSRP (also known as KSRP) is a single-stranded nucleic acid binding protein independently discovered by Levens and Black laboratories[1,2]. Davis-Smyth and coworkers described KHSRP (named FBP2 in their paper) as a factor interacting with an enhancer element upstream of the c-myc oncogene promoter[1]. Min and coworkers reported that KHSRP is a component of a multi-protein complex that binds to a splicing enhancer element of c-src pre-mRNA[2]. The official name of the protein (KHSRP, for KH-Type Splicing Regulatory Protein, used in this review) reflects both the presence in the molecule of four distinct hnRNPK-Homology (KH) domains and its splicing function.

In the last twenty years additional roles of KHSRP in the post-transcriptional control of gene expression have been discovered. Considering that several recent reviews have exhaustively addressed the mechanisms by which KHSRP exerts its numerous functions[3-6], this review is

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CONFLICT OF INTEREST

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mainly focused on how KHSRP-dependent regulation of RNA metabolism affects distinct cell functions in different tissues and can impact on pathological conditions. However, for scholarly reasons we briefly summarize below the molecular functions of KHSRP that have been most extensively investigated and that will be cited in the following Sections of this review.

1. Implication of KHSRP in pre-mRNA splicing

Biochemical studies performed more than fifteen years ago revealed that KHSRP is a component of a multiprotein complex (also including hnRNPF, hnRNPH, and Polypyrimidine Tract Binding Protein) that binds specifically to a G+U-rich intronic splicing enhancer element downstream of the neuron-specific *SRC* N1 exon and is required for proper splicing[2]. Subsequently, Russo and coworkers observed that KHSRP, together with hnRNPH and nucleophosmin, interacts with intron 3 of human *RPL3* gene thus promoting the expression of an alternative isoform of RPL3[7].

2. Implication of KHSRP in mRNA decay

Upon the initial reports, KHSRP has been extensively studied as a factor required for rapid decay of inherently labile transcripts[3,4]. The AU-rich element (ARE) is the landmark cis-acting motif responsible for rapid mRNA decay in mammalian cells and can be found in the 3' untranslated regions (UTRs) of many short-lived transcripts[3,4]. We and others have shown that KHSRP interacts with AREs and recruits the RNA exosome (and other nucleases) on labile transcripts that encode cytokines and transcriptional regulators of cell identity and fate thus promoting their decay[3,4].

3. Implication of KHSRP in microRNA biogenesis

Expanding the array of KHSRP functions on RNA metabolism, the protein has been proved to promote maturation of select microRNAs (miRNAs) from precursors. KHSRP binds to the terminal loop (enriched in G nucleotides) of a cohort of miRNA precursors and interacts with both Drosha and Dicer complexes in nuclear and cytoplasmic compartments, respectively[3]. The miRNAs whose maturation is favored by KHSRP exert important functions in controlling cell proliferation, immune response, metabolic homeostasis and cell fate determination in response to extracellular stimuli[3,5,6]. We and others proposed that the terminal loop of miRNA precursors constitutes a pivotal structure where miRNA processing co-activators (such as KHSRP) and miRNA processing co-repressors function in a coordinated way to convey proliferating and differentiating cues into changes of miRNA expression[3,5,6].

4. Implication of KHSRP-long noncoding RNA interaction in gene expression control

We have recently reported that long non-coding (nc) RNAs (lncRNAs) represent an unanticipated class of RNAs that interact with KHSRP[8]. KHSRP directly binds to H19 lncRNA in undifferentiated multipotent mesenchymal C2C12 cells and this interaction favors KHSRP-mediated destabilization of labile mRNAs such as myogenin[8]. AKT1 and

AKT2 activation, which occurs during the early steps of myogenesis, induces KHSRP dismissal from H19 lncRNA and, as a consequence, myogenin mRNA is stabilized while KHSRP is repurposed to promote maturation of myogenic miRNAs, thus favoring myogenic differentiation[8]. Our unpublished data indicate that other lncRNAs associate with KHSRP in different cell types. This allows to hypothesize that the regulatory role played by H19 in C2C12 cells might be operated by different lncRNAs in other cell types.

Table I summarizes the diverse molecular functions of KHSRP and how they are affected by post-translational modifications.

1. Role of KHSRP in the immune response

The expression of cytokines and chemokines undergoes a finely tuned control. Deregulated levels of these immune modulators can cause or aggravate many pathological conditions of high social impact such as auto-immune diseases, neurodegenerative disorders and cancer. Thus, it is conceivable that several layers of transcriptional and post-transcriptional control exist to maintain homeostatic balance of immune modulators. Post-transcriptional control of cytokines and chemokines expression is operated through distinct mechanisms involving *i)* specific RNA elements (including the AREs) that control mRNA decay and/or its translation *ii)* miRNA-mediated gene silencing. KHSRP controls the expression of several immune modulators by acting at both these post-transcriptional levels.

After our initial observation that KHSRP associates with the RNA exosome to control rapid decay of *FOS*, *TNF* and *IL2* mRNAs[9], a report from Winzen and coworkers identified a number of modulators of the immune response as KHSRP mRNA targets in a pull-down-based screening (among them *IL8*, *CXCL2*, and *CXCL3*)[10]. They also reported that cell stimulation with IL1 reduced the interaction of KHSRP with *IL8* mRNA leading to mRNA stabilization[10,11]. The analysis of KHSRP knockout mice revealed that IFNA and IFNB are up-regulated in cells derived from *Khsrp*−/− mice because of enhanced mRNA half-life and this regulation explains the robust response of *Khsrp*−/− mice to viral infections (see Section 2)[12]. Further, Li and coworkers observed that the expression of TNF and IL1B significantly increased in astrocytes derived from *Khsrp*−/− mice when compared to wild-type littermate[13]. Considering that IFNs and cytokines play a crucial role in the pathogenesis of autoimmune diseases[14], the elucidation of KHSRP function in restraining their expression in immune cells may lead to the development of therapeutic strategies for these pathologies.

NOS2A (also known as iNOS) is an important component of the inflammatory response pathway that is encoded by an unstable mRNA whose expression is modulated by cytokines[15]. Linker and coworkers reported that ZFP36 (also known as TTP, an RNA-binding protein (RBP) that usually promotes decay of ARE-containing labile mRNAs) enhances *NOS2A* mRNA stability by interacting with KHSRP and hijacking the KHSRP-exosome complex away from *NOS2A* mRNA in colorectal adenocarcinoma cells[15]. More recently, the same group has reported that KHSRP represents a pivotal element for the anti-inflammatory function of resveratrol, a natural phenol produced by plants in response to injury or upon invasion by bacteria or fungi[16]. Resveratrol has been proved to have beneficial effects on cancer or cardiovascular diseases[15]. Resveratrol shortens the half-life of

the transcripts encoding *NOS2A*, *IL8* and *TNF*, by directly interacting with KHSRP and preventing the MAPK14 (also known as MAPK p38)-mediated inhibition of KHSRP decay promoting activity[15]. These findings underline the relevance of Thr692 phosphorylation by MAPK14 in the regulation of KHSRP function (see Section 4) and hint to KHSRP modulation as a target of therapeutic intervention in inflammatory diseases.

KHSRP can negatively control gene expression also by down-regulating ARE-containing mRNA translation. Dhamija and coworkers reported that as many as 50 mRNAs that are targets of KHSRP are enriched in polysomes after KHSRP knock-down and among these mRNAs are those encoding IL6, IL23A, and TNF[17]. Also studies performed in *Khsrp*−/− astrocytes support a role of KHSRP in TNF and IL1B translation[13].

We and others have reported on the role of KHSRP as a factor required for optimal biogenesis of select miRNAs from their primary transcripts[6]. Among these, miR-155 is able to regulate inflammatory and immune responses through down-regulation of multiple targets[18]. We showed that KHSRP promotes miR-155 biogenesis in murine macrophages in response to lipopolysaccharide treatment[19]. Gene expression analysis of cells in which either KHSRP was knocked-down or miR-155 was silenced showed that the expression of a large number of transcripts encoding cytokines and chemokines, such as IL1B, IL12B, and CXCL11, was significantly affected[19]. Remarkably, the effect of KHSRP silencing was reverted by forced expression of mature miR-155[19].

Patients suffering from granulomatosis with polyangiitis (Wegener's granulomatosis), eosinophilic granulomatosis with polyangiitis (Churg-Strauss syndrome), and microscopic polyangiitis express anti-endothelial cell antibodies that cause endothelial cell dysfunction[20]. Recently, Regent and coworkers observed that anti-KHSRP antibodies are present in 50% of patients suffering for these diseases[20]. This finding suggests a possible implication of KHSRP in endothelial cell physiology and pathology. Although intriguing, the relevance of pathological conditions dependent on the presence of anti-KHSRP autoantibodies deserves further confirmation.

2. Role of KHSRP in viral infections

Mammalians respond to viral invasion and replication through specialized innate immune sensors in cells that detect viral components and trigger downstream signaling pathways that can ultimately result in the activation of systemic immune response[5]. Several innate immune sensors recognize cytoplasmic viral RNA and lead to the production of Interferons that, in turn, trigger various antiviral pathways aimed at halting viral replication and spread[21]. In the last few years several studies have revealed that viral RNA, interacting with cellular RBP, can affect their physiological functions thus impacting on cellular metabolism of various RNAs[22].

Studies from Lin and coworkers revealed that KHSRP, competing with factors that enhance IRES (internal ribosome entry site)-mediated translation of enterovirus 71 (EV71), down-regulates IRES-dependent EV71 translation[23]. This effect is achieved through direct interaction of KHSRP with the IRES present in the 5’UTR of EV71. Interestingly, more recent studies from the same laboratory demonstrated that EV71 infection induces KHSRP

cleavage through multiple mechanisms, including caspase activation, proteasome activity, and autophagy induction[24]. Thus, enterovirus-induced cleavage of KHSRP reduces the level of a negative regulator of viral protein translation envisaging a negative feedback loop. Further, the study from Chen and coworkers also demonstrated that the truncated form of KHSRP lacking the C-terminal domain (KHSRP1-503), instead of being a negative regulator, becomes a positive regulator of EV71 IRES-mediated translation. The C-terminal domain lacking in KHSRP1-503 includes Thr692, an amino acid that undergoes phosphorylation by MAPK14. We and others reported that Thr692 phosphorylation impairs KHSRP decay-promoting activity on a variety of target mRNAs[6,11]. It would be interesting to directly verify whether Thr692 phosphorylation influences also the IRES modulating activity of KHSRP.

Avian influenza virus (AIV) often results in a virus-induced cytokine deregulation called “cytokine storm" typically characterized by the presence of elevated levels of pro-inflammatory cytokines and IFNs[25]. Recently, Liu and coworkers reported that KHSRP expression levels are strongly induced by the low pathogenic H9N2 viral strain in a spontaneously immortalized cell line of chicken embryo fibroblasts while it is not regulated by the highly pathogenic H5N1 viral strain infection[26]. The subtype-dependent host response observed in this study offers new insights into the potential roles of KHSRP in the control and modulation of replication and virulence of different subtypes or strains of avian influenza A virus[26]. Further, considering that KHSRP has been implicated in MAPK14 signaling pathway and that the regulation of this pathway has been reported to be associated with host defense to AIV infection, it would be interesting to investigate whether KHSRP phosphorylation by MAPK14 occurs during AIV infection[6,11,16].

Taking advantage of *Khsrp* knock-out mice, the Chen laboratory described an additional mechanism by which KHSRP controls susceptibility to viral infection in mammalians. These animals display enhanced type I IFN levels in response to viral infections when compared to wild-type littermate[12]. Specifically, *Khsrp*-deleted mice proved to be refractory to herpes simplex virus 1 and vesicular stomatitis virus infection as a consequence of enhanced expression of IFNs[12]. This observation has been recently extended to human cytomegalovirus infected cells where viral replication was significantly reduced in cells in which KHSRP has been silenced[28].

3. KHSRP controls cell fate determination and tissue remodeling

In response to developmental and differentiative cues, cells activate a network of signaling proteins, transcription factors, epigenetic modifiers, ncRNAs, and RNA-binding proteins that modulate functional and combinatorial interactions at the genomic, epigenomic, transcriptomic, and proteomic levels. These, ultimately, culminate in the activation or the repression of gene expression resulting in cell fate determination and tissue remodeling. On this regard, in the last few years KHSRP has emerged as a regulator of cell state transitions able to orient cell fate decisions operating at different levels[6].

Starting from the evidence that mice lacking *Khsrp* gene display reduced body adiposity, Chou and coworkers demonstrated that KHSRP favors brown-like transformation in

subcutaneous/inguinal white adipose tissue (iWAT)[29]. Targeted deletion of *Khsrp* enhances brown fat-selective gene expression in iWAT through an elevation of the levels of some important regulators of the thermogenic program such as PRDM16, PPARGC1A, and PPARA[29]. Importantly, KHSRP absence results in down-regulation of miR-150 due to its defective maturation from precursors[29]. Overexpression of *Prdm16* and *Ppargc1a*, that are direct targets of miR-150, leads to brown-like transformation of iWAT[29]. It is known that brown adipose tissue oxidizes fatty acids for heat generation and energy expenditure. Thus, promoting brown-like transformation in WAT is a promising strategy for combating obesity. Therefore, modulation of KHSRP-dependent miR-150 maturation from precursors could potentially lead to the development of therapeutic tools against obesity and metabolic disorders (see also Section 6)[29].

Multipotent mesenchymal cell differentiation is subject to intense regulation via integrated signaling networks that orchestrate changes in gene expression leading to commitment toward specific cell lineages[30]. We have extensively studied multipotent mesenchymal C2C12 cells for their potential to differentiate into myofibers upon serum withdrawal (see Section 4) or into osteoblasts upon addition to the culture media of BMP/TGF-β ligands[31]. In a recent study we have demonstrated that SMAD proteins, mediators of BMP/TGF-β ligands, regulate KHSRP function[31]. SMADs interact with KHSRP and impair its ability to bind to primary myogenic miRNAs (usually referred to as myomiRs) and to promote their maturation. Prevention of myomiR maturation is instrumental to promote the differentiation of C2C12 cells into osteoblasts[31]. In line with this observation, KHSRP silencing causes a reshaping of the transcriptome largely overlapping that produced by BMP/SMAD signaling activation in C2C12 cells[31]. The observation that myomiR re-expression in KHSRP-silenced cells is sufficient to enhance myogenin expression and to abrogate the osteoblastic phenotype, allowed us to propose that the most critical consequence of KHSRP silencing in C2C12 cells is the impairment of myomiR maturation and this is sufficient to direct C2C12 cell fate decision[31].

TGF-β orchestrates wound healing by regulating cell proliferation, differentiation, extracellular matrix production, and immune modulation[32]. In the early phases of wound healing, TGF-β is induced and, among other effects, down-regulates KHSRP levels[33]. Sundaram and coworkers have demonstrated that, in normal epidermis, KHSRP promotes biogenesis of miR-198 whose primary miRNA is located in the 3'UTR of follistatin-like 1 (*FSTL1*) transcript[33]. As a consequence of KHSRP down-regulation, miR-198 maturation from precursors is abrogated and the expression of FSTL1 is enhanced. Importantly, FSTL1 stimulates keratinocyte migration whereas miR-198 expression produces the opposite effect[33]. Thus, regulation of KHSRP expression by TGF-β is required to switch the post-transcriptional control of FSTL1/miR-198 expression to a "wound healing-mode"[33]. In their paper, Sundaram and coworkers point to the consequences of the failure of the FSTL1/miR-198 switch in non-healing chronic diabetic ulcers where miR-198 levels persist high, FSTL1 expression is absent, and keratinocyte migration —as well as wound healing— fail to occur[33]. Thus, the importance of KHSRP in the pathogenesis of diabetic ulcers deserves further investigation.

4. Role of KHSRP in myogenesis, muscle functionality, and neuromuscular diseases

Experimental evidence published in the last years demonstrated that post-transcriptional control of gene expression plays a central role in myogenesis and RBPs are essential gatekeepers able to ensure proper progression of myoblasts through the myogenic differentiation program[34].

Several studies have explored KHSRP role during normal myogenesis, a complex process in which at least two enzymatic signaling cascades, MAPK14 and PI3K/AKT, are activated and play a pivotal regulatory role[6] (see also Section 3).

We initially showed that the MAPK14 phosphorylates KHSRP at Thr692 in differentiating myoblasts[27]. This, in turn, impairs KHSRP ability to associate with ARE-containing transcripts encoding myogenin and CDKN1A (also known as p21) and results in stabilization and enhanced steady-state expression of the mRNAs encoding these two important regulators of myogenesis. More recently, Amirouche and coworkers reported that MAPK14-mediated phosphorylation of KHSRP impairs its mRNA decay function thus favoring the expression of utrophin[35]. Due to its high degree of sequence identity with dystrophin and its ability to associate with members of the “dystrophin-associated protein complex”, utrophin has been proposed as an excellent substitute to dystrophin itself with high potential to alleviate the dystrophic pathology[35]. Importantly, either KHSRP knock-down or activation of MAPK14 in a mouse model of Duchenne muscular dystrophy (mdx mice) significantly increased utrophin expression[35]. Therefore, modulating either KHSRP expression or its function could be envisaged as a novel strategy to treat dystrophic disease[36].

We also found that phosphorylation by AKT1 and AKT2 promotes KHSRP-dependent maturation of myomiRs in the early phases of myogenic differentiation of C2C12 cells[37]. The analysis of the decay of unstable transcripts (e.g. myogenin) combined with the analysis of myomiR maturation from precursors enabled us to conclude that PI3K/AKT signaling activation inhibits the ability of KHSRP to promote decay of myogenin mRNA while favors KHSRP ability to promote myomiR maturation[37]. More recently, we found that H19 lncRNA favors the mRNA decay promoting function of KHSRP via a “molecular scaffold” mechanism and prevents, at the same time, KHSRP ability to interact with specific miRNA precursors and to favor their processing into the mature forms[8]. Remarkably, the cytoplasmic association of KHSRP with lncRNA H19 is abrogated by PI3K/AKT signaling activation[8].

Adult skeletal muscle regeneration in response to injury is a complex process in which PI3K/AKT signaling activation has an essential role (reviewed in ref. 38) and has been linked to induction of myomiR expression[39]. We have explored the role of KHSRP in muscle regeneration and found that the induction of myomiRs occurring during muscle regeneration is impaired in *Khsrp*−/−mice due to maturation blockade[37]. Interestingly, similarly to MAPK14, also PI3K/AKT signaling activation in mdx mice muscle is beneficial, since enhances expression of utrophin and promotes muscle regeneration[35].

Thus, it is possible to hypothesize that regulation of KHSRP functions by both MAPK14 and PI3K/AKT is required for the process of muscle regeneration.

Tadesse and coworkers reported that KHSRP expression is down-regulated in spinal cord tissues from mild spinal muscular atrophy (SMA) model mice, and this correlates with increased *Cdkn1a* mRNA levels thus suggesting the involvement of exaggerated stabilization of specific KHSRP mRNA targets in the etiology of SMA[40].

Over the last years, converging lines of evidence indicate that the expression of miR-206 is altered in several neuromuscular disorders[39]. miR-206 is essential for muscle regeneration and able to delay progression of dystrophic disease in mdx mice[41]. We have reported that miR-206 maturation from precursors is severely impaired in C2C12 cells depleted of KHSRP as well as in regenerating muscle derived from *Khsrp*−/− mice[37]. In a mouse model of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by loss of motor neurons and degeneration of target muscle fibers, deficiency of miR-206 accelerates disease progression[42]. Williams and coworkers proposed that miR-206 delays progression of ALS by stimulating compensatory regeneration of neuromuscular synapses targeting histone deacetylase 4 and fibroblast growth factors[42].

Based on the above observations, it is tempting to speculate that manipulation of KHSRP function in order to enhance its ability to promote myomiR maturation and/or affect its mRNA decay promoting function on select mRNAs could contribute to the treatment of degenerative neuromuscular disorders.

5. Role of KHSRP in neurons

Post-transcriptional regulatory mechanisms that control gene expression have been shown to be important for virtually all stages of assembling neural networks, from neurite guidance, branching, and growth to synapse morphogenesis and function[43].

Although KHSRP is known to be expressed in both neurons and glial cells[44,45], its function in the nervous system is now beginning to be understood. After the initial characterization of KHSRP as a splicing factor in neurons[2], two homologs of the human protein were identified in neurons and shown to participate in mRNA transport: the chicken zipcode-binding protein 2 (ZBP2), a protein that is required for localizing β-actin mRNA to growth cones[46], and rat MARTA1, a protein that mediates the transport of *Map2* mRNA to dendrites[47]. Also, KHSRP is located in RNA granules in peripheral nerve axons, where it interacts with —and it is regulated by— SMN, a RBP required for motor neuron survival[40]. In dorsal root ganglion (DRG)-like neuronal cells and cortical neurons, KHSRP co-localizes with the X-chromosome-linked intellectual disability protein (PQBP1) which is a component of neuronal RNA granules and regulates the formation of stress granules[48]. Furthermore, the recent finding that KHSRP is present together with Dicer and some pre-miRNAs in distal DRG axons suggests its involvement in localized pre-miRNA processing[49].

Our studies indicate that KHSRP, by controlling mRNA stability, affects axonal outgrowth during neuronal development[50]. As shown in Figure 1A, KHSRP is able to destabilize the mRNA encoding the neuronal growth associated protein GAP43[50]. Notably, KHSRP

competes with the neuronal stabilizing factor ELAVL4 (also known as HuD) which is known to increase *Gap43* mRNA stability and axonal elongation during neuronal development[51-55]. Overexpression of either full length KHSRP (Figure 1B) or a deletion mutant that lacks its nuclear localization signal was found to impair axonal outgrowth in hippocampal neurons, whereas overexpression of a mutant protein without the KH4 domain did not have any effects[50]. Further, overexpression of *Gap43* mRNA with axonal targeting sequences in the 3'UTR, but not an mRNA restricted to the cell body, is able to rescue axonal elongation in the presence of an excess of KHSRP[55]. Finally, depletion of KHSRP in hippocampal neurons led to a 2-3 fold increase in *Gap43* expression and to enhanced axonal length[50]. Altogether these findings suggest that some of the effects of KHSRP on neuronal differentiation are mediated by localized regulation of mRNA stability.

6. Role of KHSRP in lipid metabolism

Rapid changes in mRNA levels are critical for altering the pattern of protein expression in response to various metabolic stimuli in order to maintain cellular homeostasis[56]. miRNAs and RBPs that controls mRNA decay have been recently reported to be major regulators of glucose and lipid homeostasis under physiologal conditions and in the presence of metabolic disorders, such as type 2 diabetes and obesity.

We have recently provided evidence that a KHSRP/miR-145 axis can be viewed as an important negative regulator of lipolysis in epididymal white adipose tissue (eWAT)[57]. It is well known that increasing lipolysis in WAT causes an elevation in fatty acid utilization and energy expenditure, thus protecting against obesity[58]. During fast, catecholamines cause elevation of cyclic AMP levels and activation of protein kinase A resulting in stimulation of lipolysis while in the fed state lipolysis is inhibited by insulin[59]. Transcriptional activation of the gene *Pnpla2* (which encodes the lipase ATGL) by forkhead box O1 (FOXO1) and other factors enhances lipolysis in adipocytes[60]. Further, ATGL activity is modulated through interaction with the co-activator CGI58[61]. We have shown that lipolysis is increased in *Khsrp*−/− eWAT due to enhanced expression of the genes encoding the lipases ATGL and HSL (hormone sensitive lipase) as well as of FOXO1 and CGI58[57]. From a mechanistic point of view, the expression of miR-145 is decreased in *Khsrp*−/− eWAT as a consequence of impaired pri-miR-145 processing[57]. Reduction of miR-145 expression in eWAT of *Khsrp* −/− mice leads to up-regulation of FOXO1 and CGI58, thereby enhancing lipolysis.

More recently, the Chen laboratory has provided evidence that KHSRP can impinge on lipid homeostasis through a distinct additional mechanism[62]. In the liver of *Khsrp*−/− mice the expression of *Per2* (encoding a transcriptional repressor which belongs to the core component of the circadian clock) is significantly increased leading to an altered circadian clock. This leads to a reduction in liver triglycerides (TG) content in mutant mice, which become protected from diet-induced hepatic steatosis. Elevation of *Per2* mRNA levels depends on increased mRNA stability in the absence of KHSRP and results in down-regulation of important modulators of lipid metabolism. As a consequence, *Khsrp*−/− mice exhibit reduced lipogenesis and TG content[62]. Interestingly, the consequences of KHSRP deletion on *Per2* mRNA levels are evident only in the liver but not in adipose tissue, suggesting a tissue-restricted role of KHSRP in controlling *Per2* mRNA stability. Non-

alcoholic liver steatosis is the most common form of chronic liver disease associated with obesity, type 2 diabetes, and insulin resistance[63,64]. These observations point to KHSRP as a critical factor in governing hepatic lipid metabolism and it can be viewed as an unpredicted potential therapeutic target to control liver steatosis.

7. Role of KHSRP in cancer

In this Section we summarize the experimental evidence that links altered KHSRP expression or function to cell transformation. Further, in the last subsection we propose that *KHSRP* gene mutations detected in human tumors target specific protein domains (KH3 and KH4 as well as the putative nuclear localization signal [NLS]) and hypothesize a link between oncogenic mutations and protein functions.

1. Implication in the response to DNA damage

Ataxia-telangiectasia mutated kinase (ATM) plays an essential role in the maintenance of genome stability regulating the function of a large number of proteins in order to facilitate cell cycle checkpoints, to promote DNA repair, and to control many other aspects of physiological responses in the event of DNA double-strand breaks[65]. Overall, ATM activation results in a dramatic change in the gene-expression program that can be ascribed, in part, to regulation of miRNA expression[65]. A study from Zhang and coworkers demonstrated that the induction of a large population of miRNAs by ATM is controlled at post-transcriptional level as suggested by lack of significant changes in pri-miRNA transcription levels after induction of DNA damage[66]. Interestingly, all the miRNAs whose maturation was shown to be favored by KHSRP in a previous study[67] were induced in ATM-expressing cells upon DNA damage while *ATM* knock-out abrogated this effect[66]. Accordingly, KHSRP silencing abolished DNA damage-induced upregulation of the same cohort of mature miRNAs[66]. Zhang and coworkers extended this observation demonstrating that KHSRP is phosphorylated by ATM and that this phosphorylation enhances the interaction of the protein with a subset of pri-miRNAs as well as its maturation-promoting activity[66]. These results suggest that KHSRP can play a role in tumorigenesis participating in the signaling cascade that regulates cellular response to DNA damage.

2. Role in tumors

KHSRP knock-down enhances the migratory capability of glioblastoma multiforme (GBM) cells as revealed by a siRNA screening[68]. This study, performed by Yang and coworkers, also demonstrated that KHSRP down-regulation induces the formation of multifocal GBM in a mouse model[64]. Interestingly, high expression levels of KHSRP were observed in patients who survived longer after surgery suggesting that KHSRP may be used as a novel prognostic marker for GBM patients[68]. A recent independent study from Boucas and coworkers confirmed that high KHSRP transcript levels are associated with increased overall survival of patients suffering from GBM and suggested that this might result from a better response to therapy in patients expressing high KHSRP levels[69]. This last study also demonstrated that KHSRP is highly regulated in response to genotoxic stress through the MAPK14/MAPKAPK2 (the latter also known as MK2) signaling module with cells

deficient for MAPK14/MAPKAPK2 showing an overall altered interaction between KHSRP and target mRNAs[69].

Differently from GBM, silencing of KHSRP in hepatocellular carcinoma cells represses migration. Zubaidah and coworkers as well as Malz and coworkers independently reported that the coordinated activation of FUBP1 (a nucleic acids binding protein whose KH domains displays high sequence homology with KHSRP) and of KHSRP represents a pro-tumorigenic mechanism promoting cell proliferation (in the case of FUBP1) and cell motility (in the case of KHSRP) of human liver cancer cells[70,71].

From these studies it is evident that the consequences of altered levels of KHSRP are different in distinct human tumors and may reflect cell-restricted functions of KHSRP that could depend on the participation of the protein in distinct multi-protein complexes and to its ability to interact with different targets in different cells. Although intriguing, the above results necessitate both mechanistic exolanations and confirmation on a broader scale.

3. KHSRP gene translocation and mutations in cancer

Malouf and coworkers recently identified human KHSRP gene as a novel partner of TFE3 in Translocation Renal Cell Carcinoma (TRCC) tumors[72]. TFE3 is a transcription factor that transactivates expression of genes downstream of TGF-β signaling[73]. The translocation-generated fusion protein detected in TRCC tumors includes almost the entire KHSRP protein at its C-terminus and the TFE3 protein at the N-terminus. Remarkably, both the helix-loop-helix domain of TFE3 and the KH domains of KHSRP are present in the fusion protein[72]. The study also revealed that genes differentially spliced between TRCC and other renal cell carcinoma types were enriched for TFE3 targets, suggesting a putative role for KHSRP-regulated RNA splicing events in kidney carcinogenesis[72]. It will be interesting to experimentally verify whether transcripts whose alternative splicing is affected by TFE3-KHSRP fusion protein play a role either in the pathogenesis or in the natural history of TRCC and whether the KHSRP moiety in the fusion protein plays a role in the modulation of alternative splicing events.

We sought to gain insight into a potential role of missense mutations of human KHSRP gene in cancer and, to this purpose, we performed a metadata analysis of identified KHSRP mutations in public available databases (Bordo et al., unpublished). Fifty-one amino acid substitutions are reported in the COSMIC database (catalogue of somatic mutations in cancer[74]), 16 are reported in the Intogen database[75] while other 16 are reported in Biomuta database[76]. The merging of the three datasets resulted in 53 distinct amino acid mutations affecting KHSRP. The distribution of these mutations shows that 20 of them involve amino acids belonging to one of the four KH domains, two involve one of the two domains of unknown function (DUFs, as defined in Pfam[77]) and 31 instances map on the remaining regions (Figure 2). Among the KH domains, KH3 and KH4 appear to be more subject to mutations. We mapped mutations affecting KH domains on the three-dimensional structure of the corresponding KH domain and found that the majority (16 out of 19) involves either α-helices or β-strands (Figure 2). Thus, it is likely that these amino acid substitutions influence the structural stability of the corresponding KH domain. No positional bias is observed concerning the putative nucleotide binding groove of the KH domains (Figure 2)

while the linker regions connecting KH2-KH3 and KH3-KH4 host each five amino acid substitutions. Notably, although the nuclear localization signal (NLS) of KHSRP has not been unambiguously defined, 9 amino acid substitutions localize to a region spanning amino acid positions 90-120, predictably hosting the NLS of the protein. Our metadata analysis also showed that 16 mutations associate with cancer[75]. Interestingly, although KHSRP does not possess the features of a driver gene for the switch to cancer, there is an increased frequency of mutations (that affect KH3) in Small Cell Lung Carcinoma, with a PAM (Protein Affecting Mutation) of about 5%, a figure that is about four times higher than that of other cancer types[75].

8. Conclusive remarks

Among the cellular functions of KHSRP that we have described in this review, some have been more extensively investigated and broadly accepted by the scientific community. These include mRNA decay promoting function and miRNA maturation induction that have been confirmed by many laboratories in different cellular contexts and have gained the support of data derived from the analysis of knock-out mice. Conversely, although based on solid biochemical data, the pre-mRNA alternative splicing control operated by KHSRP has not been supported by in vivo experiments and clearly deserves further investigations. Similarly, the KHSRP-dependent control of mRNA translation as well as the lncRNA-mediated regulation of KHSRP activity need to be further confirmed in additional experimental settings.

As a matter of fact, given its proven ability to interact with a variety of RNA targets in distinct cellular contexts due to its modular structure, KHSRP is able to regulate a broad spectrum of cellular functions. More work is needed to understand from a mechanistic point of view the role of KHSRP in the context of large ribonucleoprotein complexes comprising other RBPs, enzymes, adaptor proteins, and ncRNAs.

Finally, results derived from the analysis of knock-out mice phenotype suggest that KHSRP deletion is favorable for certain aspects of mice physiology (Sections 2 and 6) stressing the fact that KHSRP expression and function need to be tightly regulated to ensure tissue homeostasis. Therefore, further studies are required to systematically investigate how KHSRP expression and function can be finely modulated in response to a variety of agents in the context of normal and pathological conditions.

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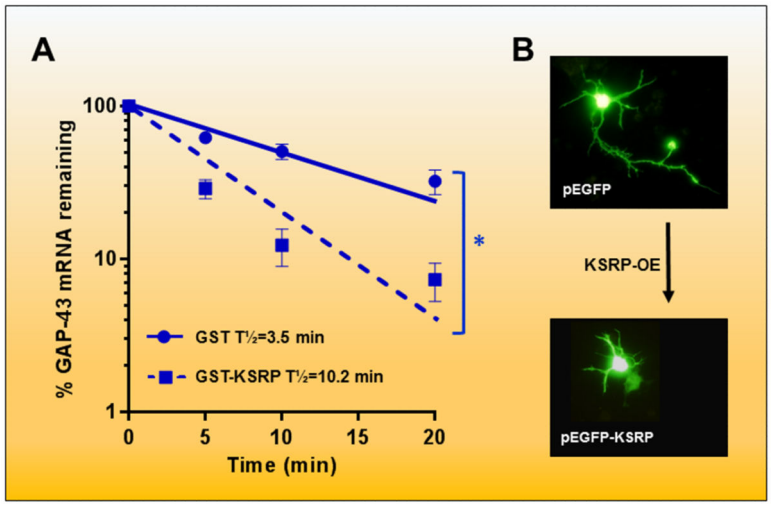

Figure 1. KSRP decreases GAP-43 mRNA stability and axonal outgrowth

(**A**) As shown by Bird and coworkers[47], addition of recombinant KSRP to S100 extracts from Ksrp $^{-/-}$ mouse brains decreases the half-life of the GAP-43 mRNA. Decay curves show the average results of 3 separate decay experiments fitted with a single rate exponential decay curve. *$p<0.05$. The effect of KSRP required both the presence of the KH4 domain in the protein and the ARE in the GAP-43 3' UTR. (**B)** Overexpression (OE) of KSRP in primary hippocampal neuron cultures impairs axonal elongation. This process is reversed by either KSRP knockdown (KD) or by OE of a GAP-43 mRNA with a 3' UTR targeting sequence for axonal localization[47].

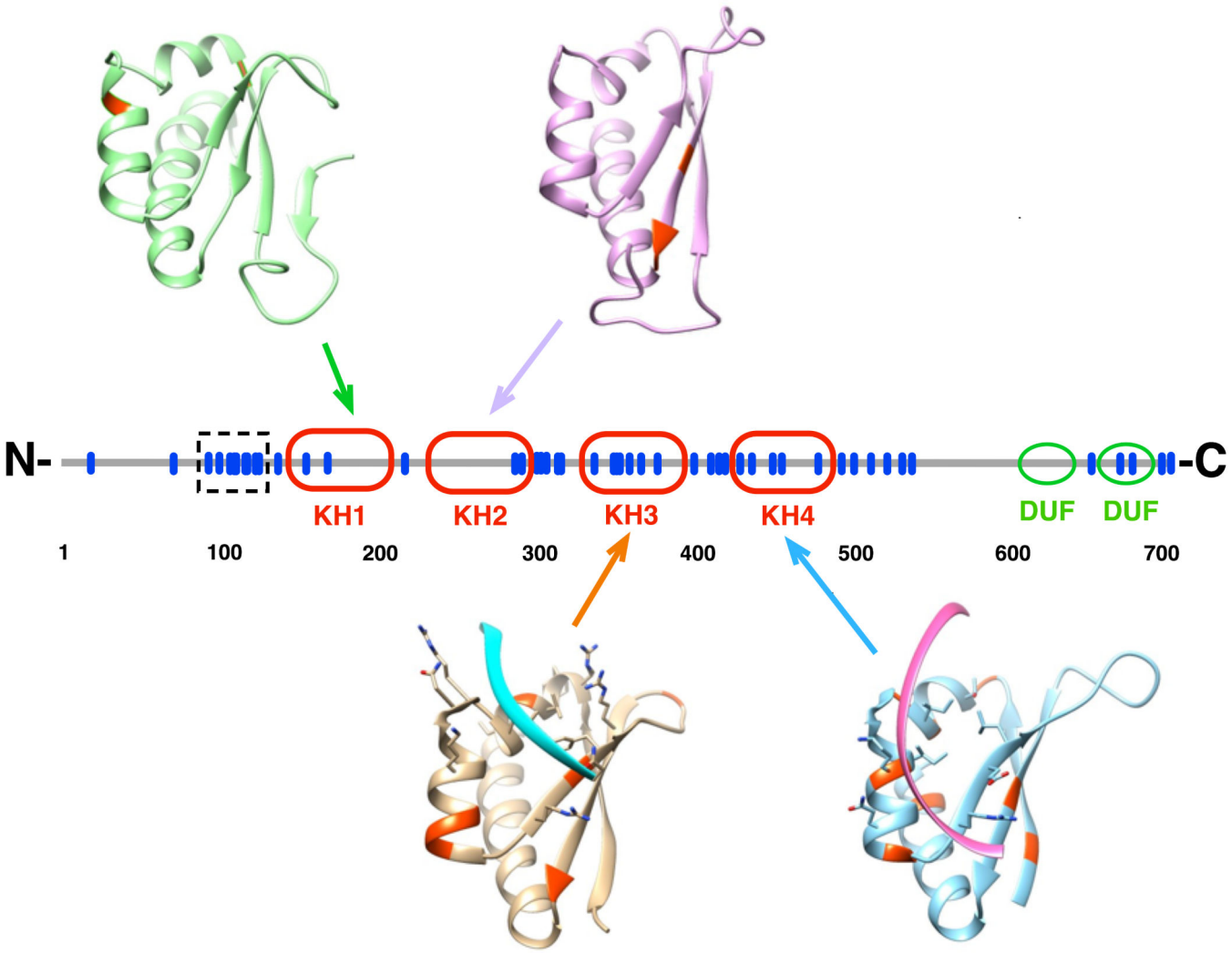

Figure 2. Missense point mutations in KHSRP

The primary sequence of KHSRP is represented as a straight line in the center, with the positions of the known missense point mutations indicated by blue bars. The position of the KH domains and that of the two domains of unknown function (DUF, as from Pfam[73]) is shown. The region spanning amino acids 90-120 containing a cluster of nine distinct mutations and putatively involved in nuclear localization is highlighted with a dotted box. The three-dimensional structures of the four KH domains are also represented as ribbon diagrams in the same orientation with respect to the RNA binding groove, with the position of the mutated residues highlighted in red. The experimentally determined position of the bound RNA is shown for KH3 and KH4. The Protein Data Bank codes for the four KH domains are 2opu (KH1), 2opv (KH2), and 1j4w (KH3, KH4).

Table I

Molecular functions of KHSRP in different cellular contexts.

Molecular function	Biological outcome	Cell type / tissue	Post-translational modification	Physiopathological context	References
mRNA decay	*FOS*, *IL2*, *TNF* mRNA stabilization	HeLa, HT1080	—	—	9
mRNA decay	*Myog*, *Cdkn1a* mRNA stabilization	C2C12 multipotent mesenchymal	MAPK14	Myogenesis	27
mRNA decay	*IL8*, *CXCL2*, *CXCL3* and others mRNA stabilization	HeLa	MAPK14	—	10
mRNA decay	*Ifna*, *Ifnb* mRNA stabilization	Fibroblasts, macrophages *Ksrp*−/− mice	—	Response to viral infections	12
mRNA decay	*Il1b*, *Tnf* mRNA stabilization	Astrocytes *Ksrp*−/− mice	—	Inflammatory response	13
mRNA decay	*NOS2A*, *IL8*, *TNF* mRNA stabilization	DLD-1 colon carcinoma	MAPK14	Inflammatory response	15, 16
mRNA decay	*Utrophin* mRNA stabilization	C2C12, mouse muscle	MAPK14, AKT	Myogenesis, Duchenne Muscular Atrophy	35
mRNA decay	*Gap43* mRNA stabilization	Neurons *Ksrp*−/− mice	—	Axonal outgrowth	50
mRNA decay	*Per2* mRNA stabilization	Liver *Ksrp*−/− mice	—	Non-alchoolic liver steatosis	62
mRNA translation	*Il1b*, *Tnf* mRNA translation	Astrocytes *Ksrp*−/− mice	—	Inflammatory response	13
mRNA translation	*Il6*, *Il23a*, *Tnf* and other mRNA translation	HeLa	MAPK14	—	17
mRNA translation	EV71 viral RNA	RD rhabdomyosarcoma cells	MAPK14	Viral infection	23, 24
miRNA maturation	Let-7 family, miR-16, miR-20b, and others	U2OS, HeLa	ATM	Cell proliferation, DNA damage response	66, 67
miRNA maturation	miR-155 targeting *Il1b*, *Il12b*, *Cxcl11* and other mRNAs	Mouse macrophages	—	Inflammatory response	19
miRNA maturation	miR-150 targeting *Prdm16*, *Ppargc1a*	iWAT *Ksrp*−/− mice	—	Control of body adiposity	29

Molecular function	Biological outcome	Cell type / tissue	Post-translational modification	Physiopathological context	References
miRNA maturation	miR-198/*FSTL1*	Human keratinocytes	—	Wound healing (non-healing diabetic ulcers?)	33
miRNA maturation	miR-145 targeting *Foxo1*, *Cgi58*	eWAT *Ksrp*−/− mice	—	Control of lipolysis (obesity?)	57
mRNA decay + miRNA maturation + interaction with lncRNA	*Myog*, miR-1, miR-133, miR-206 (myomiRs), lncRNA H19	C2C12 multipotent mesenchymal	AKT	Cell fate determination	8, 31, 37
unknown	unknown	Glioblastoma cells	—	Cell migration	68
unknown	unknown	Glioblastoma cells	MAPK14, MAPKAPK2	Cell cycle progression	69
unknown	unknown	Hepatoma cells	—	Cell motility	70,71