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Puf1p-MEDIATED mRNA DECAY AND COMBINATORIAL CONTROL OF mRNA STABILITY BY THE YEAST Puf PROTEINS

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ABSTRACT

The stability of a messenger RNA (mRNA) is a highly regulated and important aspect of gene expression. Proteins that regulate mRNA stability often bind to 3' untranslated region (UTR) sequence elements. The eukaryotic Puf proteins are one class of 3'UTR binding proteins that regulate the stability and expression of their target transcripts. Several global genome analyses have identified hundreds of potential mRNA targets of the *Saccharomyces cerevisiae* Puf proteins, however only three mRNA targets for these proteins have been characterized thus far. After direct testing of nearly forty candidate mRNAs, I have established three of these as true mRNA targets of Puf-mediated decay in yeast, *YHB1*, *HXK1* and *TIF1*. In a novel finding, multiple Puf proteins, including Puf1p, regulate *HXK1* and *TIF1* mRNAs in combination. *TIF1* mRNA decay can be stimulated individually by Puf1p and Puf5p, but the combination of both proteins is required for full regulation. This Puf-mediated decay requires the presence of two UGUA binding sites within the *TIF1* 3' UTR, with one site regulated by Puf5p and the other by both Puf1p and Puf5p. The stability of the endogenously transcribed *HXK1* mRNA, cellular levels of Hxk1 protein activity, and *HXK1* 3'UTR-directed decay are affected by Puf1p and Puf5p as well as Puf4p. *YHB1* mRNA decay is mediated by Puf5p and also requires a UGUA sequence element.

This work has discovered the first targets of Puf1-mediated decay. Since much of our knowledge of the mechanism suggests that Puf protein target recognition and mechanism of action varies with each of these proteins, I investigate the mechanism of Puf1p-mediated decay using a variety a techniques. The results of this research aid in our understanding of the similar, yet distinct, decay regulation of Puf proteins in yeast and higher organisms.

DEDICATION

I dedicate this to my husband, Jeff, for his continuous love, support and friendship. Thank you.

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I would like to start by thanking Dr. Wendy Olivas for being my advisor, mentor, and friend. She has shown great patience and care as a mentor, lending as much help and advise as I need, while still giving me the freedom to make my own decisions and judgements about my research and studies. I would also like to thank my committee members, Dr. Cindy Dupureur, Dr. Marc Spingola, and Dr. Bethany Zolman for guidance and suggestions with my project. A special thanks to Dr. Zolman who has given me extra advice and support. Also, thank you to Dr. Theresa Thiel for her help in preparing for my post-doctoral search. Our wonderful administrative assistants in the Biology department deserve special recognition, particularly Maryanne Hempen, who is essential to every Biology graduate student. It is rare to find a department that involves its graduate students in so many critical decisions. Thanks to Peter Steven for supporting and maintaining this environment.

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Last, but certainly, not least, I thank my family and friends for help and encouragement throughout my many years of school. My husband, Jeff, works

incredibly hard to help and support me in so many ways. I thank him for his patients and for chipping in during my many hours in the lab. My parents, Rick and Judy, as well as my siblings helped me learn that perseverance and self-motivation may get you where you want, but family gets you where you need to be. Also, my friends from St. Louis, for not chastising my "geek-ness", even though they do not quite understand what I do. These are remarkable people, who I hope will remain in my life long after I leave St. Louis.

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INTRODUCTION

CHAPTER I: INTRODUCTION

The information stored within the genome must first be transcribed into a messenger molecule, known as messenger-RNA or mRNA, prior to being translated into protein, the functional molecule of the cell (summarized in Figure 1.1). This process of gene expression must be highly regulated, as over-expression or under-expression of individual genes may result in disease or even death of the cell. There are many opportunities for regulation within the process of gene expression, including transcription (production of mRNA), translation (production of protein), and mRNA stability. Due to the fact that one mRNA undergoes multiple rounds of translation, in simple systems, a more stable mRNA will allow more rounds of translation and thus make more protein, while a less stable mRNA will make less protein. In more complex systems, in addition to the amount of protein made, the stability of an mRNA is essential for proper timing of gene expression. For example, in the process of development, zygotic transcription does not occur until several cell divisions after fertilization, therefore, the mRNA supplied by the oocyte will direct gene expression in the early zygote. In this example, maternal mRNAs must be stable, but translation of the mRNA will not ensue until the proper time. After which, the mRNA must be degraded to eliminate further translation of the mRNA (Schier 2007).

Since the stability of an mRNA will directly affect the amount of protein translated, understanding mRNA stability and degradation is essential to understanding gene expression. Preparation of an mRNA for proper stability begins within the nucleus at the site of transcription. Soon after initiation of transcription, the single stranded

mRNA is quickly modified by the addition of a 5' 7-methylguanosine (m^7G) cap structure. The cap structure protects the mRNA from degradation, enhances mRNA processing (splicing), facilitates nuclear export, and enhances translation. A poly-adenosine (poly(A)) tail is also added to the 3' end of each mRNA (except for histone mRNAs). The mRNA is cleaved and polyadenylated while still associated with the transcription enzyme, RNA polymerase II. In mammals, there is a very conserved cleavage and polyadenylation signal (AAUAAA), however, in yeast this signal is much less conserved. Proper cleavage and polyadenylation is essential for transcription termination, suggesting its importance in mRNA transcription. The poly(A) tail is also important in nuclear export, translation and stability of the mRNA.

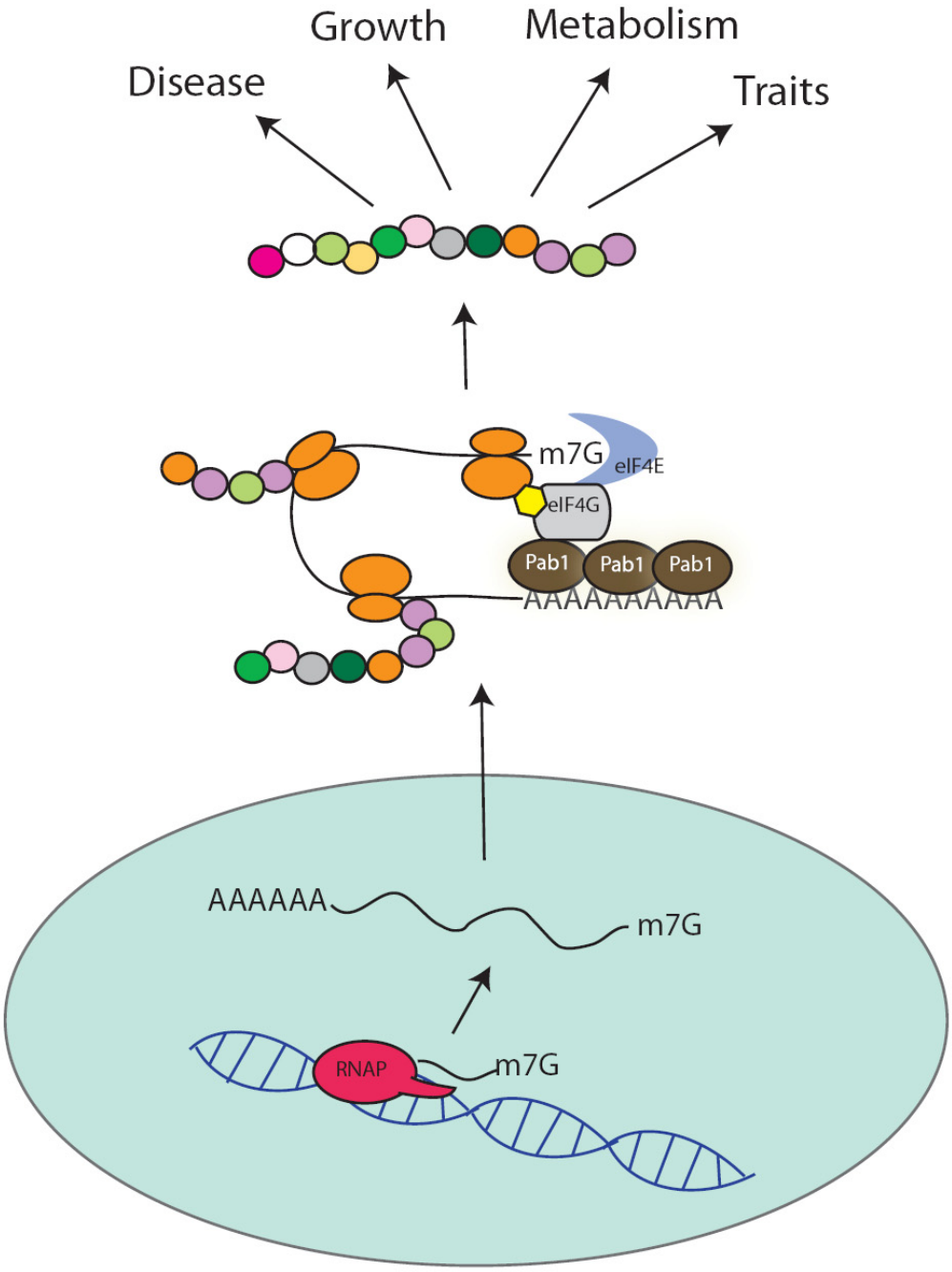


Figure 1.1 Eukaryotic gene expression.

Gene expression begins in the nucleus of a cell (light blue). A messenger RNA (mRNA) is transcribed from the DNA (dark blue) by RNA Polymerase II (RNAP). The mRNA is co-transcriptionally modified by addition of the 7-methyl guanosine cap (m^7G) and poly(A) tail. The mRNA is then exported from the nucleus to the cytoplasm where multiple rounds of translation by the ribosome (orange) occur. See text for details. Each gene produces a protein product that will be used for various cellular processes, including cell division and growth, disease prevention, cellular metabolism, and production of physical traits.

Eukaryotic mRNA Decay

The 5' cap and 3' poly(A) tail act synergistically to stabilize the mRNA and stimulate translation. While the stretch of As and nuclease resistant cap structure themselves provide some protection from degradation of the mRNA coding sequence, it is the molecules that bind these structures that afford the mRNA much of its stability and translatability. Multiple copies of poly(A) binding protein (PAB) bind to the poly(A) tail. PAB provides protection by inhibiting enzymes responsible for deadenylating the mRNA (Parker and Song 2004). The translation initiation complex is associated with the 5' cap via a direct interaction between the cap and eukaryotic translation initiation factor 4E (eIF4E), promoting translation initiation of the mRNA. Importantly, the protein complexes associated with the extreme ends of the mRNA also interact with one another, pulling the mRNA into a looped structure where the 3' end and the 5' end are in close proximity. For example, the yeast PAB, Pab1p, binds the cap-associated translation initiation factor eIF4G (Figure 1.1). This closed loop structure protects the transcript from exonucleases that may initiate degradation as well as increases translation efficiency by promoting multiple rounds of translation via ribosome recycling. Also due to the closed loop structure of the mRNA, regulatory molecules associated with the mRNA in the 3' untranslated region (3'UTR) are brought into close proximity to the cap, thus allowing for another level of control. In fact, micro RNAs (miRNAs) and regulatory proteins that regulate translation and/or stability of many mRNAs associate with the mRNA at the 3'UTR. miRNAs are nuclear encoded RNAs processed into small 20-22 nucleotide pieces by the DICER complex in most eukaryotic organisms, excluding *Saccharomyces cerevisiae*. The miRNAs will bind to complementary regions within a

target 3'UTR and repress translation of the mRNA. This is a regulatory mechanism that is more common in developmentally regulated mRNAs than once thought. miRNA-induced translational repression is thought to occur via interference with translation initiation, blocking the initiation complex from interacting with the cap (Meister 2007).

In both budding yeast *Saccharomyces cerevisiae* and mammals, the major pathway of mRNA decay begins by removal of the poly(A) tail by a deadenylase complex (Figure 1.2). In yeast, this complex includes two nucleases, Ccr4p and Pop2p, as well as a number of other factors, including Not1-5, Caf4, Caf16, Caf40, and Caf30 proteins (Parker and Song 2004). Pop2p and Ccr4 are widely conserved proteins. For most mRNAs, Ccr4p is the major deadenylase enzyme, while the Pop2p stimulates Ccr4p as well as serves a minor role in deadenylation. The Ccr4p/Pop2p complex is also inhibited by Pab1p, suggesting that this complex specifically seeks out mRNAs with a poly(A) tail lacking Pab1p. Another conserved deadenylase complex, PAN, consists of Pan2p and Pan3p. The PAN complex trims a number of A's from the poly(A) tail that varies based on the transcript (Parker and Song 2004). Thus, in one model, the poly(A) tail is shortened by PAN, while the major deadenylase complex is required for processive deadenylation of the mRNA. Unlike the Pop2/Ccr4 deadenylation complex, the PAN complex is stimulated by Pab1p, suggesting that this deadenylase prefers mRNA substrates with a Pab1p associated poly(A) tail (Figure 1.2).

After deadenylation, the mRNA is linearized and the 5' cap is removed by the decapping complex, including the proteins Dcp1p and Dcp2p. At this point, the mRNA is susceptible to rapid degradation by the Xrn1 5' to 3' exonuclease. Alternatively, after removal of the poly(A) tail, mRNA degradation may occur 3' to 5' via the exosome

complex (reviewed by Garneau et al. 2007, Parker and Song 2004). While not the focus of this dissertation, it is worth mentioning that degradation of aberrant mRNAs and some normal mRNAs occurs via nonsense-mediated decay (NMD, in the case of a premature stop codon), non-stop decay (in the absence of a stop codon) and no-go (decay of transcripts with stalled ribosomes) using additional decay factors (reviewed by Garneau et al. 2007, Doma and Parker 2006).

In many cell types, including yeast, neurons and oocytes, non-translating mRNAs and mRNA decay factors accumulate into cytoplasmic foci called processing bodies (P-bodies). The mRNA contained within these P-bodies can re-enter polysomes, and is thus thought to be in equilibrium between an mRNP aggregate and a polysome-associated translational state. P-bodies are induced by conditions that block translation (i.e. glucose depravation, stationary phase, conditional alleles of ribosomal proteins) as well as circumstances that block mRNA decay (i.e. mutation of decapping factors or Xrn1 exonuclease). Thus, when translation has ceased for reasons of stress or a reduced need for gene expression, the mRNPs are redirected to P-bodies for purposes of degradation and/or mRNA storage. After the cell returns to a translational state, the mRNA may be released to resume translation (reviewed by Parker and Sheth 2007). Decay factors not-included in the P-bodies include the deadenylation enzymes Ccr4 and Pop2. Thus, mRNA degradation within the P-body likely includes the steps of decay that occur after deadenylation. P-bodies may also be induced, independent of translation state, by destabilizing microtubules (Sweet et al. 2007). The importance of this is yet to be determined. Mammalian cells contain both P-bodies and stress granules. Stress granules are similar to P-bodies in that they are induced by stress and contain non-translating

mRNA, however they are distinct in that they do not contain mRNA decay factors (Sheth and Parker 2003, Kedersha and Anderson 2002).

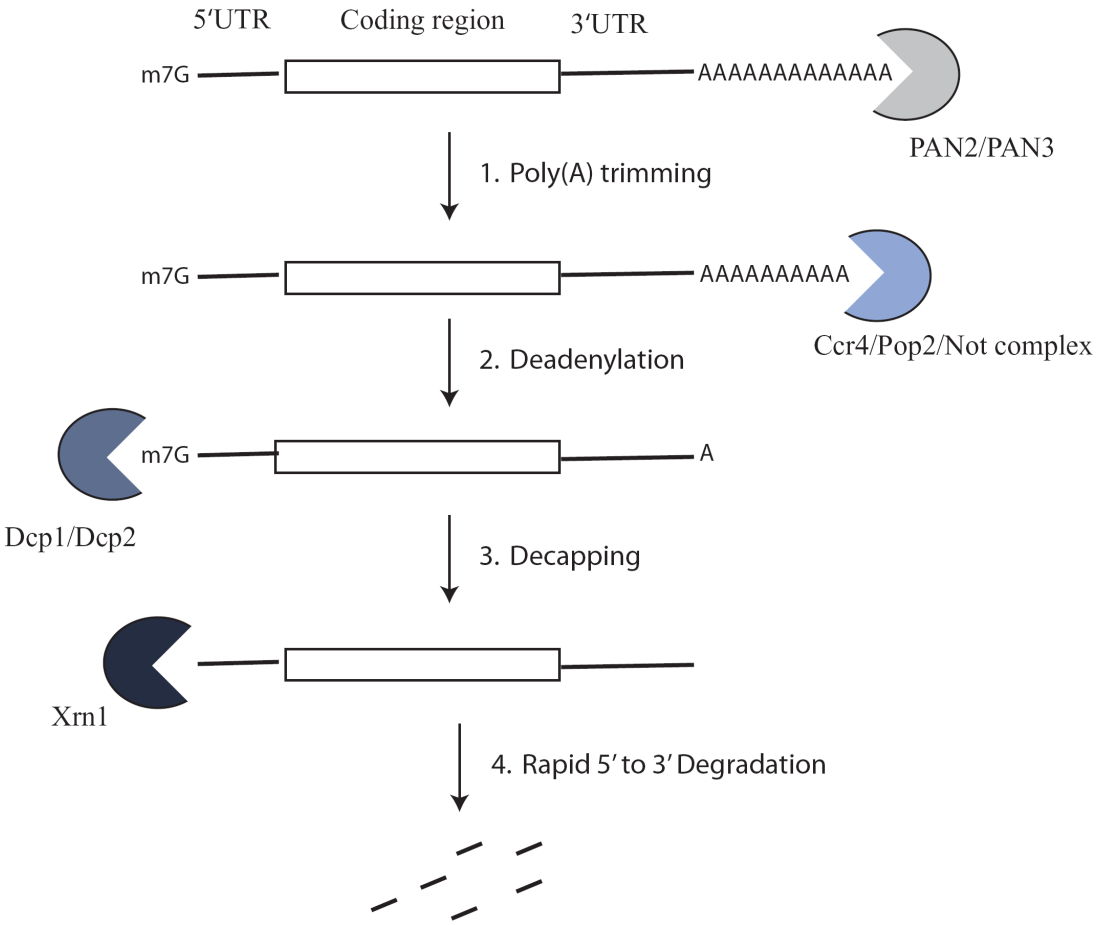


Figure 1.2. Major Pathway of mRNA decay.

Deadenylation-dependent mRNA decay occurs in four steps; poly(A) trimming, deadenylation, decapping, and 5' to 3' exonuclease digestion. The major factors involved in each step are illustrated. See text for more details.

For normal mRNAs, the stability varies greatly depending on the transcript and the cellular conditions. The focus of our research is to determine the factors that regulate transcript-specific and condition-specific mRNA stability. While the deadenylation and decapping steps of decay are highly regulated, the 5'-3' exonuclease decay is rapid and therefore unregulated. In many cases, mRNA stability determinants that influence deadenylation and decapping rates are found within the mRNA's 3'UTR. For example, AU-rich elements (AREs) are found in the 3'UTR of multiple eukaryotic mRNAs including cytokines, proto-oncogenes and transcription factors. The ARE may be bound by destabilizing factors, such as AUF, or if conditions are in favor of binding to HuR, the mRNA is stabilized (reviewed by Wilusz and Wilusz 2004). In higher organisms, maternal mRNAs in oocytes are stabilized during the early stages of development, driving developmental processes. When zygotic transcription initiates, elements within the 3'UTR provide the signal for maternal mRNA degradation (reviewed by Schier 2007). Other examples of 3'UTR elements affecting stability include the yeast *PGK1* mRNA, which has a stabilizing element within its 3'UTR (Ruiz-Echevarria et al. 2001; Decker and Parker 1993), and the *MFA2* mRNA, whose 3'UTR contains a destabilizing element (Muhlrad and Parker 1994).

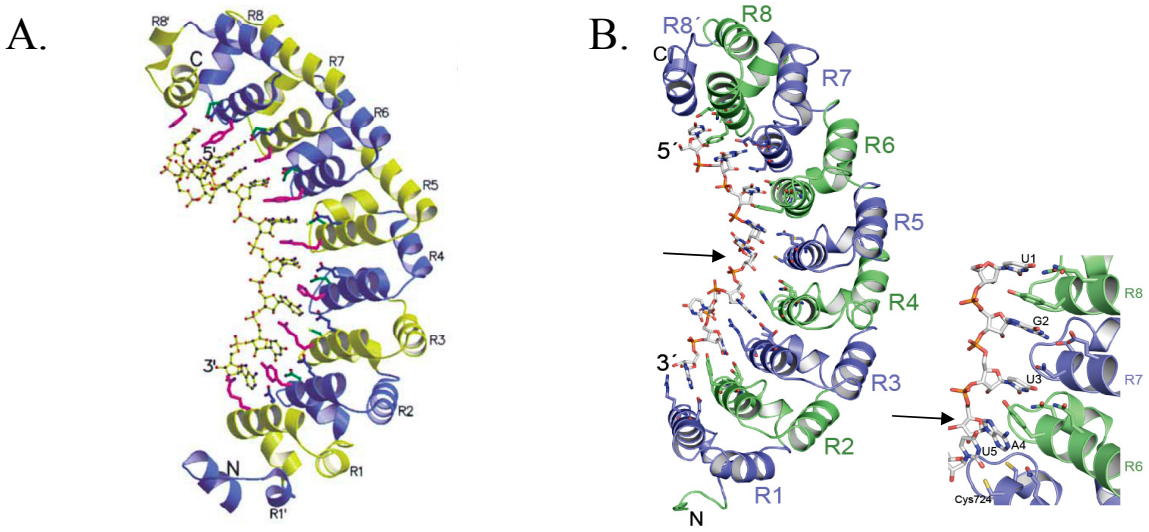
Eukaryotic Puf Proteins

Yet another example of 3'UTR stability elements, and the focus of this research, are Puf elements. All known Puf elements contain a UGUN sequence motif and are bound by a Puf protein (reviewed by Wickens et al. 2002). Puf proteins are a class of eukaryotic mRNA binding proteins that selectively bind specialized 3'UTR elements, stimulating deadenylation and inhibiting translation of target mRNAs. The Puf proteins

are characterized by a highly conserved core repeat domain (RD) consisting of eight imperfect repeats of three alpha-helices each, stacked into a crescent-shaped structure (Figure 1.3, Wang et al. 2001). Interestingly, the repeat domain of the protein, while it often makes up less than half of the total protein, is sufficient for both RNA binding and destabilization in all known cases (Zamore et al. 1997; Wharton et al. 1998; Jackson et al. 2004). A crystal structure of a human Puf, Pum1, bound to the *Drosophila* Puf protein (Pumilio) target mRNA (*hunchback, hb*) shows that the mRNA binds to the inner concave surface of the protein (Wang et al. 2002, Figure 1.3A). The mRNA-protein interactions include hydrogen bonding and base stacking interactions (Edwards et al. 2001).

The Pum1 crystal structure shows that Pufs bind 8 nucleotide 3'UTR sequences. Each of the Puf 3'UTR elements contains a UGUN sequence. The binding sites have been well dissected in several systems. It is apparent that although the UGU sequence is essential for Puf binding and regulatory activity, the surrounding sequences are variable. In yeast, all known examples show that Puf proteins bind to elements containing a UGUA sequence followed by an AU-rich sequence. Furthermore, SELEX experiments with Murine Pum2 found that this Puf also prefers UGUA sequences *in vitro* (White et al. 2001), and *in vitro* studies with the *Caenorhabditis elegans* Puf FBF-1 also found a clear preference for UGUA sequences (Bernstein et al. 2006). It is clear that the Puf elements are not interchangeable, however it is unclear what the sequence requirements are outside of the UGUA for Puf specificity. A crystal structure has recently been completed of the yeast Puf4p RD (Figure 1.3B, Miller et al. 2008). The crystal structures of Puf4p and Pum1 are remarkably similar except that while Pum1 RD directly contacts only 8

nucleotides, Puf4p RD takes on a wider angled curved structure and accommodates 9 nucleotides. The extra nucleotide of the Puf-element, U5, essentially "pops out" of the protein-RNA curve. Thus, instead of stacking interactions with Puf4p, nucleotide U5 stacks directly with nucleotide A4 and avoids hydrogen bonding with the Puf4RD, while nucleotide A6 resumes the expected RNA-protein interaction (Figure 1.3B, Miller et al. 2008). Interestingly, while only 8-9 nucleotides are apparent on the crystal structures, experimentally, it has been shown that Puf protein binding specificity depends on a 22 nucleotide region (Wickens et al. 2002; Jackson et al. 2004; Bernstein et al. 2006).



Human Pum1 *S. cerevisiae* Puf4RD

Figure 1.3. Structure of Puf proteins.

A. Crystal structure of human Pum1 repeat domain bound to *Drosophila* target *hb* mRNA (Wang et al. 2002). **B**. Crystal Structure of *S. cerevisiae* Puf4 repeat domain bound to *HO* mRNA (Miller et al. 2008). Arrows indicate extra nucleotide that is accommodated in Puf4RDp.

Puf-regulated processes

Work with the *Xenopus* Puf protein Pum2 has shown that it is involved in regulating oocyte meiotic cell cycle progression. The link between Pum2 and cell cycle regulation began with detailed analysis showing Pum2-mediated translational repression of *cyclin B1* mRNA (Nakahata et al. 2003). Later, it was shown *Xenopus* Pum2 also binds to and represses translation of RINGO/Spy mRNA, which codes for another translational regulator of *cyclin B1* mRNA (Padmanabhan and Richter 2006). More recently it has been shown that several individual cyclin mRNAs, in addition to *cyclin B1*, interact with and are translationally repressed by Pum2 in oogenesis. Interestingly, these same mRNAs are translationally regulated by cytoplasmic polyadenylation. Cytoplasmic polyadenylation element binding protein (CPEB) binds to 3'UTR cytoplasmic polyadenylation elements (CPEs) to mediate polyadenylation of maternal mRNA targets. The CPEB mediates both repression and stimulation of polyadenylation of target mRNAs. Stimulation of cytoplasmic polyadenylation will stimulate translational activation, however, the timing of CPEB-mediated repression and polyadenylation varies depending on the target mRNA. This variation is due to the number and orientation of 3'UTR CPEs as well as the combination of the CPEs with Pumilio binding elements (PBEs). The Pum2 target 3'UTRs contain one to three CPEs as well as one or two PBEs, with the number and position of each element varying in each mRNA 3'UTR. The proportion and orientation of CPEs and PBEs allows for unique levels and timing of translational repression. These unique CPE and PBE combinations are also found in many murine and human mRNA 3'UTRs. Furthermore, the CPE and PBE arrangement allows for predictable levels of translational regulation in

these higher organisms (Pique et al. 2008). The combinatorial nature of CPEB- and Pumilio-mediated translational regulation provides an example of the complexity of 3'UTR-mediated regulation. In addition, the finding that Pumilio regulates multiple mRNAs involved in oocyte cell cycle progression illustrates the importance of Puf proteins in regulating biological processes via regulating multiple mRNAs in related pathways.

There are two Puf proteins in humans. Several mRNA targets were predicted based on comparison with the *Xenopus* Pumilio mRNA target 3'UTRs. Among this group of potential target mRNAs, a significant portion code for proteins involved in cell cycle regulation (Pique et al. 2008). Evidence also suggests that human *Pum2* is involved in regulating the MAP kinase pathway, perhaps via multiple MAP kinase-related mRNAs (Lee et al. 2007). Human *Pum2* also stimulates neuronal stress granule formation while a *Pum2* knockdown prevents stress granule formation (Vessey et al. 2006), suggesting a role for Puf proteins in aggregation of non-translating mRNPs.

In *C. elegans*, the Puf proteins FBF-1 and FBF-2 regulate stem cell maintenance via regulation of *GLD1* mRNA (Crittenden et al. 2002). *C. elegans* FBF-1, FBF-2 and PUM8 are also involved in controlling the sperm-oocyte switch (Zhang et al. 1997; Bachorik and Kimble 2005). *Drosophila* Pumilio, Pum, regulates abdominal segmentation via the *hunchback* mRNA and anterior patterning via *bicoid* mRNA (Wharton and Struhl 1991, Murata and Wharton 1995, Gamberi et al. 2002). *Drosophila* Pumilio influences synaptic growth and function via regulation of the eukaryotic translation factor *eIF4E* mRNA (Menon et al. 2004). *Drosophila* Pumilio is also involved in long-term memory formation, synaptic plasticity, neuronal excitability, and

dendrite morphology (Dubau et al. 2003, Mee et al. 2004, Schweers et al. 2002, Ye et al. 2004). While the mRNAs specifically targeted by Pufs in these memory-related activities are still under investigation, the involvement of Pufs in these processes leads to many interesting and important roles for Pufs in neuronal and memory-related disease such as Alzheimer's and stroke.

Yeast Puf proteins

There are six Puf proteins in the budding yeast *S. cerevisiae* (Puf1p-Puf6p, Figure 1.4). Multiple mRNA targets have been identified for yeast Puf3 protein, each involved in mitochondrial function. The first and most well-known Puf3p target, *COX17* mRNA, codes for a copper metallochaperone for the mitochondria. *COX17* mRNA contains two Puf3p binding sites containing a UGUA within its 3'UTR. The presence of a single Puf binding element partially destabilizes *COX17* mRNA, while the presence of both is required for full destabilization (Jackson et al. 2004). Interestingly, *in vitro*, the two *COX17* Puf-elements have different binding affinities for Puf3p, however, *in vivo*, the sites equally contribute to Puf3p-mediated decay (Jackson et al. 2004). *HO* mRNA, which codes for an endonuclease involved in mate-type switching, is bound and destabilized by both Puf5p and Puf4p (Figure 1.4B, Tadauchi et al. 2001, Goldstrohm et al. 2006, Hook et al. 2007). Puf4p and Puf5p simultaneously bind their respective binding sites in the 3'UTR to fully destabilize the target mRNA (Hook et al. 2007). It is interesting that despite the fact that each Puf site contains a UGUA sequence that is essential for decay mediation, the sites are not interchangeable, suggesting once again that the regions flanking the UGUA elements are responsible for conferring specificity.

Puf6p represses translation of *ASH1* mRNA, which codes for a negative regulator of *HO* transcription (Gu et al. 2004).

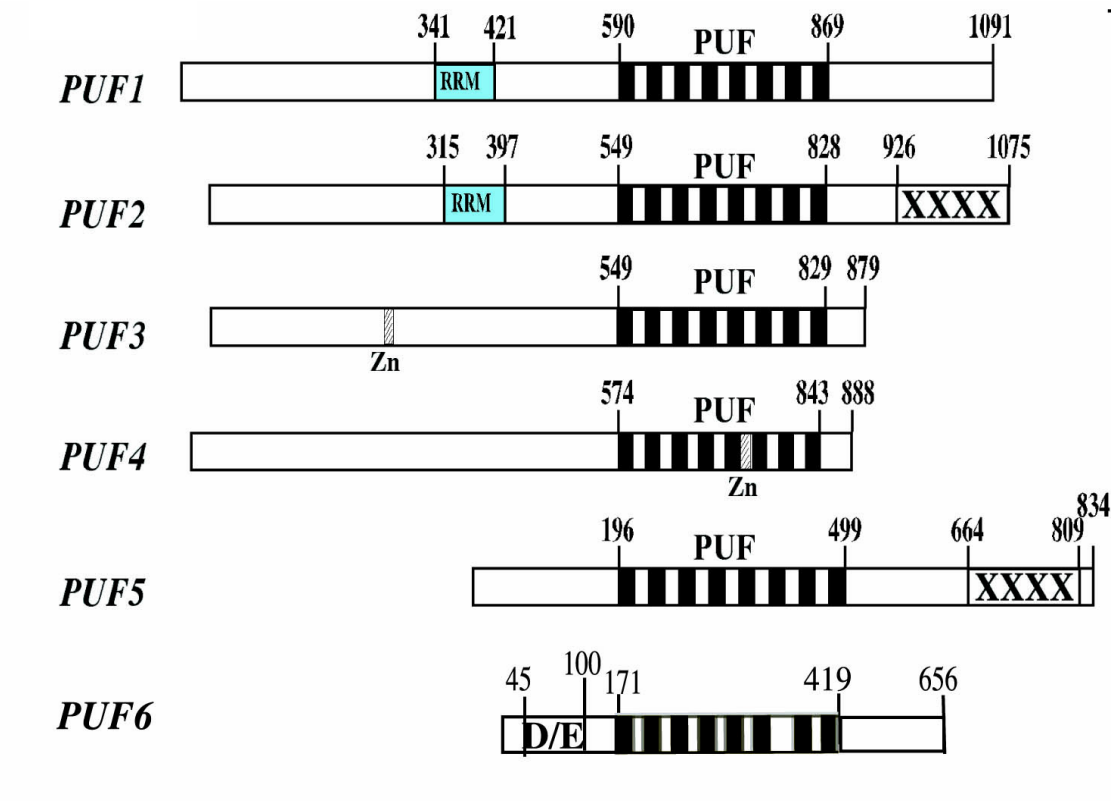


Figure 1.4. Schematic diagram of the *S. cerevisiae* Puf proteins.

Drawn to scale. The striped region represents the repeat domain of each protein. Puf1p and Puf2p contain a putative RNA recognition motif (RRM). Puf3p and Puf4p contain a zinc finger region (zn). Puf2p and Puf5p contain a region of homology (XXXX). Puf6p contains a glutamic acid and aspartic acid-rich region (D/E). Modified from Olivas and Parker 2000.

Evidence shows that Puf proteins stimulate deadenylation of target mRNAs. Both Puf4p and Puf5p have been shown to interact directly with Pop2p, a component of the deadenylation machinery, and require Pop2p to stimulate rapid deadenylation of *HO* mRNA (Goldstrohm et al. 2006, Goldstrohm et al. 2007, Hook et al. 2007). Puf5p-mediated deadenylation requires the active site of Ccr4p, and depends on the presence of Pop2p, but not on Pop2p activity (Goldstrohm et al. 2006, Goldstrohm et al. 2007). Interestingly, while Ccr4p appears to be the only deadenlylase activated by Puf5p, Ccr4p is not required for Puf5p-mediated repression of gene expression, suggesting that there are mechanisms other than deadenylation (i.e. decapping, translation) involved in Puf5p-mediated mRNA regulation (Goldstrohm et al. 2006, Goldstrohm et al. 2007). Unlike Puf5p, Puf4p requires the presence of both Pop2p and Ccr4p as well as the deadenylase functions of at least Ccr4p to repress expression and stimulate deadenylation of *HO* mRNA (Hook et al. 2007). This leads to the current model in which Puf4 and Puf5 proteins recruit the deadenlyase complex to the *HO* target 3'UTR via interaction with Pop2p to stimulate rapid deadenylation and decay of the mRNA (Figure 1.5). While Puf4p acts primarily through deadenylation, Puf5p recruits additional factors via Pop2p to represses mRNA expression through decapping and/or translation (Figure 1.5). Previous studies have shown that *CCR4* and *POP2* are required for Puf3p-mediated decay of *COX17* mRNA (Tucker et al. 2002). Studies in the Olivas lab suggest that Puf3p also interacts with Pop2p and Ccr4p, however a direct interaction between either of these factors and Puf3p is still being investigated (Lopez Leban, Houshmandi and Olivas, unpublished studies). In addition to interaction with the deadenylation factors, Puf3p also interacts with Dhh1p, a protein that stimulates both decapping and deadenylation of a

subset of mRNAs. Thus, Dhh1p is a potential link between Puf3p-mediated stimulation of decapping that is perhaps independent of Puf3p-mediated deadenylation (Figure 1.5). In summary, Puf function appears to occur via Puf binding to 3'UTR Puf-elements, which then recruits decay machinery to the mRNA, initiating rapid deadenylation and decay of the mRNA. However, each Puf protein may act differently, recruiting alternate protein partners that affect alternate aspects of gene expression.

Previous to this work, there were not any confirmed mRNA targets for Puf1p and Puf2p. The sequence homology of the yeast Pufs to one another, and to the Pufs of higher organisms suggested that Puf1p and Puf2p also accelerate decay and repress translation of a subset of mRNAs. In fact, one large-scale study of yeast Pufs 1-5 predicted multiple mRNA targets for Puf1p and Puf2p. In this study, researchers identified mRNAs that were physically associated with Pufs by immunopurifying tagged Puf1-5 proteins, then isolating the mRNA that co-purified with each protein. This study identified over 800 mRNAs that associated with at least one Puf protein, 40 for Puf1p and 146 for Puf2p. Many of the mRNAs co-purified with multiple Puf proteins, particularly the Puf1p and Puf2p targets. 36 of the 40 Puf1p targets co-purified with Puf2p (Gerber et al. 2004). The finding that many Puf1p and Puf2p targets may overlap is not surprising, considering that Puf1RDp and Puf2RDp are the most similar of the yeast Pufs, having 45% identity.

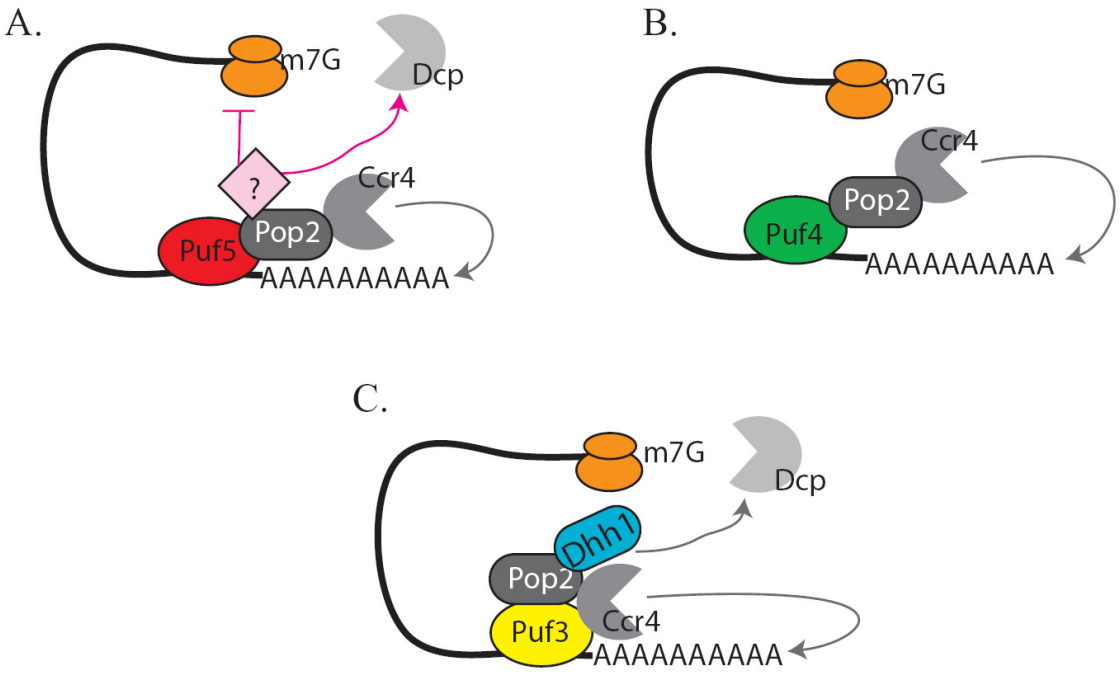


Figure 1.5. Model of Puf4p-, Puf5p-, and Puf3p-mediated repression of target mRNAs.

Puf5p (**A**) and Puf4p (**B**) are modeled to stimulate deadenylation of the *HO* mRNA via a direct interaction with Pop2p. The Ccr4p is the major deadenylase and is recruited to the mRNA via the Pop2p interaction. In addition, Puf5p represses *HO* mRNA expression by stimulating decapping or repressing translation via interaction with Pop2p. While it is unclear yet which interactions are direct, Puf3p (**C**) interacts with multiple decay factors to stimulate deadenylation and repress translation of its target mRNAs.

The Puf-RNA co-purification study not only suggested many potential target mRNAs for each Puf protein, but also suggested that Puf proteins regulate particular cellular activities by regulating subsets of mRNAs coding for related proteins. For example, Puf3p was found to preferentially associate with mRNAs encoding mitochondrial proteins, suggesting a role for Puf3p in regulating mitochondrial function (Gerber et al. 2004). Since this study, multiple mitochondrial-related transcripts have been verified as targets of Puf3p mediated mRNA decay (unpublished studies, Miller and Olivas). Similarly, Puf4p preferentially associated with mRNAs encoding nucleolar components, Puf5p with mRNAs encoding nuclear components and Puf1p and Puf2p associated with mRNAs encoding plasma membrane associated proteins (summarized in Table 1.1, Gerber et al. 2004).

The idea that Puf proteins regulate related sets of mRNAs is supported by a separate project that, through a novel computational algorithm, examined >700 existing microarray data sets to identify groups of mRNAs that are coordinately regulated under the conditions tested and also contain common 3'UTR elements. The results of this study predicted that mRNAs containing a Puf3p or Puf4p 3'UTR binding element are regulated according to cellular conditions like carbon source and growth phase (Foat et al. 2005). In the case of mRNAs with a Puf3p element, this prediction has been experimentally verified in that *COX17* mRNA is stabilized in ethanol conditions, despite the presence of Puf3p protein (Foat et al. 2005). Thus, Puf3p is a condition-specific regulator of mitochondrial related transcripts and mitochondrial function, downregulating the mRNAs when mitochondria function is not as vital (dextrose conditions) and upregulating mRNAs when fermentation is required (ethanol conditions). Despite a lack of Puf3p

activity under certain conditions including ethanol, Puf3 protein and *PUF3* mRNA are present (unpublished studies, Lopez Leban and Olivas). In addition, Puf activation and deactivation is rapid, occurring in as little as two minutes (unpublished studies, Miller and Olivas). Thus, Puf3p activity is likely be altered by condition-related post-translational modification. We have evidence that Puf3p is phosphorylated, leading to a model in which Puf activation/deactivation occurs via serine/threonine phosphorylation of the RD. In the previously described study of *Xenopus* Pum, Pum regulation of RINGO/Spy mRNA is also conditional, dependent upon progesterone levels. In this system, Pum binding to the mRNA is altered by progesterone treatment (Padmanabhan and Richter, 2006). If this model is conserved, under conditions repressing yeast Puf3p, the protein may not be able to bind target mRNAs. This possibility is currently under examination by other members of the Olivas lab.

Both the computational study and the co-purification study predicted consensus 3'UTR binding sequences for Puf3p and Puf4p (Table 1.1). A similar Puf5p consensus site was predicted from the co-purification study (Table 1.1), however, these same studies were unable to determine consensus sequences for Puf1p or Puf2p. The predicted sequences are consistent with all studied examples of yeast Puf protein mRNA targets, requiring a UGUN-containing Puf recognition sequence. The known mRNA binding sequences within the yeast Puf targets *COX17* and *HO* 3'UTR elements are no exception in that each site contains an essential UGUA sequence (Jackson et al. 2004, Tadauchi et al. 2001, Goldstrohm et al. 2006, Hook et al. 2007). These predictions aided in the identification of additional Puf3p target mRNAs (unpublished data, Miller and Olivas). These predicted and experimentally verified Puf binding sites further suggest that each

Puf 3'UTR element contains an essential UGUA core sequence. In both the predicted sequence and verified sequences for Puf3p, there is a cytosine residue present two nucleotides upstream from the core UGUA, however, this cytosine is lacking in Puf5/Puf4p target mRNA *HO*. The placement of the downstream UA sequence also appears to be distinct between Puf3p mRNA targets and Puf5p mRNA targets. Other than these observations, there is no obvious similarity or difference that makes a Puf3p site distinct from a Puf5p site.

While previous studies have been unable to classify roles for Puf1p or Puf2p in mRNA decay, *PUF1* (also known as *JSN1*) was previously found to be a suppressor of a *tub2* temperature-sensitive mutant, suggesting a role in microtubule stability (Machin et al. 1995). More recently, Puf1p was found to be associated with the mitochondria, specifically mitochondrial morphology and movement via the ARP2/3 complex (Fehrenbacher et al. 2005). It is not yet known whether these functions are independent of or related to mRNA metabolism. Since the conserved Puf repeat domain occupies only about 1/3 of the total Puf1 protein, it is possible that the protein has diverse roles related to its different functional regions.

Table 1.1. Summary of co-precipitation and computational studies with the yeast Puf proteins.

	Co-precipitation (Gerber et al. 2004)		Computational Algorythm (Foat et al. 2005)	Experimentally Verified
PUF	**Functional enrichment[a]**	**Consensus Sequence**	**Consensus Sequence**	**Target sequence**
Puf1	Plasma membrane	nd	nd	nd
Puf2		nd	nd	nd
Puf3	Mitochondrial			CUUGUAUAUAUA CCUGUAAAUAUG [b,c]
Puf4	Nucleolar			UGUGUAUAUUA[d]
Puf5	Nuclear		nd	UUGUAUGUAAU[e]

[a] Pufs association with mRNAs is enriched for mRNAs related to described locations or processes.

[b] *COX17* 3'UTR mRNA Puf-element

[c] Olivas and Parker 2000, Jackson et al. 2004

[d] Hook et al. 2007

[e] Tadauchi et al. 2001

Dissertation Overview

The research presented in this dissertation is a combination of genetic, biochemical and molecular techniques used to characterize roles of the yeast Puf proteins in mRNA decay and translational repression. As mentioned, previously published studies had not identified mRNA targets of Puf1p and Puf2p decay regulation. This work is a continuation of my Master's thesis where I developed a pool of potential target mRNAs and began work to identify bona fide targets of mRNA decay, focusing on potential targets of Puf1p and Puf2p. In this dissertation, I show work that further examines these target mRNAs, with the goal of identifying the cis-elements and trans-factors responsible for Puf-mediated decay.

Previous Studies

In my M.S. thesis project, potential mRNA targets of Puf-mediated decay were identified from a pool of mRNAs developed from the previously described computational and co-purification studies, as well as a microarray study that compared expression profiles of WT yeast and yeast deleted of *PUF1-PUF5* (Olivas and Parker 2000). I chose to examine potential mRNA targets from these studies as well as mRNAs that contain a potential Puf 3'UTR sequence element. Because there have been no mRNA targets confirmed for Pufs 1 and 2, I focused on mRNAs associated with these Pufs and on mRNAs that appeared to act coordinately with other targets in a cellular pathway. For example, *PMP1*, *PMP2*, *PMP3*, and *AST1* mRNAs were all associated with Puf1p and/or Puf2p and encode membrane-associated proteins involved in proton transport. Prior to completion of the M.S. thesis and also since its completion, I have tested 22 mRNA candidates in our decay assay, including nine associated with Puf1p and/or Puf2p, six associated with Puf5p, and eight from the *PUF* deletion microarray (Table 1.2). Decay

profiles of each candidate mRNA were then compared between a wild-type *PUF* strain (WT) and strains deleted either individually of *PUF*s 1-5 or a quintuple *PUF* deletion strain (Δ*puf1-5*). For most mRNAs, we detected no changes in half-lives in the *PUF* deletion strains under the conditions tested (Table 1.2). *COX17* mRNA, a known target of Puf3p regulation, was used as a control in these experiments. For many of the mRNAs, including an additional 18 transcripts not listed in Table 1.2, steady-state mRNA analysis was also performed comparing mRNA levels between WT and individual *PUF* deletion strains at optical density (OD) $_{600}$ of 0.4 or 1.0. Only 7 transcripts displayed any significant differences in steady-state levels - the *HXK1, TIF1,* and *YHB1* mRNAs, as well as the *PMP1*, *PMP2* and *PMP3* mRNAs (data not shown). Surprisingly, exhaustive half-life analysis of *PMP* mRNAs in the *PUF* deletion strains that had revealed changes in mRNA steady-state levels, showed no changes in mRNA decay rates (Table 1.2). Overall, these results suggest that i) there were many false positives and/or indirect target mRNAs identified by the microarray screens, ii) these mRNAs are targeted by Pufs only under particular growth conditions not yet tested, iii) there is redundant control of these mRNAs by multiple Pufs, or iv) certain mRNAs are physically associated with Pufs for a purpose not related to mRNA stability.

Table 1.2. RNAs Tested for Puf-Mediated Regulation of mRNA Stability

			OD_{600}[b]										
			0.4					1.0					
RNA	*Source*	Physically-Associated Puf[a]	puf1Δ	puf2Δ	puf3Δ	puf4Δ	puf5Δ	puf1Δ	puf2Δ	puf3Δ	puf4Δ	puf5Δ	Δpuf1-5
AME1	Olivas and Parker, 2000; Gerber et al. 2004	Puf5	-	-	-		-						
CBC2	Motif Search		-	-	-	-							
COX15	Gerber et al. 2004	Puf3, Puf5			-								
COX17	Olivas and Parker, 2000; Gerber et al. 2004	Puf3	-		+			-	-	+	-	-	
DHH1	Gerber et al. 2004	Puf1, Puf2, Puf5	-		-		-						
GCN4	Olivas and Parker, 2000							-	-	-	-	-	-
GLK1	Olivas and Parker, 2000							-	-	-	-	-	-
HXK1	Motif Search		+		-	+	+	+	-	-	+	+	+
HXK2	Olivas and Parker, 2000							-					-
MIG1	Gerber et al. 2004	Puf1, Puf2		-									
MSN2	Motif Search		-									-	-
MSN4	Gerber et al. 2004	Puf2	-	-	-	-	-	-	-		-	-	-
NOP1	Gerber et al. 2004	Puf1, Puf4, Puf5	-	-	-	-	-						
NUP100	Gerber et al. 2004	Puf2, Puf5	-	-	-	-	-						
PET117	Olivas and Parker, 2000; Gerber et al. 2004	Puf3, Puf5		-	-								
PMP1	Gerber et al. 2004	Puf2	-	-	-	-	-						
PMP2	Gerber et al. 2004	Puf2			-	-							
PMP3	Olivas and Parker, 2000; Gerber et al. 2004	Puf1, Puf2	-	-	-	-	-						
PUF1	Gerber et al. 2004	Puf1, Puf2		-									
TIF1	Olivas and Parker, 2000		-	-	-		-	+	-	-	-	+	+
TPK1	Motif Search		-	-	-	-	-						
YHB1	Olivas and Parker, 2000							-	-	-	-	+	+

[a]The Puf protein(s) shown to physically interact with particular RNAs are indicated (Gerber et al. 2004).

[b] Northern blots were prepared from transcriptional shut off experiments of WT and *PUF* deletion yeast and probed for the indicated RNA. No significant effect on stability compared to WT is indicated by (-). A significant difference in stability compared to WT is denoted by (+).

Three mRNA targets are the focus of this dissertation: *YHB1, TIF1* and *HXK1*. In the completed M.S. thesis, I showed differential steady state expression for each of these mRNAs between WT and *PUF* deletion yeast. For *YHB1* and *TIF1* mRNAs, the steady state expressions also varied between two different conditions, mid log phase (OD_{600} 0.4) and late log phase (OD_{600} 1.0). *TIF1* and *YHB1* each showed differential expression in the absence of *PUF2,* but only in the OD_{600} 1.0 condition, the more stressful condition. Based on these results and the fact that Puf3p is a conditional regulator of mRNA decay (Foat et al. 2005), we predict that all Pufs elicit some type of condition-specific regulation.

Next in the thesis, I fused the 3'UTR of each potential target to the coding region of a Puf-neutral mRNA, *MFA2* (Figure 1.6). It has been shown that the 3'UTRs of Puf targets, including *HO* and *COX17,* are sufficient to mediate decay (Jackson et al. 2004; Tadauchi et al. 2001). In these fusion analyses, I discovered that neither *YHB1* nor *TIF1* 3'UTR caused a difference in decay of the *MFA2* fusion mRNAs in *puf2*Δ yeast, however, the *MFA2/TIF1* 3'UTR fusion mRNA was slightly stabilized in *puf5*Δ versus WT yeast, suggesting a role for Puf5p in regulating *TIF1* mRNA (Figure 1.6B). I was able to show through *in vivo* experiments that both UGUA elements in the *TIF1* 3'UTR are required for full regulation of the *MFA2/TIF1* 3'UTR transcript, while mutation of only the first element leads to a partial decay phenotype. At the completion of this thesis, decay of *MFA2/TIF1* 3'UTR mRNA decay was only tested in WT, *puf2*Δ and *puf5*Δ yeast.

In addition, while I did show that *TIF1* 3'UTR was subject to Puf5p-mediated decay regulation, *PUF* deletions had no effect on decay of the *MFA2/YHB1* fusion

transcript (Figure 1.5C). However, through *in vitro* binding analysis, I showed that purified GST-Puf1RDp, GST-Puf2RDp, GST-Puf3RDp, and GST-Puf5RDp all bind *YHB1* 3'UTR specifically. Moreover, the UGUA Puf element is required for specificity of Puf binding.

HXK1 mRNA 3'UTR was also fused to the *MFA2* coding region to test for Puf-mediated decay regulation. While the *MFA2/HXK1* mRNA had a slightly longer half-life in *puf1*Δ yeast than WT yeast, the difference was slight and error was high, reflecting inconsistency in the experiments (Figure 1.6A). This construct did not have a decay phenotype in the *PUF3* or *PUF4* deletion strains. Through *in vitro* experiments, I was able to show Puf1RDp binding the *HXK1* mRNA 3'UTR.

In continuation of this earlier work, as described in the following chapters, I have further examined these mRNA targets through a variety of methods to i) confirm that they are targets of Puf mediated decay, ii) investigate the possibility that additional Pufs, not previously tested, regulate these mRNAs and iii) identify factors required for Puf-mediated decay regulation.

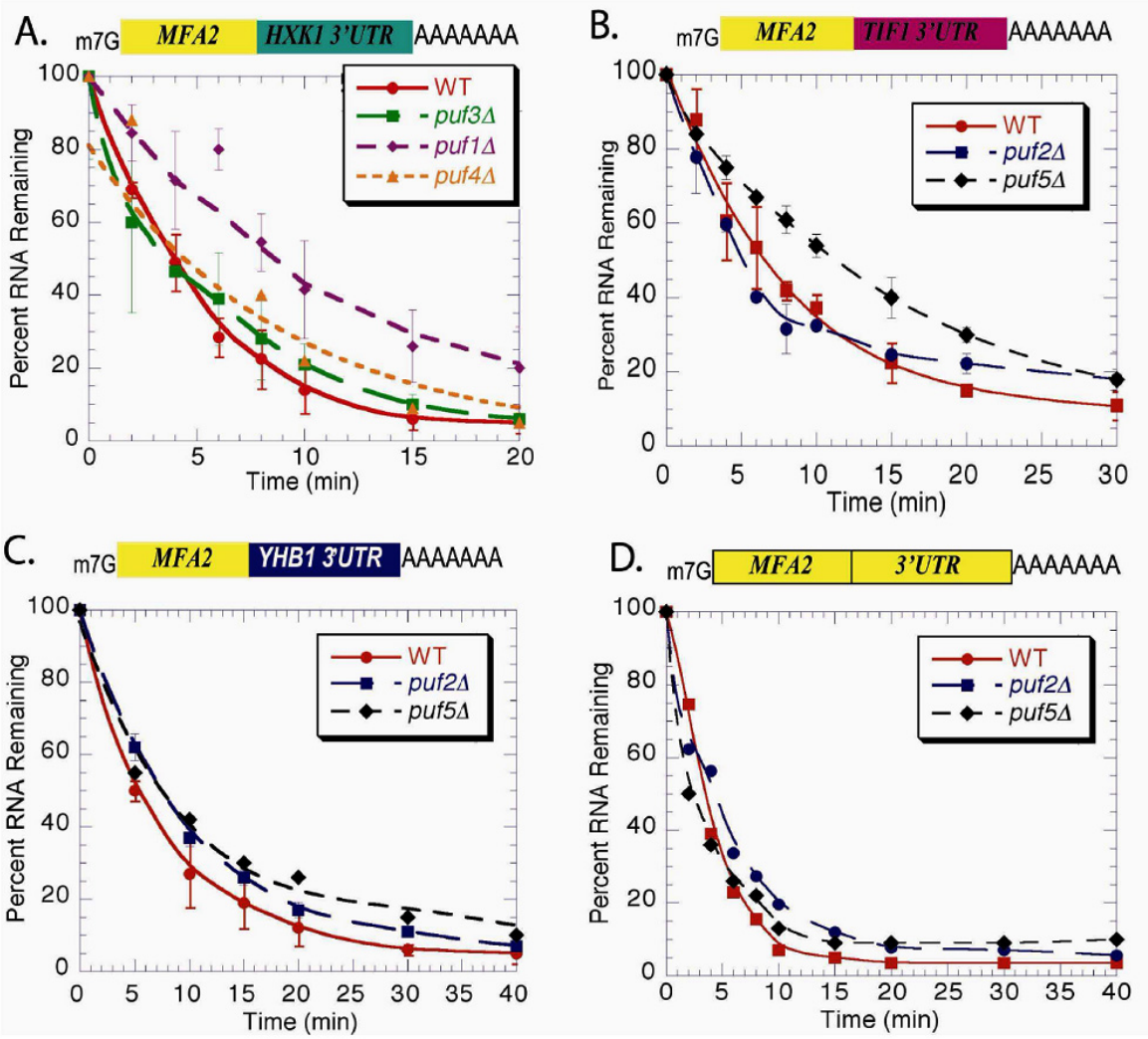


Figure 1.6. Decay of *MFA2*/3'UTR Chimera mRNAs.

Each 3'UTR of interest was fused to the *MFA2* coding region and the rate of decay monitored in WT and *PUF* deletion yeast (see Chapter 2). **A**. *HXK1* 3'UTR. **B**. *TIF1* 3'UTR. **C.** *YHB1* 3'UTR. **D**. Control, *MFA2* 3'UTR.

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CHAPTER II:

GENERAL METHODOLOGY

CHAPTER II: GENERAL METHODOLOGY

The information contained within this chapter is a general overview of experimental procedures used in the dissertation studies. More details for each method can be obtained from the chapter in which the method is used.

Yeast Strains, Plasmids and Oligonucleotides

All yeast strains used in this study are listed in Table 2.1. All plasmids used in this study are listed in Table 2.2. All oligonucleotides are listed in Table 2.3.

Table 2.1. Yeast strain used in this study.

Deletion	Strain	Genotype	Source
Wild-type	yWO3	MATa, *his4-539*, *leu2-3, lys2-201, trp1-1, ura3-52*	Hatfield et al. 1996; yRP683
Wild-type	yWO5	MATa, *leu2-3, lys2-201, trp1-1, ura3-52, cup1::LEU2/PM*	Hatfield et al. 1996; yRP840
Wild-type	yWO7	MATα, *leu2-3, ura3-52, rpb1-1*	Caponigro et al. 1993; yRP693
*pop2*Δ	yWO12	MATα, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM, pop2::URA3*	Tucker et al. 2001; yRP1617
*ccr4*Δ	yWO13	MATa, *his4-539, leu2-3, trp1-1, ura3-52, cup1::LEU2/PM , ccr4::NEO*	Tucker et al. 2001; yRP1616
*puf2*Δ	yWO14	MATa, *his4-539*, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*, *puf2::URA3*	Olivas & Parker, 2000; yRP1237
*puf2*Δ	yWO15	MATa, *his4-539*, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*, *puf2::TRP1*	This study
*puf5*Δ	yWO17	MATa, *his4-539*, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*, *puf5::TRP1*	Olivas & Parker, 2000; yRP1240
*puf1*Δ	yWO20	MATa, *his4-539*, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*, *puf1::NEO*	Olivas & Parker, 2000; yRP1243
*puf4*Δ	yWO22	MATa, *leu2-3, lys2-201, trp1-1, ura3-52, cup1::LEU2/PM, puf4::LYS2*	Olivas & Parker, 2000; yRP1245
*puf1*Δ *puf3*Δ *puf4*Δ *puf5*Δ	yWO30	MATa, *his4-539*, *leu2-3, lys2-201, trp1-1, ura3-52, cup1::LEU2/PM*, *puf1::NEO, puf3::NEO, puf4::LYS2, puf5::URA3*	Olivas & Parker, 2000; yRP1259
*puf3*Δ	yWO43	MATα, *his4-539*, *leu2-3, trp1-1, ura3-52, rpb1-1, cup1::LEU2/PM*, *puf3::NEO*	Olivas & Parker, 2000; yRP1360
*puf2*Δ	yWO48	MATα, *his4-539*, *leu2-3, ura3-52, rpb1-1, puf2::URA3*	This study
*puf5*Δ	yWO49	MATα, *leu2-3, trp1-1, ura3-52, rpb1-1, puf5::URA3*	This study
*puf1*Δ	yWO102	MATa, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*,	This study

		puf1::NEO	
Wild-type	yWO104	MATa, *his4-539, leu2-3, lys2-201, ura3-52, rpb1-1*	This study
*puf4*Δ	yWO105	MATα, *his4-539*, *lys2-201, ura3-52, rpb1-1, puf4::LYS2*	This study
*puf4*Δ	yWO106	MATa, *his4-539*, *leu2-3, lys2-201, rpb1-1, puf4::LYS2*	This study
*puf2*Δ*puf5*Δ	yWO198	MATα, *leu2-3, trp1-1, ura3-52, rpb1-1, puf2::TRP1, puf5::URA3*	This study
Δ*puf1-5*	yWO204	MATα, *his4-539, leu2-3, lys2-201, trp1-1, ura3-52*, *rpb1-1, puf1::NEO, puf2::TRP1, puf3::NEO, puf4::LYS2,puf5::URA3*	This study
*puf1*Δ*puf5*Δ	yWO208	MATa, *leu2-3, trp1-1, ura3-52, rpb1-1*, *puf1::NEO, puf5::URA3*	This study
Wild-type	yWO211	MATα, *his3, leu2, lys2, ura3*	Goldstrohm et al 2006; BY4742
*pop2*Δ	yWO212	MATα, *his3, leu2, lys2, ura3, pop2::KAN*	Goldstrohm et al 2006
*ccr4*Δ	yWO213	MATa, *his3, leu2, lys2, ura3, ccr4::KAN*	Goldstrohm et al 2006
*ccr4*Δ*pan2*Δ	yWO227	MATa, *his4-539, leu2-3, trp1, ura3-52, cup1::LEU2*, *ccr4::NEO, pan2::URA3*	Tucker et al. 2001

Table 2.2. Plasmids used in this study.

Plasmid	Description	Marker(s)	Source
pWO21	pBS-PUF2RD	*AMP*	This study
pWO22	pGEX-PUF2RD	*AMP*	This study
pWO24	pGAL-MFA2pG	*URA3, AMP*	Decker and Parker 1993; pRP485
pWO27	pGAL- MFA2/HXK1 3'UTR	*URA3, AMP*	This study
pWO48	pBS-PUF1RD	*AMP*	This study
pWO49	pGEX-PUF1RD	*AMP*	This study
pWO53	pGAL -MFA2/*tif1-1* 3'UTR	*URA3, AMP*	This study
pWO54	pGAL -MFA2/*tif1-1* 3'UTR	*LEU2, AMP*	This study
pWO58	LEU, CEN Vector	*LEU2, AMP*	Brachmann et al. 1998; pRS415
pWO61	pGAL-MFA2pG	*LEU2, AMP*	This study
pWO70	pGAL -MFA2/TIF1 3'UTR (WT)	*URA3, AMP*	This study
pWO71	pGAL -MFA2/TIF1 3'UTR (WT)	*LEU2, AMP*	This study
pWO72	pGAL -MFA2/*tif1-2x* 3'UTR	*URA3, AMP*	This study
pWO73	pGAL -MFA2/*tif1-2x* 3'UTR	*LEU2, AMP*	This study
pWO88	pGAL -MFA2/*tif1-2* 3'UTR	*URA3, AMP*	This study
pWO89	pGAL -MFA2/*tif1-2* 3'UTR	*LEU2, AMP*	This study
pWO94	pBS-HXK1 3'UTR	*AMP*	This study
pWO100	pGAL-PGK1/HXK1 3'UTR (WT)	*URA3, AMP*	This study
pWO101	pGAL-PGK1/HXK1 3'UTR (WT)	*LEU2, AMP*	This study
pWO102	pGAL-PGK1	*URA3, AMP*	Heaton et al. 1992; pRS227
pWO103	pGAL-PGK1	*LEU2, AMP*	This study
pWO109	pGAL –MFA2/*tif1-p3E* 3'UTR	*URA3, AMP*	This study
pWO110	pGAL –MFA2/*tif1-p3E* 3'UTR	*LEU2, AMP*	This study

pWO111	pHO-HIS3/HO 3'UTR	*URA3, AMP*	Goldstrohm et al. 2006; yCP33
pWO114	pG1-FLAG-PUF1	*URA3, AMP*	This study
pWO115	pG1-FLAG-PUF1RD	*URA3, AMP*	This study
pWO116	p415-GPD-PUF4	*URA3, AMP*	Hook et al. 2007
pWO117	p416-MET25-YGFP	*URA3,AMP*	Li and Kaplan 1998
pWO118	p415-MET25	*LEU2,AMP*	This study
pWO119	p416-MET25	*URA3,AMP*	This study
pWO122	pMET25-HIS3/HXK1 3'UTR (WT)	*LEU2, AMP*	This study
pWO123	pMET25-HIS3/hxk1-3x 3'UTR	*LEU2, AMP*	This study
pWO124	pMET25-HIS3/HXK1 3'UTR (WT)	*URA3, AMP*	This study
pWO125	pMET25-HIS3/hxk1-3x 3'UTR	*URA3, AMP*	This study
pWO126	pGAL-PGK1/HXK1 (WT)	*TRP1, AMP*	This study
pWO127	pGAL-PGK1/YHB1 (WT)	*URA3, AMP*	This study
pWO128	pGAL-PGK1/YHB1 (WT)	*LEU2, AMP*	This study
pWO129	pGAL-PGK1/yhb1	*URA3, AMP*	This study
pWO130	pGAL-PGK1/yhb1	*LEU2, AMP*	This study
pWO131	pMET25-HIS3/hxk1-1 3'UTR	*LEU2, AMP*	This study
pWO132	pMET25-HIS3/hxk1-1 3'UTR	*URA3, AMP*	This study
pWO133	pMET25-HIS3/hxk1-2x 3'UTR	*LEU2, AMP*	This study
pWO134	pMET25-HIS3/hxk1-2x 3'UTR	*URA3, AMP*	This study
pWO135	pADH-POP2 (WT)	*ZEO*	Goldstrohm et al. 2007
pWO136	pADH-pop2	*ZEO*	Goldstrohm et al. 2007

Table 2.3. Oligonucleotides used in this study.

Oligo	Description	Sequence
oWO21	*scRI* Probe	GTCTAGCCGCGAGGAAGG
oWO105	*HXK1* probe	CATAAGGGCATCACTCATAAG
oWO136	*PUF2RD* Up Primer	CGCGGATCCCCTCCACCATCATTATCGGATAGT
oWO137	*PUF2RD* Down Primer	TCTGCCCGGGAAACAGAAACGCCTCTGGC
oWO144	*PUF1RD* Up Primer	CCCGGATCCGAATTCGCAAATTCCGATGAATACCAAATCAATTCG
oWO145	*PUF1RD* Down Primer	CCCCCGCCGGCGCAGCTGCGAAATGCTGCTGTTATGATGCTGC
oWO153	*HXK1 3'UTR* Down Primer	CCGAAGCTTCCGAGCTATCCTACGACTTTC
oWO159	*YHB1* probe	CGCCTAAACTTGCACGGTTGAC
oWO164	*HXK1 3'UTR* Up Primer	CCCAGATCTCTTGGTATCATTGGCGCTTAATG
oWO231	*TIF1 3'UTR* Down Primer	CGCGAAGCTTCTCTATACAAGGCAGAGGG
oWO238	*MFA2* Probe	ATATTGATTAGATCAGGAATTCC
oWO239	*TIF1* 3'UTR Up Primer	CCGAAGCTTCTCTATACAAGGCAGAGGG
oWO249	*TIF1* 3'UTR Probe	CAACCTTCGTGCCGAGAGTC
oWO262	*YHB1* 3'UTR Up Primer	GGCAGATCTGTCAACCGTGCAAGTTTAG
oWO263	*YHB1* 3'UTR Down Primer	CCGAAGCTTGCTTCCATGACAGGTTCCG
oWO310	*TIF1* SDM[a] Primer #1	GGTTGAAATACCCTATACTAATTGTTTGCTTTCTCTTTTACACTAT ATCCGAACGTATCTATCTGAAATTTTTC
oWO311	*TIF1* SDM Primer #2	GAAAAATTTCAGATAGATACGTTCGGATATAGTGTAAAAGACAAA GCAAACAATTAGTATAGGGTATTTCAACC
oWO329	*HXK1* site #1 SDM Up	CTTGGTATCATTGGCGCTTAATGAAAAAAAACACATGAAATATAA ATGTGTTTTTCCCTCCC
oWO330	*HXK1* site #1 SDM Down	GGGAGGGAAAAACACATTTATATTTCATGTGTTTTTTTTCATTAAG CGCCAATGATACCAAG
oWO430	*TIF1* SDM P3E Up Primer	CTAAAAAGTTATATATGCTTCTTGTATATATATTGTTTTTCTTTTTA CATTCCTATTATTCTTCAAAAGTCCAAAAGACTC
oWO431	*TIF1* SDM P3E Down Primer	GAGTCTTTTGGACTTTGAAGAATAATAGGAATGTAAAAAGAAAAA CAATATATATACAAGAAGCATATATAACTTTTTAG

oWO447	*PGK1* Probe	CCAAAGAAGCACCACCACCAGTAGAG
oWO448	*HXK1* site #2 SDM Up	GATGCCCTTATGTTTTTTTTGCGGTCTAGTATAACAAAATATAGAC ACACACATATATATATATTTATG
oWO449	*HXK1* site #2 SDM Down	CATAAATATATATATATGTGTGTGTCTATATTTTGTTATACTAGAC CGCAAAAAAAACATAAGGGCATC
oWO450	*HXK1* site #3 SDM Up	GTAAATATAGACACACACATATATATATATTTATGACAATAATCC TAGCTAATAAACATTTTTAGATTGTTATTAG
oWO451	*HXK1* site #3 SDM Down	CTAATAACAATCTAAAAATGTTTATTAGCTAGGATTATTGTCATAA ATATATATATATGTGTGTGTCTATATTTAC
oWO466	*PUF1* Up Primer	CGGGATCCGATGGATAAAAGTAAGCAGATGAACATC
oWO467	*PUF1* Down Primer	ACGCGTCGACGGCGCCGCTTCCCTGCTAGTTGGACAC
oWO468	*PUF1RD* Up Primer (B)	CGGGATCCGATGGCAAATTCCGATGAATACCAAATCAATTCG
oWO476	*HXK1* 3'UTR Up Primer (B)	GCCAGATCTGCGGCCGCGGATCCCTTGGTATCATTGGCGCTTAAT G
oWO480	*YHB1* SDM Up	GTGGAATATTTAGATAGTAAGTAAAGATTGACAAACAATTTATAA GATGAATAAGCGCCAG
oWO481	*YHB1* SDM Down	CTGGCGCTTATTCATCTTATAAATTGTTTGTCAATCTTTACTTACTA TCTAAATATTCCAC

* SDM = site-directed mutgenesis

Steady State Transcriptional Shutoff

To determine mRNA half-lives, steady state transcriptional shutoff experiments were performed essentially as described (Caponigro et al. 1993). Yeast strains containing a temperature-sensitive lesion in RNA polymerase II (*rpb1-1*) were grown overnight at 24ºC, the temperature in which transcription is active. Upon reaching an OD_{600} of 0.4 (mid-log phase) or OD_{600} of 1.0 (late-log phase), the yeast were pelleted and resuspended in media at 37ºC, the temperature in which transcription of mRNA is inhibited. Samples were taken from the culture at the time of transcription inhibition (time 0) and at increasing minutes following incubation at 37ºC by quick centrifugation of the cells (15 sec) and quick freezing cell pellets on dry ice (Figure 2.1A). Total RNA was prepared from each sample and subjected to Northern analysis. Specific mRNAs on each Northern blot were visualized with a radiolabeled oligonucleotide complementary to the mRNA of interest. All Northern blots were normalized to the stable RNA Polymerase III *scR*I RNA (Felici et al. 1989). The time in which half of a particular mRNA species remains from the steady-state level (time 0) prior to transcription inhibition defines the half-life ($T_{1/2}$) of that mRNA. mRNA half-lives were compared between wild-type (WT) yeast and yeast deleted of one or more *PUF* genes (*puf*Δ).

In order to assay the role of only the 3'UTRs of *HXK1* and *YHB1* in Puf-mediated regulation, these 3'UTRs were fused to the *PGK1*Δ82 coding region, which itself is not regulated by Pufs, but has been shown to allow regulation by alternative 3'UTR elements (Heaton et al. 1992). The *TIF1* mRNA 3'UTR was fused to the *MFA2* coding region, which is also not regulated by Pufs, but can be put under the control of alternative 3'UTRs. Each 3'UTR was amplified by polymerase chain reaction (PCR) from genomic

DNA and inserted into the appropriate vector behind either the *MFA2* or *PGK1*Δ82 coding regions. In each of these vectors, transcription of the fusion mRNAs is under the control of the inducible *GAL10* promoter. *rpb1-1* yeast strains containing the mRNA expression vectors were grown overnight at 24ºC in media containing galactose to specifically induce transcription. At optical density (OD) $_{600}$ 0.4 (mid-log phase) or OD_{600} 1.0 (late-log phase), the yeast were pelleted and resuspended in media containing dextrose at 37ºC to not only repress transcription from RNA Polymerase II, but also specifically repress transcription from the *GAL10* promoter (Figure 2.1B). Control experiments of *PGK1*Δ82 and *MFA2* with their native 3'UTRs under the control of the *GAL10* promoter were also performed in a similar manner.

To assay the requirement of 3'UTR UGUA elements on mediation of mRNA decay, the sites were altered to eliminate Puf recognition. Sequences surrounding the *TIF1* 3'UTR UGUA site #1 were also altered to assay binding specificity of Puf proteins. Alteration of these regions was accomplished by PCR-based site-directed mutagenesis (Stratagene) or a random error in amplification. These mutated 3'UTR constructs were then treated to transcriptional shutoff experimentation. For more details, see Materials and Methods of Chapters III, V and VI.

Hxk1p Enzyme Assay

In order to assay the affect of *PUF* gene deletions on *HXK1* protein production, the activity of Hxk1p (Hexokinase) was determined using an assay based on glycolysis (Walsh et al. 1991). The reaction and its products are depicted in Chapter IV, Figure 4.2. Yeast extracts were prepared from WT and *puf*Δ yeast grown to an OD_{600} of 1.0. Fructose, Phosphoglucose Isomerase (PGI, Roche) and Glucose-6-P Dehydrogenase

(G6PDH, Roche) were added to the yeast extracts. Levels of the reaction product, NADPH, were measured by comparing absorbance λ_{340} with the control (extract alone). See Chapeter IV for more details.

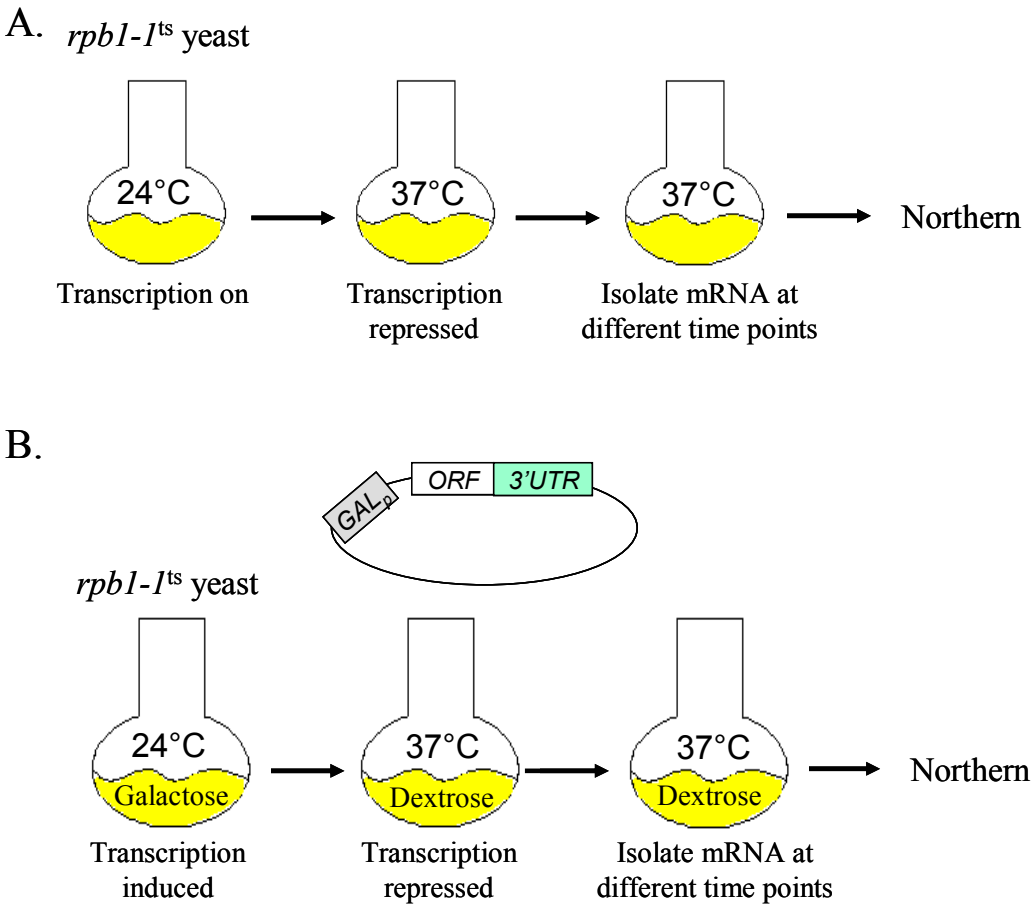


Figure 2.1. Steady state transcriptional shutoff assays.

Illustration of transcriptional shutoff assays. **A**. Transcriptional shutoff experiment with temperature-sensitive *rpb1-1* yeast strains involves shifting yeast cultures from 24ºC to 37ºC to repress transcription. **B**. Transcriptional shutoff experiments in *rpb1-1* yeast containing a fusion construct under the control of the GAL promoter, are performed by shifting from the carbon source in the culture from galactose to dextrose in addition to shifting temperature as in A. See text for more details.

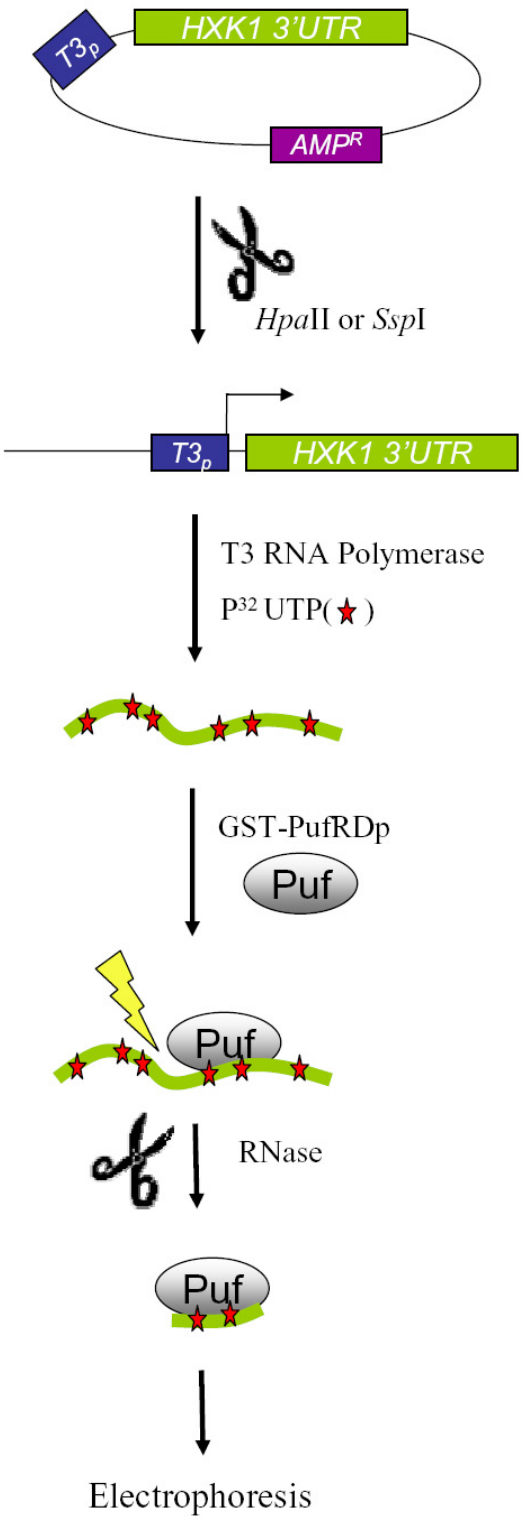
In vitro Binding

To analyze Puf protein binding to the mRNA target sequence, Puf proteins were first expressed from fusion constructs in *E. coli* to produce and purify Glutathione S-transferase (GST) tagged proteins. GST-Puf3RDp and GST-Puf5RDp were produced as previously described (Jackson et al. 2004). GST-Puf1RDp and GST-Puf2RDp expression constructs were produced by amplifying the *PUF1RD* from genomic DNA then cloning the fragment into pGEX-6p-1 (Amersham Biosceiences). The expression constructs were transformed into BL-21, protease deficient *E. coli,* for GST-fusion expression and purification. The GST-PufRDp was purified as recommended by the manufacturer (Amersham Biosciences).

The *HXK1* 3'UTR target RNA for *in vitro* binding was produced by cloning the 3'UTR fragment into pBluescript (pBS) and linearizing the plasmid with *Hpa*II (to produce full-length 3'UTR) or *Ssp*I (to produce truncated 3'UTR) prior to transcription. The radiolabeled RNA was produced by incubating the linearized DNA with T3 RNA polymerase (Ambion) in the presence of ^{32}P-UTP. The radiolabeled transcript was purified from incomplete reaction products via polyacrylamide gel electrophoresis (PAGE) and gel extraction, then incubated in the presence or absence of purified GST-PufRDp. The protein was crosslinked to the RNA using UV light and the unbound RNA digested with RNase TI. Radiolabeled protein was separated and visualized by PAGE (summarized in Figure 2.2.)

Figure 2.2. *In Vitro* Binding Assay.

Illustration of UV cross-linking experiment used to determine RNA binding ability of purified GST-PUF fusion protein *in vitro*. See text for details.



Puf1p Expression in Yeast

To express full-length Puf1p and Puf1RDp in yeast, the *PUF1* ORF and internal fragment containing *PUF1RD,* respectively, were amplified from genomic DNA. The PCR products were inserted into the vector pAV72 in frame following the FLAG tag sequence. In this vector, the *FLAG-PUF* fusion gene is under the control of the strong, constitutive GPD promoter.

Repression Assays

In order to assay Puf-mediated repression of *HXK1* 3'UTR, an assay was developed using the *HIS3* gene as a reporter. The repression assay experimental setup and rationale are summarized in Figure 2.3. The *HIS3/HXK1* mRNA expression plasmid was constructed by first cloning the weak *MET25* promoter into pRS415 (*LEU2*/CEN vector). The *HXK1* 3'UTR was then cloned into this vector. *HIS3* was removed from previously described yCP33 (Goldstrohm et al. 2006.) and cloned between the *HXK1* 3'UTR and *MET25* promoter to create a plasmid that will express *HIS3/HXK1 3'UTR* mRNA at a low level. *HIS3/hxk* mutant mRNA expression vectors were created by replacing the *HXK1* 3'UTR of the *HIS3/HXK1* 3'UTR construct with the *hxk1-1, hxk1-2x* and *hxk1-3x* mutant 3'UTRs. The mutant 3'UTRs were created by PCR-based site-directed mutagenesis (Strategene) to eliminate potential Puf elements. The *HIS3/3'UTR* constructs on the *LEU2*/CEN vector were co-transformed into yeast with pAV72 (vector), Full-length *PUF1* (pWO114), or *PUF1RD* (pWO115). In order to co-transform with the *PUF4* expression vector, the *MET25/HIS3/HXK1* fragment was transferred to a *URA3*/CEN vector. The *his3* mutant yeast strains yWO211 (WT), yWO212 (*pop2*Δ), and yWO213 (*ccr4*Δ) were used in these repression assays. Expression of the *HIS3/HXK1*

mRNA will allow growth of the *his3* mutant yeast in media lacking histidine. However, if translation of the mRNA is repressed by *PUF* overexpression, growth of the *his3* mutant yeast will be repressed (Figure 2.3). WT and mutant Pop2p was also expressed in the *HIS3* reporter system to determine the importance of Pop2p catalytic activity in Puf-mediated decay. Each Pop2 construct is expressed from a vector carrying a zeocin resistance marker, the constitutive *ADH1* promoter, and N-terminal His_6 tag (Goldstrohm et al. 2007). Transformants were selected on synthetic media containing 100mM Zeocin (Invivogen).

Five μl of a series of four 10-fold serial dilutions of each cell culture was plated on synthetic minimal media with histidine (control) or without histidine and the *HIS3* specific inhibitor 3-aminotriazole (3-AT) to minimize background levels of *HIS3*. After 3 days at 30ºC, the plates were removed and photographed.

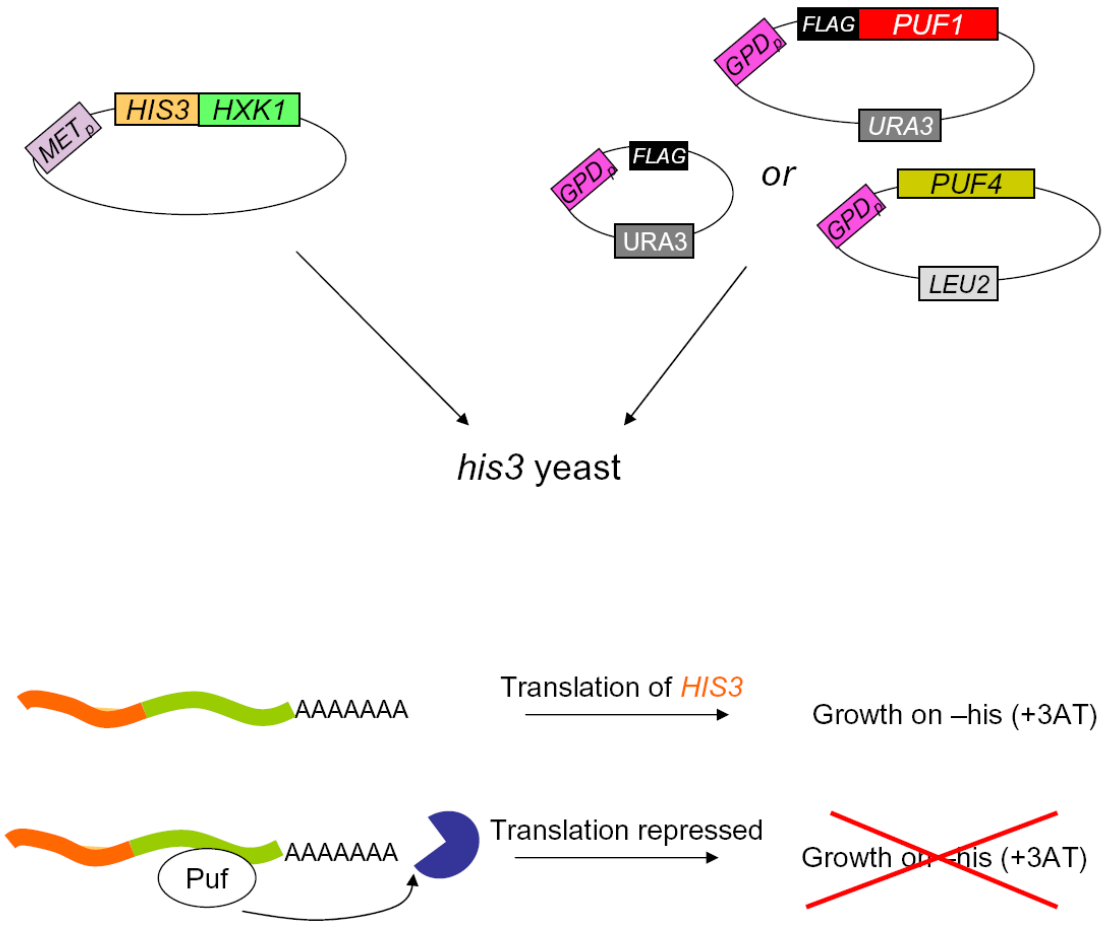


Figure 2.3. Repression Assays.

Illustration of repression assays in which *PUF* (or control vector) are co-transformed with a *HIS3* fusion construct into *his3* mutant yeast to assay Puf-mediated repression of *HIS3* fusion mRNA. See text for details.

Transcriptional Pulse-Chase

Transcriptional pulse-chase experiments were performed essentially as described (Decker and Parker 1993) in order to monitor the rate of deadenylation of the *PGK1/HXK1* mRNA. The *PGK1/HXK1* expression plasmid was transformed into WT (yWO5), *ccr4*Δ (yWO13), *puf1*Δ (yWO20), *pop2*Δ (yWO12) and *puf1*Δ*puf3*Δ*puf4*Δ*puf5*Δ (Δ4pufs, yWO30) yeast. Strains were grown overnight in media containing 2% raffinose and 0.2% sucrose, conditions that do not induce transcription from the *GAL UAS*. When the culture reached mid-log phase (OD_{600} of 0.4), the cells were pelleted and resuspended in 20ml media containing 4% galactose to induce transcription of a pulse of *PGK1/HXK1* mRNAs. After 7 min, transcription was repressed by pelleting the cells and resuspending in dextrose-containing media. Samples were collected prior to galactose addition, immediately following dextrose addition (time 0), and at increasing minutes following dextrose addition. Total RNA was extracted from yeast sampled during the course of the pulse-chase (Figure 2.4). In order to visualize the length of the *PGK1/HXK1* mRNA poly(A) tail, the mRNA was hybridized to a DNA oligonucleotide specific for the *PGK1* portion of the mRNA. The RNA of this RNA/DNA hybrid was then cleaved with RNaseH and the mRNA fragments were separated by PAGE (Figure 2.4). The RNA was transferred to a nylon membrane for Northern analysis. The 3'UTR and poly(A) tail from the *PGK1/HXK1* mRNA were visualized using a radiolabeled DNA oligonucleotide specific for the *HXK1* 3'UTR. 3'UTR mRNA lacking a poly(A) tail was created as a control by hybridization of RNA to oligo poly-d(T) in addition to the PGK1-specific oligonucleotide prior to RNaseH cleavage.

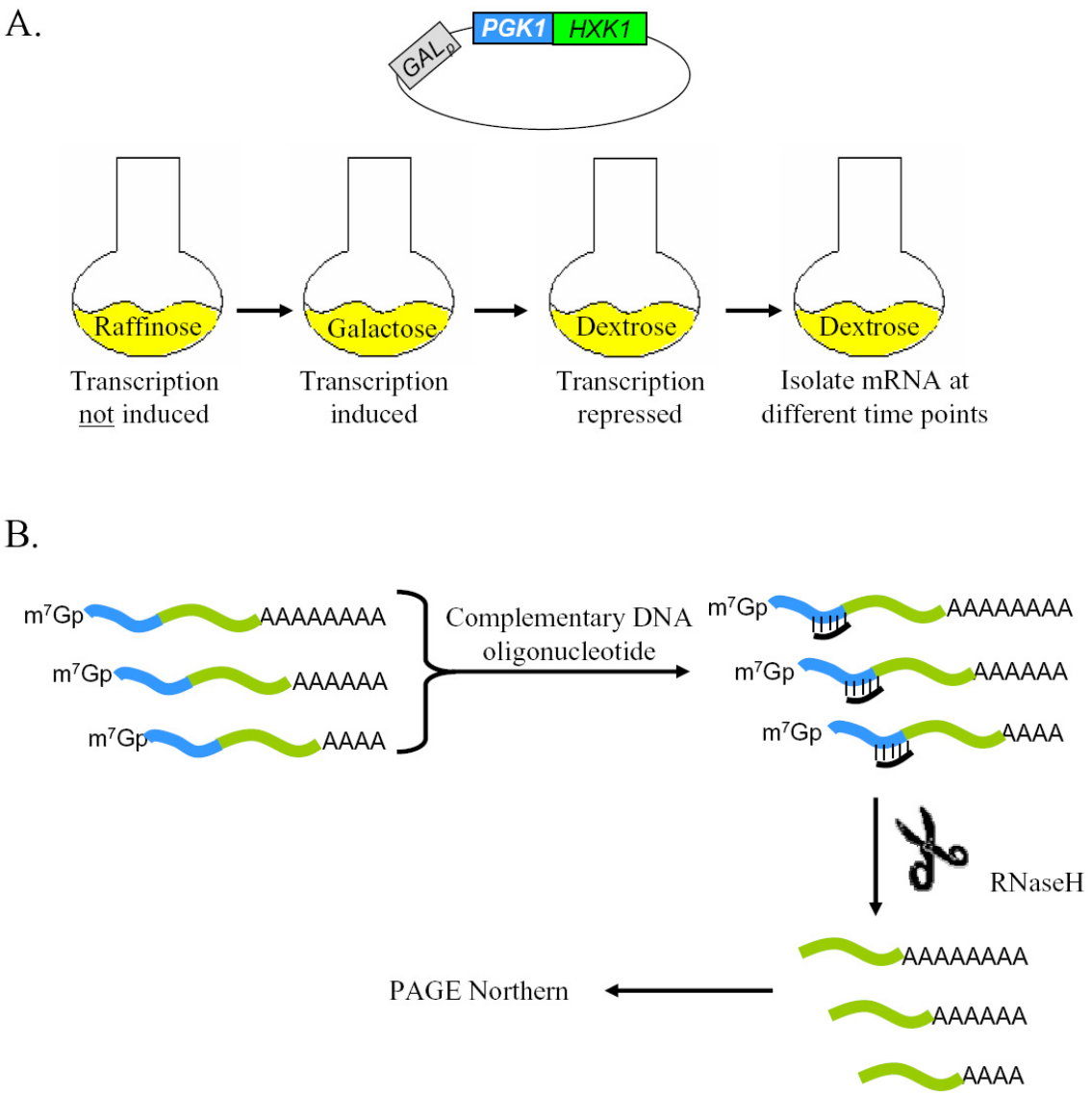


Figure 2.4. Transcriptional pulse-chase assay.

A. Transcriptional pulse-chase experiment measures the deadenylation rate of fusion mRNAs expressed from a plasmid under the control of a GAL promoter. Raffinose to galactose to dextrose carbon source shifts are necessary to grow cells under non-inducing conditions, create pulse of newly-transcribed mRNAs, and then to repress transcription from the GAL promoter. **B**. The steps necessary to visualize poly(A) tail distributions of mRNA isolated from the pulse-chase experiment in A. See text for more details.

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CHAPTER III:

TIF1 mRNA IS REGULATED BY MULTIPLE Puf PROTEINS

CHAPTER III: *TIF1* mRNA IS REGULATED BY MULTIPLE Puf PROTEINS

In my M.S. thesis work, I determined that the 3'UTR of *TIF1* mRNA is regulated by Puf5p but not Puf2p. However, since I had not yet assessed the effects of the remaining Puf proteins on the stability of the *MFA2/TIF1 3'UTR* mRNA, further testing was necessary. In this chapter I describe the results of extensive testing of Puf-mediated *TIF1* mRNA stability. In addition, I also characterize the 3'UTR sequence elements important for Puf-mediated decay and specificity through *in vivo* experimentation. The entirety of this work has been published (Ulbricht and Olivas 2008).

TIF1 mRNA is targeted for mRNA decay by Puf1 and Puf5

The *TIF1* mRNA was originally identified as a potential Puf target mRNA in the microarray screen comparing RNA levels between the WT and Δ*puf1-5* strains, with *TIF1* showing a 3.5-fold higher mRNA level in the quintuple deletion than in WT yeast (Olivas and Parker 2000). The *TIF1* 3'UTR contains two UGUA elements, potential sites of Puf interaction (Figure 3.1A). Interestingly, in our steady-state analysis of *TIF1*, altered RNA levels were detected from cells harvested at the higher cell density, OD_{600} 1.0, but not at OD_{600} 0.4 (data not shown). To investigate the role of Puf proteins in the decay of *TIF1*, I performed transcriptional shut-off assays at OD_{600} 1.0 to determine its half-life in WT versus *PUF* deletion strains. I found endogenous *TIF1* mRNA to be very stable with a half-life >30 min. Our attempts to assay the effect of *PUF* deletions on half-life were inconsistent, presumably due to the extended duration of stressful conditions in high cell densities required to assay changes in long half-lives. Therefore,

to avoid underestimating or missing changes in its decay rate in *PUF* deletion strains and also to focus on Puf protein control of mRNA decay via the potential 3'UTR binding elements in *TIF1*, I cloned the 3'UTR of *TIF1* mRNA behind the coding region of *MFA2*. Previous studies have shown that fusion of the 3'UTR of *COX17* to the *MFA2* ORF is sufficient for Puf-regulated decay of this fusion construct (Jackson et al. 2004). The *MFA2/TIF1* 3'UTR fusion was expressed from a plasmid under the transcriptional control of the *GAL* upstream activating sequence (UAS, Decker and Parker 1993). Thus, in addition to a temperature shift to disable the temperature-sensitive RNA polymerase II in these strains, transcription was also inhibited by changing the carbon source from galactose to dextrose.

In WT yeast, the *MFA2/TIF1* 3'UTR fusion mRNA decayed with a half-life of 7.0 +/- 0.6 min (Figure 3.1B). In *puf2*Δ, *puf3*Δ and *puf4*Δ yeast strains, the half-life of the *MFA2/TIF1* fusion mRNA was similar to that in WT (Figure 3.1B). However, compared to WT the *MFA2/TIF1* mRNA decayed slower in the *puf1*Δ and *puf5*Δ strains, with half-lives of 9.6 +/- 0.4 and 11.2 +/- 0.8 min, respectively (Figure 3.1B). Conversely, *MFA2* mRNA with its native 3'UTR decayed similarly in WT and each *PUF* deletion strain, including the *puf1*Δ and *puf5*Δ strains (Figure 3.1C and data not shown). Thus, both Puf1p and Puf5p stimulate mRNA decay via the *TIF1* 3'UTR.

While the difference in half-lives between WT and either single *PUF1* or *PUF5* deletion strain was small, there was a more dramatic effect on the *MFA2/TIF1* mRNA half-life in the double deletion strain, *puf1*Δ*puf5*Δ (Figure 3.1B). The half-life in this strain was 16.3 +/- 1.2 min, >2-fold slower than WT. Thus, the presence of either Puf1p or Puf5p is necessary and sufficient to accelerate mRNA decay through the *TIF1* 3'UTR,

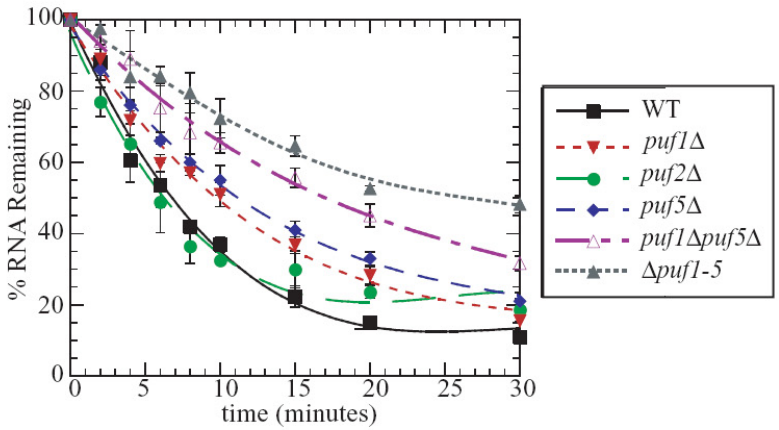
but the presence of both Pufs provides maximal decay stimulation. In contrast, the native *MFA2* mRNA decayed similarly between WT yeast and strains deleted of multiple *PUF* genes (Figure 3.1C), again indicating that Puf-mediated decay is dependent on elements in the *TIF1* 3' UTR. The *MFA2/TIF1* mRNA decayed even slower in the quintuple deletion (Δ*puf1-5*) with a half-life of 23.8 +/- 3.9 min. Therefore, it is likely that other Pufs may play small compensatory roles in the regulation of *TIF1* mRNA decay.

The results shown in Figure 3.1B and C illustrate *MFA2/TIF1* mRNA decay in yeast grown to an OD_{600} of 1.0 prior to transcription inhibition, as this was the cell density that promoted differences in *TIF1* mRNA steady-state levels. When the decay assays were performed under lower cell density (transcription inhibition at an OD_{600} of 0.4), the half-life in the WT strain (10.0 +/- 1.5 min) was extended compared to the same strain under higher cell density conditions (compare Figure 3.1B WT and 3.1D WT), indicating that Puf activity is altered under these conditions. The half-life in the *puf1*Δ*puf5*Δ strain remained similar between OD_{600} 0.4 (16.0 +/- 1.0 min) and OD_{600} 1.0 (16.3 +/- 1.2 min). Decay assays in the individual *PUF* deletion strains at OD_{600} 0.4 did not show discernable differences in decay of *MFA2/TIF1* mRNA versus WT yeast (Table 1.1). These results suggest that Puf1p and/or Puf5p activity is condition-specific, having greater activity under higher cell density conditions. Previous reports have indicated that Puf proteins are subject to condition-specific regulation. Conditions that are predicted to affect Puf activity include stationary phase and the diauxic shift (Foat et al. 2005). Each of these conditions may account for the altered Puf activity observed in the higher density cultures.

A. UAAGAACUAAGGGAGACAAAAACUUCUUAAAAAAGUAUCUUGUGAGGCUAUCUUGAUAACGUAACAUAUAUUUCUAAA
stop
AAGUUAUAUAUGCUUUUGUAUUUAAUUGUUUUUCUUUUUACAUUCCUAUUAUUCUUCAAAGUCCAAAAGACUCUC
site #1
GGCACGAAGGUUGAAAUACCCUAUACUAAUUGUUUGCUUUGUCUUUUUGUAUAUACCCGAACGUAUCUAUCUGAAAUU
site #2
UUUCAAAUUUAUAAAAAAUAAUAUAUAUAAAAGGAAAAAAAAAAAAAAUCGAUGCCCUACAAA

B. *MFA2/TIF1* mRNA (OD_{600} 1.0)

time (min) 0 2 4 6 8 10 15 20 30 40	$T_{1/2}$
WT	7.0 +/- 0.6
puf1Δ	9.6 +/- 0.4
puf2Δ	7.0 +/- 1.2
puf3Δ	8.3 +/- 0.3
puf4Δ	8.3 +/- 1.3
puf5Δ	11.2 +/- 0.8
puf1Δpuf5Δ	16.3 +/- 1.2
Δpuf1-5	23.8 +/- 3.9



C. *MFA2* mRNA (OD_{600} 1.0)

time (min) 0 2 4 6 8 10 15 20 30 40	$T_{1/2}$
WT	4.7 +/- 0.6
puf1Δ	5.8 +/- 0.8
puf5Δ	4.0 +/- 0.2
puf1Δpuf5Δ	6.2 +/- 0.6
Δpuf1-5	6.5 +/- 0.92

D. *MFA2/TIF1* mRNA (OD_{600} 0.4)

time (min) 0 2 4 6 8 10 15 20 30 40	$T_{1/2}$
WT	10.0 +/- 1.5
puf1Δpuf5Δ	16.0 +/- 1.0

Figure 3.1. *TIF1* 3'UTR is regulated by Puf1p and Puf5p.

A. Sequence of the *TIF1* 3'UTR. Underlined regions (site #1 and site #2) are proposed sites of Puf interaction. UGUA sequences are in bold. UGUN sequences are shaded gray. The length of the *TIF1* 3'UTR was estimated through PAGE analysis of the 3'UTR after removal of the poly(A) tail. **B**. Decay of *MFA2/TIF1 3'UTR* fusion mRNA in wild-type (WT), individual PUF deletion, and multiple *PUF* deletion yeast strains grown to an OD_{600} of 1.0. Representative Northern blots are presented in the left panel. Data from the Northern analyses are plotted in the right panel. Minutes following transcription repression are indicated above blots and along the x-axis of the graph. Decay was measured in the following yeast strains: WT (black, closed square), *puf1*Δ (red, closed upside-down triangle), *puf2*Δ (green, closed circle), *puf3*Δ (not graphed), *puf4*Δ (not graphed), *puf5*Δ (blue, closed diamond), *puf1*Δ*puf5*Δ (purple, open triangle) and Δ*puf1-5* (gray, closed triangle). **C.** Decay of *MFA2* mRNA with its native 3'UTR in the same yeast strains and conditions as B. **D**. Decay of *MFA2/TIF1* mRNA in WT and *PUF* deletion yeast strains grown to mid-log phase (OD_{600} of 0.4). The estimated $T_{1/2}$ is listed to the right of each representative Northern blot. For B and C, error for each data point and/or $T_{1/2}$ is the SEM ($n \geq 3$). For D, error is the range ($n = 2$).

Two UGUA elements in the *TIF1* 3'UTR are required for Puf1p and Puf5p-mediated decay

Previous co-precipitation data indicated that 32% of the mRNA targets bound to Puf5p contained the consensus sequence of (U/A)UGUA(A/U)(C/U)(A/U)(U/A/G)UA (Table 1.2, Gerber et al. 2004). The first UGUA element in the *TIF1* 3'UTR, site #1, diverges only slightly from this consensus sequence, having AU instead of UA at the 3' most positions (Figure 3.1A). The other UGUA element in the *TIF1* 3'UTR, site #2, also diverges from the consensus Puf5p binding sequence at just three positions (Figure 3.1A). These two UGUA elements were therefore likely candidates for Puf5p binding sites in the *TIF1* 3'UTR. Since no consensus binding sequence had been established for Puf1p, I could only postulate based on its similarity to other Puf proteins that it may also have affinity for these UGUA containing regions of the *TIF1* mRNA 3'UTR. If one or both of these sites are required for Puf1p/Puf5p-mediated decay, then mutations to these sites should affect the ability of Pufs to stimulate decay of the mutant mRNA. The UGUA of site #1 was mutated to CGUA by a spontaneous error in amplification (Figure 3.2A). I used PCR-based site-directed mutagenesis to mutate the UGUA of site #2 in the *MFA2/TIF1* 3'UTR expression plasmid to ACAC (Figure 3.2B). Each of these mutations has previously been shown to eliminate Puf3p binding to its target (Jackson et al. 2004).

The effect of each of these mutations on mRNA stability was measured in the WT and *PUF* deletion strains. The site #1 mutant mRNA, *MFA2/tif1-1*, decayed with a half-life of 8.1 +/- 0.5 min in WT yeast (Figure 3.2A). This half-life is only slightly greater than that of the WT *MFA2/TIF1* mRNA (7.0 +/- 0.6 min), suggesting that disruption of site #1 is not sufficient to significantly inhibit the ability of Puf1p and/or Puf5p to stimulate rapid decay of this transcript. To dissect the role of Puf1p and Puf5p in decay,

the half-life of the *MFA2/tif1-1* mutant mRNA was measured in the *PUF* deletion strains. *MFA2/tif1-1* decayed with a similar half-life in the *puf1Δ* strain (8.8 +/- 1.0 min) as in the WT strain (Figure 3.2A), indicating that Puf1p-dependent decay requires site #1. However, the *MFA2/tif1-1* mRNA decayed 2.1-fold slower in the *puf5*Δ strain, with a half-life of 17.0 +/- 0.8 min (Figure 3.2A). The decay of *MFA2/tif1-1* mRNA was similar in the *puf1*Δ*puf5*Δ double mutant as in the *puf5*Δ single mutant (Figure 3.2A). Therefore, only Puf5p is required to mediate rapid mRNA decay in the absence of site #1. Because decay of the *MFA2/tif1-1* transcript in the *puf5*Δ (Figure 3.2A) is similar to decay of WT *MFA2/TIF1* in the *puf1*Δ *puf5*Δ strain (Figure 3.2B), site #1 appears essential for the ability of Puf1p to stimulate decay of *MFA2/TIF1* mRNA, but Puf5p can still stimulate decay via another binding site within the *TIF1* 3'UTR.

Analysis of the site #2 mutant mRNA, *MFA2/tif1-2*, displayed a different decay phenotype. The half-life of this mutant mRNA in the WT strain (9.9 +/- 0.7 min) was longer than the WT mRNA in the WT yeast strain (7.0 +/- 0.6 min), but similar to the WT mRNA in either the *puf1*Δ (9.6 +/- 0.4 min) or *puf5*Δ (11.2 +/- 0.8 min) strains (compare Figure 3.2B to Figure 3.1B). This result suggests that whereas site #2 contributes to decay regulation, Puf1p and/or Puf5p can still partially stimulate decay through another site, likely site #1. Moreover, decay regulation through site #2 must be mediated by Puf5p, since Puf1p-dependent decay depends solely on site #1. To determine whether it is only Puf1p, or both Puf1p and Puf5p that stimulate decay through site #1, the mRNA half-life of *MFA2/tif1-2* was analyzed in each single deletion strain, the double *puf1*Δ *puf5*Δ strain, and the Δ*puf1-5* strain. While deletion of either *PUF1* or *PUF5* had no further stabilizing effect on the mRNA, with half-lives of 9.3 +/- 1.3 min

and 9.1 +/- 0.6 min, respectively, the half-life in the double *puf1*Δ *puf5*Δ mutant strain was slowed to 14.1 +/- 0.8 min. Moreover, the half-life in the Δ*puf1-5* strain (16.7 +/- 1.0 min) was similar to the *puf1*Δ *puf5*Δ mutant half-life (Figure 3.2B). These results indicate that Puf1p and Puf5p are each capable of regulating mRNA decay via the *TIF1* site #1, and other Puf proteins have little affect on this decay.

If no other sites are involved in Puf1p and Puf5p regulation of the *TIF1* 3' UTR, the combination of site #1 and #2 mutations should eliminate decay regulation. As expected, decay of this double site mutant mRNA, *MFA2/tif1-2x*, in WT yeast appeared unregulated by Pufs, with a half-life of 17.0 +/- 2.0 min (Figure 3.2C). This decay is similar to the half-lives of both WT *MFA2/TIF1* mRNA in the *puf1*Δ *puf5*Δ strain (16.3 +/- 1.2 min, Figure 3.1B) and of *MFA2/tif1-2x* in the *puf1*Δ *puf5*Δ strain (15.8 +/- 2.8 min, Figure 3.2C). Together, this data provides evidence that these two UGUA sites are the primary targets for Puf1p/Puf5p-mediated decay stimulation. Unexpectedly, decay of the *MFA2/tif1-2x* mutant mRNA was accelerated in the single deletion *puf1*Δ and *puf5*Δ strains, with half-lives of 10.0 +/- 1.3 and 10.1 +/- 0.8 min, respectively (Figure 3.2C). One possible explanation for these results is that various Puf proteins may be able to bind the 3'UTR at alternate locations when the two UGUA sites are mutated. Alternatively, Puf proteins may normally bind these alternate sites, but only upon mutation of the UGUA sites does this binding have a functional effect on the mRNA. Studies with the *C. elegans* FBF-1 protein have found that this Puf can bind to different UGUN sequences, where N is A, U or G (Bernstein et al. 2005). The *TIF1* 3'UTR contains two UGUU sites, a UGUC and a UGUG (Figure 3.1A, shaded gray). Indeed, Puf proteins can be somewhat promiscuous in their binding, with multiple Puf proteins able to bind the same

site, albeit with different affinities, but only specific Pufs are able to promote an in vivo decay effect (Houshmandi and Olivas 2005, and data not shown). In the case of the *TIF1* 3' UTR, the mutation of sites #1 and #2 may have altered the structure or sequence contexts of these alternate sites for better access by Pufs or other regulatory factors. If either Puf1p or Puf5p is absent, this may tilt the balance of other proteins gaining access to these alternate sites, thereby impacting the stability of the mRNA. In fact, decay of the *MFA2/tif1-2x* mRNA in the Δ*puf1-5* strain (half-life of 12.8 +/- 1.9 min) is faster than the WT mRNA in the Δ*puf1-5* strain (half-life of 23.8 +/- 3.9 min), supporting a hypothesis that mutation of sites #1 and #2 has altered the intrinsic stability of the mRNA in the absence of Pufs.

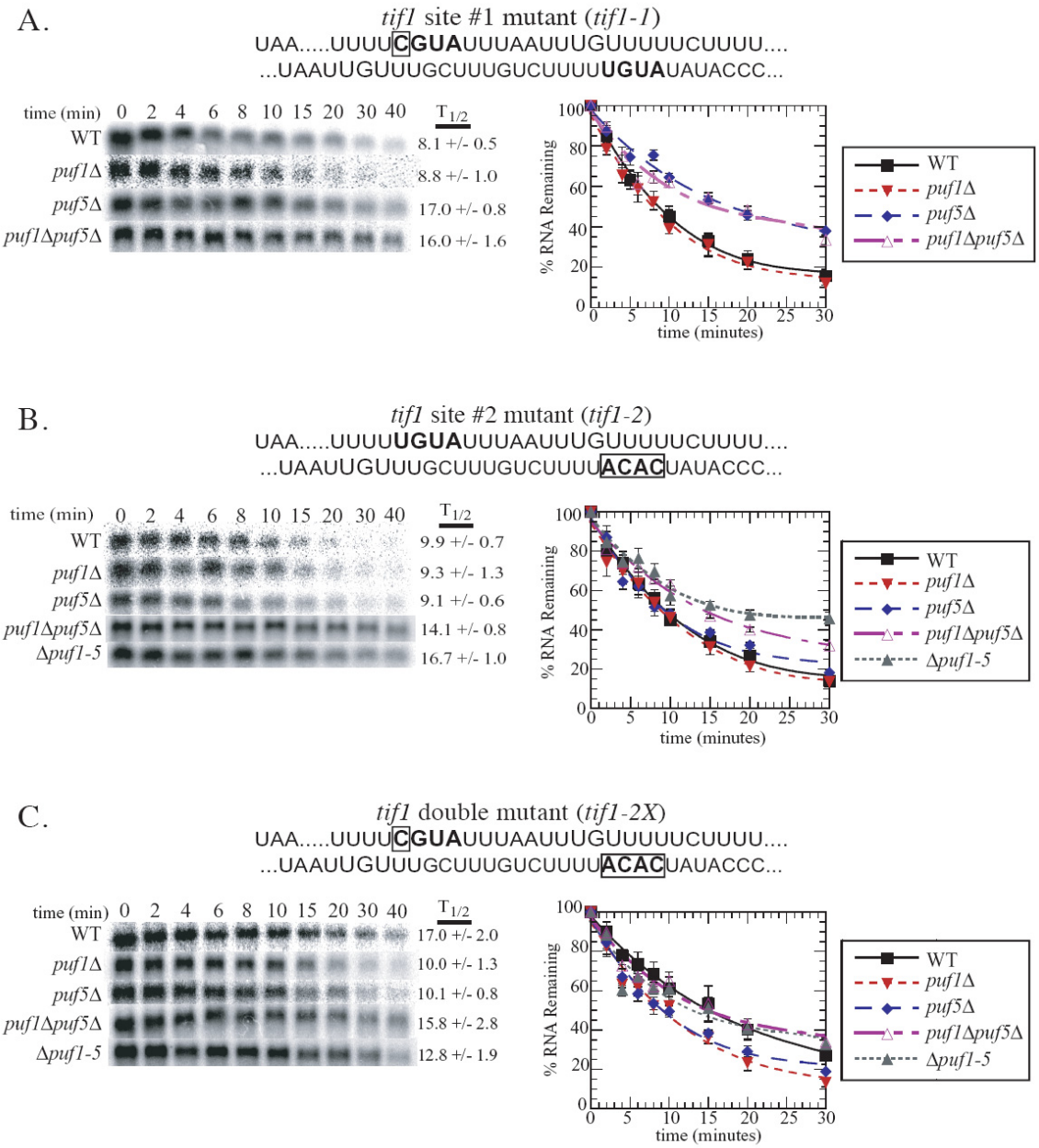


Figure 3.2. Two UGUA sites are required for Puf1p and Puf5p regulated decay of *MFA2/TIF1* mRNA.

A. Decay of *MFA2/tif1-1* 3'UTR fusion mRNA, where site #1 was mutated (boxed). **B**. Decay of *MFA2/tif1-2* 3'UTR fusion mRNA, where site #2 was mutated (boxed). **C**. Decay of *MFA2/tif1-2x* 3'UTR fusion mRNA where both sites #1 and #2 were mutated (boxed). Representative Northern blots for each mRNA in each strain are presented in the right panels. The estimated $T_{1/2}$ is listed to the right of each Northern blot. Data from the Northern analyses are plotted in the left panels. Minutes following transcription repression are indicated above each set of blots and along the x-axis of the graphs. Error for each time point and $T_{1/2}$ is the SEM ($n \geq 3$). Decay was measured in the same strains as in Figure 3.1.

Specificity of *TIF1* mRNA can be altered to include regulation by Puf3p

Upon comparison of the *TIF1* UGUA sites important for Puf1p and Puf5p regulation to the UGUA sites important for *COX17* regulation (Jackson et al. 2004) I found that the experimentally-verified 12 nt *COX17* mRNA Puf3p element (site #1) differs from *TIF1* 3'UTR site #1 by only 4 nt (Figure 3.3A, Jackson et al 2004). To determine if these 4 nt determine the specificity of Puf3p for its target mRNAs, I first altered the *TIF1* site #1 UGUA site in the MFA2/*tif1-2* construct to resemble the *COX17* Puf3 element. If these 4 nt are responsible for recruiting Puf3p, I expect that the stability of the new construct, named *MFA2/tif1-P3E*, will be regulated by Puf3p. In fact, the half-life of *MFA2/tif1-P3E* mRNA is extended 2-fold to 15.7 +/- 1.8 min in *puf3*Δ yeast compared to WT yeast (7.2 +/- 1.3 min, Figure 3.3B). Thus, by altering only 4 nt surrounding the *TIF1* 3'UTR UGUA, I have enabled regulation by Puf3p. Interestingly, the *MFA2/tif1-P3E* mRNA half-life in the *puf1*Δ*puf5*Δ strain is 13.0 +/- 1.1 min, similar to that of the *puf3*Δ yeast (Figure 3.3B), suggesting that Puf1p and/or Puf5p maintain their ability to regulate this mRNA despite the changes to the binding site. In fact, the *MFA2/tif1-P3E* mRNA half-life in yeast lacking all five *PUF*s is >30 min (Figure 3.3B), further suggesting that Pufs other than Puf3p stimulate decay of *MFA2/tif1-P3E* mRNA. Decay of the mRNA in the *puf5*Δ strain is 10.1 +/- 1.7 min, intermediate to WT and *puf1*Δ*puf5*Δ yeast (Figure 3.3B), suggesting that Puf1p and Puf5p both contribute to the decay of *MFA2/tif1-P3E*. Therefore, binding site recognition by Puf1p and Puf5p appears to be fairly flexible.

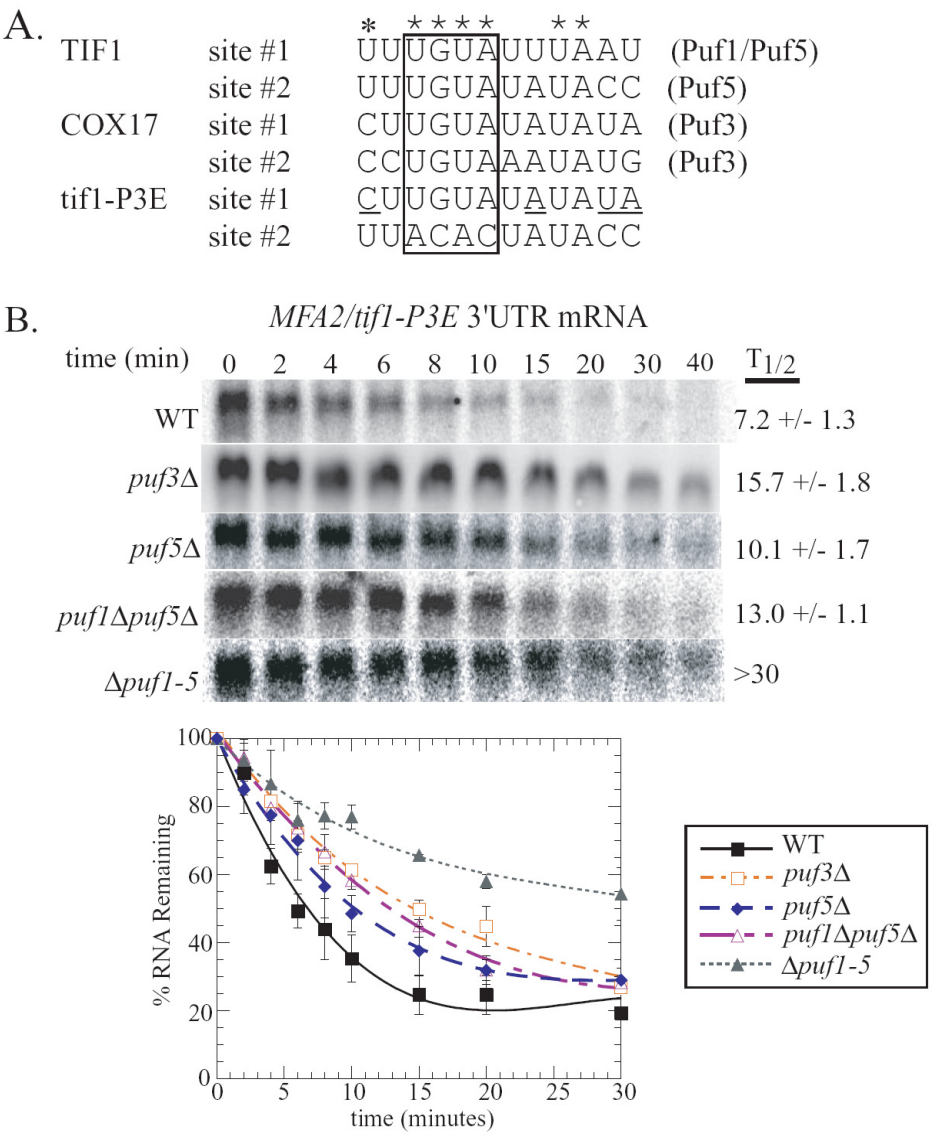


Figure 3.3. *TIF1* 3'UTR can be modified for regulation by Puf3p

A. Shown are *TIF1* and *COX17* 3'UTR 12 nt Puf elements, including and surrounding the core UGUA (boxed). The Puf responsible for regulating each site is listed to the right of each site. A star above the nucleotide position denotes that this position is identical in all four Puf sites. The asterisk indicates the nucleotide position that is identical in both Puf3 sites, but differs in both Puf1 and Puf5 sites. The four positions mutated from *tif1-2* 3'UTR to produce *tif1-P3E* 3'UTR are underlined. **B**. Decay of *MFA2/tif1-P3E* mRNA in WT and *PUF* deletion strains. Representative Northern blots from each strain are presented in the top panel. The estimated $T_{1/2}$ is listed to the right of each Northern blot. In the bottom panel, the average of the data from the Northern blots was plotted. Minutes following transcription repression are indicated above each set of blots and along the x-axis of the graphs. Error for each data point and $T_{1/2}$ is the SEM ($n \geq 3$). Symbols for each strain are the same as in Figure 3.1, except *puf3*Δ (orange, open square).

Materials and Methods

Site-Directed Mutagenesis

In vitro site-directed mutagenesis was performed to mutate *TIF1* 3'UTR UGU regions using the QuickChange XL Site-directed Mutagenesis Kit (Stratagene). To mutate *TIF1* 3'UTR UGUA site #2, primers oWO310 and oWO311 were used in PCR based mutagenesis of pWO53 and pWO70 as recommended by the manufacturer (Stratagene). To create MFA2/*tif1-p3E* 3'UTR in pWO109, site-directed mutagenesis was carried out with primers oWO430-431 in pWO88. All resulting mutants were confirmed by sequencing.

In Vivo Decay Analysis

Decay of steady-state mRNA was monitored in strains containing the temperature-sensitive *rpb1-1* RNA Polymerase II allele, in which transcription is rapidly repressed following a shift from 24°C to 37°C. All yeast transformations were accomplished by LiOAc high efficiency transformation (Gietz and Schiestl, 1995).

Transcriptional shut-offs of the *MFA2/TIF1* mRNA were performed in yeast strains containing pWO70 or pWO71. These plasmids express a fusion RNA containing the *MFA2* coding region and *TIF1 3'UTR* with transcription regulated by the *GAL UAS*. pWO70 was made by PCR amplification of the *TIF1 3'UTR* from genomic DNA with primers oWO231 and oWO239. The PCR product was ligated into pWO24 between *Bgl*II and *Hind*III sites, replacing the 3'UTR of *MFA2* with that of *TIF1*. Similarly, the *Bgl*II/*Hind*III fragment was ligated into pWO54 (see below) to make pWO71.

Transcriptional shut-off assays of the *MFA2/tif1 mRNA* mutants were performed similarly to that of *MFA2/TIF1*. Creation of the *MFA2/tif1-1* mutant (pWO53) occurred

via a spontaneous error 84 nt from the stop codon in the PCR amplification of the *TIF1* 3'UTR and was confirmed by sequencing. Other *MFA2/tif1* mutants were made by site-directed mutagenesis. To make pWO54, pWO61, pWO73, pWO89 and pWO110, the fragment containing the *GAL-MFA2 3'UTR* fusion was cut from pWO53, pWO72, pWO88 and pWO109 with *Pvu*II and ligated into pWO58, which contains the *LEU2* marker. pWO53, pWO70, pWO72, pWO88 and pWO109 (*URA3* marker) were transformed into yWO7 (WT), yWO43 (*puf3*Δ), yWO102 (*puf1*Δ) and/or yWO105 (*puf4*Δ) while pWO54, pWO71, pWO74, pWO89 and pWO110 were transformed into yWO48 (*puf2*Δ), yWO49 (*puf5*Δ), yWO205 (Δ*puf1-5*) and/or yWO208 (*puf1*Δ*puf5*Δ).

Control shut-off experiments of the native *MFA2* mRNA was performed using pWO24 and pWO61. pWO61 was created by digesting pWO24 with *Pvu*II and ligating the product containing *GAL-MFA2* into pWO58. pWO61 was transformed into yWO48, yWO49, yWO205 and yWO208, while pWO24 was transformed into yWO7, yWO102 and yWO105, yWO205 and yWO208.

Transcriptional shut-off experiments were performed essentially as described (Caponigro et al. 1993) with the following modifications to the OD_{600} 1.0 experiments; 200ml cultures were grown to an OD_{600} of 1.0 in synthetic media with 2% galactose. Half of each culture was harvested and resuspended in 20ml of 37°C media containing 8% dextrose shutting off transcription via both the temperature-sensitive inactivation of RNA-pol II and the carbon source inactivation of the *GAL* promoter. Northern blots were probed with the following ^{32}P end-labeled oligonucleotides complementary to 3'UTR sequences: oWO238 (*MFA2*), oWO249 (*TIF1*), and oWO105 (*HXK1*). Total RNA was isolated from yeast as described (Caponigro et al. 1993) and Northern blots were

prepared (NytranSupercharge membrane, Schleicher and Schuell). All blots were normalized for loading to *scRI* RNA, a constitutively expressed RNA Polymerase III transcript (Felici et. al. 1989). All quantification of RNA was accomplished using ImageQuant software (Molecular Dynamics).

Discussion

TIF1 mRNA decay is stimulated by both Puf1p and Puf5p. Two UGUA sites within the 3'UTR are required for Puf-mediated decay. I hypothesize that each UGUA site can recruit its respective Puf protein (Puf1p or Puf5p for site #1, or Puf5p for site #2), which can individually stimulate decay. However, occupation of both sites promotes an even greater rate of decay. This mechanism is similar to both yeast Puf3p binding to two sites in the *COX17* mRNA (Jackson et al. 2004), and *Drosophila* Pumilio binding two sites in the hunchback mRNA (Wharton and Struhl 1991; Curtis et al. 1997). In each case, occupation of one site promotes partial decay stimulation, while activity at both sites is required for maximum decay control.

Since Puf5p can bind both sites in the *TIF1* 3' UTR, it is curious why Puf1p also is needed for decay control. A simple explanation is that the ability of two different Pufs to stimulate decay may ensure that there is sufficient protein in vivo to occupy both sites. Alternatively, since the activity of Puf proteins is dependent on growth conditions (Foat et al. 2005), the ability of two Pufs to act on *TIF1* allows for decay regulation under different conditions that might uniquely inactivate one Puf or the other, and/or allow the tweaking of the rate of decay under different conditions. In fact, I have already shown that Puf-mediated decay of *TIF1* is primarily detected under high versus low cell density. This result seems logical, as *TIF1* encodes the translation initiation factor eIF4A, and at

low cell density the cells are actively growing and would require high levels of such translation factors. In contrast, as cell growth begins to slow at higher cell density, translation would also be slowed, thus creating a need for decreased stability of the *TIF1* transcript. Interestingly, Puf protein control of translation factors may be a common theme, as *Drosophila* Pumilio has been shown to bind and downregulate the translation factor eIF4E at the neuromuscular junction (Menon et al. 2004).

I have shown that Puf1p decay regulation of *TIF1* mRNA requires the recognition of UGUA elements in the 3' UTR, supporting a conserved role of this element for Puf binding. While the global analysis of mRNAs associated with Pufs was unable to detect a consensus binding motif in Puf1p-associated mRNAs (Gerber et al. 2004), it is possible that the sequences surrounding the UGUA site are not as well conserved, or that there were many false positives in the screen that skewed the analysis. The *TIF1* site #1 that is regulated by Puf1p does not match any of the known 10-11 nt Puf3p, Puf4p or Puf5p consensus motifs, though it is only 1-3 nt different from any one of those motifs. In fact, while both sites #1 and #2 are regulated by Puf5p, each site is 2-3 nt different from the consensus Puf5p binding motif. As a demonstration of the flexibility of the Puf recognition elements, I show that while altering *TIF1* mRNA site #1 to sequences identical to the 12 nt Puf3p-binding motif from *COX17* mRNA allows the regulation by Puf3p, these changes do not eliminate the ability of Puf1p and Puf5p to regulate the mRNA. Work with the *C. elegans* FBF-1 protein predicted that Pufs require at least 22 nt of sequence surrounding the core UGU, and the base identity at each of these positions can contribute to binding specificity (Bernstein et al. 2005). Thus, RNA recognition by

Pufs likely entails an optimal sequence context that can tolerate certain combinations of base changes.

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CHAPTER IV:

HXK1 mRNA IS REGULATED BY MULTIPLE Puf PROTEINS

CHAPTER IV: *HXK1* MRNA IS REGULATED BY MULTIPLE Puf PROTEINS

While I was able to suggest in my M.S. thesis work that *HXK1* mRNA is regulated by Puf proteins via analysis of steady-state mRNA levels in WT and the various Puf deletion strains, decay analysis of a *MFA2/HXK1* 3'UTR fusion mRNA showed high error reflecting inconsistent results (Figure 1.5). *In vitro* attempts to determine if Puf proteins can bind *HXK1* UGUA sites were also indeterminate due to the relatively low activity of the GST-tagged Puf protein purified from *E. coli*. In this chapter, I first describe alternate *in vivo* methods to determine the Pufs that regulate *HXK1* mRNA via *HXK1* 3'UTR binding. Next, I describe *in vitro* experiments to determine the *HXK1* 3'UTR and what elements are important for binding and regulation. The work from this chapter has been published (Ulbricht and Olivas 2008).

HXK1 mRNA decay is regulated by Puf1p, Puf5p and Puf4p

To analyze other potential targets of Puf-regulated decay, the same Northern blots of mRNAs from transcriptional shut-off experiments that illustrated decay of *MFA2* and *MFA2/TIF1* mRNAs (Figure 3.1, RNA harvested at OD_{600} of 1.0) were probed for the endogenously transcribed *GLK1, HXK2, MSN2*, and *MSN4* mRNAs. Like *TIF1* and *COX17*, the *GLK1* and *HXK2* mRNAs were identified as differentially expressed in the original *PUF* deletion microarray, *MSN4* was found physically associated with Puf2p, and *MSN2* was a functionally related gene (Table 1.2). All of these mRNAs contain potential Puf binding elements in their 3'UTRs. However, the half-lives of these transcripts were not significantly affected by *PUF* deletions (Table 1.2). I also tested the decay of *HXK1* mRNA. While this mRNA was not identified in any of the microarray

experiments, Hxk1p function, regulation, and expression are related to the *GLK1, HXK2, MSN2,* and *MSN4* genes. The hexokinases Hxk1p and Hxk2p are involved in regulating transcription of the *GLK1*, *HXK2* and *HXK1* genes in response to glucose (Rodriguez et al. 2001). The transcription factors Msn2p and Msn4p activate transcription of the *GLK1* and *HXK1* genes in response to stress (Boy-Marcotte et al. 1998). The *HXK1* 3'UTR contains multiple conserved Puf binding elements, further suggesting it may be a target of Puf-mediated decay.

Decay of *HXK1* mRNA was markedly slower in the *puf1*Δ, *puf4*Δ and *puf5*Δ strains relative to WT, *puf2Δ* and *puf3*Δ strains (Figure 4.1A). The decay pattern of *HXK1* mRNA is irregular, increasing in abundance after temperature shift for 4 min in WT, but ~10 min in the *puf1*Δ, *puf4*Δ and *puf5*Δ strains before finally decreasing in abundance (Figure 4.1A). Other mRNAs, including *TIF1* and *MFA2*, probed on these same blots showed no delay in decay, indicating a successful inhibition of RNA Polymerase II transcription. A similar pattern has been observed for certain mRNAs that are particularly responsive to cell stress or involved in the heat shock response (Adams and Gross 1991, Taylor et al. 2005, Aragon et al. 2006). From these decay patterns, it appears as though *HXK1* mRNA is stabilized in the *puf1*Δ, *puf4*Δ and *puf5*Δ strains, and even more so in the Δ*puf1-5* strain. It is notable that the *HXK1* decay patterns observed from higher optical density (OD_{600} 1.0, Figure 4.1A) remain consistent at a lower optical density (OD_{600} 0.4, data not shown). These results suggest that Puf1p, Puf4p and Puf5p are destabilizing *HXK1* full-length mRNA in vivo under both conditions tested.

Due to the irregular decay pattern, the above experiments alone cannot completely eliminate the possibility that Pufs have some effect on *HXK1* expression unrelated to

mRNA decay. Exclusion of *HXK2, GLK1, MSN2* and *MSN4* mRNAs as targets of Puf-mediated decay rules out many possible indirect effects Pufs may play via *HXK1* regulators, however there remains other possible factors. To better determine Puf-specific effects on *HXK1* mRNA decay, I fused the 3'UTR of *HXK1* to a truncated *PGK1* coding region (see Materials and Methods). In my master's Thesis I tried a similar method of fusing the *HXK1* 3'UTR to the *MFA2* coding region, however, results were inconsistent. Because the *PGK1* coding region is more stable than *MFA2*, I expected that any destabilizing effects of Puf proteins on the *PGK1/HXK1* fusion mRNA would be better detected than on the *MFA2/HXK1* fusion mRNA. The expression of this *PGK1/HXK1* mRNA is regulated by the GAL UAS, eliminating any transcriptional variations that may occur at the endogenous *HXK1* locus and any translational or stability affects of the *HXK1* coding region or 5'UTR. As expected from the endogenous *HXK1* mRNA decay results, the *PGK1/HXK1* 3'UTR fusion mRNA decayed similarly in the WT and *puf3*Δ strains with half-lives of 5.1 +/- 0.6 and 4.7 +/- 0.8 min, respectively (Figure 4.1B). Also expected from our previous results, the *PGK1/HXK1* mRNA decayed slower in both the *puf1*Δ and *puf5*Δ strains, with half-lives of 14.1 +/- 1.1 and 10.7 +/- 0.6 min, respectively (Figure 4.1B). The *PGK1/HXK1* mRNA half-life was also affected by deletion of *PUF4* (7.5 +/- 0.2 min), although not to the same extent as in the *puf1*Δ and *puf5*Δ strains (Figure 4.1B). Unexpectedly, the *PGK1/HXK1* mRNA half-life was also slightly prolonged in the *puf2*Δ (7.9 +/- 0.6 min). The *PGK1/HXK1* mRNA half-life was greatly increased in the Δ*puf1-5* strain (28.3 +/- 5.8 min) whereas the control *PGK1* mRNA decayed similarly in both WT and Δ*puf1-5* strains (Figure 4.1B and 4.1C). Thus, like the *TIF1* mRNA, decay of *HXK1* mRNA is accelerated by both Puf1p and

Puf5p. However, in a unique fashion, Puf4p and Puf2p also stimulate *HXK1* mRNA decay.

Because the half-life of *PGK1/HXK1* mRNA is 2- to 3-fold longer in the *Δpuf1-5* strain than any individual *PUF* deletion strain, we can assume that more than one Puf protein is acting on *HXK1* mRNA under these conditions. Previous studies have shown that Puf5p acts in combination with Puf4p to regulate *HO* mRNA (Hook et al. 2007), and our studies have shown that Puf5p acts in combination with Puf1p to regulate decay of *TIF1* mRNA. To determine whether Puf5p acts in combination with other Puf proteins to regulate *HXK1* mRNA decay, I tested decay of *PGK1/HXK1* mRNA in *puf1*Δ*puf5*Δ and *puf2*Δ*puf5*Δ yeast. Compared to the single *puf5*Δ strain (10.7 +/- 0.6 min) and *puf1*Δ strain (14.1 +/- 1.1 min), the half-life was indeed extended in the *puf1*Δ*puf5*Δ double deletion strain (17.9 +/- 1.3 min, Figure 4.1C). Therefore, similar to *TIF1* mRNA, regulation of *HXK1* mRNA by Puf5p is functioning in combination with Puf1p. The half-life of *PGK1/HXK1* mRNA was not significantly affected in the *puf2*Δ*puf5*Δ strain (12.7 +/- 1.2 min) compared to the *puf5*Δ strain (10.7 +/- 0.6 min, Figure 4.1C). Thus, it appears that Puf2p and Puf5p do not act in combination to stimulate *HXK1* mRNA decay. However, it is possible that due to the small role of Puf2p-mediated decay, an additive change in half-life in the double deletion is difficult to detect. Because the half-lives in the *puf2*Δ*puf5*Δ strain (12.7 +/- 1.2 min) and in the *puf1*Δ*puf5*Δ strain (17.9 +/- 1.3 min) were significantly less than the Δ*puf1-5* strain (28.3 +/- 5.8 min), I postulate that Puf4p acts in combination with Puf5p and Puf1p to regulate *HXK1* mRNA.

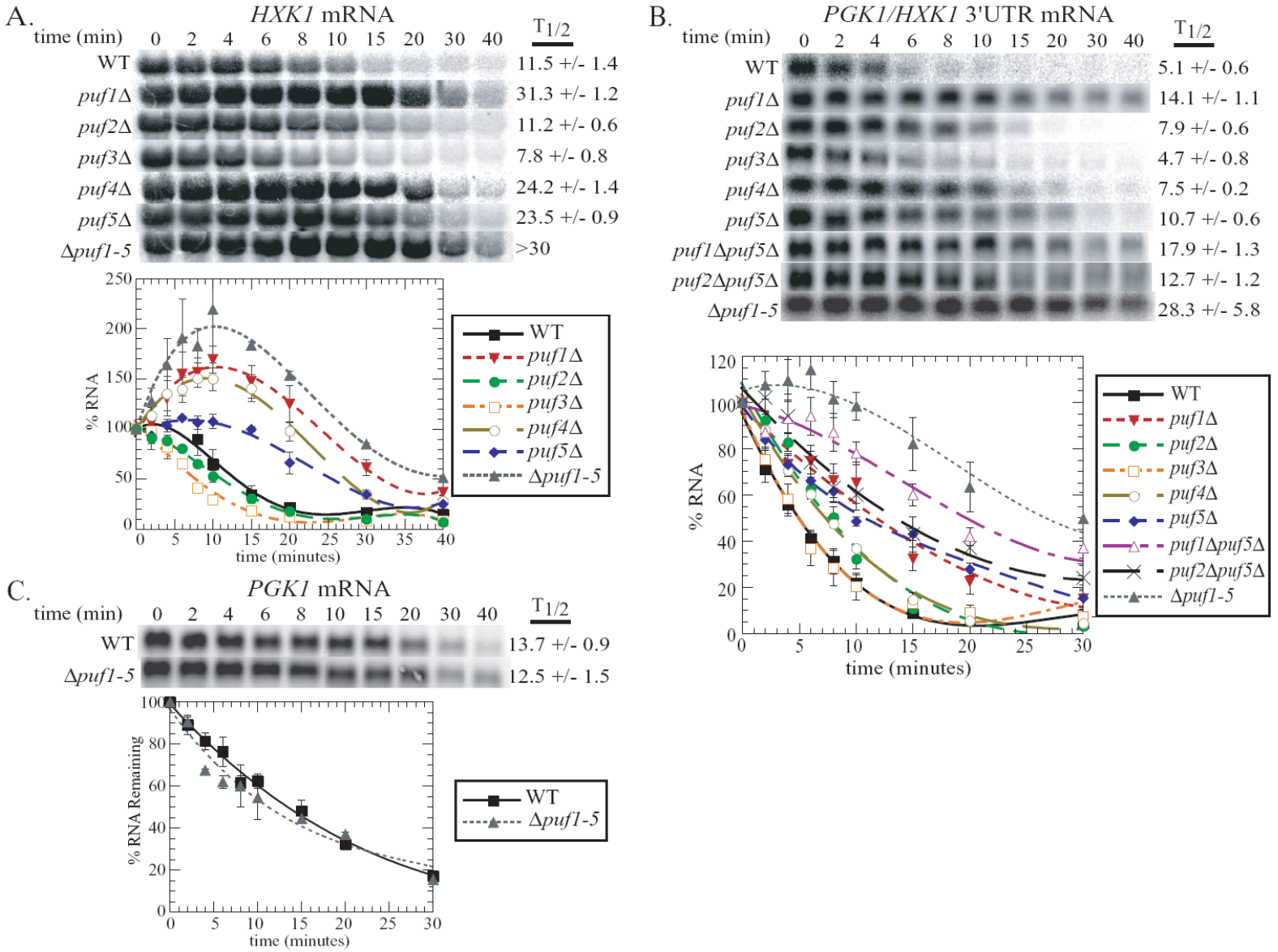

A.
HXK1 mRNA
time (min) 0 2 4 6 8 10 15 20 30 40
T1/2
WT 11.5 +/- 1.4
puf1Δ 31.3 +/- 1.2
puf2Δ 11.2 +/- 0.6
puf3Δ 7.8 +/- 0.8
puf4Δ 24.2 +/- 1.4
puf5Δ 23.5 +/- 0.9
Δpuf1-5 >30
% RNA
time (minutes)
WT
puf1Δ
puf2Δ
puf3Δ
puf4Δ
puf5Δ
Δpuf1-5
B.
PGK1/HXK1 3'UTR mRNA
time (min) 0 2 4 6 8 10 15 20 30 40
T1/2
WT 5.1 +/- 0.6
puf1Δ 14.1 +/- 1.1
puf2Δ 7.9 +/- 0.6
puf3Δ 4.7 +/- 0.8
puf4Δ 7.5 +/- 0.2
puf5Δ 10.7 +/- 0.6
puf1Δpuf5Δ 17.9 +/- 1.3
puf2Δpuf5Δ 12.7 +/- 1.2
Δpuf1-5 28.3 +/- 5.8
% RNA
time (minutes)
WT
puf1Δ
puf2Δ
puf3Δ
puf4Δ
puf5Δ
puf1Δpuf5Δ
puf2Δpuf5Δ
Δpuf1-5
C.
PGK1 mRNA
time (min) 0 2 4 6 8 10 15 20 30 40
T1/2
WT 13.7 +/- 0.9
Δpuf1-5 12.5 +/- 1.5
% RNA Remaining
time (minutes)
WT
Δpuf1-5

Figure 4.1. *HXK1* mRNA is regulated by multiple Puf proteins.

(A) Northern blot analyses of endogenously transcribed *HXK1* mRNA. Northern blots from Figures 3.1 and 3.2 were re-probed for *HXK1* mRNA. (B) Decay of *PGK1/HXK1* mRNA. (C) Decay of the control *PGK1* mRNA. Representative Northern blots are presented in the top panels. The estimated $T_{1/2}$ is listed to the right of each Northern blot. Graphical representation of the average of the data from the Northern blots is presented in the lower panels. Minutes following transcription repression are indicated above each set of blots and along the x-axis of the graphs. The following strains were used in these studies; WT (black, square), *puf1*Δ (red, inverted triangle), *puf2*Δ (green, closed circle), *puf3*Δ (orange, open square) *puf4*Δ (olive, open circle), *puf5*Δ (blue, diamond), Δ*puf1-5* (gray, triangle), *puf1*Δ*puf5*Δ (purple, open triangle) and *puf2*Δ*puf5*Δ (black x). Error for each time point and $T_{1/2}$ is the SEM ($n \geq 3$).

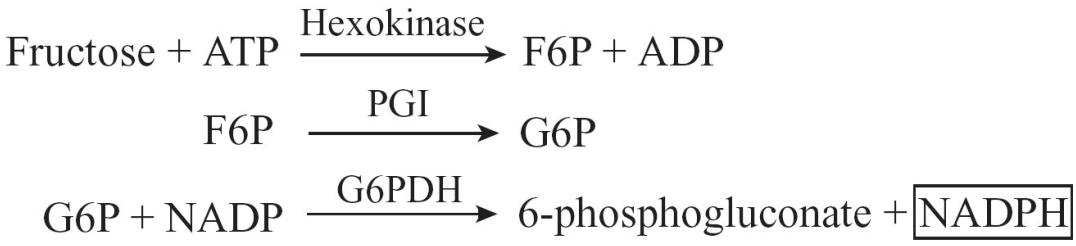
Hxk1p is upregulated in *PUF* deletion yeast

To test the effects of Puf proteins on *HXK1* gene expression at the cellular level, the activity of Hxk1p was measured in WT and *PUF* deletion strains. The yeast hexokinases Glk1p, Hxk1p and Hxk2p functionally overlap in that they each phosphorylate glucose. Hxk1p and Hxk2p also phosphorylate fructose (Walsh et al. 1991; Gancedo et al. 1977). However, Hxk1p prefers fructose to glucose phosphorylation 3:1, whereas Hxk2p phosphorylates fructose and glucose equally (Walsh et al. 1991). Thus, the stabilization of *HXK1* mRNA should result in an increase in Hxk1p and fructose phosphorylation. I measured the amount of fructose phosphorylation based on the coupled reactions of fructose phosphorylation by hexokinase (Hxk1p) and the reduction of NADP to NADPH by G6PDH (Figure 4.2A). The production of NADPH is measured by a change in absorbance at 340nm. Using these methods, the relative activity of Hxk1p in yeast extracts from WT, *puf1*Δ, *puf2*Δ, *puf4*Δ and *puf5*Δ strains was determined. As seen in Figure 4.2B, Hxk1p activity was upregulated 2.4- and 2.3-fold, respectively, in *puf1Δ* and *puf5Δ* strains, and 3.3-fold in the *puf4Δ* strain versus wild-type levels. However, there was not a significant difference in activity between the WT and *puf2*Δ strains. These results show that an increased level of protein activity correlates to increased transcript stability in the absence of Puf1p, Puf4p or Puf5p. Hxk1p activity was slightly elevated in the *puf4*Δ strain compared to the *puf1*Δ and *puf5*Δ strains, however this difference is not significant.

It is interesting that despite destabilization of *PGK1/HXK1* mRNA by Puf2p, neither the *HXK1* full-length mRNA decay nor the Hxk1p activity appears to be affected by Puf2p. Moreover, the slight stabilization of *PGK1/HXK1* mRNA in the absence of

PUF4 does not coordinate with the more drastic effects of the *puf4*Δ seen in tests of the full-length *HXK1* mRNA and Hxk1p activity. I suspect that these apparent discrepancies can be explained by unknown effects of the *HXK1* promoter, coding region and/or 5'UTR. Thus, it is possible that in addition to mRNA decay, Pufs play direct or indirect roles in transcription, translation and/or cellular availability of the *HXK1* transcript.

A.



B.

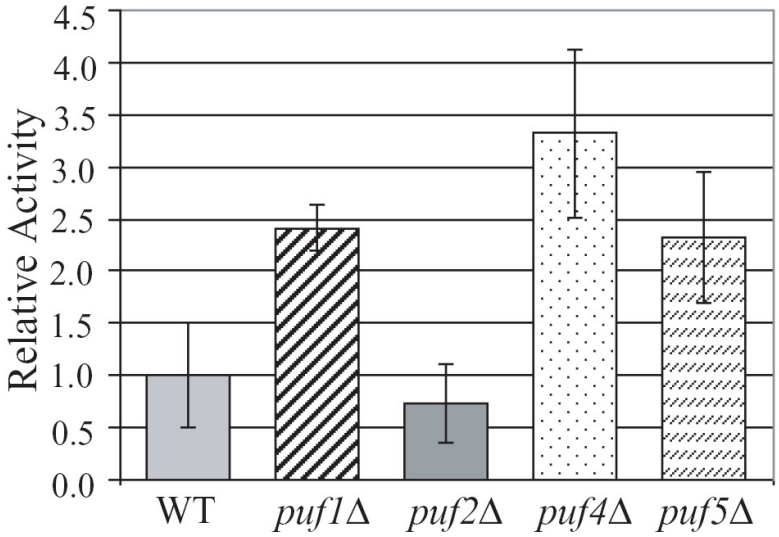


Figure 4.2. Hxk1p activity is upregulated in *PUF* deletion yeast.

A. Outline of NAD-linked assay to measure activity of Hxk1p (hexokinase). Hxk1p phosphorylates fructose to make fructose-6-phosphate (F6P). F6P is isomerized to Glucose-6-Phosphate (G6P) by phosphoglucosisomerase (PGI), then Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the reduction of NADP into NADPH. Thus, the amount of NADPH produced is dependent on the abundance of Hxk1p in cell extracts. NAPDH production is measured by the change in absorbance at 340nm. **B**. Relative Hxk1p enzyme activity in the absence of Puf proteins. The enzymatic activity was determined from *puf1*Δ (diagonal stripes), *puf4*Δ (dotted), *puf5*Δ (hatched) and *puf2*Δ (gray) strains and compared to WT (white) yeast. WT and *PUF* deletion yeast were grown to an OD_{600} of 1.0, harvested, and the lysates were subjected to the described enzyme assay. Enzyme activity (U/ml) was calculated and expressed relative to WT. Error bars represent SD (n>3).

PufRDs bind to the *HXK1* 3'UTR in vitro

To assay whether Puf proteins bind to the *HXK1* mRNA 3'UTR, in vitro binding assays were performed with in vitro-transcribed and radiolabeled *HXK1* mRNA 3'UTR incubated with Puf repeat domains tagged with glutathione S-transferase (GST) purified from *E. coli* (Figure 4.3A). The repeat domains (RD) of multiple Puf proteins, including yeast Puf3RDp and Puf5RDp, are sufficient for both in vitro binding and in vivo regulation of their targets (Jackson et al. 2004, Houshmandi and Olivas 2005). Following incubation, RNA-protein reactions were UV crosslinked and RNase treated, resulting in the RNA label attached to the Puf protein if bound to the RNA. Figure 4.3B demonstrates that GST-tagged Puf1RDp, Puf2RDp, Puf3RDp, and Puf5RDp bind to full-length *HXK1* 3'UTR. None of these proteins, except for Puf3RDp, were able to bind the *COX17* 3' UTR (the known target of Puf3p), demonstrating specificity of binding to the *HXK1* 3' UTR (data not shown). Puf4RDp was not tested because I was unable to purify stable protein from *E. coli*. The *HXK1* 3'UTR contains three UGUA elements as candidate Puf binding sites. Restriction digest of the *HXK1* template with *Ssp1* truncates the 3'UTR to contain only one UGUA Puf binding element (Figure 4.3A). This truncated RNA was still able to interact with GST-tagged Puf2RDp, Puf3RDp, and Puf5RD, but not Puf1RDp (Figure 4.3B). These results verify that the *HXK1* 3' UTR is capable of binding Puf proteins. Moreover, Puf1RDp likely requires one or both of the latter two UGUA sites in the 3' UTR for activity. This data also reinforces the idea that Puf protein binding is fairly promiscuous, with Puf proteins such as Puf3RDp able to bind this target in vitro, without comparable function in vivo. Similar results were seen with in vitro

binding to the *TIF1* 3' UTR, where Puf1RDp, Puf2RDp, Puf3RDp, and Puf5RDp were all able to bind (data not shown).

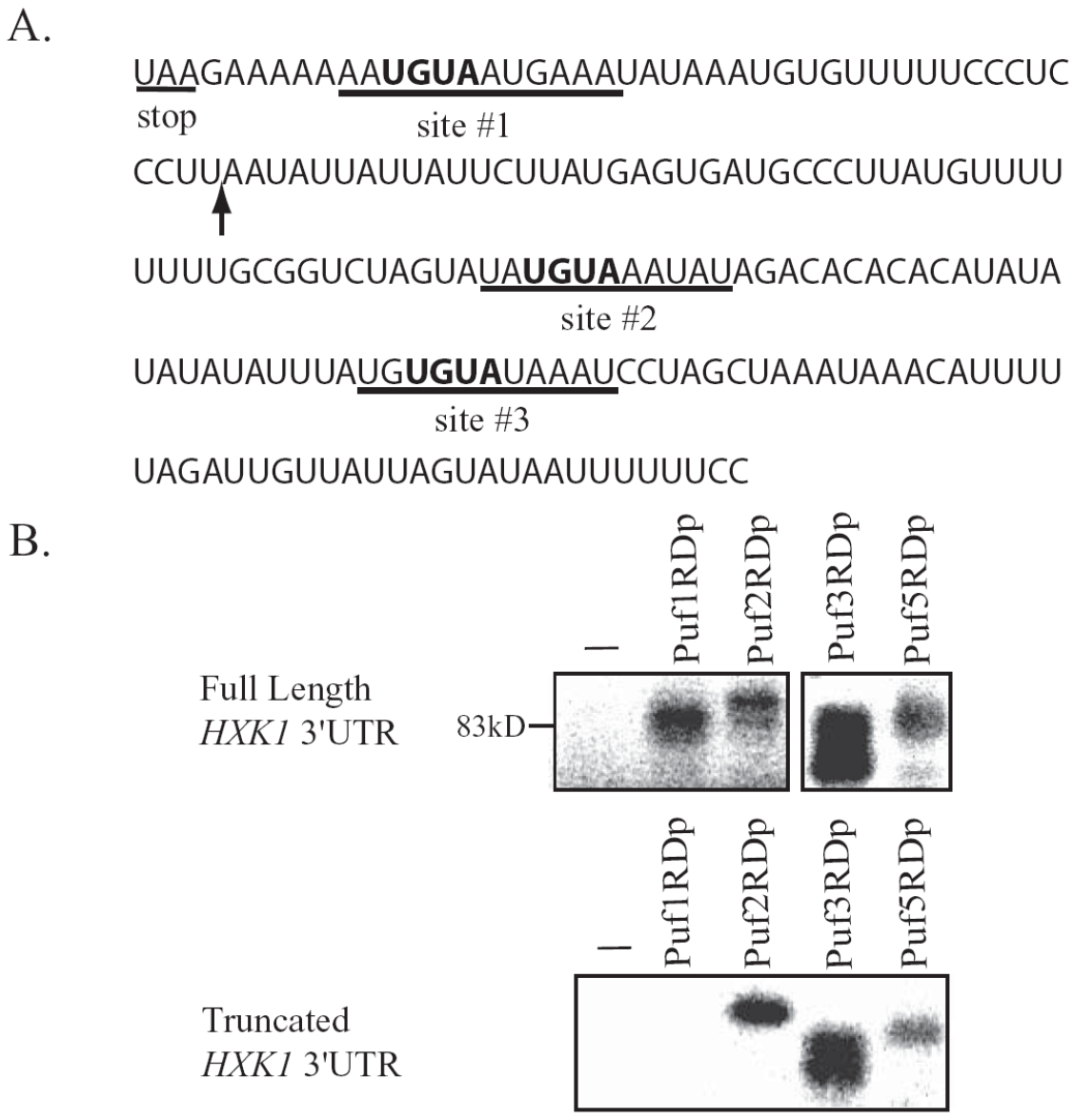


Figure 4.3. Puf repeat domains bind to *HXK1* 3'UTR in vitro.

A. Sequence of the *HXK1* 3' UTR. UGUA-containing regions are underlined and labeled as site #1, site #2 and site #3. Truncated *HXK1* 3'UTR (lower panel) was transcribed from template cut with *SspI,* whose location is indicated by an arrow. The length of the 3'UTR was estimated through PAGE analysis of *HXK1* 3'UTR after removal of the poly(A) tail. **B**. In vitro binding assays of radiolabeled transcripts in the presence or absence (-) of GST-PufRDp were UV-crosslinked and treated with RNase. Radiolabeled proteins shown in the SDS-polyacrylamide gel represent an interaction between the GST-PufRDp and the transcript.

Materials and Methods

In Vivo Decay Analysis

Steady-state transcriptional shut-off experiments, and Northern blot preparation was performed essentially as described in Chapter III, Materials and Methods, page. Decay of endogenously transcribed *HXK1* mRNA was detected by stripping the *MFA2* control or *MFA2/tif1* Northern blots (Chapter III) and re-probing for *HXK1* mRNA. Northern blots were probed with the following ^{32}P end-labeled oligonucleotides complementary to *PGK1* or *HXK1* 3'UTR sequences: oWO105 (*HXK1*) and oWO447 (*PGK1*Δ82).

The *HXK1* 3'UTR was fused to *PGK1*Δ82 to create the *PGK1/HXK1* 3'UTR fusion construct. *PGK1*Δ82 is a truncated version of the stable *PGK1* coding region that has been shown to allow regulation of its mRNA decay rate by 3'UTR regulatory sequences (Heaton et al. 1992). To create the *PGK1/HXK1* 3'UTR construct, the *HXK1* 3'UTR was amplified from genomic DNA using primers oWO164 and oWO153. The *Bgl*II site at the 5' end of the 530 nt product was first filled with Klenow (New England Biolabs). The product was then inserted between the Klenow-filled *Cla*I site and *Hind*III site of pWO102 (PGK1Δ82) to create pWO100. The *PGK1/HXK1* fragment was removed from pWO100 (*Sac*I/*Hind*III) to pWO61, a *LEU2* expression vector, to create pWO101. pWO100 and pWO101 express the *PGK1*Δ82 coding region fused to the *HXK1* 3'UTR under the control of the *GAL* UAS.

Control shut-off experiments of the native *PGK1* mRNA were performed using pWO102 and pWO103 (*PGK1*Δ82). pWO103 was created by inserting the *PGK1* fragment from pWO102 into *Sac*1/*Hind*III sites of pWO61. pWO103 was transformed

into yWO48, yWO49, yWO205 and yWO208, while pWO102 was transformed into yWO7, yWO102 and yWO105, yWO205 and yWO208.

Protein Purification

The *GST-PUF3RD* and *GST-PUF5RD* constructs in pGEX-6P-1 (Amersham Biosciences) were previously created (Jackson et al. 2004). The *GST-PUF1RD* fusion construct was created by PCR-amplification of an 1140 nt region of genomic *PUF1* (amino acids 551-934) using the primers oWO144 and oWO145. The PCR product was inserted into pBluescript (Stratagene) between *Bam*HI and *Not*1 to yield pWO48. pWO48 was digested with *BamH*I and *Pvu*II then cloned into pGEX-6P-3 (Amersham Biosciences) between *BamH*I and *Sma*I to create pWO49, the GST-Puf1RDp expression vector. To create the *GST-PUF2RD* fusion construct, nucleotides 1453-2712 were amplified from genomic *PUF2* (encoding amino acids 485-904) with primers oWO136 and oWO137. This product was inserted into pBluescript between the *BamH*I and *Xma*I sites, creating pWO21. The *BamH*I-*Xma*I digestion product of pWO21 was then ligated into pGEX-6P-3 to yield the GST-Puf2RDp expression vector pWO22. Each construct was verified by sequencing. The GST fusion constructs were transformed into BL-21 protease deficient *E. coli* and purified as recommended (Amersham Biosciences). Eluates were dialyzed in 50mM Tris-HCl pH 8.0 and verified by western analysis with anti-GST antibodies.

In Vitro Binding Assays

In vitro transcribed RNA containing the 3'UTR of *HXK1* mRNA was made by first amplifying the *HXK1* 3'UTR with primers oWO153 and oWO164, then ligating the fragment into pBluescript between *BamH*I and *Hind*III sites. The plasmid (pWO94) was

digested with *Hpa*II or *Ssp*I prior to transcription. RNA was transcribed using T3 RNA polymerase (Ambion) in the presence of ^{32}P UTP to produce transcripts 117nt and 58nt in length. The resulting transcripts were treated with DNaseI (Promega) then purified by separation on denaturing polyacrylamide gel, elution from gel slice and ethanol precipitation. Binding was performed essentially as described (Olivas and Parker, 2000) with radiolabeled transcript (100,000 c.p.m.) in the presence or absence of GST-Puf1RDp (2μM), GST-Puf2RDp (2μM), GST-Puf3RDp (3μM) or GST-Puf5RDp (2μM).

Hxk1p Enzyme Assay

Yeast strains yWO7 (wild-type), yWO48 (*puf2*Δ), yWO49 (*puf5*Δ), yWO102 (*puf1*Δ) and yWO105 (*puf4*Δ) were grown in synthetic media with 2% dextrose to OD_{600} of 1.0, harvested, and washed twice with media alone. Extracts were prepared as described (Kawasaki and Fraenkel, 1982). Total protein was determined (Bio-Rad Protein Assay, Bio-Rad). Detection of fructose phosphorylation by hexokinase was monitored as described (Walsh et al 1991) by adding extract containing 50μg total protein to reaction buffer (5mM triethanolamine, 10mM MgCl2 (pH7.4), 0.3mM NADP, 1mM rATP, 5mM fructose) with 2μg phosphoglucose isomerase (Roche) and 4□g glucose 6-P dehydrogenase (Roche). Enzyme activity (U/mg) was calculated according to the change in absorbance at 340nm with extract alone as the standard.

Discussion

HXK1 mRNA appears to be regulated by at least three Puf proteins (Puf1p, Puf4p and Puf5p) at any one time. This finding, in combination with the fact that *HXK1* contains three UGUA sites, suggests a simple model in which one Puf binds to each site simultaneously. The RNA-protein crosslinking studies show that Puf1p cannot bind

truncated *HXK1* 3' UTR, suggesting that Puf1p binds sites other than site #1. The relative levels of stabilization may suggest that Puf1p and Puf5p bind with greater affinity than Puf2p and Puf4p, or that their relative activity under these conditions vary. It is notable that there was a substantial increase in *HXK1* mRNA abundance after temperature shift, even though other transcripts on the same Northern blots showed successful transcriptional repression. Furthermore, deletion of *PUF1*, *PUF4* or *PUF5* dramatically increased both the magnitude and duration of this phenotype, with the quadruple *PUF* deletion having the largest effect. A microarray study in stationary phase *S. cerevisiae* showed that more than 800 mRNAs, many of them involved in stress response, increased in abundance after induction of oxidative stress. This increase in abundance was not due to new transcription, but to accumulation of extraction-resistant species of mRNAs prior to initiation of additional stressors (Aragon et al. 2006). *HXK1* mRNA was identified in this study, suggesting that its increase in abundance in our study may be due to accumulation in an extraction-resistant storage form (Aragon et al 2006). Since the increase we observe in *HXK1* mRNA abundance is dependent on Puf proteins, then in this scenario, Pufs may play a role in storage and/or localization of *HXK1* mRNA. Previous studies with Puf1p have found it to localize to punctate structures on the peripheral plasma-membrane and the mitochondrial edge. Puf1p localization is thought to be related to its role in the ARP2/3 complex (Machin et al. 1995, Fehrenbacher et al. 2005). In an attempt to analyze the influence of Puf1p on mRNP localization and mRNA storage, I overexpressed *PUF1* in a yeast strain containing a *GFP-DCP2* fusion commonly used to visualize P-bodies. P-bodies are sites of mRNA storage during phases of cell stress and translational repression. They are the most obvious sites where a

hypothetical mRNP shuttling protein (i.e. Puf1p) would store repressed mRNAs and would likely show a difference in size or quantity upon mis-expression of the shuttling protein. However, I was unable to see a difference in GFP-Dcp2p localization or P-body size in WT versus *PUF1* overexpression during mid-log phase, late-log phase, or osmotic stress (data not shown). These negative results cannot rule out the possibility that Puf1p plays a role in mRNP localization, especially considering Puf1p regulates only a small portion of the transcript pool and these transcript-specific effects on mRNP localization may be not be observable by microscopy. An alternative explanation of the *HXK1* decay pattern is attributed to the fact that the *rpb1-1* allele has been observed to allow transcription, to some extent, of heat-shock genes as well as some stress responsive genes (Adams and Gross 1991). Thus, since *HXK1* is a stress responsive gene, and the reporter transcript with the *HXK1* 3'UTR under the control of an alternative promoter largely lacks this phenotype, it is likely that transcription is not fully repressed from the endogenous *HXK1* promoter. In this scenario, Pufs may indirectly affect *HXK1* transcription. In either case, it is clear that Pufs indeed affect decay of *HXK1* mRNA.

It is unclear why Puf1p/Puf5p regulation of *TIF1* mRNA is dependent on cell density, while their regulation of *HXK1* in conjunction with Puf4p is not. One hypothesis is that there is a specific stabilizer of *TIF1* mRNA in actively growing cells at low density, and this stabilizer overpowers any effects of the Puf proteins. At higher cell density when translation needs to be downregulated, this stabilizer may become inactive, allowing the Puf proteins to stimulate decay. Alternatively, condition-specific Puf protein activity may be different on distinct mRNA targets due to disparate protein interactions on different 3'UTRs and/or conditionally-regulated activities of other

proteins involved in Puf-mediated decay. In addition, a factor may allow for specificity of Puf binding and/or activity in vivo. In line with this idea, we have observed that Pufs bind promiscuously in vitro where such a specificity factor is lacking.

Hxk1p has been established to be important to cellular metabolism, but what is the benefit to the cell for *HXK1* regulation by three different Puf proteins at one time in addition to its many levels of transcriptional regulation? One explanation is that during phases of stress, transcription is largely repressed, and the regulation of gene expression can still be regulated at the level of mRNA stability and translation. As already mentioned, *HXK1* is a stress-responsive gene and likely undergoes more regulation during stressful phases than other, non-stress related mRNAs. In addition, one well-known consequence of Hxk1p activity is metabolism of ATP. In order to conserve ATP during stressful phases, yet maintain the ability to metabolize any available carbon source, the cell will facilitate precise regulation of Hxk1p protein production via all available mechanisms, including transcription, mRNA stability and/or translational efficiency.

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CHAPTER V:

REGULATION ON mRNA DECAY BY Puf1p

CHAPTER V: REGULATION OF mRNA DECAY BY Puf1p

While the role of Puf1p in mRNA decay remains relatively unknown, Puf1p (also known as Jsn1p) was previously determined to play a role in mitochondrial motility (Fehrenbacher et al. 2005) and microtubule stability (Machin et al. 1995). The goal of the research in this chapter is to first determine if, like Puf5p and Puf3p, it too partners with components of the deadenylation machinery to stimulate decay of its target mRNAs. I also investigate the domains of the Puf1 protein important for decay regulation. The information gleaned from these experiments will help in understanding the diverse roles Pufs may play in regulation of gene expression.

Factors required for *PUF1*-mediated repression

Studies with Puf4p and Puf5p show that each binds Pop2p and Ccr4p, however their requirements for these two deadenylation factors differ. Puf4p requires both Pop2 and Ccr4p, while Puf5p requires Pop2p but not Ccr4p for repression of *HO* mRNA (Goldstrohm et al. 2006, Goldstrohm et al. 2007, Hook et al. 2007). Thus, while the protein partners and methods to stimulate decay and repress translation are similar among yeast Puf proteins, they are not identical. Considering that Puf4RDp and Puf5RDp are more similar to each other than to Puf1RDp, it is possible that Puf1p uses partners and methods different from each of these.

To begin testing possible protein partners required for Puf1p-mediated mRNA decay, I fused the *HXK1* 3'UTR to the *HIS3* coding region under the control of the *MET25* promoter. Transcription of the *HIS3/HXK1* 3'UTR fusion mRNA from the *MET* promoter can be tightly controlled with the level of methionine in the media to allow growth of *his3* mutant yeast on media lacking histidine, yet transcribe a low enough level

of the mRNA that will allow for detectible changes in stability of the *HIS3/HXK1* mRNA. I also created a *PUF1* overexpression construct by cloning the *PUF1* gene into a plasmid behind the constitutive *GPD* promoter. I co-transformed the *HIS3/HXK1* fusion construct along with the *PUF1* expression construct or a control empty vector into *his3*Δ yeast. In the absence of Puf-overexpression, the yeast grow equally well on media with or without histidine, however the overabundance of Puf1p represses expression of *HIS3* by destabilizing *HIS3/HXK1* 3'UTR mRNA, preventing growth on media lacking histidine (Figure 5.1). The *HIS3* competitive inhibitor, 3-aminotriazole (AT), was added to each plate lacking histidine to increase the stringency and reduce background in the *his3*Δ yeast strain.

Next, I transformed both the *PUF1* overexpression and *HIS3/HXK1* 3'UTR mRNA expression constructs into *pop2*Δ and *ccr4*Δ yeast. Growth on media lacking histidine in either of these strains indicates that a factor required for Puf-mediated repression has been deleted. As seen in Figure 5.1, growth is no longer repressed in *pop2*Δ yeast overexpressing *PUF1*, suggesting that Pop2p is required for Puf1p-mediated repression of *HXK1* gene expression. However, in the *ccr4*Δ yeast, *PUF1* overexpression continues to repress growth, suggesting that Puf1p, like Puf5p, can repress *HXK1* mRNA expression independent of Ccr4p deadenylase (Figure 5.1).

Hook et al. used a similar *HIS3* reporter system to determine that *PUF4* overexpression represses *HO* 3'UTR (2007). *PUF4* was not able to repress *HIS3/HO* in yeast lacking *POP2* or *CCR4*, suggesting that each of these factors is required for Puf4p-mediated suppression of gene expression. Since *HXK1* is also a target of Puf4p mediated decay, I expected *PUF4* to similarly repress *HIS3/HXK1* expression. In my experiments,

PUF4 overexpression repressed growth of WT yeast containing the *HIS3/HXK1* fusion on media lacking histidine similar to *PUF1* overexpression (Figure 5.1). To determine if the Puf4 protein uses the same partners to repress each of its targets, or if the protein acts slightly different based on the target of regulation, I also tested *HIS3/HXK1* repression in *pop2*Δ and *ccr4*Δ yeast overexpressing *PUF4*. Puf4p no longer repressed *HIS3/HXK1* mRNA expression in the absence of *POP2* or *CCR4* (Figure 5.1). Thus, Puf4p likely acts similarly on each of its mRNA targets.

Puf1p Domains Involved in mRNA Regulation

While the repeat domain of Puf proteins takes up only 1/3 to 1/2 of each protein (Figure 1.4), previous studies with Puf proteins from multiple organisms have found that the RD is sufficient for both mRNA regulation and binding. It is interesting that despite the presence of a relatively well conserved RD, yeast Puf1p and Puf2p also contain a putative RNA recognition motif (RRM) outside of the RD (Olivas and Parker 2000). The importance of the RRM domains is unexplored in these proteins. To investigate if the RRM plays a role in recognition of the target mRNA, I determined if the Puf1RDp is sufficient to repress *HIS3/HXK1* expression in WT yeast. As seen in Figure 5.1, Puf1RDp repressed *HIS3/HXK1* as well as the Full-length Puf1p in WT yeast. Thus, the Puf1 RRM is not necessary to stimulate *HXK1* decay.

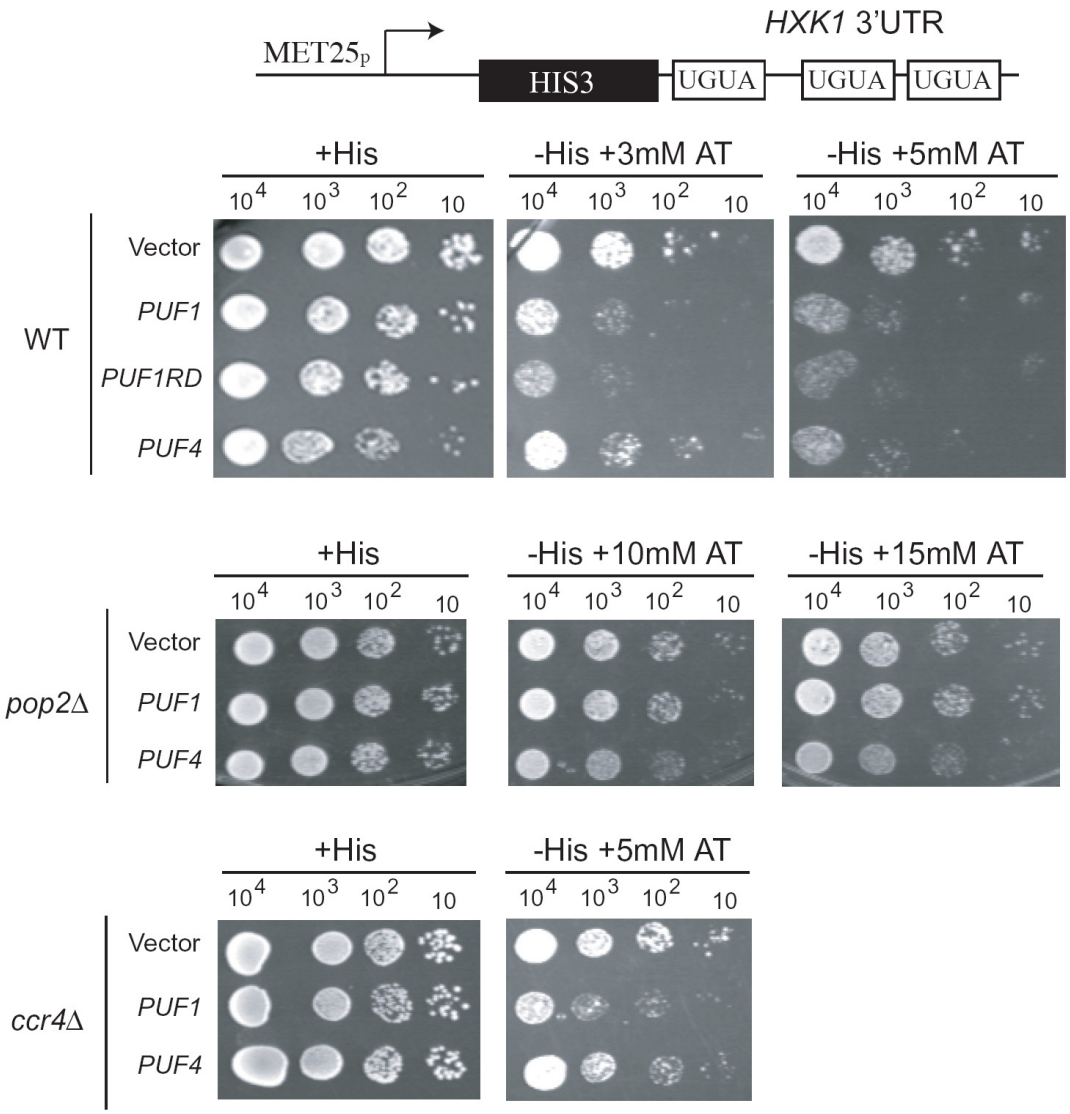

Figure 5.1. Puf1p-and Puf4p-mediated repression requires deadenylation factors.

Empty vector, *FLAG-PUF1, FLAG-PUF1RD* or *PUF4* were co-expressed in *his3* mutant yeast with the *HIS3/HXK1* fusion construct (illustrated above). Growth was assayed on synthetic media containing histidine (+His), or lacking histidine (-His) in the presence of 3-15mM AT. The concentration of AT in each plate is indicated above. The number of cells plated is also indicated above each yeast plate. Experiments were performed in WT (top panels), *pop2*Δ (middle panels) and *ccr4*Δ (bottom panels) yeast strains.

HXK1 elements required for Puf-mediated decay

There are three UGUA elements within the *HXK1* 3'UTR (see Chapter IV for full 3'UTR sequence). Each of these are potential Puf-recognition elements. The in vitro binding data (Figure 4.3) suggests that Puf1p recognizes one of the two downstream UGUA elements, however the requirement for the UGUA elements versus other elements in the 3'UTR was not determined in this experiment. In order to assay the requirement for the UGUA elements in Puf1p- and Puf4p-mediated decay regulation, I subjected the *HXK1* 3'UTR to in vitro mutagenesis to create *HIS3/hxk1* mutant 3'UTR reporter constructs with mutations at site #1 (*hxk1-1),* sites #1 and #3 *(hxk1-2x)*, and all three sites *(hxk1-3x)*. In these mutants, the UGUA sequences are altered to ACAC, eliminating Puf-recognition of these sites. Using the *HIS3* reporter system to determine Puf-regulation of the mutant 3'UTRs, I found that *PUF1* slightly represses the *HIS3/hxk1-1* construct, but is not able to repress the *HIS3/hxk1-2x* and *HIS3/hxk1-3x* constructs, thus Puf1p-mediated decay of *HXK1* requires site#3 and/or site#2 (Figure 5.2). I also tested the ability of Puf1RDp in repressing the *HIS3/hxk1-3x* construct. It too could not repress the triple mutant (Figure 5.2), providing further evidence that the repeat domain is sufficient for specificity and repression of Puf1p.

I also tested the ability of Puf4p to repress the *HIS3/hxk1-1, HIS3/hxk1-2x,* and *HIS3/hxk1-3x* constructs. Unlike Puf1p, Puf4p represses both *HIS3/hxk1-1* and *HIS3/hxk1-2x* constructs (Figure 5.2). In fact, repression of the mutant constructs is greater than repression of the WT 3'UTR (Figure 5.2). Thus, Puf4p-mediated repression of the *HXK1* 3'UTR does not require site#1 or site#3. Puf4p no longer represses the

HIS3/hxk1-3x reporter (Figure 5.2), suggesting that site #2 is important for Puf4p-mediated decay.

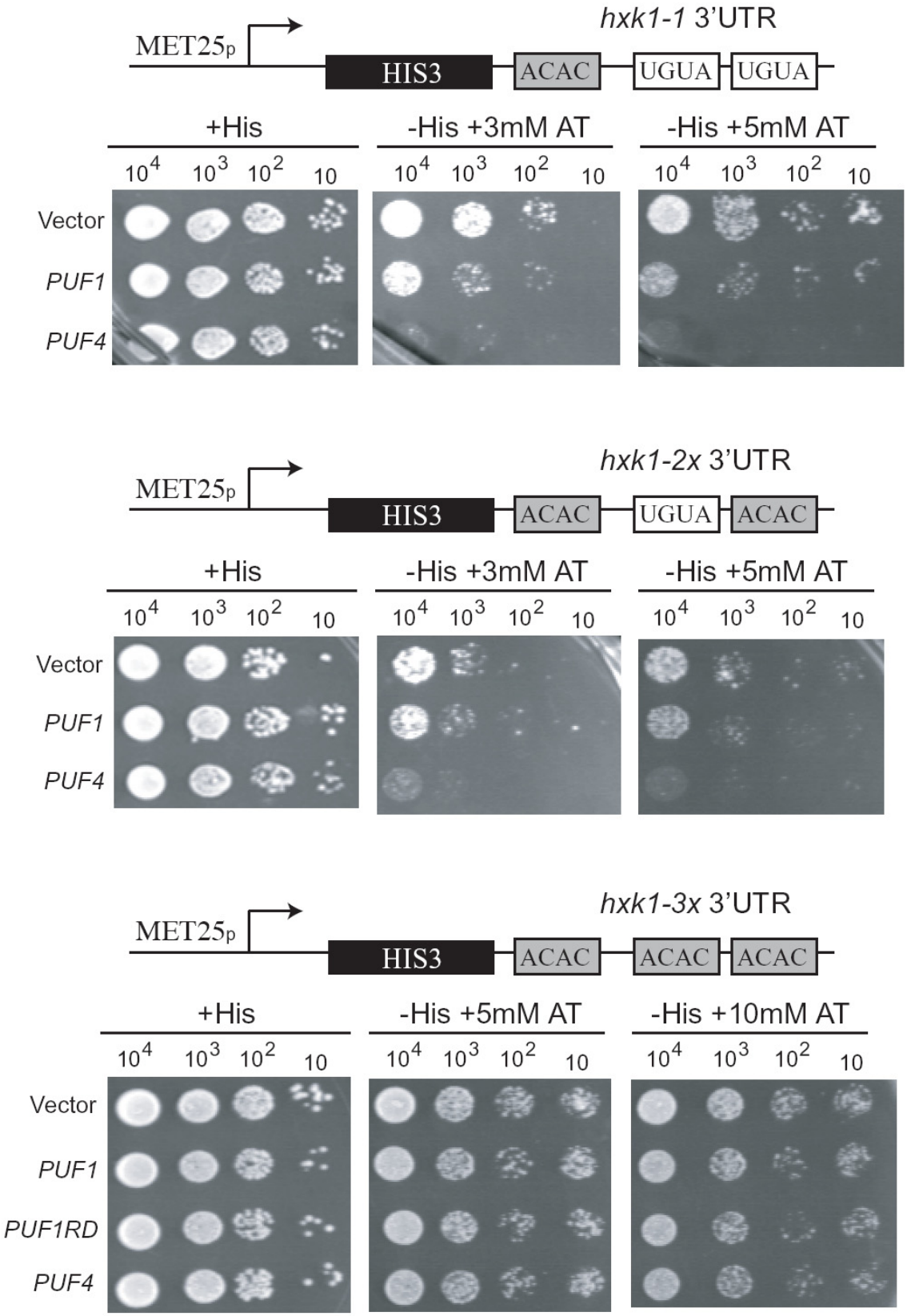
hxk1-1 3'UTR
MET25p
HIS3
ACAC
UGUA
UGUA
+His
-His +3mM AT
-His +5mM AT
10^4 10^3 10^2 10
Vector
PUF1
PUF4
hxk1-2x 3'UTR
MET25p
HIS3
ACAC
UGUA
ACAC
+His
-His +3mM AT
-His +5mM AT
10^4 10^3 10^2 10
Vector
PUF1
PUF4
hxk1-3x 3'UTR
MET25p
HIS3
ACAC
ACAC
ACAC
+His
-His +5mM AT
-His +10mM AT
10^4 10^3 10^2 10
Vector
PUF1
PUF1RD
PUF4

Figure 5.2. *HXK1* 3'UTR elements required for Puf1p and Puf4p recognition.

The three UGUA sequences within the *HXK1* 3'UTR were mutated to ACAC sequentially to eliminate Puf recognition at these sites. The *HIS3/hxk1* mutant constructs were then assayed for Puf-mediated repression in the *HIS3* reporter assays in WT yeast overexpressing *PUF1, PUF1RD* or *PUF4*. The mutated sites are indicated (gray) above each panel of results. Top panel, *hxk-1*; middle panel, *hxk1-2x*; bottom panel, *hxk1-3x*. Growth was assayed on synthetic media containing histidine (+His), or lacking histidine (-His) in the presence of 3-10mM AT . The concentration used is indicated above each panel of results. The number of cells plated is indicated over each yeast plate.

Factors Required for Deadenylation and Decay of *HXK1* mRNA

Deadenylation is not only the first step in the decay process for the majority of mRNAs, but it is a highly regulated process in yeast cells. In order to assay the deadenylation rate of *HXK1,* I performed a transcriptional pulse-chase experiment. In this pulse-chase, yeast containing the *PGK/HXK1* expression construct under the control of the *GAL* promoter are grown overnight in raffinose-containing media, conditions in which the promoter is not induced. The yeast are harvested at an OD_{600} of 0.4 and shifted to galactose-containing media, inducing transcription and creating a pulse of fully-adenylated *PGK1/HXK1* mRNAs. By inhibiting transcription with dextrose-containing media after only 8 min, it is possible to monitor the deadenylation rate of this newly transcribed population of *PGK1/HXK1* mRNA. To visualize the length of the poly(A) tail over the course of the pulse-chase, a DNA oligonucleotide is hybridized to mRNA prepared from cell harvested at various time points following transcription induction and repression. RNaseH is then used to digest the RNA of the RNA/DNA hybrid near the stop codon. After separation by polyacrylamide gel electrophoresis (PAGE), the length of the RNA region 3' of the cut, including the poly(A) tail, is imaged using a radio-labeled complementary probe.

In WT yeast, as expected, the *PGK1/HXK1* mRNA is fully adenylated at the time in which transcription is inhibited (Figure 5.3, WT, time 0). At 2 min after transcription inhibition, a heterogeneous population of poly(A) tails length is apparent, represented by a smear spanning the region between fully adenylated tails and deadenylated mRNA (Figure 5.3). The mRNA and its tail almost completely disappear only 4 min after transcription repression. In *puf1*Δ yeast, the majority of the mRNA remains fully-

adenylated 2 min after transcription repression. The heterogeneous poly(A) smear appears at 4 min and disappears by 10 min after transcriptional inhibition. Thus, Puf1p accelerates the deadenylation rate of *HXK1* mRNA. Because *HXK1* is a target of multiple Puf proteins, I expected a more dramatic deadenylation defect from a strain deleted of multiple *PUF* genes. In a yeast strain deleted of *PUF1, PUF3, PUF4* and *PUF5*, similar to the single *puf1*Δ strain, the mRNA remains fully adenylated at the 2 min time point and appears as a heterogeneous population by 4 min. In contrast to the WT and *puf1*Δ yeast, the heterogeneous poly(A) mRNA species in the Δ4pufs strain remains throughout the course of the experiment (Figure 5.3). Thus, multiple Puf proteins are affecting the deadenylation of *HXK1* mRNA.

I then assayed the requirement for the deadenylation factors Ccr4p and Pop2p in decay of *HXK1* mRNA. In *ccr4*Δ yeast, adenylated *PGK1/HXK1* mRNA remained for the entirety of the experiment (Figure 5.3). Since Ccr4p is the major deadenylase in yeast, we expect all mRNAs to have deadenylation defects in the *ccr4*Δ strain. In fact, the *HXK1* mRNA appears similar to other mRNAs tested in this strain in that, while deadenylation is largely absent, the poly(A) tail undergoes a small amount of shortening or trimming at 10min. Previous studies have shown that the *PAN* nuclease complex is responsible for poly(A) trimming (Tucker et al. 2002). Trimming is commonly observed in the *ccr4*Δ strain and is eliminated by the *ccr4*Δ*pan2*Δ double knockout (Tucker et al. 2002). Therefore, the small amount of *PGK1/HXK1* deadenylation observed was expected. Unexpectedly, we noticed a slight increase in the poly(A) tail length at 15 min after transcription inhibition, and poly(A) shortening is no longer apparent in the remaining time points (Figure 5.3). These results are unique to *HXK1* mRNA and

suggest that either transcription is still occurring, or that the small amount of deadenylation is being reversed by an adenylase enzyme. However, it remains a possibility that the subtle difference in length could be an artifact of the experiment. Since the control pre-induction lane (Figure 5.3, -8 min) shows that transcription of this mRNA is tightly controlled, it is unlikely that transcription is occurring during the chase. If the poly(A) tail is being extended, extension would likely be counteracted by *PAN* nuclease trimming, thus, poly(A) extension activity should become more apparent in a *ccr4*Δ*pan2*Δ double mutant. I performed the transcriptional pulse-chase experiments in the *ccr4*Δ*pan2*Δ and found that deadenylation was completely inhibited, suggesting that Pan2p is essential for poly(A) trimming of *HXK1*. Longer poly(A) tails did not accumulate throughout the pulse as we might expect if there is a cytoplasmic adenylase involved (Figure 5.3), however, since enzymes often have reversible activity, it is possible that Pan2p is not only trimming the poly(A) tail, but adding adenosines to the tail at the same time. It must also be noted that even though polyadenylation cannot be observed in the chase of this experiment, it is possible that the poly(A) tail extension occurred prior to time point 0, and thus, a longer tail after time 0 cannot be observed. To investigate the later possibility, the poly(A) tail length in *ccr4*Δ*pan2*Δ yeast should be directly compared to the poly(A) tail length in WT yeast.

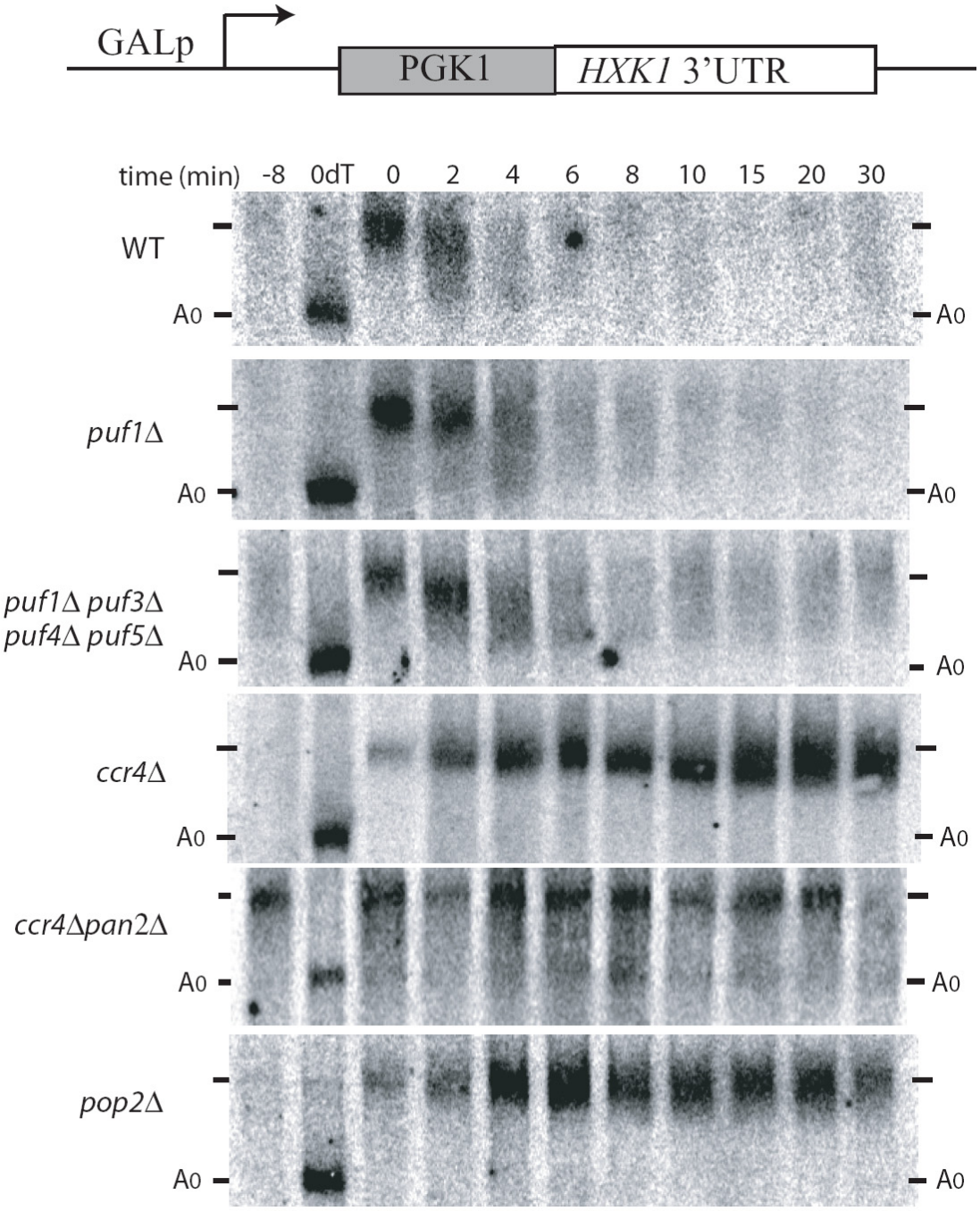

Figure 5.3. *PGK1/HXK1* mRNA deadenylation rates.

PGK1/HXK1 mRNA was subjected to pulse-chase analysis (see text) in WT, *puf1*Δ, *puf1*Δ*puf3*Δ*puf4*Δ*puf5*Δ, *ccr4*Δ, *ccr4*Δ*pan2*Δ, and *pop2*Δ yeast in order to determine the dependence of *HXK1* deadenylation rate on each factor. The time relative to transcriptional repression is indicated above each lane. Oligo d(T) was hybridized to the time 0 mRNA to visualize the length of the 3'UTR without the poly(A) tail (0dT lane). The fully adenylated and deadenylated (A_0) species are indicated by a line to the right and left of each panel.

Ccr4p is the major deadenylase enzyme in yeast. However, like Crr4p, Pop2p is also a deadenylase enzyme and a component of the deadenylase complex. Therefore, I also tested the importance of Pop2p in degradation of *PGK1/HXK1* mRNA. Previous studies in yeast deleted of *POP2* have shown that deadenylation is slowed, but not prevented (Tucker et al. 2001). However, *HXK1* deadenylation is completely inhibited in the absence of Pop2p (Figure 5.3). Even at the latest time point in our experiments, the fully adenylated poly(A) tail is present, suggesting that even trimming is prevented in the absence of Pop2p. Thus, *HXK1* mRNA decay appears to be unique, occurring in a manner completely dependent on Pop2p.

While deadenylation is dependent on Pop2p, it remains possible that the deadenylase activity of this protein is not required, but that Pop2p serves as a physical anchor for other required decay factors. In order to investigate this possibility, I obtained a *pop2* construct that has a mutation to the deadenylase active site as well as a WT *POP2* expression construct. If Pop2p deadenylase activity is required, exogenous expression of WT *POP2* will allow repression of *HIS3/HXK1* in a *pop2*Δ yeast strain, while the *pop2* catalytic mutant will not rescue *HXK1* deadenylation and repression. As shown in Figure 5.4, using my *HIS3/HXK1* reporter assay WT *POP2* and mutant *pop2* equally repressed *HIS3/HXK1* expression. Thus, Pop2p catalytic activity is not required for Puf1p-mediated repression. These results, together with those in Figure 5.3, suggest that the presence of Pop2p is required for Puf1p-mediated deadenylation and decay.

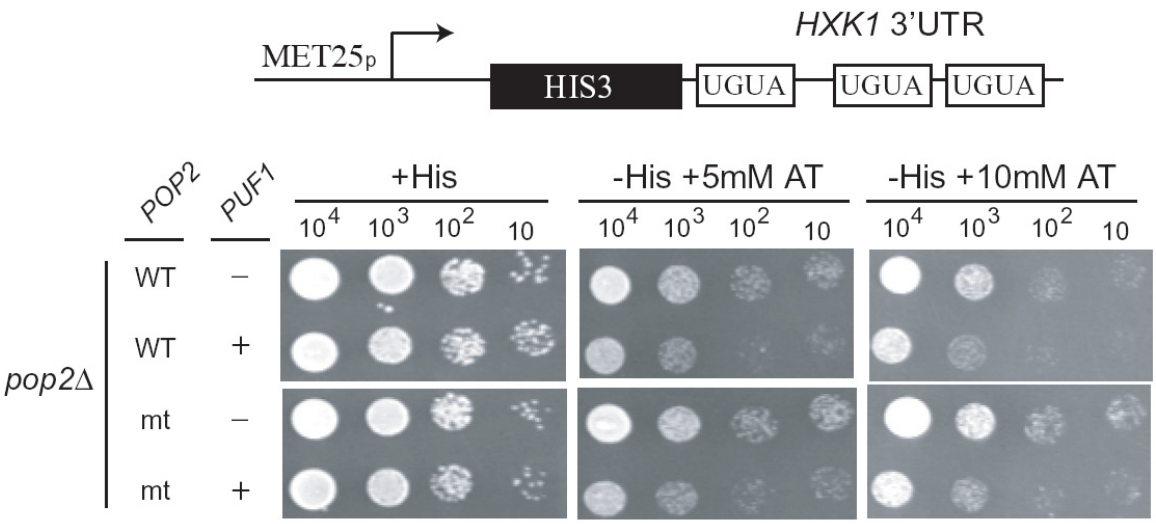

Figure 5.4. Pop2p deadenylase activity not required for Puf1-mediated repression.

Wild-type (WT) *POP2* or mutant (mt) *pop2* was expressed in *pop2*Δ, *his3* yeast containing empty vector (-) or *FLAG-PUF1* (+) and the *HIS3/HXK1* fusion construct (illustrated). Growth was assayed on synthetic media containing histidine (+His), or lacking histidine (-His) in the presence of 5 or 10mM AT. The number of cells plated is indicated above each yeast plate.

Materials and Methods

Puf1p Over-Expression in Yeast

Full-length *PUF1* was amplified from genomic DNA using oWO466 and oWO467. *PUF1RD* was amplified from pWO48 with the primers oWO468 and oWO145. The PCR products were inserted into the *BamH*I and *Sal*I sites of pAV72 behind the FLAG peptide under the control of the constitutive GPD promoter to create pWO114 and 115.

Repression Assays

The *HIS3/HXK1* (pWO122) mRNA expression plasmid was constructed by first removing the *MET25* promoter from pWO117 and cloning it in pRS415 (*LEU2*/CEN vector) using *Sac*I/*Xba*I creating pWO118. The *HXK1* 3'UTR was amplified from genomic DNA using primers oWO476 and oWO152. The product was cloned into the *BamH*I and *Hind*III sites of pWO118. *HIS3* was removed from pWO111 using flanking *Not*I sites and placed into p416/*MET25/HXK1*. The *HIS3/HXK1* 3'UTR construct containing the *URA3* marker (pWO124) was created by similarly cloning the *MET25* promoter, *HIS3* coding region and *HXK1* 3'UTR into pRS416. The *HIS3/hxk* mutant expression vectors were created by replacing the *HXK1* 3'UTR of pWO122 and 124 with the *hxk1-1, hxk1-2x,* or *hxk1-3x* 3'UTRs. pWO122 (WT), pWO123 (*hxk1-3x*), pWO131 (*hxk1-1*) and pWO133 (*hxk1-2x*) were cotransformed into *his3* yeast with pAV72 (vector), full-length *PUF1* (pWO113), or *PUF1RD* (pWO114). pWO124 (WT), pWO125 (*hxk1-3x),* pWO132 (*hxk1-1*) and pWO134 (*hxk1-2x*) were cotransformed with p415-GPD/*PUF4* (pWO116) into *his3* yeast. The yeast strains yWO211 (WT), yWO212 (*pop2*Δ), and yWO213 (*ccr4*Δ) were used in these repression assays.

Yeast transformed with WT *POP2* (pWO135) or mutant *pop2* (pWO136) expression constructs along with *PUF1* (or control vector only) and *HIS3/HXK1* constructs were grown in the presence of 100μM Zeocin (Invivogen) for selection of the Zeocin resistant marker on pWO135 and 136.

Five μl of each 10-fold serial dilution was plated on synthetic minimal media containing 50μM methionine and histidine (control) or 3AT in given concentrations. Total protein was prepared from and analyzed by Western analysis (anti-FLAG, Sigma) to verify FLAG-Puf1p and FLAG-Puf1RD expression.

Site-Directed Mutagenesis

In vitro site-directed mutagenesis was performed to mutate *HXK1* 3'UTR Puf binding sites using the QuickChange XL Site-directed Mutagenesis Kit (Stratagene). *hxk1-1, hxk1-2x,* and *hxk1-3x* were created by sequential mutagenesis using primers oWO329-330 (site #1), oWO448-449 (site #2), and oWO450-451 (site #3) as recommended by the manufacturer. All resulting mutants were confirmed by sequencing.

Transcriptional Pulse-Chase

pWO100 was transformed into WT (yWO5), *ccr4*Δ (yWO13), and *puf1*Δ (yWO20) yeast. pWO126 was transformed into yWO12 (*pop2*Δ) and yWO30 (*puf1*Δ*puf3*Δ*puf4*Δ*puf5*Δ). Transcriptional pulse-chase experiments were performed essentially as described (Decker and Parker 1993). Overnight cultures were grown in minimal selective media with 2% raffinose and 0.1% sucrose to an OD_{600} of 0.4. The cells were harvested and resuspended in 20ml minimal selective media containing 4% galactose and incubated at 30ºC for 7 min. The culture was harvested once again and resuspended in 20ml minimal selective media containing 4% dextrose and incubated at

30ºC. Aliquots were taken from the culture after transcription activation and repression and cells were harvested. Total RNA was prepared from yeast cells as described (Caponigro et al. 1993). The *PGK1/HXK1* mRNA poly(A) tail length was visualized by RNaseH cleavage of the mRNA by hybridization with oWO447. The mRNA was separated by electrophoresis on a 6% polyacrylamide gel and transferred to nylon membranes (NytranSupercharge membrane, Whatman). The cleaved mRNA was visualized using a radiolabeled DNA oligonucleotide complementary to the *HXK1* 3'UTR (oWO105). mRNA lacking the 3'UTR was visualized by hybridization to oligo d(T) prior to RNaseH cleavage. All blots were normalized for loading to *scRI* RNA, a constitutively expressed RNA Polymerase III transcript (Felici et. al. 1989). All quantification of RNA was accomplished using ImageQuant software (Molecular Dynamics).

Discussion

Previous work indicated that Puf-mediated repression of gene expression is diverse. Even the closely-related yeast Puf3p, Puf4p and Puf5p have slightly different decay factor requirements (Goldstrohm et al. 2006; Goldstohm et al. 2007; Hook et al. 2007; Houshmandi, Lopez Leban and Olivas, unpublished studies). In this chapter, my goal was to begin deciphering the mechanism of Puf1p-mediated repression of gene expression and compare this function to Puf3p, Puf4p and Puf5p. The results have illuminated new information about a relatively unknown protein (Puf1p) and have given us additional information on the diverse processes that regulate gene expression.

The studies have found that *HXK1* deadenylation and decay, independent of Puf-stimulation, is dependent on Pop2p. Previous studies determined that Ccr4p is sufficient

for deadenylation in vitro and that Pop2p shows no activity under these conditions (Tucker et al. 2002). Interestingly, *pop2*Δ yeast have deadenylation defects in vivo, however, these defects vary from transcript to transcript (Tucker et al. 2001, Tucker et al. 2002). Pop2p effects on deadenylation are likely because Pop2p stimulates the activity of Ccr4p. Pop2p could directly stimulate Ccr4p by increasing affinity for the mRNA. Alternatively, Pop2p may stimulate Ccr4p indirectly by processes that include recruiting additional factors required for Ccr4p activity or mRNA binding (Tucker et al. 2002). Applying these principles to my results suggests that, while the absence of either Pop2p or Ccr4p leads to *HXK1* deadenylation defects, it is likely that Ccr4p is the deadenylase, and that Ccr4p requires Pop2p for efficient deadenylation of *HXK1* mRNA.

The deadenylation factor Pop2p is required for Puf1p-mediated repression of gene expression, yet Puf1p only moderately stimulates deadenylation of *HXK1* mRNA. Furthermore, the deadenylase Ccr4p is not required for Puf1p-mediated suppression. These results lead to the conclusion that Puf1p-mediates translational repression through decapping and/or translation initiation in addition to deadenylation, but still in a manner dependent on Pop2p. That the catalytic activity of Pop2p is not required for Puf1p-mediated repression presents a model, similar to that proposed for Puf5p, where Pop2p mediates interactions between Puf1p and the deadenylation factor Ccr4p, as well as decapping and/or translational initiation factors (Figure 5.5A). Alternatively, Pop2p may be required to directly or indirectly stimulate the activity of the translation/decay factors (Figure 5.5A). This model is contrary to the current model for Puf4p-mediated repression, in which Puf4p-repression occurs entirely via Pop2p-dependent acceleration of deadenylation (Figure 5.5B).

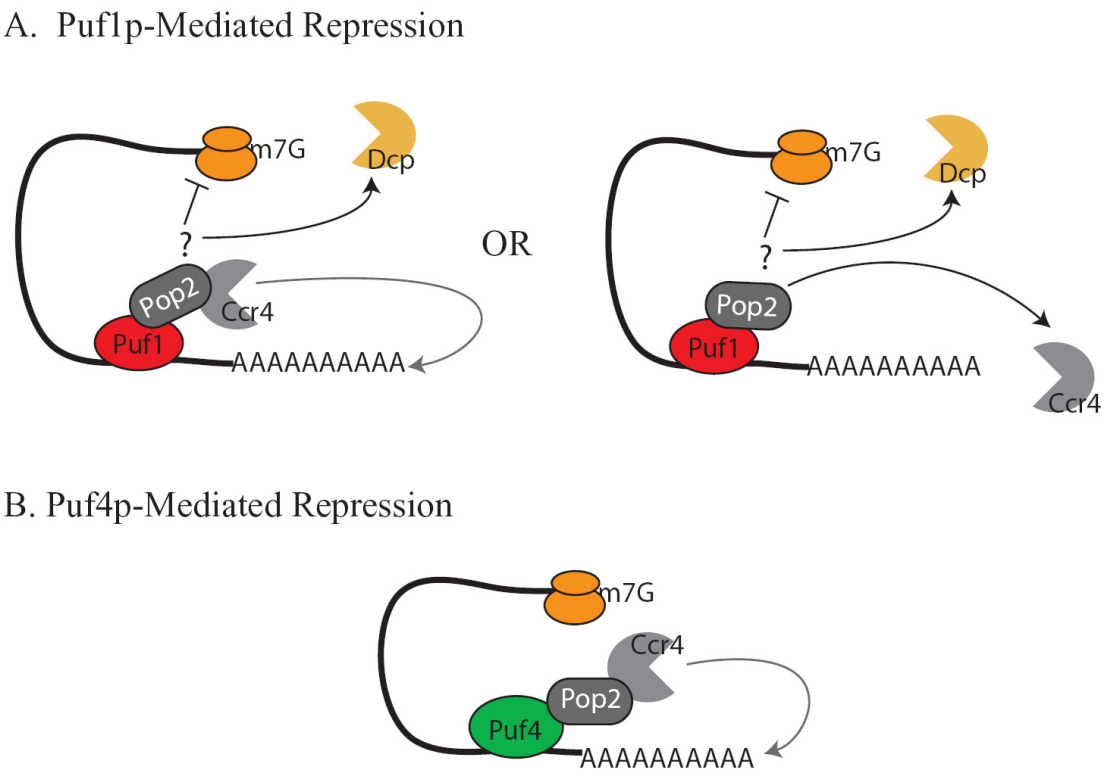

Figure 5.5. Model of Puf protein activity on *HXK1* mRNA.

A. Puf1p binds to *HXK1* 3'UTR to repress expression in a manner dependent on Pop2p. Puf1p likely influences deadenylation indirectly via Pop2p, either by using Pop2p to recruit Ccr4p to the mRNA (left) or by recruiting Pop2p, which then stimulates the activity of Ccr4p (right). In addition to deadenylation, Puf1p affects additional aspects of degradation and/or translation in a Pop2p-dependent manner. This repression may occur either via directly recruiting factors through Pop2p or through an indirect relationship between Pop2 and decapping and/or translation initiation factors. **B**. Puf4p-mediated repression of *HXK1* mRNA is similar to that of *HO* mRNA, acting via deadenylation.

While Pop2p commonly serves a role in deadenylation, the absolute dependence of *HXK1* deadenylation and decay on Pop2p is unique. It is well known that the dependence on decay factors, especially Pop2p, varies with the transcript, however, it is interesting that *HXK1* mRNA shows a dependence on Pop2p even though other Puf-regulated mRNAs, like *COX17,* do not show such dependence (Tucker et al. 2002). Since the activity of Puf3p and the *HXK1*-regulator Puf proteins appears to be diverse, this could suggest that the activity of Puf proteins varies with the decay factors native to the target mRNA, perhaps suggesting that Puf proteins increase affinity of the existing decay factors for the mRNA, rather than recruit decay factors to the mRNA that are not ordinarily present.

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CHAPTER VI:

YHB1 mRNA IS A TARGET OF Puf-MEDIATED DECAY

CHAPTER VI: *YHB1* MRNA IS A TARGET OF Puf-MEDIATED DECAY

Like *HXK1* and *TIF1*, *YHB1* is a stress-responsive gene. *YHB1* was also identified as differentially expressed in the microarray comparing steady-state mRNA levels between WT yeast and yeast deleted of all five *PUF* genes (Olivas and Parker, 2000). In my M.S. thesis, results suggested that endogenously transcribed *YHB1* was stabilized by Puf2p in a condition-specific manner, however, work since then has been unsuccessful in verifying that Puf2p inhibits decay of *YHB1* mRNA (data not shown). In this chapter, I use alternate methods to further analyze the Puf proteins involved in *YHB1* mRNA stability.

YHB1 mRNA decay is Puf-regulated

Previous studies found that a fusion mRNA containing the *YHB1* mRNA 3'UTR fused to the *MFA2* coding region had no apparent change in mRNA half-life upon deletion of individual *PUF* genes. While I speculated several possible explanations for this result, including requirement of *YHB1* sequences in the coding region or 5'UTR, the most likely explanation is that the *MFA2* transcript default decay is too rapid for noticeable changes in Puf-mediated decay through the *YHB1* 3'UTR, since the WT *YHB1* is more stable than *MFA2*. However, other mRNAs with longer default half-lives, like *PGK1*, are better suited for our tests. In support of this idea, I showed in Chapter IV that the *HXK1* 3'UTR allowed regulation of a *PGK1/HXK1* fusion mRNA (Figure 2.1), even though it was unable to allow consistent Puf-mediated regulation of a similar *MFA2/HXK1* fusion mRNA (Figure 1.5). Therefore, I fused the *YHB1* 3'UTR to the *PGK1* coding region and tested the decay of the fusion mRNA in WT yeast compared to

various *PUF* deletion yeast, controlling transcription via both the *GAL* promoter and the *rpb1-1* temperature-sensitive RNA Polymerase. While the *PGK1/YHB1* mRNA had a half-life of 17.5 (+/- 0.5) min in WT yeast, it decayed >2-fold longer in Δ*puf1-5* yeast, with a half-life of >30min (Figure 6.1B). Thus, at least one Puf protein is acting to destabilize the *YHB1* 3'UTR. I then tested decay of the *PGK/YHB1* mRNA in the individual deletion yeast strains to determine the Puf protein(s) responsible for destabilizing the mRNA. As shown in Figure 6.1B, *PGK1/YHB1* mRNA had an extended half-life in yeast deleted of *PUF5* ($T_{1/2}$ >30min, Figure 6.1B). Since the *PGK1/YHB1* mRNA half-life in the *puf5*Δ strain resembles that of the Δ*puf1-5* yeast strain, it appears that Puf5p is sufficient to destabilize *YHB1* mRNA (Figure 6.1B). The half-life of *PGK1/HXK1* mRNA was similar to WT in *puf4*Δ yeast (15.2 +/- 2.5min, Figure 6.1B). *PGK1/YHB1* mRNA was stabilized in *puf2*Δ yeast (23.5 +/- 0.3min) compared to WT, however, this stabilization was to a lesser extent than in *puf5*Δ or Δ*puf1-5* yeast (Figure 6.1B). These results suggest that Puf2p may have a destabilizing affect on the *YHB1* mRNA. This conclusion is jeopardized considering that similar changes in *PGK1/HXK1* mRNA half-lives were also observed in WT versus *puf2*Δ yeast in Chapter IV (Figure 4.1B), but these changes were not apparent in *HXK1* endogenous decay or Hxk1 protein levels (Figure 4.1A and 4.2), ruling out Puf2p as a significant contributing factor to *HXK1* mRNA decay. Therefore, future work should be done to determine if Puf2p indeed destabilizes *YHB1*.

While my previous work suggested that Puf2p may stabilize *YHB1* mRNA, I was unable to verify this with endogenous *YHB1* mRNA, and *PGK1/YHB1* mRNA is clearly not stabilized by Puf2p under these conditions. However, the half-life of *PGK1/YHB1*

mRNA is decreased in both *puf3*Δ (12.0 +/- 0.0min) and *puf1*Δ (9.9 +/- 2.1min) yeast compared to WT (17.5 +/- 0.5min), suggesting that Puf3p and Puf1p may somehow stabilize *YHB1* mRNA (Figure 6.1B). In my Thesis, I showed that Puf5p as well as Puf3RDp and Puf1RDp are able to specifically bind to the *YHB1* Puf element in vitro. Since both Puf3p and Puf1p have destabilizing activity on multiple other mRNA targets, I hypothesize that these proteins stabilize *PGK1/HXK1* mRNA indirectly. In one scenario, these proteins may passively interact with the *YHB1* Puf element in vivo as they do in vitro, but without significant destabilizing activity. In this model, in the absence of either Puf1p or Puf3p the Puf element is more available for specific interaction with Puf5p, thus allowing more efficient destabilization by Puf5p in *puf1*Δ or *puf3*Δ yeast.

While I consider it a single element, the *YHB1* 3'UTR actually contains two UGUA sequences in tandem (Figure 6.1A). This UGUAUGUA sequence element is similar to the *HO* 3'UTR element that, through multiple lines of evidence, has been shown to be required for Puf5p binding and decay mediation (Tadauchi et al. 2001, Goldstrohm et al. 2006, Goldstrohm et al. 2007). In my Master's thesis I showed that purified repeat domains of Pufs 1, 2, 3 and 5 are capable of specific binding to this region in vitro. These studies also showed that each UGUA sequence is required for specific binding of the PufRDs to the *YHB1* 3'UTR. Therefore, I expected that mutating the *YHB1* 3'UTR Puf- element to ACAAACAA via site-directed mutagenesis would eliminate Puf-mediated decay of the *PGK1/yhb1* mutant transcript (Figure 6.1A). The *PGK1/yhb1* mutant mRNA decayed similarly to the WT transcript in the *puf5*Δ or Δ*puf1-5* yeast, suggesting that this element is required for Puf5p-mediated decay of the *YHB1*

mRNA and confirming that *YHB1* is a direct target of Puf5p-mediated decay regulation (Figure 6.1B).

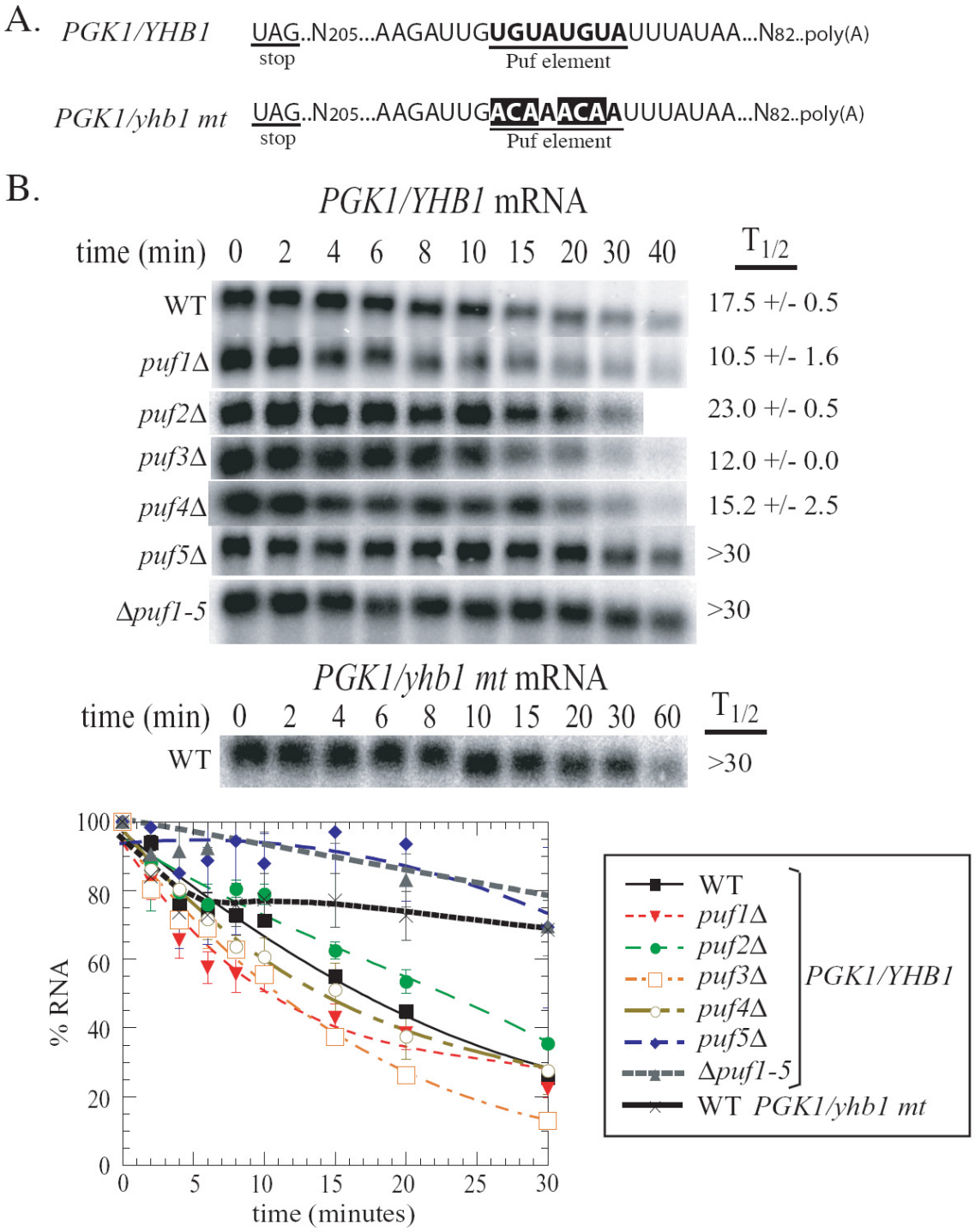

Figure 6.1. *PGK1/YHB1* mRNA decay is regulated by Puf5p.

A. 3'UTR sequences of *PGK1/YHB1* WT mRNA (top) and *PGK1/yhb1* mutant (*mt*, bottom). The stop codon and Puf-elements are underlined. Sequences mutated by site-directed mutagenesis in *PGK1/yhb1 mt* are indicated in black boxes. **B.** Representative Northern blots (upper panels) as well as a graphical representation of the data (bottom panel) illustrating decay of *MFA2/YHB1*

(filled square*)* and *PGK1/yhb1 mt* (x) mRNA in wild-type (WT, black), individual PUF deletions (*puf1*Δ, red; *puf2*Δ, green; *puf3*Δ, orange; *puf4*Δ, olive; *puf5*Δ, blue) and multiple *PUF* deletion (Δ*puf1-5*, gray) yeast strains grown to an OD_{600} of 1.0. Minutes following transcription repression are indicated above blots and along the x-axis of the graph. The estimated $T_{1/2}$ is listed to the right of each representative Northern blot. Error for each data point and $T_{1/2}$ is the SEM ($n \geq 2$).

Materials and Methods

In Vivo Decay Analysis

Steady-state transcriptional shut-off experiments, and Northern blot preparation was performed essentially as described in Chapter III, Materials and Methods. The *YHB1* 3'UTR was fused to *PGK1*Δ82 to create the *PGK1/YHB1* 3'UTR fusion construct essentially as described in Chapter IV, Materials and Methods. The *PGK1/YHB1* 3'UTR construct was created by amplifying the *YHB1* 3'UTR from genomic DNA using primers oWO262 and oWO263. The Klenow-filled (New England Biolabs) *Bgl*II site at the 5' end of the product was inserted between the Klenow-filled *Cla*I site and *Hind*III site of pWO102 (PGK1Δ82) to create pWO127. The *PGK1/YHB1* fragment was removed from pWO127 (*Sac*I/*Hind*III) to pWO61, a *LEU2* expression vector, to create pWO128. pWO127 and pWO128 express the *PGK1*Δ82 coding region fused to the *YHB1* 3'UTR under the control of the *GAL* UAS.

Control shut-off experiments of the native *PGK1* mRNA were performed using pWO102 and pWO103 (*PGK1*Δ82) essentially as described in the Chapter IV, Materials and Methods.

Transcriptional shut-off experiments were performed essentially as described (Caponigro et al. 1993) with the following modifications; 200ml cultures were grown to an OD_{600} of 1.0 in synthetic media with 2% galactose. Half of each culture was harvested and resuspended in 20ml of 37°C media containing 8% dextrose shutting off transcription via both the temperature-sensitive inactivation of RNA pol II and the carbon source inactivation of the *GAL* promoter. Total RNA was isolated from yeast as described (Caponigro et al. 1993) and Northern blots were prepared (NytranSupercharge

membrane, Schleicher and Schuell). Northern blots were probed with the following ^{32}P end-labeled oligonucleotides complementary to complementary to *PGK1* (control) or *YHB1* 3'UTR sequences: oWO159 (*HXK1*) and oWO447 (*PGK1*Δ82). All blots were normalized for loading to *scRI* RNA, a constitutively expressed RNA Polymerase III transcript (Felici et. al. 1989). All quantification of RNA was accomplished using ImageQuant software (Molecular Dynamics).

Site-Directed Mutagenesis

In vitro site-directed mutagenesis was performed to mutate *YHB1* 3'UTR Puf binding site using the QuickChange XL Site-directed Mutagenesis Kit (Stratagene). Primers oWO480 and oWO481 were used in PCR based mutagenesis of pWO127 and pWO128 as recommended by the manufacturer (Stratagene) creating pWO129 and pWO130, respectively. All resulting mutants were confirmed by sequencing.

Discussion

YHB1 mRNA decay is stimulated by Puf5p. The single UGUA element present within the *YHB1* 3'UTR is required for Puf-mediated decay. Previous data suggested that *YHB1* is stabilized by Puf2p, however this data remains unconfirmed. In fact, decay of *PGK1/YHB1* mRNA in *puf2*Δ yeast suggests that Puf2p may destabilize *YHB1* mRNA. This conflicting evidence could reflect indirect effects on *YHB1* mRNA or perhaps conditional regulation that experiments in this chapter have not captured. In an unexpected twist, Puf1p and Puf3p have stabilizing affects on the *PGK1/YHB1* mRNA. I hypothesize that these effects are passive or indirect, where the presence of Puf1p or Puf3p inhibits Puf5p-mediation of *YHB1* mRNA decay.

Steady-state data from my thesis suggests that *YHB1* levels are differentially regulated by Puf proteins based on conditions, including carbon source and growth phase. Thus, varying the growth conditions may alter Puf5p-mediated decay or enhance regulation by alternative Puf proteins (including Puf2p). Each of the decay experiments performed in this chapter were performed under the late-log phase conditions. Future work will be devoted to assaying *PGK1*/*YHB1* mRNA decay under alternate cellular conditions.

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CHAPTER VII:

SUMMARY AND FUTURE DIRECTIONS

CHAPTER VII: Summary and Future Directions

Several global microarray studies have identified hundreds of candidate mRNA targets of the yeast Puf proteins. In this work, a closer examination of a subset of candidates has established three mRNAs, *TIF1, HXK1* and *YHB1*, as direct targets of Puf-mediated decay regulation. For each of these mRNAs, multiple Puf proteins are involved in regulation. For *TIF1* mRNA, Puf1p and Puf5p are both required for full decay stimulation. For *HXK1* mRNA, Puf1p, Puf4p and Puf5p all play a part in decay stimulation and ultimately regulate Hxk1p function. For both of these mRNA targets, the absence of one Puf regulator is sufficient for a partial decay phenotype. The *HO* mRNA is the only other documented example of a transcript that is regulated by more than one yeast Puf protein, with both Puf4p and Puf5p required for maximal stimulation of deadenylation (Goldstrohm et al. 2006, Hook et al. 2007). With just these three examples, it is intriguing that Puf5p is the common Puf acting together in some combination with Puf1p and/or Puf4p. It is also clear from the decay phenotypes of individual *PUF* deletions in our studies and previous studies (Hook et al. 2007) that these Pufs are not simply acting redundantly, but coordinately to regulate their targets. Since these mRNAs are the only verified targets of Puf1p/Puf5p or Puf1p/Puf4p/Puf5p, and all show combinatorial control by at least two Pufs, such a mechanism is likely a common theme in mRNA decay regulation by the yeast Pufs. Moreover, combinatorial control may be a conserved mechanism of action in higher eukaryotes as well. In *C. elegans*, the Puf proteins FBF-1 and FBF-2 each act to control the sperm/oocyte switch via regulation of GLD-1 mRNA (Crittenden et al. 2002), while FBF-1 and PUF-8 act similarly to control a different step of this pathway (Bachorik and Kimble 2005).

My analysis of nearly 40 candidate targets of Puf protein regulation resulted in only three verified mRNAs that are under Puf-mediated decay control. It seems unlikely that we can account for this small percentage simply by categorizing all the remaining candidates as false positives or indirect targets identified in the microarray screens. Instead, many of the candidate mRNAs may indeed be direct targets of Puf-mediated decay, but the regulation of decay only occurs under particular growth conditions due to either differential activity of the Pufs or differential activity of other regulatory factors. The conditions under which Puf3p and Puf4p are active to regulate mRNA stability were computationally predicted based on steady-state microarray data of candidate target mRNAs (Foat et al. 2005). However, it is not known how growth conditions might affect the activity of the other Pufs. It is also possible that candidate targets are bound by Pufs for processes other than mRNA decay. For example, the *PMP* mRNAs were not only bound by particular Pufs (Gerber et al. 2004), but we showed they had changes in steady-state levels in some *PUF* deletions. However, we could not detect any changes in their half-lives under these conditions, suggesting that Pufs may be acting in some other step of their gene expression. The repeat domains of Pufs appear to be sufficient for mRNA binding and decay regulation (Wharton et al. 1998; Jackson et al. 2004), yet these domains usually compose less than half of the protein. The large regions outside of the Puf repeat domain have no known function, but may be acting in other cellular pathways. Together our work establishes the importance of direct testing using conventional approaches to evaluate candidate mRNA targets of Puf regulation derived from global microarray screens. This analysis not only identifies the bona fide targets of Puf-

mediated decay stimulation, but provides insight into the mechanisms by which Puf proteins act individually or in combination to regulate mRNA decay.

The data accumulated in this work has provided additional examples of Puf-mediated decay and identified additional sites to which Pufs bind in order to regulate their mRNA targets. In an attempt to survey the specificity of each yeast Puf protein, I have summarized the information known regarding the sites important for Puf1p-, Puf5p- and Puf4p in Table 7.1. The Puf1p binding sites appear to be the most diverse. Because Puf1p sites overlap with other Puf sites, including Puf5p, it is possible that the specificity of Puf1p is more flexible than the other Puf proteins. Comparison of the Puf5p-mediated sites brings a clearer picture into focus, where there are three nucleotide positions conserved (asterisks) in addition to the core UGUA sequence (Figure 7.1). These nucleotides include a downstream UA sequence (shaded gray), predicted to be important (Berstein et al. 2005). We expect these positions to show little variability as we identify additional targets of Puf5p-mediated mRNA decay. Since we have very few known binding sites for Puf4p, there are few conclusions that can be made about this protein, however the sites of this protein also appear to be variable. The Puf4p crystal structure indicates that Puf4p-recognition of its target sequences is flexible in the fourth and fifth nucleotide position (Miller et al. 2008), perhaps accounting for the adjustable position of the downstream UA in the Puf4p recognition sites (Figure 7.1). Overall, the culmination of this data suggests that Puf protein recognition of its targets is largely flexible.

mRNA	PBE	Sequence	Regulator(s)	Ref.
		★★★★ ★		
TIF1	site #1	UUUGUAUUUAAU	Puf1/Puf5	This study
HXK1	site #1	AAUGUAAUGAAA	Puf1?	This study
	site #2	UAUGUAAAUAUA	Puf4/Puf1?	This study
	site #3	UGUGUAUAAAUC	Puf1	This study
		★★★★★ ★★		
TIF1	site #1	UUUGUAUUUAAU	Puf1/Puf5	This study
	site #2	UUUGUAUAUACC	Puf5	This study
YHB1	site	UGUGUAUGUAUU	Puf5	This study
HO	site #2	GUUGUAUGUAAU	Puf5	Tadauchi et al. 2001
		★★★★ ★		
HXK1	site #2	UAUGUAAAUAUA	Puf4/Puf1?	This study
HO	site #1	UGUGUAUUAGUU	Puf4	Hook et al. 2007

Figure 7.1. Summary and comparison of 3'UTR Puf elements.

The Puf binding elements (PBEs) experimentally verified to be important for Puf1p-, Puf5p- or Puf4p-regulation are listed. Each PBE is labeled (site#1, site#2 or site#3) as referred to in the text of Chapters III, IV, and V. The Puf protein that regulates each site is listed in the column labeled "Regulator(s)". A question mark next to the Puf regulator indicates that this Puf protein is suggested to regulate at this site from experimental data, however, it remains unconfirmed. Puf1p-specific sites are grouped in the top panel, Puf5p sites in the middle panel and Puf4p sites in the bottom panel. The core UGUA element is boxed in each sequence group. Nucleotides conserved between all Puf-sites in each group are indicated by an asterisk above each panel. The downstream UA sequence is shaded gray.

Several observations now argue that much of 3'UTR based control of mRNAs will be combinatorial in nature. As we and others have shown, three out of six mRNA targets of yeast Puf regulation are controlled by multiple Puf proteins. In higher eukaryotes, 3'UTRs often contain multiple Pumilio protein and CPEB binding sites, allowing combinatorial activity of these two proteins (Pique et al. 2008). Similarly, mRNAs in metazoan cells are regulated by multiple different miRNAs. The complicated nature of this combinatorial type of regulation implies that the effects of any given trans-acting factor may be minimized in an experiment since there are other contributing factors. These considerations may be complicating much of 3'UTR analysis.

Future Directions

In order to better understand the nature of Puf1p-mediated decay, future work will be done to identify the biochemical interactions of Puf1p with the decay factors. The data presented here suggests a model in which Puf1p interacts directly with Pop2p and indirectly with Ccr4p. These interactions can most easily be visualized via co-immunoprecipitation experiments. Since other projects in the Olivas lab have performed similar experiments co-immunoprecipitating Myc-tagged decay factors with FLAG-Puf3p, the system is available to perform these experiments with FLAG-Puf1p. Another biochemical experiment that will be valuable is to confirm the Puf1p interaction with the WT *HXK1* versus the *hxk1* mutant mRNA in vivo. This can be accomplished by immunopurifying the FLAG-Puf1p from a yeast strain expressing both endogenous *HXK1* and *PGK1/hxk1* mutant mRNAs. Using reverse-transcription (RT) PCR to amplify *HXK1* and *hxk1-3x* 3'UTRs from RNA extracted from the pull-downs will allow a comparison of the amount of mRNA bound to the Puf1 protein. Perhaps the more

interesting aspect of this experiment is to compare the mRNA binding ability of the repeat domain to the full-length Puf protein in order to determine if the putative RRM upstream of the repeat domain is involved in target binding or target specificity.

It is clear that Puf1p, Puf5p, and Puf3p influence aspects of decay and/or translation other than deadenylation, yet little evidence has shown a direct connection between the Puf proteins and decapping or translations initiation. Therefore, future experiments assaying the involvement of the decapping proteins in Puf1p-mediated decay will be useful. Using the *HIS3* repression system I have already established, one could assay the requirement for *DCP1* or *DCP2* in repression of the *HIS3* fusion mRNA. As an alternative, if Puf proteins are involved in decapping or translation initiation, we might expect Puf1p to interact with the cap either directly or indirectly.

Broad Perspectives

I entered into this project with the idea that identifying targets of each Puf protein would allow classification of the distinct specificity for each protein. I also expected that, because the proteins are very similar to each other, they would also repress their targets in a similar manner. This work has clearly shown that regulation of mRNA decay by Puf proteins, like other 3'UTR-regulatory mechanisms, is more complex than expected. I have found that a single mRNA can be regulated by multiple Puf proteins and that, while elements important for Puf-mediated decay are similar and sometimes overlapping, they are also distinct. Furthermore, the mechanism by which the proteins repress their mRNA targets is also similar yet distinct. Multiple Pufs in higher organisms likely also act in a combinatorial fashion. This idea should be kept in perspective when examining the role of the Puf proteins in processes such as memory formation and cell cycle control, as more

than one Puf may be playing a part in regulation. The sequence specificity of the yeast Pufs may also be applied to higher organisms, perhaps aiding in the identification of additional targets in human systems.

The complex nature of Puf-mediated repression and post-transcriptional regulation of gene expression in general brings us to a biological realization; gene expression is neither on nor off, but in a continuum of repression and enhancement. The ultimate result of the complex specificity and diverse mechanisms of Puf protein regulation is precise and flexible expression patterns specific to each protein.

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