RNA Recognition and Regulation of the AU-rich RNA Binding Proteins: HuR, TTP and BRF1

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

Posttranscriptional gene expression is controlled and coordinated by RNA binding proteins (RBPs), many of which recognize specific RNAs through cis-regulatory RNA elements. One of the most highly studied classes of cis-regulatory RNA elements is the AU-rich elements (AREs). AREs are bound by a class of RBPs called ARE binding proteins (ARE-BPs), of which there are over a dozen in humans including HuR, tristetraprolin (TTP) and butyrate response factors 1 and 2 (BRF1 and BRF2). TTP, BRF1 and BRF2 belong to a family of tandem C3H zinc finger proteins that destabilize ARE-containing mRNAs. HuR acts to enhance the stability and translation of ARE-containing mRNAs, a function that is rare among ARE-BPs. While each of these ARE-BPs regulates the expression of ARE-containing mRNAs, some ARE-BPs themselves are also encoded by ARE-containing mRNAs, raising the possibility that each of these ARE-BPs may regulate one another's expression. In order to determine how these ARE-BPs influence each others expression and how this affects the regulation of global gene expression programs we have focused on three different aspects of these ARE-BP networks: control, response to stimuli, and global effects.

To address of network control of ARE-BPs we have focused on how HuR regulates a network of mRNAs including TTP, BRF1 and HuR's own mRNA. We demonstrate that HuR can bind to TTP's, BRF1's and its own mRNA. Furthermore, by

employing overexpression and siRNA knockdown approaches we demonstrate that these mRNAs and their corresponding 3'UTR luciferase reporters are resilient to fluctuations in HuR levels and that the degree of this resiliency is cell type and condition specific.

To address the temporal responses within an ARE-BP network we focused on how each of the members of the TTP family of ARE-BPs reacts following the induction of the other family members by using epidermal growth factor (EGF) stimulation. Here we show that induction of TTP family member mRNAs during EGF stimulation is partially attributable to changes in mRNA stability. Furthermore, we also show that TTP and BRF1 are able to bind each of the TTP family member mRNAs and subsequently affect their expression by altering their mRNA degradation rates. In addition, we demonstrate that the unique temporal induction patterns of the TTP family member RBPs is correlated with the EGF stimulated induction of TTP-bound mRNAs, suggesting that a network comprised of TTP family members is able to influence the timing of complex gene expression patterns.

Finally, to address the influence of these networks on regulation of global gene expression programs we have focused on how HuR recognizes AREs and whether it can globally recognize multiple classes of ARE-containing mRNAs, including the canonical class of AREs recognized by the TTP family members. To investigate how the three RNA recognition motifs (RRMs) of HuR contribute to ARE recognition we generated a series of RRM point mutants and test their ability to disrupt RNA recognition of each of the RRMs. To identify different classes of ARE-containing mRNAs we examined these mutants with a global RNA binding site detection method called photoactivatable ribonucleoside crosslinking immunoprecipitation (PAR-CLIP). Together these techniques suggest that the RRMs of HuR cooperate to recognize mRNA targets and that HuR's ability to bind RNA is coupled to the cellular distribution of HuR, and thus, are important in its role for regulating expression of bound mRNAs.

Together these studies indicate that ARE-BP posttranscriptional networks are highly interconnected and display complex regulatory interactions depending on cell type and stimuli. Furthermore, these networks can create complex behaviors such as timing of expression events or resiliency to fluctuations in protein levels. Finally, the components of these ARE-BP networks target partially overlapping sets of mRNAs to impact global gene expression patterns that ultimately coordinate the cellular responses to external stimuli.

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List of Abbreviations

3′UTR- 3′ Untranslated Region

4SU- 4-Thiouridine

Act D- Actinomycin D

AMPK- AMP-activated Protein Kinase

ARE- AU-rich Element

ARE-BP- AU-rich Element RNA Binding Protein

BRF1- Butyrate Response Factor 1

BRF2- Butyrate Response Factor 2

CDS- Coding Sequence

EGF- Epidermal Growth Factor

eIF4E- Eukaryotic Translation Initiation Factor 4E

EJC- Exon Junction Complex

ELAV- Embryonic Lethal Abnormal Vision

EMSA- Electrophoretic Mobility Shift Assay

GAPDH- Glyceraldehyde 3-Phosphate Dehydrogenase

HelN-1- Human ELAV Like Neuronal 1

IP- Immunoprecipitation

LPS- Lipopolysaccharide

PABP- Poly(A) Binding Protein

PAR-CLIP- Photoactivatable Ribonucleoside Crosslinking Immunoprecipitation

RBP- RNA Binding Protein

RIP-Chip- RNA Binding Protein Immunoprecipitation followed by Microarray

RRM- RNA Recognition Sequence

SELEX- Systematic Evolution of Ligands by Exponential Enrichment

SLBP- Stem Loop Binding Protein

SPR- Surface Plasmon Resonance

TTP- Tristetraprolin

TZF- Tandem Zinc Finger

VEGF- Vascular Endothelial Growth Factor

ZFP36- Zinc Finger Protein 36

1. Introduction

1.1 Regulation of Posttranscriptional Gene Expression in Eukaryotes

Jacob and Monod, who initially described the Operon Model and the concept of gene regulation in their highly influential 1961 paper, also addressed the nature of "genetic" or non-coding regulatory elements by proposing the concept of the promoter in 1964 (Jacob et al.,, 1964). In that paper they concluded that regulation of gene expression by non-coding regulatory elements may act on the level of DNA rather than RNA by suggesting that the possibility of RNA elements "raises numerous difficulties," without elaborating on what those difficulties might be. Of course, today, as proposed in the paper by Jacob and Monod, promoters are defined solely as DNA elements. But it is now widely accepted that in addition to DNA promoters, gene expression can also be regulated at the level of RNA, in spite of the "difficulties" that are, indeed, complex. Considering what we now know about RNA regulation of gene expression it is tempting to speculate that the "numerous difficulties" that were alluded to by Jacob and Monod were about "complexity."

For example, the regulation of eukaryotic gene expression at the level of RNA, or posttranscriptional gene expression, may seem complex as it begins shortly after transcription initiation while the RNA polymerase is still transcribing DNA into RNA and continues ultimately until degradation of the RNA. In between transcription and decay most mammalian mRNAs progress through the following steps: 5′ end capping, splicing, 3′ end processing, polyadenylation, nuclear export, quality control, localization, and translation (Moore, 2005). Some RNAs even undergo specialized processing or regulatory events such as RNA editing. Many of the earlier steps of gene regulation are connected to, or influence later steps, such as how deposition of the Exon-Junction Complex (EJC) following splicing enhances export and translation (Gatfield et al.,, 2001; Nott et al.,, 2004). Some regulatory events even reverse or mix the order of progression such as when translationally competent mRNAs are re-localized under stress conditions to sites called Stress Granules, where they are stored in a translationally arrested state (Anderson and Kedersha, 2009). mRNAs are escorted and controlled through these regulatory events by RNA Binding Proteins (RBPs), one of the largest and most conserved classes of proteins in the mammalian proteome (Keene, 2007).

1.2 Tandem C3H Zinc Finger Proteins

RBPs are frequently grouped and classified with other RBPs of similar sequence and function into families. Some RBP families are very large and contain hundreds of RBPs others are quite small and consist of only of few. One of the more interesting of the smaller RBP families is the tristetraprolin (TTP) family of RBPs. This family of RBPs is characterized by the highly conserved tandem CCCH zinc finger (TZF) domain which contsists of two motifs, each containing three cystidine residues and one histidine that are separated by tightly restricted distances to facilitate the coordination of a zinc ion. The TZF domains of TTP family RBPs recognize a class of RNA sequences called AU-rich elements (AREs) with a strict specificity for AREs that contain the AUUUA pentamer sequence (Lai et al.,, 2005). TTP family members promote the deadenylation and degradation of ARE containing mRNAs which they recognize through highly specific interactions with the TZF domain (Lai et al.,, 2000). The TTP family of proteins are highly conserved and are found in most species from yeast to human with nearly all species containing multiple family members (Thompson et al.,, 1996; De et al.,, 1999; reviewed in the following: Blackshear 2002; Carrick et al., 2004; Vergara and Thiele 2008; Stumpo et al., 2010).

In mammals, there are three family members, TTP, ZFP36L1 and ZFP36L2 with a fourth, ZFP36L3, that is specific to rodents. The family members contain nearly identical TZF domains and thus not surprisingly recognize similar mRNA sequences. The family members also contain overlapping functional characteristics in promoting mRNA instability and perform nearly identically in *in vitro* decay assays (Lai et al., 2000). However, the TTP family of RBPs exhibit partially-overlapping but distinct cell type, tissue-specific, and stimuli-induced expression patterns (Stumpo et al., 2010). Both TTP and ZFP36L1 were initially identified in different screens for mRNAs that were rapidly and transiently induced by insulin, serum or other mitogens (Lai et al., 1990; Varnum et al., 1989; Gomperts et al., 1990). Furthermore, the TTP family members are all phosphoproteins that can rapidly relocate from the nucleus to the cytoplasm or to cytoplasmic granules called p-bodies in response to many of the same stimuli that induce their expression, thus suggesting that the regulation of these proteins are tightly controlled and coordinated at many levels (Taylor, Thompson et al., 1996; Stoecklin et al., 2004; Vergara et al., 2011).

TTP family RBPs promote mRNA degradation by directly recruiting and activating multiple components of the decay machinery including deadenylases (CCR4-NOT complex), decapping enzymes (DCP1, DCP2 and Rck/p54), components of the exosome (PM-SCL75 and RRP4), and the 5′ → 3′ exonuclease, XRN1 (Chen et al., 2001; Lykke-Andersen and Wagner, 2005; Fenger-Gron et al., 2005; Hau et al., 2007; Sandler et al., 2011). The deadenylase, PARN, has also been shown to enhance TTP mediated deadenylation suggesting that, in addition to recruiting the CCR4-NOT complex, it can interact with multiple deadenylation pathways (Lai et al., 2003). It has also been shown that the TTP family members can co-localize with many of these components in stressed cells to cytoplasmic sites of RNA triage called p-bodies or sites of RNA storage called stress granules (Stoecklin et al., 2004; Murata et al., 2005; Kedersha et al., 2005; reviewed in Anderson and Kedersha, 2009).

While the TTP family members biochemically and mechanistically behave similarly they have different but overlapping physiological roles. TTP (also known as ZFP36, TIS11, Nup 475 and GOS 24) deficient mice are normal at birth but develop numerous immunological complications including severe arthritis, cachexia, myeloid hyperplasia, dermatitis and high titers of anti-nuclear autoantibodies (Taylor et al., 1996; Kaplan et al., 2011). Neutralization of TNFalpha with antibody treatment or breeding with TNF receptor knockout mice relieve most of these symptoms indicating that excessive TNFalpha production causes many of the complications in the TTP knockout mice (Carballo et al., 1998; Carballo et al., 2001). In support of the mouse phenotypes (notably, arthritis and autoimmune disfunction) it has also been shown that a polymorphism in the coding region of TTP is significantly associated with rheumatoid arthritis, a systemic autoimmune disease, in African-Americans (Carrick et al., 2006). ZFP36L1 (also known as BRF1, TIS11b, ERF1, Berg36, and cMG1) knockout mice are embryonic lethal with trophoblast layer defects in the placenta due to intra- and extraembryonic vascular defects potentially due in-part to elevated production of VEGF-A (Stumpo et al., 2004; Bell et al., 2006). ZFP36L2 (also known as BRF2, TIS11d, and ERF2) null mice died shortly after birth due to severe hematopoetic defects resulting from markedly reduced numbers of hematopoetic progenitor cells in fetal liver and embryonic yolk sacs (Stumpo et al., 2009). Conditional double knockout of ZFP36L1 and ZFP36L2 in lymphocytes (CD2-Cre) caused T-cell acute lymphoblastic leukemia (T-ALL) which was preceded by disturbed thymic development due to aberrant passage of thymocytes through the beta-selection checkpoint. Corresponding with these effects the levels of Notch1, an ARE-containing oncogene, were elevated in the double knockout thymocytes providing a possible mechanism for the observed carcinogenesis (Hodson et al., 2010).

In summary, mammalian TTP family RBPs share many characterictics including: the family-defining domain structure, RNA recognition specificities, transiently inducible gene expression, localization to similar subcellular compartments, enhancing mRNA decay of targeted mRNAs, interaction and recruitment of multiple components of the RNA degradation machinery, and hematopoetic related deficiencies in knockouts. However, the family members also differ in several key aspects: cell type restricted expression patterns, stimuli and kinetics of induced gene expression, posttranslation modifications by different regulators (presumably), and physiological process regulated.

1.3 Hu Proteins

One of the most intensely studied families of RBPs is the Hu family, also known as the ELAV (embryonic lethal abnormal vision) family named after the Drosophila Hu gene homolog, *elav* (Campos et al., 1985). Hu proteins are characterized by having three RNA recognition motifs (RRMs) domains and an unstructured hinge region between the second and third RRMs. In mammals there are four Hu family members: HuR, HuB, HuC and HuD. All four family members share a high similarity in their protein sequence (ranging from 70% to 85% identity) with the RRMs being the most similar regions (Samson, 2008).

The initial discovery of Hu proteins was made in Drosophila, where *elav*, was identified in an EMS screen as one of many sex-linked behavioral mutants (Homyk et al., 1980). *elav* DNA was later cloned and suggested to be an RBP based on partial homology to a RNA binding motif consensus sequence (Robinow et al., 1988). Two other members of the Hu family have also been identified in Drosophila: *rbp9* and *fne*, with all three Drosophila members being specifically expressed in developing and mature neuronal cells (Kim and Baker, 1993; Chalvet and Samson 2002). Following the discovery of *elav*, three mammalian Hu proteins (HuD, HuB/Hel-N1, and HuC/PLE21) were discovered as the antigens for autoantibodies produced in small cell lung carcinoma-associated paraneoplastic neurologic syndrome (Szabo et al., 1991; King et al., 1994; Sakai et al., 1994). The final member of the family to be identified was HuR, which was discovered by degenerate PCR cloning (Good, 1995).

Given the high sequence similarity, it is not surprising that all four mammalian Hu family members recognize similar, if not identical, single stranded uridylate-rich RNA sequences and have been suggested to similarly regulate the expression of target mRNAs by increasing mRNA stability and translation (Levine et al., 1993; Jain et al., 1997). Hu proteins have also been suggested to have roles in other posttranscriptional regulatory events including splicing, polyadenylation and nuclear export (Hinman and Lou, 2008). While the paralogous Hu proteins share similarities in molecular function they differ in their cell type and temporal expression patterns (Antic and Keene, 1997;

Wakamatsu and Weston, 1997; Okano and Darnell, 1997). HuR, the most commonly studied of the Hu family members, is ubiquitously expressed, while the other three Hu proteins (HuB, HuC and HuD) are predominately expressed in neuronal cell types and show distinct expression patterns during neuronal differentiation and synaptic plasticity (Tiruchinapalli et al., 2008). The developmentally regulated expression patterns of the three neuronal Hu proteins are similar to the expression patterns for the three Drosophila *elav*-related proteins (Antic and Keene, 1997). As expected given their cell type distribution, the neuronal Hu proteins are essential for proper neuronal development, maintenance and activity while the ubiquitously expressed HuR is involved in a large and diverse variety of cellular processes including cell cycle control, apoptosis, inflammation, myogenesis, adipogenesis and a number of stress responses (Atasoy et al., 1998; Wang, Caldwell et al., 2000; Lal et al., 2005; Mazroui et al., 2008; Myer et al., 1997; Katsanou et al., 2005; Papadaki et al., 2009; van der Giessen et al., 2003; Figueroa et al., 2003; Jain et al., 1997; Gantt et al., 2005; Wang, Furneaux et al., 2000; Gallouzi et al., 2000; Mazan-Mamczarz et al., 2003).

1.4 RNA Elements Recognized by Hu Proteins

Hu proteins affect cellular processes such as neuronal development or stress response by regulating the expression of bound mRNAs through sequence-specific RNA cis-regulatory elements. The first RNA cis-regulatory elements identified for a Hu protein were for binding of HuB, or Hel-N1 (human *elav*-like neuronal 1), which was shown by *in vitro* selection and direct binding to recognize “a preponderance of uridylate residues in short stretches interrupted by other nucleotides” (Levine et al., 1993). Interestingly, many of the HuB-selected sequences where similar to the instability elements found in the 3′ UTRs of cytokines and oncogenes, called AU-rich elements (AREs) (Caput et al., 1986; Shaw and Kamen, 1986).

Numerous other studies have additionally defined similar Hu protein recognition elements using in vitro methods. HuB was found to bind a 29 nucleotide RNA element in the 3′UTR of c-myc mRNA that contained a single AUUUG core sequence (Gao et al., 1996). Recombinant HuR was also shown to bind the c-fos 3′UTR sequence and the sites of HuR association were mapped to AUUUA pentamers surrounded by U-rich regions by using a RNase T1 protection assay (Ma et al., 1996). Another study showed that excess unlabeled AU-rich probes that were mutated at uridylate residues failed to compete with labeled (AUUU)4A probes for binding to HuR and concluded that HuR binds to AREs “loosely defined as the five-nucleotide sequence AUUUA embedded in a uracil-rich region” (Myer et al., 1997). Many other studies have similarly focused on binding of Hu proteins to sequences that contain AUUUA pentamer sequences or other similar AU-rich sequences (Gao et al., 1994).

While Hu proteins can undoubtedly bind to ARE sequences there is evidence that Hu proteins can bind to other kinds of RNA sequences as well. In a competition assay similar to that used by Myer, et al., it was shown that excess poly(U) RNA, but not other ribohomopolymers, could compete with the VEGF and c-myc AREs for binding to either HuC or HuD (King, 2000) This suggested that the uridylates are more important for binding than are adenylates in the AREs, agreeing with previous studies that mutated uridylates within AREs (Fan et al., 1997). The Hu proteins, HuR and HuD have also been shown to interact with UC-rich elements in the 3'UTRs of androgen receptor mRNA and MARCKS mRNA (Yeap et al., 2002; Wein et al., 2003). UC-rich sequences are similar to AREs in that they consistof predominately short stretches of uridylates (usually three in a row) surrounded by other nucleotides. However, in the case of UC-rich elements the other surrounding nucleotides are usually cytosines instead of the adenosines found in AREs.

Several studies have also addressed the specificity of Hu proteins' recognition of RNA in the context of RNA secondary structure. Through base pairing of complementary regions within a single RNA molecule extensive secondary structures consisting of combinations of single and double stranded RNA, such as hairpin loops can form, potentially affecting protein binding. In some instances RNA secondary structure can be an important determinant of specificity for recognition of RNA elements by RBPs, as for example in the case of Stem-loop Binding Protein (SLBP) that recognizes elements in histone mRNA 3'UTRs based on a specific sequence found within a particular hairpin loop structural context (Williams and Marzluff, 1995). Several studies investigating whether HuR recognizes sequences within secondary structures or within single stranded RNA have produced conflicting results (Lopez de Silanes et al., 2004; Meisner et al., 2004; Kazan et al., 2010). One of the early studies to address this issue simply asked whether multiple mRNAs that could be enriched using HuR RNA Immunoprecipitation followed by microarray (RIP-Chip) analysis could be used to train a covariance model to identify common secondary RNA-binding structures (Lopez de Silanes et al., 2004). The motifs generated from this model consisted of 17 to 20 nucleotide U-rich motifs with apparent stem-loop structures. These putative motifs were found in nearly all HuR bound mRNAs (as identified in the RIP-Chip analysis) and were shown to be enriched in the 3'UTRs compared to the 5'UTRs and coding sequences (CDS).

More recent studies have questioned this previous observation that HuR binds RNA elements located in secondary structures. One such study identified a U-rich HuR binding motif sequence (NNUUNNUUU) in single stranded confirmation using single molecule confocal fluorescence fluctuation analysis (Meisner et al., 2004). HuR was able to bind this U-rich motif when secondary structure was absent but was unable to recognize this same motif when it was contained within an extensive secondary structure. This binding event was described to occur in an "all-or-none" fashion depending on the absence or presence of secondary structure. Furthermore, non-binding to this motif in the context of structure was reversed by the addition of complementary oligonucleotides that disrupted the nearby structured regions, thus inducing a single stranded conformation (Meisner et al., 2004). A more recent study used an algorithm designed to find RBP-specific sequence and structural preferences from *in vitro* and *in vivo* RNA affinity selected data (ie SELEX and RIP-Chips) (Kazan et al., 2010). This "context-based" approach was able to correctly distinguish structured and unstructured motifs for previous empirically defined RBP specific motifs. This algorithm predicted that HuR bound to unstructured U-rich RNA. Furthermore, these studies generally agreed with the observation that the co-crystal structure of the first two RRMs of HuD bound to RNA in a single stranded conformation (Wang and Tanaka-Hall, 2001).

The Lopez de Silanes et al., study that predicted HuR binding in vivo to a motif with secondary structure has not to date been independently confirmed. Furthermore, the background signal remaining in the covariance training model used showed equal probability for all nucleotides while the sequences (and especially the UTRs) of the training set were greatly skewed for U's and A's, possibly leaving a likely over-estimate of the enrichment of the structured motifs (unpublished observations, Marshall Thompson, Keene laboratory). Moreover, no other studies have found evidence for Hu proteins recognizing secondary structural elements, while several studies have demonstrated Hu proteins strong preference for unstructured RNA elements. In conclusion, these findings suggest that Hu proteins likely recognize single-stranded U-rich sequences, just like the majority of other RRM containing proteins. On the other hand, it should be noted that extensive RNA secondary structure may prevent access to U-rich motifs thus affecting HuR's ability to bind mRNA.

Recognition of U-rich motifs is a conserved feature of Hu proteins, although this in not terribly surprising given the high conservation of the RRM domains among Hu family members. The Drosophila Hu proteins, *elav* and *fne*, have been shown to bind sequences containing short stretches of uridylates separated by adenosines, which is highly similar to the RNA elements recognized by mammalian Hu proteins (Borgeson and Samson, 2005). Furthermore, the Xenopus Hu homolog, ElrA, has been shown to bind to sequences containing the U-rich cytoplasmic polyadenylation element (UUUUUAU) in Cyclin E1's 3'UTR (Wu, Good, and Richter, 1997; Slevin et al., 2007). The yeast protein Pub1p, which is arguably a Hu protein homolog, has also been demonstrated to bind global populations of mRNAs that are enriched for U-rich sequences (Duttagupta et al., 2005). While the ability of Hu proteins to recognize similar RNA-binding elements is a highly conserved feature, any given mRNA that contains one of these elements is not necessarily well conserved. This process of phylogenetic remodeling of mRNAs bound by particular RBPs has been referred to as "rewiring" and may provide a platform for evolutionary adaptation and plasticity (Keene, 2007).

The minimal size of the ARE binding site recognized by a single molecule of Hu protein has been addressed by several studies using different ARE sequences. It was first determined that HuB could recognize a nucleotide sequence in human c-myc mRNA of approximately 29 nucleotides (Levine et al.,, 1993; Gao and Keene, 1996). And soon after its discovery, HuR was found to recognize sequences in the c-fos ARE at least as small as 13 nucleotides but this required between 21 and 27 nucleotides for maximal binding (Ma et al., 1996). It was later shown that HuD could recognize an AU-rich sequence at least as small as 10 nucleotides made up of multiple overlapping AUUUA pentamers but required 15 nucleotides for maximal binding (Park et al., 2000; Park-Lee et al., 2003). Interestingly, another study found that HuR only required 13 nucleotides to bind a similar AUUUA repetitive sequence but that it appeared to bind as a dimer to an 18 nucleotide sequence suggesting that only 9 nucleotides was required for each molecule of HuR to form RNP complexes (Fialcowitz-White et al., 2007). These studies used different ARE sequences and broadly removed multiple nucleotides making it possible that these "minimal" sites contained nucleotides that were not bound by Hu proteins. Furthermore, some of these studies did not distinguish between monomer and dimer complexes, thus making it likely that some of the maximal binding sites include multiple binding events. However, a comprehensive mutational analysis of a U-rich RNA demonstrated that nine nucleotides were essential for complex formation with HuR, while eight nucleotides showed no binding (Meisner et al., 2004). This is the same size minimal binding site seen for each molecule of a Hu dimer to fully bind its target mRNA. Thus, the minimal size of the binding site for Hu proteins when complexed with U-rich/ARE sequences appears to be nine nucleotides, but this may be dependent on the exact nucleotide sequence of the element. Interestingly, a recent unbiased genome-wide survey of *in vivo* HuR binding sites demonstrated that HuR's RNA footprint was as small as 9 nucleotides but that a significant fraction of the footprints were much larger (Mukherjee et al., 2011).

In summary, Hu proteins bind AREs with and without the AUUUA pentamer sequence and the U's are probably more important than the A's for binding. Hu proteins can also recognize U-rich sequences surrounded by other nucleotides including C's. This description of the Hu binding sequence is nearly identical to the initial description that HuB recognizes "a preponderance of uridylate residues in short stretches interrupted by other nucleotides" (Levine et al., 1993). These U-rich RNA elements bound by Hu proteins are about 9 nucleotides in length but Hu proteins may recognize longer sequences through RNA-dependent multimerization. It has also been demonstrated that Hu proteins most likely bind to single stranded, unstructured sequences and that the Hu proteins recognition of these ARE/U-rich sequences have been conserved.

1.5 AU-rich Elements

AREs were originally discovered as highly conserved, partially overlapping, repetitive sequences usually consisting of the five-nucleotide sequence, AUUUA, in the 3' UTRs of several labile cytokine and oncogene mRNAs (Caput el al, 1986; Shaw and Kamen, 1986). These ARE sequences were shown to be cis-regulatory elements capable of decreasing the mRNA stability of a reporter (Shaw and Kamen, 1986). While no consensus sequence was identified for the ARE at the time, it was noted that the AUUUA pentamer was frequently found in these UTRs. Due to their presence in a number of cytokine messages it was believed that AREs were immune regulatory elements. It has since been reported that up to 8% of the genes in the human genome contain at least one putative ARE in their 3′ UTR (Bakheet et al., 2003). Globally, ARE-containing transcripts are typically unstable and are enriched in several different functional categories including: growth regulatory/cell proliferation factors, development regulators, signaling proteins, gene expression trans factors (such as transcription factors and other RBPs), and cytokines (Bakheet et al., 2003).

Initially, when AREs were discovered as instability elements it was presumed that the elements were bound and regulated by yet to be identified, trans-acting factors, later called ARE binding proteins (ARE-BPs). Early studies to identify these ARE-BPs found that multiple protein bands could be identified by crosslinking to *in vitro* transcribed radiolabeled ARE probes. These putative trans-acting factors were termed AUBFs (AU binding factors) for crosslinked protein bands, and were called AU-A, AU-B and AU-C, and in other studies ARE-BPs, however, their identities were unknown at the time (Malter 1989; Bohjanen et al., 1991; Vakalopoulou et al., 1991; Bohjanen et al., 1992). Later it was shown that AU-A is the ubiquitously expressed Hu protein, HuR, while AU-B/C is the ARE-BP, tristetraprolin (TTP) (Raghavan et al., 2001). Interestingly, while it was shown that these ARE-BPs could bind the same sequences, they also had different preferences for highly related sequences. AU-B was reported to bind to AUUUA multimers but not monomeric AUUUA motifs or other AU-rich motifs whereas AU-A bound AUUUA multimers *and* “other U-rich sequences, including a poly(U) sequence” (Bohjanen et al., 1991; Bohjanen et al., 1992). The sequences preferred by AU-A, later identified as HuR, are strikingly similar to the sequences independently identified as targets of Hu proteins (Levine et al., 1993).

Currently, at least a dozen ARE-BPs have been identified, and potentially many more will be discovered (reviewed in Barreau 2005). ARE-BPs are a diverse set of RBPs that can regulate the expression of target mRNAs by an equally diverse set of mechanisms. While ARE-BPs, by definition, recognize similar RNA sequences there is no single common RNA binding domain among the ARE-BPs; several ARE-BPs contain RRMs, while others contain zinc-fingers or KH domains. Despite the differences in domains, ARE-BPs can often recognize the very same ARE sequence and potentially compete with one another. It has been shown that the ARE-BPs, HuR and AUF1, can bind the same ARE sites in p21 and Cyclin D1 mRNA and compete with each other in the cell for binding to these transcripts (Lal et al., 2004). Interestingly, this study also showed that HuR and AUF1 could uniquely bind to sites not recognized by the other ARE-BPs. This suggests that there are likely to be multiple ARE sequences with distinct yet partially overlapping ARE-BP binding patterns.

Since these AREs seem to recruit different ARE-BPs based on differences in their sequences it would stand to reason that some characteristic of the ARE sequence could predict binding or function, a so-called "ARE code". Several studies attempting to find the ARE code have classified ARE subtypes based solely on the presence and number of AUUUA pentamer repetitions (Chen and Shyu, 1995; Bakheet et al., 2001). However, it is unclear whether these ARE classes predict any functional feature. This likely reflects a lack of sophistication in the classifications and a pre-determined bias for AUUUA pentamer sequences rather than the absence of an ARE code capable of governing RBP binding and functional outcome. It is not unreasonable to think that there are some sequence properties of AREs that affect which factors bind or determine the fate of the mRNA containing the ARE, but it will probably require an unbiased search utilizing global binding and functional data sets to find the elusive ARE code. Further complicating the search for an ARE code is that the precise biding or functional events encoded by a particular ARE may be condition and cell type specific, and it is likely to be a subset of the elusive "RNP code".

ARE-BPs target ARE-containing mRNAs for posttranscriptional regulation, and oftentimes this includes a combination of the regulation of mRNA stability and/or mRNA translation. Initially, AREs were identified as mRNA instability elements that are frequently found in labile messages (Shaw and Kamen, 1986). The initial and rate limiting step of mRNA degradation is the deadenylation of the 3′ polyA tail which is initiated by the recruitment of deadenylases. In mammals there are at least a dozen different deadenylases with distinct but overlapping functions (reviewed in Goldstrohm and Wickens, 2008). Following deadenylation mRNAs can be targeted for degradation by one of two pathways, exosome-dependent 3′ → 5′ decay or XRN1-dependent 5′ → 3′ decay. The later is proceeded by mRNA decapping by Dcp1/Dcp2 (Hsu and Stevens, 1993; Muhlrad et al., 1994; Muhlrad et al., 1995). Interestingly, similar but distinct ARE sequences from GM-CSF and c-fos mRNAs both initiate rapid degradation but undergo deadenylation with different kinetics suggesting that different ARE sequences can recruit different deadenylation and/or decay pathways (Chen et al., 1995).

Besides influencing mRNA degradation AREs have also been suggested to regulate translation. ARE-mediated translational regulation can be both coupled to and independent of ARE-mediated mRNA decay depending on the ARE sequence and other factors such as cell type. An example of coupled translation and mRNA decay is seen with the GM-CSF and c-fos AREs that have been shown to increase mRNA stability when translation is inhibited by either pharmacological methods or by preventing ribosome association with initiation codon mutants or by extensive stem-loop structures (Savant-Bhonsale and Cleveland 1992; Winstall el al. 1995). However, in one case, ARE-dependent translation was uncoupled from mRNA turnover. In this case, an ARE in the 3′UTR of the p53 mRNA was shown to increase translation in response to UV treatment without a concomitant increase in mRNA levels, nuclear mRNA export, or protein stability (Mazan-Mamczarz et al., 2003).

Most ARE-BPs function as negative regulators of posttranscriptional gene expression by decreasing mRNA stability and/or translation. However, the Hu proteins are rare among ARE-BPs in that they commonly function as positive posttranscriptional regulators of gene expression by causing increases in mRNA stability and/or translation (Jain et al., 1997; Fan and Steitz 1998; Peng and Shyu 1998). It has been proposed that the Hu proteins increase expression of ARE-containing transcripts by competing with destabilizing ARE-BPs for binding thus relieving the negative regulation and causing an apparent increase in expression (Levine et al., 1993; Lal et al., 2004). This mechanism is especially attractive considering that many ARE-containing mRNAs are labile under normal conditions but are stabilized in a stimulus-dependent manner suggesting that their increase in stability is caused by a release from repression.

Given that Hu proteins are rare among ARE-BPs as positive regulators it is important to understand which ARE sequences and ARE-containing mRNAs are recognized by Hu proteins.

1.6 mRNA Targets of Hu Proteins

As discussed above, RBPs bind mRNAs and influence their expression by regulating various posttranscriptional processes. The specificity with which RBPs bind mRNA range across the spectrum from specialized RBPs, such as Stem Loop Binding

Protein (SLBP) which only targets the histone messages (Townley-Tilson et al.,, 2006), to general RBPs such as Poly A binding protein (PABP) or the cap binding RBP, eIF4E, which are assumed to bind to all mRNAs. In between the specialized and general RBPs are the group-specific RBPs that bind to sets of mRNAs that are larger than a few but smaller than the entire global set (Keene, 2001). The Hu proteins are one of the best defined families of this group-specific class of RBPs when it comes to RNA targeting (Keene, 2007).

Numerous studies have shown that Hu proteins recognize several hundreds to thousands of mRNAs and this is consistent with the observation that at least 8% of UTRs contain an ARE sequence (Gao et al., 1994; Tenenbaum et al., 2000; Lopez de Salines et al., 2004a; Lopez de Salines el al., 2004b; Mazan-Mamczarz et al., 2008a; Mazan-Mamczarz et al., 2008b; Mukherjee et al., 2009; Bolognani et al., 2010; Calaluce et al., 2010; Masuda et al., 2011; Mazan-Mamczarz et al., 2011; Fan et al., 2011; Mukherjee et al., 2011; Kishore et al., 2011; Lebedeva et al., 2011). The particular mRNAs associated with Hu proteins are dependent on cell type and condition and can be substantially relocalized within the cell in response to cellular signals and stresses (Tenenbaum et al., 2000; Mukherjee et al., 2009). Furthermore, Hu proteins recognize subsets of mRNAs that are structurally and functionally related such as members of the Wnt pathway, p53 feedback regulators, growth regulatory factors, and other posttranscriptional regulatory factors. Importantly, these subsets of related transcripts may be coordinately regulated at the posttranscriptional level in response to cellular stimuli thereby affecting cellular responses such as growth activation or stress recovery (Gao et al 1994; Tenenbaum et al 2000; Keene and Tenenbaum 2002; Mukherjee et al 2009; Mazan-Mamczarz et al 2011). These observations helped form the organizing principles of the posttranscriptional RNA operon/regulon theory (Keene, 2001; Keene and Tenenbaum 2002). The biochemical mechanisms and cellular pathways controlling and coordinating Hu protein association with and regulation of mRNA subsets in response certain cellular conditions are beginning to be elucidated and will be reviewed in later sections. Due to their central role in recognition of specific elements, the RRMs will undoubtedly play a major role in the dynamic association and regulation of Hu proteins mRNA targets.

1.7 Mechanisms of Hu Proteins Recognition of RNA

The most prominent features of Hu proteins are the three highly conserved RRM domains that together, account for roughly three quarters of the amino acid residues of full-length Hu proteins. RRMs are a vast and ancient RNA-binding domain that are found in most, if not all, eukaryotes and are even present in some bacteria and viruses. RRM containing proteins are the seventh most common protein family in the human proteome, and the largest among RNA-binding motif families, with over 700 putative RRM containing proteins in humans according to the Pfam database (Lander, Linton, et al., Nature 2001; Finn, Mistry, et al., NAR 2010). The abundance of RRM containing proteins is not limited to higher eukaryotes as it is the sixth most common motif in the yeast (*S.cerevisiae*) proteome (Lander, Linton, et al., Nature 2001). The RRM is a single stranded RNA binding domain of approximately 90 amino acids (Query et al., 1989; Kenan et al., 1991). The most conserved features of RRMs are the RNP-1 (RNA consensus sequence) and RNP-2 motifs, respectively, an 8 and 6 amino acid sequence with characteristic aromatic and basic residues that are necessary for binding to RNA (Adam et al., 1986; Sachs et al., 1986; Merrill et al 1988; Chambers et al., 1988). However, there are other conserved features of RRMs besides the RNP motifs that are also important to RNA recognition, indicating that the entire ~90 amino acid RRM is required to function as a sequence specific RNA binding domain (Query et al., 1989). Structural studies have determined that RRMs form four antiparallel beta-sheets situated against two perpendicular alpha-helices with the residues important for RNA binding (including the RNP motifs) found in the beta-sheets (Nagai et al., 1990; Hoffman et al., 1991). This general structure is a common and defining feature of all RRMs and highlights the importance of the entire ~90 amino acid sequence in providing RNA binding specificity and in some cases, adjacent amino acids are needed for optimal binding affinity (Query et al.,, 1989).

RRM-containing proteins frequently contain multiple RRMs with sometimes as many as 6 RRMs found in a single protein (Finn et al., 2010). Hu proteins contain three functional RRMs with the first two being separated from the third by a linker or hinge region. Numerous studies have investigated the contributions that each of the three Hu

RRMs with respect to RNA recognition in the context of the full-length Hu proteins, yielding sometimes different and somewhat controversial results. The first study to address the role of individual RRMs in recognition of RNA showed that RRM3 of HuB could bind to c-myc 3'UTR while RRM1 did not bind this sequence and neither did a construct containing RRM1 and most of RRM2 (Levine et al., 1993). While RRM3 is important for HuB binding to c-myc 3'UTR it was shown that RRM3 of HuD did not associate with c-fos 3'UTR (Chung et al., 1996). However, RRM1 and RRM2 of HuD were shown to bind together to this same sequence of the c-fos 3'UTR, but with a greater than 100 fold reduction in affinity compared to full-length protein (Chung et al., 1996). Another HuD study found that only RRM1 among the individual RRMs could bind to an element containing multiple AUUUA elements, but also with a large decrease in affinity compared to full-length protein (Park et al., 2000). Yet another study showed that both RRM1 and RRM3, but not RRM 2, of HuC could be crosslinked to an *in vitro* selected AU-rich RNA, and RRM1 binding was reduced compared to full-length HuC and RRM3 binding was barely detectable (Abe et al., 1996). Finally, it was shown that all three individual RRMs of HuD could recognize poly(U) with RRM1 and RRM3 binding stronger than RRM2 but still with less affinity than full-length HuD (Park-Lee et al., 2003). Interestingly, RRM1 and RRM2 produced smears when binding to poly(U) in a gel shift assay but RRM3 produced a discrete band suggesting that different types of complexes are formed between the RRMs and the RNA (Park-Lee et al., 2003). These studies suggest that each of the individual Hu RRMs in isolation from the other RRMs are sufficient to bind RNA, albeit with greatly reduced affinity and in a sequence dependent manner.

To dissect the combinatorial effects of multiple Hu RRMs on recognition of RNA several studies have deleted one of the RRMs leaving the other two intact, which is in contrast to the experiments described above in which single isolated RRMs were investigated. Each of the HuD RRMs individually bound to c-fos 3'UTR RNA at least 100-fold less tightly than full-length HuD but the RRM3 deletion (RRM1 and RRM2 together, missing RRM3) bound to c-fos 3'UTR only about 7-fold less tightly (Chung et al., 1996). Thus, indicating that RRM1 and RRM2 can act together to recognize RNA with a much higher affinity than either alone, but also suggesting that RRM3 is necessary for fully efficient recognition of c-fos 3'UTR RNA. Supporting this observation that all 3 RRMs help recognize the same element, it was observed that both the RRM1 deletion (i.e. RRM2 and RRM3 together) and the RRM3 deletion (i.e. RRM1 and RRM2 together) of HuR could bind the c-fos ARE in super-shift assays, although less of the complex was super-shifted in the RRM1 deletion (Chen, 2002). Also in agreement, it was shown in another study that HuD's affinity for the (UUAU)4U sequence was greatly reduced by deletion of RRM1 or RRM2 (Park et al., 2000). However, deletion of RRM3 caused a much smaller reduction in affinity. In addition to analyzing the steady-state binding, this study also investigated the association and disassociation rates using surface plasmon resonance. Surprisingly, while RRM3 only had a relatively small effect on steady state affinity, its removal caused a dramatic increase in both the on and off rates of the complex, indicating that RRM3 slows the recognition kinetics while reducing the rate of disassociation, thereby, masking its effect in steady-state analyses (Park et al., 2000). These studies suggest that all three RRMs cooperate to bind U-rich RNA with RRMs 1 and 2 providing the majority of binding activity and RRM3 potentially acting to stabilize the complex formed between the RNA and RRMs 1 and 2. This cooperative binding of the Hu RRMs in recognizing U-rich RNA is interesting considering that the individual RRMs were suggested to have differences in sequence preference. This may indicate that each of the RRMs are responsible for interacting with distinct nucleotides within the U-rich RNA element thus when viewed in isolation the RRMs appear to bind distinct elements, but in combination, they cooperate to provide further specificity. However, it is also possible that for certain RNA sequences not investigated in these studies the RRMs do not cooperate. A global and unbiased approach will be required to definitively determine the influence of each of the RRM's contributions and their cooperative properties in recognizing RNA elements.

The previous studies indicate that the RRMs may have some differences in sequence preferences but still all recognize poly(U) or U-rich sequences with other nucleotides interspersed. However, a few controversial studies have found that RRM3 recognizes poly(A) RNA. For example, HuD was reported to bind poly(A) sequences of greater than 70 nucleotide length in *in vitro* nitrocellulose filter binding assays. RRM3 alone was reported to bind these poly(A) sequences while the RRM3 deletion mutant could not (Ma et al., 1997). It was also shown that full-length recombinant HuC and RRM3, but not the RRM3 deletion, could be isolated with poly(A)-Sepharose beads (Abe et al., 1996). These results are contradictory to previous observations that HuB RRM3 bound to c-myc 3'UTR in mobility shift assays despite the use of excess poly(A) as a non-specific competitor (Gao et al., 1996). Furthermore, unbiased motif searches of sequences recognized by Hu proteins (e.g. SELEX) have not selected for sequences similar to poly(A). A potential explanation for this discrepancy is that HuR RRM3 has been reported to contain an ATP binding pocket that can bind ATP independently of its RNA binding ability. While this study did not demonstrate binding of RRM3 to poly(A) RNA it raised the possibility that RRM3 may have adenosine binding functions independent of RNA recognition (Meisner et al., 2009) and that this may have something to do with the previously observed poly(A) binding (Abe et al., 1996; Ma et al., 1997). Moreover, the authors essentially argued that binding of RRM 3 to poly(A) was not detectable in their assay.

Therefore, it seems that each of the Hu protein RRMs contributes uniquely to the recognition of unique RNA elements. However the recognition of RNA by Hu proteins may be more complex than a one-to-one interaction between protein and RNA. Several reports have indicated that increasing amounts of Hu proteins can shift RNA complexes to higher order structures characteristic of multimers (Levine et al., 1993; Gao and Keene, 1996; Ma et al., 1996; Soller et al., 2005). While there is some disagreement in the literature, most studies indicate that multimerization of Hu proteins is dependent on its binding to RNA. Furthermore, it has been demonstrated that increasing the size of the RNA-binding substrate can cause an increase in the number of multimers formed for either Drosophila ELAV or human HuR (Soller et al., 2005; Fialcowitz-White et al., 2007). Interestingly, as more multimers form with the increasing size of the RNA substrate there is a concomitant decrease in the dissociation constant (K_d), and this suggests the possibility of cooperativity in the formation of multimers such that binding of a single Hu protein molecule increases the affinity of a second Hu protein molecule binding.

Intriguingly, among the three Hu protein RRMs, RRM3 appears to play a distinctly important role in the formation of RNA-dependent Hu protein multimers. The first study to investigate the role of Hu protein RRMs in multimerization noted that RRM3 could compete with full-length HuB to block the formation of RNA-dependent multimers in a concentration dependent manner (Gao et al., 1996). Later studies demonstrated that HuR RRM3 was important, but not necessary, for multimerization when it was shown that RRM3 deletions greatly reduced the efficiency of multimerization but still were capable of forming multimers at much higher concentrations (Fialcowitz-White et al., 2007; Meisner et al., 2007). It has also been observed that neuronal Hu proteins could form RNA-dependent heteromultimers (multimers between two different neuronal Hu proteins) and that RRM3 was necessary for efficient multimerization and that RRM3 could interact with both itself and with RRM3 deletions in a yeast-two hybrid (Kasashima et al., 2002; Toba et al., 2008). While RRM3 clearly is important for RNA-dependent multimerization it has also been shown that part of the hinge region adjacent to RRM3 can promote dimerization (Devaux et al., 2006; Fialcowitz-White et al., 2007). As previously mentioned, HuR may also be capable of forming homodimers in the absence of RNA. However, these RNA-independent homodimers do not require RRM3 and are induced through a disulfide bond between cystines in the non-RNA binding surfaces of RRM1 (Benoit et al., 2010). Cumulatively, these studies indicate that Hu proteins are capable of forming multimeric complexes potentially both in the presence and absence of RNA with multiple Hu protein domains contributing to multimerization. In conclusion, of these Hu protein domains, RRM3 appears to play the key role in RNA-dependent multimerization. The biological significance of Hu protein multimerization with RNA is unknown, however it is possible that multimerization may promote cooperativity thus encouraging Hu protein-RNA interactions at lower concentrations.

Structural analysis of Hu protein RRMs bound to RNA has significantly advanced the understanding of the interactions necessary for recognition and specificity. The co-crystal structure of the first two RRMs of HuD bound to RNA demonstrated the basis for recognition of uridylate residues with a possible tolerance for cytosines at particular positions with a predicted consensus recognition sequence of N-U/C-U-N-N-U/C-U-U/C (Wang and Tanaka Hall, 2001). Notably this predicted motif is very similar to the experimentally determined NNUUNNUUU motif (Meisner et al., 2004). The HuD structure shows that RRMs 1 and 2 recognize an eight-nucleotide sequence that is single stranded when bound but is bent in the middle and this conformation may be important for specific recognition. The interaction between the RNA and the RRMs is extensive with roughly half of the RNA buried in the protein. This interaction is asymmetric with RRM1 making the majority of the contacts with the RNA and RRM2 making fewer but significant contributions. This is consistent with the previous demonstrations that RRM1 is the primary recognition domain. Not surprisingly the amino acid residues that made direct contact with the RNA were all identically conserved in other mammalian Hu proteins suggesting that the various Hu proteins recognize the very same elements, this too is consistent with the empirical evidence. Overall, this structure suggests that RRMs 1 and 2 cooperate to recognize single stranded U-rich sequences of eight nucleotides in length, and this is similar to the experimentally determined minimal size of nine nucleotides for full-length Hu proteins. Thus suggesting that RRM3 may not add significantly to the specificity of recognition of U-rich sequences. However, as discussed previously, RRM3 may recognize elements different than the U-rich sequences recognized by RRM1 and RRM2. It is also possible that RRM3 helps lock in RRM1 and RRM2 to U-rich sequences by reducing the disassociation rate as previously demonstrated.

1.8 Regulation by Hu Proteins

The consequences of Hu proteins associating with target mRNAs are vast and varied. The first demonstrated effect of a Hu protein on gene expression was seen for HuB, which was shown to increase the stability and translation of glucose transporter 1 (GLUT1) mRNA in differentiating preadipocytes (Jain et al., 1997). Since then, many studies have shown that Hu proteins can increase the expression of a large and ever-increasing number of target mRNAs. Nearly all of these studies have focused on the positive influence of Hu proteins on mRNA stability and translation. However, Hu proteins have also been demonstrated to affect other posttranscriptional processes such as 3′ end processing, splicing, nuclear export of mRNAs and microRNA mediated repression (reviewed in Hinman and Lou, 2008). More recent studies suggest that HuR's role in nuclear RNA processing may be more widespread than is apparent from the 20-year collective focus of Hu protein research literature (Mukherjee et al., 2011; Lebedeva et al., 2011). Furthermore, previous studies often focused on how Hu proteins positively influence expression of target mRNAs, but a few studies have shown that Hu proteins may also act as repressors of gene expression depending on the transcript or the cellular context (Kullmann et al., 2002; Meng et al., 2005; Katsanou et al., 2005; Leandersson et al., 2006; Yeh et al., 2008; Kim et al., 2009).

HuR's effects on cellular functions are as diverse as its effects on posttranscriptional regulation, and occasionally are conflicted. For example, knockout of HuR in mice results in early embryonic lethality with multiple defects ranging from disrupted placental development to atrophy of hematopoietic organs (Katsanou et al., 2009; Ghosh et al., 2009). In addition, overexpression of HuR in transgenic mice also lead to defects in thymic development and male gametogenesis (Levadoux-Matrin et al., 2003; Katsanou et al., 2005). In addition to its roles in development, HuR has been demonstrated to influence a wide set of cellular processes including cell proliferation, cell cycle progression, apoptosis, migration, differentiation, inflammatory response, senescence, stress response and carcinogenesis. Much like HuR's effects on gene expression, HuR can ironically influence both sides of regulation of these cellular processes. For example HuR has been shown to promote apoptosis in certain cell types while in other cell types it was shown to prevent apoptosis (Lal et al., 2005; Durie et al., 2011; Mazrouri et al., 2008; von Roretz and Gallouzi, 2010; von Roretz et al., 2011). These diverse and apparently conflicting functions of HuR are most likely a reflection of a complicated, yet exquisitely sensitive regulatory process that controls the functions of HuR, as evidenced by the dependence of HuR function on cell type, stimuli, transcript.

Given the complicated relationship between Hu proteins and their functions it is no surprise that the contributions of each of the three Hu RRMs towards Hu-dependent function is quite complex. Several studies have demonstrated that each of the RRMs is necessary for proper cellular and biochemical function. It was shown that mutations disrupting RNA-binding ability in any of the three RRMs of Drosophila ELAV were unable to rescue viability in *elav* null flies, thus indicating that each RRM is essential for ELAV function (Lisbin et al., 2000). Multiple RRMs were also suggested to be involved in HuB regulation of gene expression. Full-length HuB prevented 3' end processing of U-rich containing mRNAs, but RRM3 alone or RRM1+2 (RRM3 deletion), were unable to block the processing (Zhu et al., 2007). Another study showed that HuD's ability to induce neurite extension in PC12 cells was severely impaired by the deletion of RRM1, and RRM3 or regions of the linker region between RRM2 and RRM3, but deletion of RRM2 had no impact on HuD's ability to induce neurite extension (Kasashima et al., 1999). Furthermore, it was also shown that in addition to inducing neurite extension HuD's ability to stabilize GAP-43 mRNA was also dependent on RRM3. The role of the other RRMs was not addressed thus making it possible that they may still be required for induction GAP-43 mRNA stability (Anderson et al., 2000). Similarly, RRM3 of HuR was shown to be necessary for HuR-mediated stabilization of a c-fos ARE reporter but the role of the other RRMs was not addressed (Fan and Steitz, 1998). Together these studies suggest that at least RRM3 is required for several different Hu protein-induced phenotypes and Hu protein regulation of mRNA stability of multiple RNAs. The other two RRMs are also required for at least some Hu protein-induced phenotypes and may be involved in all.

However, other studies have suggested that individual RRMs are dispensable for the normal functions of Hu proteins. Notably, it was shown that the combination of any two of HuR's three RRMs are sufficient for stabilization of c-fos ARE (Chen et al., 2002). Another study found that RRM3 alone was insufficient for HuC-dependent induction of neurite extension in PC12 cells and that deletion of RRM3 only mildly impacted the ability of HuC to induce neurite extension (Akamatsu et al., 1999). Contrary to the experiments detailed in the previous paragraph these studies indicate that RRM3 is not necessary for function. There are relativity few reported differences between these groups of studies, but one of the most intriguing differences is that while all of these studies used overexpressed Hu proteins, the levels to which the constructs were overexpressed varied greatly between experiments. This is interesting because, as previously noted, the RRM3 deletion constructs were typically able to bind similar sequences as full-length protein but at a reduced affinity. Thus, at higher overexpression levels RRM3 deletion constructs may be able to compensate for the reduced ability to bind, but at lower levels the differences in affinity between the wild type and RRM3 deletions are revealed as functional effects on the cell. In support of this idea, the studies that found RRM3 to be necessary for function were designed to only overexpress the mutants at about 1 to 2 fold more than endogenous levels, whereas at least one of the studies that found RRM3 to be dispensable for function designed their experiment to overexpress the mutants about 10 fold above endogenous levels (Fan and

Steitz, 1998; Anderson et al., 2000; Akamatsu et al., 1999). This does not imply that all of the Hu RRMs are necessarily required for each of Hu functions, but instead that levels of overexpression may need to be taken into account when investigating the functional necessity of the RRMs.

1.9 Regulation of Hu Proteins

As previously mentioned, the mRNAs associated with Hu proteins can change in response to various cellular stimuli. These changes in mRNA association do not correlate with changes in total mRNA levels, thus suggesting that binding of Hu proteins to mRNA must be regulated (Tenenbaum et al., 2000; Mukherjee et al., 2009). Moreover, Hu proteins can be regulated via several different signaling pathways that affect gene expression, localization, posttranslational modification and others. This regulation of Hu proteins, in turn, not only can change the mRNA species associated with Hu proteins, but they also can potentially influence the regulatory output directed by Hu proteins, therefore, altering the posttranscriptional expression of target mRNAs.

Hu proteins have been shown to exhibit regulated expression of mRNAs and their encoded protein levels as well as the regulated production of alternatively processed transcripts. As the name suggests, the neuronal Hu proteins (HuB, HuC and HuD) differ from the ubiquitously expressed HuR in their cell type expression patterns. Numerous studies have shown that expression of the neuronal Hu proteins is induced during neuronal development in both cell culture models and in brain tissue (Good

1995, Gao and Keene 1996, Okano and Darnell 1997). Furthermore, it has been shown that levels of neuronal Hu proteins can be increased in response to stimuli such as spatial learning (Pascale et al., 2004). Presumably, this regulated expression of the neuronal Hu proteins is, at least in part, transcriptionally controlled. In addition to regulation of expression levels, the neuronal Hu proteins also undergo alternative splicing, with each neuronal Hu protein producing multiple splice variants differing in sequence of the hinge region (Gao and Keene 1996; Okano and Darnell 1997). Furthermore, HuB and HuD transcripts have also been demonstrated to be alternatively processed by A-to-I RNA editing. The sites of RNA editing occur both within the CDS of HuB and HuD and in the 3'UTR of HuD, the edited sites in the CDS encode for amino acid substitutions (Enstero et al., 2010). The effects of these editing events on HuB and HuD are unknown, however, it is possible that editing of these transcripts can alter the expression and/or the functions of HuB and HuD proteins.

While HuR mRNA and protein are present in nearly all cell types, the levels of mRNA and protein can be dramatically increased in response to cellular stimulation such as anti-CD3/CD28 activation of T cells and proliferative signals in fibroblasts (Atasoy et al., 1998). It has also been shown that HuR mRNA and protein levels can be reduced following nitric oxide treatment or guanylyl cyclase activation (Akool et al., 2003; Kloss et al., 2003). The mechanism(s) responsible for these increases in HuR expression are unknown. However, in neurons, the ubiquitously expressed HuR is regulated by a known mechanism, alternative polyadenylation, where the transcript is cleaved and polyadenylated further downstream at the mRNA 3'UTR than in other cell types, resulting in an extended 3'UTR sequence (Atasoy et al., 1998; Al-Ahmadi et al., 2009). Incidentally, regulatory elements contained within this extended 3'UTR have been shown to be important for regulation of HuR expression, although the authors of those studies failed to attribute these regulatory elements to the neuronal-restricted, alternatively processed transcript (Pullmann et al 2007; Al-Ahmadi et al., 2009). It is likely that during many of these conditions that affect the expression of Hu proteins there are resulting concomitant changes in the mRNAs associated with and regulated by Hu proteins.

However, most conditions that regulate Hu proteins, especially HuR, do not change the levels of Hu mRNA or protein but instead change its subcellular distribution. HuR is localized predominately to the nucleus during standard growth conditions but can be dramatically redistributed to the cytoplasm following numerous signals, stresses, or progression through cell cycle stages (Fan and Steitz 1998; Peng et al., 1998; Atasoy et al., 1998; Gallouzi et al 2000; Wang et al., 2000; reviewed in Doller et al 2008). The region of HuR protein responsible for its nuclear localization in normal growth conditions was mapped by deletion to a 33 amino acid sequence contained within the hinge region between RRM2 and RRM3. This 33 amino acid sequence was shown to be sufficient for both nuclear import of a reporter and for allowing cytoplasmic shuttling of a reporter in heterokaryons. Since this sequence was capable of both nuclear import and export to the cytoplasm it was named the HuR nucleocytoplasmic shuttling sequence (HNS) (Fan and Steitz 1998). The HNS is the most variable regions between the Hu proteins and is the region that is different among the neuronal splice variants. Interestingly, the neuronal Hu proteins are localized predominately to the cytoplasm with only small amounts detectable in the nucleus suggesting that the differences in the HNS sequence of the neuronal Hu proteins might be responsible for their differences in subcellular localization (Gao and Keene, 1996; Antic and Keene, 1998; Toba et al., 2002).

As previously noted, HuR is predominately localized to the nucleus in normal growth conditions, however, it has been shown to regulate cytoplasmic events such as mRNA stability and translation during these same normal growth conditions. It has been suggested that HuR binds ARE-containing mRNAs in the nucleus and escorts them to the cytoplasm to provide ongoing protection from the RNA degradation machinery and to allow translation under normal conditions (Keene, 1999). This cycle requires that HuR continually shuttles between the cytoplasm and nucleus even in the absence of stimuli which is supported by the observation that HuR can transit from the nucleus of one cell through the cytoplasm and into the nucleus of another in heterkaryon experiments (Fan and Steitz, 1998). Furthermore, it has been shown that HuR interacts with both import and export machinery. Nuclear import of HuR is partially controlled by interaction of transportin 1 and/or transportin 2 with the HNS (Rebane et al., 2004;

Guttinger et al., 2004). However, HuR can also interact with importin alpha-1 to increase nuclear import when the later is phosphorylated by AMPK during ATP depleting stresses (Wang et al., 2004; Zou et al., 2008).

In addition to regulation of nuclear import, nuclear export of Hu proteins can be regulated as well. HuR can be exported from the nucleus in a CRM1-dependent manner through interactions between the distal portion of HuR, which includes the HNS and RRM3, and the nuclear ligands pp32 and APRIL. While HuR does not directly interact with CRM1, pp32 and APRIL contain nuclear export sequences (NES) that do interact directly with CRM1, thus acting as an adaptor between HuR and CRM1. Interestingly, the CRM1 inhibitor Leptomycin B (LMB) was unable to inhibit HuR shuttling in heterokaryon experiments but it did increase HuR bound polyadenylated transcripts in the nucleus (Brennan et al., 2000). While nuclear export of HuR was not sensitive to LMB during normal growth conditions it was shown that LMB could inhibit stimuli-dependent increases in cytoplasmic accumulation. It was demonstrated that the cytoplasmic increase in HuR following heat shock was mediated by increased interaction with pp32 and APRIL and that this increased interaction was sensitive to LMB, thus indicating that CRM1-mediated export of HuR is condition specific and that another pathway may direct HuR export under normal conditions (Gallouzi et al., 2001). It has also been suggested that HuR's relatively increased cytoplasmic levels in oral cancer cells versus normal cells is not CRM1 dependent and may occur through an unnamed alternative export pathway, however, differences in import between the cancer cells and normal were not ruled out either (Hasegawa et al., 2009). Interestingly, RRM3, in addition to the HNS is required for interaction with pp32 and APRIL in CRM1 dependent export while the HNS alone was shown to be sufficient for nucleocytoplasmic shuttling in heterokaryons, thus, further suggesting that there may be alternate, CRM1-independent nuclear export pathways.

Intriguingly, transportin 2, which interacts with HuR, has been shown to be a nuclear export factor for mRNA raising the possibility that it may function as the HuR CRM1-independent nuclear export pathway (Shamsher et al., 2002). Furthermore, transportin 2 interaction with HuR is disrupted by heat shock allowing for the possibility that transportin 2 could function in export under unstressed conditions while CRM1 directs nuclear export during heat shock (Gallouzi and Steitz, 2001).

As previously mentioned, there are numerous conditions that alter HuRs cellular localization including, but not limited to: heat shock, oxidative stress, nutrient deprivation, cytokine/growth factor treatment, hypoxia, cellular differentiation, cell cycle progression and Pol II inhibition. These stimuli activate numerous different signaling pathways, some of which have been demonstrated to directly modify HuR. These posttranslational modifications of HuR include phosphorylation, arginine methylation, ubquitination and caspase-mediated cleavage.

Phosphorylation of serine/theronine residues are the most common of the known posttranslational modifications of Hu proteins, and they are mediated by at least 5 different kinases including Chk2, Cdk1, PKC-alpha, PKC-delta and p38 MAPK (Figure 1). Two other kinases, AMPK and casein kinase 2 (CK2), have been shown to regulate HuR localization but have not been shown to directly phosphorylate HuR (reviewed in Doller et al., 2008; Chemnitz et al., 2009). AMPK has been demonstrated to increase HuR nuclear import at least in part through phosphorylation of importin alpha-1 (Zho et al., 2008). CK2 has been shown activate HuR CRM1-dependent nuclear export through phosphorylation of the HuR export adaptor APRIL (Chemnitz et al., 2009). Of the kinases that directly phosphorylate HuR, both protein kinase C (PKC) alpha and delta have been shown to alter cellular distribution of HuR in a phosphorylation-dependent manner, with PCKalpha being down stream of AMPK (Doller et al., 2007; Doller et al., 2008; Doller et al., 2010). The G2-phase kinase, Cdk1, has been shown to increase nuclear levels of HuR through phosphorylation of residue S202 located in the linker region of HuR causing increased association with the nuclear phosphopeptide binding protein, 14-3-3theta (Kim et al., 2008). The DNA damage kinase Chk2 has been shown to phosphorylate HuR at several residues and this often correlates with increased cytoplasmic export (Abdelmohsen et al., 2007). However, it has been reported recently that gamma irradiation, which activates Chk2, can phosphorylate HuR and alter its function without effecting its cellular distribution (Masuda et al., 2011). This is in direct conflict with a previous study that showed that gamma irradiation increases cytoplasmic localization of HuR (Lafarga et al., 2009). While one study observed an effect on cellular distribution found HuR localization at 30 minutes after irradiation (Masuda et al., 2011), the other study that noted a difference looked at 2 and 4 hours post irradiation (Lafarga et al., 2009). Furthermore, there was a more pronounced cytoplasmic distribution at the later time point suggesting that gamma irradiation may require longer than 30 minutes to alter HuRs cellular localization. Interestingly, the Lafarga study found that gamma irradiation directly phosphorylated HuR on one of the same residues investigated in the Masuda paper, namely T118, but showed that this phosphorylation was dependent on p38 MAPK rather than Chk2. p38 MAPK and the downstream kinase MK2 have previously been reported to increase cytoplasmic levels of HuR through unidentified mechanisms (Tran et al., 2003; and refrences in Doller et al., 2008). It is likely that other kinase signaling pathways modify HuR and concomitantly altered its cellular distribution as evidenced by the discovery that serine residue 242 alters HuRs localization but the kinase responsible has yet to be identified (Kim et al., 2008).

Interestingly, many of these signaling events not only redistribute HuR, but alter the stability of target mRNAs, and furthermore, some of these signaling events even change the subpopulations of mRNAs that are associated with HuR (reviewed in Doller et al., 2008). The posttranslational modifications that alter HuR association with mRNAs are unsurprisingly located within each of the RRM domains, with the exception of S100 that is located in the small linker between RRM1 and RRM2. Also unsurprisingly, all of the modified residues that are located within the HNS induce changes in HuR localization, but have no known effects on RNA binding, which is consistent with the known function of the HNS. Interestingly, at least three of the modified residues (T118, K182 and S318) found in RRMs also effect HuR localization possibly hinting at roles of the RRMs in subcellular of Hu proteins (Figure 1). The diversity of the functions and locations of posttranslational modifications of Hu proteins suggests that these signaling events may produce a vast, varied and tunable response by which Hu proteins can alter gene expression to produce desired cellular and phenotypic responses.

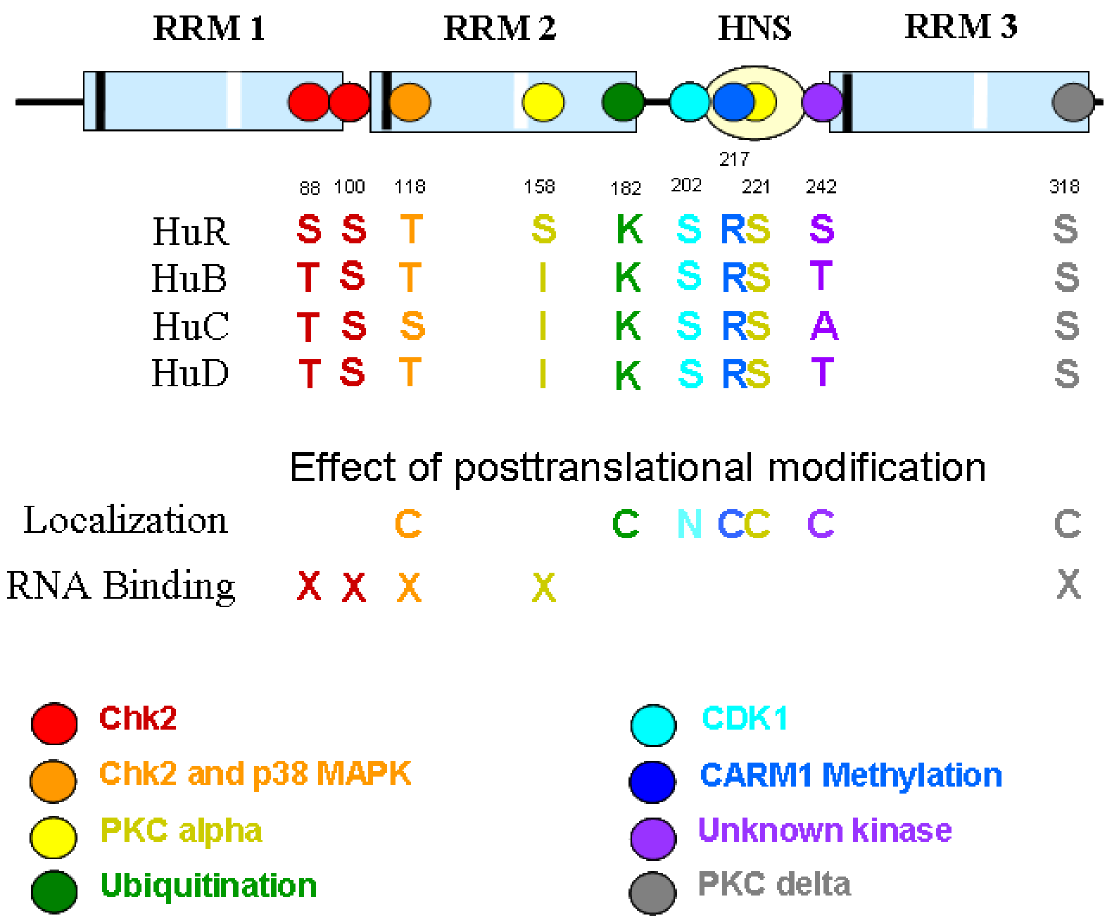

Figure 1: Summary of HuR's posttranslational modifications and the effects of these modifications.

The line and box model at the top of the figure is a scale representation of HuR's primary amino acid sequence. The light blue boxes indicate the RRMs with the vertical black lines and white lines representing RNP-2 and RNP-1, respectively. The colored circles represent sites of posttranslational modification and are coded by color to indicate the pathways known to modify the residue. The numbers indicate the amino acid position of the site of modification. The identity of the amino acid residues for each of the Hu protein family members at each of the given sites is indicated below the numbers. The effects associated with posttranslational modification at the given sites are listed below that, with 'C' representing an increase in cytoplasmic localization of Hu protein, 'N' representing an increase in nuclear localization and 'X' representing a change in RNA binding ability.

1.10 Hu Protein Autoregulation

One of the most intriguing aspects of Hu protein regulation is that it has been suggested that Hu proteins are capable of regulating their own posttranscriptional expression, this process of self regulation is often referred to as autoregulation. The first reported indication that Hu proteins may regulate their own expression was suggested from the observation that mouse HuB could bind multiple regions of its own 3′ UTR sequence, however this study did not investigate any functional effects of this binding event (Abe et al., 1996). It was also shown that immunoprecipitation of HuR could greatly enrich its own mRNA, even in the presence of EDTA. Furthermore, *in vitro* biotinylated RNA derived from a sequence in HuR's own 3′UTR was able to pull down HuR protein (Pullmann et al., 2007). Incidentally, that sequence, although the authors made no mention of it, is from an alternative 3′UTR that is predominately found in neuronal tissue and is much longer than the normal 3′UTR (Atasoy et al., 1998). Thus it is likely that there are multiple regions in the mRNA that are bound by HuR because the HuR IP that brought down its own mRNA was performed in HeLa cells, which presumably do not contain the alternative 3′UTR. Another study also found that HuR could also interact with its own mRNA through a sequence in the neuronally expressed alternative 3′UTR, however they used a different element than the one previously reported to interact with HuR. Interestingly, they also showed that HuR could increase expression of a reporter containing this sequence but it had no effect on a reporter containing only the upstream, common 3′UTR sequence found in most cell types (Al-Ahmadi et al., 2009). These studies suggest that HuR is capable of regulating itself through a feed-forward loop only when the longer, alternative, neuronally expressed 3′UTR is present. Intriguingly, the Al-Ahmadi study also showed that the 3′UTR sequence that can be regulated by HuR was inherently unstable under normal conditions when HuR protein is readily detectable, thus possibly indicating that while HuR can stabilize this reporter mRNA that it normally does not stabilize its own endogenous message.

Further evidence for Hu protein autoregulation has been performed in flies with the homologs *elav* and *fne*. It was noticed that a single copy of *elav* missing part of the 3′UTR was able to rescue *elav* null flies while two copies of *elav* without a full 3′UTR were lethal (Samson, 1998). However, two copies of *elav* containing the full 3′UTR were viable thus suggesting that sequences in the 3′UTR regulate the amount of *elav* produced and that excess *elav* was lethal. Interestingly, it was also observed that there was an inverse correlation between expression from the endogenous *elav* locus and expression of an *elav* minigene missing the 3′UTR and this was dependent on the presence of the 3′UTR in the endougenous *elav*. This suggested that *elav* could feed-back and down regulate its own expression through sequences in the 3′UTR (Samson, 1998; Borgeson and Samson, 2005). Similarly, another fly Hu protein family member, *fne*, has been shown to reduce its own endogenous levels as well as those of *elav* (Samson and

Chalvet, 2003). These fly studies show a negative feed-back by Hu proteins on their own mRNA while the mammalian studies showed that HuR is capable of forming a positive feed-forward loop that is cell type restricted to neurons. This difference in Hu protein autoregulatory circuits may be a species specific difference although given the neuronal restriction of the mammalian circuit the difference may also be cell type dependent.

2. Investigation of HuR regulated ARE-BP expression

2.1 Background and Significance

HuR is an ubiquitously expressed RBP that is traditionally thought to increase mRNA stability and translation of bound transcripts. HuR binds to these transcripts through specific interactions with single stranded U-rich RNA elements. Frequently, these RNA elements are referred to as AU-rich elements (AREs) and the RBPs that bind them are called ARE-BPs. HuR is one of the most intensely studied ARE-BPs, of which there are more than a dozen in mammals and include both TTP and BRF1. TTP and BRF1 are from the same family of RBPs that are characterized by tandem CCCH zinc finger RNA binding domains. While HuR can increase the translation and mRNA stability of ARE-containing mRNAs, TTP and BRF1, as well as almost every other ARE-BP, decreases the expression of ARE-containing mRNAs.

It has previously been observed that the mRNAs of other RBPs are frequently overrepresented in the mRNA targets of HuR (Mukherjee et al., 2009). Similar observations have also been made for the mRNA targets of other RBPs. The concept that individual RBPs may regulate the expression of other RBPs or transcription factors and thus influence a wide range of gene expression programs is referred to a the regulators-of-regulators concept (Mansfield and Keene, 2009).

Other studies have shown that several ARE-BPs can bind to the 3'UTRs of other ARE-BP mRNAs as well as their own 3'UTR (Pullmann et al., 2007). This suggests that

ARE-BPs as a family may participate in a regulators-of-regulators network to feedback and control the expression of each others mRNAs. We have proposed to investigate whether HuR can regulate the expression of the ARE-BPs TTP and BRF1 as well as its own mRNA.

2.2 Materials and Methods

2.2.1 Cell Culture

HEK293T cells were cultured in DMEM supplemented with 10% FBS. NIH/3T3 cells were cultured in DMEM supplemented with 10% BCS. Jurkat cells were obtained from ATCC and cultured in RPMI supplemented with 10% FBS. RAW264.7 cells were obtained from ATCC (catalog number TIB-71) and cultured in DMEM supplemented with 10% FBS and were passaged by dislodging cells with a cell lifter.

Transfections were performed with Lipofectamine 2000 (Invitrogen) using a slightly modified protocol. Briefly, 1ug of total DNA was diluted into 100ul of Opti-MEM I (Gibco) and mixed with 2.5ul of Lipofectamine 2000 diluted in 100ul of Opti-MEM I and incubated at room temperature for 30 min. The DNA/Lipofectamine 2000 complexes were added to cells.

Jurkat cells were activated with 50ng/ml PMA and 2ug/ml PHA (Calbiochem) for 12 hours. Cells were treated with 50uM resveratrol for 2 hours prior to PMA/PHA activation. RAW264.7 cells were treated with 0.1 ug/ml of lipopolysaccharide (Sigma).

2.2.2 Plasmids and primers

Luciferase constructs were created by directionally inserting 3'UTR fragments into the pLuc vector using NotI and ApaI resticition sites. The 3'UTR fragments that were inserted into pLuc were created by PCR cloning into pGEM-T.

2.2.3 RNA Immunoprecipitations

Lysates were prepared as previously described (Keene et al., 2006). Immunoprecipitations were performed using 25ul packed bead volume of Protein A Sepharose beads coated with either a monoclonal anti-HuR (3A2) antibody, or mouse IgG. Each primer set was normalized to GAPDH and this ratio was compared to the same primer set ratio for NMS IPed samples to give the fold enrichment.

2.2.4 RNA electrophoretic mobility shift assay

Radiolabeled RNA (100 pmoles) was incubated with indicated amounts of recombinant HuR in binding buffer (15 mM HEPES pH 7.4, 150mM KCl, 5mM MgCl2 and 10% glycerol) with 1ug tRNA as a non-specific RNA competitor for 30 min at 4 degrees. Loading buffer was added to the complexes and immediately loaded and run at 200V in a cold room on a pre-chilled non-denaturing 10% acrylamide TBE gel.

Recombinant HuR protein was made by growing pGEX-HuR in BL21 *E.coli* to an optical density of A(600) = 0.6 and then protein synthesis was induced with 1mM IPTG for 5 hours at 30 degrees. GST-fusion proteins were purified using the GST spin purification kit (Pierce).

2.2.5 Luciferase assays

Luciferase constructs were generated by inserting 3'UTR fragments into the pLuc vector. Equimolar amounts of the various UTR luciferase reporters were transfected at a ratio of 2:1, Firefly (20 pmoles) to Renilla (10 pmoles), to account for differences in length of UTR sequence. siRNAs were transfected 24hours prior to luciferase transfection using Lipofectamine 2000 (Invitrogen) with 20 pmoles siRNA per well. Luciferase assays were performed using the Dual-Luciferase Reporter Assay system (Promega) according to manufacturer's instructions. Cells were washed with PBS and lysed in 500ul of 1x Passive Lysis Buffer.

2.3 Results

2.3.1 Association of HuR with mRNAs of ARE-BPs

We began our study by investigating whether HuR protein interacted with the mRNAs of a small, select group of ARE-BPs including TTP, BRF1 and HuR's own mRNA. Each of the three mRNAs contain within their 3'UTRs multiple U-rich sequences, including several canonical AREs consisting of AUUUA pentamers. Many of these ARE sequences are located in conserved regions of the 3'UTRs suggesting that the elements are functional and have been evolutionarily selected based on their regulatory function. The presence of these conserved ARE sequences in the 3'UTRs further suggest that ARE-BPs, such as HuR, may be capable of interacting with each of the mRNAs encoding TTP, BRF1 and HuR. To investigate the ability of HuR to bind to each of these mRNAs *in vivo* we initially immunoprecipitated (IPed) endogenous HuR using a monoclonal antibody and looked for the mRNA of the three ARE-BPs by using RT-PCR. We observed that HuR could interact with its own mRNA and that of BRF1 (figure 2). Furthermore, HuR's association with both mRNAs was not effected by the addition of either EDTA or 1M urea suggesting that the interactions were robust and not due to indirect protein-protein interactions. The insensitivity to EDTA also indicates that enrichment of HuR mRNA in the HuR IP was not due to isolation of nascent HuR polypeptide and the associated mRNA template.

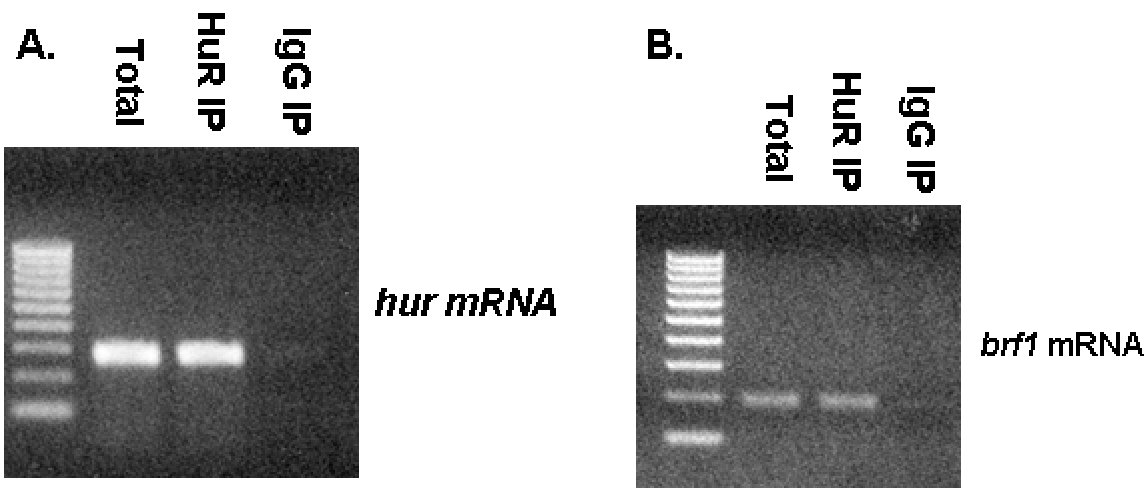

Figure 2: HuR associates with HuR's and BRF1's mRNA in HEK 293T cells.

Panel A) and B) respectively show end point HuR and BRF1 PCR products from HuR or control IgG RNA IPs. Panel A) was amplified for 22 cycles and panel B) was amplified for 28 cycles.

While we could indentify interactions between HuR protein and HuR or BRF1 mRNAs we did not initially observe any interaction with TTP mRNA, however TTP mRNA was not barely detectable in the total mRNA (not shown). Thus it is possible that HuR could interact with TTP in other cell types or in other conditions when TTP mRNA is expressed at higher levels.

Since HuR is known to interact with single stranded U-rich sequences we sought to demonstrate that HuR interacted *in vitro* with the U-rich elements in the 3'UTR of HuR's own mRNA. RNA probes consisting of roughly a couple hundred nucleotides centered around conserved U-rich regions were generated from two different portions of HuR's own mRNA that contained U-rich sequences. RNA EMSA assays were performed by incubating 100 pmoles of labeled RNA with recombinant HuR at 4 degreees in the presence of the non-specific competitor yeast tRNA. We saw that recombinant HuR protein could shift both of the U-rich RNA probes from HuR's own 3'UTR (figure 3). Increasing amounts of recombinant HuR caused quantized increases in the shifted complexes, indicative of multimerization. This is consistent with previous observations that Hu proteins can form RNA-dependent multimers (Gao et al., 1994). This suggests that there are multiple RNA elements that are recognized by HuR with in each of these 3'UTR fragments.

Since interaction between HuR and TTP mRNA was unable to be observed in 293T cells we decided to investigate whether the ARE sequence of TTP mRNA could interact with recombinant HuR in RNA gel shift assays. A 90 nucleotide sequence of TTP's 3'UTR that contained multiple ARE elements was used in RNA EMSAs as described above. Surprisingly, we did not observe any shift of the TTP 3′UTR ARE probe with increasing amounts of recombinant HuR protein (figure 4). This suggests that HuR does not bind with high affinity to ARE sequences in TTP's 3′UTR, although it is possible that HuR may still be capable of interacting with TTP mRNA through other sequences in the 3′UTR. Since we were able to observe an interaction with multiple U-rich sequences in HuR's own 3′UTR but no interaction with AREs in TTP's 3′UTR this demonstrates that these RNA EMSAs are capable of determining specific interactions with very little non-specific background binding.

Together this data suggests that HuR is able to recognize multiple U-rich elements in the 3′UTR of HuR's own mRNA. Thus, HuR can not only bind these elements *in vitro* but can also bind the mRNAs within a cellular context, as seen in the RNA IPs. In agreement with these results, previous studies have shown interaction of HuR with its own mRNA, however the particular elements investigated for binding were different than used in the present study (Pullmann et al., 2007; Al-Ahmadi et al., 2009). Our results also indicate that HuR does not directly interact with ARE sequences in TTP's 3′UTR contrary to previously published results (Lin et al., 2007). However, the ARE sequences used in the previous study are larger and contain distinctly different RNA sequences raising the possibility that HuR can interact with some regions of TTP's 3′UTR but not other U-rich regions.

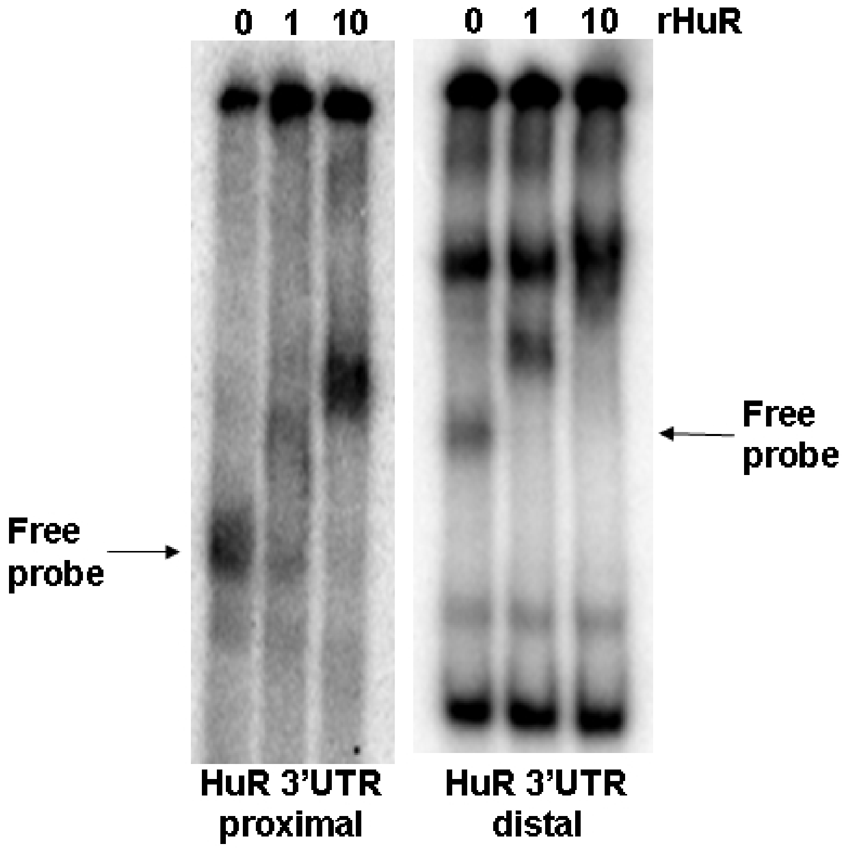

Figure 3: HuR can bind to multiple elements in HuR's 3'UTR.

RNA electrophoretic mobility shift assays performed with 100 pmoles of indicated P32 labeled RNA probes. Amounts of recombinant HuR added in ng are indicated at the top of the gel. After 30 minute incubation complexes were separated on native 10% polyacrylamide TBE gels. Free probe is indicated by arrows and shifted complexes are listed above.

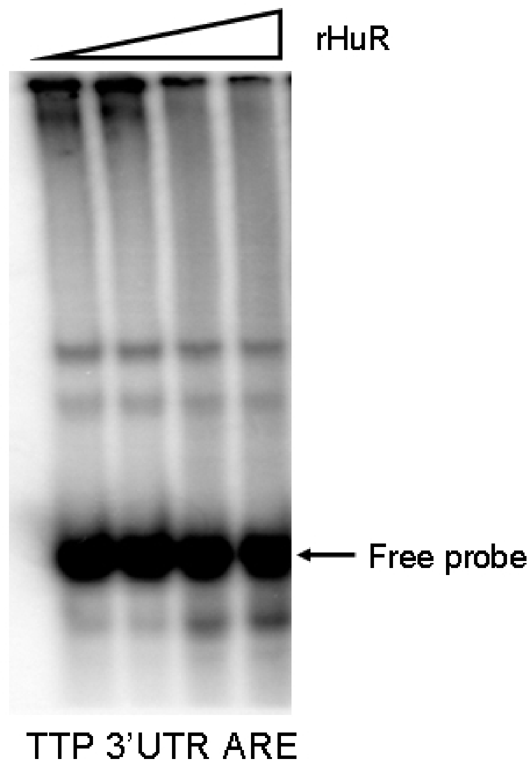

Figure 4: Recombinant HuR does not bind TTP 3'UTR ARE RNA *in vitro*.

RNA electrophoretic mobility shift assay performed with 100 pmole of p32 labeled TTP 3’UTR ARE probe. Increasing amounts from 1 to 100ng of GST-purified recombinant HuR were added.

2.3.2 Effects of HuR on the mRNA stability and translation of ARE-BPs

Since the three selected ARE-BPs, TTP, BRF1 and HuR, each contain AREs in their 3’UTRs and at least some of them were capable of interacting with HuR we investigated the effect HuR would have on the posttranscriptional expression of each of the mRNAs. 3’UTRs are thought to be involved predominately in regulation of mRNA stability and translation of mRNAs, so we focused on these aspects of HuR’s multi-faceted regulatory abilities. To achieve this we set out to measure total mRNA and protein levels of each of the three ARE-BPs in cells overexpressing HuR. Unfortunately, as mentioned above, we could not detect TTP protein in resting cells and furthermore antibodies for BRF1 are not specific enough. This meant that we could only investigate changes in mRNA of the three ARE-BPs and only protein levels of HuR in resting cells. It should be noted that while the extremely low levels of TTP mRNA in 293T cells was not enough to be detected in RIP assays that it was enough to detect total levels by RT-PCR. Since overexpression of HuR presents unique challenges in detection of endogenous HuR, namely that most reagents will detect both and thus regulatory effects may be masked by exogenously introduced protein. Thus, to study the effects of exogenous HuR overexpression on endogenous HuR protein we used a tagged version that migrated several KDa higher than endogenous HuR. Also to study the effect of HuR overexpression on mRNA levels we generated a primer set that included sequence from the 3'UTR that was not present in the overexpression construct. Overexpression of HuR did not result in robust changes in total mRNA or protein of any of the investigated ARE-BPs levels (figure 5). However, overexpression of HuR did result in a mild increase of TNF mRNA indicating that over expression of HuR was functional.

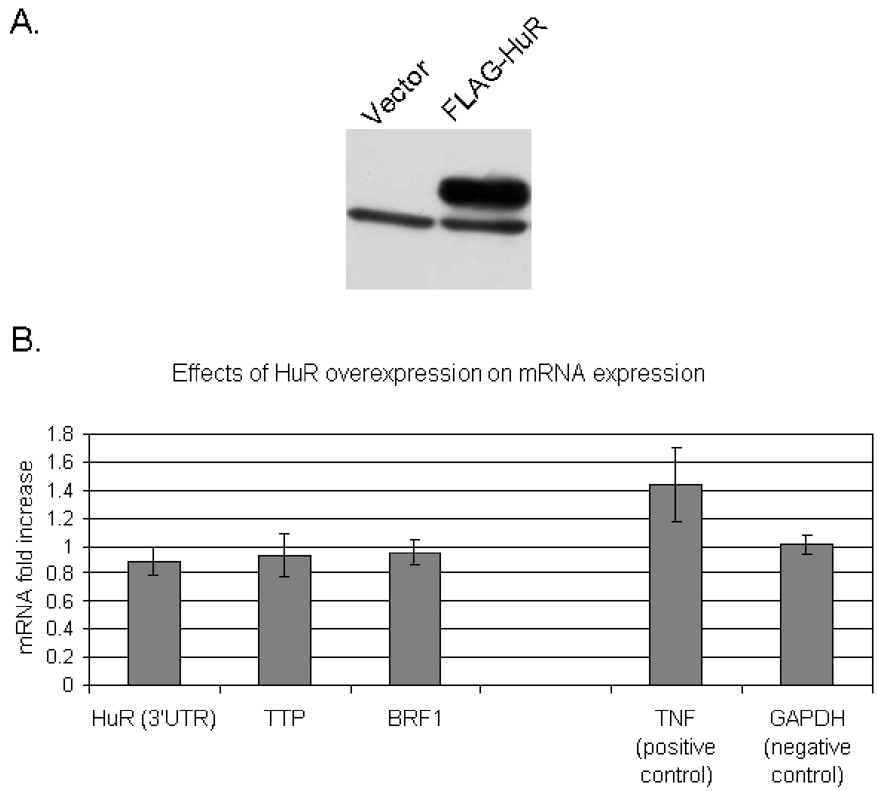

Figure 5: HuR overexpression does not dramaticaly alter endogenous HuR protein levles or mRNA levels of ARE-BPs.

Panel A) is a Western blot of HEK293T lysates transfected with either a FLAG tagged HuR construct or a vector only control and was immunoblotted with a monoclonal HuR antibody (3A2). Panel B) is a bar graph demonstrating effects of overexpressing HuR on mRNA levels of indicated genes as detected by RT-PCR. Values represent the ratio of normalized amounts for HuR overexpressed samples to control vector transfected samples to determine fold induction, and represent the average value over six independent experiments. Fold induction values above 1 indicate an increase in mRNA, while values below 1 indicate a decrease.

Changes in steady state mRNA levels, as those we investigated after overexpression of HuR, are a combination of changes in transcription rates and mRNA decay rates. Since HuR has purported roles in influencing mRNA stability we decided to see if there may be changes in the mRNA stability that were masked by opposite changes in transcription. To achieve this we treated cells overexpressing HuR with Actinomycin D to inhibit transcription for various times up to 6 hours and harvested RNA. We then measured the levels of TTP mRNA and BRF1 mRNA and normalized to GAPDH, a relatively stable mRNA that does not diminish dramatically after transcriptional inhibition. We did not notice any robust changes in the decay rates of TTP or BRF1 mRNA levels, although we only saw a mild change in the decay rate of TNFalpha mRNA (not shown).

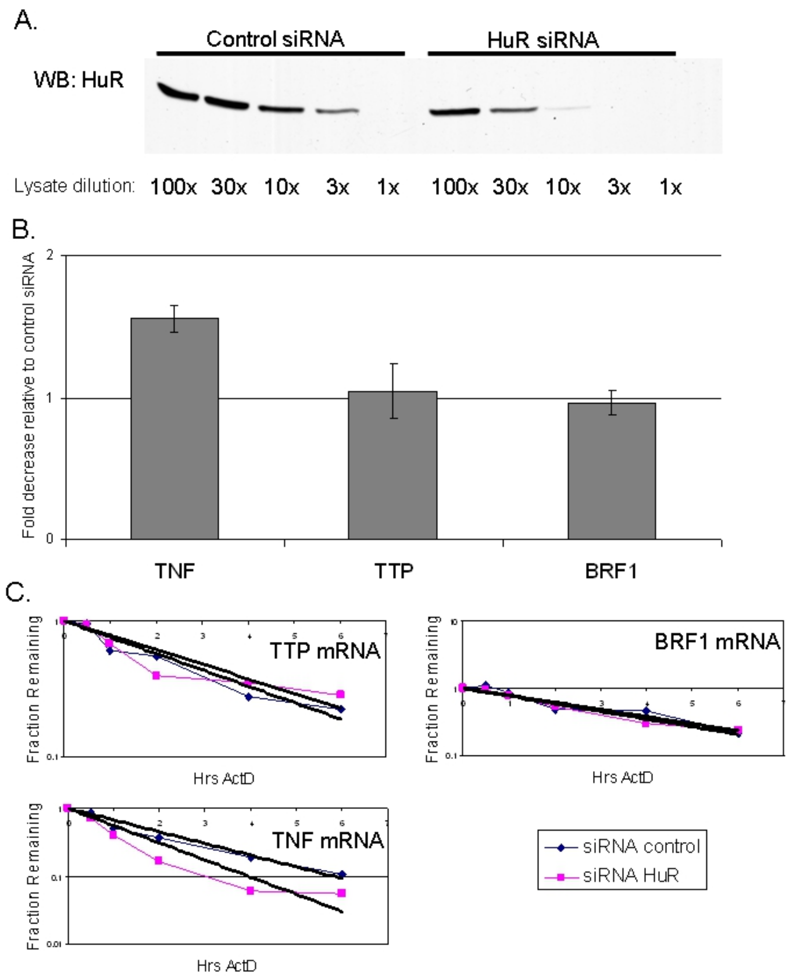

Figure 6: HuR knockdown does not dramatically alter levels of TTP and BRF1 mRNA in HEK 293T cells.

Panel A) is a HuR Western blot of siRNA knockdown. Lysates from HuR or scrambled oligo (control siRNA) knockdowns were diluted along a range of 100 fold. Intensity of band in 30x of HuR siRNA lane is somewhere between 10x and 3x of control siRNA indicating between a 67% and 90% knockdown. Panel B) indicates fold decrease of indicated mRNA as detected by RT-PCR following HuR siRNA knockdown compared to control siRNA knockdown. Values represent average of 3 independent experiments. Values above 1 indicate a decrease in expression in knockdown cells and values below 1 indicate an increase. Panel C) shows Actinomycin D decay curves of indicated mRNAs from cells treated for 72 hours with either HuR siRNA or scrambled oligo control siRNA. Values are percent remaining of transcript at given time after Actinomycin D treatment compared to starting levels (t=0).

HuR is an abundant protein, with some reports claiming over 4 million molecules of HuR protein per cell (Lebedeva et al., 2011). Therefore, HuR may already be present at levels high enough to bind most potential targets and thus overexpression may not significantly alter its function impact on most targets. While overexpression of HuR in these cells may not be enough to drive changes in expression of the ARE-BPs, we may be able to notice differences following a decrease in the level of HuR. To achieve this, we knocked down HuR levels using siRNA and investigated its effects on BRF1 or TTP mRNA levels and mRNA stability. HuR is a very stable protein, so to achieve significant knockdown, cells were treated with siRNA for 72 hours. This resulted in about a 25% reduction in HuR levels compared to a scrambled oligo control (figure 6A). However, this too did not produce robust effects on the total levels or stability of BRF1 or TTP mRNA (figure 6B, C). To ensure that knockdown of HuR was capable of producing an effect on stability of other HuR targets we calculated changes in half-lives for the well known HuR target TNFα. Knockdown of HuR mildly decreased the half-life of TNFα thus indicating that knockdown could produce observable changes in mRNA stability for at least one target mRNA. The knockdown experiments agree with the HuR overexpression experiments that HuR does not drastically effect the expression of TTP, BRF1 or HuR in unstressed HEK293T cells.

2.3.3 Effects of HuR on ARE-BP 3'UTR reporters

Hu proteins can effect translation in addition to mRNA stability of ARE containing mRNAs. Since detection of endogenous protein was difficult for TTP and BRF1 due to low expression or low antibody specificity we decided to generate a set of luciferase reporters to measure the effects HuR would have on mRNA stability and translation of transcripts containing the 3'UTR sequences of TTP, BRF1 and HuR. The luciferase reporters we generated contained the coding sequence of firefly luciferase under the control of gene-specific 3'UTR sequences. In addition to creating full-length 3'UTR sequence reporters we also created reporters that contained only isolated fragments of the 3'UTRs with the hope that this may allow us to zero in on regions under the control of HuR without having to worry about interference from potentially compensatory effects mediated by other elements. To control for off-target effects of HuR overexpression/knockdown on the luciferase system we also used a firefly luciferase reporter that lacks a 3'UTR sequence. Furthermore, we used a second luciferase construct that expresses a renilla luciferase gene, this allows us to normalize across our samples for differences in loading or transfection. Finally, we also created a reporter containing the ARE from the reputable HuR target, TNFα, to act as positive control for HuR function.

We first tested the effects of overexpression of HuR on the luciferase reporters. We observed that overexpression of HuR moderately decreased the amount of luciferase produced from the TNFα ARE reporter (figure 7A). Furthermore, the HuR 3'UTR reporters were also for the most part modestly decreased following HuR overexpression, with some showing up to a 20% reduction in expression, however some of these effects were small enough that there were not resolvable from the experimental variance of the assay (figure 7A). To ensure that HuR was overexpressed in these experiments we ran Western blots on these experiments and noted that exogenous HuR was expressed at least as robustly as endogenous HuR and oftentimes to a much greater extent. The effect of overexpression of HuR on the TTP and BRF1 3'UTR reporters was also very slight with nearly all the changes in expression being indistinguishable from experimental variance. However, the trends seen for the TTP and BRF1 3'UTR reporters looked different than the trends seen for HuR 3'UTR reporters, most notably TTP and BRF1 reporters displayed a slight increase while the HuR 3'UTR reporter slightly decreased with overexpression of HuR. While the observed effects of overexpression of HuR on the TNF and HuR 3'UTR reporters was slight, the direction of change was opposite of the conventional role that HuR plays as a positive regulator of posttranscriptional gene expression. However, it has been reported that in certain conditions or in particular cell types HuR can occasionally act to suppress posttranscriptional gene expression of a few transcripts (Katsanou et al., 2005; Kim et al., 2009).

To confirm HuR's non-canonical role in repressing the expression of some of the 3'UTR reporters we decided to investigate the effects of HuR siRNA knockdown on the expression of the luciferase reporters. Shockingly, these very same reporters that were repressed by overexpression of HuR, were also slightly repressed by HuR knockdown (figure 8A). Furthermore, HuR knockdown, just like HuR overexpression, caused a moderate increase in the expression of the TTP and BRF1 3'UTR reporters, with only the full-length BRF1 reporter increasing enough to be distinguishable from experimental noise. The only consistency between the overexpression and knockdown experiments is that HuR 3'UTR is regulated in the opposite direction than TTP and BRF1 3'UTR, otherwise these experiments appear to contradict each other with both overexpression and knockdown causing identical directional changes in expression. This paradox raises the possibility that the assay may not be reliable.

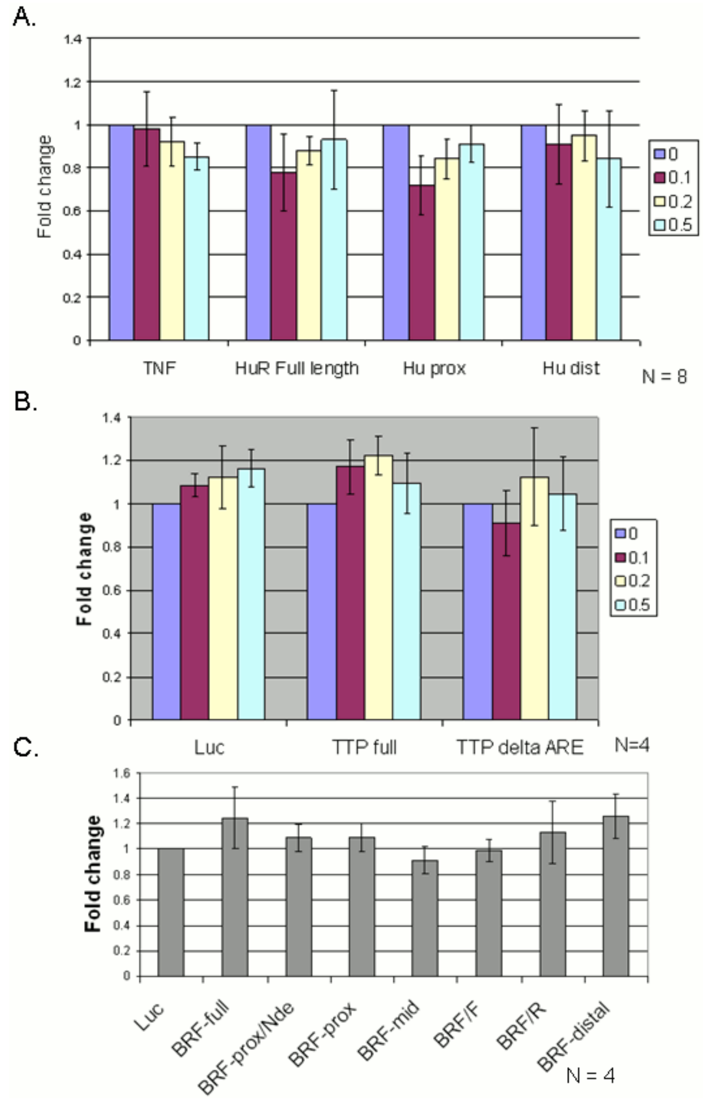

Figure 7: Overexpression of HuR has mild effects on ARE-containing reporters.

Graphs show effects of overexpressing HuR on the relative fold change of indicated 3'UTR reporters. All luciferase values are normalized to renilla luciferase, a loading control. Fold change is expressed as the fold difference relative to the vector only control for each luciferase construct. Panel A) TNF and HuR 3'UTR reporters, panel B) TTP 3'UTR reporters and panel C) BRF1 3'UTR reporters. 'Full' reporters indicate full-length 3'UTR sequence, all others indicate a fragment of the 3'UTR sequence. Panel A) and B) show increasing amounts of HuR overexpression in ug, panel C) is for 0.1ug HuR.

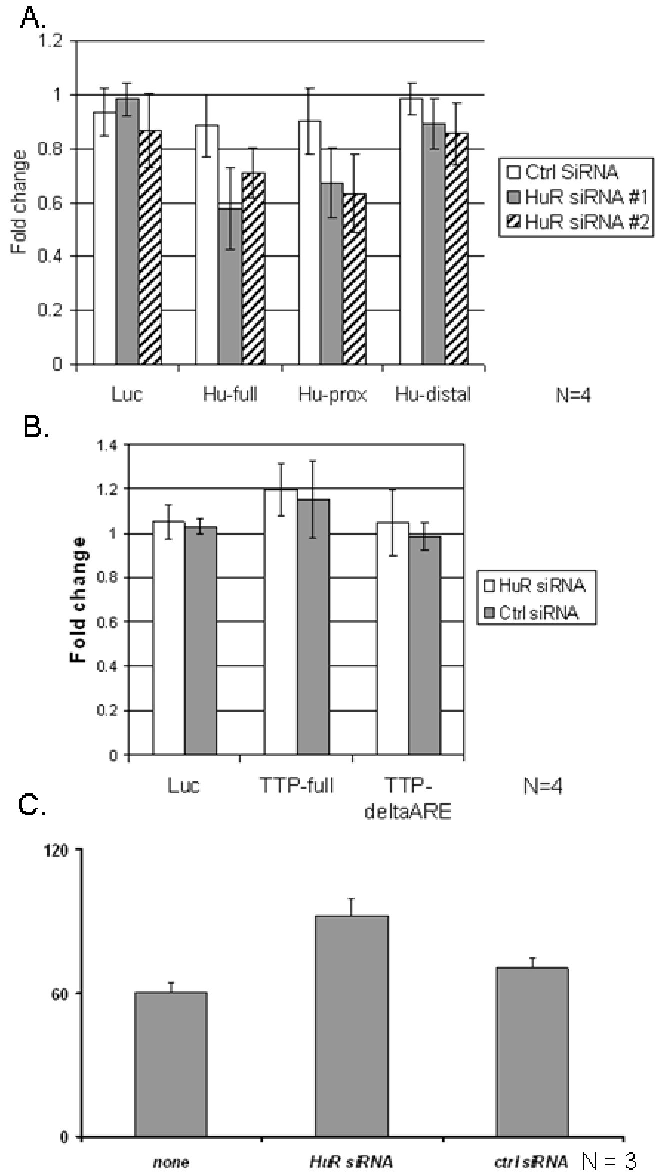

Figure 8: HuR knockdown has mild effects on ARE-containing 3'UTR luciferase reporters.

Graphs show effects of knocking down HuR on the relative fold change of indicated 3′UTR reporters. All luciferase values are normalized to renilla luciferase, a loading control. Fold change is expressed as the fold difference relative to 'no siRNA' control for each luciferase construct. Panel A) HuR 3′UTR reporters, panel B) TTP 3′UTR reporters and panel C) BRF1 full-length 3′UTR reporter. 'Full' reporters indicate full-length 3′UTR sequence, all others indicate a fragment of the 3′UTR sequence.

To test the reliability of the assay we took these same reporters with the same cellular and experimental setup and asked whether other RBPs besides HuR could alter the expression of the reporters. Since these reporters contain several U-rich and ARE sequences we decided to see if overexpression of TTP could regulate the expression of the reporters. The overexpression of TTP protein dramatically decreased (many fold) the expression of TNFα ARE, TTP 3'UTR, and to a lesser extent the BRF1 3'UTR reporter (figure 9). TTP is a well known activator of mRNA degradation of ARE-containing mRNAs and thus the results from these reporters agreed with the previously reported function of TTP. Furthermore, it has previously been reported that TTP can specifically regulate the mRNA stability of TNFα and it's own mRNA in an ARE dependent manner also in agreement with our observed effects. Since TTP, an ARE-BP, is able to repress the expression of these reporter constructs this demonstrates that the reporters are not unresponsive to overexpression of ARE-BPs and that the luciferase assay is functional.

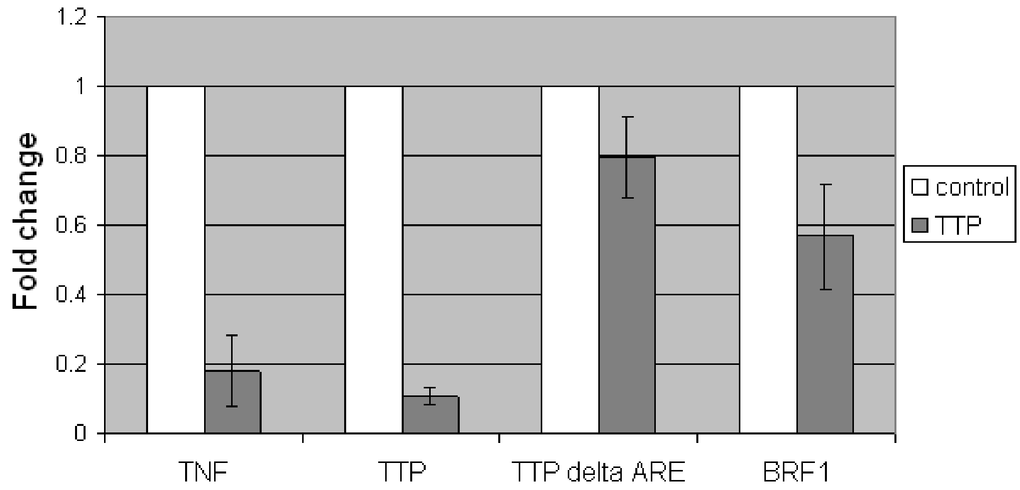

Figure 9: TTP and BRF1 3'UTR Luciferase reporters respond to overexpression of another ARE-BP.

Graph show effect of overexpressing TTP on the relative fold change of the indicated 3'UTR reporters. All luciferase values are normalized to renilla luciferase, a loading control. Fold change is expressed as the fold difference relative to vector only control for each luciferase construct. The 'TTP delta ARE' reporter is the same as the TTP reporter except that it is missing the ARE sequence.

Since the luciferase assay was properly functional we next focused on the HuR overexpression and siRNA constructs. Re-sequencing of the constructs failed to find any mutations but we still rebuilt the constructs from scratch and saw similar results in the luciferase assay as before (not shown). Using completely different overexpression vectors (different backbone, promoter, etc) and siRNAs targeted to completely different sequences within HuR, we once again saw nearly identical results to the previous luciferase experiments (not shown). Finally, we varied the amount of overexpression and the duration of knockdown to change the amount of HuR in the system. This produced some slight changes in the expression of the luciferase constructs but nothing that was distinguishable from the other induced changes (figure 7A, B). The sum of these experiments demonstrates that the odd effects seen by HuR manipulation on the luciferase constructs was not due to sequence, vectors, reagents, amounts used, or duration.

2.3.4 Effects of HuR on ARE-BP 3'UTR fragment reporters

The lack of evidence for large effects on gene expression by modulation of HuR levels in spite of its interaction with endogenous or reporter mRNAs combined with the incongruent results of knockdown and overexpression of HuR are puzzling. One possible explanation for this is that the full-length mRNA and 3'UTR sequences contain other cis-regulatory elements that could mask HuR dependent effects by opposite and compensatory effects. To test whether this is the case we generated reporter constructs that isolated potential regulatory elements from the rest of the 3'UTR sequence for each of the ARE-BP 3'UTRs.

Much like the full-length 3'UTR luciferase reporters the 3'UTR fragment reporters were not drastically responsive to HuR overexpression or knockdown (figure 7 & figure 8). However, if one lowers expectations for robust, statistically significant or meaningful changes for a minute, it is easy to see that some of these 3'UTR fragments undergo changes more like the full-length 3'UTR reporters while the reciprocal fragments are much less responsive. For example, the proximal 3'UTR fragment of HuR shows a pattern of HuR-mediated repression that is similar to full-length 3'UTR but the distal 3'UTR fragment, which contains the other half of the full HuR 3'UTR sequence, is relatively much less responsive to HuR overexpression, especially at 0.1 ug HuR (figure 7A). This is also true when comparing the full-length BRF1 3'UTR with the distal fragment versus the proximal and mid fragments (figure 7C). For TTP the loss of the ARE abolishes what little response was observable for the full-length 3'UTR reporter. These 3'UTR fragment results are suggestive of potential 'regulatory' sites but unfortunately these fragments are not any more responsive to HuR overexpression or knockdown than full-length 3'UTR reporters.

2.3.5 Dependence of cell type and stimuli on effects of HuR on ARE-BP reporters

In the experiments described previously, the results are consistent and reproducible, however the results are different from HuR's dogmatic role as a positive and potent regulator of mRNA stability and translation. These differences between the observed function and the literature reported function may be due to differences in cell type or cellular conditions. So we sought to test whether effects of modulating HuR levels on the ARE-BP luciferase reporters was cell type and/or stimulus-dependent. In the previously mentioned studies we used HEK293T epithelial cells, so we decided to examine a fibroblast line, NIH3T3, as well as two different types of immune cells including Jurkat T-cells and RAW264.7 macrophages. These cell lines were selected because ARE-BPs are known to be important regulators of innate and adaptive immune responses though both professional and non-professional mediators of inflammation, thus this limited but diverse cohort of cells represents a likely set for finding HuR-dependent responses. We introduced the full-length 3'UTR reporters for HuR, TTP and BRF1 into each cell line and overexpressed either HuR or a vector control and compared the HuR overexpressed cells to the control cells to determine the fold change caused by HuR overexpression. HuR overexpression caused a slight decrease in the expression, depending on the cell line, of each of the reporters (figure 10). HuR's negative regulation of the HuR 3'UTR reporter is reminiscent to the effects we observed in the HEK293T cells, however HuR's negative regulation of the other 3'UTR luciferase reporters (TTP and BRF1) had the opposite effect that we observed for those reporters in the HEK293T cells. But somewhat depressingly, as in the HEK293T cells, the effects seen by HuR overexpression in each of the cells lines were not robust enough to easily distinguish the effects on the 3'UTR reporters from the effects on the luciferase control. This diverse panel of cell lines suggests that overexpression of HuR protein levels does not drastically alter the expression of TTP, BRF1 or HuR 3'UTR reporters in untreated, normal growth conditions for a wide range of cell lines.

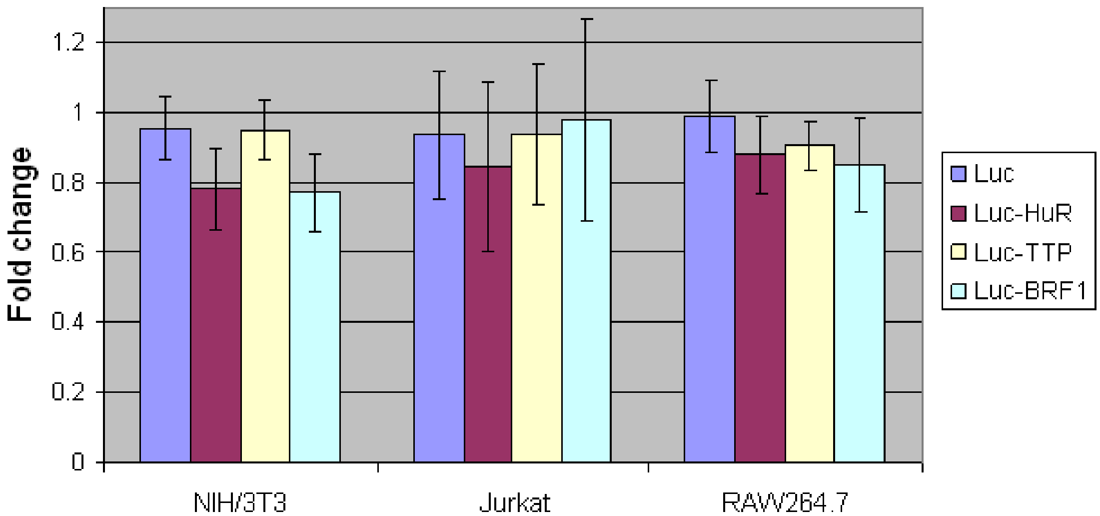

Figure 10: Effect of HuR overexpression on ARE-containing 3'UTR reporters in various cell lines.

This is a chart of effects of HuR overexpression on the expression of full-length 3'UTR luciferase constructs in three different cell lines. Fold change is calculated by comparing HuR overexpressing cells to the control cells for each cell line and luciferase construct.

While HuR does not significantly alter the expression of the reporters in untreated cells it is possible that HuR regulation of these ARE-BP 3'UTR reporters requires activation of other signaling pathways. Several lines of evidence suggest that this may be a fruitful approach. First, binding of Hu proteins to different subsets of mRNAs has been shown to be a dynamic and regulated event in response to external cellular stimuli (Tenenbaum et al., 2002; Abdelmohsen et al., 2007; Mazan-Mamczarz et al., 2008; Mukherjee et al., 2009; Masuda et al., 2011). Furthermore, TTP and BRF1 are often induced at the level of mRNA and protein following a wide variety of stimuli, including immune cell activation, and oftentimes this induction includes contributions from increases in mRNA stability. This is particularly interesting given HuR's rare role among ARE-BPs as a promoter of mRNA stability. Finally, there is evidence that HuR's function can be regulated in response to extracellular stimuli and it has been shown that multiple signaling pathways can directly modify residues of HuR (Li et al., 2002; Doller et al 2007; Abdelmohsen et al 2007; reviwed in Doller et al., 2008).

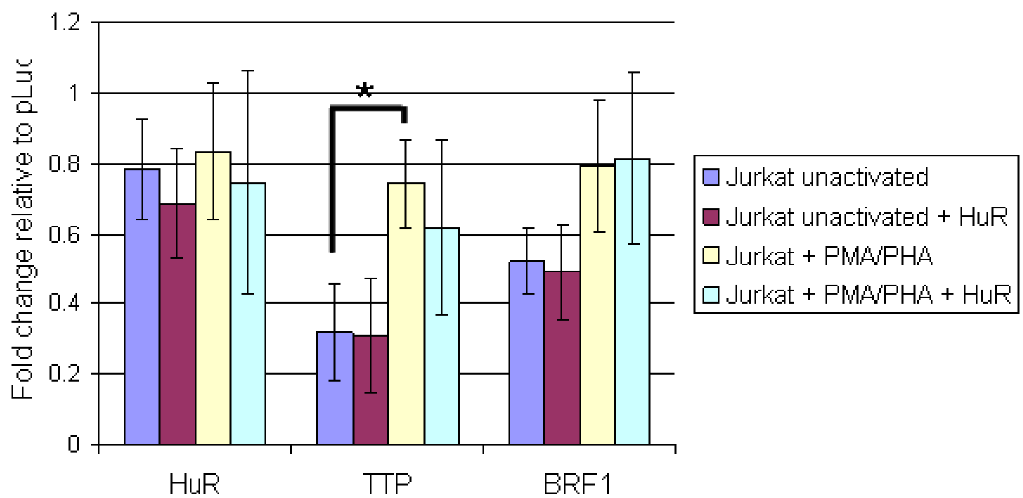

Figure 11: Jurkat activation on luciferase constructs.

Fold change of 3'UTR luciferase reporters compared to a control firefly luciferase reporter (pLuc) for each condition in Jurkat cells. All firefly luciferase values were normalized to renilla luciferase to control for loading. *$p<0.05$

A previous study has demonstrated that activation of Jurkat cells with PMA and PHA resulted in changes in mRNAs bound by HuR with some of the mRNAs increasing association and others decreasing (Mukherjee et al., 2009). Importantly these changes were not simply a result of changes in transcriptomic levels of the mRNAs. To investigate whether these conditions could also effect HuR dependent posttranscriptional regulation we introduced the ARE-BP 3'UTR reporters into Jurkat cells that were subsequently activated with PMA/PHA. Interestingly, both the TTP and BRF1 3'UTR reporters were responsive to PMA/PHA treatment and showed increased expression relative to a untreated conditions. However, overexpression of HuR during these conditions did not alter the expression of the ARE-BP 3'UTR luciferase reporters in any detectable way (figure 11). This was not true for all reporters though, we were clearly able to demonstrate that overexpression of HuR during Jurkat activation could antagonize resveratrol mediated repression of other 3'UTR reporters, including the well-known HuR targets TNFα and CSF2 (figure 12). This suggests that HuR overexpression has no discernable effect on 3'UTR reporters of the three ARE-BPs: TTP, BRF1 and HuR, in Jurkat activation.

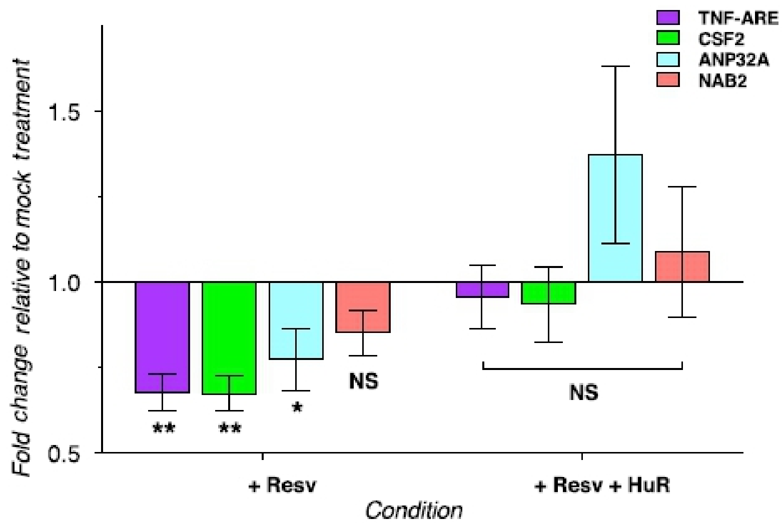

Figure 12: Overexpression of HuR during Jurkat activation is functional.

Fold change of 3'UTR luciferase expression in PMA/PHA activated Jurkats following either resveratrol treatment or reseveratrol treatment with HuR overexpression compared to mock treated cells. All firefly luciferase values were normalized to renilla luciferase. All 3'UTR reporters are from mRNAs that were identified as HuR targets from Jurkat RIP-Chips. *$p<0.05$, **$p<0.01$ Figure adapted from Mukherjee et al., 2009.

Besides Jurkat activation we also tested the effects of lipopolysaccharide (LPS) on RAW264.7 macrophages. After treatment of RAW264.7 macrophages with LPS, TTP and BRF1 undergo massive and rapid induction at both the mRNA and protein levels. First, we investigated whether HuR could associate with TTP or BRF1 mRNAs in either untreated or LPS stimulated RAW macrophages by RIP RT-PCR. We observed that HuR could associate with both TTP and BRF1 mRNAs in untreated or LPS stimulated RAW cells. LPS stimulation caused an increase in the association of both TTP and BRF1 mRNAs with HuR, and TTP went from barely detectable above background in untreated cells to robustly interacting with HuR in LPS stimulated RAW cells. Since HuR can associate with both TTP and BRF1 mRNAs we decided to investigate whether HuR could effect the posttranscriptional expression of either of the mRNAs. This was achieved like it was in the Jurkat experiments, where we introduced the 3'UTR reporters to the cells and activated with the appropriate stimuli. Also like the Jurkat experiments, we also observed an increase in expression of the luciferase 3'UTR reporter constructs for TTP and BRF1 following LPS stimulation compared to untreated macrophages. Overexpression of HuR in this context of LPS stimulated RAW macrophages showed a small, albeit statistically insignificant, increase in the expression of the TTP and BRF1 3'UTR reporters (figure 13B). Ecstatically, knockdown of HuR in RAW cells activated with LPS produced a relatively robust decrease in the expression of the TTP and BRF1 3'UTR reporters (figure 13B). HuR knockdown in untreated RAW macrophages only causes a slight, barely perceptible decrease in the expression of the TTP and BRF1 3'UTR reporters (not shown). The difference in the scale of the effects of HuR knockdown on the TTP and BRF1 3'UTR reporters in unactivated versus LPS activated RAW macrophages suggests that HuR is required for efficient posttranscriptional expression of TTP and BRF1 specifically in LPS stimulated RAW macrophages. Since knockdown of HuR produces a robust decrease on the expression of the TTP and BRF1 reporters but HuR overexpression only mildly increases the expression of the reporters, this may suggest that HuR could already be near saturation such that overexpression does not significantly alter the expression of TTP and BRF1.

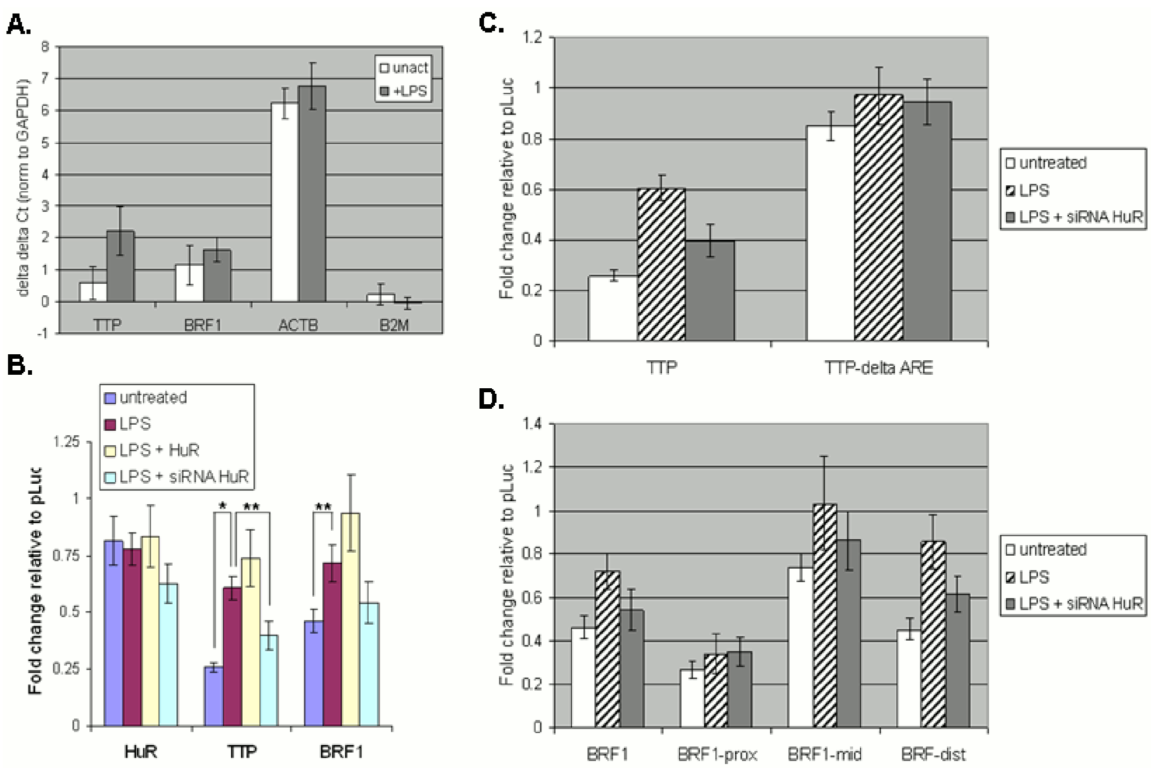

Figure 13: HuR associates with and regulates posttranscriptional expression of TTP and BRF1 mRNA during LPS activation of RAW macrophages.

Panel A) is a HuR RIP RT-PCR of indicated mRNAs from LPS activation or unactivated RAW macrophages. Deltadelta Ct is a log base 2 enrichment of GAPDH normalized mRNA compared to IgG negative IP. ACTB (Beta Actin) is a robust HuR target mRNA and B2M (Beta-2 Microglobulin) is a non-target. Panel B) Fold change of 3'UTR luciferase reporters compared to a firefly luciferase control (pLuc) for each condition in RAW macrophages. *$p<0.01$, **$p<0.05$ Panel C) and D) same as panel B) except with 3'UTR fragment reporters. All firefly luciferase values were normalized to renilla luciferase to control for loading.

With the observed change in the 3'UTR reporters in response to HuR knockdown in LPS stimulated RAW cells we decided to investigate whether they were also dependent on the presence of ARE or U-rich sequence elements. Constructs missing ARE/U-rich elements (TTP-deltaARE, BRF1-prox) were much less responsive to LPS stimulation in RAW macrophages than either the full-length 3'UTR reporters or the reporters containing only the ARE/U-rich elements (BRF1-dist) (figure 13C, D). This was especially true for the TTP 3'UTR reporter where the ARE deletion reporter did not demonstrate any of the LPS induced increases seen with the full-length reporter, suggesting that ARE in the most distal region of the TTP 3'UTR contains a posttranscriptional regulatory response element responsible for nearly all of the LPS stimulated posttranscriptional induction. Multiple elements in BRF1's 3'UTR were able to contribute to the overall induction caused LPS stimulation and none of the constructs were fully capable of recapitulating the induction of the full-length 3'UTR reporters. This indicates that the ARE/U-rich elements of TTP and BRF1 can increase their expression in response to LPS stimulation in macrophages and that HuR is necessary for maximal induction through these elements.

2.4 Discussion

We have demonstrated here that the ARE-BP, HuR, can bind to the mRNAs of other ARE-BPs, including TTP, BRF1 and HuR's own mRNA and that these interactions, at least in part, occur between HuR and the ARE/U-rich elements in the 3'UTRs. Since, HuR conventionally enhances mRNA stability and translation we investigated HuR's regulation of these processes for each of the ARE-BPs. However, when we altered levels of HuR protein either by overexpression or knockdown the effects on either the stability or translation of each of the mRNAs was rather modest, usually in the range of 10-25% change. The most robust effects we observed were in RAW macrophages stimulated by LPS and even here the effects were well below 2 fold. Otherwise, the changes we observed frequently were not large enough to be readily distinguished from experimental variance. The modest effects of HuR on these mRNAs is not a quirk of the assays, as this result was reproduced regardless of the assay, cell type, stimuli, region of 3'UTR or method for modulating HuR's expression. Furthermore, these assays were more than sensitive enough to measure changes in posttranscriptional gene expression driven by the overexpression of other ARE-BPs, such as TTP (figure 9). This suggests that modulating the levels of HuR protein may only result in modest changes in the mRNA stability and/or translation of these ARE-RBPs.

One of the goals of this study was to investigate the role HuR plays in regulating the posttranscriptional expression of its own mRNA, a process referred to as autoregulation. We demonstrated that HuR could interact with multiple elements within its own 3'UTR but that the effect this binding had on the regulation of mRNA stability or translation was rather modest. Furthermore, these modest changes were in the opposite direction as expected, for example overexpression of HuR appeared to repress the translation and/or mRNA stability of HuR mRNA. Three other studies have specifically addressed HuR's autoregulatory capabilities (Pullman et al., 2007; Al-

Ahamdi et al., 2009; Yi et al., 2010). Two studies found that HuR could bind its own mRNA, but the demonstrated interaction between HuR and the RNA element was only found to occur in an extended 3'UTR sequence (Pullmann et al., 2007; Al-Ahmadi et al., 2009). This extended 3'UTR sequence is produced by alternative 3' end processing at a downstream polyadenylation cleavage site in a cell type restricted manner (Atasoy et al., 1998). This alternative extended 3' UTR sequence is not readily detectable in most of the cell lines used in this study, so the data presented here does not necessarily disagree with the previous studies. However, it should be noted that one of the studies did use a luciferase reporter with the common, non-extended 3'UTR sequence as a control, and consistent with our observations they also saw little to no effect of HuR overexpression on this 3'UTR reporter (Al-Ahmadi et al., 2009). The third HuR autoregulatory study suggested that HuR overexpression caused a roughly four fold increase in endogenous HuR protein and that HuR could interact with the distal region of the shorter, commonly expressed 3'UTR variant (Yi et al., 2010). They also demonstrated that Hu overexpression caused no concomitant increase in HuR mRNA but caused an increase in endogenous protein that was dependent on the nuclear export factor, CRM1. Suggesting that HuR's regulates its own expression by enhancing nuclear export. While our results do not agree with those of Yi et al.,, we primarily focused on HuR's effects on mRNA stability and translation and did not investigate other posttranscriptional processes such as nuclear mRNA export. The differences could also be cell type specific as different cell lines were used.

The majority of studies that investigate HuR's regulation on individual mRNAs frequently report large changes in stability or translation in response to HuR knockdown or overexpression. However, a number of recent reports suggests that the select mRNA targets that demonstrate large changes may be the exceptions, rather than the rule for the typical effects caused by HuR. For example, several studies that have investigated HuR's role in regulating the gene expression of individual targets, such as Wnt5a, VEGF, IL8 and many others, have reported minor effects on mRNA stability or translation (Leandersson et al., 2006; Garcia-Dominquez et al., 2011; Filippova et al., 2011). Another study carried out in this laboratory using similar, but distinct reagents in a completely independent manner by another researcher also came to a strikingly similar conclusion that HuR overexpression only mildly changed the 3'UTR dependent posttranscriptional expression of HuR (unpublished results, Kyle Mansfield). Furthermore, two recent global studies have demonstrated that the majority of HuR target mRNAs undergo subtle changes in total mRNA and protein levels following HuR knockdown (Mukherjee et al., 2011; Lebedeva et al., 2011). These studies showed that the subtle changes in gene expression are highly specific and that the mRNAs that change following HuR knockdown are highly enriched for Hu target mRNAs. Interestingly, these subtle effects of HuR on mRNA stability and translation are not unlike the subtle differences driven by miRNAs and other posttranscriptional regulators (reviewed in Leung and Sharp, 2010).

Small changes in gene expression can potentially have important functional and physiological consequences. It has long been known that nonlinear systems, such as gene expression programs, can produce vastly different outcomes based on exceedingly small changes to the system, a phenomena that is commonly referred to as the butterfly effect (Lorenz 1963). These systems are often defined by recursive and interconnected circuits such as the connections seen between the ARE-BP network of HuR, TTP and BRF1. Furthermore, regulators of gene expression, such as RBPs and transcription factors, are sensitive to small changes in concentration and that this can result in changes of many other factors (targets). Many of the targets of RBPs include other gene expression regulators, such as other RBPs, and the regulation of these factors can further shape the expression landscape (Hogan et al., 2008; Mukherjee et al., 2009; Mittal et al., 2011). Thus, initial small changes in concentration of gene expression regulators can result in a drastically altered cellular outcome.

The subtle changes in expression of HuR target mRNAs after knockdown or overexpression may be a direct and intrinsic feature of HuR's regulatory capacity. For example, it is possible that HuR is part of a network that is resilient and compensatory thus balancing and tempering its effects on target mRNAs. One such network could look like the ARE-BP network suggested in this chapter, consisting of HuR, TTP and BRF1. Since each of the members contain both AREs and can regulate the expression of ARE-containing mRNA it is possible that expression of each of the members alters the expression of the others in a compensatory manner thus resulting in resiliency of the network. We attempted to address this by introducing reporter constructs that separated the regulatory sequences from the functional proteins. However, we could never rule out compensatory effects from other ARE-BPs or RBPs that were not included in the network model or even rule out effects from the endogenous three member ARE-BP network (although TTP and BRF1 were mostly undetectable in untreated cells suggesting that there functional input was essentially removed from the system).

Another unexpected result from this study besides the small effects mediated by HuR was the observation that HuR overexpression seemed to cause a decrease in the expression of at least HuR's own 3'UTR reporter. This is not without precedent though, it has previously been observed that HuR can decreases expression of a few select mRNAs in an mRNA and cell type dependent manner (Kullmann et al., 2002; Meng et al., 2005; Katsanou et al., 2005; Leandersson et al., 2006; Yeh et al., 2008; Kim et al., 2009). However, this is further complicated by the observation that HuR knockdown also decreased the expression of the mRNAs and the 3'UTR reporters. This may indicate that overexpression of HuR either triggers a different functional outcome of bound mRNAs than knockdown or more likely, overexpression of HuR is acting like a dominant negative in this case.

In this study we have demonstrated a modest role for HuR in regulating the mRNA stability and translation of TTP's, BRF1's and HuR's own mRNA. However, it is possible that HuR is also regulating other aspects of posttranscriptional gene expression of these messages. Mounting evidence is beginning to point to roles for HuR in regulating many other steps of posttranscriptional gene expression including splicing, polyadenylation, and cellular localization (reviewed in Hinman and Lou, 2008). In support of this it has recently been demonstrated that a substantial fraction of HuR binding sites are located in intronic pre-mRNA regions (Mukherjee et al., 2011; Lebedeva et al., 2011). Interestingly, these intronic binding sites, in addition to potentially regulating splicing, were demonstrated to influence mRNA stability thus suggesting that many of these posttranscriptional steps may be interconnected (Mukherjee et al., 2011). Therefore, it is possible that HuR can, in concert, regulate multiple posttranscptional steps of the three ARE-BPs mRNAs discussed here. However, it should be noted that the only intronic binding site seen in any of the three ARE-BPs from the previously mentioned global study was on the reverse strand of one of HuR's introns, suggesting that splicing may not be involved in regulating the expression of these particular mRNAs. Although other posttranscriptional steps may be involved as it has previously been suggested that HuR can regulate the expression of it's own mRNA by increasing its nuclear export and thus increasing its translation independent of any change in mRNA levels (Yi et al., 2010). This mode of regulation appears to be different than the regulation we have observed in that the they did not observe a change in mRNA stability and the increase in total protein was much larger than the effect we observed. It has also been shown that HuR can regulate the choice of polyadenylation sites that occur in neuronal cells (pers. comm., Kyle Mansfield). This may indicate that different forms, or at least different degrees, of regulation of HuR's own mRNA can occur in different cellular contexts.

The degree to which HuR regulates the expression of these three ARE-BPs is in part dependent on cell type and condition, as we observed a slightly more robust effect of HuR knockdown in LPS stimulated RAW macrophages than in any of the other tested cell lines or conditions. This indicates that HuR's regulatory capabilities are dependent on cell type or condition specific factors and signaling pathways. In fact, it is well known that HuR's function is modulated by many different signaling pathways including the cell-cycle kinase CDC2, the DNA damage response kinase CHK2, and several members of the protein kinase C family (reviewed in Doller et al., 2008). In addition to signaling specific regulation, the Hu family members have shown cell type differences in their expression and regulation. In particular, the Hu family is especially sensitive to alternative regulation in neuronal cells, which are the only cell type in which all four Hu protein family members are expressed at some point during development. It is likely that cell type specific regulation modulated by HuR is dependent on the presence and/or the interaction with other cell type specific factors. For example, HuR's function has been demonstrated to be influenced by interactions with microRNAs and/or the RISC complex (Bhattacharyya et al., 2006; Kim et al., 2009; Glorian et al., 2011; Mukherjee et al., 2011). Since the expression of particular microRNAs is cell type dependent and their effects are transcript specific this may suggest a way for Hu proteins to have highly specialized effects on different mRNAs in different conditions. It will be important going forward to identify other cell type specific factors that can influence HuR's function.

As previously mentioned, small changes in gene expression, especially of regulators such as RBPs and transcription factors, may have a large impact on the functioning of a cell. This is especially true for interconnected regulatory networks such as the ARE-BP network consisting of HuR, TTP and BRF1. This concept, called regulators-of-regulators, suggests that these interconnected regulatory networks are capable of controlling large gene expression programs and even potentially establishing temporal patterns (Keene and Tenenbaum, 2002; Pullmann et al., 2007; Mansfield and Keene, 2009). This is particularly interesting in light of the small differences in efficacy of HuR mediated regulation between different cellular conditions and their potential to create different cell type expression patterns. The regulators-of-regulators concept is of course not limited to this particular ARE-BP network, but may involve many other factors or networks. Interestingly, it has been observed that regulatory factors are overrepresented in the mRNA targets of a large number of RBPs, not just HuR (Mukherjee et al., 2009). This concept of regulators-of-regulators is much more common among posttranscriptional factors than for transcription factors (Mittal et al., 2011). This enrichment of interconnected networks in posttranscriptional regulation suggests that regulation by RBPs is highly coordinated and dependent on other RBPs though complex feedback/feedforward mechanisms.

A universal property of interconnected networks, from simple electrical circuits to complex ecological networks, is that connection and feedback between components can produce unexpected, non-linear qualities that cannot be predicted by the sum of the individual components, this concept is referred to as emergence (Bhalla and Iyengar, 1999; Barabasi and Oltvai, 2004). Examples of emergent properties of gene expression networks include timing of responses, insensitivity to fluctuations and 'molecular memory'. The precise properties of a particular network depends on the structure and connections of the network (reviewed in Alon, 2007). Thus the high connectedness of RBP networks suggests that they play an important role in processing cellular information to produce emergent behaviors. To understand how particular RBP networks, and the connections that form them, process cellular information to create biological behaviors it will be important to understand how components of networks, like the one presented here between HuR, TTP and BRF1, regulate each other.

3. Coordination of EGF-induced gene expression by autoregulation of TTP family of RNA Binding Proteins

3.1 Background and Significance

The TTP family of RNA binding proteins consists of three members in humans that contain a characteristic tandem CCCH zinc finger (TZF) domain. These RBPs promote deadenalyation and degradation of mRNAs containing AU-rich elements (AREs). The founding member of the family, tristetraprolin (TTP) or Zinc Finger Protein 36 (ZFP36), was initially discovered as an early induced mRNA in response to several broad classes of stimuli including many mitogens, hormones, cytokines and growth factors such as epidermal growth factor (EGF) (Varnum et al., 1989; Gomperts et al., 1990; Lai et al., 1990; DuBois et al., 1990; Nakajima and Wall, 1991). The other two members of the TTP family, butyrate response factor 1 and butyrate response factor 2 (BRF1 and BRF2) or ZFP36-like 1 and ZFP36-like 2 (ZFP36L1 and ZFP36L2) respectively, were later discovered as inducible genes that contained the same TZF domain as TTP (Varnum et al., 1991, Gomperts et al., 1992). Induction of TTP and BRF1 mRNAs are characteristic of other early immediate genes in that they usually undergo massive, rapid and transient increases in response to stimuli. Less is known about the induction of BRF2 mRNA.

The TTP family of RBPs was initially suspected to encode transcription factors due to the presence of zinc finger motifs and this misconception still persists even though a large number of studies have conclusively demonstrated that they bind RNA and regulate mRNA stability. Part of the reason this misconception of TTP family member function persists is that, like transcription factors, TTP family members can effect the abundance of specific mRNAs, however the TTP family members do this by degrading mRNA rather than synthesizing it. This too is a common misconception that mRNA abundance can act as a simple correlative proxy for transctiptional activity without accounting for mRNA degradation. Numerous studies have shown that mRNA turnover significantly shapes total mRNA abundance and that changes in mRNA stability contribute as much, if not more, to regulatory changes in mRNA levels as transcription does (Wang et al., 2002; Fan et al., 2002; Garcia-Martinez et al., 2004; Cheadle et al 2005; reviewed in Morris et al., 2010).

Furthermore, it has been shown that global changes in mRNA stability can coordinate the co-expression of mRNAs encoding functionally related proteins (Grigull et al., 2004; Puig et al., 2005; Amorim et al., 2010). This mRNA stability regulated co-expression is orchestrated by selective, combinatorial and dynamic binding of RBPs to the mRNAs encoding the functionally related proteins. These organizational RBP-mRNA modules are known as posttranscriptional RNA operons (PTROs) (Keene and Tenenbaum, 2002). PTROs allow cells to efficiently execute and coordinate multiple responses to specific stimuli. For example, in response to DNA damage cells must initiate DNA repair, activate cell cycle arrest and prime the cell for apoptosis in case the DNA damage is too severe to be repaired. Many of these DNA damage response pathways are regulated by HuR-mediated dynamic reorganization of a PTRO containing mRNAs that encode proteins responsible for these diverse functions (Mazan-Mamczarz et al., 2011). It is likely that regulation of multiple PTROs with distinct spatio-temporal patterns are required for complex responses, such as DNA damage.

It has been noted that mRNAs bound by many RBPs are enriched for the mRNAs encoding other gene expression regulators, including other RBPs and transcription factors, suggesting that coordinating or layering the expression of other sets of mRNAs is an important aspect of many PTROs (reviewed in Keene, 2007; Mukherjee et al., 2009). These layered gene sets and PTROs may be important for ensuring progression of gene expression programs that produce or limit the order and timing of cellular responses. This implies that it is not only important to understand how and which PTROs are regulated by RBPs but also how the RBPs themselves are regulated in response to stimuli in the context of PTROs. In this study we sought to investigate the role the TTP family of RBPs plays in promoting the progression and coordination of the gene expression program initiated by EGF activation. We have demonstrated that the TTP family member mRNAs are induced in distinct temporal patterns following EGF stimulation and that changes in mRNA stability accompany this induction. We have also shown that the TTP family members are capable of regulating their own expression and that of the other family members through changes in mRNA stability. Finally, we show that this family-wide network and its mRNA targets, that encode functionally related products, are expressed in a temporally ordered fashion during EGF activation.

3.2 Materials and Methods

3.2.1 Cell Culture

HeLa cells were cultured in DMEM supplemented with 10% FBS. For EGF stimulation we plated 0.1 x10^6 cells per well of a 12-well plate and grew in low serum media containing 0.1% FBS for 24 hours. Finally, we stimulated with recombinant EGF at a final concentration of 20ng/ml for the times indicated. Transfections were performed with Lipofectamine 2000 (Invitrogen) using a slightly modified protocol. Briefly, 1ug of total DNA was diluted into 100ul of Opti-MEM I (Gibco) and mixed with 2.5ul of Lipofectamine 2000 diluted in 100ul of Opti-MEM I and incubated at room temperature for 30 min. The DNA/Lipofectamine 2000 complexes were added to HeLa cells at least 2 hours prior to starvation.

3.2.2 Realtime PCR

RNA was isolated with Trizol (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 1ug of total RNA using the iScript cDNA synthesis kit (Bio-Rad) with a 1 hour extension at 42 degrees C. Realtime PCR was performed using the Roche Lightcycler using SYBR Green. All values were normalized to GAPDH or RPL8 to control for across sample variation.

3.2.3 Decay curves

Cells were treated with 5ug/ml Actiomycin D for the times indicated. RNA was isolated and quantified as described above. The fraction of mRNA remaining after treatment was determined by comparing the mean value of three independent biological triplicates to the appropriate 0 hr samples. Exponential regression was used to determine the half-lives.

3.2.4 RNA immunoprecipitation

Lysates were prepared as previously described (Keene et al., 2006) from cells expressing either FLAG-tagged BRF1 or HA-tagged TTP, with the notable exception that no EDTA was added to the NT2 wash buffer and EDTA-free protease inhibitors replaced protease inhibitors. Immunoprecipitations were performed using 25ul packed bead volume of Protein A Sepharose beads coated with either anti-FLAG, anti-HA, or normal mouse serum. Control IPs were performed from each of the lysates to compensate for possible changes to gene expression caused by expression of the constructs. Each primer set was normalized to GAPDH and this ratio was compared to the same primer set ratio for NMS IPed samples to give the fold enrichment.

3.2.5 Dual Luciferase Assay

Luciferase constructs were generated by inserting 3′UTR fragments into the pLuc vector. Equimolar amounts of the various UTR luciferase reporters were transfected at a ratio of 2:1, Firefly (20 pmoles) to Renilla (10 pmoles), to account for differences in length of UTR sequence. siRNAs were transfected 24hours prior to luciferase transfection using Lipofectamine 2000 (Invitrogen) with 20 pmoles siRNA per well. Luciferase assays were performed using the Dual-Luciferase Reporter Assay system (Promega) according to manufacturer's instructions. Cells were washed with PBS and lysed in 500ul of 1x Passive Lysis Buffer.

3.2.6 Comparison of TTP RIP-Chip targets to EGF induced genes

Novel analysis was performed on previously reported data sets for TTP RIP-Chip targets (Stoecklin et al., 2008) and EGF induced genes (Amit et al., 2007). Gene sets for EGF induced mRNAs were generated for each time point of EGF stimulation with each set consisting of mRNAs that reached peak expression at that time. P-values for the enrichment of TTP targets in the EGF induced gene sets were calculated using hypergeometric distribution.

3.3 Results

3.3.1 Temporal expression patterns of TTP family members during EGF treatment

We investigated the temporal expression of the TTP family members induced by epidermal growth factor (EGF) treatment. This was achieved by measuring mRNA levels using RT-PCR from Hela cells at eight times (0.25, 0.5, 0.75, 1, 1.5, 2, 4 and 6 hrs) following recombinant EGF treatment. We observed that each of the family members was inducible following EGF treatment but displayed different peak levels and times. TTP mRNA peaked first at 1 hr with a massive increase of greater than 20-fold, however this increase was only transient and rapidly disappeared to near baseline levels despite the continued presence of EGF in the media. ZFP36L1 also increased rapidly reaching near maximal levels at 1 hr, however ZFP36L1's increase was much more subdued with a roughly 2.5 fold increase and this increase was not transient, maintaining near maximal levels for the remainder of the time course. Finally, ZFP36L2 peaked the latest of the family members reaching its highest levels at the end of the time course while steadily rising throughout (figure 14). A previous study of global mRNA changes following EGF induction confirms similar induction patterns for the TTP family member mRNAs (Amit et al., 2007). Higher concentrations of EGF had small effects on the peak levels but did not dramatically alter the patterns of induction suggesting that the differences in kinetics and patterns of induction were not due to differential responses to varying concentrations of EGF (not shown). The proteins levels of TTP and ZFP36L1 roughly mimicked the increases of the mRNA suggesting that regulation of mRNA levels could, at least in part, result in correlative changes in protein levels.

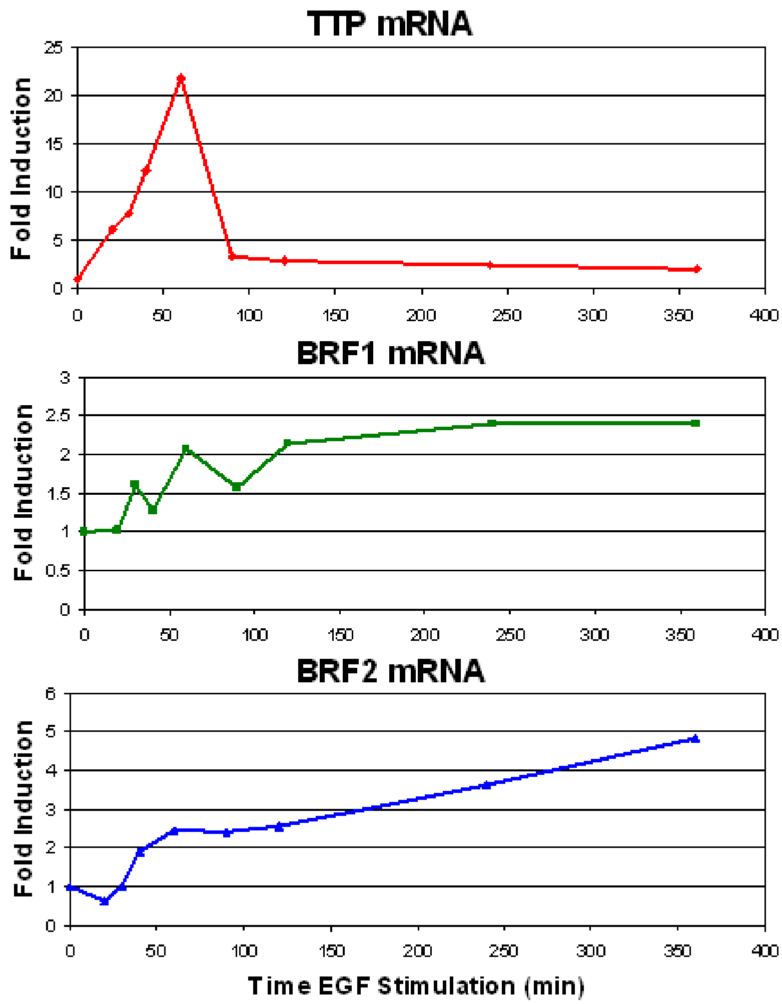

Figure 14: EGF induction timecouse of TTP family member mRNAs.

These graphs show the fold induction of mRNA for each TTP family member mRNA following EGF stimulation of HeLa cells as determined by RT-PCR. The fold induction was calculated by comparing the GAPDH (a non-responsive mRNA) normalized values for the given time point to the values at t=0. The values were averaged across three independent experiments.

3.3.2 EGF treatement increases mRNA stability of TTP family members

The massive, rapid and transient increase of TTP's mRNA is similar to that seen for immediate early genes (IEGs). IEGs are known to be highly unstable mRNAs that are rapidly induced in response to numerous stimuli through a combination of increased transcription and increased mRNA stability. We decided to investigate whether each of the TTP family member mRNA's stability was increased during EGF treatment. This was achieved by measuring the percent mRNA remaining by RT-PCR after transcriptional inhibition with Actinomycin D. mRNA half-lives were calculated by exponential regression of six time points following Actinomycin D treatment with each time point normalized to the relatively stable GAPDH mRNA. In untreated HeLa cells TTP and BRF1 mRNA are quite unstable with half-lives of significantly less than 1 hour while BRF2 mRNA was more stable than the other two family members but was still relatively unstable with a half-life of about one and a half hours (figure 15). Although Actinomycin D treatment has been reported to produce side effects, the observed short half-lives of the TTP family members are in agreement with previous studies on their basal stability suggesting that the side effects are negligible in this instance.

To investigate whether changes in mRNA stability may play a role in the induction of the TTP family members we measured the half-lives as described above at the times of peak induction following EGF treatment. All TTP family members showed an increase in mRNA stability at their corresponding peak expression. Stability of TTP and BRF1 mRNA increased about 5 fold with half-lives between 3 and 3.5 hours at 1 hour after EGF treatment. Likewise, BRF2 mRNA stability increased about 3 fold at its peak, 8 hours after EGF treatment. Since total mRNA levels result from the combination of synthesis and degradation, and EGF treatment increases the mRNA stability of the TTP family members this demonstrates that at least part of the induction of TTP family members mRNA levels is due to changes in stability.

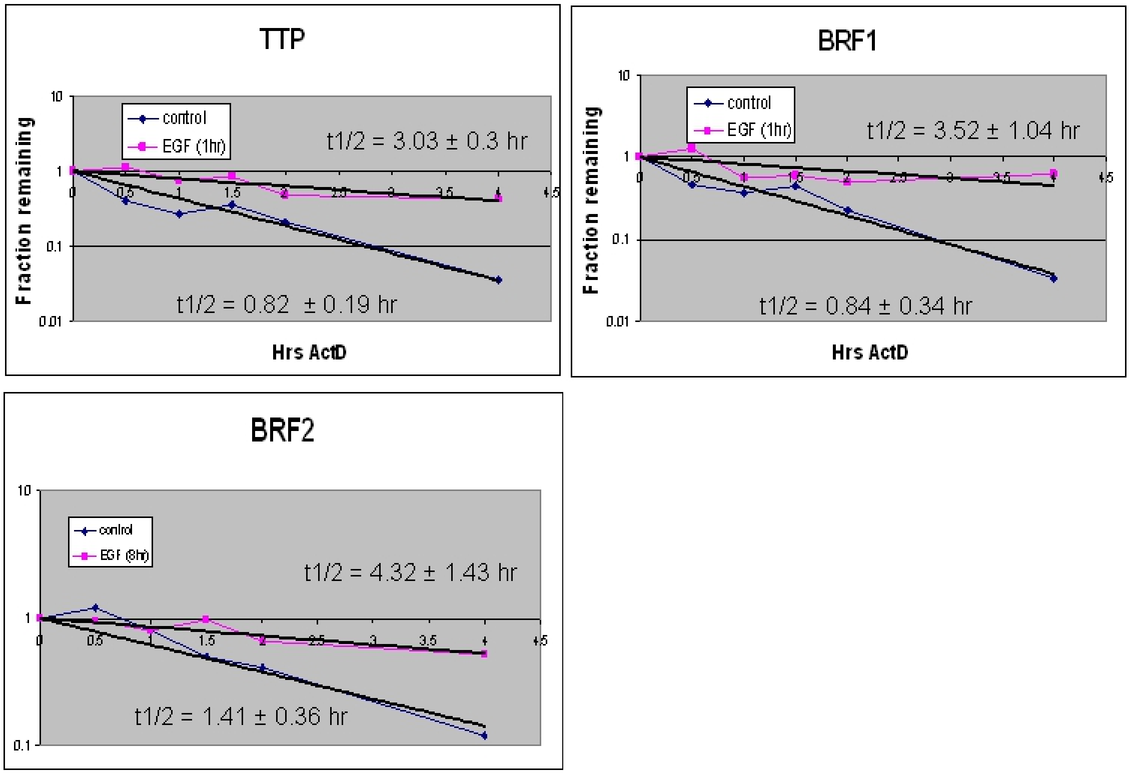

Figure 15: mRNA Decay Curves Demonstrating that EGF Stimulation Increases Half-lives of TTP Family Member mRNAs.

mRNA decay curves were generated by treating HeLa cells with Actinomycin D for various lengths of time. HeLa cells were either treated or not with EGF prior to treatment with Actinomycin D. mRNA from each time point was normalized to GAPDH mRNA and expressed as a fraction remaining from the 0hr ActD samples. Half-lifes were determined by fitting the data by exponential regression. All values are an average of 4 independent experiments.

3.3.3 TTP family members bind to their own mRNAs

Since the TTP family members underwent changes in mRNA stability in response to EGF stimulation and these RBPs are known to influence mRNA stability of targeted mRNAs, we decided to investigate whether these RBPs could bind to their own messages. TTP family members bind ARE-containing mRNAs with specific preference for sequences that at least contain the AUUUA pentamer sequence. Functional studies have suggested that TTP family members prefer to bind and regulate sequences with the UUAUUUAUU nonamer sequence, or the pentamer sequence flanked by two U's on either side. Each TTP family member contains multiple pentamer sequences (five or more) and at least one nonamer sequence within their 3'UTRs. These ARE sequences are frequently clustered together and are located in highly conserved regions suggesting that these sequences are constrained by natural selection due to their importance as regulatory elements (figure 16).

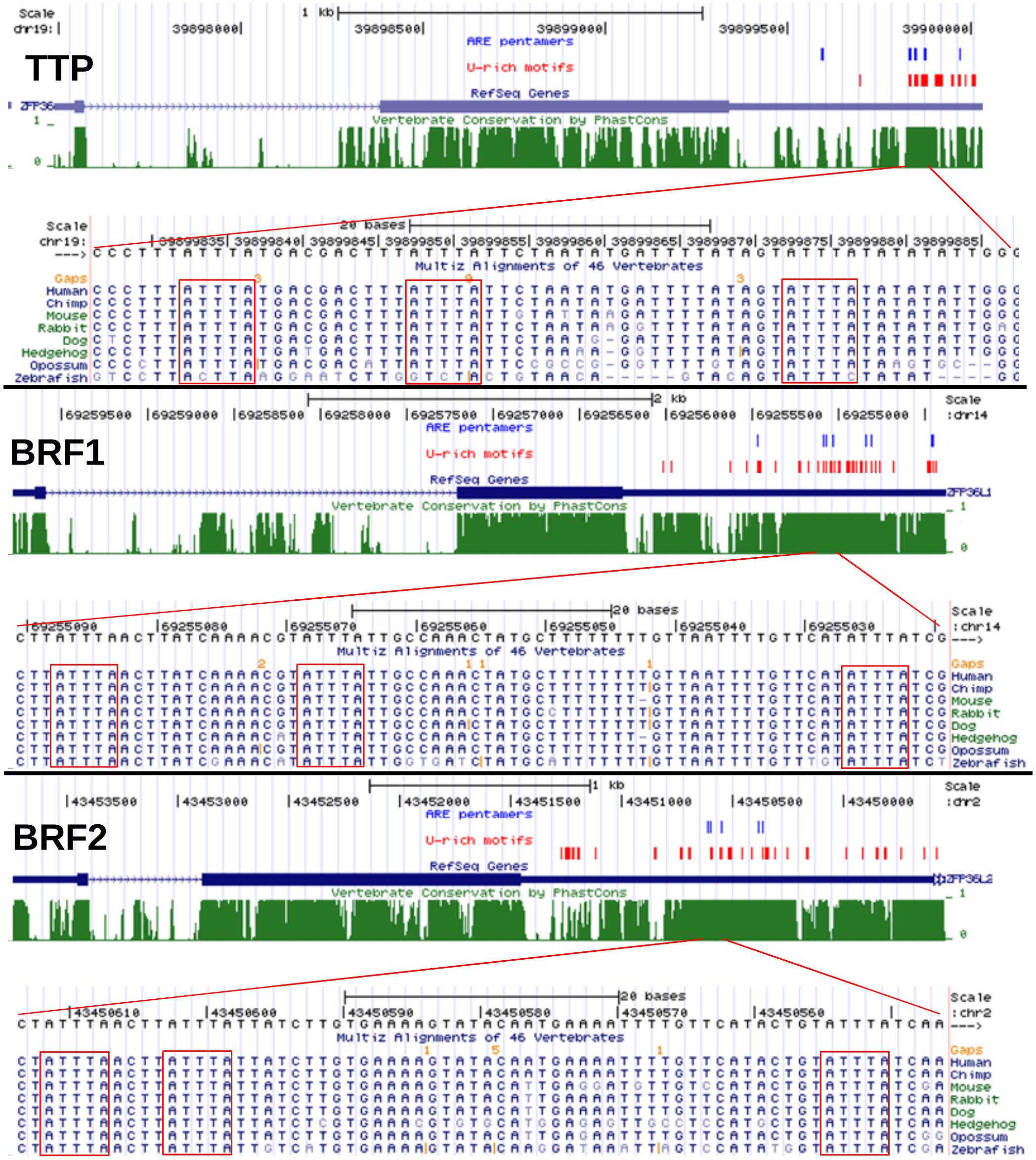

Figure 16: Conservation of AU-rich elements in the 3'UTRs of TTP family member mRNAs.

These diagrams are customized UCSC Genome Browser snapshots, with custom tracks for ARE pentamers (blue tick marks) and U-rich motifs (red tick marks) added. ARE pentamers are defined as ATTTA while U-rich motifs are defined as sequences of at least 7 bases in length in which at least two thirds of the bases are 'T'. The zoomed in sequences indicated by the diverging red lines are examples of select sequences that contain multiple conserved pentamer sequences (red boxes).

While these mRNAs contained elements that are characteristic of those recognized by TTP family member RBPs this does not necessitate that they will interact with with TTP family member RBPs in a cell. Therefore, to ensure that the TTP family member RBPs interacted with the mRNAs in a cellular context we immunoprecipitated FLAG-tagged versions of TTP and BRF1 and quantified the associated mRNAs by RT-PCR. The levels of each of the family member mRNAs was normalized to the non-target mRNA GAPDH and compared to a negative IP (FLAG-GFP) to determine fold enrichment. To first assess whether the IPs were working we verified by Western blot that the FLAG-tagged proteins were isolated in the IPs. We also demonstrated that the pulldowns were able to enrich a known target of both TTP and BRF1, namely VEGF mRNA (figure 17A). Therefore, we were confident that the IPs were pulling down the expressed proteins and that each could bind target mRNAs enriched over non-target mRNAs so we decided to investigate whether the TTP family member mRNAs were isolated in the IPs. We saw that each of the TTP family members mRNAs were significantly enriched in the IPs, with BRF2 mRNA being the most enriched of the family member mRNAs in both TTP and BRF1 IPs (figure 17B). It is likely that BRF2 mRNA is more enriched in the IPs because its mRNA is expressed higher than either TTP or BRF1 mRNA in untreated cells. Furthermore, each mRNA was enriched to similar levels in both the TTP and BRF1 IPs indicating that both RBPs recognized each of the three mRNAs similarly well which is not surprising given the striking similarity of their RNA binding domains.

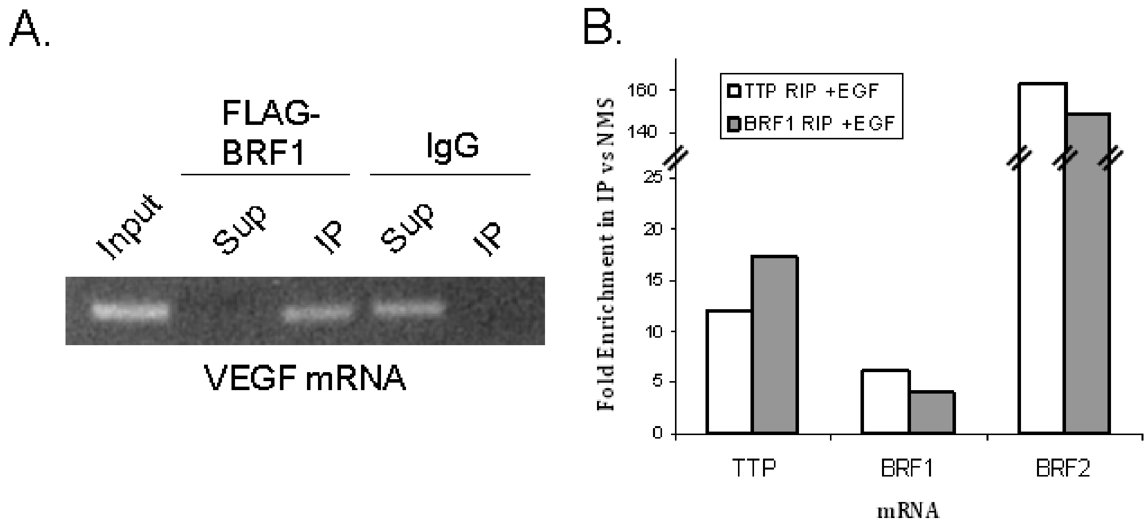

Figure 17: RNA Immunoprecipitation of TTP family member mRNAs by TTP and BRF1.

Panel A) shows end point PCR of VEGF mRNA isolated either in IPs or left in supernatants from FLAG-BRF1 IPs or in a control IgG IP. Panel B) shows fold enrichment of mRNAs in either TTP of BRF1 IPs following EGF stimulation as detected by RT-PCR. The putative target mRNAs (TTP, BRF1 and BRF2) were normalized to GAPDH and compared to normal mouse sera (NMS) control IPs.

One trivial explanation for the association of TTP or BRF1 with their own mRNA is the potential immunoprecipitation of nascent polypeptide fragments that are still associated with translating ribosomes and thus the template mRNA (it should be noted that the epitope tags are N-terminal). This is of particular concern in these experiments because EDTA, which disrupts translating ribosomes, was not used due to the requirement for zinc for these zinc finger RBPs to bind mRNA. Thus, to exclude the possibility that the mRNA we were isolating was only associated via nascent polypeptides we used a second set of RT-PCR primers designed for the 3'UTR that would detect endogenous mRNA but not the tagged version we were immunoprecipitating. Amplification with this primer set showed similar levels of enrichment suggesting that the majority of the mRNA we were immunoprecipitating was not associated via nascent polypeptides. Furthermore, IPs were performed from cells transfected with reporters containing either the full-length 3'UTR of TTP mRNA or a similar reporter of TTP's 3'UTR without the ARE sequence. We observed that the full-length 3'UTR reporter was enriched in both the TTP and BRF1 IPs but the reporter missing the ARE sequence was not enriched in either, thus showing that TTP and BRF1 interact with TTPs 3'UTR through the ARE.

3.3.4 TTP and BRF1 regulate mRNA stability of TTP family members

To determine whether TTP and BRF1 could destabilize the TTP family member mRNAs we overexpressed the RBPs and examined the decay curves following Actinomycin D treatment. Initially, we overexpressed the RBPs in untreated cells and determined the mRNA levels (steady state) using the mRNA specific primers (targeted to the 3'UTR) as described previously. Overexpression of both RBPs, TTP and BRF1, caused a decrease in steady state mRNA levels for the TTP family member mRNAs to nearly undetectable levels. This suggests that TTP and BRF1 RBPs cause decreases in mRNA expression of the TTP family member mRNAs presumably through a decrease in mRNA stability. Given the relatively low levels of TTP family member mRNAs in untreated cells it is not terribly surprising that overexpression of TTP or BRF1 would reduce the mRNA to nearly undetectable levels. Unfortunately, because the mRNA levels were approaching the limits of detection this indicates that decay could not be measured directly in untreated cells. However, since EGF treatment increases mRNA levels of the TTP family members thus making them easier to detect in a decay experiment, we decided to investigate the effect of TTP and BRF1 overexpression on TTP family member mRNA following EGF stimulation. We observed that overexpression of TTP and BRF1 could decrease the half-lives of TTP and BRF1 mRNAs following 1 hour of EGF treatment, thus demonstrating that TTP and BRF1 can regulate their own and each others mRNA stability (figure 18A). This is in accord with previous studies showing that TTP can regulate its own expression at the level of mRNA stability (Brooks et al., 2004; Tchen et al., 2004; Lin et al., 2007).

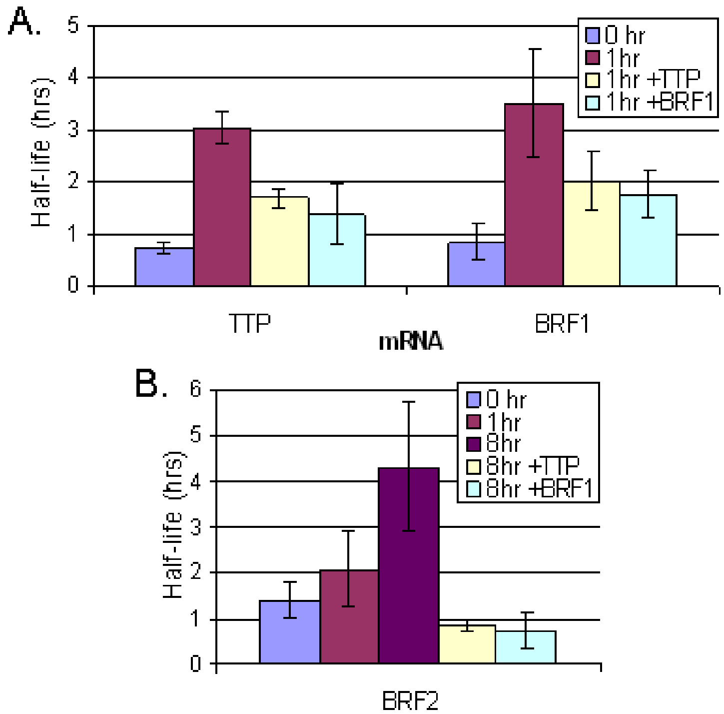

Figure 18:Effects of overexpression of TTP and BRF1 on TTP family member mRNAs following EGF induction.

The graphs display mRNA half-lives determined from Actinomycin D decay curves. HeLa cells overexpressing either TTP, BRF1 or empty vector were treated with EGF for indicated times prior to Actinomycin D treatment. Decay curves were calculated by exponential regression of GAPDH normalized expression values from six Actinomycin D treatment time points (0, 0.5, 1, 1.5, 2, 4hrs). Half-life values are averaged over four independent experiments.

While overexpression of the RBPs reduced the half-lives of TTP and BRF1 mRNA compared to the elevated half-lives seen at peak expression, the half-lives were not completely reduced to levels seen prior to EGF treatment. Interestingly, TTP and BRF1 overexpression massively decreased the mRNA stability of BRF2 mRNA 8 hours after EGF treatment, even when compared to untreated cells. This indicates that TTP or BRF1 can regulate mRNA turnover of each of the family members at precisely the point when changes in mRNA degradation overtakes changes in mRNA synthesis during EGF stimulation.

3.3.5 TTP and BRF1 regulate the posttranscriptional expression of TTP in an ARE-dependent manner

Given that TTP family member RBPs regulate mRNA stability by binding to AREs we decided to investigate whether the AREs in TTPs 3'UTR were necessary for its regulation. To study the dependency of TTP's and BRF1's regulation on the AREs of TTP's mRNA we created firefly luciferase reporters containing either the full-length 3'UTR or a 3'UTR missing the distal end containing the AREs as well as a control reporter containing no 3'UTR. Cells were transfected with these reporters and assayed for luciferase production 24 hours later. By comparing reporters that differ only in 3'UTR sequence we eliminate gene expression changes that are not dependent on the 3'UTR sequence and thus are directly measuring posttranscriptional effects. These posttranscriptional effects may be due to altered mRNA stability or translation rates or both. Furthermore, the luciferase produced is cumulative over the course of the experiment so if cells containing reporters are treated following transfection the amount of luciferase produced before the treatment are also included in the final value. Luciferase values of the 3'UTR reporters were first normalized to a renilla luciferase reporter, that was co-transfected with each firefly luciferase reporter (3'UTR reporters), to control for transfection efficiency. We then compared these ratios to the luciferase construct without a 3'UTR under each condition to correct for condition specific effects on the general luciferase system.

To test the dependence of TTP and BRF1 mediated mRNA stability on the presence of AREs in the 3'UTR of TTP mRNA we overexpressed TTP or BRF1 in cells containing either the full-length TTP 3'UTR reporter or the ARE-deleted 3'UTR reporter. To ensure that the reporter system was responsive to TTP or BRF1 RBPs we verified that a firefly luciferase reporter containing the TNFalpha ARE (a well studied target of both RBPs) responded to both overexpression on knockdown of TTP and BRF1 RBPs. Furthermore, we observed that both RBPs could decrease the amount of luciferase produced from the reporter with the full-length 3'UTR but not the one missing the AREs. We also used siRNAs to knockdown expression of TTP and BRF1 independently and saw no noticeable effect on either reporter in untreated cells. However, when cells containing the reporters and the siRNAs were treated with EGF for 4 or 8 hours we noticed increased production from the full-length 3'UTR but no change in the 3'UTR reporter with the AREs deleted. Thus, in conditions when endogenous TTP family members protein is low, overexpression of the RBPs decreases the expression of the ARE-containing reporter but not the ARE-less reporter. Furthermore, in conditions when TTP family members proteins are elevated due to stimulation of EGF, knockdown increases the expression of the reporters in an ARE dependent fashion. Importantly, the deleted ARE region in these reporters is the same region we demonstrated to interact with the RBPs, thus suggesting that the effect is due to a direct interaction.

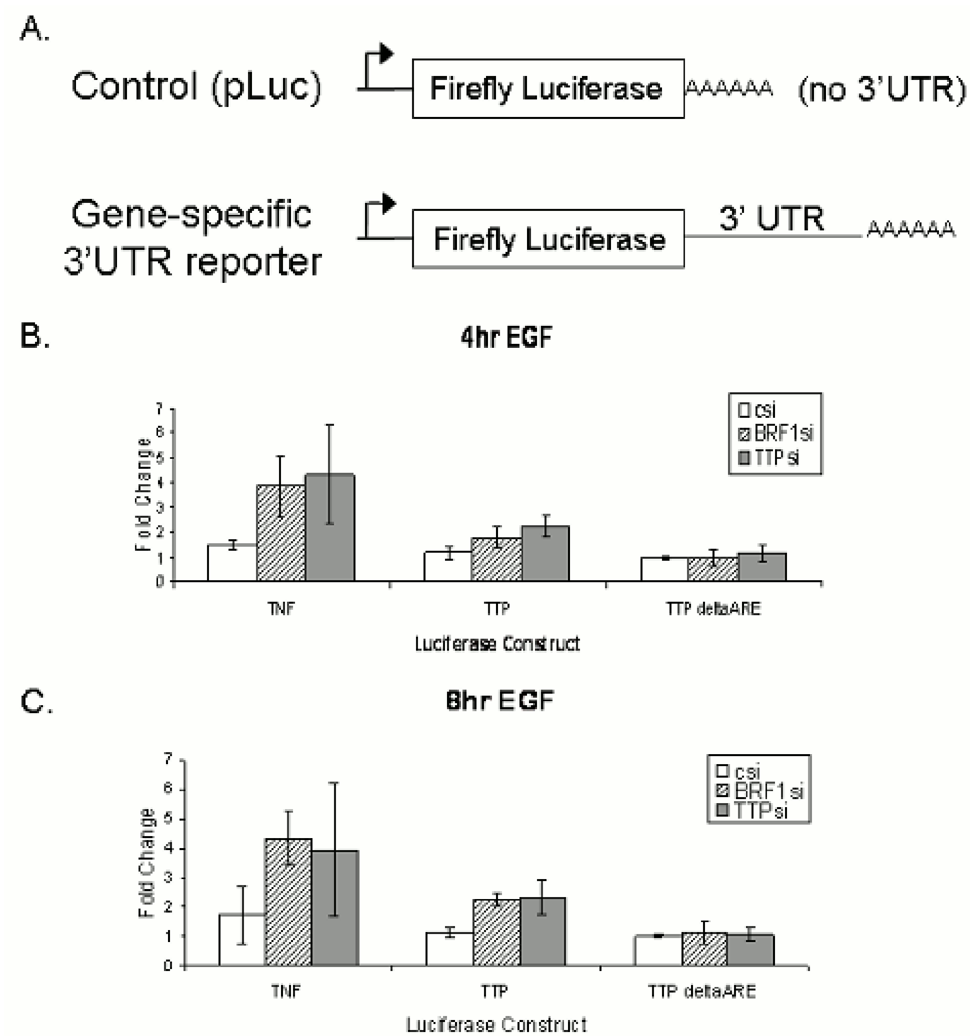

Figure 19: ARE dependence of TTP and BRF1 mediated regulation on TTP expression.

Panel A) is a representation of the firefly luciferase reporters used in this study. Panels B) & C) show effects of TTP or BRF1 siRNA knockdown on firefly luciferase constructs that were stimulated with EGF for either 4hr (B) or 8hr (C). Fold change of luciferase constructs were calculated by comparing normalized gene-specific 3'UTR reporter raw light units (RLUs) to normalized pLuc RLUs. Data represents n=3.

3.3.6 EGF-induced early genes are enriched for TTP targets

During the course of EGF treatment many other mRNAs are induced along with the TTP family member mRNAs, including several known TTP-regulated genes such as jun and c-fos mRNA. The induction of TTP family members alongside TTP targeted mRNAs suggests a possible role of TTP family members in degrading these transiently induced mRNAs. To expand this observation beyond the few anecdotal examples given, we obtained a global data set of mRNAs induced in an EGF stimulated time course (Amit et al., 2007). Since it was shown that many of the transcripts induced at early time-points following EGF stimulation are enriched for mRNAs containing AREs in their 3'UTRs, we decided to investigate whether these ARE-containing mRNAs were actually *in vivo* mRNA targets of TTP. To define *in vivo* TTP target mRNAs we obtained a data set of RIP-Chip identified TTP associated mRNAs from an LPS stimulated mouse macrophage cell line (Stoecklin et al., 2008). We grouped the EGF-induced transcripts according to their peak expression time and determined the percent of the transcripts from each peak expression group that were TTP targets, as defined by the TTP RIP-Chip. Enrichment of TTP targets was determined to be statistically significant according to the hypergeometric distribution using the whole genome as the null set.

Interestingly, the early EGF induced transcripts were greatly enriched for TTP target mRNAs, with transcripts that were induced at 20-40 minutes, 60 minutes and 120 minutes showing a statistically significant enrichment of TTP targets compared to the whole genome (figure 20). The time points with the most enrichment of TTP targets were 60 minutes and 120 minutes, this correlates with the rough peak expression times of TTP (60 minutes) and BRF1 (120 minutes) mRNA. This suggests that some of the same mechanisms that cause the TTP family members to peak (and their subsequent decrease) may also regulate the global mRNA response to EGF. Since the TTP family member mRNAs were shown to undergo ARE-BP dependent mRNA decay following EGF stimulation it indicates that many of the early induced EGF transcripts may also undergo changes in ARE-BP dependent mRNA decay.

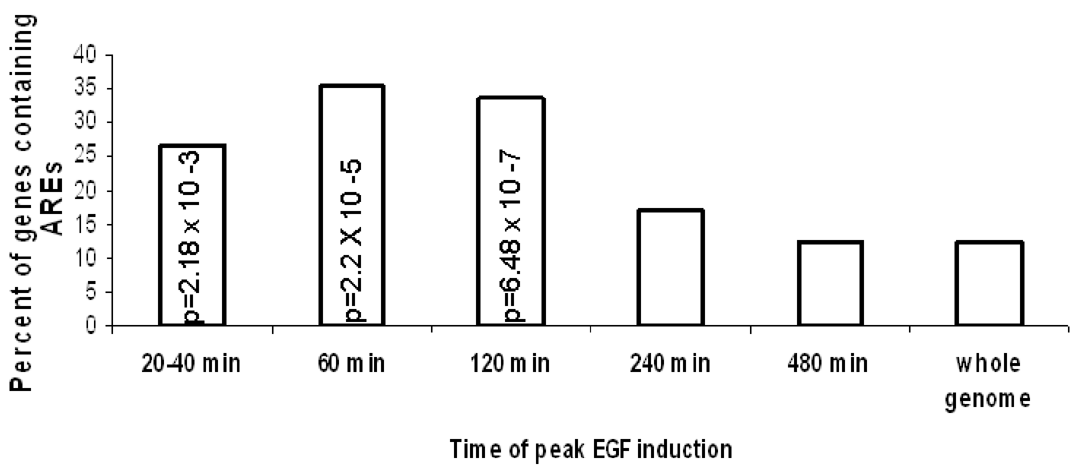

Figure 20: Early induced EGF genes are enriched in TTP target mRNAs.

EGF induced genes were grouped according to time of peak induction following EGF stimulation of HeLa cells, data set from Amit et al., 2007. TTP targets were identified by RIP-Chip from LPS stimulated RAW macrophages, data set form Stoecklin et al., 2008. The two data sets were compared to find the percent of EGF induced transcripts, for each time point, that were TTP targets, as independently identified by RIP-Chip. p-values were determined by hypergeometric distribution.

3.3.7 Correlations between TTP family member expression levels in activated cells

Since the TTP family member mRNAs show distinct temporal patterns of induction following EGF treatment we decided to investigate whether other cell lines and stimuli also show different expression patterns for the TTP family members. We looked at the correlation of mRNA levels between each of the TTP family members in publically available global gene expression datasets from Novartis (Su, Wiltshire, et al., 2004). This dataset contains many tissues and cell types in which the TTP family members are not expressed so we restricted the datasets to include only cells that were activated, this smaller data set mostly includes professional phagocytes such as marcophages, dendritic cells and mast cells that were activated with several stimuli over several time points. Using logistic regression we determined that TTP mRNA levels correlated negatively with BRF1 and BRF2 mRNAs levels, while BRF1 and BRF2 levels had a slight positive correlation (figure 21). Since this data set includes multiple time points of activation this indicates that the TTP family members have distinct expression profiles with TTP mRNA having a reciprocal relationship with the other TTP family member mRNAs.

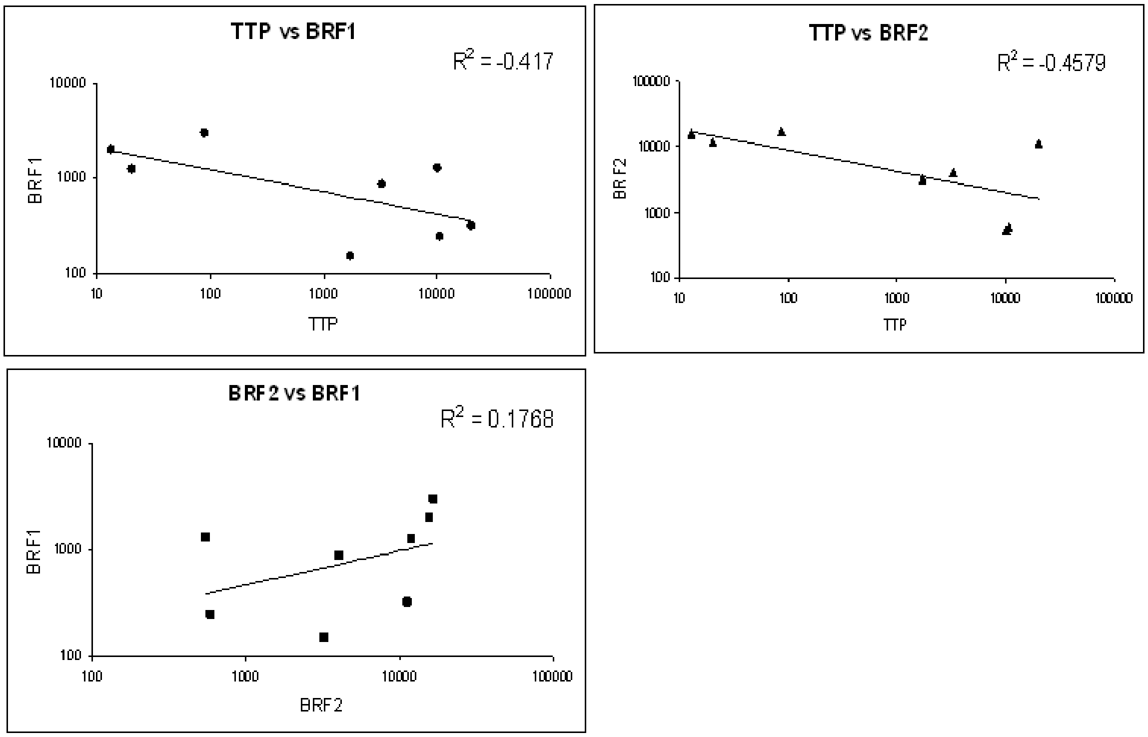

Figure 21: Correlations between TTP family members in activated cells.

These graphs show the correlation between pairs of TTP family member mRNAs across multiple activated and unactivated cells types. Values on the x- and y-axes are arbitrary expression intensity values for Affymetrix probes representing the given gene (expression data from Novartis's BioGPS, Su et al., 2004).

3.4 Discussion

We have shown that the TTP family member mRNAs are induced in a progression following EGF treatment with distinct peaks and kinetics. This pattern is controlled, at least in part, by both their basal decay rates and their changes in mRNA stability. The early induced family members, TTP and BRF1, have very unstable mRNAs in untreated cells while the late peaking BRF2 mRNA is relatively more stable in untreated cells. Interestingly, the relationship between rapid induction and short basal half-lives have been shown to be a general principle of global gene expression programs (Elkon et al., 2010). It has long been mathematically predicted that mRNA instability prior to induction causes transcripts to respond faster to induction than stable mRNAs, even in the absence of changing stability rates (reviewed in Ross, 1995). Thus, the inherent instability of TTP and BRF1 mRNA in untreated cells predicts (and in fact, causes) rapid rates of induction, like those observed following EGF treatment.

Changes in mRNA expression can result from a combination of changes in synthesis (transcription) and/or changes in decay (mRNA stability), with either an increase in synthesis or a decrease in decay resulting in elevated total mRNA levels. Since the TTP family member mRNAs are relatively unstable prior to EGF treatment but undergo increases in mRNA stability following EGF treatment, this change in mRNA stability can at least partially account for the increase in mRNA expression. This increase in mRNA stability most likely acts in concert with an increase in transcription to further enhance and amplify the increase in total mRNA levels, as it is known that TTP mRNA induction typically involves activation of its transcription (Lai et al., 1995; Lai et al., 1998).

Besides regulation of mRNA turnover and transcriptional regulation, the TTP family members are also regulated at other levels in response to various stimuli, including phosphorylation, localization, and protein stability. Both TTP and BRF1 can be phosphorylated in response to multiple stimuli by several different kinases. Some kinase signaling networks, such as MAPK, can phosphorylate and regulate both TTP and BRF1 while other networks appear to distinctly interact with one or the other (Ming et al., 2001; Maitra et al., 2008). All family members contain serine-rich regions that are likely substrates for many kinases, however these regions are less well conserved than other domains of the TTP family members suggesting that the TTP family members may be responsive to different kinases and pathways. Interestingly, while these phosphorylation events made be partially distinct among the family members, the effects of these differential phosphorylation events appear to control many of the same interactions and responses of the family members. For example, phosphorylation of both TTP and BRF1 by MAPKAP kinase 2 and protein kinase B respectively, induce protein-protein interaction with 14-3-3 proteins (Johnson, Stehn et al., 2002; Chrestensen et al., 2004; Stoecklin et al., 2004; Schmidlin et al., 2004). Furthermore, phosphorylation can influence nuclear versus cytoplasmic protein localization and/or localization to cytoplasmic granules for all family members. Importantly, many of these signaling events coordinate the regulation of most of these events simultaneously, including: mRNA induction, cellular localization, mRNA binding capacity, mRNA decay promoting functions, protein-protein interactions and protein stability (Stoecklin et al., 2004; Benjamin et al., 2006). Extensive regulation of TTP family member RBPs at many levels, through many distinct signaling networks allows for regulation at one level, such as mRNA induction, to often be accompanied by multiple regulatory events thus ensuring the proper coupling of their regulatory activities. This over-determined, intrinsically-coupled regulation indicates the important role these RBPs play in progression of these pathways.

It is interesting to note that the TTP family member RBPs, which are potent regulators of mRNA stability, are themselves regulated at the level of mRNA stability during EGF stimulation. It has previously been demonstrated that TTP regulates its own mRNA in an ARE-dependent feedback loop (Brooks et al., 2004; Tchen et al., 2004; Lin et al., 2007). We have presented here that the TTP family members, as a whole, are all sensitive to TTP and BRF1-mediated, ARE-dependent mRNA decay. Thus while the individual RBPs can regulate their own stability they also are capable of regulating each others stability in a TTP family-wide regulatory feedback loop. Since TTP family members are negative regulators of global gene expression, their induction is frequently believed to be important in limiting the extent and duration of global gene expression patterns. In this context, autoregulation of individual family members is a way to degrade and inactivate the individual family member at the same time that it is degrading and shutting off the early induced transcripts. Thus the autoregulation of individual family members is merely acting to shut down its own regulatory control of the gene expression program. So what would be the purpose of a family-wide regulatory feedback loop? One possible explanation is that the different TTP family members could bind and regulate different sets of mRNAs, thus they would degrade different transcripts at different times producing a staggered pattern of ARE-containing mRNA peaks. This possibility is unlikely given the similarities in RNA binding domains of the three TTP family member RBPs. Furthermore, the RNA elements recognized by each of the TTP family members are identical and they are known to share many common mRNA targets. Another potential and more likely explanation is that the TTP family-wide autoregulatory feedback loop serves to keep the earlier activated ARE-containing mRNAs repressed at later time points, thus preventing inappropriate reactivation of earlier expressed immediate early genes. For example, after TTP mRNA is degraded in an individual feedback loop to ensure that its levels are maintained at low levels the other TTP family members, which are expressed at later time points, feedback and prevent re-accumulation of TTP mRNA. This would allow sufficient time to proceed for full recovery and resolution of gene expression patterns before resetting induction pathways. This hypothesis for TTP family member autoregulation would suggest that the progressive and partially overlapping activation peaks of each of the family members is necessary for proper timing of recovery and resolution.

This individual and family-wide autoregulatory feedback would also predict that the global set of TTP family member targeted mRNAs would peak early and resolve at later time points. Interestingly, many previous studies have noted that ARE-containing mRNAs are often enriched in genes that rapidly peak following a wide range of stimuli, including EGF (Raghavan et al., 2002; Yang et al., 2003; Raghavan et al., 2004; Amit et al., 2007; Hao and Baltimore, 2009; Sparna et al., 2010). Furthermore, in addition to being enriched for ARE-containing transcripts, we have demonstrated that these EGF induced transcripts are enriched for *in vivo* targets of TTP as defined by RIP-Chip (Stoecklin et al., 2008). It is interesting to note that many of these TTP targets are rapidly but transiently induced, in a fashion that is similar to TTP's own mRNA. Thus it is likely that TTP is involved in the rapid clearance of many of these ARE-containing transcripts following EGF stimulation, much like it is involved in clearance of its own mRNA. Furthermore, the other TTP family member RBPs may ensure sustained repression of these ARE-containing transcripts at later time points similar to their potential role in repressing TTP's expression.

AREs are found in a larger than expected number of mRNAs encoding cell proliferation genes, signaling components, gene expression regulatory factors, and response factors, such as immune response, stress response, and host defense response (Bakheet et al., 2006). Many genes from these groups are required for rapid and efficient response to various stimuli but their sustained expression may interfere with progression and resolution of the response and in extreme cases may be detrimental to cellular health. As an example, extended expression of many of the immune response genes can lead to chronic inflammation and ultimately to destruction of bystander tissue or in some cases unchecked inflammation can also promote carcinogenesis. Therefore, it is sensible to ensure the destruction of these potentially harmful genes is encoded into the gene expression program that activates their expression or put another way, the negative regulators of these potentially harmful genes are activated along side to ensure a limited response.

It is possible that TTP family member regulatory feedback loops are an evolutionarily conserved feature given that most eukaryotes contain multiple (typically 2 to 4) TTP family member orthologs (*Schizosaccharomyces pombe* is one of the few exceptions with only one family member). In species as distant as yeast to humans, the TTP family members have been shown to have similar molecular functions and ARE binding specificities (Puig et al., 2005). Furthermore, another common characteristic of TTP family members is that their mRNA expression is often inducible and/or developmentally restricted in a wide range of species. Two yeast TTP homologs, CTH1 and CTH2, are rapidly induced by iron chelation with distinct peak expression patterns (Puig et al., 2008). This induction is also transient, much like the induction we observed for TTP mRNA following EGF stimulation. Additionally, it was shown that CTH1 and CTH2 cooperate to coordinate gene expression during iron deprivation but that they had distinct roles in regulating this coordination (Puig et al., 2008). The distinct roles of CTH1 and CTH2 in iron depletion was shown to be at least in part due to a combination of differences in induction and differences in mRNA targeting. Interestingly, the 3'UTRs of the family members, especially BRF1 and BRF2, are ultra-conserved and in particular the ARE regions are nearly identical from humans through zebrafish while the promoter regions are less well conserved (Bejerano et al., 2004). Other organisms, such as *C.elegans* and *S.cerevisiae*, also contain ARE sequences in the 3'UTRs of their respective TTP family member homologs. Finally, it has been suggested that yeast CTH2 can regulate the expression of yeast CTH1 (Puig et al., 2008). The combination of highly conserved TTP family member function and AREs in their 3'UTRs along with the presence of multiple family members per species raises the possibility that autoregulation and potentially family-wide feedback regulation is an universal regulatory aspect of TTP family member RBPs.

The precise role of TTP family member RBPs in EGF stimulated cellular responses is unknown, however there is extensive overlap in EGF induced responses and TTP family member dependent functions. For example, EGF treatment promotes cell proliferation and inhibits apoptosis whereas TTP overexpression reduces proliferation and increases caspase-3 mediated apoptosis (SuSwarm et al., 2008). Furthermore, knockdown of TTP dramatically increases EGF-stimulated cell migration in a transwell migration assay (Amit et al., 2007). This suggests that TTP expression impairs or mutes EGF induced responses, fitting the role of TTP turning off early EGF induced programs thus preventing sustained activation and allowing for recovery and resolution. Intriguingly, aberrant and sustained activation of the EGF receptor can result in cancer and furthermore, oncogenic mutations in EGF receptors (EGFRs) that cause constitutive activation of signaling have been found in several types of tumors including lung cancer and colon cancer subtypes (reviewed in Zhang, Berezov et al., 2007). It has also been observed that TTP mRNA expression is suppressed in many different types of tumors and overexpression of TTP restores sensitivity to proapoptotic stimuli and slows proliferation in cancer cell lines (Brennan et al., 2009). Thus sustained EGF signaling and/or loss of TTP-mediated attenuation are associated with cancer and cancer phenotypes. It may also suggest that TTP activation could be a promising therapeutic target in cancers with sustained EGFR activation, especially in recurrent EGFR inhibitor-resistant tumors.

4. Recognition of global RNA populations by the RRMs of HuR

4.1 Background and Significance

RNA recognition motif (RRM) containing RBPs are the largest group of single stranded RNA binding proteins in eukaryotes with over 700 members in humans alone (Finn et al., 2010). These diverse and evolutionarily conserved RBPs recruit a large variety of RNAs through binding to specific RNA elements to participate in a variety of functions ranging from splicing to translation. The RNA elements recognized by RRM containing RBPs usually are all single stranded but otherwise do not necessarily share common sequence characteristics. RRMs consist of two highly conserved subdomains, one consisting of an eight amino acid sequence called RNP-1 and a second upstream six amino acid sequence called RNP-2. RNP motifs were originally noted for their similarity between several diverse RBPs and for their ability to bind to RNA (Sachs et al., 1986; Adam et al., 1986). These RNP motifs are required but not sufficient for full RNA binding specificity of RRM domains (Query et al., 1989).

The AU-rich Element (ARE) RBP, HuR, contains three RRMs with the first two separated from the third by a linker region. Most reports indicate that each of HuR's RRMs, including RRM3, recognize similar but distinct U-rich elements (Levine et al., 1993; Gao and Keene, 1996). However, two reports have suggested that RRM3 can recognize poly(A) sequences (Abe et al., 1996; Ma et al., 1997). In some cases, individual RRMs have been suggested to act independently in recognizing distinct mRNAs (Levine et al., 1993). However, to date, no study has identified globally which RNA targets of HuR are recognized by which RRM(s) *in vivo*.

Until recently, technical limitations of global RBP target identification has prevented determining the contribution of HuR's RRMs in recognizing global mRNA populations and the RNA sequence elements directing these interactions. The standard technique for global RBP RNA target identification is RNA immunoprecipitation followed by microarray or sequencing and is known as RIP-Chip or RIP-Seq (Tenenbaum et al., 2000; Keene et al., 2006). While RIP-Chip excels at identifying *in vivo* interactions between RBPs and mRNAs it is inadequate for identifying individual binding sites with high nucleotide resolution. Other global RNA target identification techniques with better binding site resolution are unsuitable due to numerous deficiencies including low yield, sequence bias and potentially unwanted activation of DNA damage pathways (Ule et al., 2003; Licatalosi et al., 2008; Konig et al., 2010). However, a novel technique, called photoactivatable ribonucleoside crosslinked immunoprecipitation (PAR-CLIP) avoids these pitfalls while providing nucleotide level resolution of RNA-RBP interaction sites (Hafner et al., 2010; Mukherjee et al., 2011). We present here a global investigation of the contribution of HuR's RRMs to the recognition of mRNA populations using PAR-CLIP.

In addition to providing RNA recognition specificity, HuR's RRMs have also been suggested to influence various aspects of HuR-mediated posttranscriptional regulation. HuR is customarily thought to increase posttranscriptional expression of target mRNAs by stabilizing mRNA and/or enhancing translation, however HuR has also been observed to negatively impact gene expression in a condition and transcript specific manner (Katsanou et al., 2005; Kim et al., 2009). In addition to regulating cytoplasmic posttranscriptional gene expression (i.e. mRNA stability and translation) HuR has also been demonstrated to regulate nuclear posttranscriptional processing events, such as polyadenylation and splicing. HuRs ability to regulate both cytoplasmic and nuclear posttranscriptional processing events is dependent on its ability to shuttle between the two cellular compartments.

In most normal growth conditions HuR is a predominately nuclear protein with a weak cytoplasmic presence, however HuR can drastically redistribute to the cytoplasm in response to various stimuli or cellular events (Atasoy et al., 1998). Shuttling of HuR between the nucleus and cytoplasm has been suggested to require a subdomain contained in the linker region between the second and third RRMs, called the Hu nucleocytoplasmic shuttling (HNS) sequence (Fan and Steitz, 1998; Keene 1999). However, another study has suggested that only the third RRM is necessary for HuR's predominately nuclear localization in unstressed conditions (Chen et al., 2002). Other studies have suggested that the combination of both the HNS and the third RRM are necessary for HuR's cellular redistribution in response to numerous stresses (Brennan et al., 2000; Gallouzi and Steitz, 2001). Mechanistically, most of these studies have focused on protein-protein interactions between HuR's domains and nuclear ligands that mediate nuclear transport. However, to date, no studies have addressed how HuR's binding to RNA may impact its cellular distribution in either stressed or unstressed conditions.

It has been proposed that both nucleocytoplasmic shuttling under normal conditions and stress dependent cellular redistribution of HuR function to escort target mRNAs from the nucleus to the cytoplasm where HuR enhances their mRNA stability and translation (Keene, 1999). Using RNA binding impaired HuR mutants we report here that nuclear localization of HuR is inversely correlated with its ability to bind RNA.

4.2 Materials and Methods

4.2.1 Cell lines and plasmids

Stable cell lines expressing N-terminal FLAG tagged wild type or mutant HuR were generated by site specific recombination into Flp-In TREx HEK 293 cells. Stable cell lines were cultured in DMEM containing 10% tet-reduced FBS (Clontech), 15ug/ml blasticidin and 100ug/ml hygromycin for selection. Expression of the FLAG tagged inserts were induced with 1ug/ml doxycycline for 24 hours.

HuR RRM deletion mutants were generated using PCR fragment cloning and HuR RRM point mutants were created using site-directed mutagenesis. These mutants were then inserted into pCDNA5/FRT/TO using NotI and XhoI restriction sites.

4.2.2 RNA immunoprecipitations

Immunoprecipitations were prepared as previously described (Keene et al., 2006). Briefly, 5 x10^7 cells were collected, washed with PBS, and lysed in 500ul of polysome lysis buffer supplemented with protease inhibitors. Immunoprecipitations were performed using 25ul packed bead volume of anti-FLAG M2 affinity gel (Sigma). Lysates from cells expressing FLAG-EGFP were used as control IPs. In RIP RT-PCRs each primer set was normalized to GAPDH for each sample and this ratio was compared to the similar ratio from the EGFP IP to obtain fold enrichment.

4.2.3 End-labeling of Crosslinked RNA

Cells were washed with PBS and crosslinked in a UV Stratalinker 2400 at 250 mJ/cm2. Cell were lysed and immnoprecipitated as described above. IPed complexes were digested on the beads with RNase A (10ug/ml) for 1 hour at 37 degrees to remove uncrosslinked RNA. The complexes were then CIP treated and end labeled with P32 by PNK. After extensive washing with NT2 buffer the samples were boiled in 1x Laemmli loading buffer and electrophoresed on a 10% polyacrylamide gel.

4.2.4 RNA electrophoretic mobility shift assay

Radiolabeled RNA (100 pmoles) was incubated with indicated amounts of recombinant HuR in binding buffer (15 mM HEPES pH 7.4, 150mM KCl, 5mM MgCl2 and 10% glycerol) with 1ug tRNA as a non-specific RNA competitor for 30 min at 4 degrees. Loading buffer was added to the complexes and immediately loaded and run at 100V in a cold room on a pre-chilled non-denaturing 5% acrylamide TBE gel.

4.2.5 PAR-CLIP

PAR-CLIP was performed as previously described (Hafner et al., 2010). Specifically, twelve 15 cm2 plates were plated and cultured with 4-thio-uridine for 18 hours. The plates were washed with PBS and crosslinked with 150 mJ/cm2 of UV 365nm. The crosslinked cells were harvested in 5 ml of NP40 lysis buffer. The rest of the procedure was performed precisely as detailed in Hafner et al., The small RNA libraries for Solexa sequencing were made as previously described (Hafner et al., 2008). Oligonucleotides used to generate the libraries were as follows:

3′ adapter AppTCGTATGCCGTCTTCTGCTTG-L,

5′ adapter rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrC,

5′ primer AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA, and

3′ primer CAAGCAGAAGACGGCATACGA

4.2.6 Immunoflourescence

Cells were plated onto poly-D-lysine coated cover slips and allowed to grow for 24 hours prior to treatments. Cells were rinsed with PBS and fixed with 4% paraformaldehyde for 15 min. The fixed cells were then permeabilized with -20 degree methanol for 10 minutes. Cells were blocked with 5% horse serum for 1 hour, followed by 1 hour incubation of anti-FLAG M2 (1:200 dilution). Seconday antibody and DAPI were incubated for 45 min and coverslips were mounted with mounting media containing N-propylgalate. Cells were visualized using a Leica DMRA2 Compound Microscope.

4.3 Results

4.3.1 HuR RRM point mutants

To investigate the contribution of each of HuR's RRMs toward recognition of global mRNA populations we sought to independently disrupt the ability of each RRM to bind RNA. Previous studies investigating the role of the HuR's RRMs in recognizing cognate mRNA targets have deleted entire RRMs. However, the approach has several issues. Deletion of entire RRMs eliminates all RNA-independent functions of the deleted RRM, distorts the structure and/or function of the other domains and disrupts any potential interactions between each of the RRMs, such as multimerization. To avoid these issues we sought to create a series of point mutants that would cripple the ability of each RRM or combinations of RRMs to bind RNA. We narrowed the focus of identifying candidate residues to the conserved RNP motifs that have been previously shown to be important for RNA recognition by other RRM-containing RBPs (figure 22) (Sachs et al., 1986; Adam et al., 1986). The RNP motifs the aromatic residues that are responsible for base-stacking interactions were of particular interest because these are important for RNA specificity and for inducing conformational changes in the RNA that allow the RNA to fit into the RNA binding pocket of HuR (Wang and Tanaka-Hall 2001). Thus mutation of these aromatic residues were likely to drastically effect RNA binding ability of the RRMs without severely altering the structure of the protein. To ensure that mutation of aromatic residues to alanines were able to disrupt the ability of the RRM to bind RNA we first tested their ability to affect binding in individual RRMs in isolation from the rest of HuR (i.e. – deletion of the other domains). We made mutants to either RNP-1 (F65A, F289A) or RNP-2 (Y26A, Y249A) for both RRM1 and RRM3 and looked for binding to cellular RNA through UV crosslinking followed by polynucleotide kinase end-labeling (figure 23A). We demonstrated that the individual RRMs, without the point mutations, are able to bind RNA, however as expected they do not bind RNA as well as full-length HuR does. Mutations to either RNP-1 or RNP-2 mostly abolished the ability of the individual RRMs to crosslink to RNA, with the RNP-2 (RRM1-Y26A and h+RRM3-Y249A) mutations showing slightly better ability to disrupt RNA binding (figure 23B).

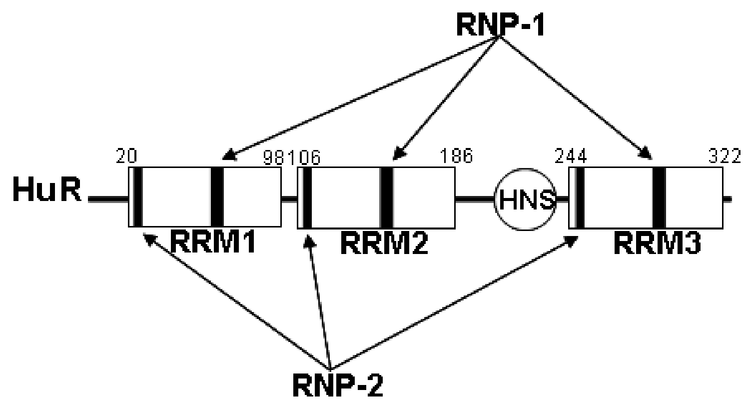

Figure 22: Domain structure of HuR.

This diagram represents the primary amino acid sequence of HuR with the boxes and the circle indicating the position of the RRMs and the HNS (Hu nucleocytoplasmic shuttling sequence) respectively. The numbers indicate the amino acid number for the beginning and end of each of the RRMs. The vertical black bars represent the RNP motifs as indicated by the labels.

Next, we introduced the RNP-2 mutations into each of the RRMs of full-length HuR creating 3 single point mutants (Y26A, Y109A and Y249A), one double point mutant (Y26A/Y109A) and a mutant with all three RRMs mutated (Y26A/Y109A/Y249A). To investigate whether these RNP mutants could effect full-length HuR's ability to bind RNA we performed RNA immunoprecipitation and probed for a diverse panel of known Hu targets and non-targets by RT-PCR. Surprisingly, each of these mutants, including the triple RRM mutant, were able to bind RNA. However, most of the mutants showed drastically reduced binding to mRNA when compared to wild type HuR. The RRM3 mutant (Y249A), which still had two functional RRMs, showed only a modest decrease in binding while the double RRM mutant (Y26A/Y109A), showed a significant decrease in binding. The triple RRM mutant (Y26A/Y109A/Y249A) was the most severely impaired in its ability to bind mRNA but still associated with amounts of mRNA that were slightly greater than control IPs. To determine if the low levels of binding seen in the full-length mutants were due to non-specific, RRM-independent interactions we performed RNA immunoprecipitations in the presence of 3M UREA. RRM-containing proteins are able to bind RNA even in the presence of high concentrations of UREA which will disrupt most other protein-protein interactions or non-specific binding to regions outside the RRMs. While UREA washes caused a slight decrease in association of RNA with all the HuR RRM mutants, this decrease was of a similar scale as to that seen for wild type HuR (not shown). This suggests that the RNP-2 point mutations severely cripple RNA binding of HuR but do not completely eliminate binding and that this residual binding is not due to indirect or non-specific interactions.

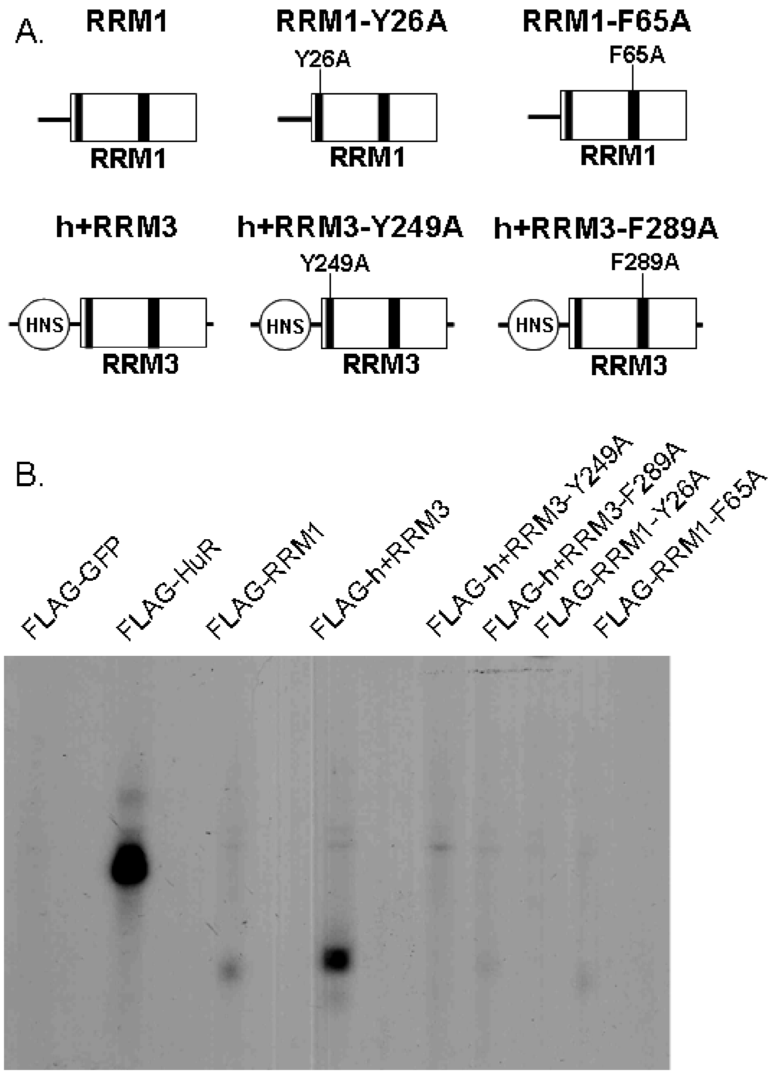

Figure 23: HuR RNP point mutants disrupt ability of individual RRMs to bind RNA.

Panel A) depicts constructs used in this experiment and the position of the mutants. Panel B) is an SDS-PAGE gel of HuR RNP point mutants that were UV crosslinked, immunoprecipitated, RNase treated and P32 PNK end-labeled. The gel was directly exposed to autoradiographic film.

Another possibility for seeing drastically reduced yet still detectable binding is that Hu proteins have been reported to form homomultimers with itself (Gao and Keene

1996; Soller and White 2005). Thus it is possible that the mutant proteins maybe forming dimers with endogenous wild type HuR and therefore the mutants maybe indirectly dragging down mRNA through this interaction with HuR. To test this we performed immunoprecipitations, using the same protocol as used in RNA immunoprecipitations, with FLAG-tagged wild type or mutant HuR and immunoblotted with an antibody directed against an endogenous HuR epitope. Under these conditions we did not see evidence for dimerization of either wild type or mutant HuR with endogenous HuR (not shown). Therefore, we can conclude that the small amounts of RNA seen with the conditions in the mutant IPs are most likely not due to dimerization.

4.3.2 Preference of HuR RRMs for different mRNA transcripts

It has previously been suggested that the RRMs of HuR recognize not only different RNA sequences but different mRNAs altogether with RRMs 1 and 2 cooperating to recognize the same transcripts and RRM3 acting independently (Levine et al., 1993). However, many other studies have come to different and somewhat controversial conclusions. Using the RRM mutants we have decided to address the contribution of HuR's RRMs to the differential recognition of individual target mRNAs. Initially, we detected mRNAs bound to wild type full-length HuR or to individual RRMs (i.e. – deletions) by RNA immunoprecipitation followed by RT-PCR using a small panel of literature validated targets and several non-targets. Each of the individual HuR RRMs were able to enrich at least several of the target mRNAs, albeit less efficiently than full-length HuR (figure 24). Furthermore, the individual RRMs demonstrated specificity by showing no binding for several of the other HuR target mRNAs. Specifically, RRM1, RRM3, and RRM1+2 were all able to bind to the highly enriched HuR target, beta-acitn (ACTB) mRNA but only RRM1 and RRM1+2, and not RRM3, bound to the classical ARE-containing mRNA, c-fos. Interestingly, another well known target, HuR's own mRNA, showed a different pattern of enrichment in the RIPs with RRM1+2 and RRM3 but not RRM1 binding to HuR's own mRNA (figure 24). This indicates that the isolated RRMs (deletions) provide different specificities for mRNA recognition.

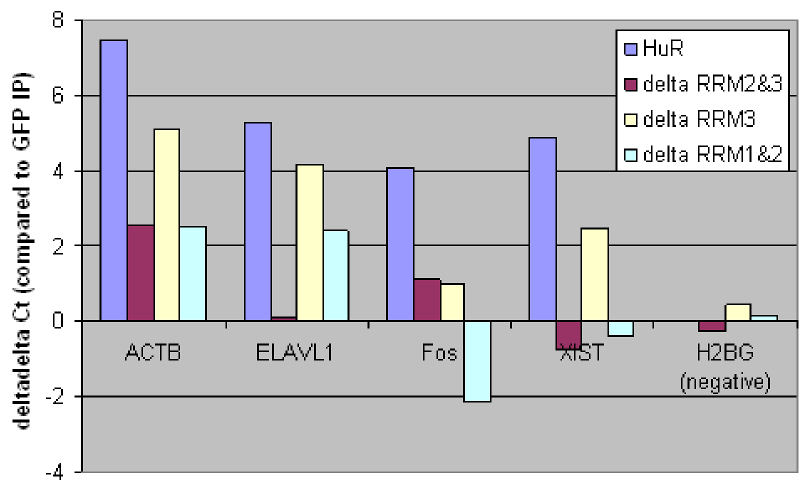

Figure 24: RIP-RTPCR demonstrating that HuR RRM deletion mutants provide different mRNA recognition specificities.

A graph of RNA Immunoprecipitation followed by RT-PCR for individual HuR RRMs. Y-axis indicates the log base 2 enrichment of GAPDH normalized mRNAs compared to a negative IP (GFP IP), a value known as deltadelta Ct (for change in change of cycle threshold).

These individual RRM RIPs agree with previous reports, however both these RIPs and the previous studies only investigated the role of the individual RRMs in isolation from the remainder of HuR, thus ignoring cooperation or contribution by the other RRMs. The observation that each of the individual RRMs bind much less efficiently to the tested targets than full-length HuR suggests that the RRMs can cooperate to recognize target mRNAs and that all RRMs are needed for maximal binding. Furthermore, the co-crystal structure of RRMs 1 and 2 of HuD, a highly similar paralog of HuR, complexed with mRNA has shown that RRM1 and RRM2 both cooperate to bind to the same mRNA sequence (Wang and Tanaka-Hall, 2001). Furthermore, the co-crystal structure demonstrated that the combination of both RRM1 and RRM2 created a biding pocket between the two that fits an RNA of a particular single-stranded conformation. This additional constraint of RNA conformation produced by the combinatorial effects of both RRM1 and RRM2 produces an emergent property of RNA recognition that is not predictable from analyzing the individual specificities of RRM1 and RRM2. Therefore we decided to analyze the capacity of the HuR RRM point mutants to bind mRNA in the natural structural context of full-length HuR. The full-length RRM point mutants, as expected, showed reduced association with target mRNAs, however relative differences in specificity for particular mRNAs were much less pronounced than those seen for the HuR RRM deletions, with many of the relative differences being small enough that they were not readily distinguishable from experimental variance (figure 25). The lack of differences in relative mRNA specificity for the RRM point mutants suggests that the RRMs cooperate to recognize target mRNAs.

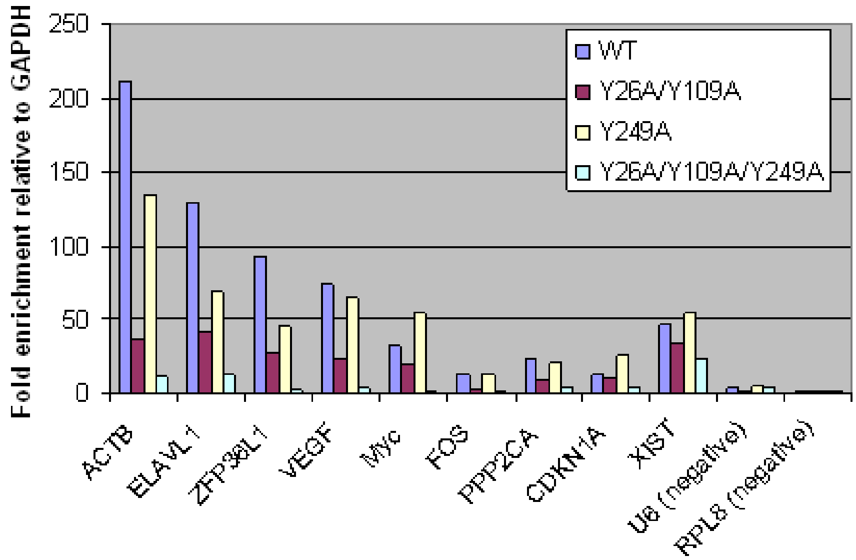

Figure 25: RIP-RTPCR demonstrates that HuR RRM point mutants reduce binding for a number of HuR target mRNAs.

The graph shows fold enrichment of HuR target mRNAs in RNA immunoprecipitations of with indicated HuR RRM point mutants of full-length HuR. GAPDH normalized values for each mRNA were compared to a EGFP control IP to determine fold enrichment. U6 and RPL8 are non-targets for comparison of background binding.

4.3.3 PAR-CLIP of HuR RRM point mutants

The binding of the full-length HuR RRM point mutants was only investigated for a few known targets, so it is possible that there are differences in specificity for other mRNAs that were untested in these experiments. To determine if the HuR RRM point mutants bind different global mRNAs we decided to use photoactivatable ribonucloside crosslinked immunoprecipitation (PAR-CLIP) (Hafner et al., 2010). Briefly, this technique utilizes 4-thiol-uridie (4SU) which is introduced into the media and incorporated into RNA, 4SU-labelled RNA is then crosslinked to protein using UV light at 365nm wavelength. The RNA is then partially digested with the sequence specific RNase T1 and immunoprecipitated with an antibody for the protein of interest. This cosslinked, partially digested RNA is then sequenced with high-throughput sequencing. This protocol allows for several advantages, first UV 365 is far less harmful to cells (it does not damage DNA) than UV 254 which used in other RNA crosslinking protocols (Ule et al., 2003). UV 365 also allows for a greatly improved recovery (100 to 1000-fold) over traditional UV 254 crosslinking. Finally, crosslinked 4SU nucleotides creates a signature at the site of crosslinking that is read in the sequencing results as a T to C conversion, thus allowing for easy identification of the direct site of interaction. With the ability to detect individual binding sites and direct sites of interaction on a global scale this technique is ideally suited to use with the RRM point mutants to identify how the RRMs of HuR contribute to the recognition of global RNA populations.

4.3.4 RNA binding dependent cellular localization of HuR

HuR is a nucleocytoplasmic shuttling protein that, under most conditions, is predominately nuclear but drastically redistributes to the cytoplasm following various treatments. A sub-domain contained in the hinge region between the second and third RRMs, called the Hu nucleocytoplasmic shuttling (HNS) sequence, has been shown to be necessary for HuRs shuttling between the cytoplasm and the nucleus (Fan and Steitz, 1998; Keene, 1999). Most of the studies involving HuR's localization have focused on the HNS or its interaction/co-localization with other factors but none have addressed whether localization of HuR is RNA dependent. To address this directly, we performed both immunofluorescence and cell fractionation with the full-length HuR RRM point mutants. Using the FLAG-tagged mutants and wild type HuR we paraformaldehyde fixed untreated, normal growing cells and immuno-stained for the FLAG tag. We observed that the wild type construct was predominately nuclear with a small fraction in the cytoplasm, as expected, indicating that the procedure is working properly. The RRM3 point mutant (Y249A) was predominately nuclear with a staining pattern nearly identical to wild type HuR (figure 26). The RRM1+2 double point mutant (Y26A/Y109A) also was predominately nuclear, however it also appeared to have a slight increase in cytoplasmic staining compared to wild type HuR or the RRM3 mutant (Y249A). Interestingly, the RNA-binding impaired triple RRM mutant (Y26A/Y109A/Y249A) was predominately cytoplasmic instead of nuclear (figure 26). Since the triple RRM mutant (Y26A/Y109A/Y249A) caused a dramatic redistribution of HuR while neither the RRM3 (Y249A) nor the RRM1+2 (Y26A/Y109A) point mutants caused a dramatic redistribution this suggests that the nuclear localization of HuR is dependent on the cooperation of all three HuR RRMs to bind RNA.

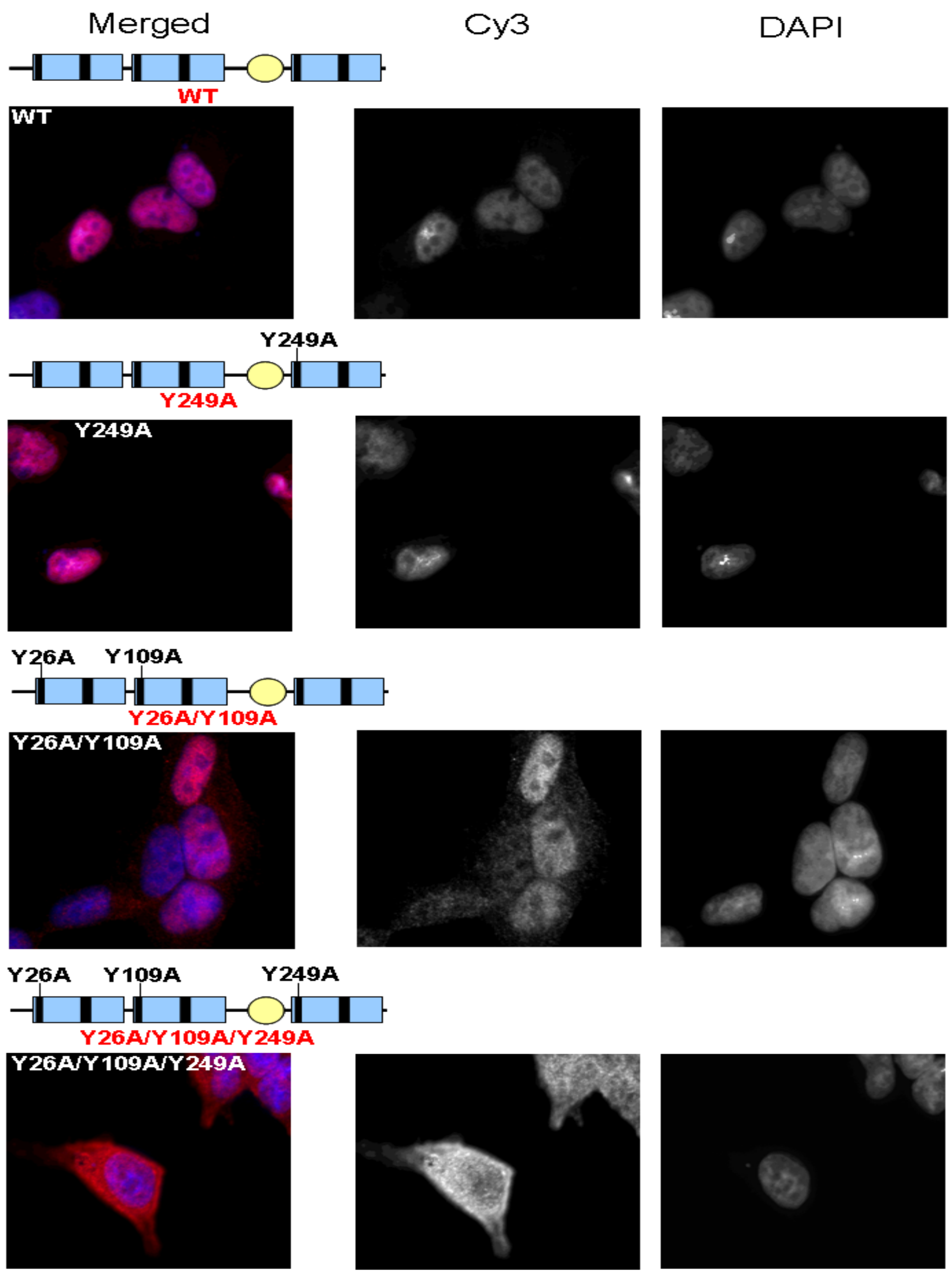

Figure 26: HuR triple RRM point mutant cause a dramatic redistribution of HuR cellular localization.

Images are from immunofluorescent staining of formaldehyde fixed HEK293 cells stained with anti-FLAG (red) and DAPI (blue). Diagrams depict the position of point mutations used in this experiment.

4.3.5 RNA binding dependent nucleocytoplasmic shuttling of HuR

Since the HuR triple RRM point mutant (Y26A/Y109A/Y249A) alters the cellular distribution of HuR we sought to identify the mechanism of this mislocalization. To test whether the cytoplasmic accumulation of the triple RRM point mutant (Y26A/Y109A/Y249A) was due to impaired nuclear import we examined localization under conditions in which nuclear import of HuR is normally increased due to activation of the import machinery. It has previously been demonstrated that disruption of cellular energy reserves and activation of AMPK cause a increase in nuclear localization of HuR protein through interaction with phosphorylated importin-1 alpha (Wang et al., 2004). Under normal conditions HuR is already predominately nuclear so activation of AMPK does not cause a drastic redistribution of HuR, however several cell lines with basally high levels of cytoplasmic HuR do respond to AMPK activation with a partial increase in nuclear HuR levels (Wang et al., 2002). By treating cells with cytochrome c reducatase inhibitor, Antimycin A (AMA), and subsequently activating AMPK we observed a partial increase in the nuclear levels of the HuR triple RRM point mutant (figure 27). This indicates that crippling HuR's ability to bind RNA does not effect its ability to be imported into the nucleus, this is further supported by the observation of a small portion of HuR triple RRM point mutant (Y26A/Y109A/Y249A) in the nucleus under unstressed conditions.

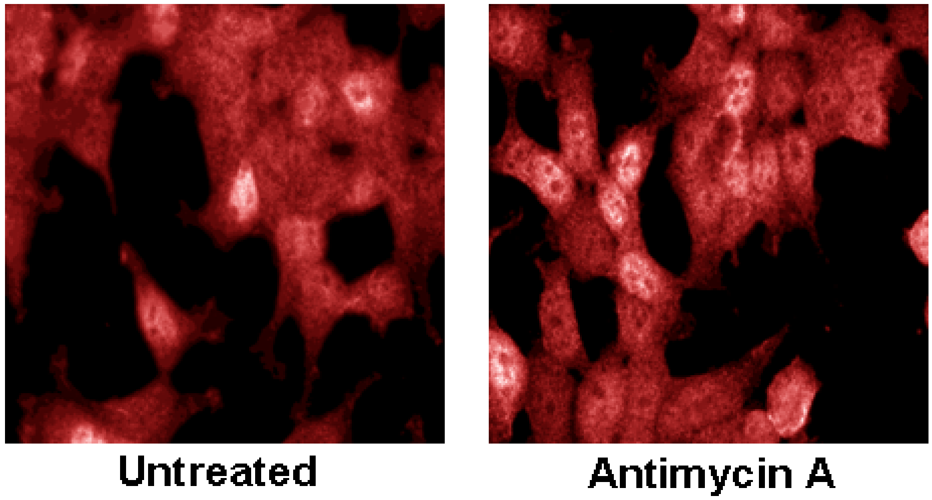

Figure 27: AMA treatment induces nuclear localization of HuR triple RRM point mutant.

Immunofluorescent staining of HEK293 cells treated with Antimycin A or mock treatment for 5 hours. Staining with anti-FLAG antibody.

There are at least two distinct pathways that regulate the nuclear export of HuR: the leptomycin B (LMB) sensitive, CRM1 pathway which regulates stimuli responsive increases in HuR cytoplasmic levels, and another unidentified pathway, which may involve the soluble carrier protein karyopherin beta 2B also known as transportin 2, that supports nuclear export of HuR during unstressed conditions (Gallouzi and Steitz, 2001). Karyopherin beta 2B is known to interact with components of the TAP/NXF1 export pathway which is responsible for the majority of mammalian mRNA nuclear export, furthermore TAP/NXF1 has been demonstrated to interact with the neuronal HuR paralog, HuD (Saito et al., 2004). To test if HuR nuclear export is dependent on its ability to bind RNA we examined the effects of LMB on cellular localization of the HuR triple RRM point mutant. We observed that there was no discernable difference in nuclear to cytoplasmic localization of the triple mutant after 8 hours of LMB treatment in otherwise unstressed cells (not shown). This suggests that RNA independent export of HuR in unstressed cells in not mediated through the CRM1 pathway.

4.4 Discussion

RRMs are large domains of roughly 90 amino acids that recognize specific nucleotides through extensive protein-RNA interactions (Query et al., 1989). Different RRMs can recognize different RNA sequences by making contact with as many as 6 nucleotides or as few as 2 nucleotides per RRM (reviewed in Clery et al., 2008). RRM domains share a general structure usually consisting of 4 β-sheets and 2 α-helices, however the primary amino acid sequence can be quite variable. RRMs contain two sub-domains called RNP-1 and RNP-2 which are important for RNA recognition and usually contain characteristic hydrophobic and aromatic residues, with the later forming base-stacking interactions with the RNA (reviewed in Kenan et al., 1991). We have demonstrated that mutation of aromatic residues in RNP-1 or RNP-2 of HuR's RRMs severely impair its ability to recognize RNA. These mutations do not completely ablate HuR's ability to bind RNA, but this is not surprising given the large size and extensive interactions between RRMs and their RNA targets (Wang and Tanaka-Hall 2001). Furthermore, the observation that point mutantion of RNP-1 or RNP-2 do not completely disrupt HuR's ability to bind RNA suggest that these mutants have not drastically altered the structure of the protein, but have nonetheless reduced the ability of HuR to recognize targets by several fold. Similar point mutants to aromatic residues in RNP motifs have previously been shown to disrupt RNA recognition of other RRM containing RBPs, including PABP and U1A, suggesting that these residues are necessary for RNA recognition of many RRM-containing proteins (Deardorff and Sachs 1997; Shiels et al., 2002).

RRM-containing proteins are the largest group of single stranded RNA binding proteins in eukaryotes, and many RRM-containing proteins have multiple RRM domains with some containing as many as 6 putative RRMs (Finn et al., 2010). It is unclear why so many RRM-containing RBPs have multiple RRM domains, however since individual RRMs can recognize specific yet distinct RNA sequences from other RRMs it is possible that multiple RRMs may allow an RBP to recognize multiple RNAs through different sequences. This has been suggested to be the case for Hu proteins, which contains three highly conserved functional RRMs that each appear to have different sequence specificities and affinities (Levine et al., 1993; Chen et al., 2002). Similarly, we observed that individual RRMs of HuR, when studied in isolation from the other RRMs, bound to distinct RNAs. This agrees with previous observations that RRM3 bound to different transcripts than RRM1+2 (Levine et al., 1993). However, when we investigated whether full-length HuR RRM point mutants bound to different transcripts we did not see robust differences in specificity. This difference in mRNA specificity between the individual HuR RRMs and the full-length RRM point mutants cannot be explained by the point mutants causing too subtle of a change to be observed because when we introduce the point mutants in the context of the individual RRMs we see an easily detectable disruption in RNA recognition. This suggests that other residues in individual RRMs cooperate with the other two RRMs to provide specificity, but importantly these other residues are not sufficient for RNA binding in isolated RRM point mutants. This may indicate that the each of the RRMs combine to confer a "structural" specificity, or a binding pocket that fits a particular RNA conformation. This hypothesis is supported by the co-crystal structure of RRMs 1 and 2 of HuD which showed that RNAs with a particular conformation, specifically a 'turn' in the RNA, mediated by intramolecular RNA interactions, bound the binding pocket of HuD and thus may contribute to the RNA specificity (Wang and Tanaka-Hall, 2001).

Since the individual RRM's contribution to mRNA specificity may also depend on the presence of other RRMs this implies that previous studies that have used RRM deletions in studying HuR mRNA specificity may have misinterpreted the implications for the contributions by the individual RRMs. However, the RRM deletions from many of the previously described studies are similar to the products produced when caspases cleave HuR in the lilnker region between RRMs 2 and 3 to produce two peptide fragments, one containing RRM 3 and the other containing RRMs 1 and 2 (Mazroui et al., 2008). This suggests that the two apoptotic cleavage fragments of HuR may have different mRNA binding specificities than full-length HuR and thus may influence HuR's function in apoptosis.

Although the full-length HuR RRM point mutants did not show much difference in mRNA specificity for the few mRNAs tested it is possible that the RRMs contribute to recognition of different mRNAs not tested in these experiments. Therefore it will be important to investigate the contribution of each RRM in the context of the full-length RBP with a global RNA target identification technique, such as PAR-CLIP. In addition to recognizing distinct mRNAs the RRMs might also recognize different and distant RNA elements within the same mRNA. To investigate this on a global level it is necessary to use a technique that has nucleotide-level resolution, such as PAR-CLIP, which ideally suited for this purpose.

Using the full-length HuR RRM point mutants we show for the first time that HuR nuclear/cytoplasmic distribution is dependent on HuR's binding to RNA. In unstressed, normal growing cells HuR is predominately localized to the nucleus, however when we disrupt its ability to bind mRNA by mutating all three RRMs simultaneously, HuR becomes predominately cytoplasmic. Interestingly, mutation of RRM3 alone does not significantly alter the cellular distribution and mutation of RRMs 1 and 2 together only mildly increase cytoplasmic localization. However, when all three RRMs are mutated HuR's localization is strikingly altered suggesting that cooperation of all three RRMs is required for HuR's nuclear localization in normal conditions. The degree to which the mutants cause cytoplasmic localization inversely correlates with their ability to bind mRNA, with the RRM3 mutants that only modestly inhibit RNA binding showing strong nuclear accumulation, the RRM1+2 double point mutants that cause an intermediate decrease in RNA binding ability coincidentally cause a slight shift in cellular localization, and finally the triple RRM mutants that severely impair RNA binding showing strong cytoplasmic accumulation.

We also demonstrate that AMPK activation can increase nuclear localization of the HuR triple RRM mutant. This suggests that the HuR triple RRM mutant is not deficient for nuclear import and that cytoplasmic accumulation of the HuR triple RRM mutant is either a result of increased nuclear export or of reduced nuclear retention. We did not observe a change in cytoplasmic localization of the HuR triple RRM mutant by pharmacological inhibition of CRM1 in unstressed cells suggesting that the mutant is not exported in a CRM1-dependent fashion. However, it remains to be seen whether the export of the triple RRM mutant during stress is dependent on CRM1. This would be interesting because it has previously been shown that various stresses increase indirect interaction between HuR and CRM1 in a manner that is dependent on both the HNS and RRM3 (Gallouzi and Steitz, 2001). Furthermore, it is not known how RNA binding impaired HuR is exported in unstressed conditions. It has been suggested that there are other nuclear export pathways that may be involved in exporting HuR, specifically karyopherin beta 2B has been suggested to interact with HuR and may regulate its export under unstressed conditions (Gallouzi and Steitz 2001; Saito et al., 2004). In addition karyopherin beta 2B is also known to interact with TAP/NXF1, the major mRNA export pathway (reviewed in Nakielny and Dreyfuss 1999). It remains to be seen if RNA binding dependent nuclear export of HuR requires karyopherin beta 2B in unstressed conditions, but the connection between karyopherin beta 2B and the major mRNA export pathway is intriguing.

Previous studies on the cellular localization of HuR have identified the HNS region between RRMs 2 and 3 as being both necessary and sufficient to impart nucleocytoplasmic shuttling on reporters in hetrokaryon experiments (Fan and Steitz, 1998). These studies never addressed the role of RNA binding in HuR shuttling and furthermore by deleting large portions of HuR and inserting them into a shuttling reporter may have disrupted long distance interactions between the HNS and other regions of HuR that may play roles in nucleocytoplasmic shuttling. Therefore it remains possible that the HNS can be necessary for shuttling of HuR but that shuttling is also dependent on HuR's ability to bind RNA. The possibility that RNA binding ability could contribute to regulation of HuR nuclear export suggests the intriguing possibility that RNA elements may also contribute to regulation of HuR nuclear export. This might occur through a mechanism where exports factors interact with other RBPs bound to HuR targeted mRNAs and indirectly influence HuR's nuclear export.

Furthermore, the HNS region has been previously shown to interact with several different proteins that influence its nuclear export (Brennan et al., 2000; Gallouzi et al., 2001). Since we have shown that HuR's ability to bind RNA alters its localization it will be interesting to see how RNA binding ability effects the interaction between the HNS on the protein ligands that mediate export.

Another curious observation is that RNA binding impaired HuR displays a cellular localization similar to wild type HuR following RNA polymerase II inhibition by either actinomycin D (ActD) or 5,6-dichloro-1-beta-D ribofuranosylbenzimidazole (DRB). This suggests the possibility that under normal conditions HuR is retained in the nucleus by binding to actively transcribing mRNA in a co-transcriptional manner and that by disrupting HuRs ability to bind RNA, either through mutation or by pharmacological inhibition of transcription, HuR is no longer passively retained in the nucleus and is exported to the cytoplasm. Recent studies have shown that HuR can bind to pre-mRNA and regulate splicing, thus supporting the hypothesis that HuR can bind RNA co-transcriptionally (Izquierdo 2008; Izquierdo 2010; Mukherjee et al., 2011; Lebedeva et al., 2011).

Interestingly, this potential connection between HuR's ability to bind RNA and it's cellular localization has a corollary with another ARE-BP, the yeast TTP homologue CTH2. CTH2 has been suggested to bind RNA co-transcriptionally as well but its localization in logarithmically growing cells is predominately cytoplasmic (Vergara et al., 2011). When CTH2's ability to bind RNA is disrupted with point mutations to it's C3H zinc finger RNA binding domain, CTH2 also redistributes and becomes predominately nuclear in localization. While the dependency of CTH2's localization on RNA binding ability is similar to HuR's it's cellular distribution is reciprocal, this may suggest distinct roles for each of these ARE-BPs in mediating co-transcriptionally regulated export of mRNAs.

HuR cellular localization has long been known to be responsive to many different stimuli and recently many of the signaling pathways regulating these responses have been identified (reviewed in Doller et al., 2008). Regulation of HuR localization by many of these pathways has been shown to require specific serine or threonine residues of HuR and several pathways even require multiple residues. The posttranslationally modified residues that regulate cellular redistribution of HuR have been found in each of the RRMs as well as in the linker region between RRMs 2 and 3 (reviewed in Doller et al., 2008) (figure 1). This suggests that multiple regions of HuR can influence its localization and that this may involve long-distance intramolecular interactions. Interestingly, several of these pathways have been shown to not only regulate the cellular distribution of HuR but also effect its ability to bind to at least a subset of mRNAs (reviewed in Doller et al., 2008). There has only been a single report of a stimuli that effects HuR's ability to bind mRNA but that does not affect its cellular localization, however this study failed to investigate whether these effects occurred simultaneously as they used a very early time point to look a localization and a much later time point to check mRNA binding (Masuda et al., 2011). Given this strong connection between HuR's stimulus-dependent cellular distribution and its ability to bind mRNA and the observation that residues located in the RRMs are important for cellular localization, it is tempting to speculate that stimulus-dependent localization and RNA binding of HuR are mechanistically linked. Tools such as the RNA binding deficient HuR mutants that we have created may be useful for elucidating the mechanisms by which localization and RNA binding are linked in response to external stimuli.

5. Conclusion

We have shown that modulation of HuR protein levels has a slight effect on the translation and mRNA stability of TTP, BRF1 and HuR itself and that the amplitude of this effect is dependent on cell type and stimuli. This raises the question: are there other conditions that may produce larger more robust effects on this network? HuR's function is known to be affected by many different signaling pathways, usually by regulating its cellular localization or its ability to bind RNA (reviewed in Doller et al., 2008). Interestingly, regulation of these pathways rarely produce changes in the levels of HuR expression. However, there are two notable exceptions to this: neuronal differentiation and cell cycle progression (Atasoy et al., 1998; unpublished observations, Kyle Mansfield). In neuronal differentiation, when HuR levels decrease the other Hu protein family members increase suggesting that the total amount of Hu protein (including the neuron-specific family members) may remain rather stable throughout neuronal differentiation. This is particularly interesting given our observation of resiliency in the ARE-BP network containing TTP, BRF1 and HuR to changes in amounts of HuR protein. It may suggest that while the mechanism between neuronal and non-neuronal cells is different, the outcome of sustaining constant total Hu protein levels is conserved. This leaves cell cycle regulation of HuR expression in the unique position with no apparent compensation for decreased Hu protein levels. This response may involve a different regulatory mechanism. Therefore, it will be interesting to see whether cell cycle events alter HuR autoregulation and whether HuR is still resilient to fluctuations in its protein levels. It is unknown whether TTP and BRF1 are also regulated during cell cycle progression.

While the decrease in HuR levels did not produce robust effects on the translation or mRNA stability of TTP, BRF1 or HuR, it is possible that HuR can regulate these transcripts during other posttranscriptional processes. As previously mentioned, HuR was been found to regulate many different posttranscriptional processes such as splicing and polyadenylation (reviewed in Hinman and Lou 2008; Mukherjee et al., 2011). Unpublished results from the Keene lab suggest that HuR (as well as the three neuronal Hu members) can bind near the poly A signal of HuR's mRNA and this binding corresponds with an increase in processing at an alternative polyadenylation cleavage site (unpublished observations, Kyle Mansfield). It remains to be seen if HuR's effects on polyadenylation of its own message can occur in cell types other than neuronal cells.

Another interesting observation of ours was that overexpression of HuR caused a slight decrease in expression of a HuR 3'UTR reporter but also caused a slight increase in the expression of TTP or BRF1 reporters. This result was complicated by the conflicting observation that siRNA knockdown of HuR also caused a slight decrease in the expression of all three of the 3'UTR reporters (HuR, TTP and BRF1). The effects of overexpression and siRNA knockdown of HuR on the TTP and BRF1 reporters are consistent with HuR's traditional stimulating effects on posttranscriptional regulation. The conflicting reports of HuR knockdown and overexpression affecting HuR's 3'UTR reporters may be the result of a dominant negative effect of overexpression. However, this potential dominant negative effect is specific for the regulation of HuR's 3'UTR reporter because the responses of the 3'UTR reporters of TTP and BRF1 mRNAs concur with the knockdown data. Therefore this potentially artifactual result is sequence specific which suggests that some property of the sequence is responsible for the observed differential regulation. This naturally raises the question of what the underlying mechanism of these sequence characteristic might be and how this interaction results in different outcomes. One possibility is that other factors such as other RBPs or microRNAs are involved, and possibly in competition with, HuR for certain sequences (Kim et al., 2009; Meisner and Filipowicz 2011). While the slight change in amplitude of the observed here does not warrant an in depth analysis of these sequences under these particular cellular conditions, it suggests that these apparent conflicting and confusing results may arise in other biological settings. Given the accumulating evidence that HuR can differentially affect the outcome of some transcripts dependent on cell type, condition or sequence, it is likely that more robust versions of events observed here will emerge in the future.

Interestingly, transcripts that are induced by EGF at early time points (20 min – 2 hr) are enriched for mRNA targets of TTP. These time points coincide with the induction and peak of TTP's own mRNA suggesting that TTP can degrade the early EGF-induced transcripts much like it degrades its own message. Thus, it would stand to reason, that changes in mRNA decay rates would determine some of the global EGF induced gene expression patterns. There are several approaches to address this proposal but one of the most interesting, and cost effective, is a relatively new technique called 4-thiouridine (4SU) pulse labeling (Dolken et al., 2008). Briefly, this technique utilizes the fact that 4SU can be added to cell culture media and incorporated into nascent RNA and then specifically isolated through biotinylation and strepavidin pulldown. The 4SU labeled, nascently transcribed RNA is identified and compared to total mRNA using microarrays or deep-sequencing, thus allowing for the determination of the mRNA synthesis rate. The decay rate is then inferred by comparing the changes in total mRNA levels to the mRNA synthesis rates after the application of a stimulus. This technique would allow the dissection of the contributions of mRNA synthesis and mRNA decay in relation to the changes in global mRNA expression levels following EGF stimulation.

Additionally, since TTP targets are enriched in early EGF-induced transcripts it suggests that TTP may regulate the expression and temporal ordering of EGF induced transcripts. In order to test whether TTP regulates the expression and timing of early EGF induced transcripts we utilized siRNA knockdown of TTP. However, siRNA knockdown was not responsive enough to overcome the rapid and massive increase of

TTP following EGF stimulation. Since siRNA knockdown is insufficient to address this problem, it will require the use of TTP null cells, such as mouse embryonic fibroblasts (MEFs) from TTP knockouts (KO) (Carballo et al., 1998). Since, TTP is likely to regulate both the peak levels of induction as well as timing of the decrease. it will require an experimental setup that includes multiple time points, including a few after the early peak phase (after 2 hr). In addition to determining TTP's effect on the timing of EGF-induced gene expression it would also be interesting to determine the effects of BRF1 and BRF2 on these gene expression patterns. Since BRF1 and BRF2 peak at later times post induction it is expected that they would regulate the later gene induction patterns in a fashion similar to that of TTP when regulating early induced genes.

While each of the individual TTP family members may contribute to the regulation of EGF induced gene expression patterns, the TTP family-wide network likely also contributes to the gene expression patterns, but possibly in ways that are different from the individual family members. Since we have suggested that the TTP family network regulates the temporal ordering of EGF induced gene expression, one would expect that by inhibiting combinations of TTP family members, the temporal progression of EGF induction would not proceed faithfully. To study this it will require double and triple KO cells, of which only the double BRF1 and BRF2 KO has been made in a conditional, cell type specific manner (Hodson et al., 2010).

While mRNA decay may affect certain aspects of EGF stimulation, it is also obvious that transcription may be important for this induction. Ensuring the appropriate timing of EGF induced expression programs likely requires coordination of transcriptional and posttranscriptional events. There are several possible mechanisms that one can imagine may link the timing of transcriptional programs to posttranscriptional programs, but one of the more interesting possibilities involves feedback networks consisting of both transcription factors and RBPs. Hints of this can be seen in TTP's documented regulation. Induction of TTP family members was previously shown to be controlled, in part, by transcription factors such as NF-kappaB (Lai et al., 1995; Schichl et al., 2009). Furthermore, several transcription factors have been suggested to be important for the induction of EGF gene expression programs, including NF-kappaB (Biswas et al., 2006). Thus, it is possible that transcriptional programs induced by NF-kappaB also include negative regulators such as TTP to shut down the expression programs.

We observed that mutation of HuR's RRMs impair their ability to bind RNA and combining mutations of individual RRM mutants leads to further reduction in binding to whole transcripts. It is unknown how this occurs but there are several potential mechanisms by which the RRMs may cooperate to bind RNA, and thereby, affect its regulatory properties. There are three possibilities, one involves RRM1+2 recognizing wholly different mRNAs than RRM2 while the other two potential mechanisms involve RRM1+2 cooperating with RRM3 to recognize the same RRM (figure 28). In discussing the potential mechanisms of RRM cooperation we will consider RRMs 1 and 2 as a single RNA element binding unit because co-crystal structural studies have previously demonstrated that RRMs 1 and 2 of HuD combine to recognize a single RNA element (Wang and Tanaka-Hall 2001). Of the two mechanisms involving cooperation between RRM1+2 and RRM3 one possibility is that RRM1+2 and RRM3 recognize distinct RNA elements that are present in the same RNA but may be separated by an undefined distance. The other possible mechanism involves a model in which both RRM1+2 and RRM3 cooperate to recognize the same RNA element with the RRMs surrounding the RNA from all sides. PAR-CLIP is uniquely suited to address this question due to its global scale and nucleotide resolution to resolve which possible mechanism best describe the contribution of HuR's RRMs to the global recognition of RNA.

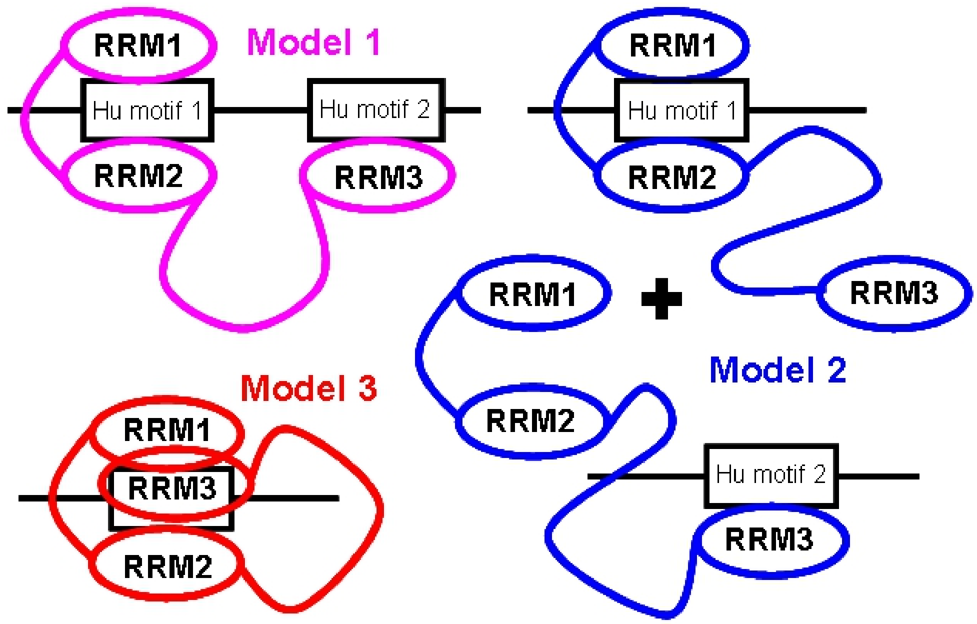

Figure 28: Possible models of cooperation of HuR's RRMs in recognizing mRNA targets.

This figure presents possible models for how HuR's RRMs may cooperate to recognize RNA elements (Hu motif 1 and 2) in either the same or different mRNAs. Model depicts a single mRNA that contains two different RNA elements, one that is recognized by RRM1+2 and another that is recognized by RRM3. Model 2 depicts the case where two different RNA elements are present in two distinct mRNAs. Model 3 depicts cooperation between all three RRMs to recognize a single RNA element on a single mRNA.

While the mechanism for cooperation between HuR's RRMs is unknown, the RRM mutants created here will be a powerful tool for future studies dissecting HuR's RNA-dependent and RNA-independent functions. An example of using the RRM mutatnts as tools to parse mechanisms of HuR's function is seen in the observations reported here about HuR's RNA-dependent cellular localization.

The surprising observation that the HuR RRM mutants alter its cellular distribution suggests a previously unnoticed dependence of HuR's localization on the ability to bind RNA. However, it is unclear how HuR's RNA binding ability controls localization. If a mechanism can be deciphered for this observation it may reveal tantalizing clues about HuR's function and how regulatory interactions and modifications work mechanistically to control HuR's function. In attempting to find the mechanism of HuR's RNA-dependent localization we found that the HuR RRM mutants impaired for RNA binding may still be imported into the nucleus suggesting that HuR's RNA binding ability is linked to nuclear export rather than import. Of the two suggested HuR nuclear export pathways we have so far attempted to block one, the CRM1 export pathway. So far we have no evidence that the HuR RRM mutants are sensitive to inhibition of this pathway. It will be interesting to see in the future whether RNA-dependent export of HuR is mediated by the karyopherin beta 2b pathway. Furthermore, it will also be interesting to see whether disrupting HuR's binding ability can impact other known HuR cellular localization events, such as Pol II dependent nuclear localization or stress induced localization to cytoplasmic stress granules.

Posttranscriptional regulation of gene expression is a rich and complex process that allows cells to respond to a wide variety of external and internal stimuli to meet the needs of a given situation and ultimately determine the function and the fate of the cell in those conditions. The myriad of environmental signals, stresses, cues, conditions, or combinations thereof that a cell may encounter are innumerable, but a cell still must be able to respond rapidly and appropriately to all potential variations in concentration, duration or location of these events. If a cell were to respond to each environmental condition by activating a single gene to produce the desired result, the mammalian genome would quickly run out of responders to diverseenvironmental conditions. Instead, cells respond by activating multiple genes in a variety of combinations and concentrations. Furthermore, these activated genes interact with and regulate other related genes through multiple surfaces and interacting elements to produce networks capable of non-linear responses. These complex networks not only allow cells to respond to a myriad of environmental conditions but also to produce complex behaviors such as oscillations, bistabilization, pulses, filtering or temporal ordering (Alon, 2007).

Networks are made up of multiple components, which are called nodes, and nodes interact through connections called edges (Le Novere et al., 2009). Edges describe how nodes affect other nodes in direction, intensity and outcome. The topology of nodes and edges determines how a network responds to perturbation and networks with slightly different topologies can produce drastically different outcomes. Therefore, there are several important aspects in understanding the functions of networks: the nodes, the control of nodes by other nodes (the edges), the response of the network to perturbation and the global targets of the nodes that determine the cellular function.

We focused on an ARE-BP network that consisted of nodes that included HuR, TTP and BRF1. In order to understand the function of this ARE-BP network we investigated the how HuR controls the expression of TTP, BRF1 and itself, how TTP, BRF1 and BRF2 respond to EGF stimulation, and if HuR recognizes distinct global populations of mRNAs. In investigating this network we learned that it is highly interconnected and can display complex cell type and stimulus-dependent regulatory functions. Furthermore, we learned that this network exhibits complex and distinct behaviors in response to different stimuli such as involvement in temporal ordering and resiliency to fluctuations in protein levels. Finally, we learned that the RRMs of HuR can cooperate to recognize specific sites in whole transcripts, and that surprisingly, these are coupled to its cellular distribution.

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Publications:

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