UNIVERSITY OF CALIFORNIA, SAN DIEGO

Investigating the role of TTP in mRNA decay and pre-mRNA processing

A dissertation submitted in partial satisfaction of the

requirements for the Doctor of Philosophy

in

Biology

by

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2012

This Dissertation of Boris Reznik is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2012

DEDICATION

This thesis is dedicated to my parents, Joseph and Izabella.

EPIGRAPH

“Don’t panic”

Douglas Adams, The Hitchhiker’s Guide to the Galaxy

“Enjoy the little things in life, for one day you'll look back and realize they were big things.”

Kurt Vonnegut

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ABSTRACT OF THE DISSERTATION

Investigating the role of TTP in mRNA decay and pre-mRNA processing

by

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The AU-rich element (ARE) is a cis-encoded determinant within mRNA 3' untranslated regions (UTRs) that contributes to mRNA translation and stability in the cell. Tristetraprolin (TTP) is an RNA binding protein that specifically binds to mRNAs containing AREs and activates their rapid decay. TTP is rapidly activated following external stimulus and modulates the gene expression program of the responding cell. To better understand TTP-mediated mRNA decay activity, I identified the RNA binding protein hnRNP F as an RNA-independent interactor of TTP. I further characterized this interaction and observed that hnRNP F stimulated the decay of a subset of TTP-associated mRNAs, thus implicating hnRNP F in mRNA decay activated

by TTP. Several possible mechanisms were tested but it remains to be determined how hnRNP F stimulates the decay of TTP-associated mRNAs.

In addition to mRNA degradation, TTP appears to regulate gene expression through non-mRNA decay activities. I observed that TTP expression stimulated the 3' end processing of ARE-containing reporter mRNAs, leading to alternatively polyadenylated mRNAs with shortened 3' UTRs. This resulted in stabilization and loss of regulation of these mRNAs by TTP. The 3' end processing required an ARE and an upstream polyadenylation signal, and RNA binding of TTP was necessary but not sufficient for this activity. Thus, TTP appears to be a multifunctional protein, which in addition to mRNA decay activity promotes the 3' end processing of mRNAs, altering the 3' UTR composition and mRNA regulation in cells.

Chapter 1. Introduction to mRNA decay pathways and TTP-mediated mRNA decay

Chapter 1. 1

Regulated and quality control mRNA turnover pathways in eukaryotes

Introduction

Gene expression can be regulated at multiple levels, including transcription, RNA processing, RNA localization, translation, and finally, RNA turnover. RNA degradation may occur at points along the processing pathway or during translation as it undergoes quality control by RNA surveillance systems. Alternatively, mRNAs may be subject to regulated degradation, often mediated by cis-encoded determinants in the mRNA sequence that through the recruitment of trans factors, determine the fate of the mRNA. The aim of this review is to highlight mechanisms of regulated and quality control RNA degradation in eukaryotic cells, with an emphasis on mammals.

Regulated mRNA decay pathways

AU-rich element mediated mRNA decay

The AU-Rich Element (ARE) is one of the best characterized cis-encoded mRNA destabilizing elements (Chen, & Shyu 1995; Bevilacqua et al 2003; Gingerich et al 2004; Barreau et al 2005; Clark et al 2009). It is found within the 3' untranslated region (3' UTR) of a number of unstable mRNAs.

The ARE is loosely defined as an AU-rich sequence that often contains repeats of the ARE pentamer (AUUUA) or nonamer (UUAUUUAUU), and ranges in length from 50-150 nucleotides (Bevilacqua et al 2003). It has been estimated that upwards of 8% of mammalian mRNAs contain AREs (Bakheet et al 2006), and it appears that AREs are enriched in mRNAs coding for growth factors, cytokines, transcription factors, RNA metabolism proteins and early response genes (Gingerich et al 2004; Barreau et al 2005).

AREs exert their effect on post-transcriptional gene expression through the recruitment of trans factors. Several AUBPs (AU-rich element binding proteins) have been identified and characterized. AUBPs may destabilize or stabilize ARE mRNAs or modulate the translation of the ARE mRNA. AUBPs shown to destabilize ARE mRNA include the TTP-family of zinc finger proteins (TTP, BRF1, and BRF2), as well as KSRP and AUF1 (Barreau et al 2005). Stabilizing AUBPs include the Hu family of proteins (HuR, HuB, HuC, and HuD) and, under certain conditions, AUF1 (Barreau et al 2005).

AUBPs are not known to possess intrinsic nucleolytic activities. Instead, they activate mRNA decay through the recruitment of cellular mRNA decay machineries. Biochemical studies of the AUBPs TTP, BRF1 and KSRP demonstrated that they associate with a variety of decay factors, including deadenylases, decapping factors, as well as 5'-to-3' and 3'-to-5' exonucleolytic factors (Baou et al 2009). Meanwhile, stabilizing AUBPs, such as HuR, may outcompete destabilizing AUBPs for ARE binding to promote RNA stability (Brennan, & Steitz 2001).

Many AUBPs display tissue or cell type specific expression patterns. For example, among the stabilizing AUBPs, HuR is more ubiquitously expressed than the other Hu-family proteins, which show tissue specific expression mostly limited to neuronal cell types (Barreau et al 2005). AUBPs are often modified in response to external signaling cues or changing environmental stimuli and this may alter their ability to regulate ARE mRNAs. For instance, when upstream kinase pathways are activated, destabilizing AUBPs such as KSRP, TTP and BRF1 are phosphorylated and this limits their ability to activate RNA decay (Garneau et al 2007). During conditions of genotoxic stress, HuR relocalizes from the nucleus to the cytoplasm, enhancing its stabilizing effect on ARE mRNA (Gorospe 2003). Thus ARE-mRNA decay can be regulated by external cues, thereby controlling the expression of ARE-mRNA encoded proteins.

GU-rich element mediated decay

The GU-rich element (GRE) is another cis-encoded determinant known to regulate mRNA stability. The GRE sequence, UGUUUGUUUGU, was originally identified through computational analysis studies of unstable mRNAs in primary T cells (Vlasova, & Bohjanen 2008). More recently, GREs were also found enriched among unstable transcripts in muscle cells (Lee et al 2010). The RNA binding protein CUGBP-1 has been shown to mediate GRE mRNA instability (Vlasova, & Bohjanen 2008). While the mechanism of GRE-mediated decay is not well established, human CUGBP-1 and the Xenopus

homolog of CUGBP-1, called EDEN-BP, promote deadenylation of mRNA (Vlasova, & Bohjanen 2008). Additionally, CUGBP-1 is thought to mediate alternative splicing (Lee, & Cooper 2009). Putative GRE-containing mRNAs include those important for transcription, apoptosis, RNA-processing, cell division, signaling and cellular metabolism in developing muscle cells, T cells, and HeLa cells (Vlasova, & Bohjanen 2008; Rattenbacher et al 2010; Lee et al 2010). Future studies should reveal the specific mechanism of GRE-mediated decay and whether this is a regulated mRNA decay pathway. Moreover, CUGBP-1 has been implicated in muscular dystrophy (Lee, & Cooper 2009). It will be important to learn whether the decay function of CUGBP-1 plays a role in this disease.

RNA induced silencing complex (RISC)-mediated mRNA decay

MicroRNAs (miRNAs) and short interfering RNAs (siRNAs) are small non-coding RNAs that generally downregulate the expression of target mRNAs. These small non-coding RNAs are typically ~22 nucleotides in length and assemble into a ribonucleoprotein (RNP) effector complex called the RNA induced silencing complex (RISC). Through RNA-RNA base pairing interactions, the small RNAs guide RISC to target mRNAs for silencing (Carthew, & Sontheimer 2009; Liu, & Paroo 2010; Fabian et al 2010). When siRNAs or miRNAs form perfect base pairing with the target RNA, RISC triggers endonucleolytic target RNA cleavage, which is followed by exonucleolytic decay of the fragments. Upon imperfect base pairing, which is

typical for miRNA-mRNA pairs in most eukaryotes with the exception of plants, the target mRNAs are subject to translational repression and/or degradation initiated by deadenylation followed by decapping and exonucleolytic decay (Mallory, & Bouché 2008; Carthew, & Sontheimer 2009; Liu, & Paroo 2010; Fabian et al 2010). Why some miRNA targets are degraded while others appear to be silenced only through translational repression remains unclear.

A large fraction of human mRNAs are predicted to be targets for miRNA regulation, but there have only been several hundred miRNAs identified (Siomi, & Siomi 2010). A single miRNA is able to regulate multiple mRNAs due to the allowed mismatches in base pairing between the miRNA and target mRNA interaction. Evidence suggests that only the critical “seed region” of the miRNA, nucleotides 2-8, must base pair with full complementarity to the target mRNA sequence, and looser base pairing rules apply for other regions of the miRNA.

Production of miRNAs is required for viability of organisms, but individual miRNAs vary in expression according to tissue or cell type, developmental stage, or environmental stimulus (Chang, & Mendell 2007). Individual miRNAs are often regulated at the level of transcription, processing and degradation, thus allowing for regulation of RISC target mRNAs in various cell types and physiological conditions (Chang, & Mendell 2007; Garzon et al 2009; Kai, & Pasquinelli 2010). Because a large number of mRNAs are likely subject to miRNA regulation, it is not surprising that miRNAs have been

implicated in many biological processes as well as numerous diseases and pathologies (Chang, & Mendell 2007; Garzon et al 2009).

Other mRNA decay pathways - Histone mRNA decay and Staufen-mediated decay

Histone mRNA is unique in that it is the only known mRNA that is not polyadenylated. Instead of a poly(A) tail it contains a stem loop structure at the 3' end which is bound by the stem loop binding protein (SLBP) (Marzluff et al 2008). Histone mRNA and protein levels fluctuate during the cell cycle and peak at S phase when more histones are required to package newly synthesized DNA. At the conclusion of S phase, histone mRNA is rapidly degraded in a process that requires SLBP, translation of the histone mRNA, and the decay factor Upf1, which interacts with SLBP (Marzluff et al 2008). Upf1 is an essential component of the nonsense-mediated mRNA decay (NMD) pathway (discussed later) and is thought to promote mRNA decay when it is recruited during translation termination. The degradation of histone mRNA involves oligouridinylation at the 3' end, a modification believed to stimulate decapping and exonucleolytic decay of the mRNA (Marzluff et al 2008).

Similar to SLBP, Staufen 1 (Stau1) is an RNA binding protein that shows specificity for double stranded RNA stem loop structures and interacts with Upf1. Like SLBP, Stau1 also promotes the decay of bound mRNAs in a manner dependent on translation and the presence of Upf1 (Maquat, & Gong

2009). While Stau1 substrate mRNAs are still being identified and validated, some may be important for the process of muscle cell differentiation (Maquat, & Gong 2009).

Quality control RNA decay pathways

RNAs are subject to quality control at many stages of biogenesis, including transcription, processing, localization, and incorporation into ribonucleoprotein (RNP) complexes. It is believed that the activity of RNA quality control pathways along these steps is driven by a constant kinetic competition between the surveillance machinery and the functional reactions of the RNA (Doma, & Parker 2007; Houseley, & Tollervey 2009). As RNAs progress through the stages of maturation, abnormal RNAs are more likely to suffer from compromised reaction kinetics, and thus will have a greater chance of being recognized by the RNA surveillance machinery.

RNA quality control begins in the nucleus shortly after the start of transcription. Through a number of different mechanisms, the integrity of transcribed and processed nuclear RNA is assessed, and this includes the monitoring of RNA polymerase II transcripts, rRNAs, snRNAs, snoRNAs and tRNAs (Doma, & Parker 2007). RNAs may be subject to a variety of processing steps and modifications, including 5’ capping, splicing, trimming and excising, nucleotide modification, and incorporation into RNP complexes. Aberrant RNAs are less likely to be processed correctly or in a kinetically favorable manner, and therefore are more likely to be recognized and

degraded by the nuclear RNA decay machinery, consisting mostly of 5'-to-3' and 3'-to-5' exonucleases. 3'-to-5' exonuclease activity is enhanced by oligo(A) addition onto the 3' end of RNAs by a dedicated polyadenylation complex called TRAMP (Houseley et al 2006). In contrast to the generally stabilizing effect of a poly(A) tail, the oligo(A) tail added onto nuclear RNAs by TRAMP promotes their decay, likely by serving as a bait for exonucleases. However, some aberrant RNA molecules escape nuclear surveillance and are exported to the cytoplasm, where cytoplasmic quality control pathways, often linked to translation, act to clear these RNAs.

Nonsense-mediated mRNA decay (NMD)

mRNAs with nonsense mutations contain premature translation termination codons (PTCs) and can potentially make C-terminally truncated proteins that, depending on the nature of the protein product, may be detrimental to cellular fitness. For instance, truncated proteins can lead to dominant negative effects by not performing their intended function and titrating away interacting proteins. PTC-containing mRNAs are estimated to comprise 3% to 10% of cellular mRNAs (Isken, & Maquat 2007), and they may arise through mutations in genomic DNA, DNA recombination events, or errors in transcription and RNA processing (Isken, & Maquat 2007; Rebbapragada, & Lykke-Andersen 2009). A large fraction of PTC-containing mRNAs in mammalian cells are thought to be produced by unproductive alternative splicing (McGlincy, & Smith 2008). PTC-containing mRNAs are detected and

degraded in a quality control process linked to translation termination called the nonsense-mediated mRNA decay (NMD) pathway.

One important question is how PTC-containing mRNAs are distinguished from those with normal termination codons. NMD is dependent on translation and evidence suggests that PTC recognition is mediated by the ribonucleoprotein (RNP) complex bound to the mRNA downstream of the termination codon during translation termination (Amrani et al 2006; Rebbapragada, & Lykke-Andersen 2009; Nicholson et al 2010). Termination codons in mammalian transcripts are usually found in the last exon proximal to the poly(A) tail, and the local mRNP environment at translation termination consists of poly(A)-binding protein (PABP) and the remaining 3' UTR bound proteins. This 3' UTR mRNP is believed to enhance translation termination and prevent NMD (Amrani et al 2006; Rebbapragada, & Lykke-Andersen 2009; Brogna, & Wen 2009; Nicholson et al 2010). However, PTC-containing mRNAs exhibit an altered mRNP environment. Because translation is terminated distal to the 3' end of the mRNA, more factors sitting on the mRNA body are retained and the distance between the termination site and PABP is lengthened. This altered protein environment allows for NMD factors to be recruited to the terminating ribosome and trigger mRNA decay (Rebbapragada, & Lykke-Andersen 2009; Silva, & Romão 2009; Brogna, & Wen 2009; Nicholson et al 2010). An exon-junction complex (EJC), which is deposited upstream of exon-exon junctions after pre-mRNA splicing and is normally displaced from the mRNA during translation, strongly enhances NMD

in mammalian cells when positioned downstream of a termination event (Chang et al 2007; Silva, & Romão 2009).

One of the core NMD components, Upf1, is thought to facilitate the transition from PTC recognition to mRNA decay through interactions with translation termination factors and cellular RNA decay factors (Mühlemann, & Lykke-Andersen 2010). PTC-containing mRNAs are degraded through either of two pathways. The first pathway initiates through decapping and deadenylation, followed by exonucleolytic decay. Alternatively, the mRNA is degraded by endonucleolytic cleavage catalyzed by an NMD factor, Smg6, followed by exonucleolytic decay of the mRNA fragments (Nicholson et al 2010; Mühlemann, & Lykke-Andersen 2010).

NMD is considered a surveillance system that safeguards against translation of aberrant mRNAs, but there is also evidence to suggest that some non-PTC-containing mRNAs are regulated through NMD (Neu-Yilik et al 2004). It will be interesting to see how involved the NMD pathway may be in regulated mRNA degradation in cells.

Nonstop decay

mRNAs that lack termination codons are also targeted for rapid decay. These mRNAs could make abnormal proteins with C-terminal extensions which may be detrimental to the cell. Such mRNAs are thought to be produced by events such as aberrant polyadenylation or endonucleolytic cleavage within the mRNA coding region, and are recognized and rapidly degraded in a

translation dependent manner via the nonstop mRNA decay pathway. Evidence from yeast suggests that when ribosomes translate through the poly(A) tail and stall at the very 3' end of a nonstop mRNA, Ski7, a component of the cytoplasmic exosome homologous to translation release factor 3, interacts with the stalled ribosome and triggers the rapid degradation of the nonstop transcript (Isken, & Maquat 2007). Another class of aberrant mRNAs, where the normal termination codon is not recognized and translation terminates at a downstream site in the 3' UTR have been described (Kong, & Liebhaber 2007). These mRNAs are rapidly degraded in a translation and deadenylation dependent manner but the mechanism of how they are distinguished from normal mRNAs and the prevalence of this as a form of RNA quality control remain to be determined.

No-go decay (NGD) and Non-functional rRNA decay (NRD) pathways

Some aberrant mRNA molecules may contain obstacles, such as strong secondary structure, which prevent translation elongation of ribosomes and leads to stalled ribosome complexes and surveillance by the No-Go mRNA decay (NGD) pathway. NGD clears the stalled ribosomes and rapidly degrades the mRNA transcript (Doma, & Parker 2006). In yeast, NGD is dependent on active translation and the activity of two proteins, Dom34 and Hbs1, which resemble translation release factors eRF1 and eRF3, respectively (Doma, & Parker 2006). These factors are believed to interact with the stalled ribosome and assist in the disassembly of the stalled complex, and trigger

mRNA decay through endonucleolytic cleavage and subsequent exonucleolytic decay (Doma, & Parker 2006).

Stalled ribosome complexes also lead to the rapid degradation of rRNA from the small ribosomal subunit. Although rRNAs are usually very stable, studies from yeast have demonstrated that mutant 18S rRNAs deficient in codon recognition are rapidly degraded via the non-functional rRNA decay (NRD) pathway (LaRiviere et al 2006; Cole et al 2009). The mutant rRNAs assemble into small ribosome subunits but induce translation defects that lead to ribosomal stalling. Decay of the 18S rRNA, like NGD, is dependent on Dom34 and Hbs1, but unlike NGD, the rRNA is degraded by exonucleases and no endonucleases are thought to participate (Cole et al 2009). Separate studies of NGD and the NRD of 18S rRNA have shown that stalled ribosomes are cues that activate surveillance pathways to clear the stalled ribosome complex and degrade aberrant RNA molecules. An interesting question to address in the future is if both the mRNA and rRNA of the same stalled ribosome complex are degraded concurrently (Cole et al 2009).

The large ribosomal subunit rRNA is also subject to quality control inspection through a NRD pathway. For example, yeast 25S rRNA with a mutation in the peptidyl-transferase center renders the ribosome incapable of catalyzing peptide bond formation. rRNAs with this mutation are rapidly degraded when compared to wild-type 25S rRNA (LaRiviere et al 2006; Cole et al 2009). In contrast to the decay of 18S rRNA, the 25S rRNA decay is not dependent on translation, does not get incorporated into the translational pool

of ribosomes, and is likely recognized and degraded at some step during assembly (LaRiviere et al 2006; Cole et al 2009), although the exact mechanisms of detection and decay remain to be determined.

miRNA decay

Because miRNAs are important regulators of post-transcriptional gene expression in many organisms, the maintenance of appropriate miRNA levels is critical, and misregulation of miRNAs has been implicated in pathologies such as cancer (Chang, & Mendell 2007). miRNAs are subject to regulation through transcription, processing, and degradation, and recent studies from plants and worms suggest that miRNA degradation is mediated by both 3'-to-5' and 5'-to-3' exonucleases (Kai, & Pasquinelli 2010). Incorporation of miRNAs into RISC complexes, or binding to target mRNAs is thought to stabilize miRNAs (Kai, & Pasquinelli 2010). Additionally, modifications of the miRNA 3' end, such as methylation or non-templated nucleotide addition (of either adenosines or uridines), have varying effects on the stability (both stabilizing and destabilizing) of the miRNA (Kai, & Pasquinelli 2010). It remains to be determined how prevalent miRNA degradation as a post-transcriptional mechanism to regulate miRNA levels is in mammalian cells.

tRNA decay

tRNAs act in concert with ribosomes to decode mRNAs and direct amino acid incorporation during protein synthesis. The biogenesis of tRNAs

includes multiple modification and processing steps such as intron splicing, trimming of 5' and 3' ends, 3' CCA nucleotide addition, and extensive base modifications (Hopper, & Phizicky 2003). tRNA molecules are highly processed and thus subject to regulation at multiple points by RNA surveillance pathways. At least two characterized quality control steps ensure the fidelity of cellular tRNAs. In the nucleus, improperly processed tRNAs are subject to 3'-to-5' exosome decay stimulated by poly(A) addition by the TRAMP complex, or 5'-to-3' exonucleolytic decay (Doma, & Parker 2007; Houseley, & Tollervey 2009). In the cytoplasm, tRNAs lacking proper base modifications may be rapidly degraded through a 5'-to-3' exonuclease activity (Chernyakov et al 2008). The exact mechanisms of recognition of these aberrant tRNAs by the quality control machinery remains unclear and a subject for further investigation.

Conclusions and future directions

RNA steady state levels in cells are determined by two factors, the rate of RNA synthesis and the rate of RNA turnover. RNA turnover is not only important in the maintenance of cellular homeostasis, but it is also required to complement changes in the transcriptional program that occur when the cell responds to challenges such as changing environmental conditions or various stimuli. While progress has been made in recent years to shed light onto some of the underlying mechanisms that mediate RNA degradation, there are many unresolved questions left to explore. For instance, some basic understanding

of the cis-encoded destabilizing elements and the trans factors that mediate regulated mRNA decay are known. But there is still much to learn about the various decay pathways that operate in the cell and the mechanisms by which they are regulated to coordinate post-transcriptional gene expression.

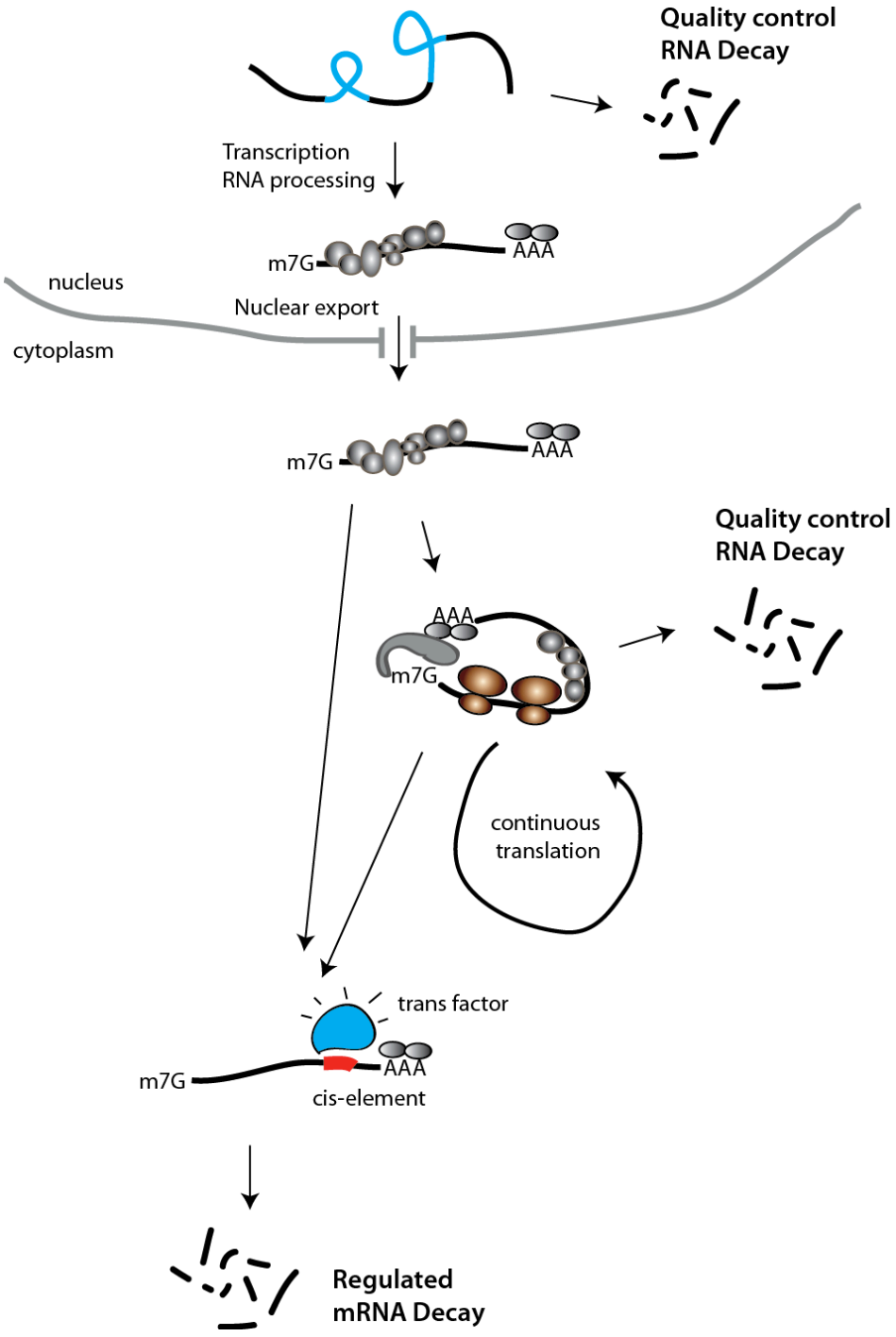


Figure 1.1: Regulated and quality control RNA decay. During transcriptional processing in the nucleus, or translation in the cytoplasm, aberrant RNAs are recognized and degraded by one of several quality control pathways. Alternatively, mRNAs might be targeted for regulated degradation by trans factors, which recognize mRNA cis-elements.

Chapter 1.2 Introduction to TTP-mediated ARE-mRNA decay

TTP activates ARE-mRNA decay

Tristetraprolin is an RNA binding protein that specifically activates the decay of mRNAs containing AREs. mRNA decay is often initiated by deadenylation from the cytomplasmic deadenylases, Pan2/3 and the Ccr4/Caf1/Not complex (Garneau et al 2007; Yamashita et al 2005). TTP substrate mRNAs show evidence for deadenylation-initiated mRNA decay (Chen, & Shyu 1995; Carballo et al 2000) and TTP interacts with components of the Ccr4/Caf1/Not complex (Lykke-Andersen, & Wagner 2005; Sandler et al 2011) and the Pan2/3 deadenylases (Clement et al 2011). TTP associates with other cellular decay factors including decapping enzymes (Fenger-Grøn et al 2005), exonucleases (Chen et al 2001; Lykke-Andersen, & Wagner 2005), and the RNA-induced silencing complex (RISC) (Jing et al 2005). Thus, TTP activates ARE-mRNA decay through recruitment of cellular decay enzymes.

TTP was originally identified in a screen for rapidly and transiently induced genes in in response to mitogens (Varnum et al 1991). Initially thought to be a transcription factor, it was later shown that TTP is instead a potent activator of mRNA decay (Carballo et al 1998). This was facilitated by studies of the TTP knockout mouse, which displayed a systemic inflammatory phenotype and pathologies attributed to elevated levels of the cytokine, tumor necrosis factor-alpha (TNFα) (Taylor et al 1996). TTP appears to directly

target TNFα mRNA decay since the mRNA was stabilized two-fold in the TTP knockout mouse and TTP directly binds the ARE from the TNFα mRNA (Carballo et al 1998). The mRNA coding for granulocyte-macrophage colony stimulating factor (GMCSF) was also confirmed as a TTP substrate in using these knockout mice (Carballo et al 2000). Since then, TTP has been confirmed to regulate the decay of many ARE-containing mRNAs including IL-2, IL-3, IL-8, IL-10, Cox-2, and others (Balakathiresan et al 2009; Stoecklin et al 2000; Ogilvie et al 2005; Barreau et al 2005) . More recently, genome wide studies have identified hundreds of TTP-associated mRNAs (Stoecklin et al 2008; Emmons et al 2008) and potential mRNAs that are downregulated by TTP mRNA decay activity (Lai et al 2006), although many of these candidates need to be confirmed. A surprising finding of these studies was that many of the mRNAs that associated with TTP did not appear to have AREs in the 3' UTR (Emmons et al 2008), although TTP-binding and activation of mRNA decay remains to be confirmed for many of these putative targets.

Regulation of TTP mRNA decay activity

The mRNA decay activity of TTP can be modulated by post-translational modifications and tight regulation of TTP expression. In most tissues, TTP is expressed below the level of detection by Western blot, except for the liver and sex organs (Lu, & Schneider 2004). TTP mRNA is rapidly induced in response to stimulus and is then rapidly downregulated (Lai et al 1990). Similarly, TTP protein is rapidly induced, transiently expressed, and

then downregulated in a poorly understood mechanism mediated by the proteasome (Deleault et al 2008; (Brook et al 2006).

TTP-mediated mRNA decay activity is attenuated by phosphorylation (Taylor et al 1995; Mahtani et al 2001; Chrestensen et al 2004), and the phosphorylation status of TTP changes throughout the TTP expression time course (Mahtani et al 2001). This cycle modulates TTP mRNA decay activity, possibly allowing for transient stabilization and translation of mRNAs when TTP is phosphorylated, and subsequent mRNA decay when TTP is dephosphorylated. Though TTP has multiple phosphorylation sites in vitro (Chrestensen et al 2004), the best characterized sites, at serines 52 and 178, are phosphorylated by the p38 MAPK signaling pathway and attenuate TTP mRNA decay activity in cells (Stoecklin et al 2004; Chrestensen et al 2004; Brook et al 2006). 14-3-3 binding to TTP via these phosphorylated residues might attenuate TTP mRNA decay activity (Stoecklin et al 2004), and other studies suggest that phosphorylation reduces TTP mRNA decay activity by inhibiting TTP-mediated deadenylase recruitment (Clement et al 2010; Marchese et al 2010). The subcellular localization and protein stability of TTP might also be regulated by p38 phosphorylation, since treatment with a p38 MAPK inhibitor localizes TTP to the nucleus from the cytoplasm and renders TTP susceptible to proteasomal decay (Brook et al 2006). PP2A phosphatase activity dephosphorylates TTP and could be another step to regulate TTP mRNA decay activity and TTP protein degradation (Sun et al 2007; Frasca et al 2010).

TTP-mediated mRNA decay activity is evolutionary conserved

The TTP protein is 328 amino acids long and contains a central RNA-binding domain with two zinc finger motifs. Two human paralogs of TTP, BRF1 and BRF2, share high similarity in the RNA binding domain (68% amino acid identity) but are less similar in other regions (Lykke-Andersen, & Wagner 2005). In vitro, BRF1 and BRF2 can activate mRNA decay of the same ARE-mRNAs as TTP (Ciais et al 2004; Stoecklin et al 2002; Ming et al 2001; Lykke-Andersen, & Wagner 2005; Hodson et al 2010), but at the organismal level they may regulate non-overlapping mRNAs (Lai et al 2006). Individual BRF1 and BRF2 knockout mice are embryonic lethal (Stumpo et al 2004; Ramos et al 2004), but tissue specific knockout mice show immunological defects resulting in T cell leukemia and decreases in hematopoietic progenitor cells (Stumpo et al 2009; Hodson et al 2010), implicating BRF1 and BRF2 in the post-transcriptional regulation of critical mRNAs of the immune system.

Post-transcriptional regulation by AREs and TTP is evolutionarily conserved from mammals to yeast. CTH2 is the homolog of TTP in *S. cerevisiae*, and features high conservation of the zinc finger RNA binding domain. CTH2 regulates ARE-mRNA decay in response to stress, such as low iron concentration, and coordinates the decay of mRNAs involved in iron-dependent processes (Puig et al 2005). The *Drosophila* homolog of TTP, TIS11, degrades ARE-containing mRNAs in response to external stimuli, including mRNAs coding for anti-microbial peptides through a deadenylation-

initiated mRNA decay pathway (Lauwers et al 2009; Wei et al 2009). Interestingly, the activity of TIS11 appears to be attenuated by the p38 MAPK signaling in *Drosophila* (Wei et al 2009), suggesting that this pathway that modulates TTP and its homologs is evolutionarily conserved.

Acknowledgements

Chapter 1.1, in full, is a reprint of the material as it appears in Reznik B and Lykke-Andersen J. Regulated and quality-control mRNA turnover pathways in eukaryotes. *Biochemical Society Transactions*. 2010. The dissertation author was the primary author of this paper.

Chapter 2. hnRNP F is a novel TTP-interacting protein that stimulates ARE mRNA decay

2.1 Introduction

mRNA decay is an important process that maintains proper gene expression in cells. Tristetraprolin (TTP) is an RNA binding protein that promotes the rapid mRNA decay of transcripts containing AU-rich elements (AREs) in the 3' UTR (Sandler, & Stoecklin 2008; Blackshear 2002; Carballo et al 1998). TTP does not appear to have catalytic mRNA decay activity of its own but it interacts with the cellular decay machinery, including deadenylases, decapping factors, and exonucleases to activate decay of target mRNAs (Chen et al 2001; Lykke-Andersen, & Wagner 2005; Fenger-Grøn et al 2005; Sandler et al 2011). The two mammalian homologs of TTP, BRF1 and BRF2, appear to have similar RNA binding properties and decay activity as TTP (Ciais et al 2004; Stoecklin et al 2002; Ming et al 2001; Lykke-Andersen, & Wagner 2005; Hodson et al 2010).

Post-transcriptional regulation of ARE-containing mRNAs is complex since there are predicted to be many ARE mRNAs, upwards of 8% of mammalian transcripts (Bakheet et al 2006), that can associate with a variety of AU-rich element binding proteins (AUBPs). At least 20 confirmed and putative ARE-BPs have been identified thus far (Barreau et al 2005). The proper coupling of mRNA to AUBP is important for homeostasis and normal physiology, and misregulation is often associated with detrimental effects to

health and fitness. For example, TTP knockout mice display severe autoimmune pathologies and systemic inflammation that is attributable to increased levels of the cytokine tumor necrosis factor-α (TNFα) due to slower decay of its mRNA in macrophages from these animals (Carballo et al 1998; Taylor et al 1996). In fact, many characterized ARE mRNAs encode for cytokines, growth factors, transcription factors, and early response genes (Barreau et al 2005; Gingerich et al 2004), so regulation of these mRNAs is critical. Most often, association of ARE mRNAs with AUBPs promotes the degradation of transcripts, but some AUBPs stabilize mRNAs, or regulate translation of the mRNA (Barreau et al 2005). How TTP and other AUBPs bind to specific substrate mRNAs amidst all the potential ARE-containing mRNAs and other AUBPs in the cell is an important and not well understood question in the field.

The first and best characterized targets of TTP mRNA decay activity are the cytokine mRNAs, TNFα and GMCSF, identified and characterized in the initial studies of the TTP knockout mouse (Carballo et al 2000; Carballo et al 1998; Taylor et al 1996). Since then, additional TTP substrate mRNAs have been discovered (Stoecklin et al 2008; Emmons et al 2008; Lai et al 2006), and study of the tandem zinc finger RNA binding domain of TTP has demonstrated high binding affinity for the ARE nonamer sequence UUAUUUAUU, and iterations of this sequence (Lai et al 2005; Brewer et al 2004; Michel et al 2003; Worthington et al 2002). While TTP has been shown to bind and regulate the decay of ARE mRNAs, there is evidence from global

transcript analyses to suggest that TTP may regulate many non-ARE containing mRNAs. For example, only 23 of 250 stabilized mRNAs in fibroblasts derived from TTP knockout mice contain predicted TTP binding sites (Lai et al 2006) and only ~ 10% of the 400 TTP-associated mRNAs identified in human dendritic cells appeared to contain an ARE (Emmons et al 2008). In a third contrasting study, most of the 128 mRNAs associated with TTP in mouse macrophage cells did contain an ARE or a minimal ARE pentamer sequence (AUUUA) (Stoecklin et al 2008). Thus, it appears that TTP RNA binding is diverse and possibly not limited only to mRNAs containing AREs, and the profile of TTP-associated mRNAs may vary by tissue or cell-type. Additionally, not all TTP-bound mRNAs require TTP for degradation (Stoecklin et al 2008), demonstrating the complexity of TTP regulation of mRNA.

Given the abundance of predicted ARE mRNAs, potential competition for binding to these transcripts from other AUBPs, and the possibility that TTP associates with a large amount of non-ARE mRNA in the cell, much remains to be known about how TTP binds to and regulates target mRNAs. The purpose of this study was to identify TTP-interacting proteins that might help to better understand the activity and mechanisms of TTP-mediated mRNA decay. In this chapter, I characterize the interaction between TTP and hnRNP F, and demonstrate that hnRNP F binds to and stimulates the decay of a subset of TTP substrate mRNAs. I also describe attempts to elucidate the mechanism by which hnRNP F stimulates ARE mRNA decay.

2.2 Results

TTP and BRF1 associate in an RNA-independent complex with hnRNP F

To gain mechanistic insight into the specificity of TTP/BRF-family proteins in ARE-mRNA decay, we decided to isolate proteins that biochemically associate with TTP and BRF1. Human embryonic kidney (HEK) 293T cell lines were generated that stably express Flag-tagged TTP and BRF1 proteins under control of tetracycline, which allows titration of exogenous protein expression levels. Immunoprecipitation (IP) experiments were performed from RNase-treated extracts of cells expressing Flag-tagged TTP and BRF1 at levels similar to endogenous BRF1 (data not shown). Interacting proteins were separated in SDS-PAGE gels and visualized by silver staining. As seen in Figure 2.1, TTP and BRF1 displayed remarkably similar profiles of co-purifying proteins, and none of the co-purifying proteins were observed from cells expressing no FLAG-tagged proteins (compare lanes labeled 'TTP' and 'BRF1' with those labeled 'none'). Interacting proteins were identified through mass spectrometry analysis as 14-3-3ε, hnRNP F (heterogeneous nuclear ribonucleoprotein F), and CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase). In addition, Tubulin α, Tubulin β, and Hsp70 (Heat shock protein 70) were observed, but these proteins were excluded from further study as they are commonly observed to co-purify with exogenous Flag-tagged proteins (Fenger-Grøn et al., 2005). The

identification of 14-3-3ε, a previously characterized TTP-interacting protein (Johnson et al 2002), validated our IP conditions and experimental protocol.

To verify identified protein interactions co-IP experiments were performed with exogenously expressed tagged proteins from RNase-treated HEK293T cell extracts. As seen in Figure 2.2A, Myc-tagged CAD, hnRNP F and 14-3-3ε all co-purify with Flag-tagged TTP and BRF1, but not with HuR, an ARE-mRNA stabilizing protein that served as a negative control (compare lanes 6, 7 to lane 8). As an additional negative control, the RNA binding protein hnRNP A1 did not co-IP with either TTP or BRF1 (bottom panel). Thus, exogenously expressed CAD, hnRNP F and 14-3-3ε exist in RNA-independent complexes with TTP and BRF1.

hnRNP F is an RNA binding protein belonging to the hnRNP superfamily of proteins and has previously been implicated in multiple steps of mRNA metabolism and gene expression regulation (Veraldi et al 2001; Min et al 1995), including translational repression in *D. melanogaster* (Kalifa et al 2006) and translational regulation of transported mRNA in neurons (White et al 2012). We therefore focused our attention on this protein. To test whether TTP and hnRNP F can be observed in an endogenous complex with one another, we took advantage of a mouse macrophage cell line, RAW264.7 (hereafter called RAW), in which TTP expression is induced upon treatment with lipopolysaccharides (LPS), which mimics bacterial infection (Figures 2.2B, 2.2C, left panels). As seen in the co-IP experiments in Figures 2.2B and 2.2C, endogenous hnRNP F can be detected to co-purify with TTP when TTP is

expressed after 2 and 6 hours of LPS treatment, but not prior to LPS treatment when TTP is expressed only at low levels (compare lanes 5, 6 with 4). The antibody against hnRNP F recognizes the close homologs hnRNP H1 and hnRNP H2 (96% shared amino acid identity with each other and 70% amino acid identity with hnRNP F (Alkan et al 2006), which also appear to co-purify with TTP (compare upper and lower bands in top panel of Figure 2.2B). By contrast, the negative control RNA-binding protein HuR did not co-purify with TTP (lower panels), and hnRNP F and TTP did not co-purify with unspecific antisera (Figures 2.2B and 2.2C, lanes 7-9). Thus, TTP can be observed in an RNA-independent complex with hnRNP F, and at least one of its close hnRNP H homologs, under conditions in which TTP is induced in a macrophage cell line.

hnRNP F stimulates decay of an exogenous TTP/BRF-target ARE-mRNA

We next tested whether hnRNP F affects the decay of a TTP/BRF ARE-mRNA substrate. For this, we used a heterologous mRNA reporter, β-BRSK1-3' UTR, which contains the 3' UTR from the BRSK1 gene. This 3'UTR was chosen because it contains a 37-nucleotide ARE sequence surrounded by multiple G-rich stretches predicted to be hnRNP F binding sites (Caputi, & Zahler 2001). As seen in the RNA-IP (RIP) assays in Figure 2.3A, the β-BRSK1-3'UTR reporter mRNA co-purifies with both Flag-tagged hnRNP F (lanes 4 and 5) and TTP (lanes 6 and 7). As expected, the negative control b-globin mRNA, which is identical to the β-BRSK1-3'UTR except for the BRSK1

3’ UTR, did not associate with hnRNP F or TTP (lower band), and background RNA binding to beads was minimal (lanes 8 and 9).

To test whether hnRNP F plays a role in the degradation of the β-BRSK1-3’UTR reporter mRNA, pulse-chase mRNA decay assays were performed in HeLa Tet-off cells. The β-BRSK1-3’ UTR reporter mRNA was stabilized ($p<0.01$) following hnRNP F knockdown, degrading with a half-life of 123±12 minutes as compared to 77±6 minutes for the control siRNA treated cells (Figure 2.3B). Less than 10% of cellular hnRNP F remained in the siRNA-treated cells, whereas hnRNP H levels were unaffected (Figure 2.3C). We were unable to test the effect of co-depletion of hnRNP F and hnRNP H proteins as this impaired cell growth (data not shown). When cells were treated with an siRNA targeting all three TTP/BRF proteins, the reporter mRNA degraded with a half-life of 220±43 minutes (Figure 2.3B, bottom panel). Thus, hnRNP F stimulates the degradation of an exogenous ARE-mRNA that binds both hnRNP F and TTP.

Is hnRNP F RNA binding important for ARE mRNA decay?

I next wanted to test whether hnRNP F binding is required for stimulating the decay of ARE mRNAs. To address this question, I tested if hnRNP F stimulated the decay of a β-ARE_{GMCSF} reporter ARE-mRNA that has no predicted hnRNP F binding site. When tested in RIP assays, the β-ARE_{GMCSF} mRNA was pulled down in the Flag-TTP RIP (Figure 2.4A, lanes 2, 6), but was not enriched in the Flag-hnRNP F RIP (Figure 2.4A, lanes 1, 5),

even when Myc-TTP was co-expressed (Figure 2.4A, lanes 3, 7). However, the decay of this reporter mRNA was stimulated by hnRNP F, as seen by its stabilization after hnRNP F knockdown (Figure 2.4B). A similar reporter mRNA, β-$ARE_{TNFα}$, containing the ARE from TNFα mRNA, was stabilized by hnRNP F knockdown (Figure 2.4C) to a half-life of 105±6 minutes compared to 78±9 minutes for the control siRNA knockdown.

The ability of hnRNP H to stimulate mRNA decay of these reporters was also tested with an siRNA that targeted both hnRNP H1 and hnRNP H2. hnRNP H appeared not to change the half-life of the β-ARE_{GMCSF} mRNA and β-$ARE_{TNFα}$ mRNA reporters relative to the control siRNA (Figure 2.4B, C). The siRNA targeting TTP/BRF proteins stabilized the decay of both reporters (Figure 2.4B, C). The hnRNP H proteins were not knocked down as efficiently as hnRNP F (Figure 2.4D), so it is difficult to tell whether the lack of effect on mRNA stability is due to insufficient knockdown. It remains to be determined if hnRNP H stimulates ARE mRNA decay.

hnRNP F also stimulated the decay of the β-ARE_{GMCSF} mRNA when exogenous TTP was co-expressed. The decay of this reporter was stabilized to a measured half-life of 63 minutes after hnRNP F knockdown, compared to 43 minutes for the control knockdown when TTP was co-expressed (Figure 2.5A, lanes 5-8 and 13-16). This occurred despite higher Myc-TTP protein expression in the hnRNP F siRNA-treated cells (Figure 2.5B).

Another ARE mRNA reporter tested for hnRNP F-stimulated decay contained the entire 3’ UTR of the TNFα mRNA, β-TNFα-3’UTR. Although it

was not determined if hnRNP F associates with this reporter mRNA, endogenous TNFα mRNA was not enriched for in hnRNP F/H IPs (see below). The β-TNFα-3'UTR mRNA reporter was significantly stabilized ($p<0.05$) following hnRNP F knockdown (Figure 2.6A) and decayed with a half-life of 130±25 minutes compared to a half-life of 58±11 minutes for control siRNA-transfected cells (Figure 2.6A and plotted in Figure 2.6B). This mRNA also showed stabilization ($p<0.05$) following knockdown of the TTP/BRFs proteins, with a measured half-life of 106±26 (Figure 2.6A).

Experiments on the effect of hnRNP F depletion on exogenous ARE-mRNAs were performed with a single siRNA. To test whether exogenous hnRNP F can rescue endogenous hnRNP F depletion, rescue experiments were performed by adding back exogenous hnRNP F expressed from an mRNA resistant to the used siRNA. Addback of hnRNP F appeared to rescue mRNA decay of the β-BRSK1-3'UTR (Figure 2.7A, compare lanes 11-15 with 6-10), although it was unclear if exogenous Myc-hnRNP F expression in the addback was similar to endogenous hnRNP F levels (Figure 2.7B, lanes 1-5) since the migration of Myc-hnRNP F appeared to overlap with an unknown band just below hnRNP H (Figure 2.7B, lane 5; denoted by arrowhead).

hnRNP F/H associate with a subset of endogenous TTP-bound ARE mRNAs

We next wanted to test whether endogenous hnRNP F/H exists in complex with endogenous TTP substrate mRNAs. RIP experiments from LPS-

stimulated RAW cells were performed and bound mRNAs were tested by quantitative RT-PCR. The enrichment of associated mRNA was quantified as the ratio of bound ARE mRNA over GAPDH mRNA, since the pulldown of GAPDH mRNA for both TTP and hnRNP F/H was minimal. We specifically monitored for association of TTP target mRNAs as determined by prior studies from global mRNA decay analysis in TTP -/- mouse embryonic fibroblasts (Lai et al 2006) or TTP RIPs (Stoecklin et al 2008; Emmons et al 2008). RIPs performed on endogenous hnRNP F/H (Figure 2.8A) and TTP (Figure 2.8B) revealed that a subset of TTP-associated mRNAs were also enriched for in the hnRNP F/H RIP, although to a smaller extent (compare Figure 2.8A and 2.8B). Notably, LIF and USP46 mRNAs were enriched in both hnRNP F/H and TTP RIPs. By contrast, TNF-α and IL10 mRNAs, both well-characterized TTP substrates, were not enriched in the hnRNP F/H RIP. KLHL2 and TTP mRNA were also enriched in the hnRNP F/H and TTP RIPs, though KLHL2 was enriched to a lesser extent in the TTP RIP than other mRNAs.

The hnRNP F/H and TTP RIPs were repeated in another cell line, NIH3T3 fibroblasts, where TTP expression is induced with serum addition after starvation (Varnum et al 1991). These cells did not detectably express TNF-α or IL10 mRNAs (data not shown). However, monitoring for previously described TTP-associated mRNAs expressed in NIH3T3 cells yielded similar results, in which a subset of TTP-associated mRNAs also associate with hnRNP F/H. These include TTP, LIF, IER3 and BRF1 mRNAs (Figure 2.8C and 2.8D). By contrast, cFOS, PLK3, and MLLT11 mRNAs were enriched only

in the TTP RIP assays (Figure 2.8C and 2.8D). From these experiments we conclude that hnRNP F/H exist in complex with a subset of TTP-associated mRNAs; however, since the antibody used does not discriminate between hnRNP F and hnRNP H1/H2 it cannot be ruled out that some of these mRNAs show specificity for one or the other of these hnRNPs.

hnRNP F stimulates the decay of a subset of TTP substrate ARE-mRNAs

We next tested if hnRNP F stimulates the decay of endogenous TTP substrate mRNAs. These assays were performed in 3T3 cells, since siRNA-mediated knockdowns were more efficient in these cells than in RAW cells (data not shown). TTP and BRF1 proteins were induced following serum addition after starvation, and TTP protein levels peaked between 2-4 hours after serum add-back, while BRF1 levels peaked at closer to 6 hours (Figure 2.9A). hnRNP F and hnRNP H levels did not change after two hours serum addition (Figure 2.9B), nor did HuR levels for up to 6 hours after serum add-back (Figure 2.9A).

To monitor mRNA decay rates, cells were treated with the global transcription inhibitor Actinomycin D at three hours after serum addback, and samples were collected at subsequent time points to determine remaining mRNA levels by qRT-PCR. LIF, IER3, and cFOS mRNA decay were assessed, since these were enriched in hnRNP F/H and/or TTP RIPs, and reached maximal induction and clearance during this time course (Figure 2.9C). LIF mRNA decay was stabilized ($p<0.05$) upon hnRNP F knockdown

(knockdown efficiency shown in Figure 2.9B), degrading with a half-life of 87±6 minutes compared to 66±4 minutes for the control siRNA (Figure 2.9D). Similarly, TTP/BRF knockdown stabilized LIF mRNA to a half-life of 83±3 minutes. Two other siRNAs targeting hnRNP F stabilized LIF mRNA decay to a similar extent (Figure 2.10, siRNAs F3 and F1). In contrast to LIF mRNA, IER3 mRNA, which was enriched in both TTP and hnRNP F/H RIPs, was not stabilized upon hnRNP F knockdown (Figure 2.9E), showing a half-life of 53±6 minutes compared to 50±9 minutes for the control Luciferase siRNA. However, TTP/BRF knockdown stabilized IER3 mRNA to a half-life of 75±12 minutes. Thus, either hnRNP F levels are limiting for only a subset of mRNAs associated with TTP and hnRNP F, or the interaction observed in the hnRNP F/H RIP assays reflects an interaction of IER3 mRNA with hnRNP H only. cFOS mRNA served as a negative control, and although cFOS mRNA contains AREs and a binding site for TTP, TTP is not limiting for cFOS mRNA decay (Lai et al 2006). As expected, there was no stabilization in cFOS mRNA decay following TTP/BRF or hnRNP F knockdown (Figure 2.9F). A summary of the TTP-substrate mRNAs tested for decay following siRNA knockdown is listed in Table 2.1.

Similar endogenous mRNA decay assays performed in RAW cells were difficult to interpret since the RNAi knockdown was less efficient and varied dramatically between experiments. However, LIF mRNA appeared to be stabilized after TTP/BRF and hnRNP F knockdown (data not shown),

suggesting that the decay of this TTP-substrate mRNA is stimulated by hnRNP F in two different cell lines in response to different stimuli.

Do TTP and hnRNP F/H co-localize in cells?

TTP is primarily cytoplasmic, although it can shuttle to the nucleus (Murata et al 2002; Phillips et al 2002), whereas hnRNP F and hnRNP H are mostly nuclear, but shuttle and localize to the cytoplasm in some cell types (Kamma et al 1995; Honoré et al 1999; Van Dusen et al 2010; Honoré et al 2004) . To test whether TTP expression mediated cellular relocalization of hnRNP F/H, I performed indirect immunofluorescence assays in RAW cells during an LPS stimulation time course (Figures 2.11A, B). hnRNP F/H was mostly nuclear throughout the entire LPS time course (Figures 2.11A, B), while TTP protein was observed after 30 minutes of LPS stimulation and up until 6 hrs of LPS stimulation, and diffusely stained the cytoplasm (Figures 2.11A, B). hnRNP F/H did not appear to co-localize with TTP, and neither co-localized with markers of processing bodies (data not shown), cytoplasmic foci where mRNA decay factors and non-translating mRNAs accumulate (Erickson, & Lykke-Andersen 2011). Similar localization studies in HeLa cells for endogenous hnRNP F/H and exogenously expressed hnRNP F confirmed their primary nuclear localization and no apparent co-localization with exogenously expressed TTP or other cytoplasmic mRNA decay factors (data not shown).

Does hnRNP F stimulate TTP binding to substrate mRNAs?

To test whether hnRNP F recruits and/or stimulates TTP binding to substrate mRNAs, TTP RIPs were performed to monitor the association of ARE-mRNAs with or without hnRNP F knockdown. A difference in RIP enrichment would be expected if hnRNP F stimulates TTP binding to mRNA. The experiments were performed in RAW and NIH 3T3 cell lines. In RAW cells, most of the tested ARE mRNAs (except for TTP and IL-10 mRNAs) appeared to be slightly more associated with TTP following hnRNP F knockdown, ranging from a 1.4-fold enrichment of TNFα mRNA to a 2.7-fold enriched association of Zbtb26 mRNA (Figure 2.12A). These enrichments in TTP association were not statistically significant but the p-value for the Zbtb26 and Klhl2 mRNAs was <0.2. Interpretation of these results is difficult since hnRNP F knockdown was not as efficient or consistent in RAW cells as compared to HeLa or 3T3 cells (compare Figure 2.12B to 2.3C, 2.9B), and the amount of TTP protein pulled down varied across the repeat samples (Figure 2.12C). There did not appear to be a major difference in TTP protein expression between the control siRNA and hnRNP F siRNA transfected cells (Figure 2.12B), but this was not quantified. RIPs performed in NIH 3T3 cells showed no significant change in the enrichment of mRNAs in the TTP RIP following hnRNP F knockdown; however in these samples the TTP protein pulled down could not be visualized by Western blot since it closely migrated with non-specific bands (data not shown).

Next, I tested if TTP binding to a reporter mRNA is dependent on hnRNP F. TTP RIPs were performed in a human embryonic kidney cell line (HEK293T) that expressed exogenous TTP and the β-BRSK1-3' UTR reporter mRNA. hnRNP F depletion did not appear to change the amount of β-BRSK1-3' UTR mRNA pulled down with Flag-TTP, since roughly 5% of the input mRNA was bound to TTP in all conditions tested (Figure 2.13, compare lanes 7 to 9, and 8 to 10). Thus, no conclusive evidence was found to suggest that hnRNP F stimulates mRNA substrate binding of TTP.

Does hnRNP F repress translation of ARE mRNAs?

Some evidence suggests a role for hnRNP F in regulating translation of specific transported mRNAs, including the MBP mRNA in neuronal cells (White et al 2012), and nanos mRNA in *D. melanogaster* oocytes (Kalifa et al 2006). Two experimental approaches were taken to test whether a relationship exists between hnRNP F and ARE-mRNA translation. First, I tested whether translation of an ARE-mRNA is required for hnRNP F to stimulate its mRNA decay by monitoring the effect of a 5'UTR hairpin that blocks the recruitment of the large ribosomal subunit (Franks, & Lykke-Andersen 2007; Kozak 1989). The decay of a 5' hairpin-containing β-BRSK1-3' UTR mRNA reporter was stabilized by hnRNP F knockdown (Figure 2.14) and had a half-life of 100 minutes, compared to 70 minutes for the control knockdown. This fold stabilization (~ 1.5) was similar to the stabilization of the same reporter mRNA lacking a hairpin, which decayed with a half-life of 123 minutes after hnRNP F

knockdown compared to 85 minutes for the control knockdown (Figure 2.14). Thus, inhibition of large ribosomal subunit recruitment does not overcome the effect of hnRNP F on the β-BRSK1-3' UTR mRNA decay.

In the second approach, I tested if hnRNP F affected the translation of ARE mRNAs as monitored by polysome profile fractionation. Cytoplasmic lysates from HEK 293T cells expressing the β-ARE_{GMCSF} mRNA reporter were separated by centrifugation through sucrose density gradients and the corresponding RNA from each fraction was visualized by Northern analysis. The distribution of the β-ARE_{GMCSF} mRNA reporter appeared slightly shifted out of the heavy fractions 11 and 12, and more greatly enriched in fractions 7-10, in the hnRNP F depleted cells compared to the control siRNA treated cells (Figure 2.15A, quantified in Figure 2.15B). However it remains to be determined if this shift is significant, reproducible, and responsible for the slower mRNA decay phenotype observed when hnRNP F is knocked down.

2.3 Discussion

hnRNP F interacts with TTP and stimulates ARE mRNA decay

hnRNP F was one of three major proteins that were found in an RNA-independent complex with TTP and BRF1 (Figure 1.1). The interaction was confirmed for TTP and hnRNP F by co-IPs with exogenously expressed proteins (Figure 2.2A) and with endogenous proteins (Figure 2.2B, C), where TTP was also observed to interact with the closely related homologs hnRNP H1/H2. hnRNP F/H showed association with a subset of TTP-associated ARE-

mRNAs (Figure 2.3, 2.8), and hnRNP F stimulated the decay of a subset of TTP-associated ARE-mRNAs, including LIF mRNA (Figure 2.9) and ARE-containing reporter mRNAs (Figures 2.3-7, 2.14). However, not all ARE-mRNAs whose decay were stimulated by hnRNP F were stably associated with hnRNP F/H (Figure 2.4), and no evidence was found that hnRNP F stimulates TTP-ARE-mRNA assembly (Figures 2.12, 2.13). Thus, the mechanism by which hnRNP F stimulates TTP activity remains to be determined and could be direct or indirect.

What is the mechanism of hnRNP F stimulation of ARE mRNA decay?

Does hnRNP F stimulate TTP association with substrate mRNAs?

How does hnRNP F stimulate ARE-mRNA decay? One hypothesis was that hnRNP F stimulates TTP binding to ARE mRNAs. The association of substrate ARE mRNAs to TTP was tested when hnRNP F was knocked down (Figure 2.12) but the results were inconclusive due to technical difficulties encountered with the experiment, including inefficient knockdown of hnRNP F in RAW cells, and inability to quantify the pulled down TTP protein in 3T3 cells. Similarly, there was no observed difference in association of the β-BRSK1-3'UTR mRNA reporter with exogenous TTP upon hnRNP F knockdown (Figure 2.13). However, it is possible that exogenous expression of TTP rendered TTP non-limiting for binding to this reporter mRNA. Although endogenous hnRNP F/H enriched for only a subset of TTP-associated mRNAs in RIP assays (Figure 2.8), it cannot be ruled out that hnRNP F/H transiently

associates with primary transcripts of these mRNAs. Consistent with this idea, hnRNP F/H are primarily nuclear and are known to bind a subset of tested introns (Min et al 1995; Gamberi et al 1997; Huelga et al 2012) . Thus, it remains possible that hnRNP F is an mRNP, or pre-mRNP, component that recruits TTP through protein-protein interaction. Once at the mRNP, the chance of TTP binding to the ARE or other cis-element or trans factor in the 3' UTR could be increased. After the conditions for RIP assays are optimized a global analysis of TTP-associated mRNAs +/- hnRNP F knockdown could identify potential mRNAs for which TTP association are stimulated by hnRNP F.

Does hnRNP F promote translation repression of ARE mRNAs?

Another possibility is that hnRNP F regulates the translation of ARE mRNAs to stimulate decay. In neuronal cells, hnRNP F appears to regulate translation of the transported MBP mRNA (White et al 2012), and in *Drosophila*, the hnRNP F homolog glorund represses the translation of nanos mRNAs during oogenesis (Kalifa et al 2006). Similarly, hnRNP F might repress translation of TTP-associated mRNA and stimulate mRNA decay as a consequence. In a preliminary experiment, the polysome association of an ARE mRNA reporter appeared to be enriched in polysomal fractions after hnRNP F knockdown (Figure 2.15), but whether this slight shift is reproducible or indicative of decreased protein output was not determined. If hnRNP F represses translation then increased polysome association would be expected

when hnRNP F is knocked down. This initial result needs to be confirmed and the translation profile of endogenous TTP substrate mRNAs should also be examined. Additionally, hnRNP F stimulated the decay of a non-translating 5' hairpin-containing ARE mRNA reporter (Figure 2.14), suggesting that if hnRNP F stimulates mRNA decay via translation repression it is not at the step of large ribosome subunit recruitment. Although the mechanism of translation repression by the *Drosophila* homolog of hnRNP F, Glorund, is unknown, it has been suggested to block translation initiation (Andrews et al 2011), so this could be a step at which hnRNP F acts. Other ways to test if hnRNP F regulates the translation of ARE mRNA include monitoring the effect of hnRNP F knockdown on cellular protein levels of TTP substrate mRNAs relative to their mRNA levels, and on the translation of reporter ARE mRNAs using a luciferase based reporter assay.

Does hnRNP F stimulate mRNA decay downstream of TTP binding to mRNA?

hnRNP F could stimulate TTP mRNA decay at a step after TTP mRNA binding. This could be tested in mRNA decay assays with an MS2-TTP fusion protein tethered to MS2 binding site reporter mRNA lacking an ARE. In this system, TTP binding to RNA is dependent on the MS2 domain and not the TTP RNA binding domain. If hnRNP F knockdown slowed the decay of the reporter with tethered TTP it would indicate that hnRNP F stimulates TTP

mRNA decay at a step after RNA binding, but it would not exclude the possibility that hnRNP F also stimulates TTP binding to ARE mRNA.

Another assay to test whether hnRNP F stimulates TTP activity downstream of TTP mRNA binding is to test whether TTP association with decay factors is facilitated by hnRNP F. Co-IP experiments that probe the composition of TTP protein complexes after hnRNP F knockdown could be used to test for pull down of decay factors.

Does hnRNP F stimulate TTP activity by modulating the TTP protein?

The stimulation of TTP activity by hnRNP F may not involve translation regulation or TTP RNA binding. Potential steps for regulation of TTP activity include TTP protein induction/expression levels, post-translational modifications of TTP (Sun et al 2007; Stoecklin et al 2004; Clement et al 2011; Marchese et al 2010), subcellular localization, or possibly other steps. The levels of induced TTP protein did not appear to significantly change after hnRNP F knockdown in 3T3 cells (Figure 2.9C), although this needs to be quantified. Additionally, there was no difference in the induction or decay of TTP mRNA in 3T3 cells following hnRNP F knockdown (data not shown). TTP mRNA and protein induction did not appear altered in RAW cells after hnRNP F knockdown after 6 hours of LPS stimulation (data not shown), but the hnRNP F knockdowns may have been too inefficient to observe any differences.

Does hnRNP F stimulate TTP-mediated decay of non-ARE mRNA?

It was surprising that many of the stabilized mRNAs identified in a global mRNA decay study of TTP knockout fibroblasts did not contain AREs (Lai et al 2006). In a separate study of TTP-associated mRNAs in dendritic cells, most of the enriched mRNAs lacked AREs (Emmons et al 2008). Although TTP is best characterized to regulate ARE mRNAs there may be a large population of non-ARE mRNAs that it regulates as well. It will be important to follow up on those global studies since only some of the non-ARE mRNA TTP substrates were further characterized (Emmons et al 2008), and it is possible that hnRNP F might be a TTP co-factor in the decay of those mRNAs. It would be interesting to determine, in a proof-of-principle experiment, if hnRNP F can act as a trans factor to recruit TTP to mRNAs which lack AREs or TTP-binding sites. For instance, does hnRNP F tethered to a non-ARE mRNA reporter promote mRNA decay of the reporter in a TTP-dependent manner? Does hnRNP F recruit TTP to non-ARE substrate mRNAs in cells? It could be worthwhile to cross-reference the list of the non-ARE mRNAs stabilized in TTP knockout cells, with the list of non-ARE mRNAs associated with TTP (in dendritic cells), to hnRNP F associated mRNAs (Huelga et al 2012) for candidates of TTP non-ARE mRNA decay substrates also associated with hnRNP F. It would be interesting if the decay or TTP-association with these mRNAs showed a greater dependence on hnRNP F. The recruitment of TTP to substrate mRNAs through trans factors is not well established, but a recent study demonstrated that AUF1, another AUBP, can

interact with an RNA-binding mutant of TTP and recruit it to ARE mRNAs in vitro (Kedar et al 2011).

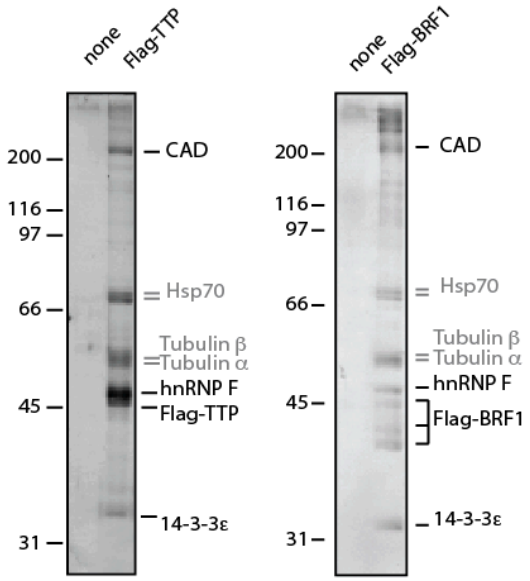


Figure 2.1: Identification of TTP/BRF1-interacting proteins. Silver-stained SDS-polyacrylamide gels showing the proteins that co-purify with stably expressed Flag-tagged TTP (left gel) or BRF1 (right gel) from HEK 293T cell line. Proteins co-purified from cells expressing no Flag-tagged protein are run alongside as a control.

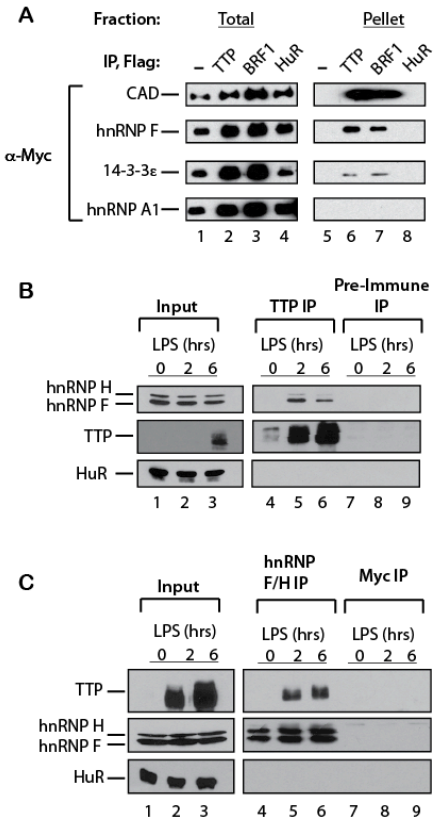


Figure 2.2: hnRNP F complexes with TTP/BRF1. (A) Western blots of anti-Flag IP from RNase treated extracts of HEK 293T cells transiently expressing Flag-tagged TTP (lane 6), BRF1 (lane 7), HuR (lane 8), or empty vector control (lane 5). Precipitates were probed for the presence of co-expressed Myc-tagged CAD, hnRNP F, 14-3-3ε, or hnRNP A1. __% of the total extract was run in lanes 1-4. **(B)** Western blot for endogenous proteins that co-IP with anti-TTP (lanes 4-6) or pre-immune sera (lanes 7-9) from RNase-treated extracts from RAW264.7 cells stimulated with 100 ng/ml LPS for the indicated times. Precipitates are compared with 1% of the input (lanes 1-3). The anti-hnRNP F/H antibody recognizes both hnRNP F (bottom band) and hnRNP H (top band). **(C)** Similar as in (B) except Western blot for proteins that co-IP with anti-hnRNP F/H (lanes 4-6) or anti-Myc (lanes 7-9).

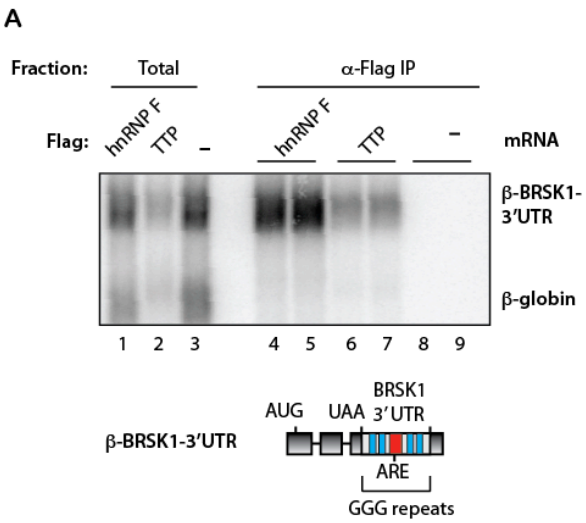


Figure 2.3: hnRNP F stimulates decay of an ARE mRNA containing hnRNP F binding sites. (A) Northern blot showing reporter mRNAs that co-precipitate with transiently expressed Flag-tagged hnRNP F (lanes 4, 5), TTP (lanes 6, 7), or empty vector (lanes 8, 9) in HEK 293T cells. Precipitates and 5% of total extract (lanes 1-3) were probed for the presence of β-globin. A cartoon schematic of the β-BRSK1-3'UTR mRNA reporter with the 3' UTR from BRSK1 mRNA containing an ARE (red bar) and G-rich sequence repeats (blue bars) downstream of the β-globin coding region. **(B)** Northern blots showing mRNA decay of the β-BRSK1-3'UTR mRNA reporter in HeLa Tet-off cells after siRNA transfection of hnRNP F, TTP/BRF1/2, or luciferase control as indicated. Transcription of the reporter mRNA was pulsed on for six hours by removal of tetracycline, and samples were collected at the times indicated after transcriptional shut-off by tetracycline addition (chase). Levels of reporter mRNA was normalized to control RNA (β-globin) and the average half-life ($t_{1/2}$) and standard error mean was determined from 6 independent experiments. **(C)** Western blot of the corresponding cell extracts from (B) were probed with anti-hnRNP F/H antibody (bottom panel) to assess siRNA knockdown of protein, and anti-Upf1 antibody for loading control (top panel). Luciferase siRNA transfected protein sample was loaded at 100%, 33%, and 11% to approximate levels of hnRNP F knockdown.

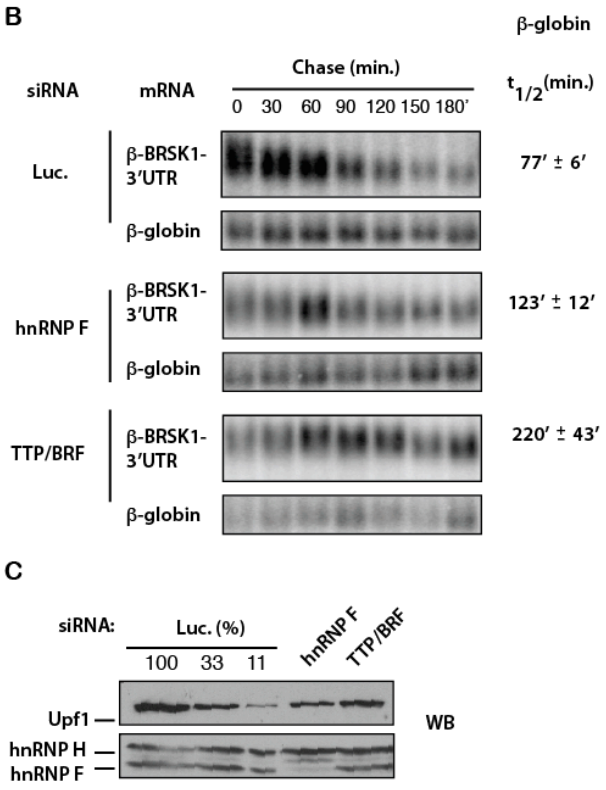
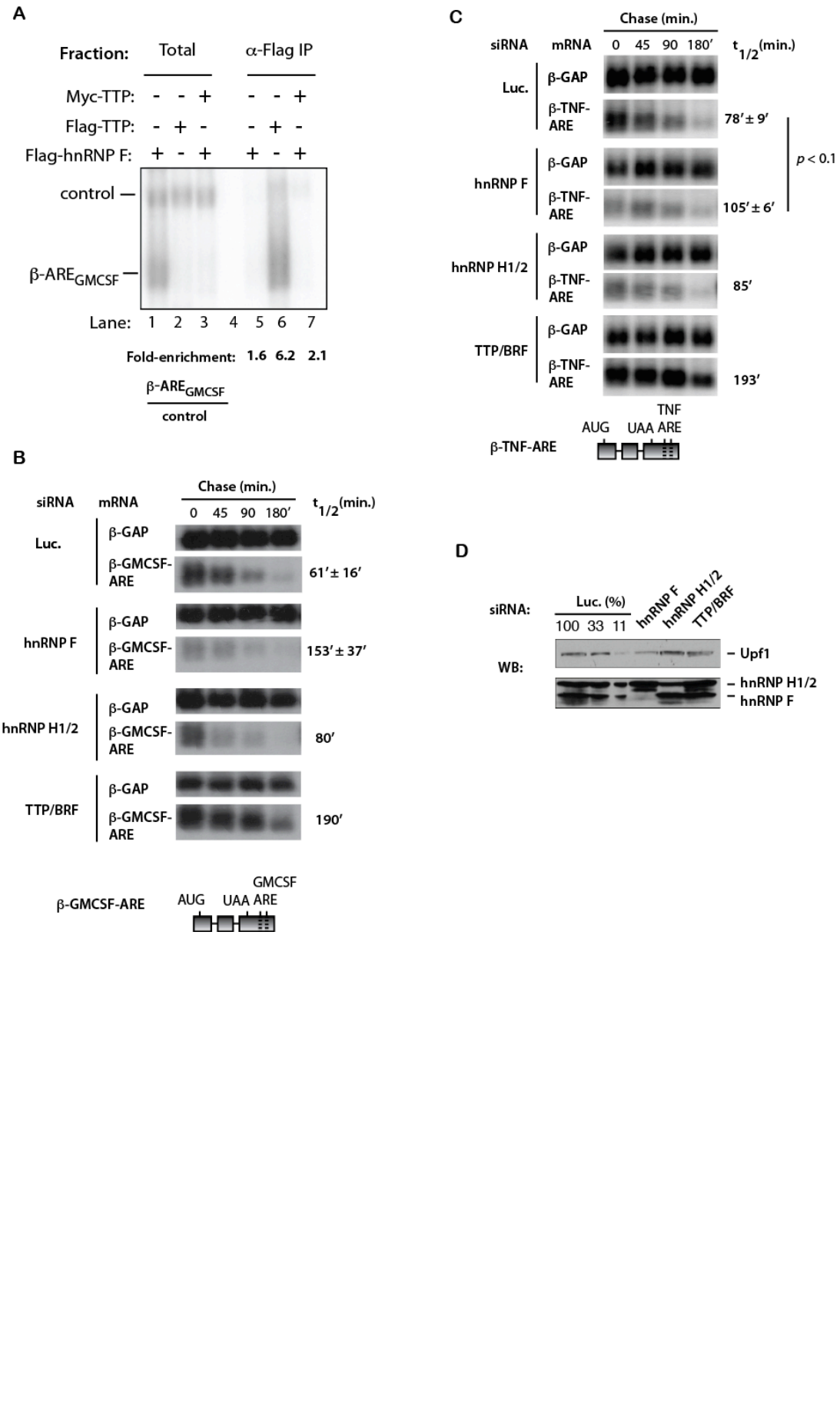


Figure 2.3: hnRNP F stimulates decay of an ARE mRNA containing hnRNP F binding sites, continued.

Figure 2.4: hnRNP F stimulates the decay of ARE-mRNA reporters that it does not enrich for in RNA immunoprecipitation. (A) Northern blot showing reporter mRNA containing the ARE from GMCSF mRNA, β-ARE_{GMCSF}, or control (β-GAP), that co-precipitates with transiently expressed Flag-tagged hnRNP F (lanes 5, 7) or TTP (lanes 6) in HEK 293T cells. Myc-TTP was co-expressed with Flag-hnRNP F in lane 7. Precipitates and 5% of total extract (lanes 1-3) were probed for the presence of β-globin. Fold enrichment was based on the percent RNA precipitated from total RNA. **(B)** Northern blots showing mRNA decay of the β-ARE_{GMCSF} mRNA reporter in HeLa Tet-off cells after siRNA transfection of hnRNP F, hnRNP H, TTP/BRF1/2, or luciferase control as indicated. Transcription of the reporter mRNA was pulsed on for six hours, and samples were collected at the times indicated after transcriptional shut-off (chase). Levels of reporter mRNA was normalized to control RNA (β-GAP) and half-life ($t_{1/2}$) and standard error mean for Luc. siRNA and hnRNP F siRNA samples was determined from two independent experiments. Cartoon of the β-ARE mRNA reporter with the ARE from GMCSF inserted in the 3' UTR is shown below. **(C)** mRNA decay as in (B), measuring the decay of a reporter containing the ARE from the TNFα mRNA. **(D)** Western blot of the corresponding cell extracts from (B) were probed with anti-hnRNP F/H antibody (bottom panel) to assess siRNA knockdown of protein, and anti-Upf1 antibody for loading control (top panel). Luciferase siRNA transfected protein sample was loaded at 100%, 33%, and 11% to better approximate levels of hnRNP F remaining after knockdown.


A
Fraction: Total α-Flag IP
Myc-TTP: - - + - - +
Flag-TTP: - + - - + -
Flag-hnRNP F: + - + + - +
control
β-ARE GMCSF
Lane: 1 2 3 4 5 6 7
Fold-enrichment: 1.6 6.2 2.1
β-ARE GMCSF / control
B
siRNA mRNA Chase (min.) 0 45 90 180′ t 1/2 (min.)
Luc. β-GAP
β-GMCSF-ARE 61′ ± 16′
hnRNP F β-GAP
β-GMCSF-ARE 153′ ± 37′
hnRNP H1/2 β-GAP
β-GMCSF-ARE 80′
TTP/BRF β-GAP
β-GMCSF-ARE 190′
β-GMCSF-ARE AUG UAA GMCSF ARE
C
siRNA mRNA Chase (min.) 0 45 90 180′ t 1/2 (min.)
Luc. β-GAP
β-TNF-ARE 78′ ± 9′
hnRNP F β-GAP
β-TNF-ARE 105′ ± 6′
p < 0.1
hnRNP H1/2 β-GAP
β-TNF-ARE 85′
TTP/BRF β-GAP
β-TNF-ARE 193′
β-TNF-ARE AUG UAA TNF ARE
D
siRNA: Luc. (%) 100 33 11 hnRNP F hnRNP H1/2 TTP/BRF
WB: Upf1
hnRNP H1/2
hnRNP F

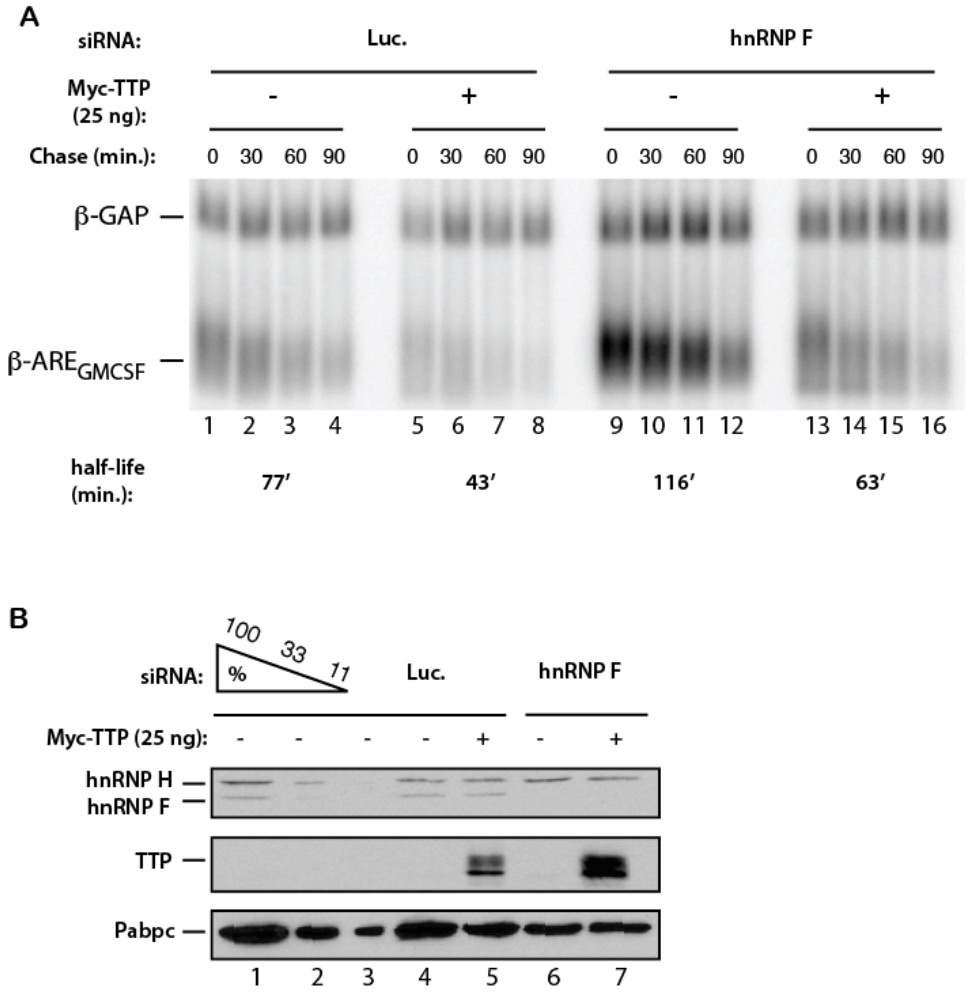


Figure 2.5: hnRNP F stimulates decay of ARE-mRNA reporter when exogenous TTP is expressed. (A) Northern blot showing mRNA decay of the β-ARE_{GMCSF} mRNA reporter in HeLa Tet-off cells after siRNA transfection of hnRNP F or luciferase control and expression of Myc-tagged TTP as indicated. Transcription of the reporter mRNA was pulsed on for six hours, and samples were collected at the times indicated after transcriptional shut-off (chase). Levels of reporter mRNA was normalized to control RNA (β-GAP) and half-life was determined **(B)** Western blot probed for hnRNP F/H, TTP, and PABP showing corresponding proteins from cell extracts in (A). Luciferase siRNA transfected protein sample was loaded at 100%, 33%, and 11% to better approximate levels of hnRNP F remaining after knockdown.

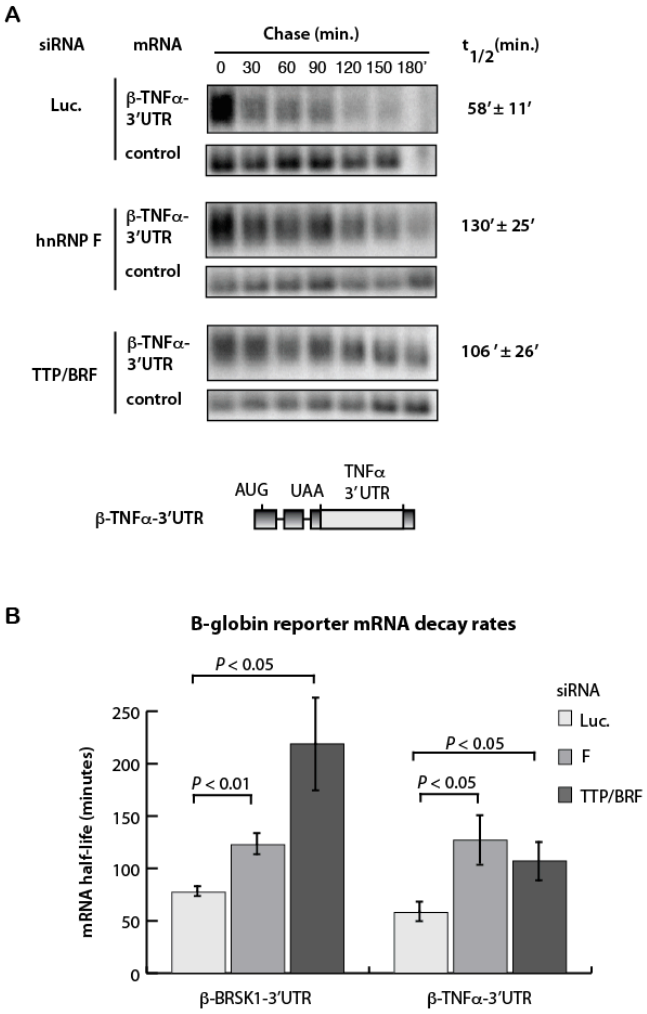


Figure 2.6: hnRNP F stimulates mRNA decay of a TNF-α 3' UTR-containing mRNA reporter. (A) Northern blots showing mRNA decay of the β-TNFα-3'UTR mRNA reporter, containing the 3' UTR of TNFα mRNA downstream of β-globin coding region, in HeLa Tet-off cells after siRNA transfection of hnRNP F, TTP/BRF1/2, or luciferase control as indicated. Transcription of the reporter mRNA was pulsed on for six hours by removal of tetracycline, and samples were collected at the times indicated after transcriptional shut-off by tetracycline addition (chase). Levels of reporter mRNA was normalized to control RNA (β-globin) and average half-life ($t_{1/2}$) and standard error mean was calculated from 4 independent experiments. **(B)** Quantification of the stabilization of mRNA decay for the β-BRSK1-3'UTR (Figure 2.3) and β-TNFα-3'UTR mRNA reporters in HeLa Tet-off cells after siRNA transfection. Significance was calculated with Students t-test (two tailed).

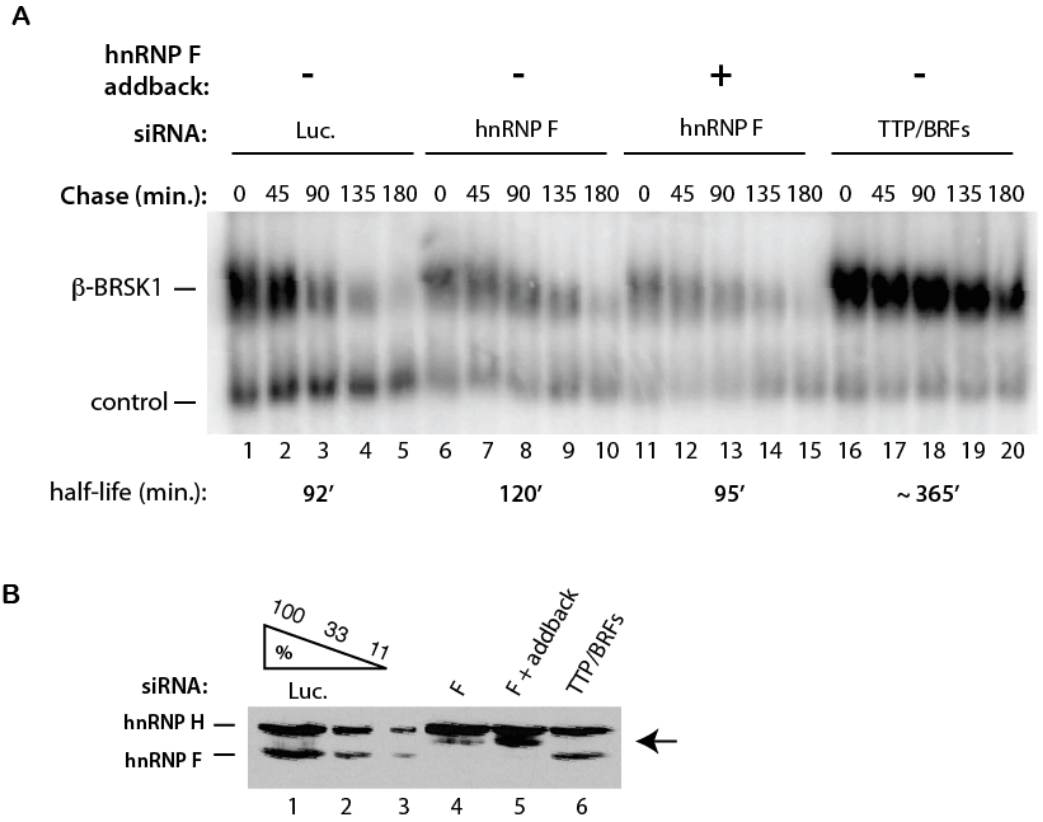


Figure 2.7: Does hnRNP F addback rescue mRNA decay observed for hnRNP F knockdown? (A) Northern blot showing mRNA decay of the β-BRSK1-3'UTR mRNA reporter in HeLa Tet-off cells after siRNA transfection of hnRNP F, TTP/BRF1/2, or luciferase control and expression of siRNA-resistant Myc-tagged hnRNP F (lanes 11-15). Transcription of the reporter mRNA was pulsed on for six hours and samples were collected at the times indicated after transcriptional shut-off (chase). Level of reporter mRNA was normalized to control RNA (β-globin) to calculate the half-life ($t_{1/2}$). Longer time points are required for a more accurate determination of half-life after TTP/BRFs siRNA transfection. **(B)** Western blot showing the corresponding protein extracts from (A). Myc-hnRNP F (expressed in lane 5, arrowhead) migrated at the same size as an unknown band in between hnRNP F and H. Luciferase siRNA transfected protein extract was loaded at 100%, 33%, and 11% to better approximate level of hnRNP F remaining after knockdown.

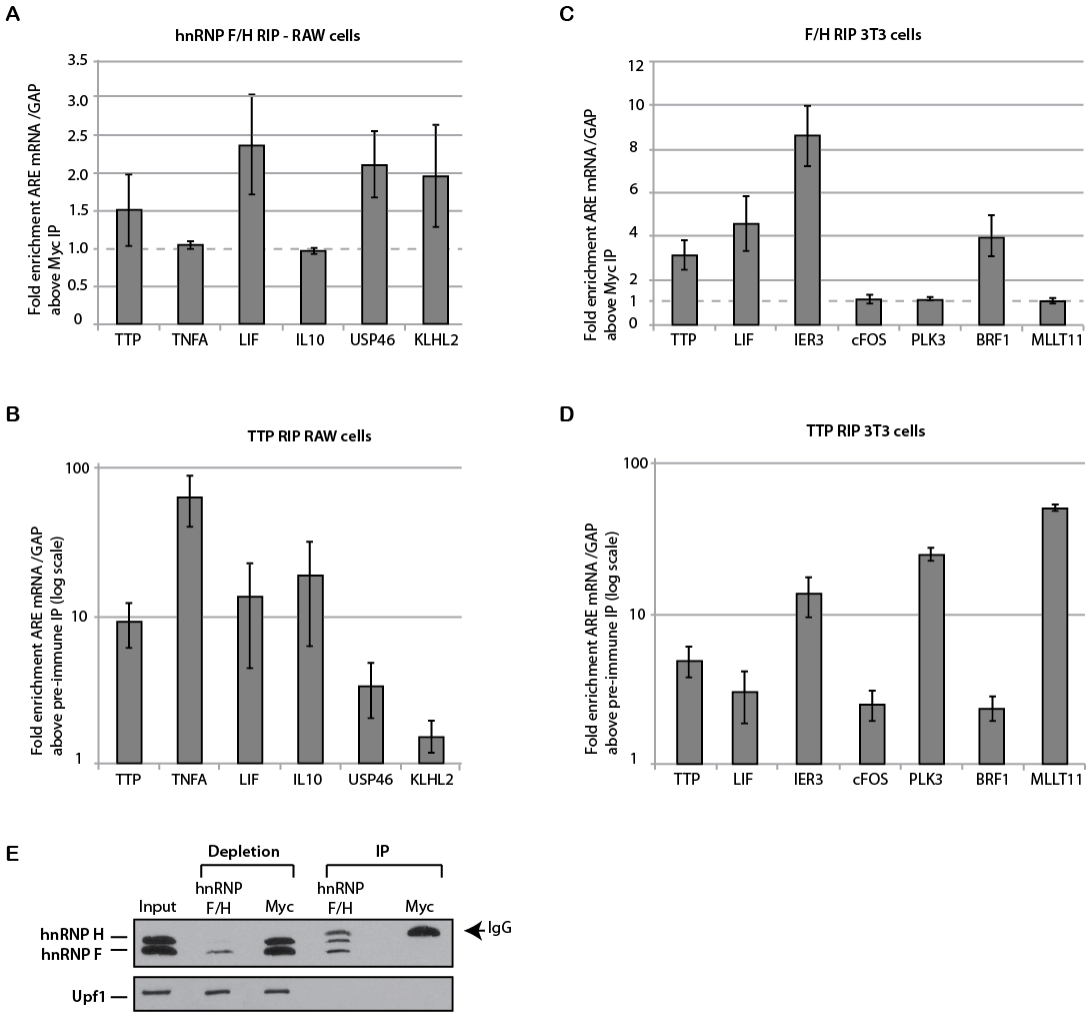


Figure 2.8: hnRNP F binds to a subset of TTP-associated ARE mRNAs. **(A)** Quantification of the enriched ARE-mRNA above GAPDH mRNA that co-precipitates with hnRNP F/H in RAW264.7 cells stimulated with 100 ng/ml LPS for 2 hours as determined by qRT-PCR. The average fold enrichment and standard error mean were calculated from three experiments. **(B)** Same as in (A) except TTP co-precipitating mRNA was quantified. **(C)** Quantification of the enriched ARE-mRNA above GAPDH mRNA that co-precipitates with hnRNP F/H in NIH 3T3 cells refed for 2 hours after 24 hours starved. Relative mRNA levels were determined by qRT-PCR. **(D)** Same as in (C) except TTP co-precipitating mRNA was quantified. **(E)** Western blot probed for hnRNP F/H showing the anti-hnRNP F/H and anti-Myc precipitates (IP) from (C). Input cell extract and hnRNP F/H-depleted and Myc-depleted extracts were probed for hnRNP F/H and Upf1.

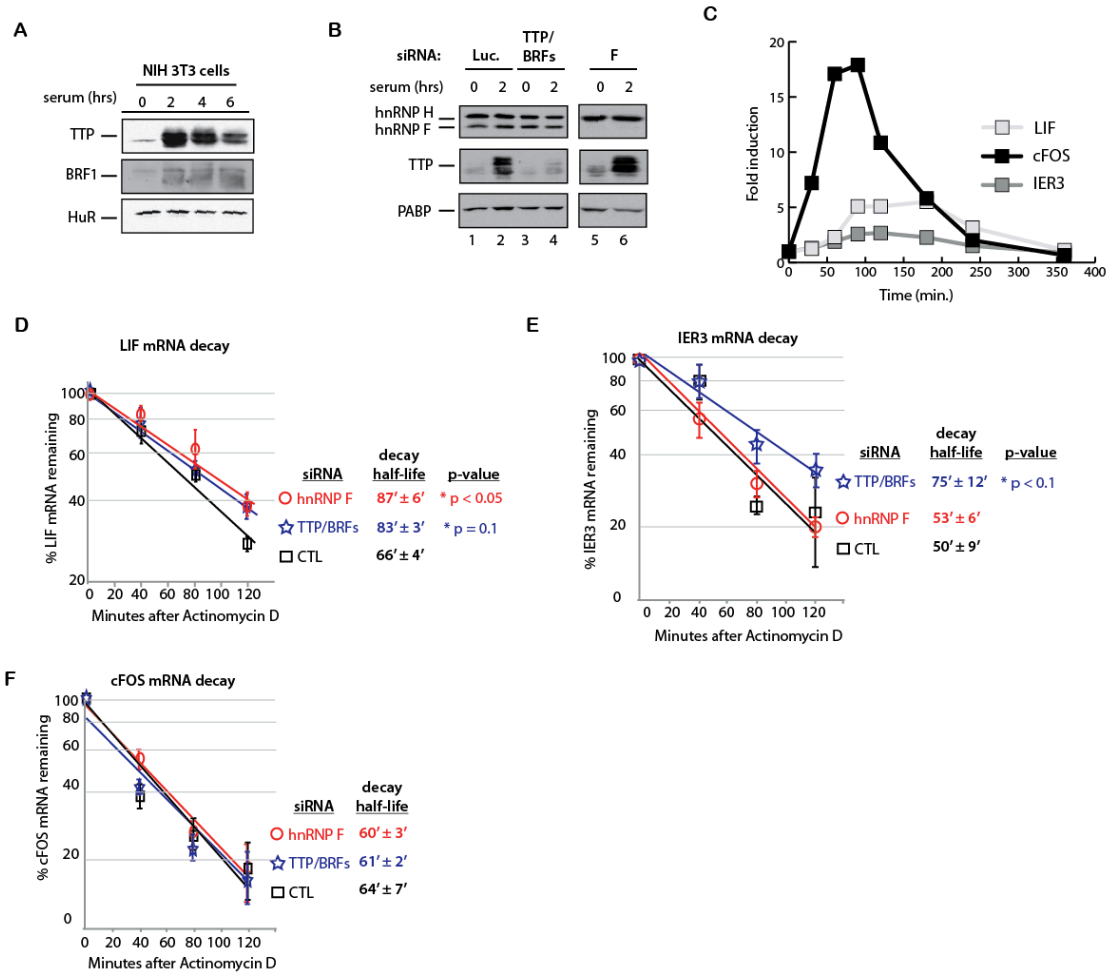


Figure 2.9: hnRNP F stimulates mRNA decay of some associated ARE mRNAs. (A) Western blot showing protein levels of TTP, BRF1, and HuR in NIH 3T3 cells re-fed for indicated time after 24 hr starvation. **(B)** Quantification of LIF, IER3, and cFOS mRNA in NIH 3T3 cells as in (A), assessed by qRT-PCR. **(C)** Western blot of protein levels in NIH 3T3 cells transfected with 80 nM of the indicated siRNA and re-fed with serum for 0 or 2 hours after 24 hours starvation. PABP was used as a loading control. **(D)** mRNA decay of LIF mRNA in NIH 3T3 cells transfected with 80 nM indicated siRNA and re-fed for 3 hr on full media after 24 hr starvation. Samples were collected at 0, 40, 80, and 120 minutes after addition of 10 µg/ml Actinomycin D addiction and LIF mRNA was normalized to GAPDH mRNA by qRT-PCR to determine relative abundance and to calculate mRNA decay half-life. The student t-test was used to measure significance from three biological repeat experiments. **(E)** cFOS mRNA decay half-life measured as in (D). **(F)** IER3 mRNA decay half-life measures as in (D).

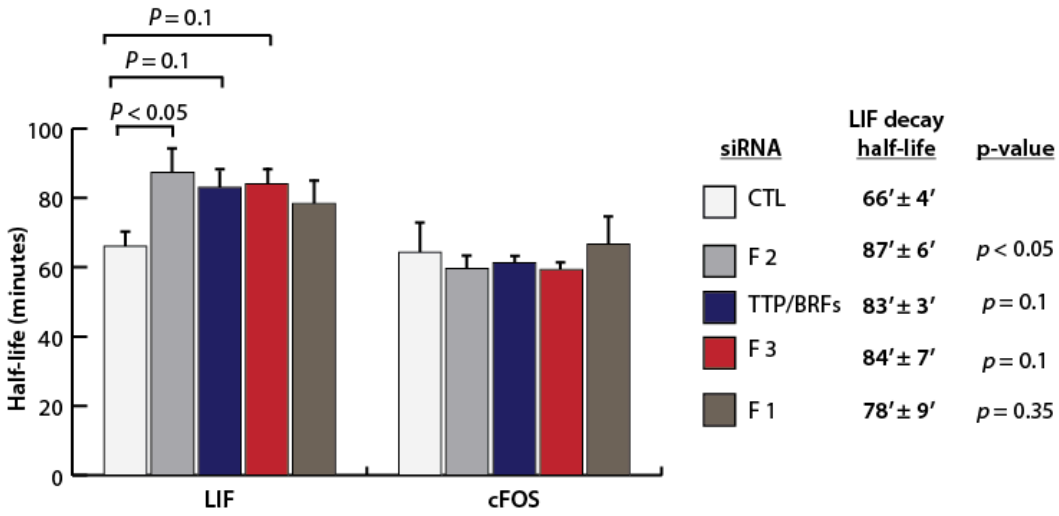


Figure 2.10: Knockdown of hnRNP F with different siRNAs slows LIF mRNA decay in NIH 3T3 cells. LIF mRNA decay half-life in NIH 3T3 transfected with the indicated siRNAs. Experiments were performed as in Figure 2.5D, with two additional siRNAs targeting hnRNP F (F3, F1).

Table 2.1: Summary of mRNA decay in 3T3 cells. Summary of mRNA decay of endogenous mRNA in NIH 3T3 cells for the experiments described in Figure 2.5D. The half-lives of mRNA decay with two additional hnRNP F siRNAs (1, 3) are listed in the table. In calculating IER3 and PLK3 mRNA half-life, one experiment was discarded since there was no stabilization observed for the positive control TTP/BRF knockdown.

mRNA	siRNA	Avg. half -life	SEM	N	*p-value*
LIF	Luc.	66′	4′	3	-
	hnRNP F (1)	78′	9′	3	$p = 0.35$
	hnRNP F (2)	87′	6′	3	$p < 0.05$
	hnRNP F (3)	84′	7′	3	$p = 0.1$
	TTP/BRF	83′	3′	3	$p = 0.1$
cFOS	Luc.	64′	7′	3	-
	hnRNP F (1)	67′	7′	3	-
	hnRNP F (2)	60′	3′	3	-
	hnRNP F (3)	60′	2′	3	-
	TTP/BRF	61′	2′	3	-
IER3	Luc.	50′	9′	2	-
	hnRNP F (1)	50′	9′	2	-
	hnRNP F (2)	53′	6′	2	-
	hnRNP F (3)	51′	2′	2	-
	TTP/BRF	75′	12′	2	$p < 0.1$
PLK3	Luc.	93′	6′	2	-
	hnRNP F (1)	102′	15′	2	$p = 0.5$
	hnRNP F (2)	231′	0′	2	$p < 0.05$
	hnRNP F (3)	156′	17′	2	$p = 0.22$
	TTP/BRF	166′	65′	2	$p = 0.43$

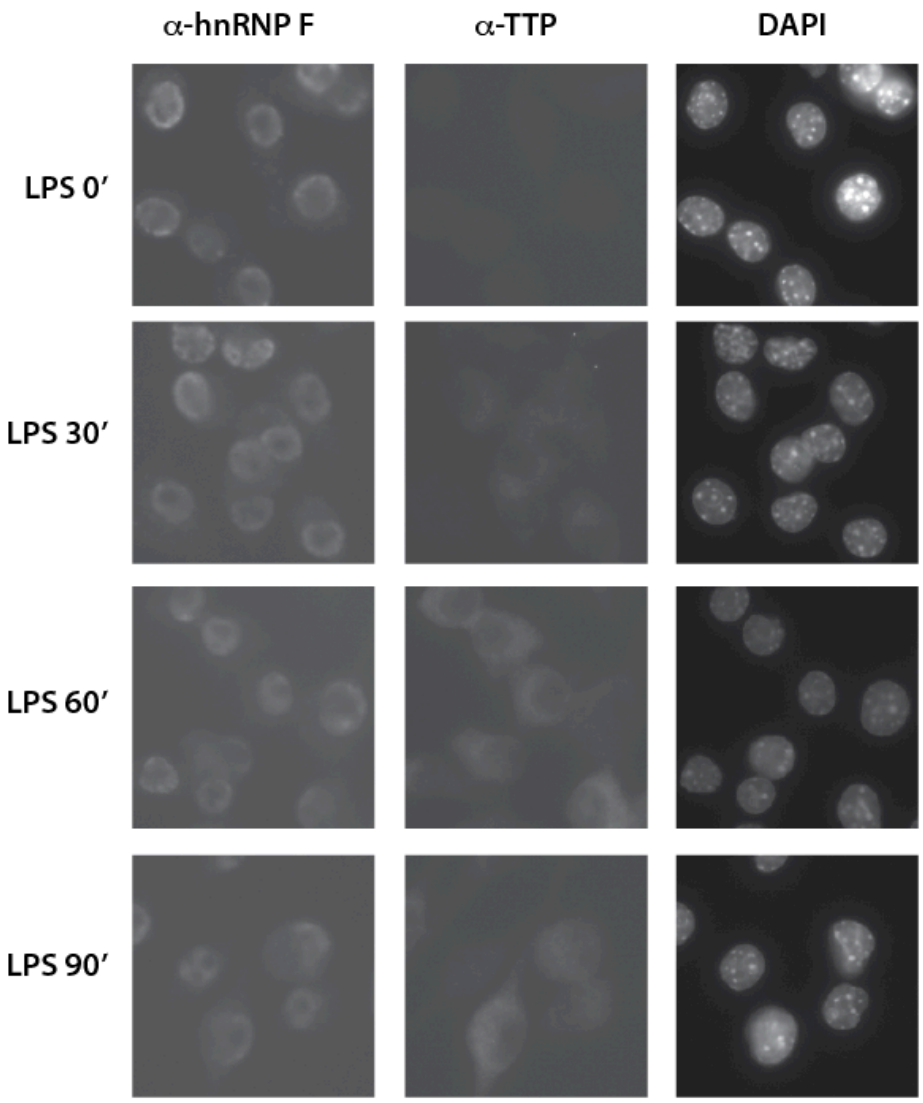


Figure 2.11A: hnRNP F/H and TTP localization in LPS stimulated RAW264.7 macrophage cells. Immunofluorescence assays of RAW264.7 cells stimulated with 100 ng/ml LPS as indicated and stained for endogenous hnRNP F/H and TTP. Nuclei were stained with DAPI.

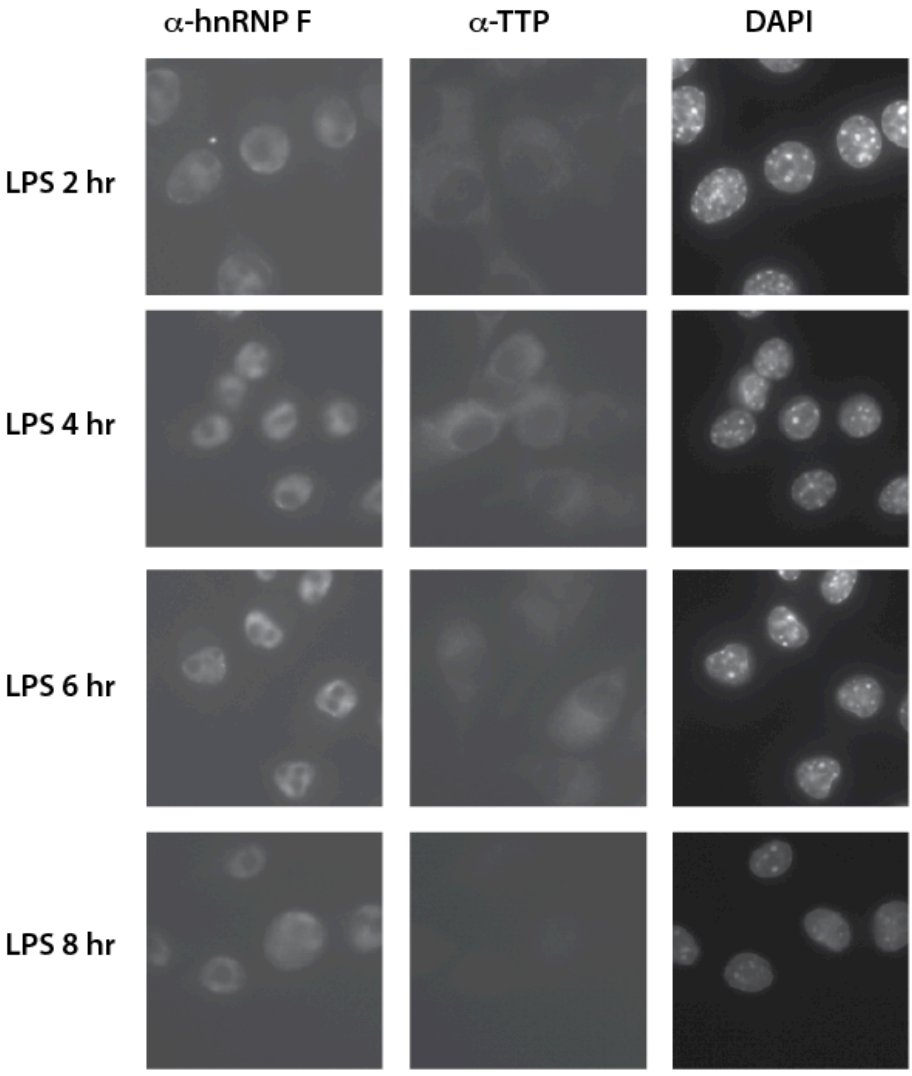


Figure 2.11B: hnRNP F/H and TTP localization in LPS stimulated RAW264.7 macrophage cells. Immunofluorescence assays of RAW264.7 cells stimulated with 100 ng/ml LPS as indicated and stained for endogenous hnRNP F/H and TTP. Nuclei were stained with DAPI.

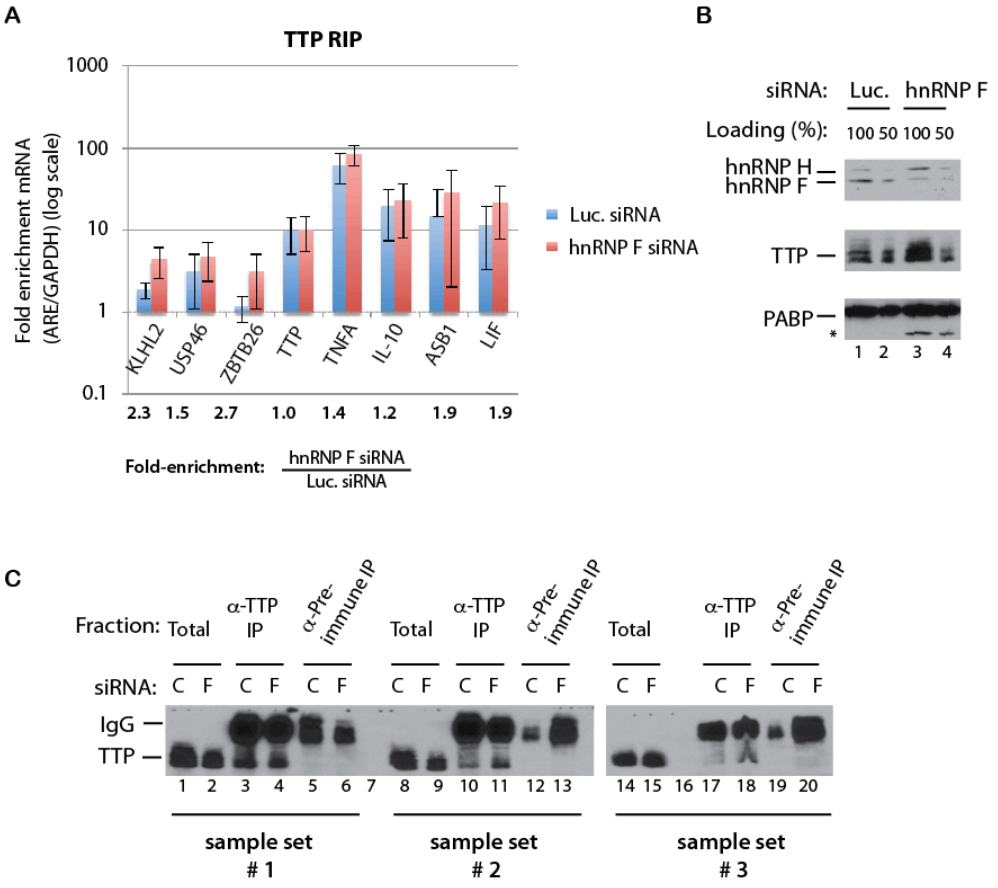


Figure 2.12: Is TTP ARE mRNA association influenced by hnRNP F? (A) Quantification of the enriched mRNA above pre-immune IP that co-precipitates with TTP in RAW264.7 cells transfected with siRNA targeting hnRNP F or luciferase control and stimulated with 100 ng/ml LPS for 2 hours as determined by qRT-PCR. The enrichment average and standard error mean were calculated for three independent experiments. **(B)** Western blot of the total cell extract prior to immunoprecipitation to show levels of hnRNP F/H, TTP, and PABP. Samples were loaded at 100% and 50% to better estimate hnRNP F depeletion. A background band (indicated by *) appeared for some PABP samples. **(C)** Western blot showing the levels of precipitated TTP from anti-TTP (lanes 3, 4, 10, 11, 17, 18) and anti-preimmune sera (lanes 5, 6, 12, 13, 19, 20) from the three sample sets in (A). IgG heavy chain migrated above TTP. Lanes 1, 8, 14, 15 contained 5% of total cell extract, while lanes 2 and 9 contained 2.5% of total extract.

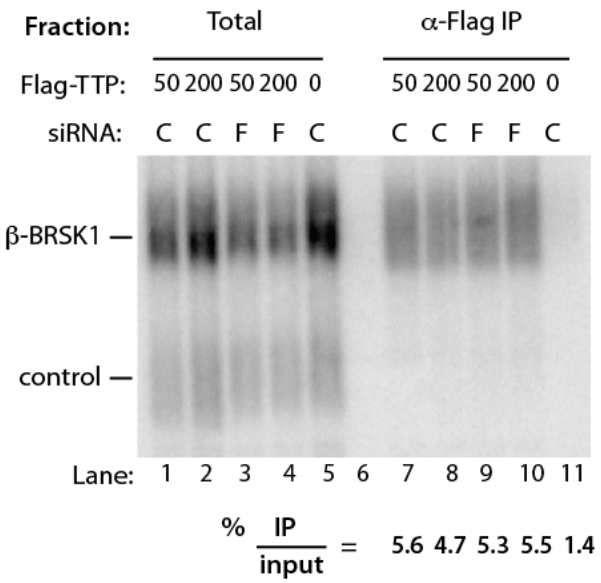


Figure 2.13: Reporter mRNA association with TTP is not altered by hnRNP F knockdown. Northern blot showing β-BRSK1-3'UTR reporter mRNA and control reporter mRNA that co-precipitate (lanes 7-11) with stably expressed Flag-tagged TTP from HEK 293T Tet-on cell lines. mRNA reporters were constitutively transcribed, and Flag-TTP was induced at two different concentrations of tetracycline (50 ng/ml or 200 ng/ml) for 24 hours prior to RIP. Cells were also either transfected with hnRNP F (F) siRNA or luciferase (C) siRNA. The percentage of associated β-BRSK1-3'UTR over the input levels was calculated below.

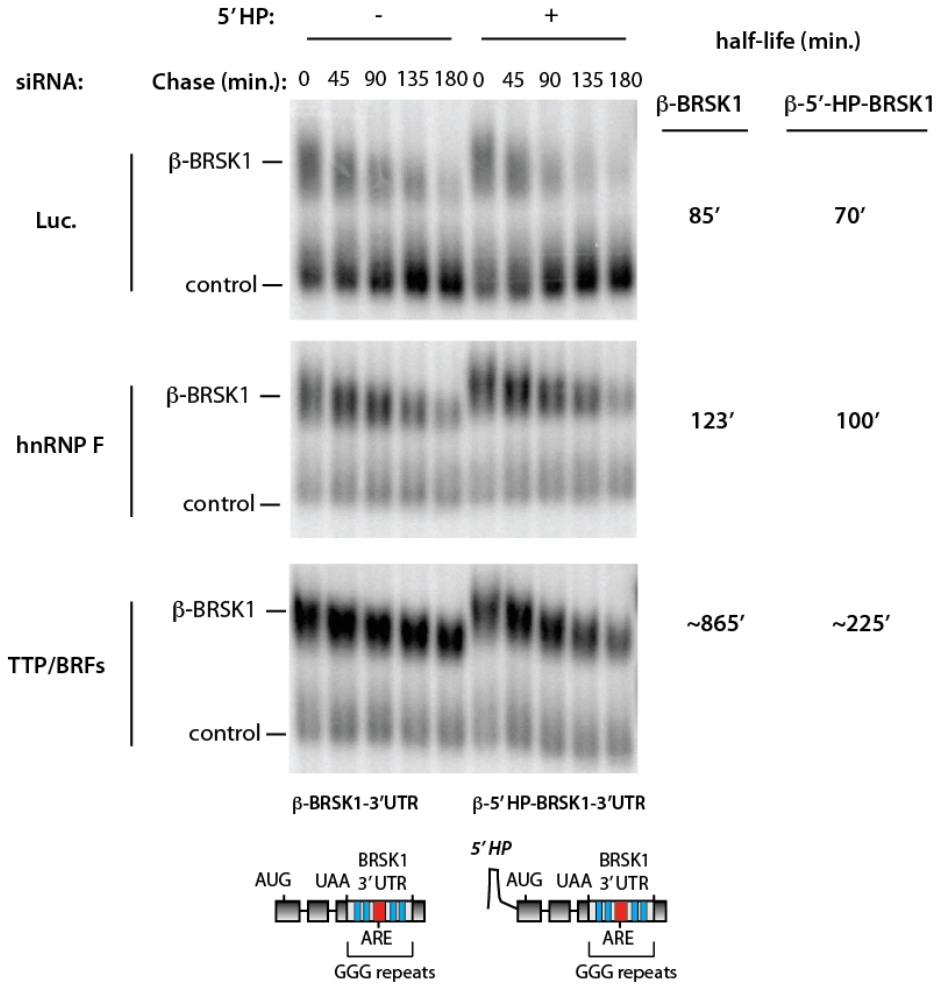


Figure 2.14: mRNA decay of a 5' hairpin-containing mRNA reporter is stimulated by hnRNP F. Northern blot showing mRNA decay of the β-BRSK1-3'UTR mRNA reporter and a similar reporter containing a 5' hairpin, β-5'HP-BRSK1-3'UTR (see cartoon below), in HeLa Tet-off cells after siRNA transfection of hnRNP F, TTP/BRF1/2, or luciferase control as indicated. Transcription of the reporter mRNA was pulsed on for six hours, and samples were collected at the times indicated after transcriptional shut-off (chase). Levels of reporter mRNA was normalized to control RNA (β-globin) to calculate the half-life ($t_{1/2}$).

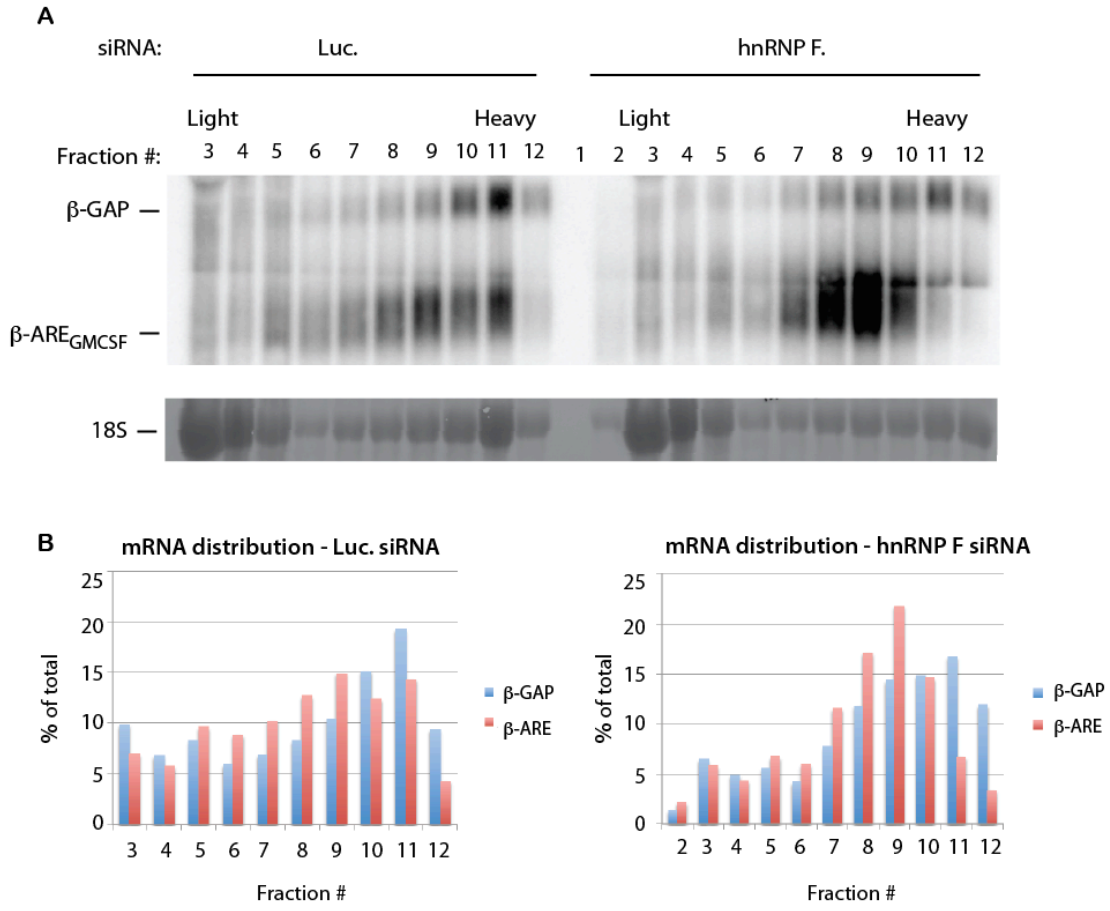


Figure 2.15: Polysome association profile or ARE-mRNA reporter following hnRNP F knockdown. (A) Northern blot of sucrose gradient polysome fractions from HEK 293T cells transfected with hnRNP F siRNA or luciferase control siRNA and constituively transcribed β-ARE$_{GMCSF}$ mRNA reporter and control reporter. β-globin hybridization probe was used to visualize reporter mRNAs and methylene blue staining was used for 18S rRNA. **(B)** Quantification of the β-ARE$_{GMCSF}$ mRNA and control mRNA reporters was calculated as a percentage of the total mRNA from all fractions for hnRNP F siRNA (right) and control (Luc.) siRNA treated cells (left).

2.4 Materials and Methods

Plasmids

The BRSK1 3' UTR was PCR amplified from human genomic DNA using the oligos – F - gga gaa GCGGCCGC TCC TGG CCA CCA ACG GGA CC and R - gga gaa TCTAGA CGG AAT CAG AGA CAC GGA CGC AGG. The amplicon was restriction digest inserted into the pcTET2-βwtβ reporter plasmid previously described(Franks, & Lykke-Andersen 2007) using NotI and XbaI restriction enzymes. The TNFα 3' UTR was PCR amplified using the oligos, F - gga gaa GCGGCCGC CGA ACA TCC AAC CTT CCC AAA CGC, R - gga gaa CTGCAG GCT CCT CTC CAG CTC TCT CCG, and restriction enzyme cloned into pcTET2-βwtβ plasmid with NotI and PstI. The BRSK1 3' UTR was restriction digest cloned into the βHP-wt reporter plasmid as previously described(Franks, & Lykke-Andersen 2007), and the β-ARE_{GMCSF} and β-$ARE_{TNFα}$ reporter plasmids were described in the same study. The pcDNA3-Myc-TTP and pcDNA3-Flag-TTP plasmids were described previously (Lykke-Andersen, & Wagner 2005). hnRNP F was PCR amplified and restriction digest inserted into pcDNA3-Myc and pcDNA3-Flag using BamHI and NotI restriction sites. siRNA resistant pcDNA3-Myc-hnRNP F-siResist was created following the QuickChange Site-Directed mutagenesis protocol (Stratagene).

Co-immunoprecipitation experiments

RAW264.7 cells seeded in 15 cm plates were cultured in DMEM/10% fetal bovine serum/1% penicillin and streptomycin. 100 ng/ml LPS (Sigma) was added to cells for 0, 2, or 6 hr and cells were washed in PBS, pelleted, and lysed in 1.4 ml lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.1% Triton-X 100, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin) and incubated on ice for 5 min. NaCl was added to 150 mM and 15 µl RNase A 10 mg/ml was added and incubated on ice for 10 min. Cytoplasmic extract was collected after a 15 min spin at 14,000 rpm in 4°C and incubated with 80 µg of Protein A sepharose in Net-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Triton X-100) conjugated to 4 uL anti-TTP antibody (or pre-immune anti sera). An equivalent amount of Protein G sepharose was used with 4 ul anti-hnRNP F/H antibody or anti-Myc antibody. After 3 hrs rotating incubation at 4°C the precipitates were washed 8X in Net-2 buffer and eluted in 50 ul of load buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) to which 200 mM DTT was added. Precipitated protein was analyzed by SDS-PAGE and Western blot.

RNA-immunoprecipitation experiments (RAW264.7 cells)

RAW264.7 cells seeded in 10 cm plates were cultured in DMEM/10% fetal bovine serum/1% penicillin and streptomycin (full DMEM). For siRNA transfections, 4 x 10^6 cells were seeded in full DMEM. The following day, culture media was changed DMEM/2% fetal bovine serum/1% penicillin and streptomycin and cells were transfected with 100nM siRNA with TransIT TKO

(Mirus) reagent as per manufacturers protocol. Two days later the media was changed back to full DMEM and an hour later LPS was added at 100 ng/ml. Cells were collected for RIP two hrs later just as for protein immunoprecipitation except the lysis buffer was supplemented with 14 µl yeast total RNA solution 10 mg/ml, 1.4 µl RNaseOUT, and 150 mM NaCl. RNase A was not added to extracts, and precipitated RNA was harvested in Trizol (Invitrogen) for RNA extraction.

RNA-immunoprecipitation experiments (NIH3T3 cells)

NIH 3T3 cells were seeded at ~20% confluency in 10 cm plates in full DMEM. For siRNA transfections, the next day 80 nM siRNA was transfected with TransIT TKO (Mirus) reagent as per manufacturers protocol. The following day cells were washed 3X with PBS and split and re-plated at ~25% confluency in DMEM/0.2% fetal bovine serum/1% penicillin and streptomycin. 24-48 hours later cells were re-fed with DMEM/20% fetal bovine serum/1% penicillin and streptomycin for 2 hrs and collected for RIP as described above (for RAW264.7 cells).

RNA-immunoprecipitation experiments (HEK 293T cells)

HEK 293T cells were seeded at ~20% confluency on 10 cm plates in full DMEM and transfected the following day with TransIT 293 transfection reagent (Mirus) and 5 µg pcDNA3-βwtβ-BRSK1-3'UTR, 1 µg pcDNA3-βwtβ mRNA reporter plasmid and either 4 µg pcDNA3-Flag-hnRNP F, or 500 ng

pcDNA3-Flag-TTP and 3.5 µg pcDNA3-Flag plasmid, or a combination of 4 µg pcDNA3-Flag-hnRNP F and 500 ng pcDNA3-Myc-TTP. Two days later the cell extracts were collected for RIP as for the RAW264.7 protocol.

Northern blot and pulse chase mRNA decay assays

mRNA decay assays and Northern blot were performed as previously described(Lykke-Andersen et al 2000). HeLa Tet-off cells (Clontech) were seeded to 12-well plates and cultured in DMEM/10% fetal bovine serum/1% penicillin and streptomycin (full DMEM). siRNAs were transfected at a final concentration of 20 nM with siLentFect Lipid Reagent (Bio-Rad) following the manufacturers protocol. Then either: 1) 24 hours later plasmid DNA was transfected in the presence of 50 ng/ml tetracycline with TransIt HeLa-Monster (Mirus) transfection, or 2) 48 hours later 20 nM siRNA was transfected with plasmid DNA using Lipofectamine 2000 reagent (Inivitrogen) in the presence of 50 ng/ml tetracycline. Two days after plasmid transfection, transcription of mRNA reporter was pulsed on for six hours with a PBS wash and replacement of full DMEM without tetracycline. Six hours later transcription was shut off with addition of 1 µg/ml tetracycline. Cells were harvested in Trizol (Invitrogen) for RNA extraction at subsequent time points as indicated, with time “0” taken 20 minutes after tetracycline addition. Some cells were taken up in 2X-SDS load buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) for protein analysis by SDS-PAGE and Western blot.

Endogenous mRNA decay assay

NIH 3T3 cells were seeded at ~20% confluency in 10 cm plates in full DMEM. 80 nM siRNA was transfected with TransIT TKO (Mirus) reagent the next day. 24 hours later the cells were washed 3X with PBS and split and re-plated at ~25% confluency in DMEM/0.2% fetal bovine serum/1% penicillin and streptomycin. 24-48 hours later cells were re-fed with DMEM/20% fetal bovine serum/1% penicillin and streptomycin for 3 hrs and then 10 ug/ml Actinomycin D was added and cells were collected at the indicated timepoints in Trizol (Invitrogen) for RNA extraction.

siRNAs

siRNAs were purchased from Dharmacon. Luciferase control siRNA: 5'-CGU ACG CGG AAU ACU UCG AUU-3' + 5'-UCG AAG UAU UCC GCG UAC GUU-3'. mTTP siRNA: 5'-GGA GGA CUU UGG AAC AUA AUU -3' + 5'- UUA UGU UCC AAA GUC CUC CUU-3'. mBRF1/2 (A) - 5'- UGC CGC ACC UUC CAC ACC ACA UU-3' + 5'- UGU GGU GUG GAA GGU GCG GCA UU -3. mBRF1/2 (B) - 5'- CUA CAA GAC GGA GCU GUG CCG UU-3' + 5'- CGG CAC AGC UCC GUC UUG UAG UU-3. Target sequences for siRNAs were as follows: human TTP/BRF1/2 – CGC TGC CAC TTC ATC CAC AAC TT, hnRNP F (1) – GGA AUG UAU GAC CAC AGA UUU, hnRNP F (3) – TGA GAA AGC TTT AGG GAA G, hnRNP F (2) – AGT CAG AAG ATG ATG TAA A, hnRNP H – TAA GCA GTA AGC GTA TTT A.

qRT-PCR

3 ug total RNA was treated with DNase I (Invitrogen) and reverse transcribed with either SuperScript II or III (Invitrogen) following the manufacturers protocol. cDNA was used for qPCR with SYBR Green PCR Master Mix (Applied Biosystems) and run on an Applied Biosystems machine. The following oligos were used: TTP-F - cggaggactttggaacataaac, TTP-R - ggagttgcagtaggcgaagtag, GAPDH-F - catggccttccgtgttccta, GAPDH-R - cctgcttcaccaccttcttgat, LIF-F- tgtgcaacaagtaccgtgtg, LIF-R- ttgcttgtatgtccccagaag, TNFA-F - accttgtctactcccaggttctc, TNFA-R - gaggttgactttctcctggtatg, IL10-F - tgctatgctgcctgctcttac, USP46-F - tgcttcaagcgctgtacttc, USP46-R - tgacgccaaccttcttcttc, KLHL2-F - accaaaggctatccgaagtg, KLHL2-R – aagccaccaacagcaaagac, IER3-F - gcgcgtttgaacacttctc, IER3-R- cagaagatgatggcgaacag, cFOS-F - gaatggtgaagaccgtgtcag, cFOS-R - gtctccgcttggagtgtatc, PLK3-F - ttgcgtcctacatggaacag, PLK3-R - actgaggatcagcttcgtgtg, MLLT11-F - tattgccagcatccactctg, MLLT11-R - cagcaccaccagcacaatag.

Polysome profile

HEK 293T cells were seeded in 10 cm plates at ~20% confluency. The next day cells were transfected with siRNA to a final concentration of 20 nM with siLentFect Lipid Reagent (Bio-Rad) as per manufacturers protocol. The following day cells were transfected with 9 µg pcDNA3-βwtβ-BRSK1-3'UTR

and 1 µg pcDNA3- βwtβ plasmids using Lipofectamine (Invitrogen). The next day cells were collected for sucrose gradient centrifugation and polysome fractionation as previously described (Damgaard, & Lykke-Andersen 2011).

Antibodies

Rabbit polyclonal anti-PABP was purchased from Abcam, mouse monoclonal anti-hnRNP F/H (1G11) was purchased from Abcam, mouse monoclonal anti-Myc-tag (9B11) was purchased from Cell Signaling, Upf1 rabbit polyclonal antisera was described previously (Lykke-Andersen, & Wagner 2005; Singh et al 2008),mouse monoclonal anti-HuR (3A2) was previously described (Gallouzi et al 2000), rabbit polyclonal anti-TTP-N-terminal was purchased from Sigma.

Indirect immunofluorescence

RAW264.7 cells were cultured in DMEM/10% fetal bovine serum/1% penicillin and streptomycin in chamber slides. LPS (Sigma) was added at 100 ng/ml and cells were fixed in 4% paraformaldehyde for 15 minutes and permeabilized and blocked with PBS/1% goat serum/0.1% Triton X-100 for 30 minutes. Cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) solution, then washed with PBS, and incubated with anti-hnRNP F/H (Abcam) and anti-TTP (N-terminal; Sigma) antibodies at 1:1000 dilutions for 1 hour. Following removal of primary antibody, cells were incubated with 4 µg/ml

secondary anti-rabbit antibody labeled with Alexa 488 fluorophore and anti-mouse antibodies labeled with Texas Red fluorophore (Molecular Probes).

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Chapter 2, in part, is currently being prepared for submission for publication of the material as Reznik B, Clement SL, and Lykke-Andersen J. hnRNP F is a novel TTP-interacting protein that stimulates ARE mRNA decay. The dissertation author is the primary investigator and author of this paper.

Chapter 3. TTP expression promotes 3' end processing of ARE mRNA

3.1 Introduction

TTP is a potent activator of AU-Rich element mediated (ARE) mRNA decay that is upregulated in response to stimuli or changing environmental conditions (Blackshear 2002; Varnum et al 1991; Varnum et al 1989; Carballo et al 1998). TTP is conserved in eukaryotes and the human genome contains two paralogs, BRF1 and BRF2, which have similar RNA binding and ARE mRNA decay activity as TTP (Ciais et al 2004; Stoecklin et al 2002; Ming et al 2001; Lykke-Andersen, & Wagner 2005; Hodson et al 2010). Homologs in *D. melanogaster* and *S. cerevisiae* also bind to and degrade ARE-containing mRNAs, and are induced in response to stimuli or changing environmental conditions just as TTP; for example, CTH2, the TTP homolog in budding yeast, promotes the rapid decay of ARE-containing mRNAs in response to iron deprivation (Puig et al 2005).

A function for TTP outside of mRNA decay had not been well established until recently when TTP was shown to negatively regulate transcription, by associating with and preventing the nuclear import of p65, a subunit of the NF-KB transcription complex (Liang et al 2009; Schichl et al 2009). This function appears to be separate from TTP mRNA decay activity, since an RNA binding mutant of TTP was able to inhibit p65 import and transcription, but does not bind to or degrade ARE mRNA. Additionally, TTP immunoprecipitates with histone deacetylase proteins (Liang et al 2009),

although whether this interaction is direct, or dependent on nucleic acids was not demonstrated.

Another non-mRNA decay function was observed for the homolog of TTP in yeast, CTH2. Recent work suggests that CTH2 is implicated in the 3' end processing of ARE mRNA transcripts (Prouteau et al 2008), when a mutant of CTH2 was found to disrupt the normal 3' end processing of ARE mRNAs. This mutant, which was also defective in activating mRNA decay, could bind to substrate ARE mRNAs, and led to the production of ARE mRNAs with extended 3' UTRs generated by usage of cryptic cleavage and polyadenylation sites downstream of the usual site, suggesting that CTH2 functions in ARE mRNA 3' end processing. Additional evidence that the TTP-family of proteins can participate in 3' end processing came when BRF1 was demonstrated to regulate Dll4 mRNA levels by preventing normal 3' end processing, and not through mRNA decay activation (Desroches-Castan et al 2011). It is proposed that BRF1 downregulates Dll4 mRNA levels by blocking the normal cleavage and polyadenylation site of the mRNA, leading to production of misprocessed transcripts and decreased mRNA levels. In the proposed mechanism, BRF1 binds an ARE pentamer near the cleavage and polyadenylation site to interfere with normal processing.

In this chapter I present evidence to suggest that TTP promotes the 3' end processing of ARE mRNA, a novel activity for TTP. I characterize some of the mRNA encoded cis-elements and domains of TTP required for 3' end processing, show that TTP RNA binding is necessary but not sufficient for this

activity, and attempt to identify endogenous targets of TTP-mediated 3' end processing. This work was started before a function for CTH2 and BRF1 in ARE mRNA 3' end processing was reported, and the hypothesis that guided the initial experiments was to determine if TTP recruited endonucleases and promoted endocleavage of ARE substrate mRNAs. As it became more apparent that I was studying an RNA processing function of TTP, rather than an mRNA decay function, reports that the TTP homologs were implicated in ARE mRNA 3' end processing supported my observations that this is a new and biologically relevant function of TTP.

3.2 Results

TTP expression results in alternative 3' end processing of ARE-containing reporter mRNAs

Two of the best characterized target mRNAs of TTP encode the important cytokines, granulocyte macrophage colony-stimulating factor (GMCSF) and tumor necrosis factor alpha (TNFα) (Carballo et al 1998; Carballo et al 2000; Lai et al 1999). The ARE from these mRNAs is sufficient to confer TTP-mediated instability to heterologous reporter mRNAs (Lykke-Andersen, & Wagner 2005; Franks, & Lykke-Andersen 2007). In previous studies with these reporters, an unanticipated and smaller than expected RNA species was observed on Northern blots following exogenous TTP expression (unpublished data). This RNA remained uncharacterized, and to learn more about the activity and function of TTP, I investigated the nature of this RNA.

I began by repeating the experiments in which the RNA species was first observed. I expressed exogenous N-terminal Myc-tagged TTP with a β-globin mRNA reporter containing the ARE from the GMCSF gene, β-ARE_{GMCSF}, and this led to the appearance of a faster migrating RNA species, referred to as β-ARE_{GMCSF}*, (Figure 3.1A, lanes 2-5). The β-ARE_{GMCSF}* RNA appeared when 20 ng and 100 ng of TTP plasmid was transfected, but not with 4 ng or no Myc-TTP. When Myc-TTP was expressed with an mRNA reporter lacking an ARE, β-wt, no additional RNA species was observed (Figure 3.1B, lanes 9-12).

The β-ARE_{GMCSF}* RNA could be either an mRNA decay intermediate or an alternatively processed mRNA. To distinguish between these possibilities, I incubated the RNA with RNase H and oligo-dT to determine if the small RNA contained a poly(A) tail. The increased mobility of β-ARE_{GMCSF}* after RNase H/oligo-dT incubation (Figure 3.1B, lane 7), indicated that β-ARE_{GMCSF}* is polyadenylated, thus eliminating the possibility that the RNA is a decay intermediate 5' fragment. To determine if β-ARE_{GMCSF}* RNA was alternatively processed I used different hybridization probes for detection in Northern blots. As seen in Figure 3.1B, a probe complementary to the coding region of β-globin mRNA detected β-ARE_{GMCSF}* and the full-length reporter (lanes 1, 2), while a probe complementary to the reporter 3' end only detected the full-length β-ARE_{GMCSF} RNA, and not β-ARE_{GMCSF}* (lanes 3, 4). Thus, β-ARE_{GMCSF}* RNA lacks the 3' region of the full-length mRNA and is

polyadenylated, suggesting that this mRNA was alternatively processed. I cloned and sequenced the β-ARE$_{GMCSF}$* cDNA and it contained the entire coding sequence of the reporter but was alternatively polyadenylated in the 3' UTR ~240 nucleotides (nt) upstream of the normal polyadenylation site (Figure 3.2). Thus, exogenously expressed TTP can promote alternative 3' end processing of an ARE-containing mRNA reporter.

TTP-mediated 3' end processing was not restricted to the β-ARE$_{GMCSF}$ reporter, and another ARE-containing β-globin reporter mRNA, β-ARE$_{TNF}$α, with the ARE from the TNF-α gene, also generated an alternatively processed mRNA when TTP was co-expressed (Figure 3.1B, lanes 5-8).

The alternatively polyadenylated β-ARE$_{GMCSF}$* reporter mRNA is stable

The β-ARE$_{GMCSF}$* mRNA is polyadenylated at an upstream site in the 3' UTR to exclude the ARE (Figure 3.2). Therefore, the β-ARE$_{GMCSF}$* RNA is predicted to no longer be subject to rapid ARE-mediated mRNA decay. The decay rate of the β-ARE$_{GMCSF}$* mRNA measured at a half-life of greater than five hours, in contrast to a half-life of less than one hour for the full length β-ARE$_{GMCSF}$ reporter mRNA in the presence of exogenous TTP (Figure 3.3, lanes 1-5). The mRNA decay rate of β-ARE$_{GMCSF}$* RNA closely resembled that of the stable β-wt reporter mRNA, which also had a half-life of greater than 5 hours (Figure 3.3, lanes 6-10). Therefore, TTP-mediated 3' end processing

can have profound effects on the post-transcriptional regulation of this mRNA reporter.

A polyadenylation signal is required for TTP-mediated alternative 3' end processing of ARE-containing mRNA reporters

The 3' end processing of pre-mRNAs is determined by cis-elements encoded within the pre-mRNA that recruit cleavage and polyadenylation factors. Most mammalian polyadenylation sites contain two sequence motifs: 1) a poly(A) signal (PAS) hexamer, most commonly AAUAAA, located 15-25 nt upstream of the cleavage and polyadenylation (CPA) site; and 2) a G/U-rich or U-rich sequence downstream of the CPA site (Di Giammartino et al 2011). The sequence of β-ARE_{GMCSF} mRNA revealed that there was a non-canonical PAS, AUUAAA, located ~ 20 nt upstream of the alternative CPA site (Figure 3.2). By contrast, no obvious downstream G/U-rich region was present, instead ~ 30 nt downstream of the CPA site lies the GMCSF ARE sequence, which contains repeats and differing iterations of the core pentamer, AUUUA.

To map the cis-elements required for TTP-mediated 3' end processing, I first asked if the predicted upstream PAS was required. The PAS was mutated from AUUAAA to GUUAGG in the β-ARE_{GMCSF} reporter (Figure 3.4A), and when Myc-TTP was expressed with the corresponding β-ARE_{GMCSF}-GUUAGG reporter, the alternatively polyadenylated RNA failed to appear (Figure 3.4B, lanes 1-4). The lack of β-ARE_{GMCSF}* RNA was not due to low TTP expression or activity, since the steady state level of β-ARE_{GMCSF}-

GUUAGG mRNA was lowered at high TTP levels, and the mRNA that remained was deadenylated, consistent with the model of deadenylase recruitment by TTP to substrate mRNAs (Sandler et al 2011; Lykke-Andersen, & Wagner 2005; Carballo et al 2000). When the PAS was mutated to the more common AAUAAA sequence, a very low level of β-ARE_{GMCSF}* RNA was present without exogenous TTP expression, and the levels of the alternatively polyadenylated RNA increased with Myc-TTP expression (Figure 3.4B, lanes 5-8), just as for the β-ARE_{GMCSF} mRNA reporter (Figure 3.1). The low level of alternatively polyadenylated β-ARE_{GMCSF}* mRNA without exogenous TTP expression may be due to endogenous TTP, or TTP paralogs, in these cells.

The same PAS mutations introduced to a reporter lacking the ARE, β-wt, failed to induce APA (Figure 3.4B, lanes 10-16), demonstrating the importance of the ARE in TTP mediated 3' end processing. Thus, the ARE and an upstream poly(A) signal are required for TTP-mediated alternative 3' end processing of the ARE-containing mRNA reporters.

RNA binding is necessary but not sufficient for TTP-mediated alternative polyadenylation

TTP binds ARE mRNAs through a central RNA binding domain (RBD) composed of two tandem CCCH zinc fingers. RNA binding is necessary but not sufficient for TTP to activate ARE mRNA decay, since TTP deletion mutants containing only the RBD do not activate ARE mRNA decay and instead increase the stability of ARE mRNA (Lykke-Andersen, & Wagner

2005). I tested if the RBD of TTP alone (Figure 3.5D) was sufficient for 3' end processing of the ARE reporter mRNA. When TTP-RBD was expressed with β-ARE_{GMCSF} reporter mRNA, the β-ARE_{GMCSF}* RNA was not observed (Figure 3.5A, lanes 1-6), even though TTP-RBD protein was expressed at comparable levels to Myc-TTP (Figure 3.5B, lanes 1-6) and TTP-RBD stabilized the β-ARE_{GMCSF} mRNA reporter in mRNA decay assays (Figure 3.5C). Thus, RNA binding of TTP is not sufficient for 3' end processing of the tested mRNA, indicating that the other domains of TTP are required for this activity.

I next tested if RNA binding of TTP is required for 3' end processing of the reporter mRNA. A single point mutation in the TTP-RBD, F126N, disrupts TTP ARE binding and activation of ARE mRNA decay (Lai et al 2002). When expressed with the ARE mRNA reporter, there was no indication that TTP-F126N stimulated alternative 3' end processing (Figure 3.5A, lanes 7-9), even though this protein was expressed at higher levels that wildtype TTP (Figure 3.5B, lanes 7-9). Therefore I conclude that RNA binding is necessary, but not sufficient for TTP-mediated 3' end processing of ARE-containing reporter mRNA.

mRNA decay activation domains of TTP promote 3' end processing of ARE reporter mRNA

The central RNA binding domain (amino acids 100-174) of TTP, is flanked by N- and C-terminal domains (Figure 3.6A), both of which are sufficient for activating mRNA decay when tethered to a target mRNA (Lykke-

Andersen, & Wagner 2005). I wanted to test which domains (besides the RNA binding domain) of TTP were required for the 3' end processing activity in the hope of identifying a TTP mutant where the mRNA decay activity was separable from the 3' end processing activity. Identification of such a mutant would strongly suggest that TTP is a multi-functional protein, and mapping of the domains required for one process but not the other could provide mechanistic insight into TTP activity.

I initially tested an array of TTP deletion mutants including TTPΔN50, TTP 1-214, TTP 1- 257, and TTPΔC13 (Figure 3.6A). These mutants were expressed in increasing amounts, and each of them led to appearance of the alternatively processed ARE mRNA reporter (Figure 3.6B, lanes 1-9, 13-16). These mutant proteins also lowered the steady state levels of the full-length β-ARE_{GMCSF} mRNA reporter, and accelerated its decay in mRNA decay assays (data not shown, and Lykke-Andersen & Wagner, 2005). A TTP mutant containing only the C-terminal domain did not lead to production of the β-ARE_{GMCSF}* mRNA nor decreased the steady state levels of the full-length reporter mRNA, as was expected since it lacks the RBD (Figure 3.6B, lanes 10-12).

Although there are three repeats of tetra-proline amino acids scattered throughout TTP, their significance in TTP function is unknown. I made TTP mutants with the proline residues mutated to valines, and tested one mutant for activity. The TTP-219P4V, with mutations in one of the three tetra-proline repeats, produced the 3' end processed mRNA reporter and accelerated the

decay the full length ARE mRNA reporter (Figure 3.6E). Similar mutants of the two other tetra-proline repeats were not tested.

Lastly, TTP mutants that lacked either the C-terminal or N-terminal domain of TTP were tested. Both of these mutants were previously shown to activate mRNA decay of ARE mRNA reporter (Lykke-Andersen, & Wagner 2005). These TTP mutants also promoted 3' end processing of two ARE-containing reporters tested, β-ARE_{GMCSF} and β-$ARE_{TNFα}$ (Figure 3.6C; expression levels shown in Figure 3.6D). Thus, for all the TTP mutants that were tested, the mRNA decay activity was not separable from the 3' end processing activity of TTP.

No alternative 3' end processing of the ARE mRNA reporter observed by TTP family members BRF1 and BRF2, and another AUBP, HuR

BRF1 and BRF2 are paralogs of TTP with similar binding to ARE-containing mRNA and decay activating properties (Hodson et al 2010; Lykke-Andersen, & Wagner 2005; Stoecklin et al 2002). Interestingly, BRF1 was recently shown to regulate the levels of a target transcript, Dll4 mRNA, by modulating its 3' end processing, but not its mRNA decay. BRF1 was shown to bind an ARE pentamer near the cleavage and polyadenylation site of the Dll4 mRNA, preventing 3' end processing to downregulate transcript levels (Desroches-Castan et al 2011). I tested if the TTP paralogs could promote 3' end processing of the β-ARE_{GMCSF} mRNA reporter mRNA, and did not observe any evidence that Myc-BRF1 was able to do so (Figure 3.7A, lanes 1-11).

Although there appeared to have been a β-ARE$_{GMCSF}$* mRNA signal with exogenous BRF1 expression, it did not appear to be higher than the background signal as quantified by intensity line plots (Figure 3.7B). When compared to the 3' end processing measured with Myc-TTP expression, which formed a small shoulder adjacent to the full length reporter in the intensity plots (Figure 3.7B), such a shoulder appeared to be absent for BRF1 expression. The expression of Myc-BRF1 did decrease steady state levels of the ARE reporter mRNA (Figure 3.7A) and accelerated its decay rate (Figure 3.7C, lanes 1-8), suggesting that exogenous BRF1 does bind the reporter mRNA. Exogenous TTP had a similar activating effect on mRNA decay (Figure 3.7C, lanes 13-16). The Myc-BRF1 and Myc-TTP proteins expressed at similar levels at 250 ng of transfected plasmid (Figure 3.7D). When BRF1 3' end processing activity was tested in repeat experiments, sometimes a faint band appeared, but it was never as strong as with TTP expression and difficult to distinguish from background (data not shown). Therefore, there was no strong evidence that BRF1 expression promotes 3' end processing of this ARE mRNA reporter. BRF2 expression did not promote 3' end processing either (Figure 3.7E, lanes 5-8), even though the steady state ARE mRNA reporter level was reduced with increased BRF2. It should be noted that the levels of the control mRNA reporter, β-GAP, which lacks an ARE and should not be targeted for ARE mRNA decay, decrease and appear to migrate slower following increasing TTP, BRF1 and BRF2 expression (Figures 3.7A, 3.7E, 3.1A, and 3.4B). Since the TTP-family of proteins can bind an array of cellular

decay factors, including deadenylases, it is likely that high expression titrates deadenylases and leads to highly polyadenylated, slower migrating mRNA, which collapses down with RNase H and oligo-dT treatment (Figure 3.1B, lanes 12, 14).

In addition to TTP/BRF proteins, I also assayed for 3' end processing activity of HuR, an ARE-binding protein that stabilizes ARE-containing mRNAs. Myc-HuR expression did not lead to accumulation of the β-ARE_{GMCSF}* mRNA (Figure 3.7A, lanes 12-16), even though HuR appears to bind to the mRNA as it stabilized the decay rate of the reporter mRNA (Figure 3.7C, compare lanes 1-4 to 9-12, although the time course was too short for accurate half-life analysis). Thus, when TTP-family members and HuR were tested, only TTP promoted strong alternative 3' end processing on the mRNA reporter.

Expression of murine TTP-AA mutant (which shares 82% amino acid identity to human TTP), that contained two serine to alanine mutations at phosphorylation sites known to constitutively activate TTP mRNA decay activity (Stoecklin et al 2004), promoted 3' end processing of the reporter mRNA and reduced steady state levels of full-length ARE mRNA reporter (Figure 3.7E, lanes 1-4).

Tethered TTP, TTP activation domain mutants, BRF1, and BRF2 did not promote 3' end processing of reporter mRNA

The simplest hypothesis to explain the effect of TTP on alternative 3' end processing of the ARE-containing mRNA is that TTP binding to the ARE, by an unknown mechanism, promotes the cleavage and polyadenylation usage of the weaker, internal PAS. This model accounts for the determined cis-elements (an ARE and a PAS), and the domain requirements (TTP RNA binding is necessary but not sufficient), for TTP-promoted 3' end processing. However, the observed 3' end processing of reporter mRNA could be an indirect effect of TTP expression. One way to reduce the indirect effects of TTP is to target it to a specific transcript. A TTP fusion protein with the N-terminal addition of the MS2 coat protein, which specifically binds the MS2-coat binding site, was tested with a reporter mRNA containing MS2 binding sites in the 3' UTR (β-6BS). The distance between the start of the MS2 binding sites to the upstream PAS was ~50 nt, the same distance as in the β-ARE reporter (Figure 3.2), but the sequence of the MS2 binding site was several hundred nucleotides, so it was longer than the GMCSF ARE.

When TTP was tethered to the β-6BS reporter, there was no evidence of 3' end processed mRNA (Figure 3.8, lanes 4-6), even though tethered TTP reduced the steady state mRNA levels when compared to expression of MS2 protein (Figure 3.8, lanes 1-3). The doublet RNA band that appeared at 450 ng of tethered TTP was presumed to be full-length and deadenylated β-6BS mRNA (Figure 3.8, lane 6), since the lower band migrated slower than an expected alternatively processed mRNA. An alternatively processed β-6BS* mRNA should run at the same size as β-ARE*, and have greater separation

from the β-6BS mRNA than the deadenylated full-length mRNA in this Northern blot. However, the samples need to be treated with RNase H and oligo-dT to conclusively discriminate between deadenylated and alternatively polyadenylated mRNA and eliminate any doubt.

Tethered BRF1 and BRF2 also did not promote alternative 3' end processing of β-6BS mRNA (Figure 3.8, lanes 7-11), but did decrease the steady state mRNA level. However, the same caveat applies to this interpretation as for tethered TTP. Similarly, tethering the TTP RNA binding mutant, F126N, and the N-terminal or C-terminal TTP activation domains, Myc-TTP-1-100 and Myc-TTP-176-326, decreased β-6BS mRNA steady state levels, but did not promote the appearance of a smaller 3' end processed mRNA (Figure 3.8, lanes 13-21).

Previous studies showed that tethered TTP, TTP N-terminal, and TTP C-terminal domains activated the rapid decay of the β-6BS mRNA reporter (Lykke-Andersen, & Wagner 2005). In this assay, the tethered proteins reduced the steady state mRNA levels, but did not appear to promote alternative 3' end processing of the β-6BS mRNA reporter. However, these preliminary results should be repeated with the appropriate RNase H and oligo-dT treated control samples.

Endogenous TTP substrate mRNAs showed no evidence of alternative 3' end processing

All experiments so far had been done using exogenously expressed TTP. It is important to determine if endogenous TTP can mediate 3' end processing of cellular mRNAs, since this would demonstrate that TTP can regulate gene expression through a novel, non-mRNA decay, mechanism. To address this question, I took a candidate gene approach. The initial candidates I tested for TTP-mediated 3' end processing are the well-characterized TTP substrate mRNAs: GMCSF, TNFα, and TTP mRNA itself (though not a substrate for TTP-activated decay it is bound by TTP protein). Although these mRNAs did not have annotated or predicted alternative polyadenylation sites, a search of the EST databases showed that some clones of GMCSF had shortened 3' UTRs (data not shown). I used the mouse macrophage cell line, RAW264.7 (referred to as RAW cells), since TTP protein is induced after lipopolysaccharide (LPS) treatment (Figure 3.9C, lanes 1-3) (Mahtani et al 2001). Following LPS treatment, TTP substrate mRNA was monitored for unusual size, multiple bands, or any other sign of alternative 3' end processing.

TNFα mRNA was induced as one major RNA species that migrated close to the expected size of ~1619 nt plus poly(A) (Figure 3.9A, lanes 2-7). TNFα mRNA peaked between two to four hours after LPS induction, and collapsed into a single mRNA species after RNase H and oligo-dT treatment (Figure 3.9A, lanes 8-13), suggesting no alternative polyadenylation. Minor bands appearing near the 3,000 bp marker were not reproducible and likely background of the hybridization probe used. TTP mRNA, migrated close to the

expected size of ~ 1765 nt, and collapsed down to a single band following RNase H and oligo-dT treatment, and did not appear to reproducibly show additional RNA species (Figure 3.9B).

The GMCSF mRNA hybridization signal was weaker than the other tested TTP targets, but the RNA species that was induced appeared to migrate faster than the expected size of ~1033 nt, which was more obvious in the RNase H and oligo-dT treatment, where it appeared to migrate closer to the 850 bp marker than 1000 (Figure 3.9C). To monitor whether the induced GMCSF mRNA was alternatively polyadenylated, or contained the full-length 3' UTR, I performed an RNase H assay with oligos complementary to sequences in the 3' UTR. Three different targeting oligos were used (Figure 3.9D, cartoon) and alternatively polyadenylated, smaller than expected GMCSF mRNA would not be targeted for RNase H cleavage by the most distal oligo C. Incubation of the induced mRNA with the individual oligos and RNase H did generate cleavage products for each oligo (Figure 3.9D), indicating that the induced GMCSF mRNA contained the full length 3' UTR and was not alternatively 3' end processed.

TNFα and GMCSF mRNA induction was monitored when TTP protein level was reduced by RNAi. While RAW cells are hard to transfect, less than 33% of TTP protein was induced in the TTP siRNA-treated samples than in the control (Figure 3.10C). Subsequent knockdowns using this protocol were inconsistent and highly irreproducible, despite efforts to systematically repeat and optimize these knockdown conditions. However, for the corresponding

RNA samples from this knockdown, there was no obvious difference in TNFα and GMCSF mRNA induction following TTP knockdown (Figure 3.10A-B), with the mRNAs migrating at the appropriate size and no additional mRNA species.

Although TNFα and GMCSF are TTP substrate mRNAs they were not the best candidates for TTP-mediated alternative 3' end processing. Ideal candidates would contain the required cis-elements found in the β-ARE_{GMCSF} mRNA reporter, i.e., an ARE sequence located downstream of a polyadenylation signal. Based on those criteria, I identified several candidates from a list of TTP-associated mRNAs in LPS-stimulated RAW cells (Stoecklin et al 2008). To monitor 3' UTR length, I attempted two different PCR-based protocols (3' RACE and adapter-ligated RT-PCR), but was unsuccessful in optimizing conditions to reliably measure 3' UTR length. I also attempted to visualize some of the candidate mRNAs by Northern blotting, but the candidates were not detectable, even though similar methodologies detected TTP, TNFα, and GMCSF mRNA.

Given the unsuccessful attempts to assay the endogenous candidate 3' UTRs, I decided to test the candidate 3' UTRs after insertion into the β-globin reporter mRNA. The entire 3' UTR of the candidate mRNA, including the downstream genomic sequence containing the GU-rich cleavage and polyadenylation elements was inserted downstream of the stop codon in the β-globin reporter mRNA. Four candidate 3' UTRs were cloned, each with 1-4 predicted alternative polyadenylation sites that were upstream of, or near to, an ARE or an ARE pentamer (Figure 3.11A). The reporters were expressed

with increasing amounts of Myc-TTP and steady state mRNA levels were assessed by Northern blotting. The β-ARE_{GMCSF} mRNA reporter was assayed as a positive control, and, as expected, the β-ARE_{GMCSF}* alternatively processed mRNA appeared with exogenous TTP expression (Figure 3.11B, lanes 1-4). The first candidate reporter, containing the 3' UTR of the mouse Rab2b gene, produced an mRNA that migrated close to the predicted size of 2400 nt (size was estimated based on 18S rRNA and β-ARE reporter mRNA migration), but did not produce additional RNA species (predicted to be 900 nt) with TTP protein expression (Figure 3.11B, lanes 5-8). Levels of the full-length reporter decreased with increasing TTP expression, as expected for a TTP target mRNA. Other faint bands were visible but were ignored since they did not show dependence on exogenous TTP for expression, and because it was difficult to distinguish them from background.

The three remaining candidate reporter mRNAs had 3' UTRs from the mouse genes Pfkfb2, Nfyb, and N4bp2l1, and did not show clear evidence of TTP-mediated 3' end processing either (Figure 3.11B, lanes 9-20). The reporters produced RNA species that migrated close to the expected full-length size, and had lower steady state levels with increased TTP expression. The mN4bp2l1 reporter did not produce additional bands (Figure 3.11B, lanes 17-20). The mPfkfb2 reporter expressed an additional, faster migrating band that ran below 18S rRNA, but was larger than the expected alternatively polyadenylated mRNA and did not show dependence on exogenous TTP for expression, so it was not further investigated (Figure 3.11B, lanes 9-12).

The mNfyb reporter expressed two additional smaller RNAs that were close in size and migrated just above the ~800 nt β-ARE reporter mRNA (Figure 3.11B, lanes 13-16). One of these mRNAs migrated at the size of the expected alternatively polyadenylated mRNA at ~ 1050 nt, but since this RNA was present without exogenous TTP expression, and difficult to interpret due to the overlapping RNA, it was not further analyzed.

In addition to testing these candidate 3' UTR mRNA reporters, I tested if the β-ARE_{GMCSF} mRNA reporter was subject to alternative 3' end processing by endogenous TTP in RAW cells. Since RAW cells were poorly transfected with plasmid DNA, the mRNA reporter was not clearly detectable with Northern analysis so whether endogenous TTP can mediate 3' end processing of this reporter remains unanswered.

3.3 Discussion

Characterization of the cis-elements and TTP domains required for TTP promoted 3' end processing of ARE mRNA reporter

The function of TTP in ARE mRNA decay was first established in studies of the TTP knockout mouse, which displayed autoimmune pathologies that were attributed to elevated levels of the cytokine TNFα (Taylor et al 1996). Levels of another cytokine, GMCSF, were also shown to be elevated, and it was demonstrated that TTP was responsible for the regulation of these mRNAs at the step of mRNA decay. Since then, other TTP-substrate mRNAs

have been identified (Emmons et al 2008; Lai et al 2006; Stoecklin et al 2008), and more is known about the mechanism of how TTP activates mRNA decay and of the regulation of TTP. However, a handful of studies suggest that TTP, and TTP homologs and family members, may function in processes besides mRNA decay, including, transcription regulation and pre-mRNA processing (Prouteau et al 2008). Thus, TTP-family proteins could be multifunctional regulators of gene expression at the steps of mRNA decay, transcription, and RNA processing. The study in this chapter contributes to this idea and for the first time implicates TTP in an mRNA processing activity, the regulation of cleavage and polyadenylation of ARE-containing mRNAs.

This study began with the observation of an unexpected RNA species on a Northern blot that was present with exogenous TTP expression (Figure 3.1). This smaller RNA species is an alternatively polyadenylated version of the ARE-mRNA reporter, cleaved and polyadenylated at an internal site in the 3' UTR. This site of alternative polyadenylation is in between two cis-elements, a weak upstream poly(A) signal (PAS), and a downstream ARE (Figure 3.2). The ARE is required for this TTP-mediated 3' end processing since no alternatively polyadenylated RNA species was observed in reporters lacking the ARE (Figures 3.1, 3.3, and 3.4). The PAS is also required since mutation of that sequence prevented TTP-mediated 3' end processing of this mRNA (Figure 3.4). These required cis-determinants were not analyzed further. It would be interesting to determine if: (1) the orientation of these two elements is important for the TTP-mediated 3' end processing, i.e., is there alternative

processing when the ARE is upstream of the PAS?; (2) the distance between these two elements was important for 3' end processing. In the ARE mRNA reporter, the distance and orientation resembles that of a normal cleavage and polyadenylation site, except that the ARE (located downstream of the PAS and cleavage site) resides where the GU- or U-rich downstream sequence usually is (Figure 3.2). If the orientation and proximity of the ARE and PAS were required, it would be interesting to test whether there is crosstalk, or interaction, between TTP and the cleavage and polyadenylation machinery (see below).

Attempting to gain mechanistic insight into the activity of TTP in 3' end processing of the ARE-mRNA reporter, I tested an array of TTP mutants, homologs, and other AUBPs for this activity. The key results from these experiments were: (1) TTP RNA binding is necessary but not sufficient to promote 3' end processing of the reporter mRNA (Figure 3.5); (2) mRNA decay and 3' end processing activities were not separable functions for the TTP mutants tested (Figures 3.5 and 3.6); and, (3) other AUBPs tested (BRF1, BRF2, and HuR) did not promote 3' end processing of the reporter, even though they affected ARE mRNA reporter stability (Figure 3.7). It is interesting that binding to the ARE by TTP-RBD, and presumably HuR, BRF1, and BRF2 (since mRNA stability was altered) was not sufficient to promote 3' end processing of the reporter. The RNA binding domains of TTP, BRF1, and BRF2 are very similar. TTP shares 68% and 69% amino acid identity with BRF1 and BRF2, respectively (Lykke-Andersen, & Wagner 2005). However

similar they are, there may be subtle differences in binding affinity to substrate mRNAs and it is possible that these proteins bind differently to the ARE in the reporter mRNA. And although they similarly activate mRNA decay of the reporter (Figure 3.7), 3' end processing activity could be more sensitive to such differences. This idea could be tested by measuring the 3' end processing activity of a fusion protein containing the RBD of BRF1, or BRF2, and the N- and C-terminal domains of TTP. Alternatively, the N- and C-terminal domains of BRF1 and BRF1 could be swapped to the TTP RBD and tested to see if the could activated 3' end processing of the ARE mRNA reporter. These domains share less conservation with TTP: the N-terminal domain of TTP shares 18% and 25% amino acid identity with BRF1 and BRF2, respectively; while the C-terminal domain shares 29% and 39% amino acid identity with the corresponding BRF1 and BRF2 domain (Lykke-Andersen, & Wagner 2005).

The inability of BRF1 to promote 3' end processing is surprising since TTP and BRF1 are considered to have similar activity in binding to and activating ARE mRNA decay, and since BRF1 was shown to influence the 3' end processing of an endogenous ARE transcript, Dll4 (Desroches-Castan et al 2011). A difference between these two examples is that for the Dll4 mRNA, BRF1 binding prevents the cleavage and polyadenylation, whereas for the case of the ARE mRNA reporter, TTP expression promotes the 3' end processing of the transcript. For the Dll4 transcript regulation it would be interesting to determine whether the RBD of BRF1 alone is sufficient to

regulate this 3’ end processing event, as this would contrast the observation with TTP and the β-ARE mRNA reporter.

TTP mutants tested for 3’ end processing activity were all able to promote the 3’ end processing of the ARE mRNA reporter (Figure 3.6), including RNA-binding mutants, mutants with truncations of pieces of the N-terminal and C-terminal domains, as well as entire N-terminal or C-terminal deletion mutants. Murine TTP was also able to promote the 3’ end processing activity (Figure 3.7). A mutant that separates mRNA decay activity from 3’ end processing was not identified, although if it turns out that the BRF1/BRF2 terminal domains do not promote 3’ end processing activity, it may be worthwhile to more closely compare the sequences of these domains to TTP to identify potential interesting domains that may promote this activity.

The possibility remains that the 3’ end processing activity of TTP on the ARE mRNA reporter is due to indirect effects. An argument against this is that similar indirect effects might be expected with BRF1 and BRF2 expression, since they are believed to have similar mRNA decay activity, and were not observed. An approach that might circumvent indirect effects is to target TTP specifically to the reporter mRNA by the tethering assay. Tethered TTP, TTP deletion mutants, and TTP homologs, reduced mRNA levels but did not appear to promote 3’ end processing of the bound reporter mRNA (Figure 3.8). This assay should be repeated with the appropriate control of RNase H and oligo-dT treatment of RNA samples. Another assay to test for direct involvement of TTP in 3’ end processing of ARE mRNA is to determine if TTP

biochemically complexes with the cellular cleavage and polyadenylation factors, including the cleavage and polyadenylation specificity factors (CPSF), which bind the mRNA upstream of the cleavage site, the cleavage stimulation factors (CstF), which bind the downstream elements, and cleavage factors (CF) (Di Giammartino et al 2011). It will be important to determine whether the activity of TTP on 3' end processing is direct or indirect, but the most important and interesting question left to answer is if endogenous TTP regulates endogenous mRNAs by modulating 3' end processing in a similar manner as the reporter mRNA.

Does TTP regulate the 3' end processing of endogenous ARE mRNAs?

It remains to be determined if the 3' end processing activity of TTP on the ARE mRNA reporter reflects a true biological function for TTP. The report that CTH2, the yeast homolog of TTP, regulates 3' end processing of specific ARE mRNAs (Prouteau et al 2008), suggests that this activity is a real biological function of TTP that is conserved across species. The implication of BRF1 in the regulation of 3' end processing of an endogenous ARE target mRNA (Desroches-Castan et al 2011), suggests that this activity is conserved in TTP homologs. It will be important to determine if endogenous transcripts are regulated by 3' end processing activity of TTP.

I attempted to visualize the 3' end processing activity of endogenous TTP on TTP substrate mRNAs in induced RAW cells, but did not see evidence for alternatively processed mRNAs for the GMCSF, TNFα, or TTP mRNA

transcripts (Figure 9), or when TTP levels where knocked down (Figure 3.10). It cannot be ruled out that the TTP knockdown was not efficient enough to observe a change in RNA processing, but the induction of two of these transcripts, GMCSF and TNFα, was previously monitored in TTP knockout cells with no reports of mis-processed or incorrectly sized mRNAs. Better candidate mRNAs to test for TTP-mediated 3' end processing activity would more closely mirror the sequence and orientation of the PAS relative to the ARE in β-ARE mRNA reporter (Figure 3.2). I identified candidates that more closely resembled these sequence elements from a list of TTP-associated mRNAs (Stoecklin et al 2008), but was unable to optimize a PCR-based assay to examine 3' UTR length (data not shown). The 3' UTRs of several candidate genes were cloned into the β-globin reporter mRNA, but expression of TTP did not show any clear changes in 3' end processing (Figure 3.11).

At this point the best method to identify endogenous mRNAs that may be regulated by TTP at the level of 3' end processing, would be to perform a global transcript analysis comparing the 3' UTRs of TTP-depleted cells to control samples. TTP knockout MEF cell lines could be used, or reliable and efficient TTP knockdown conditions could be optimized. I was unable to consistently attain good knockdown with standard chemical transfection reagents in RAW cells, but a lentivirus-mediated knockdown protocol could be tried in these cells, or a different cell line could be used. More consistent TTP knockdowns, with a significant mRNA decay phenotype, were attained in 3T3

cells (Chapter 2), and it is possible to use these cells in the global transcript analysis.

Alternative polyadenylation is one way in which mRNAs are post-transcriptionally regulated. Since 3' UTRs contain regulatory elements may determine the regulation of the mRNA, including AREs, miRNAs, and other sequence elements (see Introduction) (Reznik, & Lykke-Andersen 2010), the composition of the 3' UTR, at the level of alternative polyadenylation, can play an important part in determination of the mRNA fate. Recent reports have highlighted the important contribution and regulation of gene expression by changes in alternative polyadenylation and 3' UTR length in proliferating cells, response to stimulus, and in disease pathology (Mayr, & Bartel 2009; Ji, & Tian 2009; Ji et al 2009; Sandberg et al 2008). For example, cancer cells exhibit shortened 3' UTRs, and loss of regulation of many oncogenes, compared to non-cancer control cells (Mayr, & Bartel 2009), in contrast to the global increase in 3' UTR length observed in the developing mouse embryo(Ji et al 2009). As seen in this study, the consequences of alternative polyadenylation of the β-ARE mRNA reporter led to a loss of regulation by the ARE, and enhanced stability in cells (Figure 3.3). This study provides preliminary evidence that TTP regulates gene-specific 3' end processing, and that TTP can regulate gene expression in a non-mRNA decay step. It will be important to determine if TTP regulates specific genes in such a way in a biologically relevant manner.

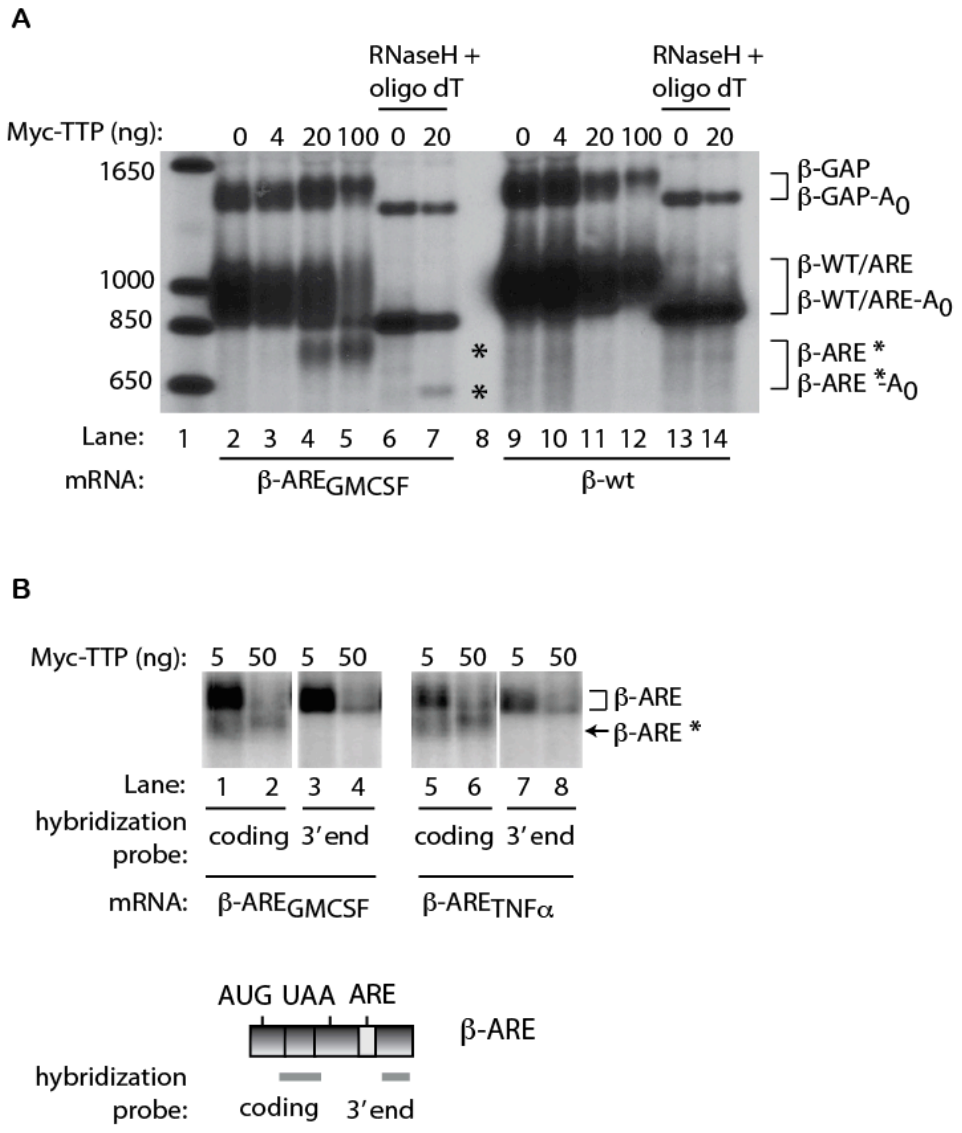


Figure 3.1: Exogenously expressed TTP promotes the alternative 3' end processing of ARE-containing reporter mRNAs. (A) HeLa cells were transfected with plasmids for indicated amounts of TTP and control (β-GAP) and reporter (β-WT/ARE) mRNA. Transcription of the reporter mRNA was pulsed for six hours and total RNA was prepared and visualized by Northern blot. RNA from empty vector (0 ng) and 20 ng Myc-TTP transfection samples was treated with RNase H and oligo-dT. The asterisks (*) denote the 3' end processed mRNA reporter, and A_0 denotes fully deadenylated mRNA. Radiolabeled DNA ladder was used to estimate size. **(B)** RNA prepared from HeLa cells transfected with Myc-TTP and β-ARE mRNA reporter plasmid was visualized by Northern blot using two different hybridization probes – directed at the coding region or a site in the 3' UTR downstream of the ARE in the reporter. Lane 8 was intentionally left empty.

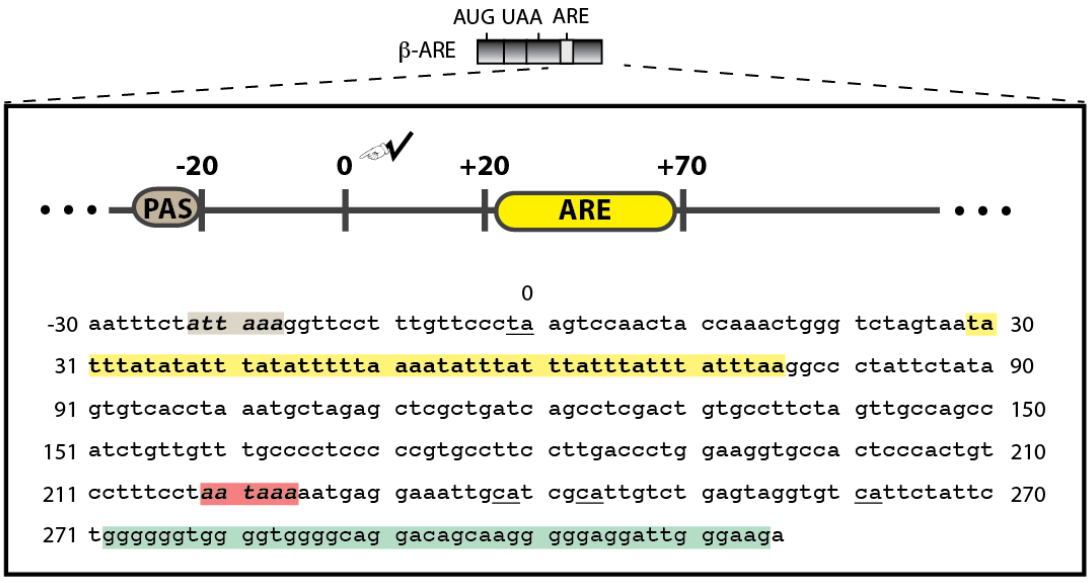


Figure 3.2: 3' UTR Sequence of the β-ARE mRNA reporter. A cartoon schematic highlighting the site of alternative cleavage and polyadenylation, and other cis-elements in the 3' UTR, determined after sequencing the β-ARE$_{GMCSF}$* cDNA. The sequence of the 3' UTR is in the boxed inset. Site 0 (underlined) denotes the site of alternative cleavage and polyadenylation, -20 nt marks the non-canonical polyadenylation signal (shaded in grey and italicized), and the GMCSF ARE sequence is highlighted in yellow. The red italicized box denotes the polyadenylation signal used by the full length reporter mRNA, the underlined di-nucleotides are the possible sites of cleavage and polyadenylation of the full length reporter, and the downstream G-rich sequence that stimulates cleavage and polyadenylation is highlighted in green.

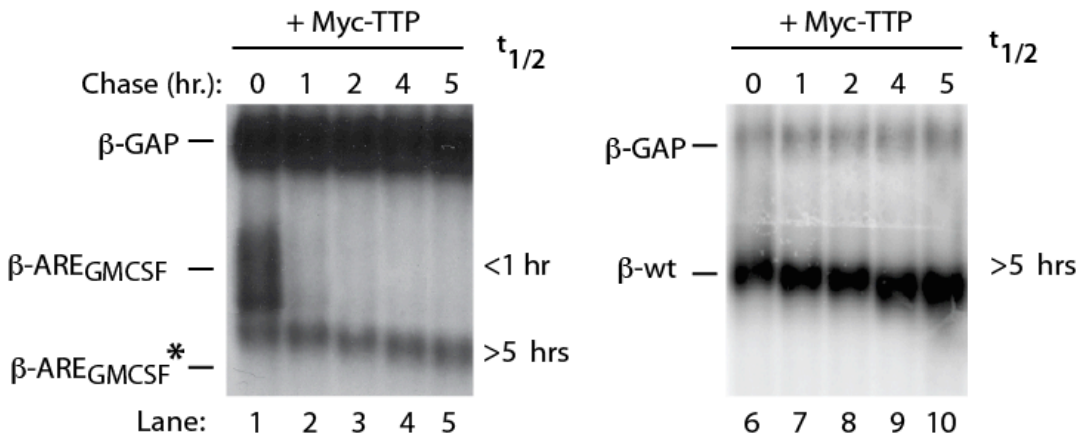


Figure 3.3: The alternatively processed mRNA reporter lacks an ARE and is stable. HeLa Tet-off cells were transfected with TTP, reporter and control mRNA plasmids. Transcription of the reporter mRNA was pulsed on for six hours by removal of tetracycline, and samples were collected at the times indicated after transcriptional shut-off by tetracycline addition (chase). Levels of reporter mRNA was normalized to control RNA (β-GAP) and half-life ($t_{1/2}$) was determined.

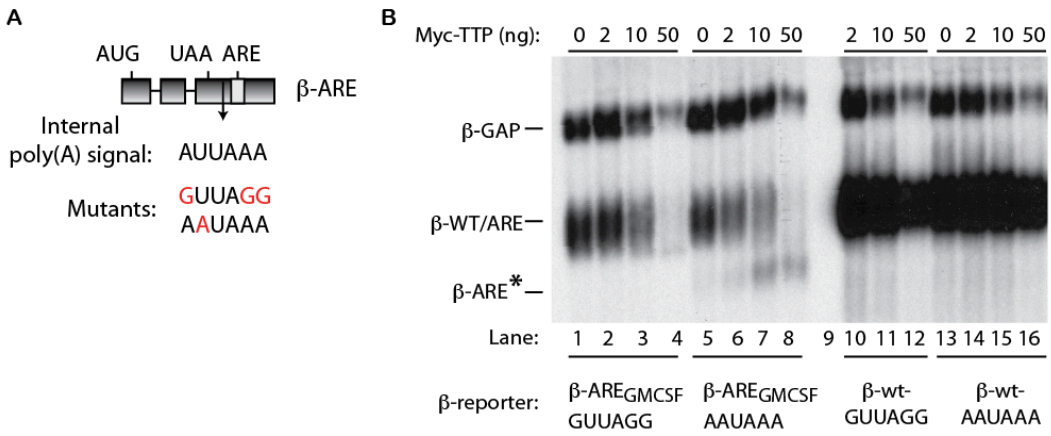


Figure 3.4: The internal polyadenylation signal is required for TTP-mediated alternative 3' end processing. (A) Cartoon depicting the β-ARE_{GMCSF} reporter with mutated sequence of the internal polyadenylation signal. Identical mutations were made in the β-wt reporter. (B) HeLa cells were transfected with plasmids for TTP and control and reporter mRNA. RNA was collected after 6 hours of transcriptional pulse of reporter mRNA and visualized by Northern analysis. The RNA sample in lane 9 was lost.

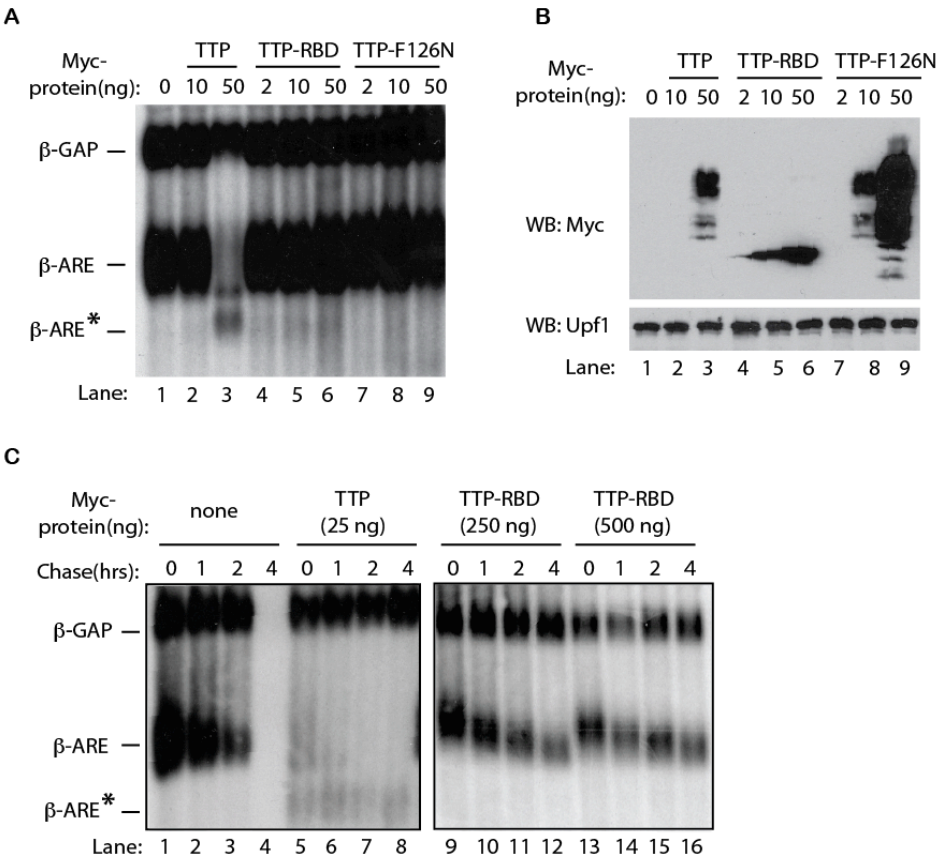


Figure 3.5: TTP binding is necessary but not sufficient for 3' end processing of the ARE reporter mRNA. (A) RNA visualized from HeLa cells transfected with reporter mRNA plasmid and TTP plasmids where transcription of the reporter was pulsed for six hours. **(B)** Top panel - Western blot probed with α-Myc antibody to determine the level of expressed TTP and TTP mutants. Bottom panel – The same samples probed with α-Upf1 antibody as a loading control. **(C)** RNA from a pulse-chase mRNA decay assay monitoring the decay of the β-ARE_{GMCSF} mRNA reporter from 0 to 4 hours after transcription shut-off. Expression of TTP-RBD domain prevented the rapid decay of β-ARE reporter mRNA.

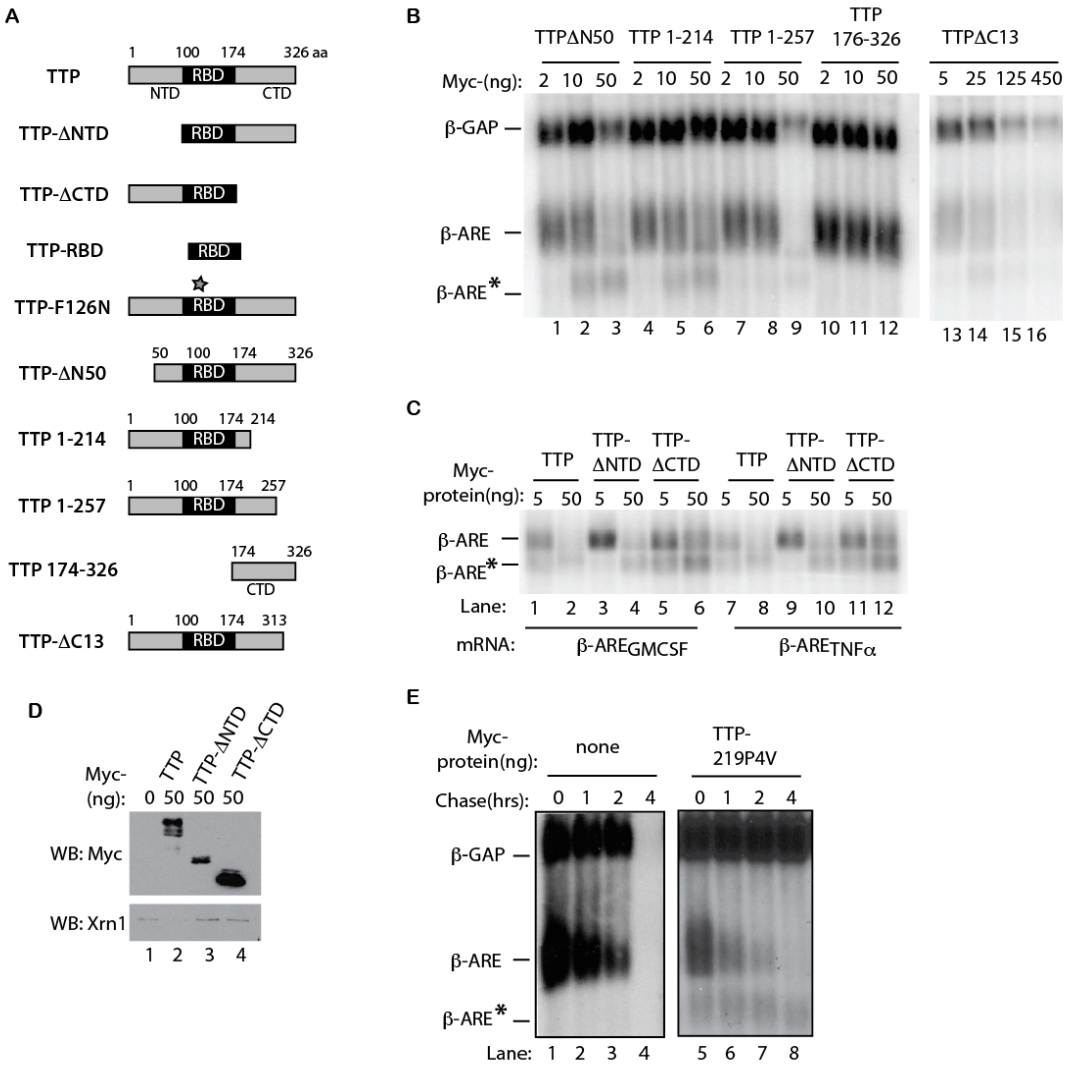
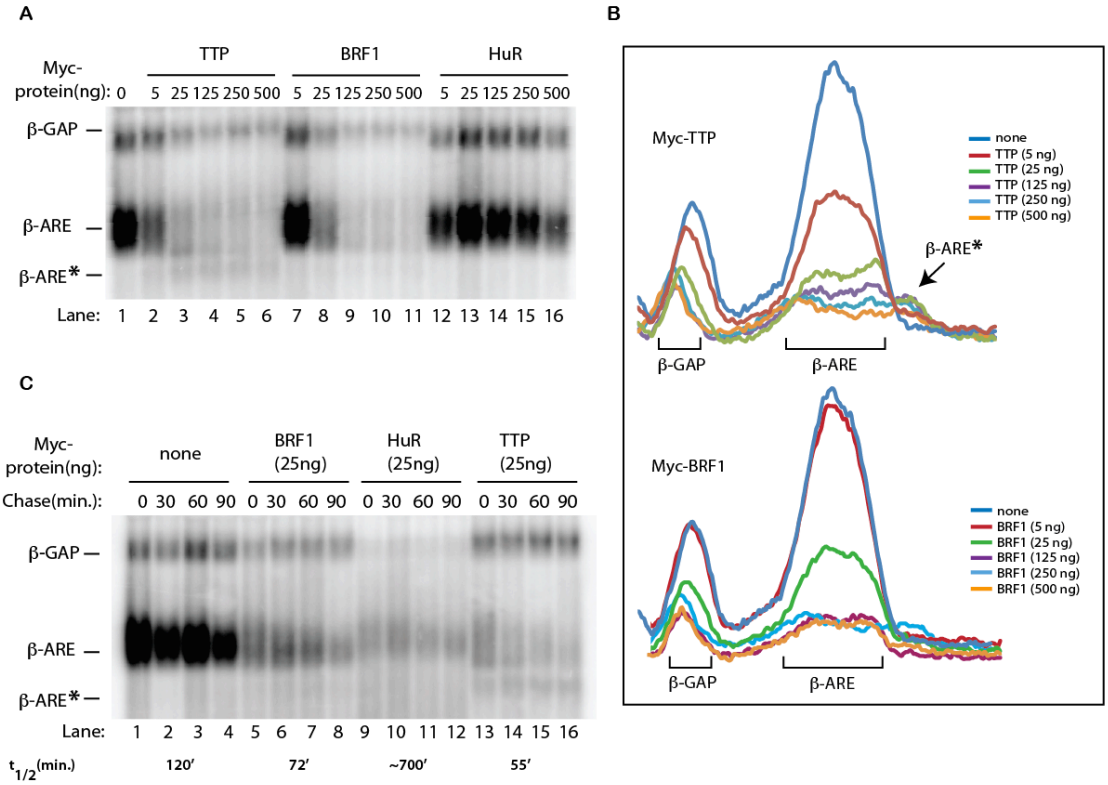


Figure 3.6: TTP mRNA decay activation domains promote 3' end processing of ARE reporter mRNAs. (A) Cartoon depiction representing the TTP deletion mutants used. **(B)** RNA visualized from HeLa cells transfected with TTP, control, and reporter mRNA plasmids collected after six hours of pulsed transcription. **(C)** RNA from HeLa cells transfected with two different β-ARE mRNA reporters and TTP plasmids. **(D)** Top panel – Western blot probed with α-Myc antibody to determine transfected protein expression. Bottom panel – Samples were probed with α-Xrn1 antibody as a loading control. **(E)** RNA visualized from HeLa cells transfected for a pulse-chase mRNA decay assay to test for mutant TTP activity. The RNA sample in lane 4 was lost.

Figure 3.7: BRF1, BRF2 and HuR expression do not lead to 3' end processing of ARE reporter mRNA. (A) RNA visualized from HeLa cells transfected with the indicated Myc-protein, control reporter, and β-ARE_{GMCSF} plasmid after six hour transcription pulse. **(B)** Intensity plot depiction of the signal of each RNA sample for the TTP and BRF1 transfected samples measured using Image-J software. **(C)** RNA from a pulse-chase mRNA decay assay in HeLa cells transfected with plasmids as in A. Amount of transfected Myc-protein is indicated. $t_{1/2}$ was measured by normalizing remaining reporter mRNA level to control reporter level. **(D)** Top panels – Expression of transfected protein was detected after probing Western blot with α-Myc antibody and α-HuR as a loading control. Bottom panels – Expression level of transfected HuR was determined after probing Western blot with α-HuR antibody and α-Upf1 antibody as a loading control. **(E)** Northern blot of RNA from HeLa cells transfected with indicated Myc-plasmid, control and reporter mRNA plasmids after six hour transcription pulse.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HuR
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β-ARE
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none
TTP (5 ng)
TTP (25 ng)
TTP (125 ng)
TTP (250 ng)
TTP (500 ng)
β-ARE*
β-GAP
β-ARE
Myc-BRF1
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BRF1 (125 ng)
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β-ARE*
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WB: Myc
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HuR
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WB: HuR
WB: Upf1
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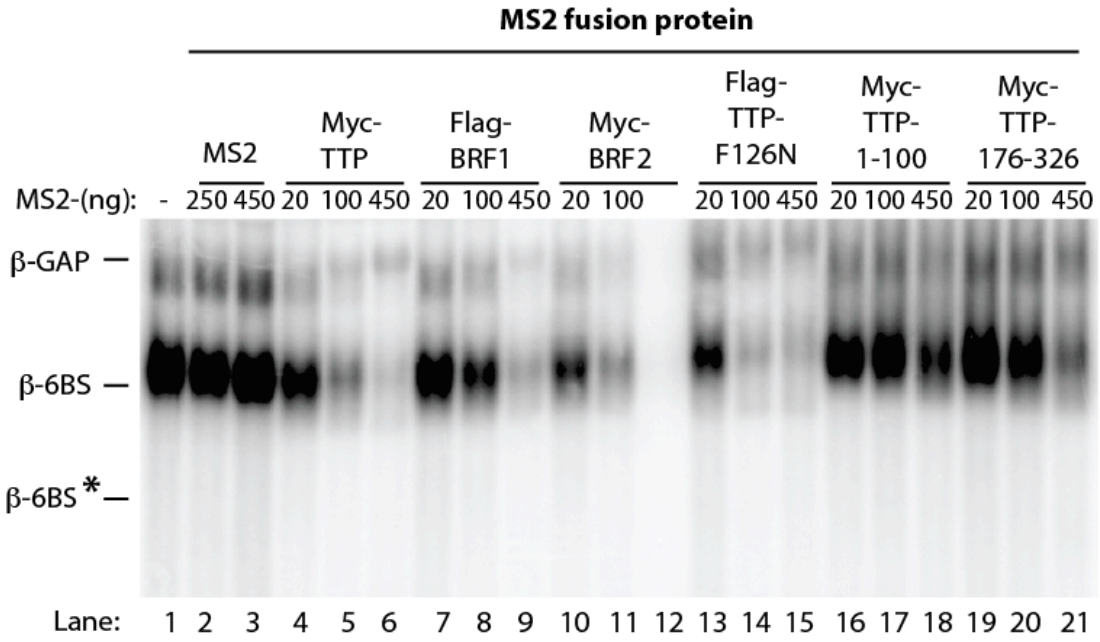


Figure 3.8: Tethering TTP and TTP-homologs did not lead to 3' end processing of reporter mRNA. HeLa cells were transfected with control reporter plasmid, β-6BS reporter plasmid, and indicated MS2-plasmid. After a six hour transcription pulse the cells were collected, the RNA was visualized by Northern blot. The estimated migration of the alternatively processed β-6BS* mRNA reporter is indicated. The RNA sample from lane 12 was lost.

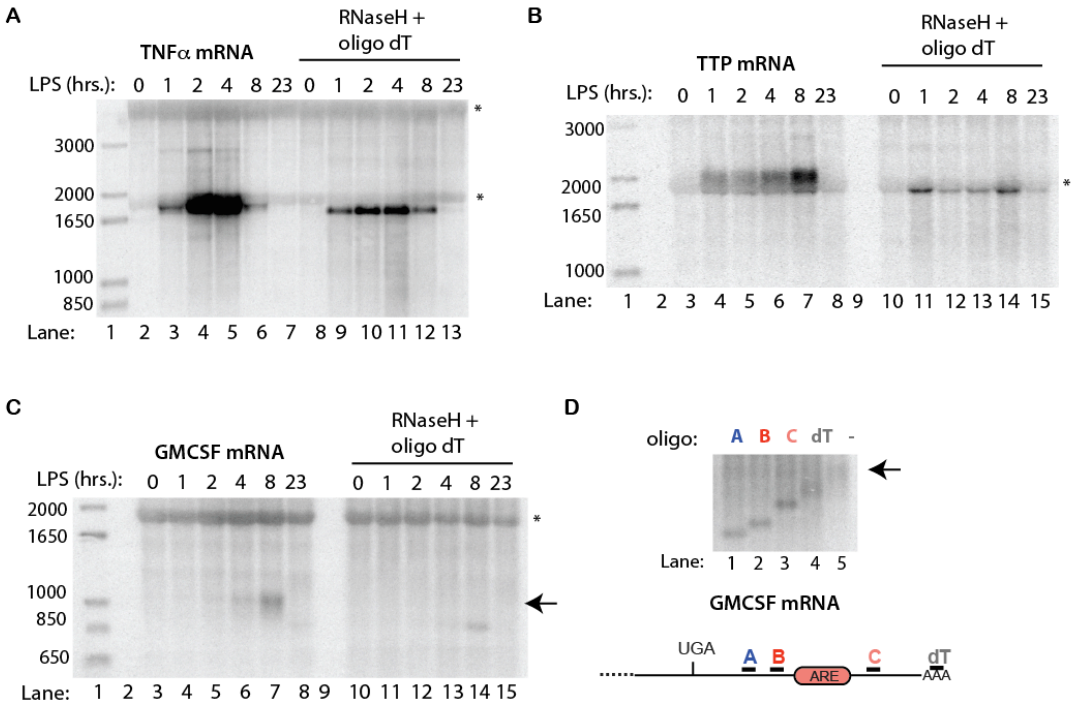


Figure 3.9: Induction of endogenous TTP-substrate mRNAs. (A) Northern blot of TNFα mRNA induction from RAW macrophage cells incubated with LPS for the indicated time, and corresponding RNA samples treated with RNase H and oligo-dT. The expected size of TNFα mRNA is 1619 nt, and DNA ladder (lane 1), 18S (~1,900 nt in size and annotated by *) and 28S rRNA (~5,000 nt and annotated by *) were used to estimate size or induced RNA. **(B)** Same experimental conditions as in (A) except the induction of TTP mRNA (expected size 1765 nt) was monitored. **(C)** Same as (A) and (B) but GMCSF (expected size 1033 nt) induction was visualized. The arrowhead indicates GMCSF mRNA, and the (*) indicates 18S rRNA. (D) Total RNA after 8 hrs LPS induction was treated with the indicated oligo and RNase H. Approximate positions of the oligos are depicted in the cartoon below. RNA in lane 5 was untreated.

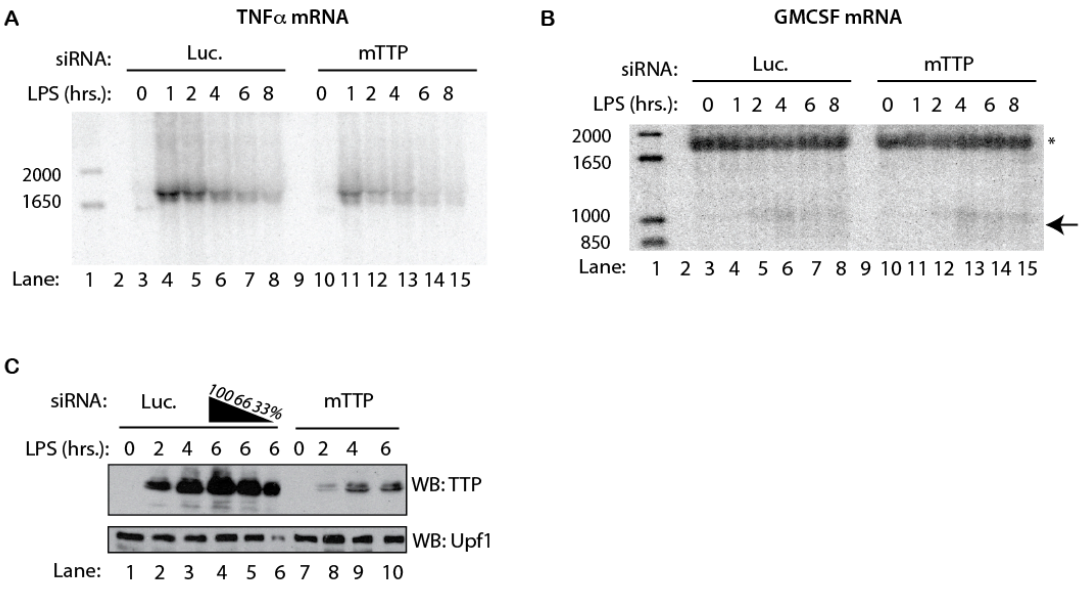
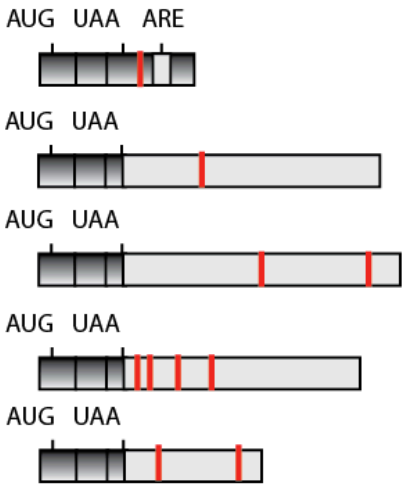


Figure 3.10: Induction of endogenous TTP-substrate mRNAs following TTP knockdown. (A) TNFα mRNA induction in RAW macrophage cells following TTP siRNA and luciferase (control) siRNA transfection. Samples were collected at the indicated times after LPS treatment and visualized by Northern blot. Lanes 2 and 9 were intentionally left empty. **(B)** Same induction as in (A) except GMCSF mRNA was monitored. The arrowhead indicates GMCSF mRNA and the asterisk indicated 18S rRNA. **(C)** Attenuation of TTP protein induction following LPS treatment in the TTP siRNA transfected cells was assessed by probing the Western blot with α-TTP antibody. Probing with α-Upf1 was used to monitor loading.

A

3′ UTR reporter	Reporter size	Expected size of APA reporter
β-ARE	800	640
mRab2b	2400	900
mPfkfb2	2500	1250, 2250
mNfyb	2300	650, 700 850, 1050
mN4bp2l1	1700	750, 1350



B

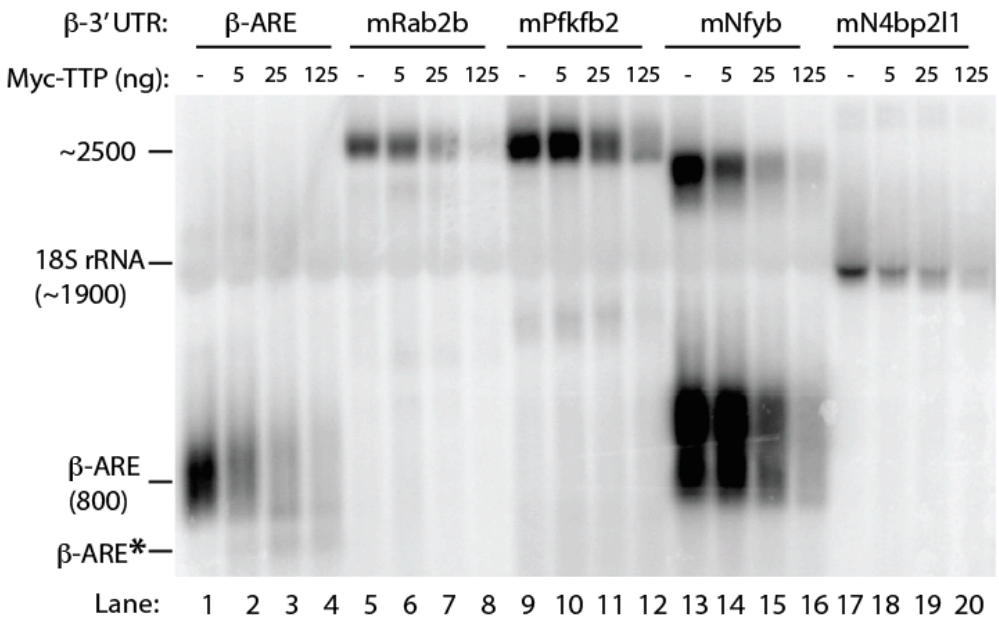


Figure 3.11: Testing TTP-mediated 3' end processing in candidate 3' UTR reporter mRNAs. (A) Cartoon depiction of the candidate 3' UTRs cloned just downstream of the stop codon in the β-globin reporter. Listed is the expected size of full-length reporter mRNA and expected size of potential TTP-mediated alternative polyadenylation (APA) variants. Red bars in the cartoon represent identified polyadenylation signals in the 3' UTR. **(B)** RNA from HeLa cells transfected with TTP and indicated reporter mRNA plasmid.

3.5 Materials and Methods

Plasmids

Plasmids for the β-ARE, β-wt mRNA, and β-6BS, reporters are derived from pcDNA3 (Invitrogen) and previously described (Lykke-Andersen, & Wagner 2005; Franks, & Lykke-Andersen 2007; Clement, & Lykke-Andersen 2008). β-ARE and β-wt mRNA internal polyA signal mutants were created following the QuickChange Site-Directed mutagenesis protocol (Stratagene). Plasmids for Myc-TTP, Myc-BRF1, Myc-HuR, Myc-TTP deletion mutants and derivatives, and MS2-proteins were described previously (Franks, & Lykke-Andersen 2007; Lykke-Andersen, & Wagner 2005). Plasmid for the pcDNA3-Myc-TTP-219P4V mutant was created following the QuickChange Site-Directed mutagenesis protocol (Stratagene). The coding region of BRF2 plasmid was PCR amplified and inserted into pcDNA3-Flag vector using BamHI and XbaI restriction sites, and sub-cloned into pcDNA-Myc-NMS2 plasmid. Plasmid for pcDNA3-Myc-mTTP-AA was described previously (Clement et al 2010).

Cloning of 3’ UTR mRNA reporters

β-3’UTR plasmids were cloned by PCR amplification of the 3’ UTR and downstream genomic sequence of the following genes using Pfu polymerase to amplify from mouse genomic DNA and the following primers: mRab2b, F-oligo with NotI restriction site, GGA GAA GCG GCC GC TGA GTG AGC GCT TTC TCT TTC CTC, R-oligo with NaRI restriction site, GGA GAA GGC GCC

TCT GGT ACA GAA TAT AAG ACC CAC C; mPfkfb2, F-oligo with NotI restriction site GGA GAA GCG GCC GC CAC TTT GCT CTC CTG ATG TGA GG, R-oligo with NaR1 restriction site, GGA GAA GGC GCC CCA TTG CTT TGA AGA TAC CAT GAC C; mNfyb, F-oligo with NotI restriction site GGA GAA GCG GCC GC AAA TTC AGT TTT CAT GAT CGG AAG G, R-oligo with XbaI restriction site GGA GAA TCTAGA GAC TAA AAT GTG AGA TGA CCT AGA GG; mN4bp2l1, F-oligo with NotI restriction site GGA GAA GCG GCC GC GCC ACC ACC AAG GAT ATT AAT GGC, R-oligo with AvrII restriction site gga gaa CCT AGG ATG AAT GGC AAA AGC ATT TGG CG. PCR amplified products were then restriction digest cloned into the pcTET2-β-wt-β reporter plasmid previously described (Franks, & Lykke-Andersen 2007).

Northern blot and mRNA decay assays

mRNA decay assays and Northern blot were performed as previously described (Lykke-Andersen et al 2000). HeLa Tet-off cells (Clontech) were cultured in DMEM/10% fetal bovine serum/1% penicillin and streptomycin (full DMEM) and transfected in the presence of 50 ng/ml tetracycline with TransIt HeLa-Monster (Mirus) transfection reagent according to the manufacturers protocol. 2 µg total plasmid DNA was transfected per well in a 6-well plate, with 100 ng β-GAP, 1.5 µg β-globin reporter, and 500 ng pcDNA3-Myc empty vector control plasmid or pcDNA3-Myc protein plasmid as indicated in Figures. Two days after plasmid transfection, transcription of mRNA reporter was pulsed on for six hours with a PBS wash and replacement of full DMEM

without tetracycline. Six hours later transcription was shut off with addition of 1 µg/ml tetracycline. Cells were harvested in Trizol (Invitrogen) for RNA extraction at subsequent time points as indicated, with time "0" taken 20 minutes after tetracycline addition. Some cells were taken up in 2X-SDS load buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) for protein analysis by SDS-PAGE and Western blot.

Hybridization probes used for Northern blot

All probes were transcribed with UTP-alpha-^{32}P and either T3 or SP6 RNA polymerase (company) using the manufacturers protocol. DNA templates for: β-globin coding probe was previously described (Clement et al 2010); β-globin 3' end probe was generated by T3 transcription from the PCR product generated from amplification of pcTET2-βwtβ reporter plasmid with oligo 1 (underlined sequence is T3 promoter element) - AAT TAA CCC TCA CTA AAG GGA GA GTG GGA GTG GCA CCT TCC, and oligo 2 - ATA GTG TCA CCT AAA TGC TAG AGC; mTTP probe was generated by T3 transcription of the PCR product from pcDNA3-mTTP, previously described (Clement et al 2010), using oligos, F - GAT CTC TCT GCC ATC TAC GAG AG, and R (underline denotes T3 promoter element) - AAT TAA CCC TCA CTA AAG GGA G CTC AGA GAC AGA GAT ACG ATT GAA G; mTNFα probe probe was transcribed with T3 from a PCR product from TNF-alpha gene with the oligos, F – ATG AGC ACA GAA AGC ATG ATC C, and R (underline denotes T3 promoter element) - AAT TAA CCC TCA CTA AAG GGA G CAG AGC AAT

GAC TCC AAA GTA; mGMCSF probe was transcribed with T3 from a PCR product from GMCSF plasmid (sandi) with oligos, F - TGT GGC TGC AGA ATT TAC TTT TC, and R (underline denotes T3 promoter element) - AAT TAA CCC TCA CTA AAG GGA G GCA TTC AAA GGG GAT ATC AGT C.

RAW264.7 cell culture, transfection, and LPS stimulation

RAW264.7 cells were grown in 10 cm plates and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) /1% penicillin and streptomycin (PS) (full DMEM). Cells were treated with lipopolysaccharide (LPS) at 100 ng/ml (Sigma) and collected in Trizol (Invitrogen). 10 ug of total RNA from each sample timepoint was resolved on a 1.2% agarose/formaldehyde gel and Northern analysis. Poly$(A)_0$ deadenylated mRNA was generated with RNase H (Invitrogen) and oligo-dT incubation, as previously described (Clement et al 2011). A similar protocol was used for RNase H incubation of the GMCSF mRNA, where 10 ug total RNA was used with the following oligos: oligo A – GGC TAT ACT GCC TTC CAA CTG, oligo B – TAT CTC TCG TTT GTC TTC CGC, and oligo C – GAC ATT CTC AAT AAA TAG AGT TGC. For siRNA transfections, 2 x 10^5 cells were seeded in a well of a 12-well plate in full DMEM. For siRNA transfections the next day, cells were switched to OMEM and transfected with siRNA at a 100 nM final concentration with 100 µl OMEM and 2 µl TransIt TKO Reagent (Mirus). Six hours later the medium was changed to DMEM supplemented with 2% FBS and 1% PS. 48 hours later the media was changed back to full DMEM and the

cells were treated with 100 ng/ml LPS, and collected in Trizol at subsequent timepoints for RNA extraction and Northern analysis. To determine protein levels after siRNA treatment, cells were harvested in 100 µl 2X-SDS load buffer for SDS-PAGE and Western blot analysis.

Antibodies

Mouse monoclonal anti-Myc-tag (9B11) was purchased from Cell Signaling, Upf1 and Xrn1 rabbit polyclonal antisera was described previously (Lykke-Andersen, & Wagner 2005; Singh et al 2008), mouse monoclonal anti-HuR (3A2) was previously described (Gallouzi et al 2000), and rabbit polyclonal anti-TTP-N-terminal was purchased from Sigma.

siRNAs

siRNAs were purchased from Dharmacon. Luciferase control siRNA: 5'-CGUACGCGGAAUACUUCGAUU-3' + 5'-UCGAAGUAUUCCGCGUACGUU-3'. mTTP siRNA: 5'-GGAGGACUUUGGAACAUAAUU -3' + 5'-UUAUGUUCCAAAGUCCUCCUU-3'.

Acknowledgements

Chapter 3, in part, is currently being prepared for submission for publication of the material. Reznik B and Lykke-Andersen J. The dissertation author is the primary investigator and author of this paper.

Chapter 4. Characterization of the putative deadenylase, PNLDC1

4.1 Introduction

mRNA modifications at the 5' end (addition of a 7-methyl-guanosine [m(7)G] cap) and the 3' end (non-templated addition of a ~200 nucleotide poly(A) tail) protect the mRNA from exonucleases, and promote the processing, nuclear export, and translation of transcripts. To maintain proper gene expression and cellular homeostasis, mRNAs generated by transcription are eventually degraded and cleared through degradation pathways. Cellular mRNA decay is often initiated by deadenylation of the poly(A) tail, and this rate-limiting step is followed by exonucleolytic decay from the mRNA ends; either in the 3' to 5' direction by the cytoplasmic exosome, or decapping and then 5' to 3' exonucleolytic decay by Xrn1 (Parker, & Song 2004; Garneau et al 2007). Alternatively, mRNA decay can be initiated by endonucleolytic cleavage (Garneau et al 2007).

Human cells contain three major classes of deadenylase enzymes – PARN [poly(A)-specific ribonuclease], the Pan2/3 complex, and Ccr4/Caf1/Not complex (Goldstrohm, & Wickens 2008; Wiederhold, & Passmore 2010). PARN is unique among the characterized deadenylases since it binds the m(7)G mRNA cap and localizes primarily to the nucleus (Yamashita et al 2005; Berndt et al 2012; Dehlin et al 2000). In vitro, PARN displays processive deadenylase activity on capped, poly(A) RNA, and is inhibited by PABP and cap-binding protein (Gao et al 2000; Balatsos et al 2006; Martînez et al 2001;

Goldstrohm, & Wickens 2008). The role of cellular PARN is ill defined and the current model of deadenylation-initiated decay does not account for PARN activity in general mRNA decay. In the current model, the Pan2/3 complex initially deadenylates mRNAs in a slow and distributive manner, followed by a second round of rapid, processive deadenylation attributed to the Ccr4/Caf1/Not deadenylase complex (Yamashita et al 2005). The exclusion of PARN from this model is likely due to two reasons: (1) the dependence on PARN for decay of mRNAs from assorted mRNA decay pathways has not been well established (Yamashita et al 2005); and (2) bulk mRNA decay is considered to be a cytoplasmic event and PARN localizes to the nucleus. However, there are reports of PARN involvement in regulated mRNA decay pathways and it interacts with mRNA decay factors such as KSRP (Gherzi et al 2004), an ARE binding protein that activates decay, Upf proteins that activate NMD (Lejeune et al 2003), and CUGBP (Moraes et al 2006), an RNA decay factor that binds to specific CUG or GU sequences of some mRNAs. Additionally, a fraction of PARN localizes to cytoplasmic exosome granules with other mRNA decay factors (Lin et al 2007). Other important roles for PARN activity include the decay of nuclear mRNAs in response to DNA damage (Cevher et al 2010), and in the maturation of snoRNAs (Berndt et al 2012). In the developing *Xenopus* embryo, PARN helps coordinate the deadenylation and decay of maternal mRNAs (Körner et al 1998), while also maintaining a subset of maternal mRNAs translationally silent through deadenylation activity but not decay. After fertilization, PARN is

phosphorylated and dissociates from these mRNAs allowing for their readenylation and translation (Kim, & Richter 2006).

The human genome contains a homolog of PARN called PNLDC1 (PARN-like domain containing 1) that is predicted to code for a functional deadenylase. It is uncharacterized with unknown activity, function and expression profile. Based on the amino acid sequence it is predicted that PNLDC1 contains deadenylase activity but may lack some distinct features of PARN, such as cap-binding and nuclear localization. The work below describes my initial attempts to test for deadenylase activity of PNLDC1, to characterize and contrast other biochemical properties of PNLDC1 to PARN, and to determine if PNNLDC1 functions in cellular mRNA decay.

4.2 Results

Sequence features of PNLDC1

PNLDC1 was identified as a homolog of PARN in the human genome (Genbank gene ID: 154197). This uncharacterized gene is predicted to code for a protein, 520 amino acids in length with 30% shared identity with PARN. Importantly, PNLDC1 retains the RNase D exonuclease domain found in PARN and other homologs of Caf1 deadenylases, and the catalytic amino acids (DEDD) required for exonuclease activity (Ren et al 2002) are also conserved (Figure 4.1). PNLDC1 appears to lack the cap-binding domain, the nuclear localization signals (NLS), and the R3H domain (important for protein stability and some RNA binding (Wu et al 2005)) found in PARN (Figure 4.1). Overall, the proteins are most similar in the amino (N-) terminal region, whereas the features in the carboxy terminal region of PARN are not conserved in PNLDC1 (Figure 4.1). Based on the primary amino acid sequence analysis, PNLDC1 was predicted to code for a deadenylase lacking the cap-binding and nuclear localization of PARN. Therefore the experiments described below were designed to test for these and other characteristics of PNLDC1.

Purified PNLDC1 lacks deadenylase activity in vitro

The processive, cap-dependent deadenylase activity of PARN was demonstrated through in vitro biochemical assays on substrate RNAs (Körner, & Wahle 1997; Martînez et al 2001; Dehlin et al 2000; Gao et al 2000). Similar

assays were used as a starting point to test for PNLDC1 deadenylase activity. N-terminal Flag-tagged PNLDC1 was expressed and purified from HEK293 cells and incubated with a capped and polyadenylated [poly$(A)_{60}$] substrate RNA, labeled with ^{32}P at the cap. Myc-PARN, expressed and purified under similar conditions, was used as a positive control for activity. Following Flag-PNLDC1 incubation with substrate RNA, no deadenylation of the poly$(A)_{60}$ RNA was observed, in contrast to the deadenylation observed with PARN incubation (Figure 4.2A, compare lanes 1-5 to 6-10). Even when a ~17-fold higher concentration of PNLDC1 than PARN was used, there was no deadenylation of the poly(A) RNA (Figure 4.2B, lanes 7-11). RNA incubated with PARN at 50 nM had two distinct bands (Figure 4.2A, lane 10), one running just below the poly$(A)_{60}$ RNA and a smaller band halfway between poly$(A)_{60}$ and poly$(A)_0$. This profile was consistent with the processive deadenylation and previous reports of PARN in vitro deadenylase activity in 20-30 nt increments (Körner, & Wahle 1997; Dehlin et al 2000). RNA incubated with lower amounts of PARN was also deadenylated (Figure 4.2A, lanes 7-9, Figure 4.2B, lane 6), but to a lesser extent (Figure 4.2A, lanes 7, 8) and was smeary (Figure 4.2A, lane 9, Figure 4.2B lane 6), appearing more like RNA deadenylated in a distributive manner. Mock treated RNA samples remained intact (Figure 4.2A and 4.2B) indicating that the sample handling and preparation remained free from RNase contamination. The in vitro

deadenylase assay was repeated with several preparations of purified PNLDC1 with similar negative results.

PNLDC1 overexpression may inhibit deadenylation and mRNA decay in cells

Cell based assays were next used as an alternative approach to test for PNLDC1 deadenylase activity and ability to influence mRNA decay. Since deadenylation initiates decay in several mRNA decay pathways, aberrant deadenylation can stabilize transcripts and alter cellular decay rates (Yamashita et al 2005; Tucker et al 2001). The role of PARN in cytoplasmic mRNA decay remains unclear: it appears to be important for the decay of some regulated ARE mRNA decay (Chou et al 2006; Lin et al 2007; Gherzi et al 2004); while dispensable for other mRNA decay pathways and substrates, such as NMD (Yamashita et al 2005), even though it associates in complexes with NMD activating proteins (Lejeune et al 2003). Since PNLDC1 may have different characteristics than PARN, I next decided to compare the activity of PNLDC1 to PARN in these mRNA decay pathways.

Cellular mRNA decay assays were performed with two different mRNA reporters, an ARE and an NMD reporter mRNA, in HeLa cells expressing exogenous Myc-PNLDC1. Myc-PNLDC1 (Figure 4.3D, lanes 5, 6) slightly stabilized the decay of the ARE-containing reporter mRNA, with a measured half-life of 124±6 minutes compared to 90±4 minutes for the empty vector control (Figure 4.3A, lanes 1-4, 10-12). This experiment was only repeated

twice but the stabilizing effect of PNLDC1 was statistically significant. The decay of an NMD reporter also appeared to be slightly stabilized following PNLDC1 expression, with a measured half-life of 209±45 minutes compared to 137±19 minutes empty vector (Figure 4.3A, lanes 13-16, 21-24), although this difference was not statistically significant. There may have also been a slight decrease in deadenylation of the NMD reporter with Myc-PNLDC1 expression compared to control transfected samples (Figure 4.3A compare lanes 21-24 to 13-16C, and plotted in Figure 4.3C). No apparent change in deadenylation was observed for the ARE reporter (Figure 4.3B).

Expression of Myc-PARN (Figure 4.3D, lanes 3, 4) did not significantly alter the decay rate of the ARE or NMD reporter mRNA (Figure 4.3A, lanes 5-8 and 17-20), although it did appear to slightly increase the deadenylation rate of the NMD reporter, but not the ARE mRNA reporter (Figure 4.3B and 4.3C). This lack of effect on NMD by PARN overexpression is consistent with previous reports (Yamashita et al 2005).

PNLDC1 does not bind the mRNA cap

A unique feature of PARN among the mammalian deadenylases is that it binds the m(7)G RNA cap. Cap-binding activates the deadenylase activity of PARN in vitro (Martînez et al 2001; Dehlin et al 2000; Gao et al 2000) and the activity of PARN is inhibited on RNAs bound by cap binding proteins (Balatsos et al 2006). The cap-binding domain of PARN was mapped to amino acids 430-537 of PARN (Wu et al 2005), a region of low conservation with PNLDC1.

Therefore, PNLDC1 was predicted not to be able to bind the cap and this was tested using cap-binding assays.

Lysates from HEK293T cells expressing Flag-PNLDC1 were incubated with m(7)GTP coupled sepharose and tested for protein binding. This standard cap-binding assay with a cap analog has previously been used to show PARN cap binding (Dehlin et al 2000). In contrast to Myc-PARN, Flag-PNLDC1 did not bind the m(7)GTP sepharose (Figure 4.4, bound lanes). The cap-binding protein Myc-eIF4e, used as a positive control, was enriched on the m^7GTP sepharose, while the general RNA binding protein, Myc-hnRNP A1, was not (Figure 4.4). As a control for background binding to sepharose, cell lysates incubated with glutathione sepharose remained unbound (Figure 4.4, GSH bound lane).

PNLDC1 localizes to the cytoplasm

PARN is primarily localized to the nucleus in mammalian cells and contains two predicted NLSs (Figure 4.1). These localization signals are not conserved in PNLDC1 and determining the localization could offer insight towards function and activity of PNLDC1. Exogenous Myc-PNLDC1 expressed in human HeLa cells showed diffuse cytoplasmic staining with concentration in some cytoplasmic foci and around the nuclear periphery (Figure 4.5, top panel). PNLDC1 co-localization studies were not performed and it remains to be tested whether these foci overlap with known cytoplasmic RNA granules such as P-bodies or stress granules. In contrast, and consistent with previous

reports, exogenous Myc-PARN and endogenous PARN concentrate in the nucleus (Figure 4.5, middle and bottom panels).

Tissue-specific expression of PNLDC1

Unsuccessful attempts to PCR-amplify PNLDC1, but not PARN, from HeLa and HEK293 cell line cDNA suggested that the expression of PNLDC1 differed from PARN (data not shown). Analysis of expressed sequence tag (EST) databases suggested that PNLDC1 expression was limited to a subset of tissues, including testis, pancreas and placenta (data not shown), while PARN expression is abundant and ubiquitous (data not shown). I confirmed the restricted tissue expression of PNLDC1 mRNA by probing a multiple tissue Northern blot; in contrast to GAPDH mRNA, PNLDC1 mRNA was only detected in testis (Figure 4.6).

Cloning a "leadzyme" reporter to better resolve poly(A) tail length

To successfully monitor deadenylation rates, small changes in poly(A) tail length must be observable. Difficulties might arise when trying to measure small size changes of long mRNA molecules. Assays currently used to measure poly(A) tail length include PCR based assays and poly(A) tail measurements of RNase H cleaved mRNAs (Murray, & Schoenberg 2008). This generates smaller RNA molecules that can more readily be resolved and accurately measured by polyacrylamide gel electrophoresis. I first tried the RNase H method to measure the deadenylation of reporter mRNAs used in

the cellular decay assays (Figure 4.3), but this was unsuccessful because of non-specific RNase H cleavage and high background RNase activity, despite several optimization attempts (data not shown). To obtain more consistent RNA cleavage, an alternative method to RNase H mediated in vitro cleavage was developed. The 35 nucleotide sequence of a lead-activated ribozyme (from now on referred to as leadzyme), which self-cleaves in the presence of Pb^{2+} (Pan, & Uhlenbeck 1992), was cloned into the 3' UTR of a β-globin reporter mRNA (Figure 4.7B). Tested in a proof of principle experiment, the leadzyme-containing reporters, β-wt-Pb and β-ARE-Pb, cleaved to the expected size following incubation with Pb^{2+} in vitro (Figure 4.7A lanes 6, 15). RNA without the hairpin or with the inverted hairpin sequence was not cleaved (Figure 4.7A lanes 2, 9, 12, 18), but all reporter RNAs were nonspecifically degraded at high lead concentration (Figure 4.7A lanes 4, 7, 10, 13, 16, 19). In cells, the leadzyme-containing ARE-mRNA degraded similarly to the "normal" ARE mRNA reporter (Figure 4.7C) (with a half-life of 64 minutes compared to 61 minutes), indicating that insertion of the leadzyme hairpin did not disrupt ARE-mediated decay of this reporter. Therefore, leadzyme-containing reporters could be used as an alternative to RNase H-mediated RNA cleavage in future studies to monitor deadenylation rates of reporter mRNAs in cells.

4.3 Discussion

This study marks the first attempt to characterize PNLDC1, a putative deadenylase and homolog of PARN. Since the exonuclease domain, but not the cap-binding domain or NLS of PARN, is conserved in PNLDC1 it was predicted to contain cap-independent deadenylase activity. Surprisingly, PNLDC1 failed to display deadenylase activity in two different assays (Figure 4.2 and 4.3) and it inhibited decay of an mRNA reporter when exogenously expressed in cells (Figure 4.3). It would be premature to conclude that PNLDC1 lacks deadenylase activity based on this work alone and further studies would need to address some critical issues discussed below.

In vitro assays were used to test for PNLDC1 deadenylase activity and cap-binding. Flag-PNLDC1, expressed and purified from HEK293 cells, failed to deadenylate a capped, polyadenylated RNA (Figure 4.2), and was unable to bind the m(7)G cap (Figure 4.4). Myc-PARN, purified under similar conditions, displayed deadenylase and cap-binding activity and was used as positive control in these assays. Without further analysis of the purified PNLDC1 protein, it is difficult to conclude if the negative results reflect the true biochemical properties of PNLDC1 or if they were due to to suboptimal purification/experimental conditions. Possible explanations for the lack of PNLDC1 activity include: (1) improper folding in the expressed cell line; (2) disrupted activity of PNLDC1, but not PARN, due to the N-terminal tag; (3) improper post-translational modifications required for PNLDC1 activity; (4) PNLDC1 may require co-factors for activity not present in HEK293 cells.

Alternatively, the results might reflect the true nature of PNLDC1 activity – it does not have any, despite the high conservation of the RNase D domain of PNLDC1 to PARN (40% shared amino acid identity). It could be that non-conserved residues in other regions of the protein render PNLDC1 inactive. However, without a positive control to test for proper PNLDC1 folding, conclusions about its activity cannot be made. One possibility is to determine if purified PNLDC1 can bind RNA in vitro, since RNA binding is a property of the RNase D domain. Another possibility is that PNLDC1 displays 3'-to-5' exonuclease activity for non-poly(A) RNA substrates, such as oligo(U) tails, which are added to some 5' endo-cleaved RNAs after miRNA targeting (Shen, & Goodman 2004). Future studies should test this possibility.

mRNA decay assays were used in an alternative approach to test for PNLDC1 deadenylase activity in human cells. Expression of deadenylases often increases the deadenylation and decay of reporter mRNAs (Yamashita et al 2005; Wagner et al 2007), but PNLDC1 expression unexpectedly increased the stability of an ARE and an NMD reporter mRNA, and appeared to inhibit deadenylation of the NMD reporter (Figure 4.3). PARN appeared to slightly increase deadenylation of the NMD reporter but did not alter the decay rate of either reporter (Figure 4.3). These preliminary results should be followed with more thorough characterization of PNLDC1 activity in cells. To rule out that the results were due to indirect effects it should be determined if PNLDC1 binds to the reporter mRNAs directly, and if PNLDC1 complexes with cellular decay factors, since it is possible that PNLDC1 overexpression titrated

these required factors away from the reporter mRNAs. Another way to test if PNLDC1 directly alters the deadenylation and decay of reporter mRNAs is through a tethered decay assay (Clement, & Lykke-Andersen 2008). Another possibility is that PNLDC1 active is tissue and cell type specific, requiring co-factors absent in HeLa cells but present where PNLDC1 is normally expressed, such as the testis. Without further characterization it is difficult to make conclusions based on the inactivity of PNLDC1 expressed in the HeLa and HEK293T cell lines.

The subcellular localization of exogenously expressed PNLDC1 was determined to be cytoplasmic in contrast to the nuclear localized PARN (Figure 4.5). This was expected since the two NLSs of PARN were not conserved in PNLDC1. It would be important to determine if PNLDC1 co-localizes with other RNA decay factors since it appeared to be concentrated in some cytoplasmic foci (Figure 4.5). Localization should be tested for processing bodies, concentrated cytoplasmic foci of mRNPs marked for mRNA decay, or stress granules, cytoplasmic granules where translationally repressed mRNPs accumulate (Balagopal, & Parker 2009). It is possible that endogenous PNLDC1 localizes differently than the exogenously expressed tagged protein and this should be determined in future studies.

Several lines of evidence suggest that PNLDC1 expression is limited to a subset of tissues and differs from PARN expression. EST database searches and the multiple tissue Northern blot (Figure 4.6) suggest that PNLDC1 mRNA is expressed specifically in testis and it would be interesting

to test this expression pattern in organisms. If confirmed then the natural question is what is the biological role of PNLDC1 in the testis? Post-transcriptional regulation of gene expression is important during spermatogenesis, since transcription is shut off following nuclear compaction and to accommodate the change in morphology of the elongating spermatid. Translation and stability of mRNAs must be tightly controlled to ensure proper spermatid maturation (Idler, & Yan 2012; Braun 1998), and it is an intriguing possibility that PNLDC1 regulates some mRNAs in parallel to the regulation of maternally deposited stored mRNAs by PARN (Kim, & Richter 2006).

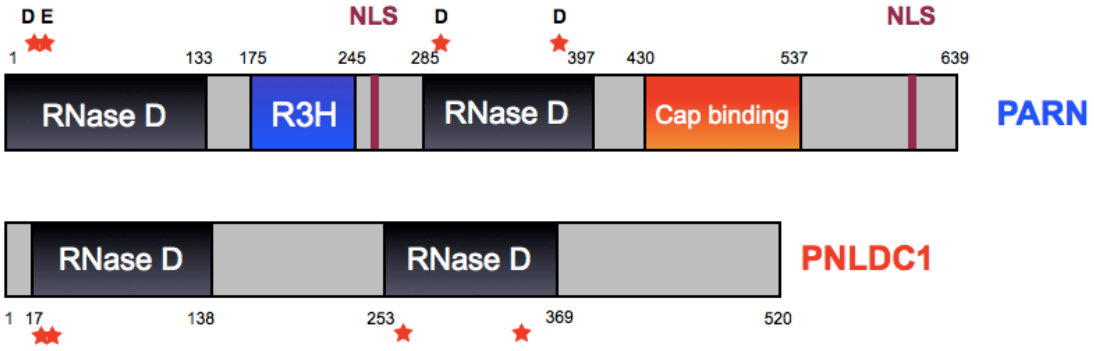


Figure 4.1: PNLDC1 is a homolog of PARN. The amino acid sequences of PNLDC1 and PARN were aligned with BLAST software. The conserved RNase D domain (black boxes) of PNLDC1 retains the DEDD amino acids (red stars) required for catalytic activity. The cap-binding domain, R3H domain and NLS of PARN are not conserved in PNLDC1.

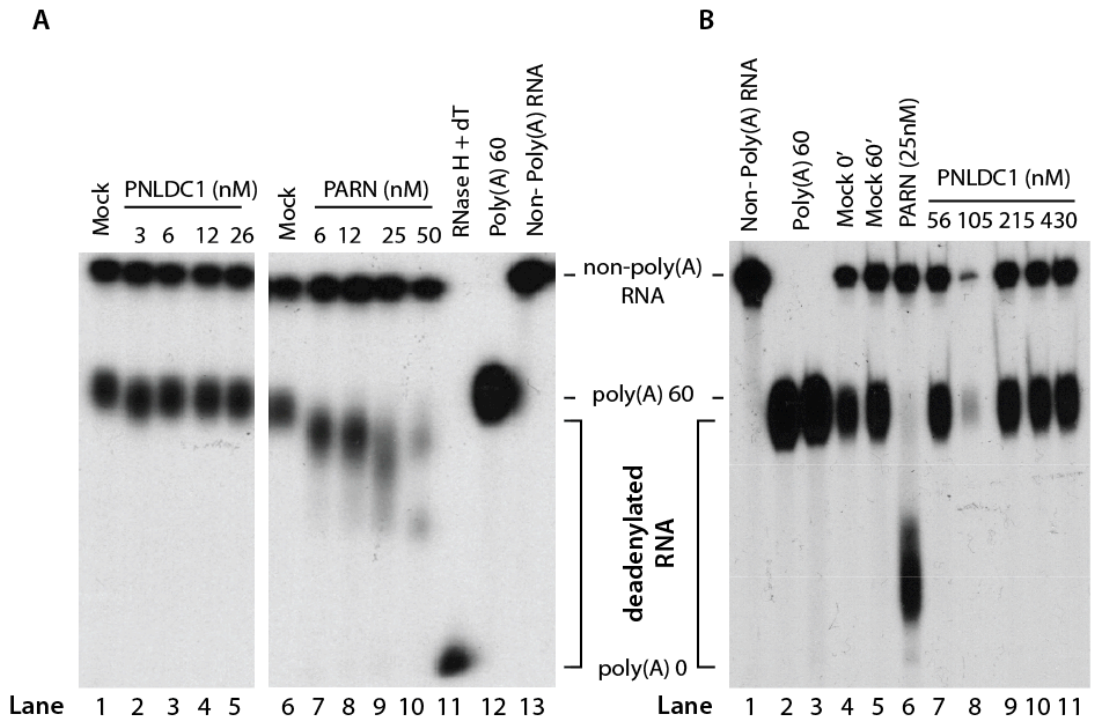
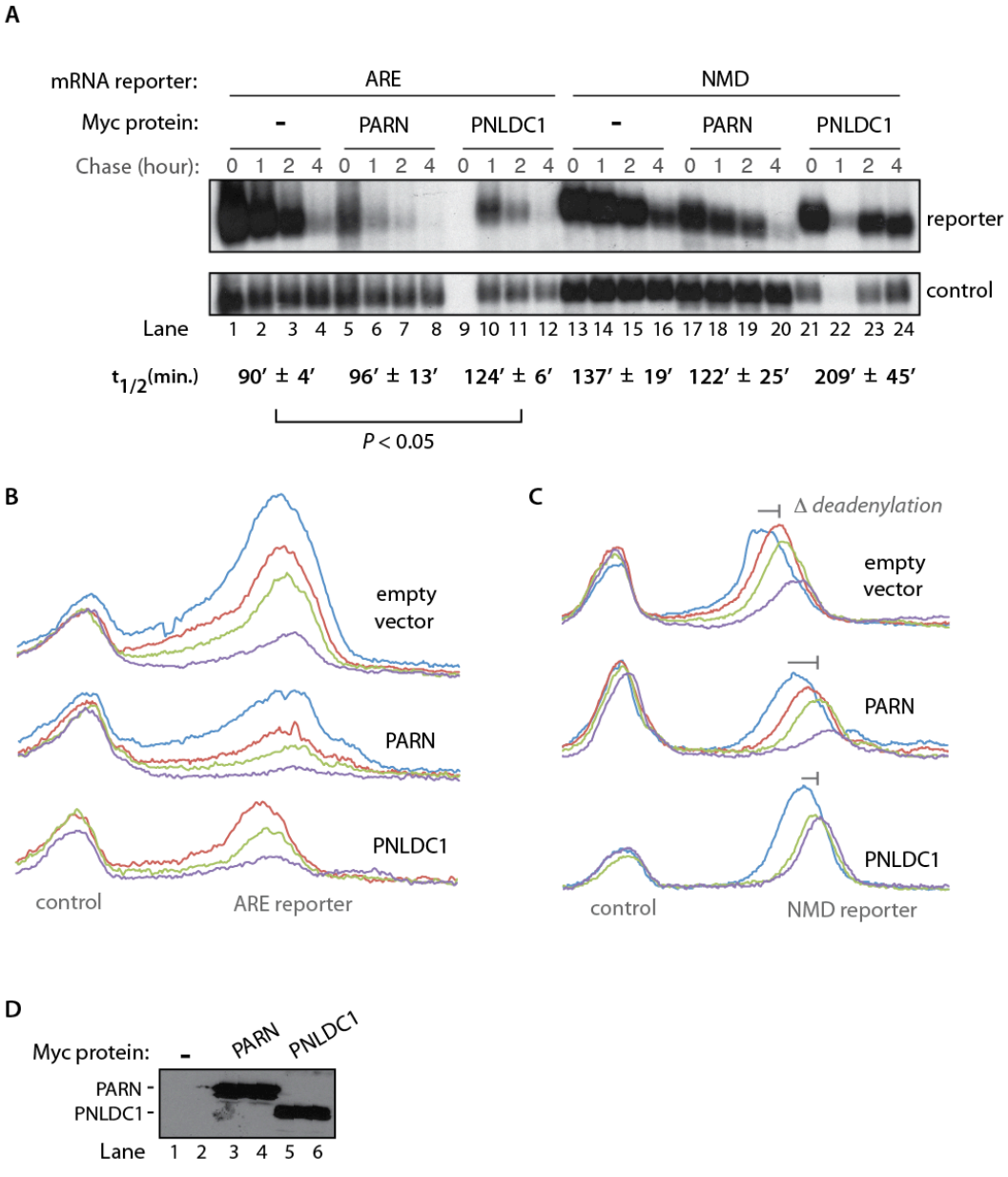


Figure 4.2: Deadenylase activity of PNLDC1. (A) Increasing amounts of purified Flag-PNLDC1 (3 nM to 26 nM) was incubated with poly(A)$_{60}$ and non-poly(A) substrate RNA for 30' at 37°C. Myc-PARN (6 nM to 50 nM) was used as a positive control. Poly(A)$_{60}$ RNA was incubated with oligo dT and RNase H to generate poly(A) 0 RNA (lane 11). **(B)** In vitro deadenylase assays were repeated as in A with higher concentrations of PNLDC1 (56 nM to 430 nM) for one hour at 37°C. (The sample in lane 8 was underloaded.)

Figure 4.3: Affect of PNLDC1 expression on decay and deadenylation of mRNA reporters. (A) HeLa cells were transfected with reporter and control mRNA plasmids and deadenylase plasmids or empty vector. Transcription of the reporter mRNA was pulsed on and samples were collected at 0, 1, 2, and 4 hours after transcriptional shut-off (chase). Reporter mRNA was normalized to control RNA and half-life ($t_{1/2}$) ± standard deviation of two biological repeats was calculated. P-value was calculated with a student t-test. (Note - the RNA sample from lane 9 was lost and lane 22 was underloaded.) **(B)** Signal intensity profile of the ARE reporter mRNA from time 0 (blue line) to 1 hour (red), 2 hours (green line), and 4 hours (purple line). Measured from the Northern blot in (A). **(C)** Signal intensity profile of the NMD reporter as in (B). Migration of the reporter mRNA peak represents deadenylation and the drawn line represents change in deadenylation from time 0 to 2 hours chase. Line colors are the same as in (B). **(D)** Western blot probed with mouse monoclonal α-Myc antibody to determine protein expression from decay assays in (A). Odd lanes were from the ARE reporter samples, even lanes were from the NMD reporter samples.


A
mRNA reporter:
ARE
NMD
Myc protein:
-
PARN
PNLDC1
-
PARN
PNLDC1
Chase (hour):
0 1 2 4 0 1 2 4 0 1 2 4 0 1 2 4 0 1 2 4 0 1 2 4
reporter
control
Lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
t1/2(min.)
90′ ± 4′
96′ ± 13′
124′ ± 6′
137′ ± 19′
122′ ± 25′
209′ ± 45′
P < 0.05
B
empty vector
PARN
PNLDC1
control
ARE reporter
C
Δ deadenylation
empty vector
PARN
PNLDC1
control
NMD reporter
D
Myc protein:
-
PARN
PNLDC1
PARN
PNLDC1
Lane
1 2 3 4 5 6

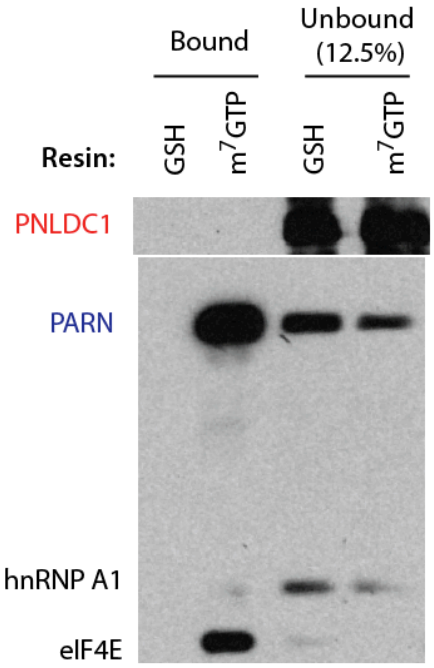


Figure 4.4: PNLDC1 does not bind the mRNA cap. Affinity tagged proteins (Flag-PNLDC1, Myc-PARN, Myc-hnRNP A1, and Myc-eIF4E) were expressed in HEK293T cells and lysates were incubated with m^7GTP coupled sepharose or glutathione sepharose (GSH). The bound fraction was compared to 12.5% of the unbound fraction on SDS-PAGE and visualized with anti-epitope antibodies.

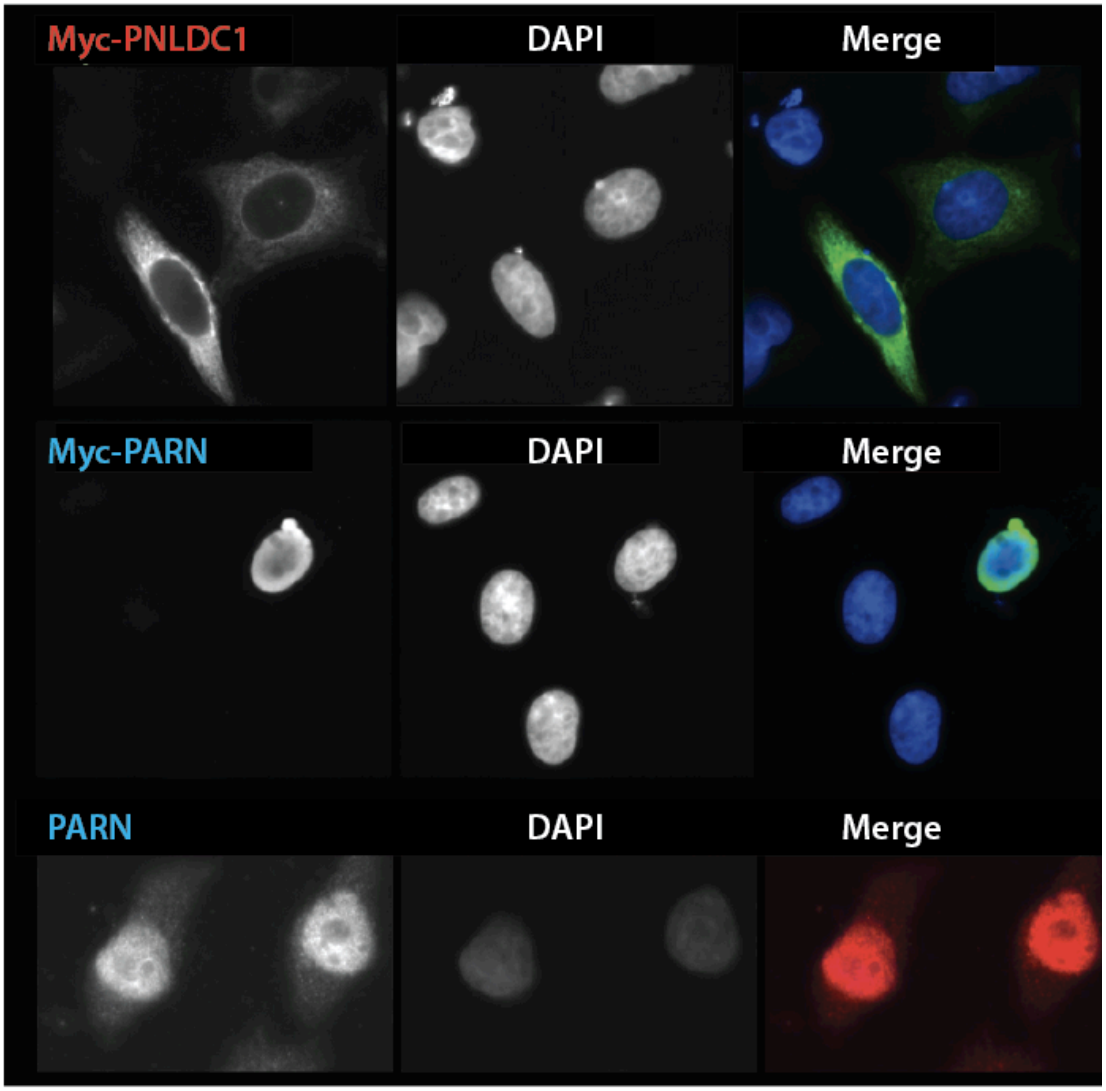


Figure 4.5: Exogenous PNLDC1 localizes to the cytoplasm. Top panel – HeLa cells expressing exogenous Myc-PNLDC1 were prepared for immuno-fluorescence microscopy and visualized with α-Myc primary antibody. Nuclei were visualized with DAPI staining (second column) and merged with the α-Myc image (third column). **Middle panel** – Exogenous Myc-PARN was visualized as above. **Bottom panel** – Localization of endogenous PARN in HeLa cells using α-PARN rabbit polyclonal antibody.

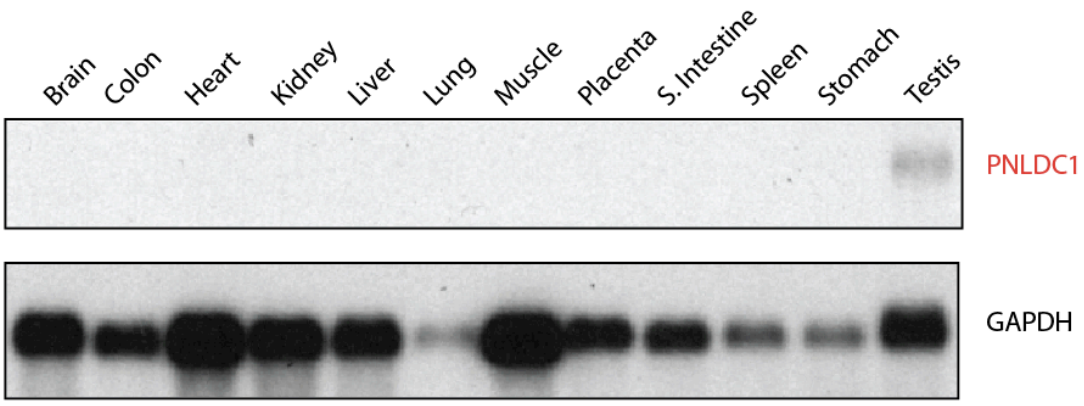


Figure 4.6: Tissue specific expression of PNLDC1. A multiple tissue Northern blot was probed for PNLDC1 mRNA stripped and then reprobed for GAPDH mRNA.

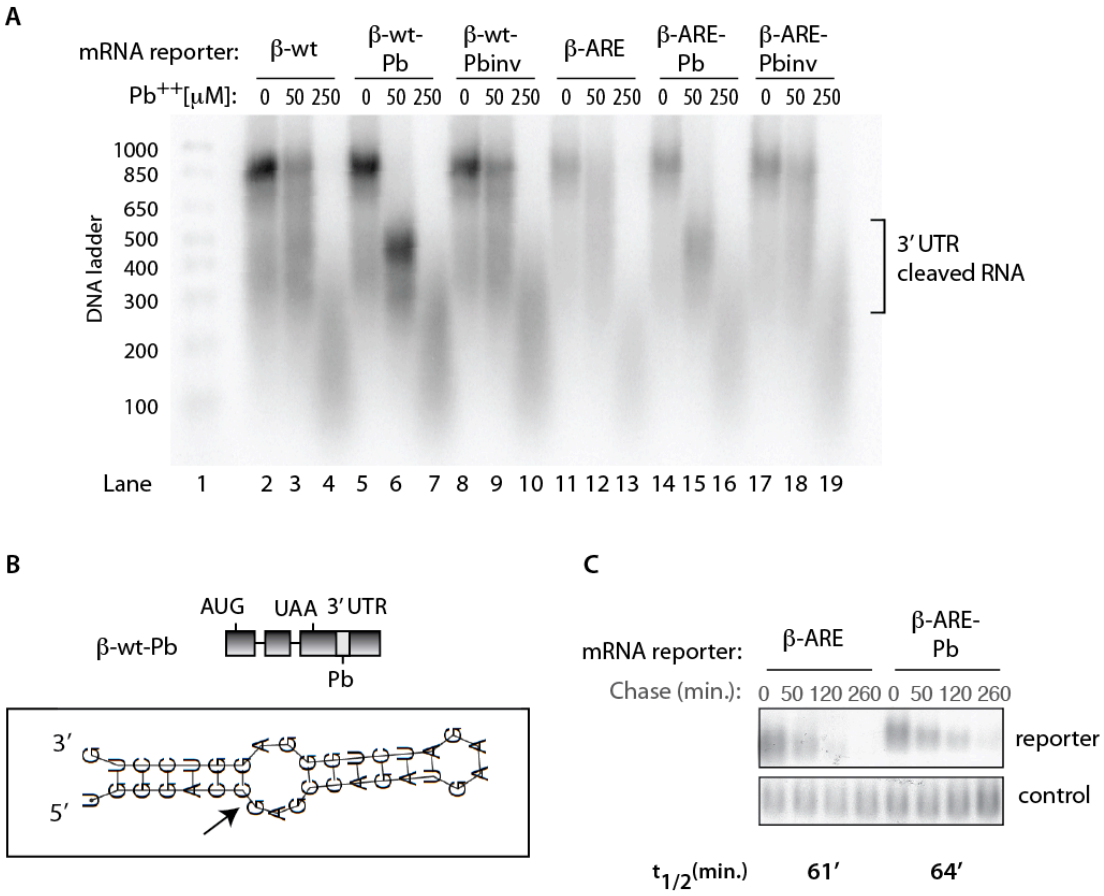


Figure 4.7: Specific cleavage of the leadzyme reporter mRNA. (A) Increasing amounts of Pb^{2+} was incubated with RNA for 90 minutes at 30°C. The cleaved RNA, containing the poly(A) tail and 3'UTR, migrated at the 300-500 bp size marker. **(B) Top** - Cartoon schematic of the leadzyme hairpin sequence inserted into the 3' UTR of the beta-globin derived reporter mRNA. **Bottom –** Predicted folding of the leadzyme sequence using RNAfold. The arrow indicates the site of Pb^{2+} catalyzed cleavage. **(C)** mRNA decay assays were performed as previously described.

4.4 Materials and Methods

Plasmids

A cDNA clone of the open reading frame of human PNLDC1 (Open Biosystems) was subcloned into pcDNA3-Myc and pcDNA3-Flag constructs using BamHI and NotI restriction enzymes. PARN plasmids were cloned as described previously (Clement 2010). pcDNA3-Myc-hnRNP A1 and pcDNA3-Myc-eIF4E were previously described (Fenger-Grøn et al 2005).

Protein purification

Flag-tagged PNLDC1 was induced from one 15 cm plate of stably integrated HEK293T stable lines by addition of 500 ng/ml tetracylcline to full DMEM. For PARN purification, one 15 cm plate of HEK293T cells was transfected with 25 µg pcDNA3-Myc-PARN plasmid. 48 hours later, cells were harvested and prepared for immunoprecipitation as described previously (Wagner et al 2007). Myc-PARN was immunoprecipitated with anti-Myc antibody (Sigma) and PNLDC1 was immunoprecipitated with 80 µl anti-FLAG M2 agarose (Sigma). Complexes were washed 8X with Net-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Triton X-100) and eluted with 1 mg/ml Flag-peptide (Sigma) or 1 mg/ml Myc peptide (Sigma) in Net-2 buffer with gentle shaking at 4°C for 2 hours. Eluates were stored at -20°C.

In vitro deadenylation assay

Preparation of RNA substrates was performed as previously described (Wagner et al 2007). In vitro deadenylase assays were performed as previously described (Wagner et al 2007) in 20 mM HEPES, pH 7.4, 2 mM DTT, 0.2 mg/ml BSA, 2 mM spermidine, 0.01 % NP-40, 2 mM $MgCl_2$, 0.5 U/µl RNaseOUT (Invitrogen), and 5 µg yeast total RNA at 37°C for the indicated time and with incubation with RNA substrate and indicated protein. RNAs were precipitated, and resolved on 6% polyacrylamide/7M urea denaturing gels and visualized by autoradiography.

mRNA decay assay

mRNA decay assays were performed as previously described (Lykke-Andersen, & Wagner 2005). HeLa Tet-off cells were cultured in DMEM/10% fetal bovine serum/1% penicillin and streptomycin (full DMEM) and transfected in the presence of 50 ng/ml tetracycline with TransIt HeLa-Monster (Mirus) transfection reagent according to the manufacturers protocol. State the amount of plasmids used for transfections here. 36-48 hours later cells were washed with PBS and changed to full DMEM without tetracycline to start transcription of the β-globin mRNA reporters. Transcription was turned off six hours with addition of 1 µg/mL tetracycline and chase timepoint 0 was collected 30 minutes later in 500 µL Trizol. Total RNA was prepared per manufacturers protocol and 10 µg was resolved on a denaturing agarose gel for Northern blotting. A ^{32}P-labeled probe hybridizing to the coding sequence of β-globin was used to visualize the RNA reporter. Blots were either exposed

to film and or phosphoscreen and later developed on a Storm phosphoscanner.

Cap binding assay

HEK293T cells seeded at 20% confluency in six well plates in full DMEM and transfected with TransIT-293 transfection reagent (Mirus) using the manufacturers protocol with the indicated plasmids. 48 hours after transfection, cells were washed in PBS, and lysed in 400 µl lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.1% Triton-X 100, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin) and incubated on ice for 5 min. NaCl was added to 150 mM and 5 µl RNase A 10 mg/ml was added and incubated on ice for 10 min. Cytoplasmic extract was collected after a 15 min spin at 14,000 rpm in 4°C and incubated with 20 µg of m7GTP-Sepharaose beads (Amersham Pharmacia) in Net-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Triton X-100). After overnight rotating incubation at 4°C, the bound proteins were washed 8X in Net-2 buffer and beads were taken up in an equivalent volume of load buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mM DTT) for analysis by SDS-PAGE and Western blot.

Indirect immunofluorescence

HeLa cells were cultured in DMEM/10% fetal bovine serum/1% penicillin and streptomycin in chamber slides. Cells were fixed in 4%

paraformaldehyde for 15 minutes and permeabilized and blocked with PBS/1% goat serum/0.1% Triton X-100 for 30 minutes. Cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) solution, then washed with PBS, and incubated with anti-Myc (Cell Signal) and rabbit anti-Parn sera antibodies at 1:1000 dilutions for 1 hour. Following removal of primary antibody, cells were incubated with 4 μg/ml secondary anti-rabbit antibody labeled with Alexa 488 fluorophore and anti-mouse antibodies labeled with Texas Red fluorophore (Molecular Probes)

Multiple tissue Northern blot

A multiple tissue Northern membrane (Origene) was sequentially probed for PNLDC1 and GAPDH using Ambion strip easy probe kit. ^{32}P-labeled hybridization probes were transcribed with SP6 RNA polymerase (Ambion) using BamHI restriction enzyme digest of pcDNA3-Flag-PNLDC1 plasmid, XhoI restriction enzyme digest of pcDNA3-Myc-PARN plasmid, and NotI restriction enzyme digest of pc-βwt-GAP3UAC plasmid for GAPDH.

Leadzyme cleavage assay

HeLa cells were transiently transfected with indicated β-globin reporter plasmid with TransIT transfection reagent (Mirus) and collected in Trizol (Invitrogen). RNA was resuspended in 15 mM MOPS pH 7.2 buffer and 10 ug was heat denatured at 80°C for two minutes, then allowed to cool slowly to 30°C on a heat block. Cleavage reaction was initiated by addition of lead

acetate to the appropriate final concentration (0, 50 μM, or 250 μM) and incubated for 90 minutes at 30°C. An equal volume of stop buffer (25 mM sodium citrate, 50 mM EDTA, 0.5% xylene cyanol, and 0.5% bromophenol blue) was added on ice. RNA was resolved on with electrophoresis on agarose formaldehyde gel and visualized by Northern blot using 32P labeled RNA hybridization probe targeting the coding region of the β-globin reporter.

Chapter 5. Conclusions and future directions

Conclusions

TTP promotes the decay of many physiologically important ARE-containing mRNAs for which misregulation would be detrimental, including mRNAs coding for cytokines, growth factors, and transcription factors (Baou et al 2009; Blackshear 2002; Stoecklin et al 2008; Emmons et al 2008) . How TTP specifically regulates a specific subset of ARE-mRNAs despite the presence of other ARE-containing mRNAs and competing AUBPs is an outstanding question in the field. Regulation by TTP is complex. Most cells express TTP at low basal levels and turn it on in response to stimulus, and TTP mRNA decay activity is modulated by post-translational modifications (Clement et al 2011; Marchese et al 2010). Additionally, accumulating evidence suggests non-mRNA decay functions for TTP (Liang et al 2009; Schichl et al 2009). My thesis research aimed to better understand the specific activity of TTP in mRNA decay, and it also surprisingly suggested a possible new role for TTP in RNA 3' end processing.

In Chapter 2, I characterized the interaction between TTP and hnRNP F, and observed that hnRNP F stimulates the decay of a subset of TTP-associated mRNAs. This implicates hnRNP F in mRNA decay. Previous studies have characterized roles for hnRNP F in RNA processing (Mauger et al 2008; Min et al 1995; Veraldi et al 2001; Huelga et al 2012) and in translation repression (White et al 2012; Kalifa et al 2006).

In addition to mRNA degradation, TTP appears to regulate gene expression through non-mRNA decay activities (Liang et al 2009; Schichl et al 2009). Chapter 3 further explores this possibility with the observation that TTP stimulates the 3' end processing of mRNAs, leading to alternatively polyadenylated mRNAs with shortened 3' UTRs. This activity requires an ARE and a poly(A) signal, and RNA binding of TTP was necessary but not sufficient to promote alternative 3' end processing. This newly identified activity of TTP appears to be conserved, since the mammalian and yeast homologs of TTP, BRF1 and CTH2, respectively, have been implicated in 3' end processing of ARE-containing mRNAs while my studies were in progress (Prouteau et al 2008; Desroches-Castan et al 2011). Thus, TTP appears to be a multifunctional protein that, in addition to its function in mRNA decay, promotes 3' end processing, which could alternatively polyadenylate ARE-containing mRNAs thereby affecting their expression.

Deadenylation is an important component of ARE-mRNA decay (Chen, & Shyu 1995) and TTP interacts with cellular deadenylases (Lykke-Andersen, & Wagner 2005; Sandler et al 2011). Chapter 4 describes my initial characterization of the putative deadenylase, PNLDC1. Although deadenylase activity was not observed in vitro, PNLDC1 inhibited the mRNA decay of reporter mRNAs, and appeared to have tissue specific expression in the testis.

Future Directions

By what mechanism does hnRNP F stimulate TTP-associated mRNA decay?

Does hnRNP F repress translation, enhance TTP-substrate mRNA binding, or stimulate a decay step after TTP RNA association? These possibilities were discussed in greater detail in the Discussion in Chapter 2, and they are not mutually exclusive. An unresolved question is whether the stimulation of ARE mRNA decay requires RNA-binding by hnRNP F? It will be important to determine if adding back of exogenous hnRNP F rescues mRNA decay after hnRNP F knockdown (Figure 2.7), so that an RNA-binding mutant of hnRNP F can be tested. This might offer valuable insight towards understanding the mechanism of hnRNP F stimulation of ARE mRNA decay. If an RNA-binding mutant does rescue TTP-associated mRNA decay, one possibility could be that hnRNP F is structural component required by TTP for mRNA decay.

Further mechanistic insight might come from additional co-IP experiments where the cell extracts are carefully fractionated to determine in which subcellular compartment TTP and hnRNP F/H interact, the nucleus or the cytoplasm. Both TTP and hnRNP F/H shuttle between the nucleus and cytoplasm (Murata et al 2002; Phillips et al 2002; Kamma et al 1995; Honoré et al 1999; Van Dusen et al 2010; Honoré et al 2004) but they did not appear to co-localize in LPS-stimulated RAW cells (Figure 2.11). A nuclear interaction between TTP and hnRNP F/H might facilitate TTP loading onto substrate mRNAs in the nucleus, as has been reported for the yeast homolog, CTH2

(Vergara et al 2011). Another possibility is that hnRNP F/H stimulates TTP-mediated mRNA decay in the nucleus. The TTP and hnRNP F/H interaction might target TTP to nuclear RNA decay substrates, such as mis-spliced pre-mRNAs or other RNAs bound by hnRNP F/H. The TTP-mediated RNA decay might be executed by the nuclear exosome, since TTP interacts with exosome components (Lykke-Andersen, & Wagner 2005; Chen et al 2001).

hnRNP F stimulation of TTP-associated mRNA decay may be due to an indirect effect since hnRNP F modulates RNA processing (Mauger et al 2008; Min et al 1995; Veraldi et al 2001; Huelga et al 2012) and translation repression (White et al 2012; Kalifa et al 2006). Although the decay of c-FOS mRNA, a non-TTP target, was not sensitive to hnRNP F knockdown (Figure 2.9), it will be important to test the decay of other mRNAs to determine if hnRNP F stimulation of mRNA decay is specific to TTP-associated mRNAs. I have already mentioned other experiments that might suggest if the effect of hnRNP F is direct or indirect, such as testing if tethered hnRNP F can stimulate the TTP-dependent decay of a targeted mRNA reporter containing MS2 binding sites. Alternatively, a closer examination of the decay of LIF and IER3 mRNA decay might be informative since only LIF mRNA decay is sensitive to hnRNP F (Figure 2.9).

Does hnRNP H stimulate ARE mRNA decay?

TTP was observed to complex with the homologs of hnRNP F, hnRNP H1 and hnRNP H2 (Figure 2.2). Future studies should determine whether the

hnRNP H proteins also stimulate ARE mRNA decay since this was unclear due to inefficient siRNA-mediated protein knockdown, despite trying three different siRNAs (Figure 2.4B and data not shown). It is a possibility that hnRNP H1/H2 does not have similar mRNA decay stimulating activity as hnRNP F, since global analyses highlighted the differences in RNA binding and their effects on alternative splicing of these two hnRNPs (Huelga et al 2012), despite similarities in their effects on splicing of selected transcripts (Martinez-Contreras et al 2006; Garneau et al 2005). hnRNP F and hnRNP H also have opposing 3' end processing activity (Veraldi et al 2001). So despite the similarities in amino acid sequence and their effects on splicing of certain transcripts, it is possible that hnRNP F and hnRNP H would have different effects on mRNA decay. TTP association with either hnRNP F or hnRNP H could be an interesting way to modulate TTP mRNA decay activity. It will be important to test if the amount or ratio of hnRNP F/H associated with TTP varies in different cell lines or changes during prolonged TTP expression in cells.

Non-mRNA decay functions for TTP

TTP expression stimulated the 3' end processing of ARE-containing mRNA reporters (Chapter 3), but it needs to be determined if TTP regulates the alternative polyadenylation of endogenous mRNAs. Several studies highlighted the global changes in alternative polyadenylation and 3' UTR length of cellular mRNAs during development, growth and proliferation, and in

disease (Mayr, & Bartel 2009; Ji, & Tian 2009; Ji et al 2009; Sandberg et al 2008). As an immediate early response gene, TTP expression is induced in response to stimuli, and TTP regulates other early response genes through mRNA decay. Could another function of TTP in the immediate early response pathway be to promote alternative 3' end processing of some mRNAs?

In a study monitoring the mRNAs induced in fibroblasts after cytokine exposure, three groups of mRNAs were observed: 1) rapidly induced mRNAs that were unstable, 2) mRNAs whose level peaked and plateaued slightly later that were less unstable, and 3) delayed mRNAs whose levels rose slowly that were stable (Hao, & Baltimore 2009). AREs were enriched in the first group of unstable mRNAs and were less prominent in the third group of stable mRNAs (Hao, & Baltimore 2009). It would be interesting to test if the stable mRNAs had shorter 3' UTRs, and if TTP promoted the alternative polyadenylation, and possible exclusion of AREs or other destabilizing elements, in the 3' UTR.

Another possibility is that the interaction between TTP and hnRNP F/H mediates alternative splicing alternative splicing. TTP association with hnRNP F/H might modulate pre-mRNA splicing. Examples of AUBPs that regulate ARE-mRNA decay and RNA processing events including KSRP, with roles in alternative splicing (Min et al 1997) and miRNA processing (Trabucchi et al 2009), and HuR, which is implicated in alternative splicing (Mukherjee et al 2011; Lebedeva et al 2011). Might TTP function in a similar manner?

The experiments in Chapter 3 demonstrated that TTP expression promoted the alternative 3' end processing of ARE mRNA. Interestingly, the

TTP-interacting proteins hnRNP F/H are also implicated in RNA 3' end processing, and hnRNP H stimulates cleavage and polyadenylation (Bagga et al 1998; Arhin et al 2002) while hnRNP F inhibits it (Veraldi et al 2001). Therefore, it should be tested if hnRNP H is required for the 3' end processing activity observed with TTP expression. If hnRNP F/H proteins are involved in TTP 3' end processing and TTP mRNA decay activities, it would be interesting to test if TTP activity correlated with hnRNP F/H association. For instance, does TTP associate with hnRNP F for mRNA decay activity and hnRNP H for 3' end processing activity? TTP might associate with either hnRNP F or hnRNP H depending on cellular localization since, in some cell types, hnRNP H expression is primarily nuclear while hnRNP F expression is mostly cytoplasmic (Honoré et al 2004).

Another possibility, analogous to the mechanism by which TTP inhibits transcription by blocking nuclear import of the p65 unit of NF-KB (Liang et al 2009; Schichl et al 2009), is that TTP regulates the cellular localization of either hnRNP F or hnRNP H through protein interactions. This could significantly change the cellular RNA processing and transcriptome in cells and have profound effects. Although TTP did not appear to co-localize with hnRNP F/H, or mediate the cellular relocalization of these proteins during an LPS time course in RAW cells (Figure 3.11), any changes in re-localization might have been obscured since an antibody that recognized both hnRNP F/H was used. Specific antibodies that recognize hnRNP F and hnRNP H should be used in the future. hnRNP F/H localization may be important in certain

types of cancer, where hnRNP F displays increased nuclear localization in tumors compared to normal tissues (Honoré et al 2004), and TTP expression is generally suppressed (Brennan et al 2009; Carrick, & Blackshear 2007). A possibility is that low TTP expression contributes to the mislocalization of hnRNP F and altered mRNA splicing observed in cancer cells (Venables 2004; Warzecha, & Carstens 2012). When exogenous TTP is expressed in cancer cells, it suppresses some tumorogenic phenotypes (Brennan et al 2009), and it would be interesting to determine the hnRNP F localization in those experiments before and after TTP expression.

CAD, another TTP and BRF1-interacting protein (Figure 2.1, 2.2), catalyzes the de novo synthesis of pyrimidine nucleotides (Nyunoya et al 1985). It should be determined if its interaction with TTP and BRF1 is important for mRNA decay, or CAD pyrimidine nucleotide synthesis activity, both, or neither. CAD is required in proliferating cells and its activity and expression appears to be upregulated in cancer (Aoki, & Weber 1981; Morin et al 2012). Is the loss of TTP expression in cancer (Brennan et al 2009; Carrick, & Blackshear 2007) related to the increase in CAD activity? Do TTP/BRF1 negatively regulate the activity of CAD, maybe through protein binding inhibition? It would be interesting to test if TTP/BRF1 expression inhibited CAD activity in protein-protein interaction dependent manner. Is CAD activity decreased in cancer cells after forced TTP expression and is this important for the suppressed tumorogenic phenotype (Brennan et al 2009)?

mRNA decay and post-transcriptional regulation in gametes

PNLDC1 mRNA appeared to be enriched in the testis (Figure 4.6) and other RNA binding proteins are also enriched in the testis and ovary, including TTP (Lu, & Schneider 2004), and hnRNP F (Kamma et al 1995). RNA processing and post-transcriptional regulation is important during spermatogenesis since transcription is shut off during nuclear compaction and sperm cell maturation (Idler, & Yan 2012; Braun 1998). Additionally, the expressed mRNAs have shorter 3' UTRs and with increased usage of alternative polyadenylation sites (McMahon et al 2006; Liu et al 2007). Could TTP, hnRNP F/H, or PNLDC1 be important for the post-transcriptional regulation of gene expression in these organs?

It is an exciting possibility that hnRNP F represses translation of specific mRNAs during spermatogenesis, or that it regulates maternal mRNAs during oogenesis or embryogenesis in a similar manner as Glorund, the homolog of hnRNP F in *Drosophila*, which maintains the translationally silent state of the maternally deposited nanos mRNA in the embryo (Andrews et al 2011; Kalifa et al 2006). Also it would be interesting to test if differences in hnRNP F/H activity or TTP/BRF1 contributes to the alternative polyadenylation observed during spermatogenesis (McMahon et al 2006; Liu et al 2007).

It will be important to determine if PNLDC1 contains deadenylase or other nuclease activity and to confirm its testis-specific expression. An interesting possibility is that PNLDC1 deadenylase activity maintains stored mRNAs in a deadenylated, translationally inactive state, analogous to PARN

deadenylation of maternal mRNAs in *Xenopus* oocytes (Kim, & Richter 2006). Conversely, PNLDC1 might not harbor deadenylase activity, which could suggest that it blocks the activity of other deadenylases if it associates with mRNAs.

What about the role of TTP and its homologs in these processes? There was no reported sterility or abnormality of the sex organs associated with the TTP knockout mouse, but BRF2 appears to be important since fertilized embryos from BRF2 knockout mice fail to develop past the two-cell stage (Ramos et al 2004). Whether this is due to a defect in decay of maternal mRNA or some other reason remains to be determined.

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