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The RNA binding protein KSRP destabilizes GAP-43 mRNA to limit axonal elongation in cultured hippocampal neurons

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Abstract

The KH-type splicing regulatory protein (KSRP) promotes the decay of AU-rich element (ARE) containing mRNAs. Although KSRP is expressed in the developing and mature nervous system, very little is known about its role in regulating gene expression in the brain. In this study, we utilized *in vitro* binding and decay studies to examine whether KSRP regulates the stability of the GAP-43 transcript, an ARE-containing neuronal mRNA whose protein product plays a role in axonal growth and synaptic plasticity. We found KSRP destabilizes GAP-43 mRNA by binding to the GAP-43 ARE,

a process that depends on the presence of the fourth KH domain in the protein. Furthermore, KSRP competed with another GAP-43 mRNA binding protein, the stabilizing factor HuD, for binding to these ARE sequences. Given that GAP-43 expression is crucial for accurate axonal outgrowth during neuronal development, we also examined the functional consequences of KSRP overexpression and depletion on the axonal outgrowth from primary hippocampal neurons. Overexpression of either full length KSRP or KSRP without the nuclear localization signal hindered axonal outgrowth in these cultures, while overexpression of a mutant protein without the KH4 domain did not have any effect. In contrast, depletion of KSRP led to a dramatic increase in axonal length. Concurrent overexpression of GAP-43 and KSRP rescued the axonal outgrowth deficits seen with KSRP overexpression, but only when the GAP-43 mRNA was targeted to axons using GAP-43 or amphoterin 3' UTR sequences. Together, our results suggest that KSRP is an important regulator of GAP-43 mRNA stability and neuronal differentiation that works in direct opposition to HuD.

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1. Introduction

1.1 The AU rich element and associated proteins

Gene expression from any given gene is dependent on a number of cellular events working in parallel. The initial transcription of a gene determines the amount of pre-mRNA ready for processing. Mature mRNA levels are dependent on the speed at which post-transcriptional processes occur, which include splicing, capping, and polyadenylation. Once processed, the transcript still needs to be exported to the cytoplasm, so nuclear export rates need to be considered. Subsequent stability of the mRNA also determines the available pool of transcript available for translation. The next step determining final protein output is the rate of translation of the mRNA by ribosomes. Once the final protein product is produced, the amount of functional protein is still determined by post-translational processing and folding events, as well as degradation processes mediated by cellular proteases. Any one of the preceding events can have a significant impact on the overall expression of a given gene.

For a number of genes, the primary factor governing final protein output is the stability of mRNA following nuclear export. Many proteins in a cell do not need to be constitutively produced, but rather are needed for specific cellular processes. Sometimes these proteins need to be produced relatively quickly to impact a cellular event, such as the production of inducible nitric oxide synthase (iNOS) following an immunologic insult to generate nitric oxide, which has anti-microbial and anti-apoptotic functions (Linker et al., 2005). In human hepatocytes, the iNOS promoter exhibits high basal activity, but the mRNA product is virtually undetectable. Following cytokine induction, the promoter

activity of iNOS is only increased by two- to five-fold, but there is a much more significant accumulation of the mRNA (Kleinert et al., 2004). This indicates that the stability of the mRNA is the major determining factor determining iNOS levels following cytokine induction.

In 1986, a conserved sequence in the 3' untranslated regions (3'UTRs) of numerous mRNAs encoding for cytokines was identified, having the adenine (A) and uracil (U) containing consensus sequence of UUAUUUAU (Caput et al., 1986). Other investigators of the time separately noticed the phenomenon, noting that many lymphokine, cytokine, and proto-oncogene mRNAs contained stretches of A and U nucleotides (Shaw and Kamen, 1986). The conserved nature of this nucleotide sequence prompted researchers to postulate that the sequence had a specific functionality. When the AU rich sequence-containing 3'UTR of granulocyte-monocyte colony stimulating factor (GM-CSF) was attached to the normally stable mRNA encoding for β-globin, the mRNA became very unstable (Shaw and Kamen, 1986). Other investigators also noted these AU rich sequences in a number of labile mRNAs, including c-fos, c-myc, β-interferon, IL-3, IL-8, and many others (Treisman, 1985, Brewer and Ross, 1988, Peppel et al., 1991, Stoecklin et al., 1994, Winzen et al., 1999). The A and U rich region in the 3'UTRs of short-lived mRNAs has since been coined the AU-rich element (ARE) (Brewer, 1991).

Since their original identification, ARE's have been categorized into three separate subgroups based on their sequence composition (Chen and Shyu, 1995). Class I ARE's contain 1-3 copies of an AUUUA pentamer surrounded by a U rich region. This type of ARE is contained in c-fos and c-myc (Treisman, 1985, Chen and Shyu, 1994).

The class II ARE, which is found in many unstable cytokine mRNAs, comprises at least two overlapping copies of a UUAUUUA(U/A)(U/A) nonamer (Shaw and Kamen, 1986). The least well defined ARE sequence is that for the class III ARE, which does not contain a canonical sequence, but is rather a span of mostly A and U nucleotides. Examples of class III ARE containing mRNAs are those encoding for c-jun and the neuronally expressed growth associated protein 43 (GAP-43) mRNAs (Kohn et al., 1996, Peng et al., 1996). While there are many mRNAs that have AU rich stretches within their 3'UTRs, they are not defined as AREs unless they confer instability to the transcript (Chen and Shyu, 1995). An estimated 5-8% of mRNAs contain typical AREs according to the ARE database (ARED), which may be an underestimate of the actual number of ARE containing genes due to the ambiguous nature of the class III ARE (Bakheet et al., 2006).

Degradation of an ARE-containing mRNA involves a number of different steps. The first process typically involved in mammalian mRNA decay is deadenylation, which is most likely mediated by the poly(A) ribonuclease (PARN) (Wilusz et al., 2001). Initial reports indicated that the next step in ARE-mediated mRNA decay (AMD) was 3' to 5' exonucleotic digestion by the exosome complex (Chen et al., 2001). Later evidence also implicates cytoplasmic processing bodies (p-bodies) in the degradation of ARE containing mRNA, which is initiated by Dcp decapping proteins followed by 5' to 3' exonucleolytic cleavage by the enzyme Xrn1 (Fenger-Gron et al., 2005). Interestingly, 3' to 5' degradation by the exosome complex is aided by decapping, indicating that 5' to 3' and 3' to 5' digestion may be functionally linked (Murray and Schoenberg, 2007). The pathway that AMD follows may depend on the type of ARE that the mRNA contains, or

may be a function of a *trans* acting factor: an ARE-binding protein (ARE-BP) (Barreau et al., 2005).

A number of ARE-BPs have been identified to date, which can serve to stabilize or destabilize bound ARE-containing transcripts. The most well-studied stabilizing ARE-BPs are the Hu family proteins HuR (also known as HuA), HuB (also known as He1-N1), HuC, and HuD (Bolognani and Perrone-Bizzozero, 2008). These proteins are the human homologues of a *Drosophila* protein that causes an embryonic lethal abnormal vision phenotype (ELAV) (Szabo et al., 1991). All the Hu family proteins contain a similar structure and sequence, and contain three RNA recognition motifs (RRMs) (Brennan and Steitz, 2001). The most well studied of the ELAV proteins, HuR, has been implicated in the stabilization of a number of mRNAs involved in multiple cellular processes ranging from immediate early gene expression (c-fos and c-myc), to cell cycle progression (Cyclin A, Cyclin B1, Cyclin D1) and differentiation (MyoD and VEGF) (Reviewed in (Brennan and Steitz, 2001, Barreau et al., 2005)). Binding of Hu family proteins to the ARE protects the mRNA from degradation, and may help to enhance translational rates by associating with polysomes (Keene, 1999). HuR is mainly nuclear but can shuttle to the cytoplasm, leading some to postulate that HuR initially binds to ARE-containing mRNA in the nucleus, protecting the mRNA from both nuclear and cytoplasmic decay processes (Fan and Steitz, 1998).

Functioning in opposition to the ELAV family proteins are a number of ARE-BPs that serve to destabilize target mRNA. The first ARE-BP identified serving to destabilize bound mRNA is the protein AUF1 (also known as hnRNP D), which was shown to promote the degradation of bound c-myc mRNA (Brewer, 1991). AUF1 has also been

implicated in stabilizing IL-3 mRNA in NIH 3T3 cells, while destabilizing IL-3 mRNA in K562 cells, providing a somewhat ambiguous role for this ARE-BP (Loflin et al., 1999, Ming et al., 2001). Other destabilizing RNA binding proteins were later identified, such as KH-splice regulatory factor (KSRP), tristetraprolin (TTP), and butyrate-regulated factor-1 (BRF1) (Reviewed in (Wu and Brewer, 2012)). KSRP mainly associates with components of the exosomal degradation machinery, while TTP and BRF1 promote mRNA degradation via both exosomes and p-bodies (Gherzi et al., 2004, Lykke-Andersen and Wagner, 2005).

How and when ARE-BPs bind to, and compete for binding to ARE-containing mRNAs will determine the functional protein output of a specific gene. For example, iNOS can be destabilized by KSRP, but HuR also competes for the same binding site in the iNOS 3'UTR (Linker et al., 2005). It seems that before cytokine induction of iNOS, KSRP is bound to the mRNA to negatively regulate its stability. Following an antigenic insult and cytokine signaling, KSRP is dislodged from the iNOS mRNA, allowing HuR to bind to and stabilize the transcript for translation (Linker et al., 2005, Wu and Brewer, 2012). This post-transcriptional "operon" of stabilizing and destabilizing proteins and their interaction with ARE-containing mRNA serves as a dynamic regulator of gene expression and provides regulation of gene expression beyond simple transcriptional and translational activation (Keene and Lager, 2005).

1.2 GAP-43 and HuD

The ARE-containing mRNA encoding for GAP-43 is the most well studied labile mRNA specific to neurons. GAP-43 was initially identified as a protein present in retinal ganglion cells during axonal regeneration following optic nerve crush (Skene and Willard, 1981). GAP-43 protein was later shown to be highly expressed in dissociated rat cerebrocortical cultures soon after plating and also in PC12 cells following neurite outgrowth induced by NGF stimulation (Perrone-Bizzozero et al., 1986, Van Hooff et al., 1989). During axonal outgrowth GAP-43 accumulates in axonal growth cones and helps to direct axonal outgrowth, and knockdown of GAP-43 leads to regression of the axon (Skene et al., 1986, Caroni and Becker, 1992). Once axons reach their post-synaptic target, GAP-43 levels diminish as a functional synapse is formed (Schreyer and Skene, 1991, Karimi-Abdolrezaee et al., 2002). Transgenic mice overexpressing GAP-43 exhibit aberrant axonal sprouting of mossy fibers in the hippocampus, while GAP-43 knockout mice have deficits in axonal pathfinding and die during development (Aigner et al., 1995, Strittmatter et al., 1995, Klein et al., 1999). Clearly, the coordinated expression of GAP-43 during development is critical to proper nervous system development.

The actual mechanism of action of GAP-43 in promoting axonal outgrowth is not completely understood, but there are a number of indications as to its cellular function. The N-terminal end of the GAP-43 protein can activate the GTP-binding protein G_0, and subsequently causes filopodial extension (Strittmatter et al., 1994). Another potential mechanism of GAP-43 mediated axonal outgrowth involves protein kinase C (PKC) activity, which is induced by NGF stimulation (Perrone-Bizzozero et al., 1993). Active PKC phosphorylates GAP-43, which leads to changes in GAP-43 binding to different

cellular molecules (Perrone-Bizzozero et al., 1993). Unphosphorylated GAP-43 binds to a large number of actin filaments, and is proposed to act as a cap to actin filaments, preventing further actin polymerization and limiting filopodial extension at the growth cone (He et al., 1997). When GAP-43 is phosphorylated by PKC, it has less affinity for actin, and is then proposed to act as a lateral stabilizer of actin filaments and promote filopodial extension (He et al., 1997, Denny, 2006). In a separate model, unphosphorylated GAP-43 binds readily to phosphatidylinositol 4,5-bisphosphate [PI (4,5) P_2], and clusters this phospholipid into rafts. Upon phosphorylation by PKC, GAP-43 releases PI (4,5) P_2, allowing PI (4,5) P_2 to bind profilin, cofilin, and gelsolin, which are molecules that act to prevent actin polymerization. With these actin-binding molecules sequestered to the membrane, actin is free to polymerize and promote filopodia extension (Laux et al., 2000, Denny, 2006). These two models could also be working cooperatively, enhancing actin polymerization and promoting axonal outgrowth.

Control of GAP-43 expression is regulated at both the transcriptional and post-transcriptional level. The promoter for GAP-43 restricts its transcription to the nervous system (Reinhard et al., 1994, Weber and Skene, 1997). Enhancer elements upstream of the GAP-43 gene in the 5' regulatory region can be bound by basic helix-loop-helix transcription factors, promoting GAP-43 transcription (Chiaramello et al., 1996). Following initial transcriptional events, GAP-43 expression is also regulated by post-transcriptional mechanisms. GAP-43 transcription occurs at similar rates in quiescent and developing rat cortical neurons, but steady state levels of GAP-43 mRNA are greatly increased in the developing neurons, indicating that this transcript is regulated by a post-transcriptional mechanism (Perrone-Bizzozero et al., 1991). Later investigations

identified a pyrimidine rich stretch in the 3'UTR of the GAP-43 mRNA that regulates its stability (Kohn et al., 1996). When this instability conferring sequence was attached to the normally stable β-globin mRNA it promoted its degradation (Tsai et al., 1997). This pyrimidine stretch, rich in U and cytosine (C) nucleotides, is classified as a class III ARE, as it is U rich but does not contain a canonical AUUUA sequence (Chen and Shyu, 1995, Kohn et al., 1996). The GAP-43 ARE is also the binding site for the stabilizing ARE-BP HuD (Chung et al., 1997).

The neuronally expressed ELAV protein HuD has been implicated repeatedly as a critical determinant regulating GAP-43 mRNA stability. Overexpression of HuD in NGF induced PC12 cells led to increased neurite outgrowth and elevated GAP-43 protein levels (Anderson et al., 2000). Transfection of a HuD overexpression construct into PC12 cells or E19 rat cortical neurons accelerated neurite outgrowth, as well as increased both mRNA and protein levels of GAP-43 (Mobarak et al., 2000, Anderson et al., 2001). Conversely, knockdown of HuD in PC12 cells led to a significant decrease in GAP-43 gene products (Mobarak et al., 2000). Stabilization of GAP-43 mRNA requires all three RRMs of HuD (Anderson et al., 2000). The first two RRMs of ELAV proteins bind to ARE sequences, while the third RRM binds to poly(A) stretches, i.e. the poly-A tail (Liu et al., 1995, Abe et al., 1996, Ma et al., 1997). HuD binds preferentially to GAP-43 mRNAs with a long poly-A tail, and inhibits deadenylation of the transcript, protecting it from the first step in AMD (Beckel-Mitchener et al., 2002).

HuD has not only been demonstrated to enhance the stability of GAP-43 *in vitro* and in cell culture, but also *in vivo* by utilizing transgenic animal models. Transgenic mice overexpressing HuD display elevated levels of GAP-43 mRNA in granule cells of

the dentate gyrus, in the lateral amygdala, and in layer V of the neocortex (Bolognani et al., 2006). Increased GAP-43 mRNA stability in transgenic HuD overexpressing mice was further confirmed by utilizing *in vitro* mRNA decay assays using S100 extracts from the mutant mice (Bolognani et al., 2006). In S100 extracts from HuD KO mice GAP-43 mRNA is much less stable than in S100 extracts from WT mice (Bolognani et al., 2007a).

HuD and GAP-43 expressions are induced by a few different cellular processes besides initial neuron outgrowth. In the peripheral nervous system, injury to the peripheral branch of a dorsal root ganglion (DRG) leads to an increase in GAP-43 mRNA levels during regeneration (Schreyer and Skene, 1993). It was later demonstrated that this was accompanied by an increase in HuD protein levels in the same DRG neurons (Anderson et al., 2003). Learning and memory processes also affect HuD and GAP-43 expression. Following Morris water maze and radial arm maze training, there is a significant increase in the levels of HuD and GAP-43 mRNA, as well as increased association of HuD protein with GAP-43 mRNA, indicating that these brain specific molecules are important not only in the development of the nervous system but also in its everyday function (Quattrone et al., 2001, Pascale et al., 2004)

1.3 KSRP

The ARE-BP KSRP was originally identified as a far upstream element (FUSE) binding protein (FBP), named FBP2, and implicated in activating transcription of the *c-myc* gene (Davis-Smyth et al., 1996). KSRP was next associated with post-transcriptional processing as a splicing factor, serving to include the neural specific N1 exon in neuronally expressed *c-src* (Min et al., 1997). It was in 2001 that the ARE

binding properties of KSRP were identified. CY Chen et al., demonstrated the exosome does not bind ARE-containing mRNAs directly, but through intermediary proteins, one of which was shown to be KSRP (Chen et al., 2001). Further experiments showed that depleting KSRP in S100 extracts from several different cell types inhibited the decay of many ARE containing transcripts (Gherzi et al., 2004). Tethering KSRP to a transcript that does not contain an ARE instability conferring element led to the rapid decay of the bound mRNA, further demonstrating that KSRP serves to promote the degradation of target mRNAs (Chou et al., 2006).

Structurally, the main important features of KSRP are the 4 KH binding domains it contains (Davis-Smyth et al., 1996, Min et al., 1997). KH domains are conserved RNA binding domains that were originally identified in the protein/RNA hnRNP K complex, where the KH domain was shown to be essential for the RNA binding activity of the protein (Siomi et al., 1993). The N-terminal end of KSRP contains a proline/glycine rich domain, and also contains a bipartite nuclear localization sequence consisting of non-basic amino acids flanked by basic amino acids (Hall et al., 2004). The 4 KH domains are located in the central portion of the protein and are separated by intervening sequences, followed by a glutamate rich domain at the C-terminal end. Also located at the C-terminal end of KSRP are four degenerate copies of the sequence DYTKAWEEYYKK, which are implicated in protein-protein interactions, although their specific function relating to KSRP protein interactions has not been identified (Hall et al., 2004).

Gherzi et al., (2004) investigated the role of each of the KH domains in binding to ARE-containing mRNA and how they impact subsequent AMD. Utilizing different

KSRP deletion mutants, KH domains 3 and 4 were shown to bind to AREs with high affinity. Mutants lacking KH domain 4 did bind to ARE-containing mRNA efficiently, leading the researchers to conclude that the fourth KH domain was the one primarily involved in ARE binding. The third KH domain of KSRP proved to be essential for binding to the exosome and PARN, and is key for the decay promoting activity of KSRP. KSRP functions most efficiently, however, when it contains all four of its KH domains, implicating the first two KH domains in aiding KSRP binding to ARE containing mRNA (Gherzi et al., 2004).

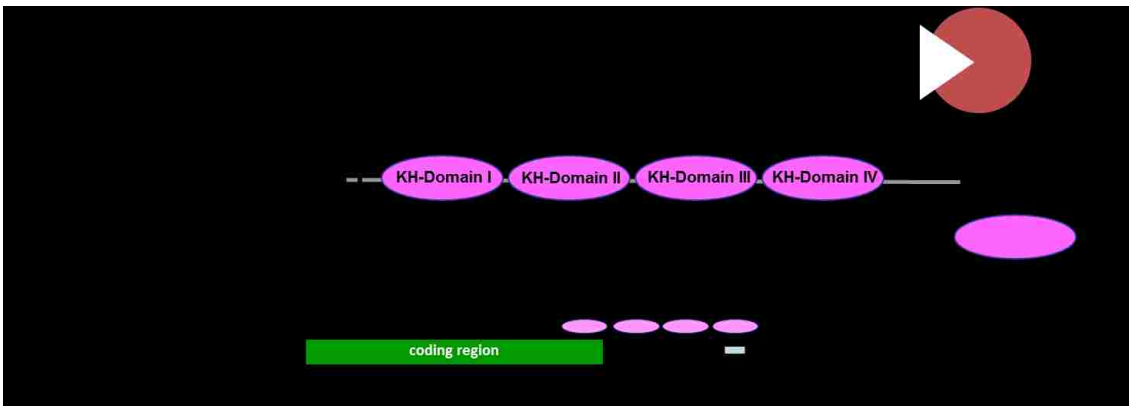

Figure 1.1 Overview of KSRP binding to the ARE of GAP-43

Shown here is a schematic of how KSRP binds to ARE-containing mRNA. The fourth KH domain of KSRP has been demonstrated to be crucial for binding to KSRP target mRNAs. The third KH domain is critical for recruiting the exosome and PARN to the bound transcript, where deadenylation and decapping events take place, followed by 3' to 5' degradation by the exosome.

Identifying a specific consensus sequence for KSRP binding has proven elusive. KSRP can promote the decay of mRNA reporter constructs containing any of the 3 ARE classes (Gherzi et al., 2004). A binding study examining the affinity of the different KH domains for RNA tetramers of varying composition failed to identify a motif that KSRP

preferred to bind, as the KH domains bound most of the tetramers with similar affinity, with the exclusion of the KH3 domain which has a slight affinity for guanine (G) rich sequences (Garcia-Mayoral et al., 2008). A subsequent experiment by the same group showed that the third and fourth KH domains bind AU rich sequences similar to the type II ARE with a relatively high affinity, but still failed to identify a consensus sequence (Diaz-Moreno et al., 2010). The most fruitful experiments to examining KSRP targets have involved immunoprecipitation of KSRP and subsequent identification of bound mRNAs by microarray analysis. One such study performed this type of immunoprecipitation experiment, and also knocked down KSRP to see what mRNAs were upregulated. Investigators analyzed the data obtained from both types of experiments to see what types of mRNAs were regulated by KSRP, and found a large number of targets, including cytokines and cellular growth factors (Winzen et al., 2007). Post-transcriptional regulation of these mRNA targets by KSRP still needs to be verified, however, which is a daunting task given the number of mRNA binding targets found.

Recently, KSRP has also been identified to function as an enhancer of microRNA (miRNA) biogenesis. The initial processing of primary miRNA transcripts occurs in the nucleus when the hairpin transcript is cleaved by the ribonuclease Drosha, producing a pre-miRNA. Following nuclear export of the pre-miRNA it is once again cleaved, this time by the ribonuclease Dicer, after which one or both strands of the RNA duplex is incorporated into the RNA-induced silencing complex (RISC). Depending on the sequence homology of a miRNA for its mRNA target, the mRNA may be translationally repressed or actively degraded [for a review of miRNA biogenesis see (Kim, 2005)]. KSRP is able to bind to Drosha, Dicer and stem loop sequences in some pre-miRNAs,

promoting the biogenesis of a subset of miRNAs, including let-7 and miR-155 (Ruggiero et al., 2009, Trabucchi et al., 2009). KSRP serves many cellular functions, and it is interesting to note that it functions in two completely different post-transcriptional regulatory processes with similar outcomes on gene expression.

1.4 Summary

Precise spatio-temporal regulation of GAP-43 expression during neuronal growth and differentiation is essential for correct nervous system development. The instability-conferring ARE in the GAP-43 3'UTR is critical in this process, as are the *trans*-acting ARE-BPs that can bind to this mRNA to modulate the stability of the transcript. HuD has repeatedly been shown to be a stabilizer of GAP-43 mRNA and functions to promote axonal outgrowth. KSRP, a negative regulator of ARE-containing mRNA expression, has the potential to bind to and promote the decay of GAP-43 mRNA. In this dissertation we examine this possibility, and show that KSRP does indeed function in neurons to modulate the expression of GAP-43.

2. Rationale, hypothesis, and specific aims

2.1 Rationale

Stabilization of the GAP-43 transcript by HuD has been extensively studied, but the opposite side of the spectrum has yet to be investigated: are there other ARE-BPs associated with GAP-43 mRNA that destabilize this transcript when neurons mature? Preliminary investigations in our laboratory demonstrated that the destabilizing factor KSRP binds to GAP-43 transcripts.

2.2 Hypothesis

KSRP functions in direct opposition to HuD, destabilizing GAP-43 mRNA during neurite development to counteract HuD's growth stimulating effect. Overexpression of KSRP is hypothesized to limit GAP-43 transcript levels and limit primary axonal outgrowth, while knockdown of KSRP will increase GAP-43 mRNA levels and axonal growth.

2.3 Specific aims

The above hypothesis was investigated with two specific aims:

2.3.1 Specific aim 1:

How does KSRP affect GAP-43 mRNA stability *in vitro*? In order to demonstrate that KSRP functions in opposition to HuD's stabilizing effect on this target mRNA, we will need to determine whether KSRP serves to promote GAP-43 mRNA degradation. We will accomplish this aim utilizing *in vitro* binding and mRNA decay assays.

2.3.2 Specific aim 2:

How does KSRP expression affect axonal outgrowth in primary neuronal cultures?

2.3.2.1 Specific aim 2A:

Will overexpression or knockdown of KSRP in developing neurons lead to limited or enhanced axonal outgrowth, respectively? To examine the involvement of KSRP in developing axons, we will transfect primary hippocampal neuronal cultures with a GFP conjugated plasmid overexpressing KSRP, or a GFP-shRNA construct to knockdown KSRP. We can then examine axon outgrowth through fluorescence microscopy.

2.3.2.2 Specific aim 2B:

In neurons overexpressing KSRP, can the resulting neuronal phenotype be rescued with overexpression of GAP-43? Performing this experiment will be critical in demonstrating that KSRP's effect on axon growth is due to its modulation of GAP-43 mRNA stability.

3. Regulation of GAP-43 stability by KSRP *in vitro*

3.1 Introduction

The regulation of gene expression by stabilization or degradation of mRNA transcripts is a well-documented process important during a number of cellular events, including cytokine production and cellular differentiation (Wilusz and Wilusz, 2004). GAP-43 is important in developing neurons, where GAP-43 protein promotes axonal outgrowth in response to growth factor signaling (Goslin et al., 1988, Perrone-Bizzozero et al., 1993). GAP-43 is also important in mature synapses, and is expressed during learning and memory processes. The mRNA for GAP-43 is unstable, a trait conferred to it by the class III ARE present in its 3'UTR. The ARE-BP HuD has been repeatedly proven to be a post-transcriptional regulator of GAP-43 mRNA, stabilizing the transcript in developing and regenerating neurons (Anderson et al., 2000, Anderson et al., 2003).

KSRP, a mRNA destabilizing protein, has been implicated in contributing to the degradation of a variety of transcripts. KSRP binds to the ARE sequence present in many labile transcripts, and targets them for degradation by binding to the exosomal degradation machinery (Chen et al., 2001). KSRP contains 4 RNA binding KH domains that are important for binding to AU rich elements, but the third and fourth domains are the most essential for its degradatory function (Gherzi et al., 2004). The fourth domain is important for binding to target mRNAs, while the third domain is essential for binding to the exosome. KSRP is highly expressed in brain tissue, but little is known about its function in the nervous system (Gu et al., 2002).

In the following *in vitro* studies, we examine the role of KSRP in regulating GAP-43 mRNA stability. We approach these studies using a combination of *in vitro* binding and decay studies to see if KSRP degrades GAP-43 transcripts. We also investigate whether or not KSRP and HuD compete for binding to the same sequence in the GAP-43 3'UTR.

3.2 Materials and methods

Biotinylated GAP-43 mRNA protein pull-down. (Performed by Dan Tanner)

Biotinylated mRNA was prepared and pull-down assays performed as described by Ambrosino et al. (2003) with minor modifications (Ambrosino et al., 2003). Briefly, a biotinylated RNA containing GAP-43 ARE was prepared from EcoRI linearized pGAP/B plasmid (Kohn et al., 1996) by *in vitro* transcription using SP6 RNA polymerase and biotin-11-UTP (Enzo Life Sciences, Farmingdale, NY). RNA (2 µg) was resuspended in 1X TENT buffer (20 mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0, 500 mM NaCl, 1% [vol/vol] Triton X-100) and then incubated with 300 µg of S100 extract prepared from rat cortices and 40 units RNasin (Promega, Madison, WI) for 30 minutes at room temperature. Control reactions did not include biotinylated RNA. Pre-washed streptavidin-coated Dynabeads (Life Technologies) were then added to each reaction and allowed to incubate for 30 more minutes at room temperature. Beads were collected by centrifugation, washed twice with TENT buffer, then resuspended in SDS-PAGE loading buffer. Samples were then run on a 10% SDS-PAGE gel. Western blotting was performed as described previously (Anderson et al., 2001) using α-KSRP AB5 antibody

provided by Dr. Doug Black and affinity purified anti-HuD antibody (Mobarak et al., 2000).

RNA electrophoretic mobility-shift assay (REMSA)

^{32}P-labeled GAP-43 ARE mRNA was prepared by *in vitro* transcription from EcoRI linearized pGAP/B plasmid using SP6 RNA polymerase and [α-^{32}P] UTP (3000 Ci/mmol, Perkin-Elmer, Waltham, MA). REMSA assay was performed as described by Li *et al*. with minor modifications (Li et al., 2004). Briefly, 100,000 CPM (45 nCi) of ^{32}P-UTP labeled GAP-43 3'ARE mRNA was incubated with increasing amounts of purified GST or recombinant GST-KSRP in a buffer containing 50 mM Tris-HCl pH 7.0, 150 mM NaCl, 0.25 mg/ml tRNA, 0.25 mg/ml bovine serum albumin, and 5% glycerol for 10 min at 37˚C. RNA-protein complexes were then run on a non-denaturing 10% polyacrylamide gel in TBE buffer for 45 min. at 200V. The gel was then dried and exposed to a phosphor screen overnight before radioactivity was measured using a Bio-Rad Personal Molecular Imager FX (Bio-Rad, Hercules, CA).

Competitive RNA binding assay

Protein G dynabeads (Life Technologies) were washed and pre-bound with HuD E-1 antibody (Santa Cruz). ^{32}P-labeled GAP-43 ARE was prepared as described above. Labeled GAP-43 ARE was incubated with 1.5 nmol GST-HuD protein and increasing amounts of GST or GST-KSRP in a binding buffer containing 10 mM HEPES pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, and 0.5% NP40. Assays were then incubated for 10 minutes at 4˚C before being exposed to UV light for 30 minutes at 4˚C. Pre-bound beads were then added to the binding assays along with 40 U of RNasin RNase inhibitor (Promega).

Samples were then mixed at 4˚C for 1 hour on a rotating mixer, then washed three times with binding buffer. Samples were then resuspended in Tris-EDTA buffer and the radiation measured by scintillation count.

***In vitro* mRNA decay assay**

KSRP KO mouse S100 extracts were prepared from cortical brain tissue as described (Ford et al., 1999). GST protein and recombinant GST-KSRP protein was expressed in BL21 *E. coli* and purified using the MagneGST™ Protein Purification System (Promega) according to the manufacturer's protocol. ^{32}P-labeled GAP-43 3'UTR mRNA was prepared and decay reactions were performed as described previously (Bolognani et al., 2006), with minor modifications: 50 fmol ^{32}P-labeled GAP-43 3'UTR mRNA was incubated with 20 µg KSRP KO S100 protein and 50 ng purified recombinant protein.

3.3 Results

3.3.1 KSRP binds to GAP-43 mRNA *in vitro*: RNA electrophoretic mobility shift

Given that KSRP is known to bind ARE sequences, initial studies used two separate *in vitro* binding assays to determine if this RBP directly binds to GAP-43 mRNA. First, we utilized REMSA using radiolabeled GAP-43 ARE. As shown in Figure 3.1A, incubation with 25 ng of GST-KSRP protein was sufficient to cause a shift in the migration of a GAP-43 ARE containing RNA, and bands were completely shifted in the presence of 100 ng KSRP protein. In contrast, there was no shift in the mobility of the mRNA when incubated with up to 10 µg of the GST protein control. Previous research

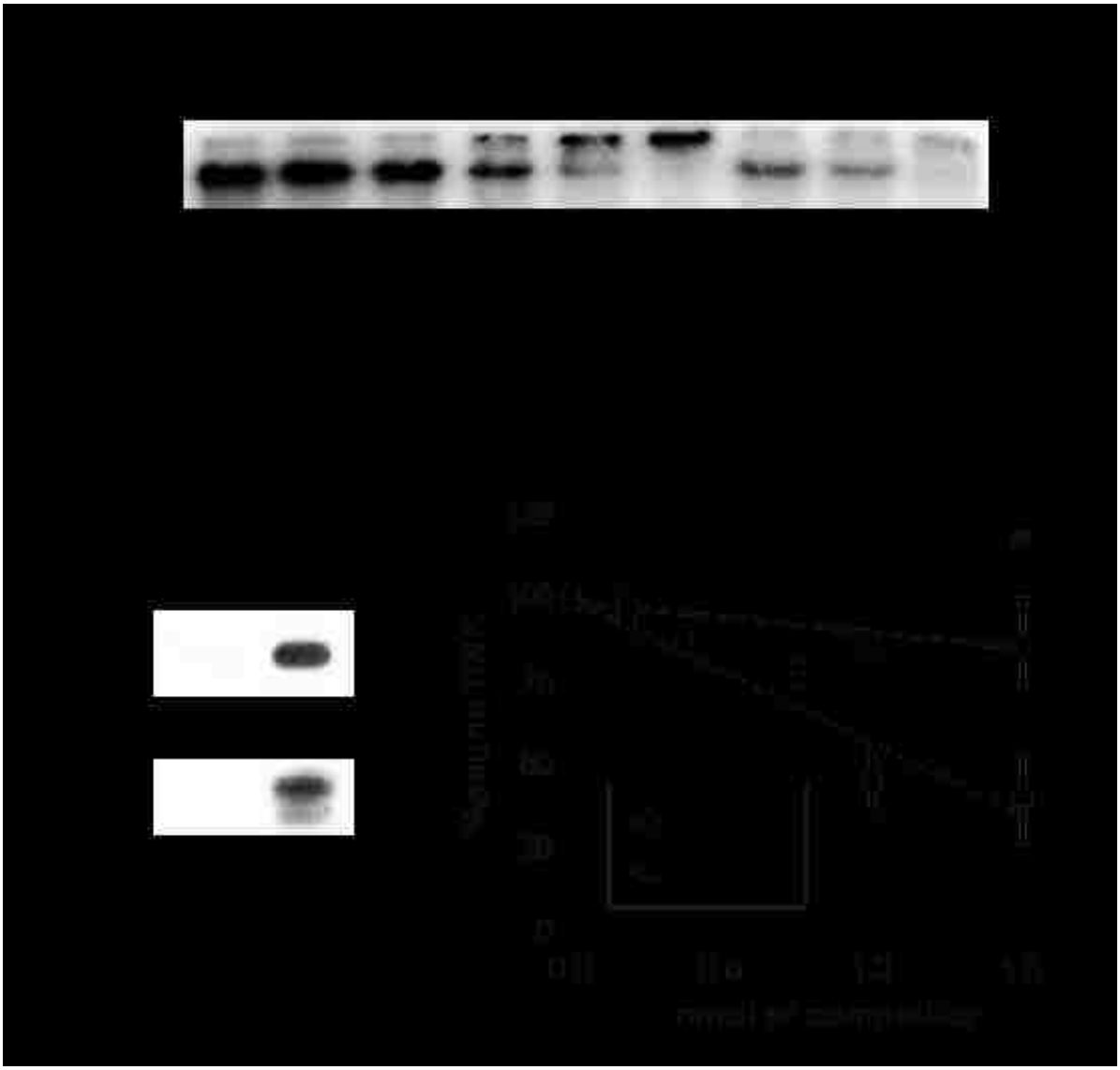

Figure 3.1 Binding of KSRP to GAP-43 ARE

A. REMSA shift assays used increasing amounts of purified GST, GST-KSRP, or GST-KSRP-ΔKH4 protein and ^{32}P-labeled GAP-43 ARE. **B.** RNA pull-down assay was performed with biotinylated GAP-43 ARE and S100 cytosolic protein extracts from mouse brain as indicated in the Methods (Performed by Dan Tanner). Western blots (WB) show the presence of KSRP and HuD proteins in the RNA pull down. **C.** Competitive binding assay of HuD and KSRP. ^{32}P-labeled GAP-43 ARE was incubated with HuD and increasing amounts of either GST or KSRP competitor protein, before HuD was pulled down and bound RNA measured by scintillation counting. **, $p < .05$ using Student's t-test (n=2).

showed that KSRP binds ARE sequences primarily via its 4th KH domain (Gherzi et al., 2004). Thus, we used a KSRP protein lacking this domain (GST-KSRP-ΔKH4) for binding GAP-43 ARE. GST-KSRP-ΔKH4 bound to the GAP-43 ARE, but with significantly less affinity than wild-type KSRP (Figure 3.1A)(Gherzi et al., 2004).

3.3.2 KSRP binds to GAP-43 mRNA *in vitro*: biotinylated GAP-43 mRNA pulldown (Experiment performed by Dan Tanner)

In a separate binding assay, biotinylated GAP-43 ARE-containing RNA was incubated with rat S100 protein extracts, and mRNA/protein complexes pulled down using streptavidin coated beads. Analysis of the bound proteins by western blot revealed that KSRP, as well as HuD (Figure 3.1B) bound the GAP-43 ARE.

3.3.3 HuD and KSRP compete for binding to the GAP-43 ARE

Given that both HuD and KSRP bind to the same ARE sequence, we then examined the ability of KSRP to compete with HuD for binding to this RNA. For these studies, ^{32}P-labeled GAP-43 ARE was incubated with 1.5 nmol of GST-HuD protein and increasing amounts of either GST or GST-KSRP. HuD was then immunoprecipitated and the amount of mRNA bound was measured by scintillation counting. Increasing amounts of GST protein did not interfere with HuD binding to GAP-43 ARE, while increasing amounts of KSRP displaced HuD from the transcript (Figure 3.1C). When equal molar amounts of KSRP and HuD were used in the assay HuD binding to GAP-43 ARE decreased by roughly half, indicating that KSRP and HuD have a similar affinity for binding GAP-43 ARE (Figure 3.1C).

3.3.4 KSRP destabilizes GAP-43 mRNA *in vitro*

We used *in vitro* decay assays to determine if KSRP has an effect on GAP-43 mRNA stability (Figure 3.2). For this, ^{32}P-labeled capped and polyadenylated RNA containing the GAP-43 ARE was incubated with purified recombinant GST-KSRP protein along with S100 protein extracts from KSRP KO mice, and decay of the labeled mRNA was measured over time. This experimental system has been shown to reliably represent the relative decay rates of several mRNAs, as the protein complexes required for mRNA degradation are present in S100 extracts (Ford et al., 1999, Chen et al., 2000). The use of S100 extracts from KSRP KO mice also ensures the absence of endogenous KSRP, which could confound the decay results (Lin et al., 2011). When GST was incubated with GAP-43 ARE, the mRNA decayed with a half-life of 10.7 ± 5.7 minutes. Addition of GST-KSRP to the decay system enhanced the degradation rate of GAP-43 ARE, decreasing the half-life of the mRNA to 3.2 ± 1.5 minutes, which is significantly different than control GST ($p < .05$) using two-way ANOVA. In contrast, addition of recombinant KSRP protein lacking the 4th RNA binding KH domain (GST-KSRP-ΔKH4) resulted in a RNA half-life that is not significantly different than control (Chou et al., 2006). These experiments indicate that KSRP binds to GAP-43 mRNA, and increases the decay rate of GAP-43 mRNA *in vitro* via the interaction of GAP-43 ARE with the 4th KH domain in the protein.

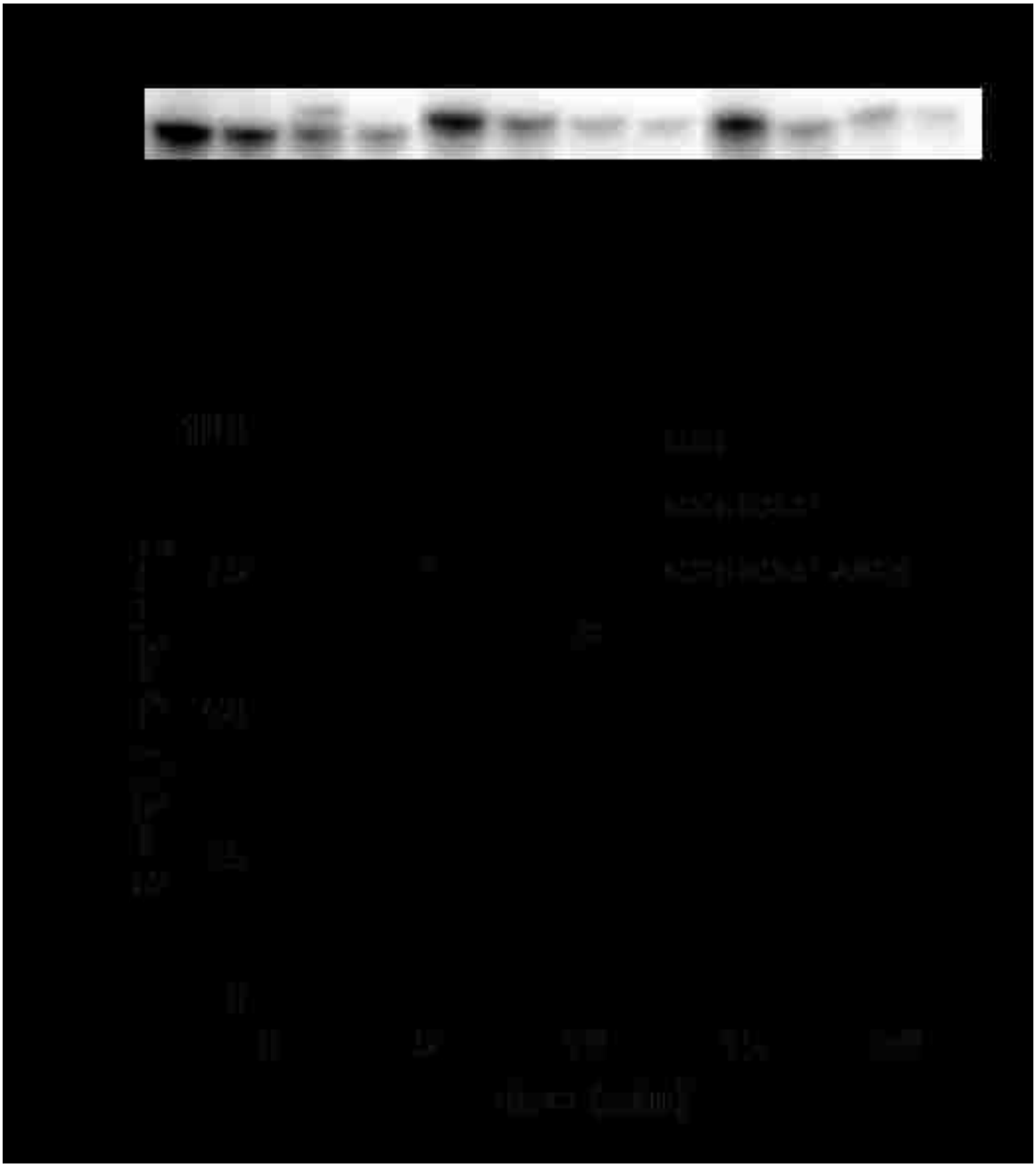

Figure 3.2 KSRP increases GAP-43 ARE decay *in vitro*

A. Representative images form mRNA decay assays. Purified recombinant GST, GST-KSRP, or GST-KSRP-ΔKH4 protein was incubated with ^{32}P-labeled GAP-43 ARE in the presence of S100 extracts from KSRP KO mice. The graph in (**B**) represents the results of 3 separate decay experiments fitted with a single rate exponential decay curve. *, $p < 0.05$ comparing GST and GST-KSRP using 2-way ANOVA with Bonferroni's post-test.

3.4 Discussion

In this study, we show for the first time that KSRP binds to and destabilizes GAP-43 mRNA *in vitro*. In the REMSA experiments, GAP-43 mRNA migration through the gel was shifted completely by 100 ng of KSRP protein, demonstrating that KSRP closely associates with GAP-43 ARE. KSRP-ΔKH4 protein also bound to the GAP-43 ARE, but with less affinity than full length KSRP protein. This was expected, based on the fact that the fourth KSRP KH domain was found to be primarily responsible for ARE binding (Gherzi et al., 2004). The KSRP-ΔKH4 protein still binds to the GAP-43 ARE sequence with some affinity, because the first two KH domains aid in binding ARE sequences (Gherzi et al., 2004). These binding studies help to confirm the results obtained from the *in vitro* decays, which showed that full length KSRP protein led to the fastest degradation rate of GAP-43 ARE. KSRP-ΔKH4 also enhanced the decay rate of the GAP-43 when compared to the GST control, but less so than the full length KSRP protein, supposedly due to the reduced affinity of the KSRP-ΔKH4 protein for the ARE sequence. Performing these decays in S100 extracts from the brains of KSRP KO mice provided by CY Chen was valuable in running a clean assay, as there was no endogenous KSRP to confound results (Lin et al., 2011).

Future experiments that could be performed to enhance the results of these decay studies would further utilize the KSRP KO mouse. *In vivo* decay assays analyzing GAP-43 mRNA stability could be performed in cortical neuronal cultures from these mice, using Actinomycin D to inhibit transcription. Cultures from wild-type and KO mice would be cultured at the same time, and at a specific time point after plating Actinomycin D would be added to the media, and neurons collected at measured time points. The

collected neurons would be flash-frozen to inhibit further RNA degradation, and measured using RT-PCR. Such a decay experiment would be relatively easy to perform. The reason such a study has not been performed to date is purely logistical: we have not had access to a breeding colony of KSRP KO mice until recently. Most likely we will perform these experiments in the near future to further strengthen our results, however, the evidence that KSRP enhances the decay of GAP-43 ARE obtained from our assays performed is already quite strong.

It is interesting to note that KSRP and HuD appear to compete for the same binding site in the GAP-43 ARE. Analyzing the actual binding affinities for each of these ARE-BPs will be integral to understanding how they affect GAP-43 mRNA stability during different cellular conditions, to see if KSRP would displace HuD from the transcript. During axonal outgrowth GAP-43 mRNA is bound by HuD, stabilizing the transcript for translation. After the axon has reached its target and the neuron matures, GAP-43 and HuD levels both drop off (Skene et al., 1986, Bolognani et al., 2007b). It would be interesting to know exactly when KSRP binds to GAP-43 mRNA after the axon has reached its target. Performing high-resolution *in situ* hybridization and immunocytochemistry at different points during neuron development, both in cultured neurons and *in vivo* could lend some insight as to the precise time-course of KSRP, HuD, and GAP-43 expression in the developing brain.

4. KSRP inhibits axonal outgrowth in cultured hippocampal neurons

4.1 Introduction

In developing neurons, GAP-43 mRNA expression is essential for axonal growth and pathfinding processes (Skene et al., 1986). GAP-43 is critical during development, as GAP-43 KO mice die before birth, and exhibit impairments in axonal targeting (Strittmatter et al., 1995). If GAP-43 is overexpressed, aberrant axonal sprouting is observed (Aigner et al., 1995, Klein et al., 1999). The ARE-BP HuD stabilizes the GAP-43 transcript, and overexpressing HuD increases the rate of process outgrowth in both NGF-stimulated PC12 cells and cultured cortical neurons (Anderson et al., 2000, Anderson et al., 2001). HuD overexpressor mice have elevated GAP-43 mRNA levels in the dentate gyrus of the hippocampus, demonstrating that HuD not only regulates GAP-43 expression *in vitro* and in cell culture, but *in vivo* as well (Bolognani et al., 2006).

Regulation of GAP-43 expression is imperative to proper neural development, and knowledge about regulatory processes impacting GAP-43 mRNA stability are evolving as we gain knowledge about ARE binding proteins and their targets. Work presented in the previous chapter of this dissertation demonstrated that the destabilizing protein KSRP is able to bind to the GAP-43 ARE *in vitro*, and serves to destabilize GAP-43 mRNA. The following experiments examine the functional consequences of KSRP expression in hippocampal cultures using KSRP overexpression vectors, as well as shKSRP vectors to achieve knockdown of this protein. We also were able to measure GAP-43 protein levels in neurons in which KSRP has been knocked down, and mRNA levels in PC12 cells transfected with shKSRP and sorted to attain an enriched population of KSRP deficient cells.

4.2 Materials and methods

Plasmids

shRNA constructs were obtained from SA biosciences (Qiagen, Valencia, CA). pAc-GFP-KSRP, pAc-GFP-KSRP 1-4, and pAc-GFP-ΔKH4 plasmids were prepared from pET15b-KSRP vector provided by Dr. Doug Black (Figure 4.1) (Min et al., 1997). Coding sequences were amplified using PCR with primers specific to corresponding regions (pAc-GFP-KSRP fwd: AAGGCCTCTGTCGACGACTACAGCACGGGAGG, rev: AGAATTCGCAAGCTTATTCATTGAGCCTGCTGCTGTC; pAc-GFP-KSRP 1-4 fwd: AAGGCCTCTGTCGACTCAATGACAGAAGAGTACAGGGTCCCAGA, rev: AGAATTCGCAAGCTTCTCGATCTTTTCCTCGATAAGCTGCTTGGC; pAc-GFP-ΔKH4 fwd: AAGGCCTCTGTCGACTCAATGACAGAAGAGTACAGGGTCCCAGA, rev: AGAATTCGCAAGCTTCTGGAGGAGGTCGTTGATGATCCGG). pAc-GFP-KSRP plasmid was cloned using Clontech's In-Fusion enzyme and pAcGFP1-C In-Fusion Ready vector according to manufacturer's protocols (Clontech, Mountain View, CA) . pAc-GFP-KSRP 1-4 and pAc-GFP-ΔKH4 were directionally cloned into the freshly prepared linearized pAc-GFP-KSRP digested with SalI and HindIII. mCherry-GAP-GAP3', mCherry-GAP-Amp3' and mCherry-GAP-γ-actin3' were provided by Dr. Jeff Twiss. GST-KSRP and GST-KSRP-ΔKH4 plasmids were provided by Dr. CY Chen (Gherzi et al., 2004).

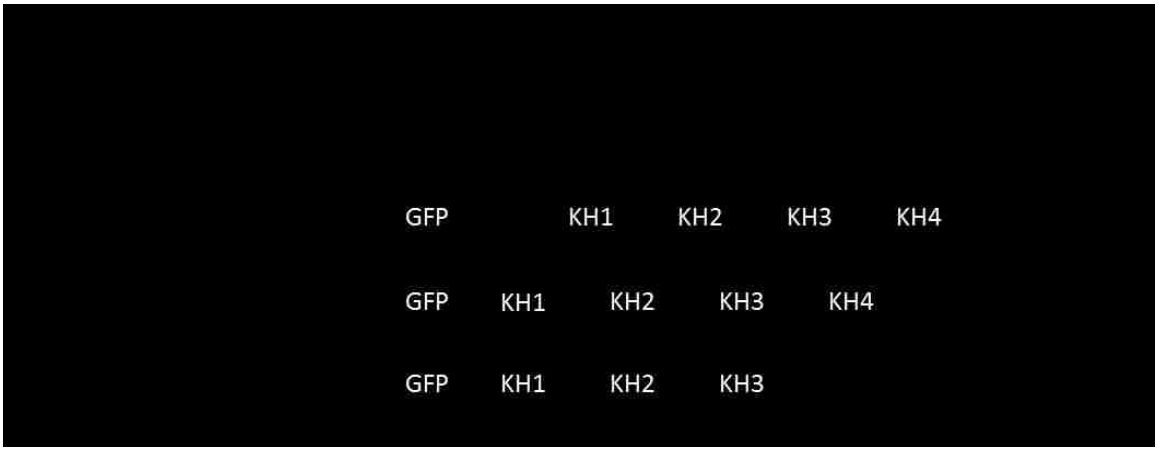

Figure 4.1 GFP-KSRP constructs cloned for KSRP overexpression studies.
GFP-KSRP-KH 1-4 lacks a NLS, while the GFP-KSRP-ΔKH4 lacks both a NLS and the 4th KH domain.

Fluorescence activated cell sorting

Fluorescence activated cell sorting (FACS) was performed in the UNM Flow Cytometry Facility by dedicated personnel. 10 cm dishes of PC12 cells near 100% confluence were incubated overnight in Opti-MEM media supplemented with 4% serum before being transfected with either control non-targeting or shKSRP GFP plasmids. Transfections were performed with 24 µg of plasmid per dish using Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. Transfection media was replaced the next day with Opti-MEM + 4% serum. Neurons were grown for 48 hours post transfection before cells were trypsinized and pelleted, then resuspended in sorting medium (cation free PBS supplemented with 0.2% fetal bovine serum, 10mM HEPES pH 7.3, 1 mM EDTA) at a concentration of 5 x 10^6 cells/ml. Cells were then sorted, using gating to collect the brightest 3% of GFP positive cells. Cells were collected in sorting medium, then pelleted by centrifugation at 10,000 x g for 1 min and flash frozen on dry ice before RNA was extracted.

Real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed as described previously (Bullock et al., 2008). Briefly, RNA was extracted from sorted PC12 cells using an RNeasy mini kit (Qiagen). cDNA was synthesized using 1µg total RNA using Superscript II reverse transcriptase (Invitrogen) according to manufacturer's protocol. Exon spanning primer pairs were designed using Primer Express 3.0 (Life Technologies) and validated using NCBI primer BLAST software. Primers used for analysis were: KSRP (forward 5'-GGACTCAGGCTGCAAAGTTC, reverse 5'-CCAGGATCATCTTTGCCTTT), GAP-43 (forward 5'-AGCCAAGGAGGAGCCTAAAC, reverse 5'-CTGTCGGGCACTTTCCTTAG), and GAPDH (forward 5'-TGTGATGGGTGTGAACCACGAGAA, reverse 5'-GAGCCCTTCCACAATGCCAAAGTT). qRT-PCR reactions were performed on an Applied Biosystems 7300 Real Time PCR System. Gene expression levels were analyzed using SYBR Green (Life Technologies). No evidence of primer dimerization was evident by dissociation curve analysis. Primers for KSRP and GAP-43 were validated against GAPDH and were within optimal amplification values (slope <|0.1|). Samples were run in triplicate, and relative levels of expression compared to GAPDH were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Primary hippocampal cultures

Primary hippocampal neuronal cultures were isolated and grown as described previously (Smrt et al., 2010). Briefly, hippocampi from E17 Sprague-Dawley rats were pooled, triturated with a fire-polished Pasteur pipette and dissociated neurons plated on

poly-D-lysine/laminin coated coverslips (BD Biosciences, San Jose, CA). Cultures were grown in Neurobasal medium (Life Technologies, Grand Island, NY) supplemented with B27 supplement (Life Technologies), Penicillin/Streptomycin (Life Technologies), 0.5 mM L-glutamine and 25 µM glutamate for 4 days *in vitro* (DIV) before being transfected with GFP plasmid constructs using Lipofectamine 2000 and Opti-MEM (Life Technologies) according to manufacturer's protocol. Cells were incubated with transfection medium for 48 hours before being fixed with 4% paraformaldehyde.

Immunocytochemistry

Fixed primary hippocampal cultures were incubated with 50 mM ammonium chloride in phosphate-buffered saline (PBS) for 20 minutes to quench paraformaldehyde autofluorescence. For KSRP and HuD colocalization studies, cells were fixed after 3 days *in vitro*. The cells were then incubated with 1% horse serum and 0.1% Triton X-100 in PBS (PBST) for 30 minutes. Neurofilament light chain (NF-L) was detected with α-NF-L antibody generated in mouse (MAB1615, Millipore, Billerica, MA) at a 1:100 concentration. KSRP was detected using α-FBP-2 antibody generated in goat (SC-33031, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 concentration. HuD was detected using E-1 α-HuD antibody (SC-28299, Santa Cruz). Cells were incubated with primary antibody for 2 hours at room temperature before being rinsed with PBST. Alexa-Fluor 546 donkey α-mouse antibody (Life Technologies), Alexa-Fluor 546 donkey α-goat antibody (Life Technologies), or Alexa-Fluor 488 donkey α-goat antibody (Life Technologies) was then incubated with the cells in 1% horse serum PBS for 1 hour at room temperature in the dark at a concentration of 1:200. The cells were then rinsed with PBST before coverslips were mounted onto glass slides (VWR, Radnor, PA) with PVA-

DABCO mounting media. Mounted coverslips were dried overnight in the dark at room temperature before imaging.

Microscopy and image analysis

For quantitation of axonal outgrowth, transfected neurons were imaged with an Olympus BX60 fluorescence microscope with a 20X objective and images collected using an Olympus DP71 camera. For neurons that were too large to be imaged in a single 20X field, multiple overlapping images of the same neuron were taken and the images merged together using Adobe Photoshop Elements (Adobe, San Jose, CA). For quantitation of KSRP shRNA knockdown and analysis of KSRP and HuD localization transfected primary hippocampal neurons were imaged with a Zeiss LSM 510 confocal microscope. Axonal outgrowth of transfected primary hippocampal neurons was measured using Neurolucida (MBF biosciences, Williston, VT). Cell bodies and primary axons were traced using the program, and overall axonal length per neuron was calculated. Quantitation of axonal outgrowth was calculated from multiple transfection experiments run in duplicate. In each coverslip, roughly 6 GFP positive neuron axons were measured for overall length, and these lengths were averaged together to generate a single n for statistical purposes. About 50 total cells were measured per condition.

4.3 Results

4.3.1 KSRP and HuD localize to separate cytoplasmic granules in developing hippocampal neurons

We examined whether HuD or KSRP colocalize to the same cytoplasmic granules in the neuronal cytoplasm. Because HuD stabilizes and KSRP destabilizes bound ARE

containing mRNA, we postulated that they would be found in separate granules in the neuronal cytoplasm where they would bind to a population of stable or labile mRNAs, respectively. In hippocampal neurons cultured for two days *in vitro*, we performed immunocytochemistry using anti-HuD and anti-KSRP antibodies to visualize their expression patterns. As shown in Figure 4.2, KSRP is localized in the extended processes of the cultured neurons, and HuD closer to the cell body. There is no evidence of overlapping KSRP and HuD, indicating that they are indeed localizing to separate loci in the neurons.

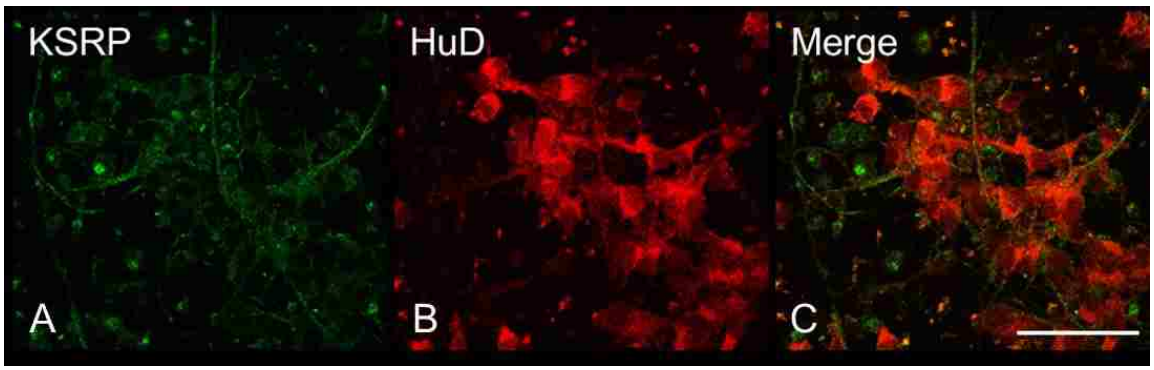

Figure 4.2 KSRP and HuD localized to separate locations in the cytoplasm of cultured hippocampal neurons

Hippocampal neurons cultured for 3 days *in vitro* were fixed and then immunocytochemistry performed to analyze KSRP (A) and HuD (B) expression. Notice the lack of yellow coloring in panel (C), indicating the expression patterns of these two proteins do not overlap. Scale bar = 50µm.

4.3.2 KSRP limits axonal outgrowth in cultured rat E17 hippocampal neurons

Having established that KSRP binds to and affects GAP-43 mRNA half-life *in vitro*, we set out to determine the physiological effect of KSRP expression in cultured rat hippocampal neurons. In this experimental system hippocampi from E17 rat embryos were isolated, dissociated, and grown for 4 days *in vitro* before being transfected with various GFP-KSRP constructs. The neurons were grown for 48 hours post-transfection

and then fixed and immunostained with anti-NF-L antibody to distinguish neurons from astrocytes (data not shown).

The effect of KSRP overexpression of is shown in Figure 4.3. Neurons transfected with control GFP vector had an average axonal length of 602.5 ± 225.1 μm. When neurons were transfected with GFP-KSRP, axonal outgrowth was significantly stunted, with neurons having an average axonal length of 156.4 ± 43.5 μm. This is significantly different when compared to GFP transfected cells using one-way ANOVA with Dunnett's post-test ($p < 0.001$). KSRP was originally identified to function in enhancing mRNA splicing (Min et al., 1997). To distinguish nuclear vs. cytoplasmic functions of KSRP in these analyses, we transfected neurons with a KSRP construct (GFP-KSRP-KH 1-4) lacking the nuclear localization signal (NLS). Overexpression of the cytoplasmic GFP-KSRP-KH 1-4 also significantly ($p < 0.01$) limited axonal outgrowth in our cultured hippocampal neurons (average length of 216.9 ± 63.5 μM), suggesting that KSRP enhancement of RNA splicing contributes minimally to KSRP's effects on axonal outgrowth. However, overexpression of GFP-KSRP-ΔKH4, which has reduced affinity for GAP-43 (see Figure 3.1A) did not limit axonal outgrowth when compared to control transfected cells. Axons of the GFP-KSRP-ΔKH4 transfected neurons were significantly longer than those of GFP-KSRP and GFP-KSRP-KH 1-4 transfected neurons ($p < 0.001$). Together, these data suggest that KSRP limits axon growth through binding to cytoplasmic RNAs.

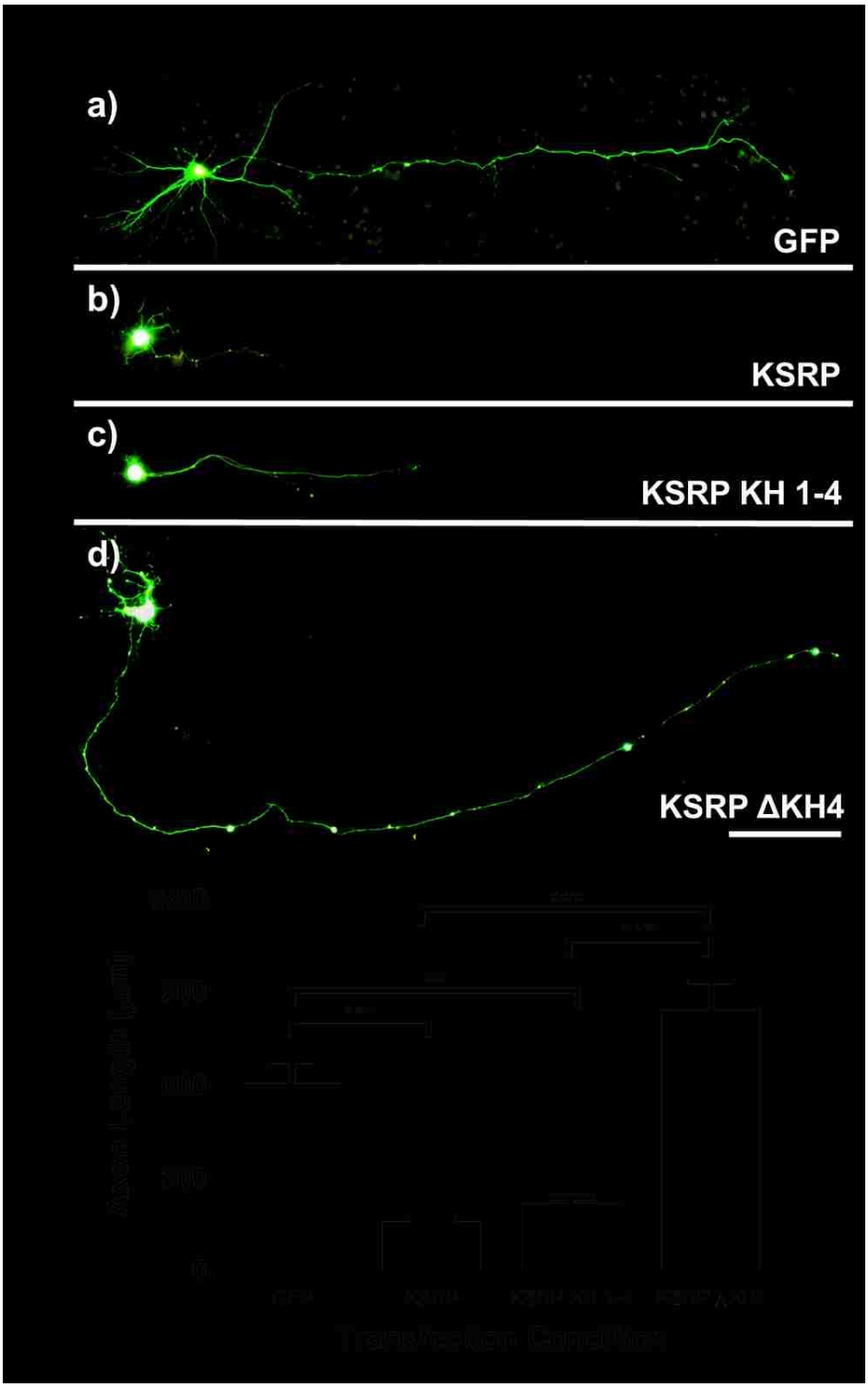

Figure 4.3 Overexpression of KSRP limits axonal outgrowth in E17 hippocampal neurons

A. Rat E17 hippocampal neurons transfected with KSRP constructs. Transfection conditions: a) GFP, b) KSRP, c) KSRP KH 1-4, d) KSRP ΔKH4. Scale bar is 100 µm. **B.** Quantitation of axonal outgrowth in KSRP transfected E17 hippocampal neurons. Averaged axonal outgrowth from several transfection experiments is shown (mean +/- SEM). **, p <.01; *** p < .001 using one-way ANOVA with Dunnett's post-test (GFP n=8, KSRP n=11, KSRP KH 1-4 n=6, KSRP ΔKH4 n=10 transfection experiments).

4.3.3 Knockdown of KSRP enhances axonal outgrowth in E17 rat hippocampal neurons

To further characterize the effect of KSRP expression on axonal outgrowth, we knocked down KSRP expression using a GFP expressing shRNA construct. Protein knockdown was confirmed by comparing immunofluorescence intensity of KSRP in shRNA transfected cells versus other untransfected cells in the same field. Expression of shKSRP reduced KSRP protein levels by roughly 60% ($p < 0.01$ using a Student's t-test) (Fig. 4.4 A and C). In contrast, control shRNA transfected cells had the same KSRP immunofluorescence intensity when compared to other cells in the same image frame

The effect of KSRP on axonal elongation was measured in the same manner as the KSRP overexpression studies, using the same time course, and using anti-NF-L counterstaining to confirm that neurons were being examined. When E17 hippocampal neurons were transfected with non-targeting control GFP-shRNA (Fig 4.4 B and D), axons grew to an average of 577.8 ± 231.9 μm, which is not significantly different when compared to GFP transfected cells (data not shown). Knockdown of KSRP with KSRP shRNA resulted in axons growing to significantly longer lengths (average of 1098.9 ± 434.4 μm, $p < 0.01$. These data indicate that a moderate knockdown of KSRP expression enhances axonal outgrowth, further implicating KSRP as an important regulator of axogenesis.

To further characterize the effect that KSRP has on GAP-43 expression *in vivo* we assayed the effect of KSRP knockdown on GAP-43 mRNA levels in a stable cell line. PC12 cells were transfected with GFP-shKSRP and GFP-control non-targeting shRNA,

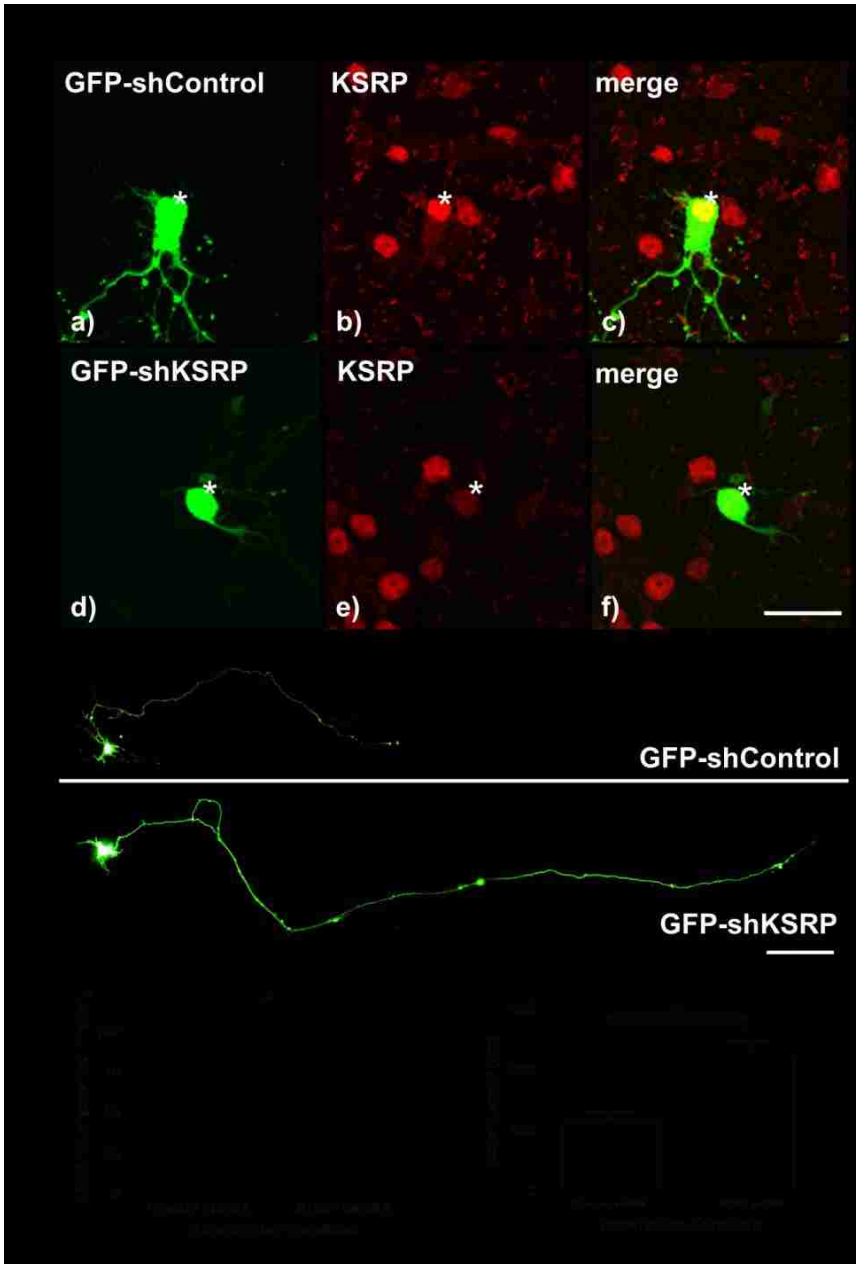

Figure 4.4 shRNA knockdown of KSRP in transfected hippocampal neurons increases axonal length.

A. Representative images of shRNA transfections. Control (a-c) or anti-KSRP (d-f) GFP-shRNA plasmids were transfected into primary hippocampal neuronal cultures, and KSRP expression was quantified by comparing KSRP immunofluorescence levels with untransfected cells in the same field. Scale bar is 25 µm. **B.** Rat E17 hippocampal neurons transfected with shRNA constructs. Transfection conditions: a) control shRNA, b) α-KSRP shRNA. Scale bar is 100 µm. **C.** Quantification of KSRP knockdown by anti-KSRP shRNA. Knockdown was measured by comparing KSRP immunofluorescence intensity in shRNA transfected cells vs. untransfected cells in the same image frame. Plotted graphs are relative mean (+/- SEM) levels of KSRP fluorescence intensity. **, $p < .01$, Student's t-test (n=7 transfections for control shRNA; n=14 transfections for shKSRP). **D.** Quantitation of axonal outgrowth in shRNA transfected E17 hippocampal neurons. Averaged axonal outgrowth from 10 separate transfection experiments is shown (mean +/- SEM). **, $p < .01$ using Student's t-test.

and incubated for 48 hours. Transfected cells were then purified using fluorescent activated cell sorting to enrich for the brightest 2.5% of transfected GFP positive cells (Appendix A, images A and B). KSRP and GAP-43 mRNA expression was then analyzed by qRT-PCR. Knockdown of KSRP by the shRNA construct was robust, lowering KSRP mRNA levels by roughly 95% (Appendix A, graph C). GAP-43 mRNA levels were increased 1.7 fold in cells with reduced KSRP (Appendix A, graph D). These results indicate that KSRP is a negative regulator of GAP-43 expression, as knockdown of KSRP can increase GAP-43 expression.

4.3.4 Rescue of limited axonal outgrowth in KSRP transfected neurons by co-expression of GAP-43

Based on the results of the overexpression and knockdown of KSRP in affecting axonal outgrowth, and the knowledge that KSRP binds to and affects the stability of GAP-43 mRNA, we sought to determine if the phenotype resulting from KSRP overexpression could be rescued by overexpressing GAP-43 in the same neurons. These rescue experiments were performed by transfecting E17 hippocampal neurons with GFP-KSRP along with different mCherry-GAP-43 constructs. We used three different GAP-43 constructs, each with a different 3'UTR. The mCherry-GAP-GAP3' plasmid contains GAP-43 coding region along with GAP-43 3'UTR, which contains the ARE and the element necessary for targeting GAP-43 to axonal growth cones, where its localized translation is required for promoting axonal extension (Donnelly et al., 2011). mCherry-GAP-AMP3' contains the GAP-43 coding region with the 3'UTR from amphoterin, which does not contain an ARE but does contain an axonal targeting sequence. As

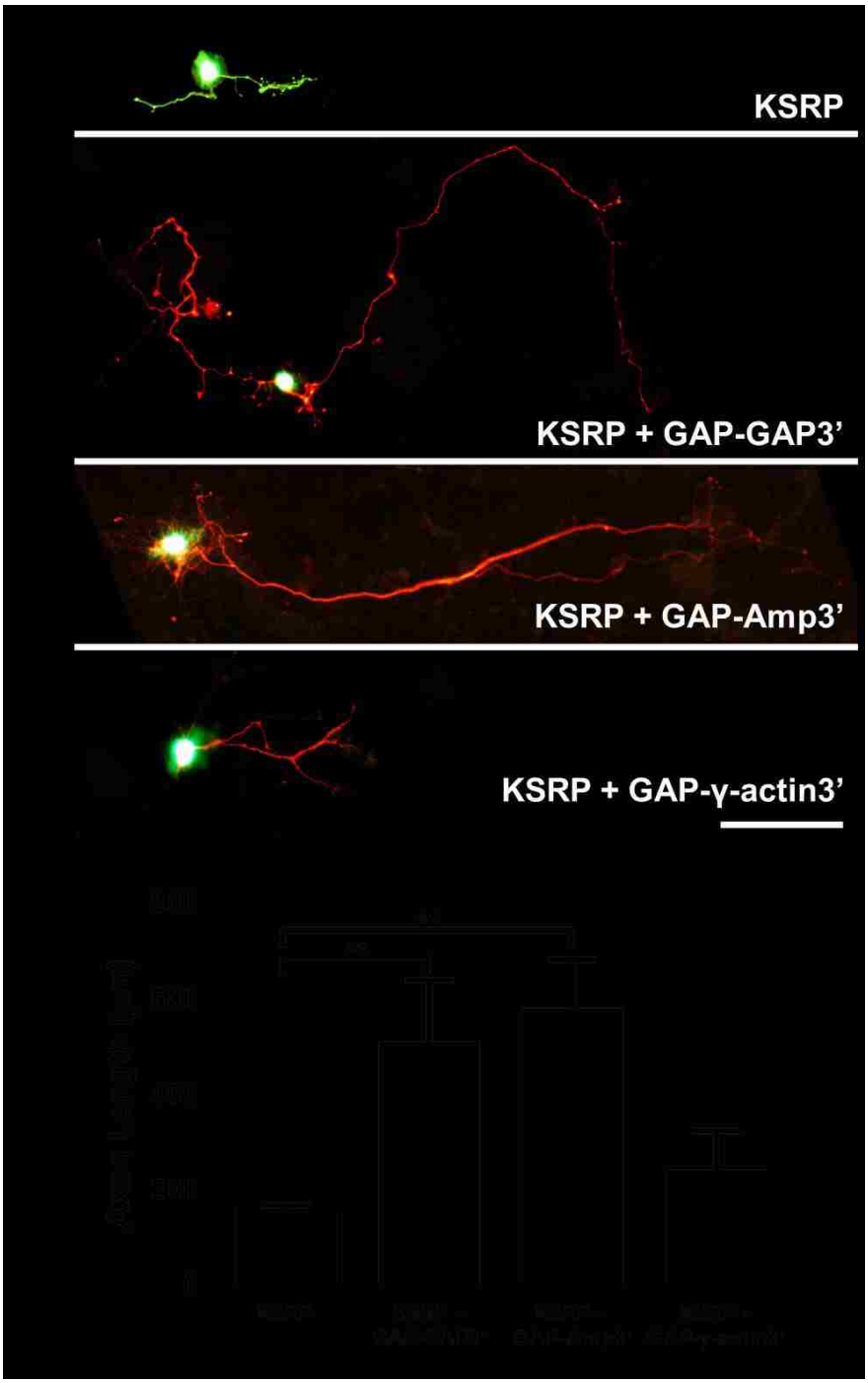

Figure 4.5 Overexpression of GAP-43 rescues limited axonal outgrowth in KSRP transfected E17 hippocampal neurons.

A. Rat E17 hippocampal neurons transfected with GFP-KSRP and various mCherry-GAP-43 constructs. Transfection conditions: a) KSRP only, b) KSRP + GAP-GAP3', c) KSRP + GAP-Amp3', d) KSRP + GAP-γ-actin3. Scale bar is 100 µm. **B.** Quantitation of axonal outgrowth in transfected neurons. Averaged data from 7 separate transfection experiments is shown (mean +/- SEM). **, $p < .01$ using one-way ANOVA with Dunnett's post-test (KSRP n=11; KSRP/GAP-GAP3', KSRP/GAP-Amp3', KSRP/GAP-γ-actin3' n=6).

control we used mCherry-GAP-γ-actin3', which has the GAP-43 coding region with γ-actin 3'UTR, and does not contain an ARE or an axonal targeting sequence.

Hippocampal neurons transfected with both GFP-KSRP and mCherry-GAP-GAP3' had axons grow to lengths significantly longer (505.6 ± 331.1 μm vs. 156.4 ± 43.5 μm, $p < 0.01$ using ANOVA with Dunnett's post-test) than neurons transfected with GFP-KSRP alone (Fig. 4.5). Neurons transfected with GFP-KSRP/mCherry-GAP-AMP3' also grew to lengths (average 577 ± 260.5 μm) significantly longer than neurons transfected with GFP-KSRP alone and comparable to the levels of control GFP-transfected neurons (Figure 4.3). In contrast, mCherry-GAP-γ-actin3' did not rescue axonal outgrowth in KSRP transfected cells, indicating that axonal targeting of GAP-43 mRNA is required for its correct function.

4.4 Discussion

Overexpression of KSRP in cultured hippocampal neurons led to a significant reduction in axon outgrowth compared to control neurons. Knockdown of KSRP had the opposite effect, resulting in axons growing out to significantly longer lengths. The overexpression of KSRP could be rescued with GAP-43 overexpression, indicating that KSRP regulates GAP-43 expression in neurons and has a significant impact on axonal outgrowth during development.

KSRP appears to function in direct opposition to HuD, which is supported by the finding that KSRP and HuD do not colocalize in the cytoplasm of cultured hippocampal neurons. This supports the notion that HuD and KSRP are binding to separate pools of mRNAs, with one group being stabilized for translation by HuD and another group

targeted for degradation by KSRP. This study only examined the locations of HuD and KSRP after neurons were cultured for 3DIV. A more detailed time course study of ARE-BP localization during neuronal development, both in cell culture and *in vivo*, will provide valuable insight into how these proteins interact and localize within the cell to post-transcriptionally regulate developmentally related ARE containing mRNA.

The shKSRP construct that we utilized led to a robust knockdown of KSRP mRNA in PC12 cells, reducing KSRP transcript levels to 5% of control levels. In cultured hippocampal neurons, shKSRP decreased KSRP protein levels by 60% based on immunocytochemical analysis of KSRP expression. The difference in knockdown levels between KSRP mRNA and protein could be attributed to the fact that turnover of RNA in a cell is much faster than protein turnover, so a quick knockdown of mRNA levels will be followed by a slower knockdown of the corresponding protein (Wu et al., 2004). In addition, some differences in knockdown efficiencies can also be attributed to the two different cells types utilized for these studies, i.e. PC12 cells vs. cultured hippocampal neurons.

One of the most exciting results from these culture studies was the rescue of the limited outgrowth in KSRP transfected neurons by concomitant overexpression of GAP-43. Especially intriguing is the fact that axonal targeting of GAP-43 mRNA is necessary for this rescue. The class III ARE of GAP-43 also contains a zip code targeting sequence required for targeting this mRNA to axons. Recent studies have shown that overexpression of GFP containing the β-actin 3'UTR, which also contains a zip code sequence, leads to decreased GAP-43 mRNA in axons, and hinders axonal outgrowth during development (Donnelly et al., 2011). Overexpression of a GAP-43 construct with

the γ-actin 3'UTR did not rescue limited axonal outgrowth caused by KSRP overexpression, demonstrating that localization of GAP-43 mRNA to axons, and most likely subsequent localized translation of GAP-43 is essential to promote axonal elongation during neuronal maturation.

5. Discussion

RNA-binding protein-mediated mRNA stabilization is an important mechanism of gene expression control during cell growth and differentiation. This is especially true in the nervous system, where the neuronal RBP HuD was shown to stabilize the mRNAs of several growth-related genes, such as GAP-43, AChE, tau, MARCKS and neuroserpin controlling neuron development and function [for review see (Bolognani and Perrone-Bizzozero, 2008)]. Despite the evident role of these post-transcriptional mechanisms in neurons, very little is known of the factors responsible for destabilizing mRNAs upon maturation. In this study, we have identified KSRP as a negative regulator of GAP-43 mRNA stability in developing neurons, which functions in an opposite manner to HuD, promoting mRNA decay and limiting axonal outgrowth.

In vitro studies examining how KSRP interacts with GAP-43 mRNA clearly indicate that KSRP binds to the highly conserved type III ARE present in the 3'UTR (Chung et al., 1996). Furthermore, KSRP and HuD compete for binding to the same site in GAP-43 mRNA with similar affinity. However, binding of KSRP to the GAP-43 ARE has the opposite effect of HuD, decreasing mRNA stability by almost 3-fold. While *in vitro* work is important in demonstrating KSRP modulation of GAP-43 transcript stability, there are a number of other factors that interact in cells to affect gene expression. To validate this *in vitro* result we performed shRNA knock-down studies and found increased levels of GAP-43 mRNA in PC12 cells. Using primary hippocampal neurons, we found that knockdown of KSRP drastically increases axonal outgrowth, while overexpression stunts growth. The proper control of axonal outgrowth during development is critical for forming functional synapses, so it seems that careful control of

both KSRP and HuD expression and their interaction with specific transcripts during differentiation are important for regulating axonal elongation and hence normal neuronal development.

Given that KSRP is highly expressed in brain tissue, it is interesting that there are relatively few studies examining how it affects neuronal development. One of these studies reported that the chick KSRP homolog zip code-binding protein 2 (ZBP2) aids the other zip-code binding protein ZBP1 binding to β-actin mRNA in the nucleus, and shuttles the mRNA to neurites for localized translation during development (Pan et al., 2007). ZBP1 expression has also been shown to be crucial for proper axonal localization of GAP-43 mRNA, because limited availability of ZBP1 depletes axonally localized GAP-43 and hampers axonal outgrowth (Donnelly et al., 2011). Knock-down of ZBP2 in a mouse neuroblastoma cell line hindered neurite outgrowth once the cells were differentiated, supposedly due to decreased loading of ZBP1 onto β-actin mRNA (Pan et al., 2007). In the present study, we observed the opposite effect in primary cultures with KSRP knocked down. This apparent discrepancy is likely due to the differences in the cell types used and the timing of the knockdown of KSRP expression, as we let the neurons establish themselves for 4 days *in vitro* before performing shRNA knockdown. Perhaps early in development KSRP functions to help load ZBP1 onto zip code containing mRNAs in the nucleus, while it can subsequently function in the cell body and processes to destabilize growth related transcripts to fine-tune neuronal development. It is important to note that the ARE in GAP-43 3' UTR also acts as a zip-code element that is critical for its correct axonal localization, as shown by our rescue study showing that

overexpression of GAP-43 lacking these sequences (mCherry-GAP-γ-Actin3') did not rescue the phenotype induced by KSRP overexpression (Donnelly et al., 2011).

One factor crucial to understanding where KSRP is localized and how it binds mRNAs during development is its phosphorylation state. KSRP can be phosphorylated by p38 MAP kinase (p38 MAPK), which inhibits KSRP binding to ARE containing mRNAs and prevents their subsequent decay (Briata et al., 2005). Phosphorylation of KSRP's KH1 domain also allows it to be bound by the regulatory 14-3-3ζ protein, after which KSRP is localized to the nucleus where it cannot bind to cytoplasmically localized labile transcripts (Diaz-Moreno et al., 2009). It is interesting to consider the possibility that during HuD stabilizing events KSRP is mainly localized to the nucleus, aiding in the export of zip-code containing mRNAs that are stabilized by HuD in the cytoplasm. These HuD stabilizing events need not be limited to initial neuron outgrowth during development, as there are other cellular processes in which HuD protein levels are up-regulated to promote stability of GAP-43 mRNA. As mentioned in the introduction, HuD levels are increased in the neuronal cell body of DRG neurons following a nerve crush injury (Anderson et al., 2003). HuD protein and GAP-43 mRNA levels are also increased during learning and memory processes (Quattrone et al., 2001, Pascale et al., 2004). In the course of our studies, we have seen that KSRP and HuD do not colocalize within the cytoplasm, lending support to the notion that these two RNA binding proteins could be interacting with separate populations of mRNAs at a given time point. Studies examining KSRP and HuD localization during these cellular events in which GAP-43 is stabilized could lend insight into how these two RBPs with opposing effects on mRNA stability operate in processes critical for neuronal growth and everyday neuronal function.

Based on the above information, I propose the following model for how KSRP and HuD function during initial axonal pathfinding and later target acquisition (Figure 5.1). As axons of developing neurons are finding their targets, KSRP is localized to the nucleus, which is regulated by p38 MAPK signaling and 14-3-3 ζ binding. This notion is supported by the fact that NGF stimulation of PC12 cells also activates p38 MAPK signaling, which leads to KSRP phosphorylation (Takeda and Ichijo, 2002, Briata et al., 2005). KSRP, localized to the nucleus in a developing neuron, would aid in loading ZBP1 to GAP-43 mRNA, which would then be exported from the cytoplasm and localized to the axon (Donnelly et al., 2011). Once exported from the nucleus, cytoplasmic HuD would bind to the GAP-43 mRNA transcript, stabilizing it and promoting its translation via association with polysomes (Bolognani et al., 2004, Tiruchinapalli et al., 2008). Once translated into a functional protein, GAP-43 would be phosphorylated by PKC activated by growth factor signaling, which would then serve to promote actin polymerization and filopodia extension of the axon terminal to reach its dendritic target (Sofroniew et al., 2001, Denny, 2006). Once the axon terminal of the developing neuron acquires its post-synaptic target and forms a functional synapse, growth factor signaling would decrease, leading to subsequent decreases in p38 MAPK and PKC activity. Cellular phosphatases would dephosphorylate GAP-43 protein at the axon terminal, which would in turn lead to decreased levels of actin polymerization. In the nucleus, active phosphatases would dephosphorylate KSRP allowing it to dissociate from 14-3-3ζ, at which point KSRP would shuttle to the cytoplasm, where it is free to bind to GAP-43 mRNA and promote exosome binding and decay of the GAP-43 transcript (Diaz-Moreno et al., 2009). This hypothetical model is based on the both

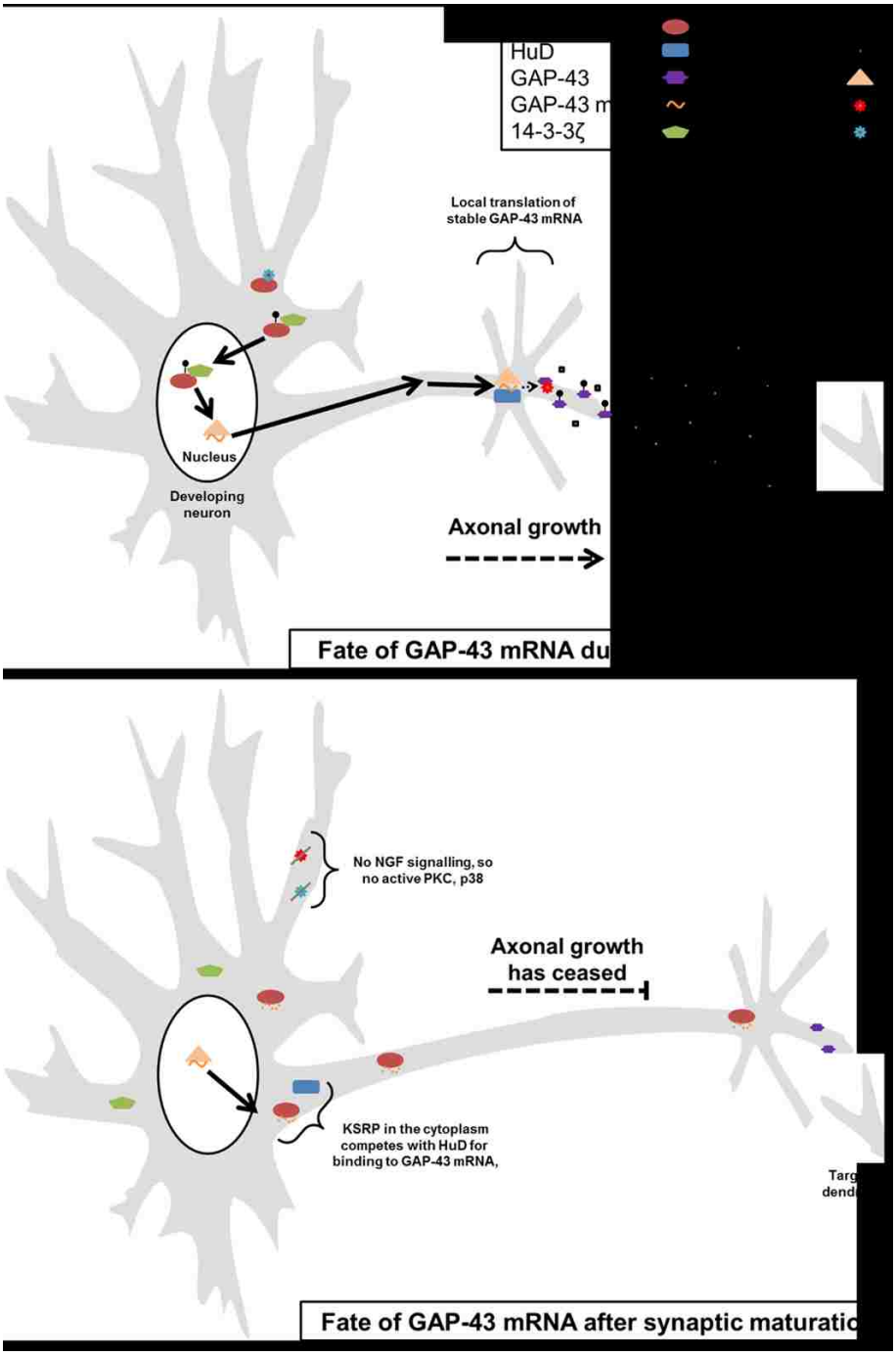

Figure 5.1 Theoritical model for GAP-43 regulation during axonal elongation

A. GAP-43 mRNA is stabilized during axonal outgrowth. Phosphorylated KSRP localized to the nucleus by 14-3-3ζ aids in binding ZBP1 to GAP-43 mRNA, which is exported from the nucleus and localized to the axonal growth cone. HuD binds GAP-43 mRNA in the cytoplasm as well, stabilizing it for translation. **B**. p38 and PKC are no longer active after the axon reaches its dendritic target and growth factor signaling ceases. Dephosphorylated KSRP is then localized to the cytoplasm, free to bind to GAP-43 mRNA and promote its degradation.

the results obtained and data from the literature. Subsequent investigations will need to tease apart the specific processes mediating axonal outgrowth associated with post-transcriptional regulation of the GAP-43 gene.

Another mechanism promoting mRNA degradation also needs to be considered when discussing KSRP's function as recent evidence demonstrates KSRP's involvement in promoting the biogenesis of a subset of miRNAs. Thus far KSRP has been shown to be a required factor in the maturation of two different miRNAs, let-7a and miR-155 (Ruggiero et al., 2009, Trabucchi et al., 2009). Knockdown of KSRP in P19 cells led to a decrease in the maturation of a number of miRNAs (Trabucchi et al., 2009), one of which (miR-21) has the potential to bind to GAP-43 mRNA with 100% sequence complementarity (MicroCosm Targets Version 5, http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). Initial investigations in our laboratory have shown KSRP potentially binds to a number of miRNA targets, and may be involved in promoting their maturation as well (Gardiner et al., unpublished observations). Perhaps one of these miRNAs can also bind to GAP-43 mRNA and promote GAP-43 transcript degradation through a decay pathway independent of AMD.

KSRP regulation of GAP-43 mRNA stability may not be the only mechanism affecting neuron differentiation and development. As shown by Winzen et al. (2007) several transcripts bind KSRP in HeLa cell extracts including a number of mRNAs that encode for growth related proteins or signaling molecules relating to cellular development. Furthermore, using UV-crosslinking and immunoprecipitation assays, we have recently demonstrated binding of KSRP to not only GAP-43 mRNA but also to other axonally localized mRNAs (Gardiner et al., unpublished observations). Therefore,

it will be important to examine the interplay of KSRP with HuD in the stabilization and transport of target mRNAs during neuronal development. HuD and KSRP show a similar expression pattern during brain development, with KSRP and HuD highly expressed early in development and their levels dropping off during adolescence (Gu et al., 2002, Bolognani et al., 2007a). Although both proteins are present in neurons, preliminary studies indicate that they are not co-localized to the same cytoplasmic granules. Along these lines, it is interesting to note that both HuD and KSRP have been shown to localize to peripheral nerve axons via binding to SMN, and improper localization of these RBPs can affect motor neuron function (Tadesse et al., 2008, Akten et al., 2011, Fallini et al., 2011, Hubers et al., 2011).

As with any scientific study, there are some limitations to the work presented in this dissertation. The *in vitro* decay study is a powerful tool for examining the stability of an mRNA, but because it takes place outside the cell it does not include other cellular proteins and factors that could be having an effect on mRNA expression. In order to strengthen our decay studies, we could perform decay studies in cells transfected with isoforms of KSRP, and inhibit transcription using actinomycin D. Following transcriptional inhibition, we could collect RNA at different time points and examine the stability of GAP-43 over time. Another drawback to the studies performed involves the cell sorting experiments. At the time this dissertation was written, the experiment had been performed once in duplicates, so the data is limited in that respect. More transfection and sorting experiments could be done to further confirm that the KSRP knockdown in PC12 cells increases the expression of GAP-43 mRNA. Lastly, performing cell culture and transfection experiments to examine the effect of KSRP on

axonal elongation is a good model system, but does not capture the complete environment of the intact brain. To truly show that KSRP restricts axonal elongation *in vivo*, *in utero* transfection experiments could be performed to truly show that excess KSRP expression limits axonal growth in the developing brain. Unfortunately, these types of studies require extensive training and specialized equipment, which are currently unavailable in the laboratory.

In conclusion, the research contained within this dissertation shows that KSRP binds to and destabilizes GAP-43 mRNA *in vitro*, and serves to limit axonal outgrowth in primary hippocampal cultures. These data add another layer of complexity to the post-transcriptional operon that regulates GAP-43 mRNA stability, showing that tight control of this gene's expression is an important factor regulating axonal growth processes.

Abbreviations Used

3'UTR	Three prime untranslated region
A	Adenine
AChE	Acetylcholine esterase
AMD	AU-rich element mediated mRNA decay
ARE	AU-rich element
ARE-BP	ARE binding protein
ARED	ARE database
BRF1	Butyrate regulated factor one
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
Dcp	Decapping protein
DIV	Days *in vitro*
ELAV	Embryonic lethal abnormal vision
FBP	Far upstream element binding protein
FUSE	Far upstream element
G	Guanine
GAP-43	Growth associated protein 43
GFP	Green fluorescent protein
GM-CSF	Granulocyte-monocyte colony stimulating factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KH	K homology
KSRP	K homology splicing regulatory protein
MAPK	Mitogen activated protein kinase

mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
NGF	Nerve growth factor
NLS	Nuclear localization signal
PARN	Poly(A) ribonuclease
PKC	Protein kinase C
RBP	RNA-binding protein
REMSA	RNA electrophoretic mobility shift assay
RRM	RNA recognition motif
RT-PCR	Real time polymerase chain reaction
shRNA	Short hairpin RNA
SMN	Survival of motor neuron protein
TTP	Tristetraprolin
U	Uracil
WB	Western blot
Xrn1	Exoribonuclease 1
ZBP	Zip code binding protein

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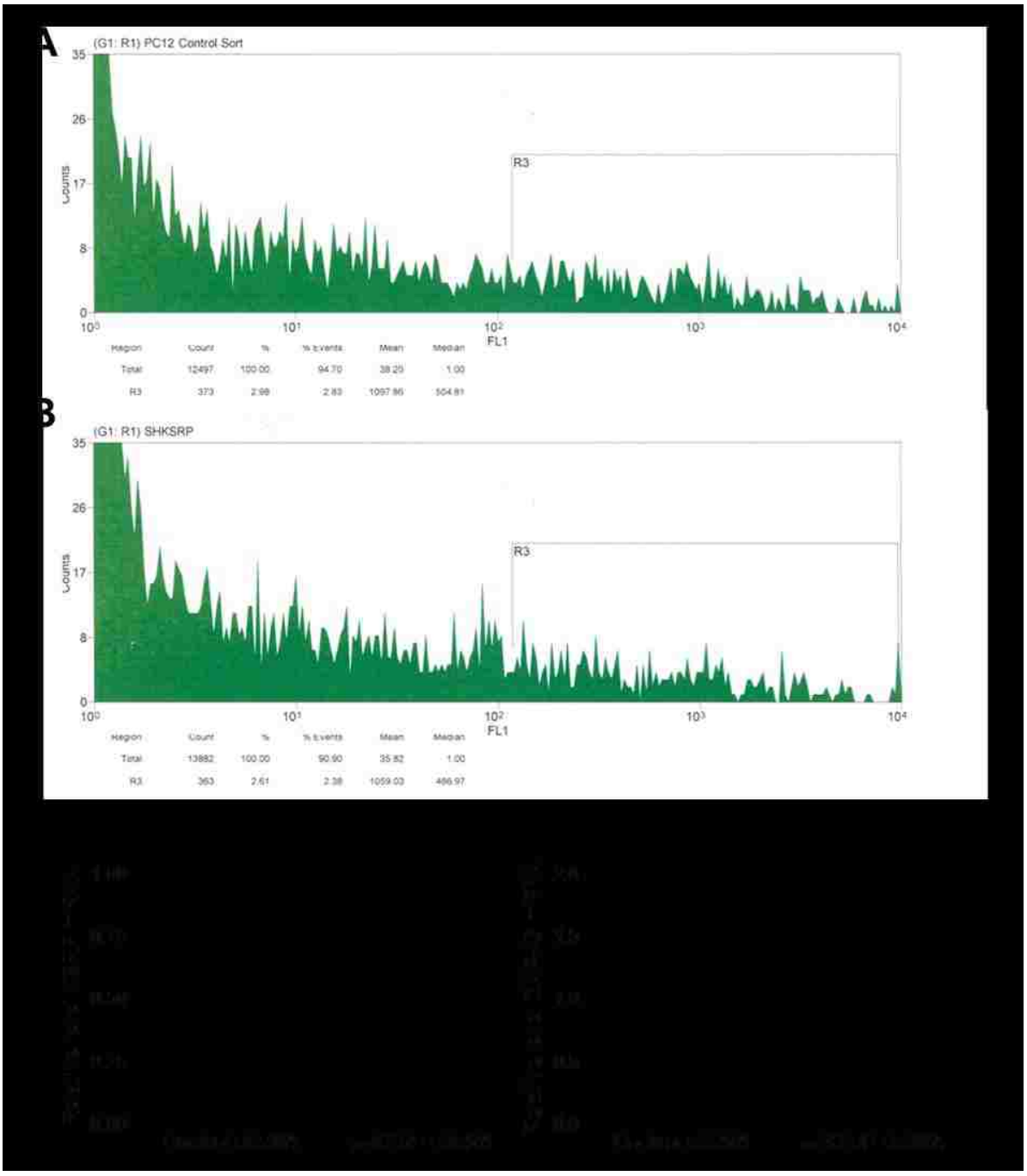
Appendix A: KSRP and GAP-43 mRNA expression in shRNA transfected PC12 cells



Appendix A: shRNA knockdown of KSRP in PC12 cells increases GAP-43 mRNA expression

A and B. Graphs of the gating used to collect GFP positive transfected PC12 cells. Control non-targeting GFP-shRNA (A) and GFP-shKSRP (B) transfected PC12 cells were enriched to collect the brightest 2.5% fraction of cells. **C.** Graph of KSRP mRNA knockdown in transfected PC12 cells. KSRP levels were knocked down ~95%. **D.** Graph of GAP-43 mRNA levels in transfected cells. PC12 cells transfected with shKSRP expressed 1.7 fold more GAP-43 mRNA than control non-targeting GFP-shRNA transfected cells.

Appendix B: Submitted short-report article to J. Neuroscience co-authored by Clark Bird

References for this article are self-contained within this appendix.

HuD Promotes BDNF Expression via Selective Stabilization of the BDNF Long 3'UTR mRNA

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Abstract

Complex regulation of the brain-derived neurotrophic factor (BDNF) governs its intricate functions in brain development and neuronal plasticity. Besides tight transcriptional control, alternative 3'end processing of the BDNF transcripts generates either a long or a short 3'untranslated region (3'UTR). The distinct RNA sequence in the BDNF 3'UTRs differentially regulates BDNF production in brain neurons, conceivably due to differential interactions with undefined trans-acting factors. In this study, we report that the neuronal selective RNA-binding protein HuD interacts with a highly conserved AU-rich element (ARE) specifically located in the BDNF long 3'UTR. Such interaction is sufficient for selective stabilization of the BDNF long 3'UTR mRNA *in vitro* and *in vivo*, leading to increased levels of BDNF long 3'UTR mRNA and BDNF protein in the hippocampal mossy fiber (MF) pathway in HuD transgenic mice. Our results identify the first trans-acting factor that enhances BDNF expression specifically through the long 3'UTR and a novel mechanism that differentially regulates BDNF production in selected neuronal populations.

Introduction

Brain-derived neurotrophic factor (BDNF) plays pivotal roles in governing a broad spectrum of brain functions including neuronal survival, neural network development, and synaptic plasticity. To accommodate such intricate functions, BDNF expression is under precise regulation. Furthermore, dysregulation of BDNF is indicated in the pathogenesis of neurological and psychiatric diseases (K. Martinowich et al., 2007; A. H. Nagahara and M. H. Tuszynski, 2011). Transcription of BDNF can be initiated from at least eight different promoters in mammals in response to various stimulation cues (Q. R. Liu et al., 2006; T. Aid et al., 2007). In addition, alternative poly-adenylation results in two pools of BDNF mRNAs; each carrying either a short or a long 3'untranslated region (UTR) regardless of which promoter drives BDNF transcription. While the entire sequence of the short 3'UTR is contained within the long 3'UTR, it is the unique sequence in the BDNF long 3' UTR that mediates differential regulation of BDNF production through multiple posttranscriptional mechanisms. The long 3'UTR, but not the short 3'UTR, mediates neuronal activity-dependent translation of BDNF (A. G. Lau et al., 2010) and dendritic localization of BDNF mRNA (J. J. An et al., 2008). Additionally, the short and long 3'UTR differentially affect BDNF mRNA stability, with the long transcript having a much shorter half-life than its short counterpart (E. Castren et al., 1998). The unique sequence in the BDNF long 3'UTR is predicted to provide binding sites for trans-acting RNA-binding proteins (RBPs) and/or microRNAs to achieve differential posttranscriptional regulation of BDNF mRNA isoforms. However, no previous studies have identified RBPs that specifically bind and regulate the BDNF long 3'UTR mRNA.

We report here that HuD, a neuronal RBP that plays critical roles in governing neuronal circuitry development and plasticity via controlling mRNA stability and/or translation (J. Deschenes Furry et al., 2006), selectively binds the BDNF long 3'UTR through a highly conserved AU-rich element (ARE) and stabilizes the BDNF long mRNA. Moreover, we show that a HuD transgene increases the levels of the BDNF long 3'UTR mRNA and BDNF protein primarily in the hippocampal mossy fiber (MF) pathway, offering a novel mechanism for posttranscriptional regulation of distinct BDNF mRNA isoforms.

Methods

Cell culture, plasmids and transfection

The ARE at the distal end of BDNF long 3'UTR was removed by PCR amplification of the pcLuc-BDNFlongUTR plasmid (A. G. Lau et al., 2010) with primers of ccgctcgagTGGATTTATGTTGTATAGATTAT (forward) and gctctagaACATGGTGAATAATATCTTTACC (reverse), and subcloned between the XhoI/XabI sites to replace the full length BDNF 3'UTR in pcLuc-BDNF longUTR (pcLuc-BDNF longUTRΔARE). Luciferase reporter constructs were co-transfected with myc-HuD (K. D. Anderson et al., 2003) or pcDNA into CAD cells using Lipofectamine 2000 (Invitrogen). The pRL-TK renilla luciferase construct was also co-transfected. Reporter expression was quantified using the Dual-Luciferase assay (Promega).

UV-crosslinking-immunoprecipitation (CLIP) and RT-PCR

Crosslinking was performed as previously described (J. Ule et al., 2003) with modifications. Briefly, cells were UV crosslinked using a Stratalinker (Stratagene, 400 mJ). The postnuclear supernatant was precleared with IgG-conjugated protein A-Sepharose beads in the presence of 0.001% SDS. Anti-c-myc antibody (SantaCruz) 1:500 conjugated to protein A beads was used for immunoprecipitation (L. Zhao et al., 2006). The immunoprecipitated complexes were proteinase K-treated before RNA extraction (L. Zhao et al., 2010). RT-PCR was performed with Primer A: ATCAGGCAAGGATATGGGCTCACT (forward) and TCCAGATCCACAACCTTCGCTTCA (reverse); Primer B: TGGCCTAACAGTGTTTGCAG (forward) and GGATTTGAGTGTGGTTCTCC (reverse); and Primer C: CAGTGGCTGGCTCTCTTACC (forward) and GGCCACAGACATTTACTTACAGTTT (reverse).

In vitro mRNA binding and decay

A 164 bp fragment containing the BDNF ARE (nt 2640- 2746) was PCR-amplified and cloned into the XbaI/XhoI sites of pBSKII (Invitrogen). Radiolabeled BDNF-ARE RNA was prepared by *in vitro* transcription using 32P-UTP as described (A. C. Beckel-Mitchener et al., 2002). RNA electrophoretic mobility shift assay [REMSA, (Y. Li et al., 2004)] used 100,000 cpm of 32P-UTP labeled BDNF-ARE RNA, increasing amounts of purified GST or GST-HuD (S. Chung et al., 1997), and 0.25 mg/ml yeast tRNA and 0.25 mg/ml of BSA to minimize non-specific interactions. Specific competition was carried out with a 10-fold molar excess of cold BDNF-ARE RNA. *In vitro* mRNA decay reactions were performed using ~200 fmol (100,000 cpm) of capped and polyadenylated radiolabeled BDNF-ARE RNA and 20 μg S100 protein from HuD-

KO mice. Reactions were supplemented with either 50 ng of GST-HuD or GST and the half-life of the mRNA was calculated as described (F. Bolognani et al., 2007).

Treatment of Primary cultures of embryonic cortical neurons

Neuronal cell cultures were prepared from E17 C57BL/6 mice (P. Washbourne et al., 2002), and were grown for 24 hours before infection with HSV-HuD or control HSV-lacZ as described (K. D. Anderson et al., 2001). After 72 h, total RNA was isolated and BDNF long 3'UTR mRNA levels were measured by RTqPCR using GAPDH as reference.

In situ hybridization and immunofluorescence

Fluorescent in situ hybridization (FISH) was performed using a digoxigenin-labeled antisense oligonucleotide complementary to nucleotides 2508-2556 in the BDNF long 3' UTR (5' GGGTGTATACAATAACTTTTATCTGCAAACACGTTAGG-CCATATTAC) as described (N.I. Perrone-Bizzozero et al., 2011). Duplicate adjacent sections from HuD-Tg mice and nontransgenic wild type littermates (WT) were analyzed in parallel and images acquired using the same condition. To assess BDNF protein levels in hippocampus, brain slices derived from 2- month old HuD-Tg mice and WT littermates were subjected to immunofluorescent staining followed by image acquisition as described previously (A. G. Lau et al., 2010). Fluorescent signals were quantified using ImageJ (NIH) and the density of IF was calculated.

Results

HuD selectively enhances expression of the BDNF long 3'UTR reporter through a highly conserved ARE

AREs are prominent motifs found in the 3'UTRs that recruit various ARE-binding proteins (ARE-BPs) to stabilize or destabilize target mRNAs (N. Xu et al., 1997). AREs can be divided into three different classes: Class I ARE contains a core penta-nucleotide AUUUA flanked by A/U, Class II ARE contains overlapping AUUUA motifs, and Class III does not contain typical AUUUA motifs but long stretches of U-rich sequences. Using the ARED-Organisms database (http://brp.kfshrc.edu.sa/ARED/), we identified a highly conserved Class I ARE specifically located in the BDNF long 3'UTR immediately up-stream of the distal poly-adenylation site (Figure 1A), suggesting that ARE-BPs may preferentially regulate stability of the BDNF long 3'UTR mRNA. HuD is a neuronal specific ARE-BP (F. Bolognani et al., 2009) that displays a functional spectrum substantially overlapping with that of BDNF (N. Perrone-Bizzozero and F. Bolognani, 2002). To directly test whether HuD can regulate mRNA expression selectively via the BDNF long 3'UTR, we co-expressed c-myc-tagged HuD (K. D. Anderson et al., 2000) with luciferase reporters carrying the short BDNF 3'UTR (S-3'UTR), the full-length BDNF long 3'UTR (L-3'UTR) or the BDNF long 3'UTR lacking the ARE (ΔARE) in the brain neuron cell line CAD. As shown in Figure 1B, c-myc-HuD significantly increased luciferase reporter expression when full length BDNF L-3'UTR is present. In contrast, c-myc-HuD did not have any effect to the BDNF S-3'UTR reporter. Furthermore, removing the ARE in BDNF long 3'UTR completely abolished the effect of HuD (Figure 1C). Importantly, myc-HuD significantly increased BDNF L-3'UTR reporter mRNA levels in an ARE-dependent manner (Figure 1D), recapitulating the HuD

response measured by luciferase activity (Figure 1C). Thus, although HuD can also promote translation initiation (A. Fukao et al., 2009), the BDNF ARE appears to mediate regulation by HuD primarily at the level of mRNA stability. The expression of each luciferase reporter with expected sequence of BDNF 3'UTRs was confirmed by RT-PCR using multiple primer pairs (Figure 1E).

To test whether HuD associates with the BDNF long 3'UTR through the ARE in transfected cells, we performed UV-cross linking immunoprecipitation (CLIP) followed by RTPCR. As shown in Figure 1F, only the long 3'UTR reporter mRNA, but not the short 3'UTR reporter mRNA was co-immunoprecipitated with HuD (left panel). Furthermore, removing the ARE in the long 3'UTR significantly attenuated the association with HuD (Figure 1F, right panel, and Figure 1G). These results suggest that selective association of HuD with the long 3'UTR mediates the biological effect of HuD, and the ARE serves as a key site for interaction with HuD.

HuD directly binds and stabilizes the BDNF long 3'UTR mRNA

To test whether HuD can directly bind to the BDNF long 3'UTR segment that contains the ARE, we performed RNA-mobility shift assays using recombinant GST-HuD or GST. As shown in Figure 2A, GST-HuD bound the BDNF-ARE segment with high affinity. A mobility shift of the RNA can be visualized even with 25 ng of GST-HuD. In contrast, GST alone did not bind the RNA at any concentrations examined. The specificity of HuD-ARE interaction was further demonstrated by the displacement of the radiolabeled RNA with a cold ARE competitor (Figure 2A, right panel).

To further examine whether interactions between HuD and the BDNF-ARE could result in mRNA stabilization, we used *in vitro* transcribed capped and polyadenylated

BDNF-ARE RNA and GST-HuD for *in vitro* decay assays (Figure 2B). Addition of GST-HuD to brain extracts resulted in a significant stabilization of the RNA, with an almost 2-fold reduction in the initial rate of decay. We next examined the effect of HuD on endogenous long-BDNF mRNA in primary cultured cortical neurons treated with HSV vectors expressing either HuD or the control LacZ gene (K. D. Anderson et al., 2001). As shown in Figure 3A, overexpression of HuD significantly increased the levels of the BDNF long 3' UTR mRNA, whereas the pan BDNF mRNA levels were unaltered, in which the short BDNF mRNA is the major isoform (A. G. Lau et al., 2010). Reciprocally, shRNA-mediated HuD knockdown reduced the levels of the BDNF long 3'UTR mRNA in both the soma and processes (data not shown), suggesting that HuD governs BDNF long mRNA expression throughout the entire neuron.

A HuD transgene selectively up-regulates BDNF long 3'UTR mRNA and BDNF protein in the hippocampal MF pathway

Finally, we tested whether elevated HuD expression can regulate neuronal BDNF production *in vivo* through the long 3'UTR. Quantitative fluorescent in situ hybridization (FISH) was performed using brain slices of HuD transgenic mice (HuD-Tg) that express myc-tagged HuD under the CamKIIα promoter (F. Bolognani et al., 2006). Non-transgenic WT littermates were processed in parallel as baseline controls. Consistent with the preferential increase of HuD in hippocampal dentate gyrus granule cells (DGCs) of the HuD-Tg (F. Bolognani et al., 2006), BDNF long 3'UTR mRNA is significantly up-regulated in the DGCs of HuD-Tg (Figure. 3B). In contrast, BDNF long 3' UTR mRNA was not increased in CA3 or CA1 neurons that do not harbor significant over-expression of transgenic HuD (data not shown). Furthermore, pan BDNF mRNA that contains 80%

of BDNF short 3'UTR mRNA is not affected in HuD-Tg, indicating preferential regulation of the BDNF long 3'UTR mRNA by HuD *in vivo*.

BDNF protein synthesized in DGCs is primarily transported to and stored in hippocampal MF axons that project through the hilus to form large synapses with CA3 dendrites (S. C. Danzer and J. O. McNamara, 2004). Consistent with the enhanced expression of BDNF long 3'UTR mRNA in DGCs (Figure 3B), significantly increased BDNF protein immunofluorescence was detected in the hilus (Figure. 4B), the CA3 strata lucidum and radiatum (Figure. 4C). Transgenic HuD expression also increased BDNF protein in cells of the DG subgranular zone (SGZ) that is enriched of adult neural stem cells (W. Guo et al. 2011), particularly in the characteristic long processes extended into the DGC layer (arrows in Figure. 4B). In contrast, BDNF protein levels in CA1 neurons are not significantly altered (Figure. 4F). Given the vigorous regulation of endogenous HuD in DGCs (F. Bolognani et al., 2007), HuD-dependent stabilization of BDNF long 3'UTR mRNA is a novel mechanism that controls BDNF production in the hippocampal MF pathway, a critical circuitry that governs hippocampal excitatory activities in physiological and pathological plasticity during epileptogenesis (S. C. Danzer and J. O. McNamara, 2004).

Discussion

Our studies identify HuD as the first RBP that selectively binds to and stabilizes the BDNF long 3'UTR mRNA but not the short BDNF mRNA isoform. Such function is mediated by a highly conserved ARE in the BDNF long 3'UTR. Furthermore, we showed that elevated HuD expression can enhance BDNF protein production in brain neurons in

culture and *in vivo*, suggesting that the quantity of HuD controls BDNF levels in neurons through preferential regulation of the long 3'UTR BDNF mRNA.

Consistent with the fact that mRNAs carrying AREs in the 3'UTR often display short half-lives (N. Xu et al., 1997; C. Y. Chen et al., 2001), the BDNF long 3'UTR mRNA is less stable than the BDNF short 3'UTR mRNA (E. Castren et al., 1998), likely due to recruitment of undefined destabilizing RBPs to the unique sequence in the long 3'UTR. Such instability provides a practical opportunity for stabilizing RBPs, represented by HuD, to differentially regulate BDNF mRNAs that carry the two distinct 3'UTRs. However, although the long 3'UTR can mediate dendritic localization of BDNF mRNA (J. J. An et al., 2008), manipulating HuD expression reduced the long BDNF mRNA isoform in both neuronal soma and processes. Thus, HuD-dependent stabilization governs the overall levels of the BDNF long 3'UTR mRNA, which is known to mediate neuronal activity-dependent translation (A. G. Lau et al., 2010). Importantly, the abundance of HuD is vigorously regulated during neuronal development and functional changes. HuD is highly abundant in the brain during embryonic and neonatal development, down-regulated in adult, but markedly up-regulated upon neuronal activation in multiple plasticity paradigms (F. Bolognani et al., 2004; A. Pascale et al., 2004; F. Bolognani et al., 2007; D. M. Tiruchinapalli et al., 2008). Moreover, marked regulation of HuD is found in various neuronal subpopulations (H. J. Okano and R. B. Darnell, 1997; N. Perrone-Bizzozero and F. Bolognani, 2002). Together with the differential levels of BDNF mRNAs that carry the long or the short 3'UTR in distinct brain regions (J. J. An et al., 2008), HuD-dependent stabilization of the BDNF mRNA

through the long 3'UTR provides a posttranscriptional mechanism that increases the complexity of BDNF regulation to accommodate brain development and function.

A particular interesting case is the regulation of HuD in hippocampal MFs. HuD protein is not normally detected in adult hippocampal DGCs (F. Bolognani et al., 2007). However, upon seizure induction, HuD is drastically up-regulated in DGCs. In contrast, although HuD is present in CA1 and CA3 neurons at rest, it is only moderately increased after seizures. Recapitulating the robust increase of HuD in DGCs in response to seizure, resting HuD-Tg mice display the highest fold increase of HuD protein in DGCs (F. Bolognani et al., 2006; N. I. Perrone-Bizzozero et al. 2011), although HuD transgene expression can be detected in all forebrain neurons. As a result, BDNF protein expression is preferentially increased in DGCs and stored in the MF pathway. Considering the well-known effects of BDNF in promoting neuronal processes growth (R. Koyama et al., 2004), the increased BDNF expression in hippocampal MFs is likely an contributing factor for the MF over-projection in HuD-Tg (N. I. Perrone-Bizzozero et al. 2011). Conceivably, the seizure-induced HuD up-regulation in DGCs could contribute to the wellcharacterized preferential increase of BDNF in the MF terminals and MF sprouting during epileptogenesis (S. C. Danzer et al., 2004; S. C. Danzer and J. O. McNamara, 2004).

Taken together, our study reveals a novel mechanism that controls BDNF expression through HuD-dependent stabilization of the BDNF long 3'UTR mRNA. Because BDNF is also under rigorous transcriptional regulation, and conventional HuD knockout results in abnormal neuronal development (W. Akamatsu et al., 2005), a definitive answer regarding the biological function of HuD-dependent BDNF mRNA

stabilization in response to neuronal activation may only be obtained once inducible knockout of HuD can be achieved. In addition, HuD was recently reported to enhance translation initiation through unwinding structural 5'UTRs (A. Fukao et al., 2009). Thus, although the ARE in BDNF long 3'UTR appears to mediate HuD's effect primarily through mRNA stability, whether HuD can also enhance BDNF translation via specific 5'UTRs upon neuronal activation is an intriguing and challenging question to be answered by future studies.

Figure legends

Figure 1. HuD enhances luciferase reporter expression through an ARE in the BDNF long 3'UTR. (A) A highly conserved cluster of Class I ARE in the BDNF long 3'UTR, with a consensus core sequence of ***AUUUA*** flanked by symmetric A or U (underlined). Triangles indicate alternative polyadenylation sites. Primers used for RT-PCR to detect reporter mRNAs are illustrated underneath. (B) HuD enhances luciferase reporter expression through the BDNF long 3'UTR but not the short 3'UTR. (C) and (D) The ARE in the BDNF long 3'UTR mediates HuD's effects to enhance reporter expression. CAD cells were cotransfected by 0.1 μg of reporter construct together with either pcDNA-HuD (HuD) or the pcDNA parental vector (PC). 20 ng of pRL-CMV Renilla luciferase construct was included in each transfection. 24 hours after transfection, cells were subjected to dual luciferase reporter assay (B and C) or mRNA extraction followed by DNAase-treatment and RT-qPCR (D). Due to the presence of endogenous BDNF mRNA, primers specific for the luciferase coding region were used to detect reporter mRNAs. (E) RT-PCR using multiple primers illustrated in (A) confirms expression of the expected RNA sequence in each reporter. (F) CLIP-RT-PCR detects

BDNF long 3'UTR reporter mRNA, but not short 3'UTR reporter mRNA, in immunoprecipitated HuD complex. Deletion of the ARE in the long 3'UTR reduced the association with HuD. Expression of the corresponding reporters in the input (inp) was shown on top panels. (G) Quantification of reporter mRNAs coimmunoprecipitated with HuD relevant to the corresponding mRNA levels in the input by RTqPCR. * indicates $P<0.05$ by Student's t-test (n=3).

Figure 2. Binding of HuD to the ARE in the BDNF-long 3'UTR increases the stability of the mRNA. (A) Specific binding of HuD to the ARE in BDNF-long 3'UTR was demonstrated by REMSA using recombinant proteins and radiolabeled RNA along with a 10-fold molar excess of cold ARE competitor. (B) In vitro decay assay of capped and poly-adenylated RNA containing the BDNF-ARE with left lane showing RNA molecular weight markers. Analysis of the rate of decay in three independent experiment revealed that the mRNA is stabilized in the presence of HuD. Half-life for GST-treated mRNA is 7.0 ± 0.90 min, and for GST-HuD-treated mRNA is 12.0±1.0 min

Figure 3. Overexpression of HuD increases the BDNF long 3'UTR mRNA in primary cultured neurons and *in vivo* (A) E17 cortical neurons were infected with HSV-HuD or HSVlacZ virus and the levels of long 3'UTR BDNF mRNA and pan BDNF mRNA were determined by qRT-PCR. (B) Fluorescent in situ hybridization of BDNF long 3'UTR mRNA in hippocampal sections derived from HuD-Tg and WT littermates. Left panels show representative images (bar= 75 μm) and the right panel displays the quantification of four pairs of HuD-Tg and WT controls. *$p<0.05$ and ** $p<0.01$ (Student's t-test, n=4).

Figure 4. The HuD transgene enhances BDNF protein levels in the hippocampal MF pathway. Representative images of BDNF immunofluorescence (green) in the hippocampal hilus (A and B), CA3 (C and D) and CA1 regions in HuD-Tg (B, D, and F) and WT littermates (A, C, and E). DAPI stained nuclei (blue) mark the aforementioned principle neuron layers. Asterisks indicate enhanced BDNF signals in the long processes of adult neural stem cells in the SGZ. Quantification of BDNF immunofluorescence in the hilus (G), CA3 strata lucidum and radiatum (H) and CA1 stratum radiatum (I) are graphically displayed. *$p<0.05$ (Student's t-test, n=3).

Figure 1

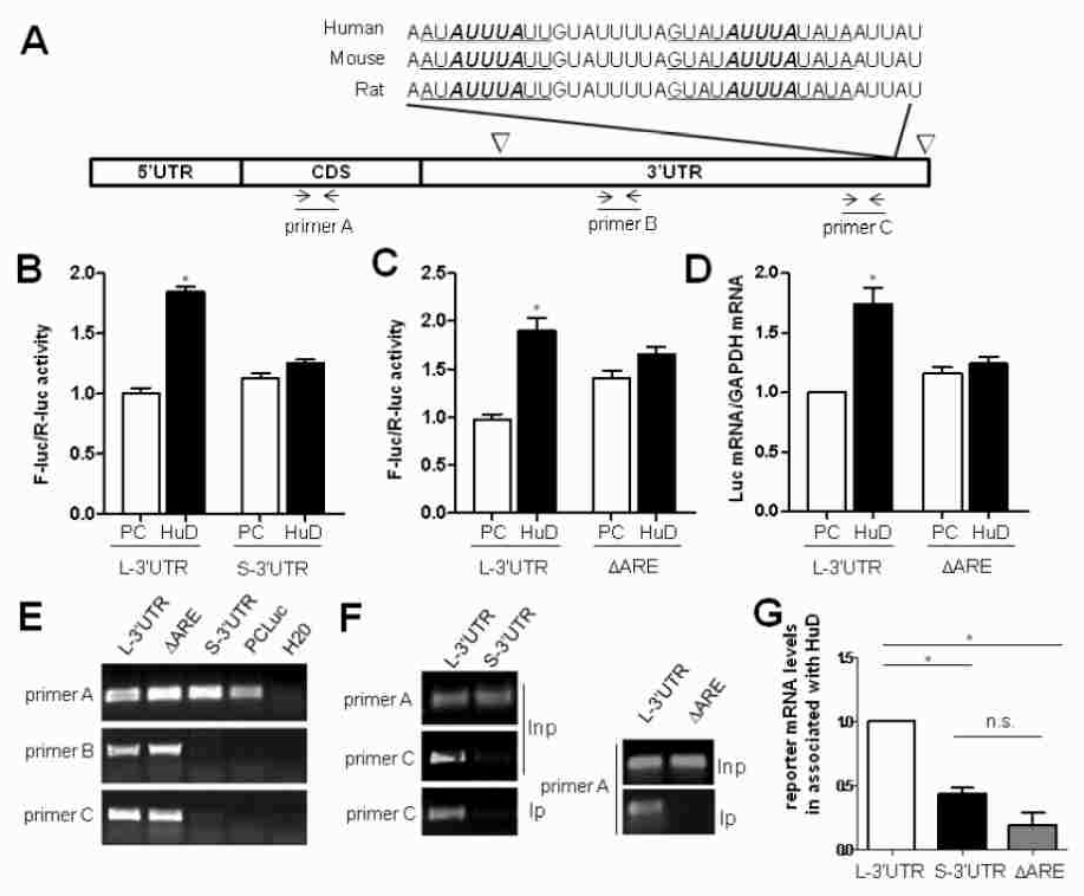


Figure 2

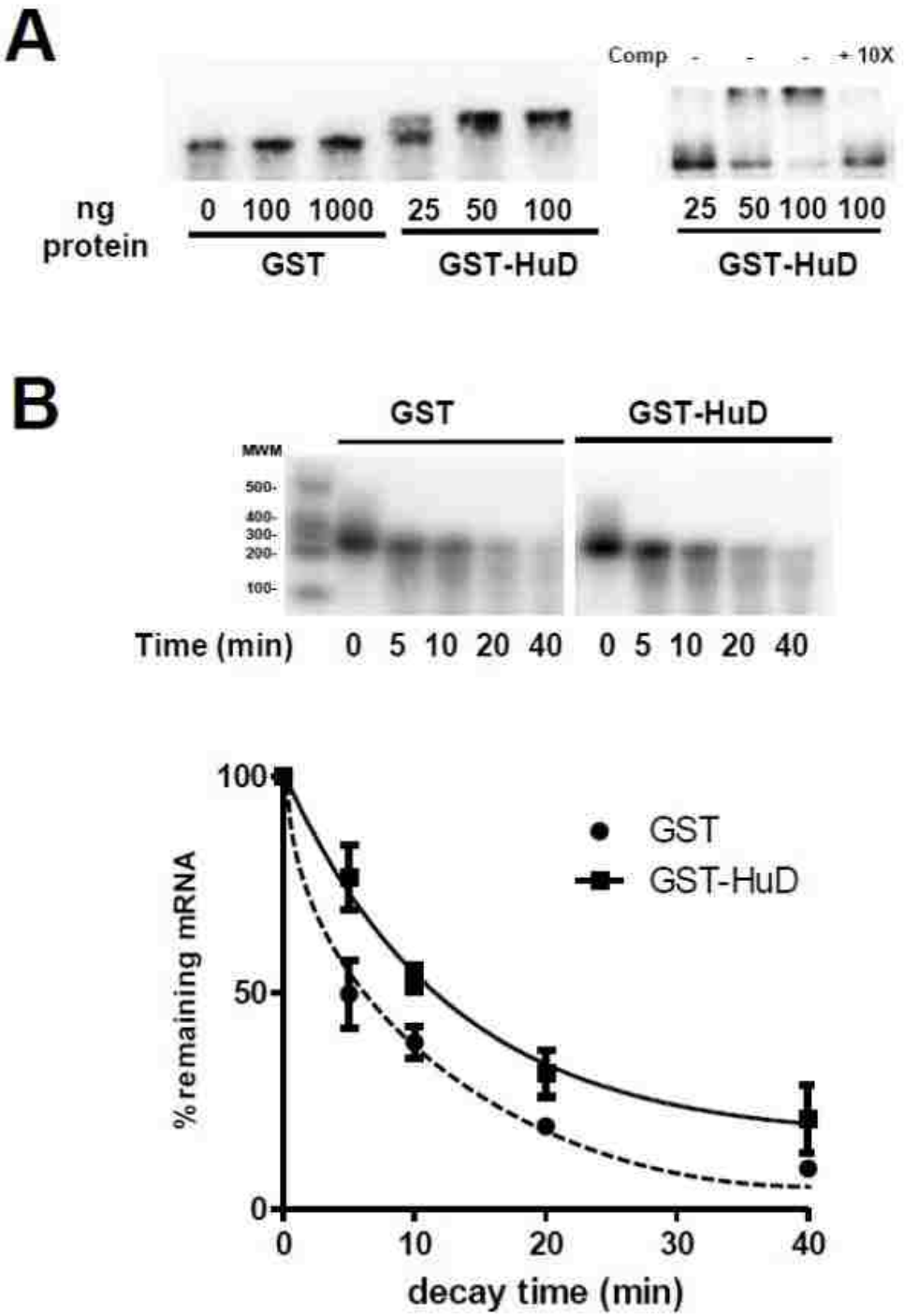


Figure 3

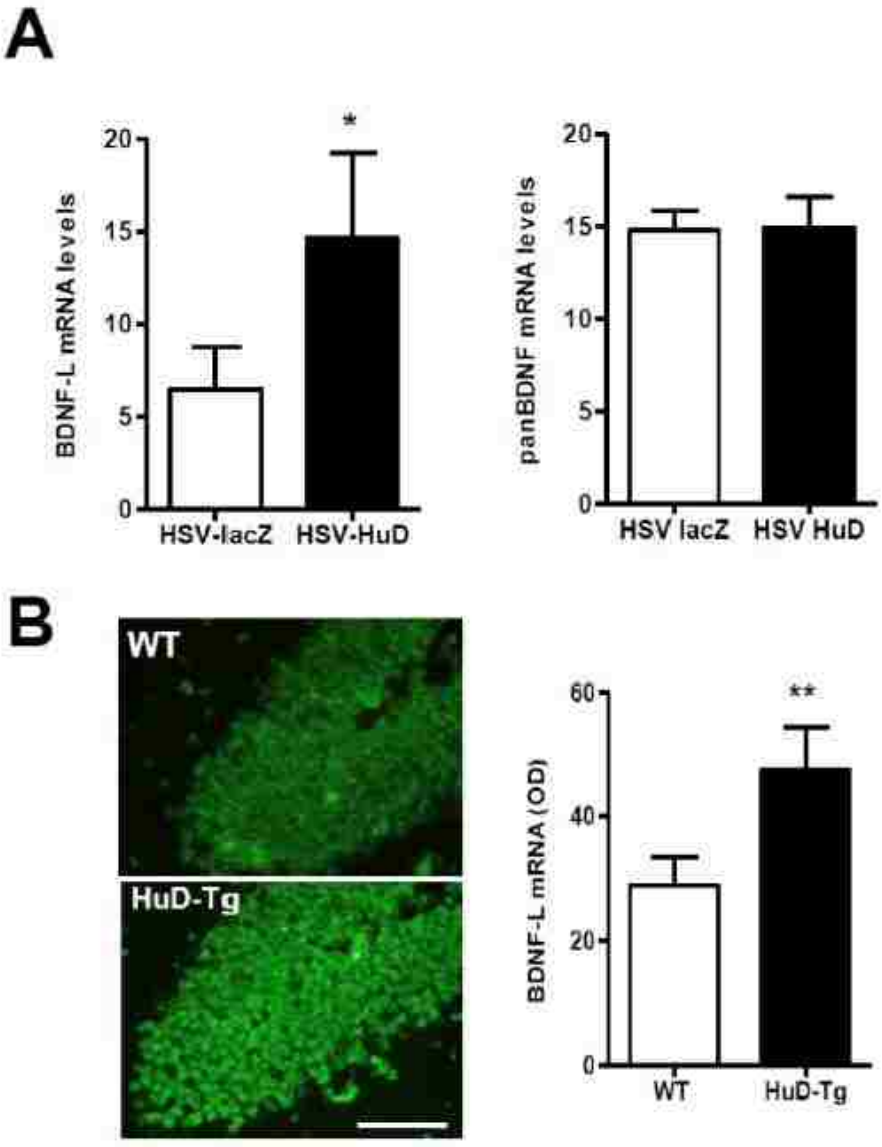
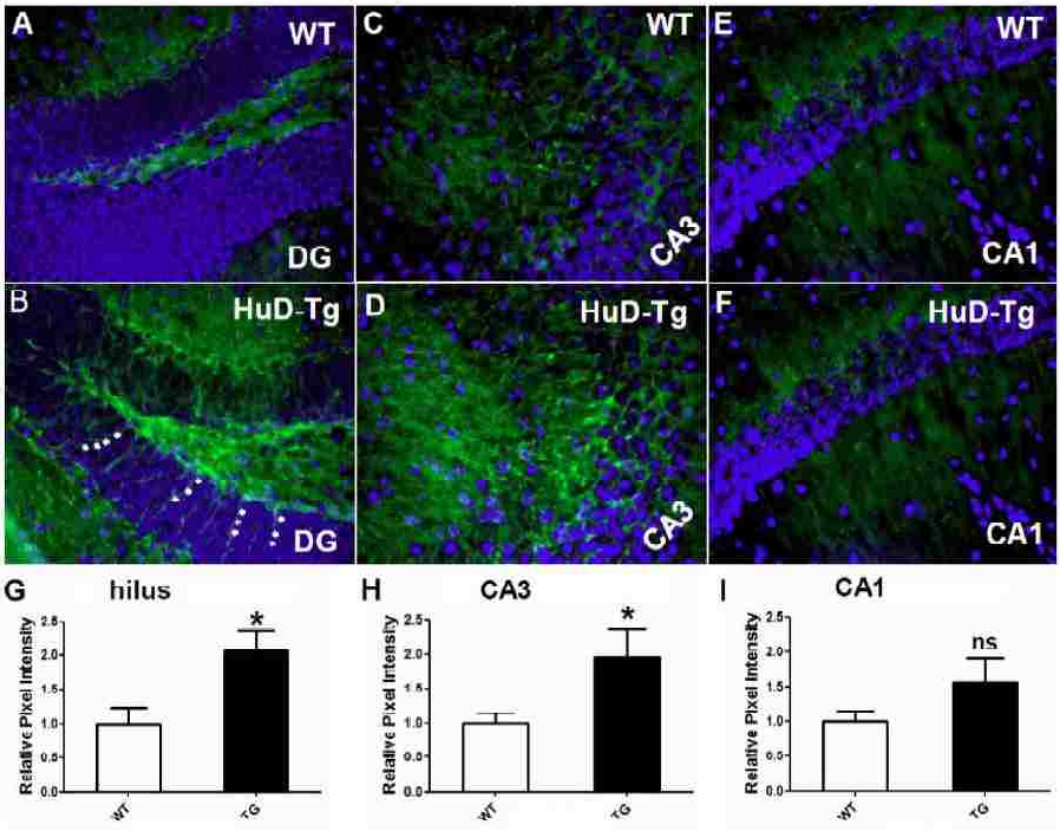


Figure 4



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Appendix C: Review article in press, Frontiers in Bioscience. Co-authored by Clark Bird

References for this article are self-contained within this appendix.

Role of HuD in neurological diseases

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TABLE OF CONTENTS

1. ABSTRACT

Hu proteins are a family of RNA-binding proteins (RBPs) that are homologs of *Drosophila* ELAV, a protein required for nervous system development. Three of these proteins (HuB, HuC, and HuD) are developmentally regulated and expressed in neurons. The fourth member, HuR is also expressed in other tissues. At the molecular level, Hu proteins are known to interact with AU-rich instability conferring sequences in the 3' UTR of specific target mRNAs, stabilizing the mRNAs. These proteins are not only the best known mRNA stabilizers but also the earliest markers of the neuronal cell lineage. Among the neuronal Hu proteins, HuD has been shown to accelerate neuronal differentiation and axonal outgrowth in neurons both in culture and *in vivo*. In addition, HuD and other Hu proteins participate in synaptic plasticity mechanisms in the mature central nervous system and promote regeneration of peripheral nerves. Furthermore, HuD has been implicated in pathological conditions from neurodegenerative disorders such as Parkinson's and Alzheimer's disease to childhood brain tumors. This review will focus on the involvement of HuD in nervous system function and pathology.

2. INTRODUCTION

Hu proteins are a family of RNA-binding proteins that were first detected as the targets of autoantibodies found in patients with paraneoplastic encephalomyelitis (1). These proteins are homologs of ELAV (embryonic lethal abnormal vision), a *Drosophila* RNA-binding protein identified because of the lethality shown by its deletion (2). Although there is only one ELAV protein in *Drosophila*, four mammalian ELAV-like Hu proteins have been identified [HuR, HuB (a.k.a.Hel-N1), HuC and HuD]. Three of these proteins (HuB, HuC, and HuD) are expressed in neurons while the fourth member, HuR, is ubiquitously-expressed in all tissues. At the molecular level, all four ELAV-like Hu

proteins contain three RNA recognition motifs (RRMs), a highly conserved 80 amino acid region that was first recognized in splicing factors and poly(A)-binding protein (3, 4). The RRMs are highly conserved among members of the family while the amino terminus and a basic domain between RRMs 2 and 3 are very diverse. RRMs 1 and 2 in Hu proteins bind AU-rich elements (ARE) found in the 3'UTRs of several unstable mRNAs involved in cell growth and differentiation (5, 6). In contrast, the third RRM is important for the interaction of the protein with long poly (A) tails (7, 8) (Figure 1). Recent studies indicate that Hu proteins are involved in various aspects of mRNA regulation, from mRNA processing and stability to translation (9-13).

3. Hu PROTEIN FUNCTION IN NEURONS

3.1. Role of Hu proteins in neuronal development

In *Drosophila*, deletion of the *elav* gene is embryonic lethal due to the failure of neurons to differentiate (2, 14). The continued expression of ELAV in adult neurons is essential for brain function, as temperature-sensitive mutants become incapacitated at non-permissive temperatures (15). In higher vertebrates and mammals, Hu proteins are one of the earliest markers expressed in neurons (16). HuR is the first protein to be expressed in chicken embryos where it is thought to be involved in cell proliferation (17). HuR is mainly nuclear and shuttles to the cytosol (18, 19) while HuD and other Hu proteins are localized to the cytoplasm (20-22). The expression of HuD coincides with the earliest stages of neuronal differentiation and is maintained through the maturation of neurons (17). Similar types of expression patterns have been observed in the developing mouse (23) and rat (24, 25) brains. The involvement of Hu proteins in different stages of

neuronal differentiation was confirmed by overexpression and knockout studies. Overexpression of HuB, HuC or HuD in PC12 cells and *in vivo* was shown to increase the rate of neuronal differentiation (22, 26-29). Down-regulation of these proteins in neural cell lines results in the opposite phenotype, with cells failing to grow neurites (29, 30). Neuronal Hu proteins also have a role in neural stem cell differentiation as seen by the phenotype of HuD KO mice, which show increased proliferation of stem cells in the subventricular zone but decreased production of mature neurons (31). Altogether, these findings support the notion that Hu proteins play a critical role in nervous system development.

3.2. Role of Hu proteins in synaptic plasticity

Although Hu proteins were initially described as early markers of development, in certain mature neurons significant levels of Hu proteins persist throughout life, particularly in the cortex and hippocampus. As shown in Bolognani *et al.*, 2004, (ref. 20) HuD is present in the soma and dendrites of pyramidal cells in the hippocampus and neocortex in close association with polysomes. In contrast this protein is not detected in mature dentate granule cells. The spatial pattern of expression of HuB is similar to that of HuD but distinct from HuC, which is normally expressed at high levels in dentate granule cells (23). The function of these proteins in mature neurons is not completely understood but several lines of evidence indicate that they are involved in synaptic plasticity mechanisms. First, HuD protein levels are increased in the hippocampus after different learning and memory tasks (20, 32, 33). Second, antisense-mediated knock down of HuC in the hippocampus impairs learning in the radial maze task in mice (33). Third, overexpression of HuD in transgenic mice leads to profound deficits in the

performance of two associative learning and memory tests, fear conditioning and the Morris water maze (34). Finally, recent analysis of the HuD targets in the mature brain (35) revealed that several of these mRNAs are associated with long-term potentiation, a phenomenon thought to underlie learning and memory (Figure 2).

3.3. Role of HuD in nerve regeneration

In addition to participating in synaptic changes in adult neurons in the central nervous system, HuD has been implicated in the response of peripheral neurons to injury. Following sciatic nerve crush, HuD protein and transcript levels increase in dorsal root ganglia sensory neurons within 7 days and remain elevated for up to three weeks (36). This increase in HuD expression is accompanied by a dramatic increase in GAP-43 mRNA levels, a known HuD target that encodes for a growth-associated protein involved in axonal outgrowth. In regenerating dorsal root ganglion neurons, HuD protein co-localizes with GAP-43 transcripts and ribosomal proteins in cytoplasmic granules (36), suggesting that HuD contributes to stabilize GAP-43 mRNA before translation. Another study also demonstrated the co-localization of HuD and GAP-43 mRNA in ribosome containing granules in axons and growth cones (37), the sites of localized GAP-43 protein synthesis (Moon, Twiss and Perrone-Bizzozero, unpublished results). The role of HuD in nerve regeneration was further investigated using a viral vector to express exogenous human HuD in rat superior cervical ganglion neurons following axotomy (38). HuD overexpression prevented the acute downregulation of acetylcholinesterase (AChE) and GAP-43 mRNAs, leading to faster response to the injury. Together, these findings demonstrate that in addition to its role in neuronal development and plasticity HuD is

important for the increased expression of growth-associated genes during nerve regeneration.

4. CONTROL OF HuD FUNCTION AND ITS TARGET mRNAs

4.1. Transcriptional, post-transcriptional and post-translational control

Analysis of the structure of the four genes encoding each of the Hu proteins demonstrated that these proteins not only have a high degree of sequence conservation but also that their genes have a similar genomic organization (39-41, for review see ref. 10). Although the transcriptional control of Hu protein expression has not been fully characterized, elements in the promoter regions of HuD, HuB and HuC were shown to control neuron-specific expression of these proteins (41-45). In addition, HuD transcription is known to be repressed by thyroid hormone (46), suggesting a potential dysregulation in both hypo- and hyperthyroidism. Hu proteins are also subjected to alternative splicing between RRMs 2 and 3. In the case of HuD, three alternatively-spliced isoforms have been identified: HuDpro (with exons 6 and 7 inclusion), HuD (with exon 6 inclusion) and HuDmex (excluding both exons 6 and 7). Among these isoforms, HuD is the most abundant protein in neurons followed by HuDpro. Interestingly, it was found that although Hu proteins block inclusion of specific exons in the mRNA encoding neurofibromatosis 1 (NF1) and calcitonin gene-related peptide (CGRP) (12), they promote inclusion of exon 6 in HuD (47), explaining the increased abundance of this isoform in neurons.

Another post-transcriptional mechanism involves the regulation of Hu protein levels by microRNAs. The effects of mir-519 and mir-125a on HuR levels have been

well established (for further details see Subramanya and Gorospe, this series). Likewise, a recent study demonstrated that HuD levels are also controlled by microRNAs. As shown by Abdelmohsen *et al*, 2010 (48), miR-375 represses HuD expression by binding to a specific and highly conserved site on the 3' UTR, decreasing both neurite outgrowth in cultures of developing neurons and dendritic density in hippocampal neurons *in vivo.*

In addition to these post-transcriptional mechanisms, HuD, like HuR, is known to be post-translationally regulated by arginine methylation (49, 50) and phosphorylation (51). Protein kinase C (PKC)-dependent phosphorylation of HuD has been shown contribute to the stabilization of the GAP-43 (29, 51, 52) and NOVA1 mRNAs (53). HuD is also methylated at an arginine residue in the hinge region by the coactivator associated arginine methyltransferase (CARM1). The physiological role of CARM1 in the regulation of HuD activity was first described in PC12 cells induced to exit the cell cycle and differentiate in the presence of nerve growth factor (49). This treatment decreased CARM1 expression and lead to a concomitant increase in the binding of HuD to the $p21^{cip1/waf1}$ mRNA (49). A similar reduction in CARM1 protein levels was recently reported in motor neuron cells induced to differentiate with retinoid and neurotrophic factors, which also resulted in the increased interaction of HuD and $p21^{cip1/waf1}$ mRNA (54).

4.2. Mechanism of HuD-mediated mRNA stabilization of neuronal mRNAs

A detailed analysis of the mechanism by which HuD stabilizes GAP-43 mRNA (55) revealed the following requirements for this function. To be stabilized by HuD, mRNAs need to be: a) capped b) polyadenylated with long poly(A) tails, and c) contain

an intact U-rich HuD binding motif in the 3' UTR. Also, HuD was found to decrease the rate deadenylation of GAP-43 mRNA, which is the first step in the reaction followed by a rapid and processive decay of the body of the mRNA (55). Analysis of HuD's protein structure indicated that all three RRMs are required for this function as a truncated protein including only RRMs 1 and 2 was not effective in stabilizing the mRNA or inducing neurite outgrowth (27).

Besides GAP-43 a number of neuronal mRNAs are known to be stabilized by HuD including those encding c-fos, the microtubule-associated protein tau, neuroserpin, $p21^{cip1/waf1}$, N-myc and c-myc, VEGF, MARCKS, NOVA1, Musashi 1 and AChE (53, 56-63). Of these targets, a few were confirmed by cell culture studies, including GAP-43 neuroserpin, tau, NOVA1, AChE and N-myc (28, 29, 53, 57, 58, 63, 64) and two, GAP-43 and AChE were confirmed *in vivo*, in the brains of HuD overexpressor mice (21, 38).

4.3. HuD targets in neurological disorders

In addition to the mRNAs listed above, it is likely that HuD binds and stabilizes additional neuronal mRNAs containing instability-conferring sequences in their 3' UTRs. Using RNA immunoprecipitation and microarrays, we have recently identified about 600 mRNAs that bind HuD in the mouse brain (35) and thus, constitute new targets of this RBP. The majority of HuD-target interactions occur via the specific binding of HuD to three novel recognition motifs, which are mostly U-rich and localized to the 3' UTR (35). In agreement with the role of HuD in neural development and synaptic plasticity, a number of its targets are significantly enriched in the following nervous system functions:

axon guidance and neurite outgrowth, long-term potentiation, cell cycle progression and neuronal differentiation (Figure 2A and ref. 35). Interestingly, HuD targets are also associated with apoptosis of dopaminergic neurons, which die preferentially in Parkinson's disease (PD), and neuronal cell death in general, suggesting the potential involvement of this RBP in neurodegenerative disorders. As shown in Figure 2B and Table 1, HuD target mRNAs are associated with a number of neurological disorders including neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease and PD, mood disorders, epilepsy, schizophrenia and mental retardation conditions such as Rett syndrome. As discussed below, there is significant evidence showing the association of polymorphisms in the HuD (*ELAVL4*) gene with PD and the correlation of *ELAVL4* gene deletions with neuroblastoma malignancy.

5. ASSOCIATION OF HuD WITH NEUROPSYCHIATRIC DISORDERS

5.1. HuD and the genetics of Parkinson's disease

Research examining the genetic factors affecting the age-at-onset (AAO) of PD identified a genetic locus on chromosome 1p that seemed to be modulating how early PD affected patients (65, 66). The *ELAVL4* gene is contained within the chromosome 1p linkage region termed PARK10, leading researchers to investigate single-nucleotide polymorphisms (SNPs) within this gene that could be contributing to the AAO of PD. Of the nine single nucleotide polymorphisms (SNPs) within the *ELAVL4* gene initially genotyped in a US study (67), two, rs967582 and rs2494876, were significantly associated with AAO. While rs967582 is located in intron 2, rs2494876 is a non-synonymous SNP located in the most 3' exon (exon 8) of the HuD gene (67). Two

additional studies, one in an Irish case-control cohort (68) and another one using an international sample of familial PD cases (69) replicated the association of rs967582 with the AAO of PD.

The non-synonymous SNP rs2494876 results in a substitution of a proline for a serine at amino acid 270, which is located in the hinge region between RRMs 2 and 3. This change could have a serious impact in the protein's secondary structure, which in turn could alter the binding of HuD to its target mRNAs and contribute to AAO of PD. The contribution of rs967582 is a little more tenuous, as this intronic SNP would not be affecting the coding sequence of HuD. Nevertheless, it is conceivable that rs967582 is linked to a different, yet unidentified, polymorphism in the *ELAVL4* gene that contributes to AAO.

While the precise nature of HuD in AAO of PD is presently unclear, it is enticing to propose that this may be related to its interaction with the mRNAs for □-synuclein (SNCA) or tau (MAPT), two targets of this RBP (Table 1) that have been implicated in the genetics of PD (70).

5.2. HuD and Alzheimer's disease

HuD stabilization deficits are implicated in the development and progression of AD specifically through a lack of stabilization of the α-secretase ADAM10 (71). A hallmark of the AD is the presence of senile plaques that contain aggregated β-amyloid, which is a product of successive cleavage of the amyloid precursor protein (APP) by □ and γ secretases (72). Cleavage of APP by ADAM10 produces soluble APP, which is non-pathogenic (71, 72).

ADAM10 and HuD protein levels are reduced in AD, suggesting that HuD could be regulating ADAM10 protein levels through post-transcriptional regulation (73, 74). Computational analysis of ADAM10 mRNA sequence demonstrated the presence of an ARE, which also contains the putative HuD binding site. Immunoprecipitation of mRNPs containing HuD demonstrated an enrichment of ADAM10 mRNA in these complexes, further implicating HuD in the regulation of ADAM10 gene expression (73).

Binding of HuD to target mRNAs is regulated in part by PKCα isoenzyme signaling (51), which may play a role in HuD regulation of ADAM10 levels. PKC isoenzyme levels are decreased in the post-mortem brain of AD patients, leading to the interesting possibility that a deficit of PKC signaling could be impairing the binding of HuD to its mRNA targets, including ADAM10 (71). As shown in Table 1, in addition to ADAM10, a number of AD associated protein mRNAs are HuD targets. Among these is the mRNA for the β-secretase BACE1, which is involved in the production of β-amyloid and a new target for the treatment of AD (72, 75). However HuD's effect on the levels of this mRNA is yet to be established.

5.3. HuD and schizophrenia

A DNA microarray study of mRNAs expressed in the prefrontal cortex of patients with schizophrenia (76) revealed that HuD mRNA levels are increased in this disorder. Clustering analyses of the data showed that not only was HuD increased in patients but also and, most-importantly, that GAP-43 was tightly co-regulated with this protein and so were other HuD targets such as neuroserpin and MARCKS. Considering that these mRNAs are also developmentally-regulated and that the expression of at least one of

them, GAP-43, is increased in these patients (77), it is enticing to propose that the observed changes in these mRNAs could be due to their HuD-induced stabilization.

5.4. HuD levels in epilepsy and drug abuse

As shown in Table 1 and Figure 2B, a number of HuD targets are associated with epilepsy. Supporting a role of HuD in this disorder HuD mRNA levels were shown to increase in rat hippocampal dentate granule cells 24 hours following kainic acid induced seizures (78) and similar findings were reported in CA1 and CA3 region of the hippocampus after pilocarpine induced seizures (79). Furthermore, HuD expression is also affected by exposure to drugs of abuse such as cocaine, as shown by the increases in the levels of this mRNA either in the whole brain (79) or in the nucleus accumbens (Perrone-Bizzozero and Neisewander, unpublished observations) 24 hours after rats received a single sensitizing injection of cocaine.

5.5. HuD and spinal muscular ataxia

Spinal muscular ataxia (SMA) is an autosomal recessive neuromuscular disease characterized by the selective degeneration of lower motor neurons of the spinal cord. SMA is caused by deletions or loss-of-function mutations in the Survival of Motor Neuron (*SMN*) gene. SMN is a protein connecting neuronal splicing and axonal transport and a recent study demonstrated that HuD co-localizes with this protein in axons (54). Furthermore, SMN was shown to recruit HuD and its target mRNAs into RNA granules, a process that depends on the presence of the Tudor domain in SMN. Finally, it was shown that HuD overexpression could compensate for the differentiation defects

observed in SMN haploinsufficient motor neurons, suggesting that increasing HuD levels in these cells could lead to better treatments for SMA (54).

6. ASSOCIATION OF HuD WITH NEUROBLASTOMAS

6.1. HuD levels in different neuroblastoma subtypes

Neuroblastomas (NB) arise from embryonic neural crest and primarily affect young children. The proto-oncogene encoding N-myc (*MYCN*) is found to be amplified as well as overexpressed in a number of these cancers (64). A number of NB-derived cell lines have been used to understand the mechanisms of carcinogenesis. Phenotypically these lines are diverse but can be classified into two main subtypes: neuroblastic N-type cells, which are non-adherent and very tumorigenic and substrate adherent S-type cells, which are not tumorigenic. N-myc is believed to maintain the proliferative, undifferentiated state of cells during development (80). HuD expression levels are high in N-type but absence in S type cells, creating the possibility that HuD may stabilize N-myc transcripts and push the cells towards a cancerous fate (81).

In vitro experiments showed that HuD binds to elements in the 3' UTR of N-myc mRNA (81). Ectopic HuD expression in stable cell lines leads to the stabilization of a reporter gene expressing the N-myc 3'UTR (64). Conversely, treatment of cells with anti-sense oligomers against HuD lead to a decrease in N-myc reporter levels, implicating HuD as the main factor regulating the stability of this mRNA (64). In addition to stabilization of the mature N-myc transcripts, HuD-induced pre-mRNA processing and stability has been reported in these cells (82).

6.2. ELAVL4 haploinsufficiency and NB malignancy

The posttranscriptional regulation of N-myc transcripts is not the only mechanism by which HuD is involved in neuroblastoma progression. About 30% of neuroblastomas with a clinically poor prognosis contain an amplification of the *MYCN* gene and a deletion in the small arm of chromosome 1 where the *ELAVL4* gene is located (83, 84). Moreover, higher HuD expression levels in neuroblastoma cells were correlated with a better clinical outcome in patients (85). Supporting a role of HuD in decreasing malignancy, overexpression of HuD in two neuroblastoma cell lines with high *MYCN* amplification was shown to decrease both cell proliferation and *MYCN* gene copy numbers (86). In contrast, knockdown of HuD levels in non-amplified SY5Y cells had the opposite effect, causing decreased HuD levels and selecting for cells with multiple copies of the *MYCN* gene (86). Although the precise mechanism of *MYCN* amplification remains to be established, one possible explanation for these findings is that cells with HuD haploinsufficiency, which normally would have lower N-myc levels, will not survive in culture unless they contain multiple *MYCN* copies, which will give them a growth advantage over non-amplified cells (86).

7. PERSPECTIVE

Altogether the data presented above supports the idea that HuD and other neuronal Hu proteins play a critical role in the post-transcriptional control of gene expression during nervous system development and plasticity. As shown in Figure 2A, HuD targets are involved in different aspects in the life of a neuron from cell cycle progression and neuronal differentiation to proper maturation and cell death. Therefore, it

is not surprising that many of these same target mRNAs are associated with various neuropsychiatric disorders, from mental retardation and schizophrenia to neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Table 1 and Figure 2B). The role of HuD in Parkinson's disease is also supported by genetic linkage studies demonstrating a significant association of two polymorphisms in the *ELAVL4* gene with AAO of PD. The recent findings that deletions in the *ELAVL4* gene are associated with increased malignancy in neuroblastoma cell lines not only highlight the function of this RBP in cell cycle arrest but also suggest that gene therapy directed at increasing HuD levels in neuroblastoma cells could lead to more effective treatments of those NB patients with the worst prognosis. Likewise, it is possible that that HuD overexpression could help rescue part of the motor neuron death phenotype in patients with SMA. Finally, the changes in HuD expression in response to epileptic seizures and cocaine exposure suggest a role of HuD in these disorders. From a biomedical perspective, the elucidation of the mechanisms controlling how HuD regulates the stability and translation of its target mRNAs and how these RNA-protein interactions respond to environmental cues has potential implications for the understanding a broad range of conditions from normal development and synaptic plasticity to neurodevelopmental disorders and neurodegenerative diseases.

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FIGURES

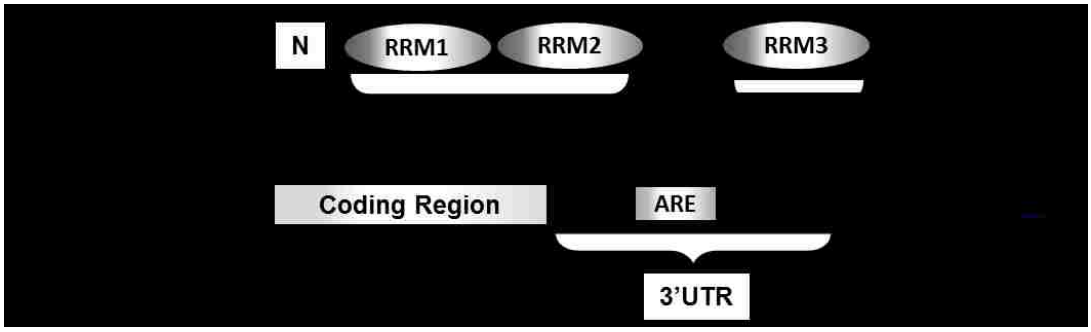

Figure 1. Diagram of the interaction of the RNA-binding protein HuD with its target mRNAs The RNA-Recognition motifs (RRMs) 1 and 2 in HuD are known to interact with AU-rich elements (AREs) in the 3' UTR of targets mRNAs while RRM3 is known to bind long poly(A) tails. See text for details.

Figure 2. Association of HuD targets with nervous system function and neurological diseases. The list of HuD targets identified by Bolognani et al, 2010 (ref. 35) was uploaded onto Ingenuity Pathway Analysis (IPA). Panel A. Top functional nervous system categories significantly enriched with HuD targets. B. Neurological and psychiatric disease categories with significant HuD target enrichment.

Table 1. List of HuD targets associated with neuropsychiatric disorders

Alzheimer's and Parkinson's disease	ACTB, ADAM10, AKAP5, APBB2, ARAP2, BACE1, BCL2L11, BECN1, CANX, CHRNA7, CNTNAP2, CRLS1, FGF12, GABRB2, GALNT13, GM2A, GNG4, GRM5, GSK3B, HIF1A, MAGI2, MAPK8, MAPT, MSI2, NALCN, NKAIN2, NPAS3, OPCML, PAK3, PLCB1, PRDX1, PRKCE, PTPRD, RAB14, RAB6A, REEP1, RIMS1, RPS6KB1, SCN2B, SERPINE2, SET, SLC1A1, SNCA, SCN2B SOD2, SPAST, STK24, STXBP6, VCL, WASF1, WDR37, XIAP, YWHAZ
Huntington's disease	ACAT1, ACTB, AHCYL1, ARPP19, ARPP21, ATP2A2, B2M, CAMKK2, CDH2, ELAVL2, ESRRG, FBXW7, FGF12, FOXG1, FOXN3, FOXP1, GABRB2, GJA1, MBNL2, NAP1L5, OSBPL8, OXR1, PCDH7, PDCL, PDP1, PLCB1, PPARGC1A, PPP1CB, PPP3CA, RAB6A, RERE, SCARB2, SCN2B, SLC1A1, TBR1, TPM3, TRAM1, XIAP, YWHAZ, ZNF706
Schizophrenia	ATF2, CALR, CDKN1B, CHRNA7, CLINT1, CNTNAP2, CUX2, ELAVL4, FZD3, GABBR1, GABRB2, GRM5, GSK3B, KIF2A, LYRM5, MAGI2, MARCKS, NCAM1, NPAS3, NPTN, PFN2, PIK3R1, PPP3CB, RIT2, SCN2B, SLC1A1, SOD2, SSTR4
Mood disorder	ATP2C1, AUTS2, B2M, CDH2, CELF2, CHRNA7, CNTNAP2, CUL3, CUX2, DIP2C, ESRRG, FAM107B, FAT1, FBXO9, FGF12, FOXN3, FRY, G3BP2, GABBR1, GABRB2, GMPS, GNAZ, GSK3B, HNRNPC, MARCKS, MBNL2, MSI2, NALCN, NCAM1, NKAIN2, NPAS3, PAN3, PDP1, PPP3CA, PRDX1, PRKCE, PRKCI, RBMS3, RERE, RGS17, RIT2, RNF111, SATB2, SCN2B, SLC1A1, SNCA, SOD2, SSR1, TBR1, TCF4, TGOLN2,TLE4, VAMP7, VKORC1L1, WASF1, ZBTB43, ZEB2
Bipolar disorder	ATP2C1, AUTS2, CDH2, CELF2, CHRNA7, CNTNAP2, CUL3, CUX2, DIP2C, ESRRG, FAM107B, FAT1, FBXO9, FGF12, FOXN3, FRY, G3BP2, GABRB2, GMPS, GNAZ, GSK3B, HNRNPC, MARCKS, MBNL2, MSI2, NALCN, NCAM1, NKAIN2, NPAS3, PAN3, PDP1, PPP3CA, PRDX1, PRKCE, RBMS3, RERE, RGS17, RIT2, RNF111, SATB2, SCN2B, SLC1A1, SNCA, TBR1, TCF4, TGOLN2, TLE4, VAMP7, VKORC1L1, WASF1, ZEB2
Epilepsy	FKBP1A GABBR1, GABRB2, KCNC2, MAPK10, PLCB1, SERPINE2, SLC1A1
Mental retardation	ATRX, AUTS2, CREBBP, CTNND2, CUL4B, PAK3
Rett syndrome	ARF6, AUTS2 CALR, DNAJB6, MREG, PKIG, SERPINE2, TCF4, YWHAE

Table 1. List of HuD targets associated with neuropsychiatric disorders

Table shows HuD target mRNAs encoding proteins linked to neurological and psychiatric diseases. HuD targets were analyzed using IPA software to identify disease categories with significant enrichment of HuD targets and the target mRNAs associated with each disorder.