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The Role of Puf3 Protein Interactions in the Regulation of mRNA Decay in Yeast *Saccharomyces Cerevisiae*

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THE ROLE OF Puf3 PROTEIN INTERACTIONS IN THE REGULATION OF mRNA DECAY IN YEAST *Saccharomyces cerevisiae*

by

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ABSTRACT

The regulation of messenger RNA (mRNA) metabolism is an important step in proper gene expression. In many eukaryotic organisms this regulation can be mediated by a group of highly conserved RNA-binding proteins known as the Puf family of proteins. The yeast *Saccharomyces cerevisiae* has several proteins that belong to this family. One of the yeast Puf proteins, Puf3p, binds and regulates the *COX17* mRNA, which codes for a protein involved in mitochondrial copper transport. Specifically, Puf3p stimulates the decay of *COX17* mRNA. However, the precise mechanism of Puf3p binding and decay regulation is yet unknown. The goal of this research is to determine the role of the Puf3p interactions required for regulation of mRNA decay in yeast, and to understand how Puf3p activity is regulated.

The studies to examine Puf3p interactions have focused on the Puf3 protein sequences required for specific binding and regulation of *COX17* mRNA decay. The data show that a specific region of the Puf3 protein, known as the Puf3 Repeat Domain, is sufficient to both bind *COX17* mRNA and also regulate its rate of decay. Furthermore, key amino acids on the RNA-binding surface of the repeat domain that promote target specificity have been identified, as well as a specific loop structure on the protein-binding surface of the repeat domain that is required for RNA decay regulation. In addition, these studies show that the repeat domain of Puf3p directly interacts with other known mRNA decay factors, more specifically, decay factors that are involved in the deadenylation and decapping steps of mRNA decay.

Additional collaborative studies have focused on the condition-specific regulation of mRNA stability in yeast. In these studies, the activity of Puf3p was found to be dependent on the available carbon source, as well as inhibited by rapamycin treatment, which in turn places the Puf3p downstream of the Target of Rapamycin (TOR) signaling pathway. Together the results from the research in this body of work will help further our understanding of transcript-specific decay mechanisms in yeast and other eukaryotes.

DEDICATION

This work is dedicated to my beautiful wife Theresa for

her love, patience, and continuous support

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First and foremost, I would like to extend my everlasting gratitude to my advisor Dr. Wendy M. Olivas for her guidance, inspiration, encouragement, and friendship. I would also like to thank my current and previous dissertation committee members Dr. Shirley Bissen, Dr. Teresa Thiel, Dr. Marc Spingola, Dr. Cynthia Dupureur, and Dr. Donald Becker for their assistance in my progression in the last four years.

I would like to thank my lab-mates: The original "PufDaddys" John Jackson, Andrew van Brunt, and Jeff Griesemer, as well as the "PowerPuf Girls" Florencia Lopez Leban, Randi Ulbricht, and Melanie Miller. It is with the help of these wonderful individuals that this work was made possible. I am proud to call them "my friends."

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Introduction

Eukaryotic mRNA Processing

Gene expression in eukaryotes is a complex process requiring specific transcriptional, as well as post-transcriptional regulation. It has become apparent that the regulation of mRNA metabolism is one important aspect of post-transcriptional regulation necessary for proper gene expression. Comprehension of the regulation of mRNA metabolism requires an understanding of the eukaryotic mRNA processing pathway. The life cycle of an mRNA (Figure-1) begins as a single-stranded copy of a gene produced via nuclear transcription. The transcript is then subjected to 5' capping, splicing, and 3' polyadenylation, which are processes that lead to the production of a mature mRNA. Although each of these processes is distinct from one another, they actually occur co-transcriptionally during the elongation of the transcript, and almost all are mediated by RNA polymerase II, more specifically the C-terminal domain (CTD) of the RNA polymerase II (Proudfoot, 2000).

Immediately after transcription begins, a unique structure called a "cap" is added to the 5' end of the transcript by the capping enzyme, a bi-functional polypeptide conducting the phosphotase and guanyltransferase activities, and a methylase (Parent et al, 2004). The structure of the cap entails a 7-methylguanyl (m^7G), which is added to the triphosphate end of the transcript and is linked to the first nucleotide by a special 5'-5' linkage. The bi-functional capping enzyme interacts with the CTD of RNA polymerase II and carries out the capping before the transcript has reached a size of approxiamtely 30 nucleotides (Parent, et al., 2004; Ho and Shuman, 1999; McCracken et al., 1997; Cho et al., 1997). The cap has been shown to play a critical role in the stability,

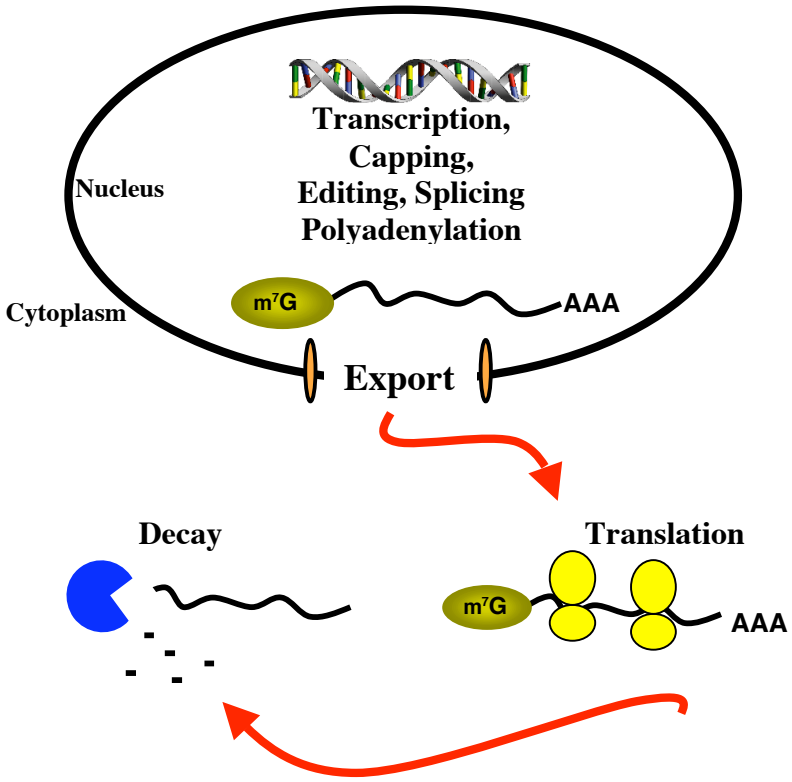


Figure - 1: mRNA Life Cycle. Simple schematic representation of the life cycle of an mRNA.

transport, as well as translation of mRNAs (Furuichi and Shatkin, 2000).

Also occurring during transcription are the processes of editing and splicing. RNA editing is a process that enables a C or A base deamination to U or I, respectively, leading to a change in structure and function of the resultant protein. In mammals a handful of mRNA editing substrates along with an essential deaminase have already been identified. However, very little is known about RNA editing in yeast. Interestingly, the mammalian deaminase has been shown to carry out the editing of its mammalian target mRNA in yeast (Dance et al., 2000). Moreover, an ortholog of the mammalian deaminase has been discovered in yeast and even though its target is yet unknown, it has been shown to edit the target of the mammalian deaminase (Dance et al., 2001). Several studies have suggested that mRNA editing is functionally linked with splicing and that the editing process may actually precede the splicing process. Additionally, although to this date the role of the RNA polymerase II CTD in RNA editing has not yet been determined, given the role of the CTD in other nuclear mRNA co-transcriptional processes, it is hypothesized that the CTD may play a role in mRNA editing as well (Howe, 2002).

Splicing is a process that enables the removal of the non-coding intervening regions (introns) from the transcript, hence bringing the coding regions (exons) together for proper expression. Splicing is catalyzed by the spliceosome, a large complex formed by five small nuclear RNAs. Although the precise role of RNA polymerase II in splicing is not fully understood, RNA polymerase II has been shown to co-immunoprecipitate with splicing regulatory proteins. In addition, the importance of its CTD has been demonstrated by the inhibition of splicing in mRNA transcribed by a CTD-lacking RNA

polymerase II (Kim et al., 1997; McCracken et al, 1997; Mortillaro et al, 1996). Splicing is less common in yeast where only 3% of genes have introns, in comparison to mammalian genes where over 90% of genes are intronic.

Polyadenylation is the other process that also occurs during transcription. In this process a stretch of adenylate residues are added to the 3' end of the mRNA, constructing what is referred to as the poly(A) tail. All mRNAs, except histone mRNAs, are polyadenylated. Polyadenylation involves an endonucleolytic cleavage at the polyadenylation synthesis site that is signaled by the AAUAAA sequence in mammals, or an A-rich element in yeast, located upstream of the cleavage site. Along with the essential polyadenylation sequence, the cleavage stimulation factor (CSF), the cleavage/polyadenylation specificity factor (CPSF), as well as the poly(A) polymerase are required for proper polyadenylation (Shatkin and Manley, 2000). The CTD of RNA polymerase II also plays an important role in polyadenylation, for without a functional CTD, polyadenylation is inhibited (McCracken et al., 1997). Additionally, the cleavage/polyadenylation factor has been shown to co-immuoprecipitate with RNA polymerase II (Dantonel et al., 1998). Although the mechanism of polyadenylation is similar in eukaryotes, the length of the poly(A) tails differ greatly. In mammals, poly(A) tails average 250 A-residues, while in yeast poly(A) tails average 100 A-residues.

Following these nuclear processes, the mature mRNA is exported into the cytoplasm. Post-transcriptional regulation continues in the cytoplasm where the ribosomes, with the help of transfer RNAs (tRNAs), translate the mRNA into the protein for which it encodes. Lastly, the mRNA enters the decay pathway.

Eukaryotic mRNA Decay

Two general mRNA decay pathways have been identified in yeast and mammals (Figure-2), the deadenylation-independent and the deadenylation-dependent pathways. The deadenylation-independent pathway includes three subpathways. First is deadenylation-independent decapping followed by 5' to 3' exonuclease digestion, as seen in nonsense-mediated decay (NMD), which recognizes and degrades mRNAs that contain premature translation stop codons. Second is nonstop decay, which recognizes and degrades mRNAs lacking translation stop codons through direct 3' to 5' exonucleolytic decay. Third is endonucleolytic cleavage of mRNA (Parker and Song, 2004). The deadenylation-dependant pathway includes two subpathways: deadenylation followed by direct 3' to 5' exonucleolytic decay, and a multi-step deadenylation-dependent decapping followed by 3' to 5' exonucleolytic decay that has been shown to be the most conserved pathway for mRNA turnover in eukaryotes, and the most common in yeast (Figure-3).

This pathway has several distinct steps and as indicated by its name, the first step in this degradation of mRNA is the deadenylation of the 3' end of the transcript. The removal of the poly(A) tail is done by a 3' to 5' deadenylase enzyme complex. Two endonucleases, Ccr4 and Pop2, and other accessory proteins make up the deadenylase complex. Ccr4 is believed to be a member of the ExoIII family of nucleases (Dlakic, 2000) and has been predicted to nucleophilically attack the phosphodieseter bond by activation of an OH group through specific glutamic acid and histidine residues. Mutations in these residues have been shown to abolish the Ccr4 activity. Furthermore, it appears that Ccr4 conducts the catalytic deadenylation function of the deadenylase

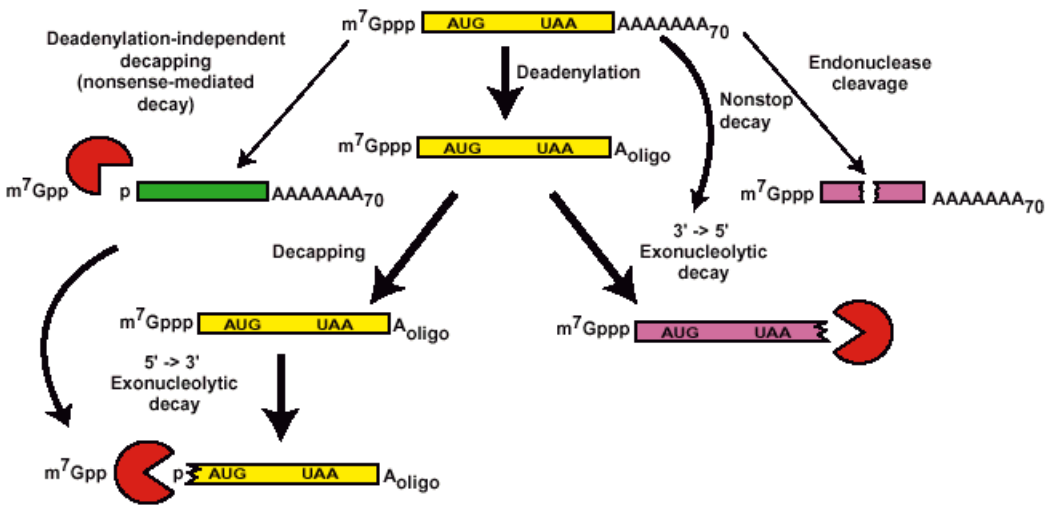


Figure – 2: Eukaryotic mRNA turnover pathways. General deadenylation-independent and deadenylation-dependent pathways (Parker and Song, 2004).

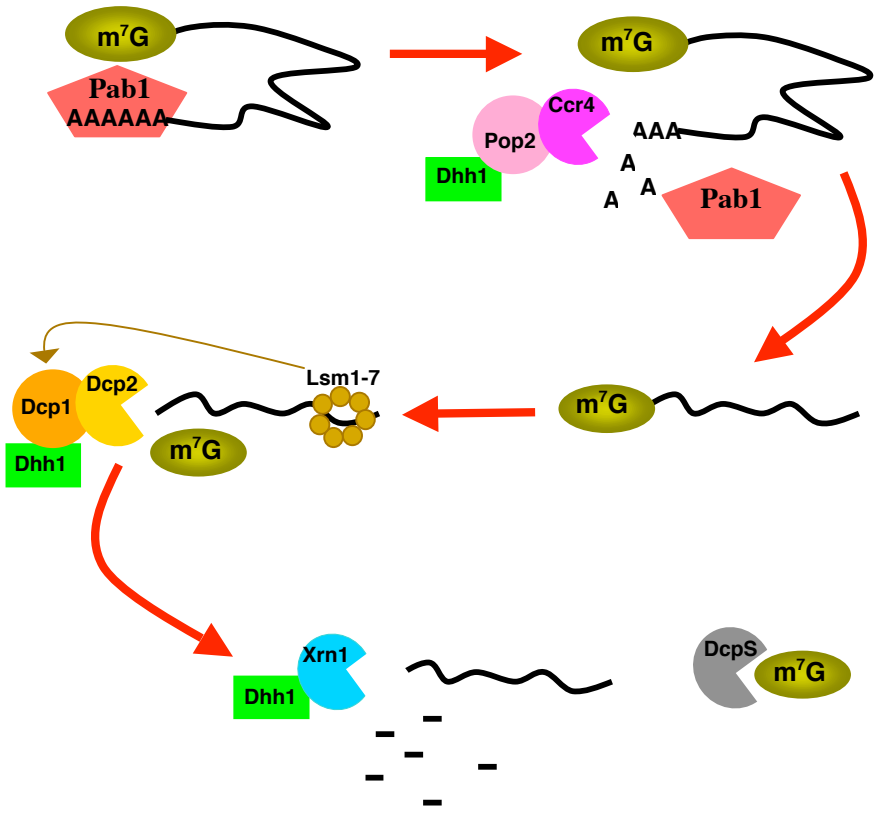


Figure – 3: Yeast mRNA decay pathway. The predominant deadenylation-dependent mRNA decay pathway in *Saccharomvces cerevisiae.*

complex (Tucker et al., 2004; Chen et al., 2002). Pop2 is a member of the RNaseD family of nucleases. It has two functions. One is as a nuclease and the other is as an enhancer of the function of the Ccr4 deadenylase, perhaps via stabilization of the deadenylase complex. Both Ccr4 and Pop2 have been found to be conserved in eukaryotes with gene variants present in the genomes of more complex eukaryotes (Parker and Song, 2004). There is one other protein of interest that has been shown to interact with the deadenylase complex; this protein is Dhh1. Dhh1 is a member of the DEAD box helicase family and has been demonstrated to associate with Pop2 of the deadenylase complex (Hata et al., 2001). This protein is also involved in other steps of the decay process and will be further discussed below.

Deadenylation of the 3' end of the transcript is the precursor to the next step in the decay pathway, which is decapping of the transcript. Studies have shown that the presence of a poly(A) tail along with the binding of the poly(A) binding proteins (Pabs) can inhibit decapping (Coller et al., 1998; Capanigro and Parker, 1995). The mode of this inhibition is still unknown, although *in vitro* experiments have shown that Pabs can bind the cap structure and inhibit decapping directly (Khana and Kiledjian, 2004). Consequently, removal of the poly(A) tail and its accompanying Pab proteins eliminates the interaction between the 5' end cap binding complex and the 3' end of the mRNA, hence, linearizing the mRNA and exposing the 5' cap structure for removal by the decapping enzyme.

In eukaryotes there are two types of decapping enzymes, Dcp1-Dcp2 and DcpS. As the name suggests, Dcp1 and Dcp2 together form the decapping enzyme Dcp1-Dcp2. The function of Dcp1-Dcp2 enzyme is the removal of the cap structure, but the exact

mode of its activity is not fully explained. It has been suggested that Dcp1-Dcp2 decapping enzyme recognizes its target by interacting with both the mRNA itself as well as the cap structure (Parker and Song, 2004). Although Dcp2 is the catalytic unit of the complex, its activity is stimulated by Dcp1; moreover, both subunits are essential, for the loss of either subunit completely inhibits decapping in yeast (Dunckley and Parker, 1999; Beelman et al., 1996). There are other secondary factors that also play a role in decapping. One such important factor is the aforementioned, deadenylase-interacting, Dhh1 protein. Interestingly, Dhh1 interacts with Dcp1 as well (Coller et al., 2001; Uetz et al., 2000) and it has been shown to be required for efficient decapping (Coller et al., 2001) by stimulating the activity of Dcp1 (Fischer and Weis, 2002). Other important secondary factors include the Lsm proteins, which also have been shown to affect mRNA decay. There are two seven-member Lsm complexes, one composed of Lsm proteins 1-7, and the other composed of Lsm proteins 2-8. The Lsm2-8 complex has been shown to be involved in the splicing machinery (Mayes et al., 1999; Stevens and Abelson, 1999). Studies of the Lsm1-7 complex have shown that mutations in Lsm1-7 inhibit decapping (Mayes et al., 1999; Bouveret et al., 2000; Tharun et al., 2000), suggesting that this complex may act as an activator of decapping (Tucker and Parker, 2000). Moreover, Lsm1 has been shown to directly associate with Dcp1 has been confirmed (Tharun et al., 2000). Other studies have suggested that the Lsm1-7 complex interacts with the 3' end of the mRNA and is involved in 3'end protection, which leads to decapping activation (He and Parker, 2000; Boeck et al, 1998).

The second decapping enzyme is referred to as the "scavenger" decapping enzyme, or DcpS. It has been shown to have two functions. One is the decapping of

capped oligonucleotides produced by 3' to 5' exosome-mediated decay. The second function is the hydrolysis of the m^7G cap produced by normal decapping of mRNAs by the Dcp1-Dcp2 enzyme.

After decapping, the mRNA goes through the final step of the decay pathway and is rapidly decayed by the 5' to 3' exonuclease Xrn1 (Muhlrad and Parker, 1994; Hsu and Stevens, 1993). It is again of interest to note that the aforementioned deadenylase complex- and Dcp1-interacting protein, Dhh1, has also been shown to associate with the Xrn1 exonuclease (Fischer and Weis, 2002).

Although it has been known for a while that mRNA decay is a cytoplasmic event, only recently more information regarding the precise location of its occurrence has come to light. Green fluorescent protein fusions of yeast mRNA decay factors were used to determine a more precise localization of these proteins in the cytoplasm. The Dcp1-Dcp2 decapping enzyme, the decapping activators Dhh1 and Lsm1-7, as well as the Xrn1 exonuclease were shown to localize in discrete cytoplasmic foci in yeast. These foci are referred to as processing bodies (P-bodies) and they are defined as the sites where decapping and 5' to 3' exonucleolytic decay of mRNAs occurs (Sheth and Parker, 2003). Further studies have shown that P-Bodies are dependent on mRNA for their formation, and their size and number vary under cellular stress as well as inhibition of translation-initiation, suggesting that P-bodies may act as storehouses for untranslated mRNAs in addition to their role in mRNA degradation (Teixeira et al., 2005).

Besides the factors discussed so far, there are additional elements and proteins that play equally important roles in mRNA decay. These factors direct the deadenylation and or the decapping steps of the decay pathway therefore regulating the mRNA decay

rates. Individual mRNAs in eukaryotic cells have been shown to have decay rate variances of more than two orders of magnitude (Cabrera et al., 1984; Singer and Penman, 1973; Spradling et al., 1975). For mRNAs encoding proteins that are needed in large volumes, slow decay rates are essential. In contrast, for those mRNAs that encode "time-sensitive" proteins, such as the mRNAs for mammalian oncogenes, cytokines, and lymphokines, it is imperative that these mRNAs are targeted for rapid degradation. Bearing in mind the complexity of the mRNA life cycle, regulation of mRNA decay rates is considered to be a faster way to control the protein levels in the cell.

One important element that plays a major role in determination of mRNA decay rates is the 3' untranslated region (3'UTR). 3'UTR sequences have been shown to control mRNA stability. Adenylate/Uridylate-rich elements (AREs) have been found in many 3'UTRs. These elements range from 50 to 150 nucleotides and their primary function is to target mRNAs for selective degradation (Grzybowska et al., 2001). For example, the mRNAs of mammalian oncogenes and the yeast *MFA2* RNA contain AU-rich destabilizing sequences in their 3'UTRs (Wilson and Treisman, 1988; Decker and Parker, 1995). In contrast, the β-globin mRNAs contain stabilizing elements in their 3'UTRs (Wang et al., 1995). In addition, other 3'UTR regulatory sequences have been identified in many organisms including *Drosophila*, *C. elegans*, and *S. cerevisiae* (Decker and Parker, 1995; Wharton and Struhl, 1991; Goodwin et al., 1993; Tadauchi et al., 2001). Although essential, these 3'UTR elements do not regulate mRNA decay rates by themselves. Proteins that bind these 3'UTR elements are also required (Derrigo et al., 2000; Grzybowska et al., 2001; Mazumder et al., 2003). These RNA-binding proteins

have been shown to be involved in mRNA metabolism and are emerging as important positive and negative post-transcriptional regulators of cellular gene expression.

The Puf Family of Proteins

One group of mRNA 3' UTR-binding proteins is the Puf family of proteins. The Puf proteins are characterized by the presence of eight consecutive repeats in their RNA binding region known as the Puf repeat domain. The Puf repeat domain is highly conserved among the Puf proteins. Each Puf repeat is of approximately 40 amino acids folded into three α-helices, with a "core consensus" that contains aromatic and basic residues (Wickens et al., 2002).

The first two members of this family to be analyzed in detail were *Drosophila* Pumilio (Pum) and *C. elegans* Fem3 Binding Factors (FBFs); hence the group is referred to as PUF proteins. The *Drosophila* Pumilio (Dm-Pum) binds specifically to two tandem sequence motifs, the Nanos Response Elements (NREs), in the 3'UTR of the *hunchback* mRNA (*hb* mRNA). The Dm-Pum bound to the NRE-containing RNA forms a quaternary complex with the Nanos (NOS) and Brain Tumor (BRAT) proteins (Wreden et al., 1997 and Sonoda et al., 2001). The complex enables the repression of Hunchback protein expression in the posterior half of the *Drosophila* embryo, and thereby permits proper abdominal development. The repeat domain of Dm-Pum alone has been shown to be sufficient to bind and rescue the defect of a Dm-Pum mutant (Wharton et al., 1998). The *C. elegans*' FBFs interact with the sequences in the 3'UTR of the *FEM3* mRNA, as well as a NOS protein to form a ternary complex. This complex represses the expression of the Fem3 protein, therefore, regulating the sperm/oocyte switch in hermaphroditic *C. elegans* (Zhang et al., 1997).

In addition to *Drosophila* and *C. elegans*, Puf proteins are widespread in eukaryotes. They have been found in animals, plants, and unicellular organisms. The variety of Puf proteins found in different organisms ranges from one to eleven Pufs. *C. elegans*, *Arabidopsis thaliana,* and *Sacharomyces serevisiae* are among those that have multiple Puf proteins. Humans and mice have two Pufs, while Drosophila has one (Wickens, et al 2002). A dendrogram of Puf proteins (Figure-4) shows that there are two distinct clusters of Puf proteins – the "Pumilio cluster" which includes the *Drosophila* Pumilio (Dm-Pum) as well as Pufs from other species and the "FBF cluster" containing nine of eleven *C. elegans* Puf proteins, which suggests a duplication and divergence burst in that species (Wickens et al, 2002)

The Dm-Pum and the closely related human Puf protein (Hs-Pum) have been crystallized and their structures have been determined (Edwards et al. 2001, & Wang et al. 2001). The crystal structures (Figure–5A-B) reveal that the Puf repeats are organized in an extended crescent shape structure with each repeat folding into a three-helix domain (Figure-5A). The structure also shows two distinct surfaces for the repeat domain, an inner side of the crescent and an outer side of the crescent. Based on the previously observed correspondence between the lengths of the inner surface of the Dm-Pum with the predicted length of an extended RNA binding site of 20-30 nucleotides for a monomer (Zamore et al, 1999), and based on the observation that interaction-disrupting mutations lie in the inner concave surface of the crescent, it was suggested that it is the inner surface of the crescent that interacts with the RNA (Edwards et al, 2001; Wang et al, 2001). In addition, based on the "core consensus" amino acid sequence of the repeats, interaction between the Puf proteins and mRNAs were suggested to consist of base

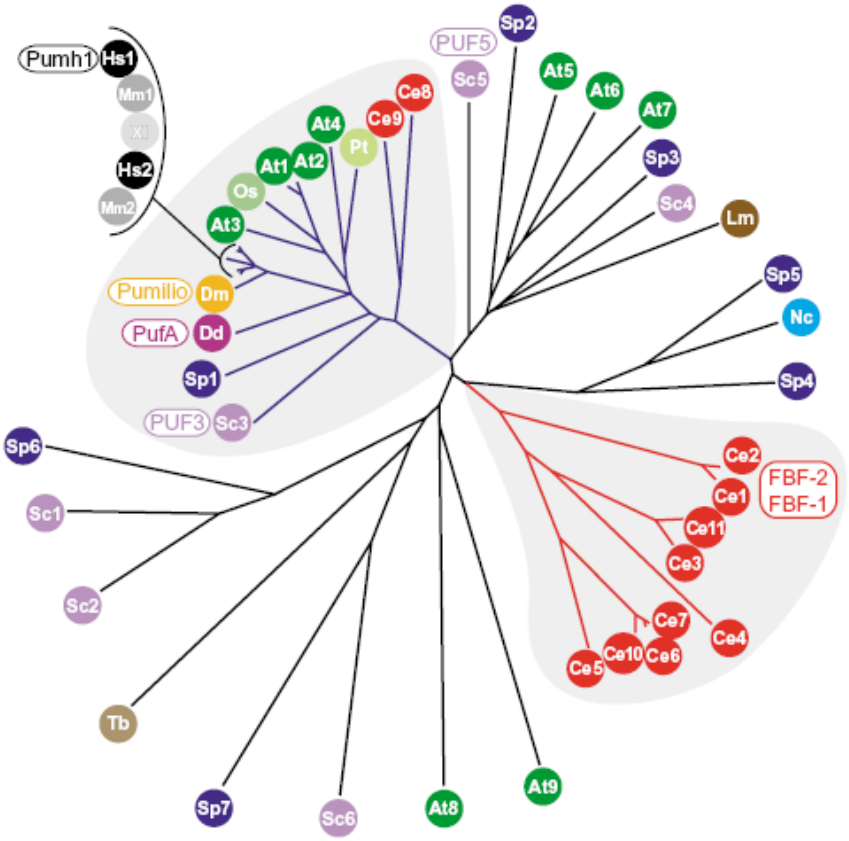


Figure – 4: The Puf protein family tree. A Dendogram of Puf proteins across eukaryotes, derived by aligning only the Puf repeat domain. The un-rooted tree suggests that many other Pufs were not included. Fungi: Sc (*Saccharomyces cerevisiae*), Sp (*Schizosaccharomyces pombe*), Nc (*Neurospora crassa*), and Dd (*Dictyostelium discoideum*). Vertebrates: Hs (*Homo sapiens*), Mm (*Mus musculus*), and Xl (*Xenopus laevis*). Plants: At (*Arabidopsis thaliana*), Os (*Oryza sativa*), and Pt (*Populus tremuloides*). Trypanosomes: Lm (*Leishmania major*) and Tb (*Trypanosoma bruce*). Ce (*Caenorhabditis elegans*). Dm (*Drosophila melanogaster*) Wickens et al., 2002.

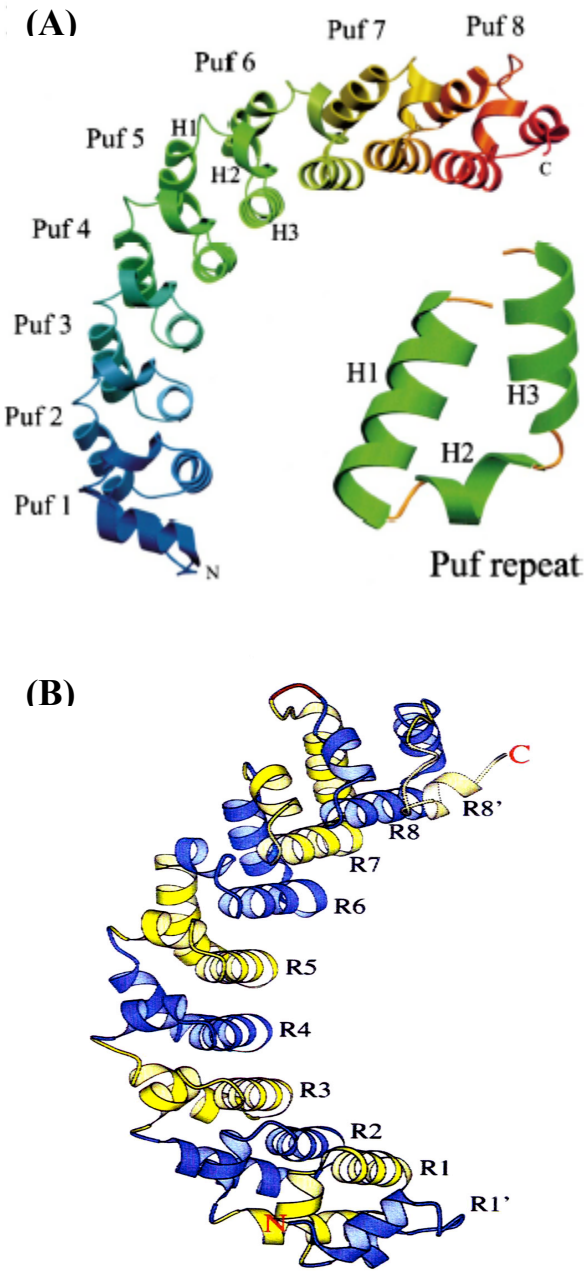
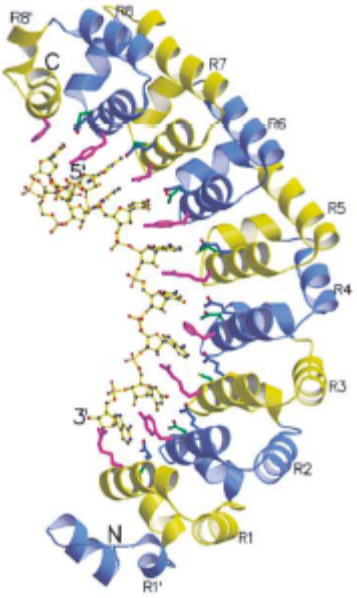


Figure – 5: Crystal Structure of the Puf Repeat Domain. The RNA binding region of (A)Dm-Pum (Edwards et al., 2001), where each puf repeat is composed of three α-elices (H1, H2, and H3); and (B) Hs-Pum (Wang et al., 2001).

stacking and charge-charge interactions with the backbone. Moreover, further mutational studies on the outer surface of the repeat domain indicated that it is the outer surface that is involved in interactions between Dm-Pum and its partners Nanos and Brat (Sonada et al., 1999; Sonada et al., 2001; Edwards et al, 2001; Wang et al, 2001). The crystal structure of the Hs-Pum bound to an RNA ligand confirmed the binding of the inner surface to RNA (Figure-6). This crystal structure of the Hs-Pum shows that nucleotides 1 through 8 of this RNA motif are contacted by protein repeats 8 through 1, respectively, showing a modular state of interaction with each repeat recognizing a specific successive base along the RNA (Wang et al. 2002).

Several studies have analyzed the interaction between Puf proteins and their target mRNAs in more detail. These studies have shown that the binding sequences of all RNA targets analyzed to date contain a shared UGUR motif required for Puf binding, with flanking sequences providing specificity (Wickens et al., 2002). Further inspection of the RNA target sequences of Dm-Pum and its most closely related Puf proteins reveals an expanded shared binding motif of UGUANAUA (Murata and Wharton 1995; Zamore et al. 1997; White et al. 2001; Nakahata et al. 2001). In contrast, the *C. Elegans* FBF binds a divergent target sequence containing UCUUGUGU (Zhang et al., 1997), where the underlined nucleotides are critical for binding.

(A)



(B)

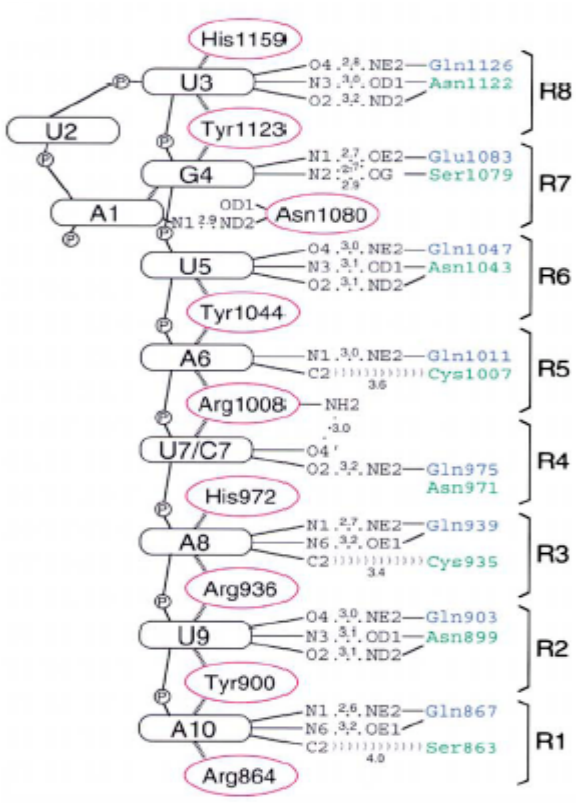
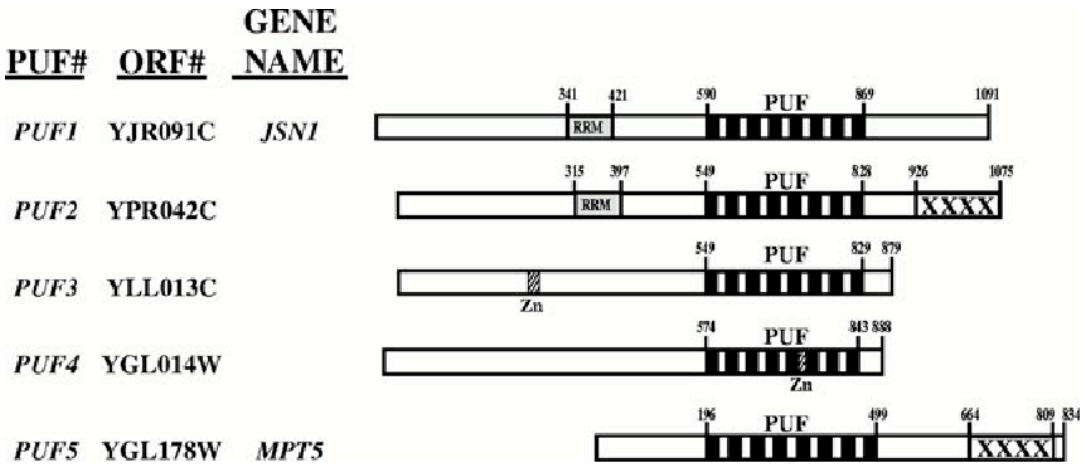


Figure - 6: Crystal structure of Hs-Pum with RNA ligand. (A) Crystal structure of Hs-Pum with *Drosophila* NRE-containing RNA. The RNA is shown as a ball and stick model. (B) Schematics of RNA-protein interactions. Residues of the α-helices of repeats 1-8 making stacking interactions with RNA bases are circled. Other residues making hydrogen bond and van der Waals interactions are indicated by dotted lines and "))))" respectively (Wang et al, 2002).

The yeast Puf proteins are a group of non-essential proteins and are conveniently named Puf 1-6. Based on several studies and sequence similarity analyses of the Puf repeat domain, it was originally believed that yeast has five proteins with the characteristic Puf repeat domain that belonged to the Puf family of proteins (Figure–7A), However, a less conserved sixth Puf has also been discovered (Figure-7B). The RNA-binding domain of the yeast Puf proteins 1-5 are highly conserved between each other as well as among other Puf family members. Only few specific targets of the yeast Puf proteins have been experimentally verified. However, several hundred candidate RNA targets that interact with one or more of the yeast Pufs 1-5 have been identified by a microarray analysis (Gerber et al. 2004). This extensive association study of the first 5 Puf proteins in yeast has also shown that Puf1, Puf2, Puf3, and Puf5 proteins are found only in fairly low abundance of 350-400 molecules per cell, while Puf4 is twice as abundant at about 900 molecules per cell. These numbers are consistent with those for other regulatory proteins such as transcription factors and kinases. This also implies that the intercellular concentration of these Pufs are within the range of 20 to 50 nM, which is one order of magnitude higher than the dissociation constants measured for several Pufs binding their target RNAs (Gerber et al, 2004). Another interesting observation is that consensus sequence motifs containing UGUR were also identified in many of the RNAs associated with Pufs 3, 4, and 5, with distinct sequences following the UGUR in the RNAs bound by each of these Puf proteins (Gerber et al, 2004). The same has also been observed in the RNA bound by the Puf6 protein (Gu et al, 2004).

Pufs are believed to be diverse in the processes they regulate. Little is known about the specific function of Puf1 and Puf2 proteins; however, they have been shown to

(A)



(B)

Figure – 7: Yeast Puf Proteins. Alignment and sequence elements of the yeast proteins of the Puf RNA-binding family. (A) Linear representations of Puf proteins 1-5 are drawn to scale, with the characteristic Puf repeat regions (denoted as eight black vertical rectangles) aligned with each protein. Puf1p and Puf2p also contain putative RRM RNA-binding domains (blue boxes), while Puf3p and Puf4p contain putative zinc finger domains (box labeled Zn). A c-terminal sequence region related in Puf2p and Puf5p is denoted by boxes labeled XXXX (Olivas and Parker, 2000). (B) Linear representation of Puf6 protein. The shaded regions indicate the seven conserved Puf repeats. D/E shows a region rich in aspartic and glutamic acids. NLS shows the position of the nuclear targeting signal (Gu et al., 2004). Puf6 is made up of 656 amino acids, with the seven Puf repeats located between amino acids 171 and 419. The D/E region is located between amino acids 45 and 100.

localize to the periphery of cells and are believed to interact with mRNAs that encode membrane-associated proteins (Gerber et al., 2004). Puf4 protein has been shown to somehow be involved in localization of Sir proteins to the nucleolus, and thus, in the regulation of aging in yeast (Kennedy et al., 1997). Furthermore, the Puf4 protein has also been shown to selectively interact with nuclear component-encoding mRNAs (Gerber et al, 2004). Although the Puf4 RNA binding domain has been shown to be essential for its role in the aging process, its mode of function is yet unknown. The Puf5 protein has also been shown to interact with mRNAs encoding nuclear components (Gerber et al, 2004). In addition the Puf5 protein is involved in the regulation of the *HO* mRNA (Tadauchi et al., 2001). The HO protein is a homothallic switching endonuclease, which has been shown to stimulate mating-type switching in yeast (Herskowitz et al., 1988). The newest member of the yeast Puf family, the Puf6 protein, has been shown to inhibit translation of *ASH1* mRNA (Gu et al., 2004). The Ash1 protein negatively regulates HO endonuclease in newly budded yeast cells (Sil and Herskowitz, 1996). The Puf6 protein is mainly localized within the nucleus, but has also been shown to co-localize with *ASH1* mRNA in the cytoplasm (Gu et al, 2004).

Altogether, while a few targets of Puf regulation have been identified, the mechanism by which the Pufs recognize their targets or induce functional changes in those mRNAs remains unclear.

Yeast Puf3 Protein

The focus of this research is on the third member of the yeast Puf proteins, the Puf3. Puf3 protein (Puf3p) is an approximately 97 KD, 880 amino acid protein. The Puf3p Repeat Domain (Puf3RD) is of approximately 1/3 the size of the entire protein and is located towards the C-terminal of the protein. A theoretical structure of Puf3RD (Figure-8) was created by Swiss-Model, a protein homology modeling server. The structure was predicted by utilizing a sequence alignment of Puf3RD with Puf proteins of known structure (Dm-Pum and Hs-Pum) to model the Puf3RD sequence on those structures (Schwede 2003, Guex 1997, Peitsch 1995). As expected, this structure is strikingly similar to those of Dm-Pum and Hs-Pum repeat domains (compare to Figure-5).

The Puf3 protein has been shown to be a cytoplasmic protein with almost exclusive interactions with mRNAs that encode mitochondrial proteins (Gerber et al, 2004). The only verified target of the Puf3p, *COX17* mRNA, was originally identified through a deletion microarray analysis (Olivas and Parker, 2000). The *COX17* gene encodes a protein that is involved in the shuttling of copper into the mitochondria for assembly of cytochrome oxidase, the terminal complex in the mitochondrial respiratory chain (Glerum et al., 1996). Upon identification of *COX17* mRNA as a possible target of Puf3 protein regulation, the role of Puf3p in the decay of *COX17* mRNA was examined. An mRNA stability assay was performed to compare the half-life of the *COX17* mRNA in a wild-type yeast strain versus that in a PUF3 deletion (*puf3Δ*) strain. Strains containing a temperature sensitive lesion in the RNA polymerase II (rpb1-1) were

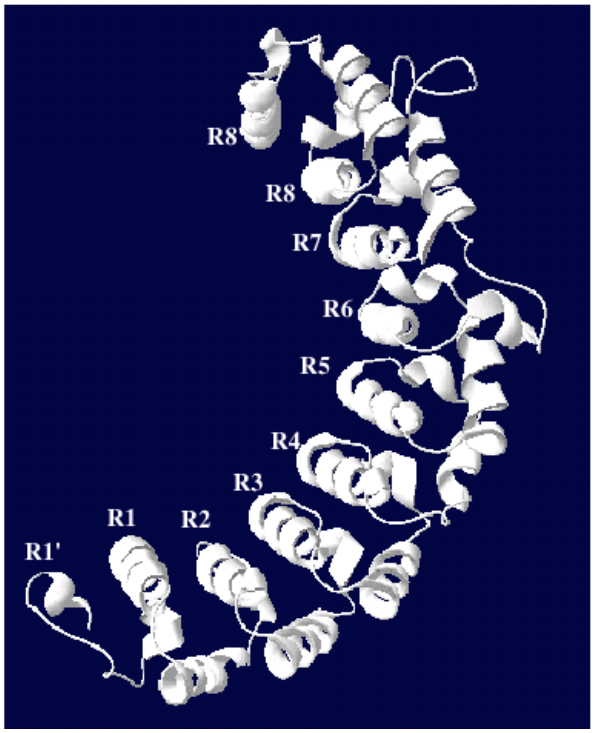


Figure – 8: Puf3 Repeat Domain Structure. Predicted Puf3RD structure created by Swiss-model is shown (Schwede, 2003; Guex, 1997; Peitsch, 1995).

used so that transcription would occur normally at a permissive temperature, but could be shut off following a shift to a higher non-permissive temperature. Samples were taken in a time course following transcription shut-off, and total RNA was extracted from each sample. A northern blot analysis of the decay of the *COX17* transcript was conducted. The results (Figure-9) indicated that the half-life of the *COX17* transcript in wild-type PUF3 cells is 3 minutes. In contrast, in the *puf3Δ* strain, the *COX17* mRNA is stabilized more than 5-fold to a half-life of 17 minutes (Olivas and Parker, 2000) indicating the involvement of Puf3p in *COX17* mRNA decay.

Possible mechanisms of Puf3p involvement in the decay machinery are through stimulation of deadenylation and/or decapping. In order to determine whether or not the role of Puf3p in the decay of *COX17* mRNA was due to an effect on its deadenylation rate, the decay was examined using a transcriptional pulse-chase assay. The *COX17* gene was first put under the control of a regulatable *GAL10* promoter in wild-type and *puf3Δ* strains, such that the transcription of *COX17* mRNA could be induced by the addition of galactose to the growth medium, then rapidly repressed by the addition of glucose. This creates a pulse of newly synthesized transcripts with approximately the same poly(A) tail lengths whose deadenylation can be monitored over time. Samples were taken in a time course following transcriptional shut-off. Northern analyses were performed on each RNA sample. The results (Figure-10) indicated that following induction of transcription in wild-type cells (Figure– 10A), *COX17* mRNA is first observed with a heterogeneous poly(A) tail of approximately 45-60 residues, presumably reflecting newly synthesized mRNAs (Figure-10A, 0 lane). The poly(A) tail of *COX17* mRNA deadenylates slowly in the first 2 minutes, then between 2 and 4 minutes shortens to a fully deadenylated state.

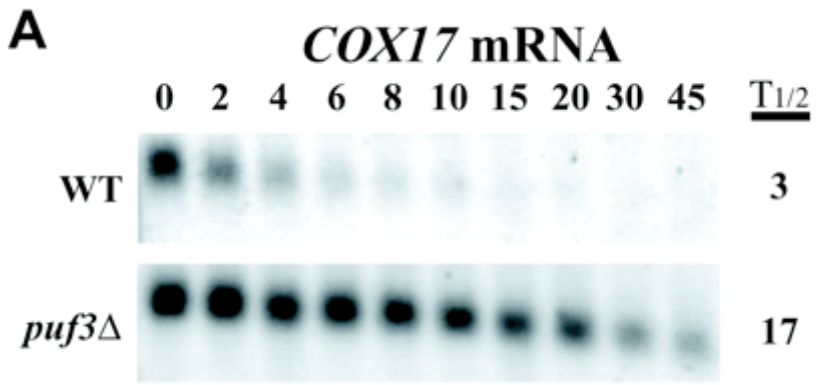


Figure – 9: Puf3p promotes rapid decay of *COX17* mRNA. Northern blot analysis of the decay of *COX17* transcript. Minutes following transcriptional repression are indicated above the blots with half-lives (T _) as determined from multiple experiments (Olivas and Parker, 2000).

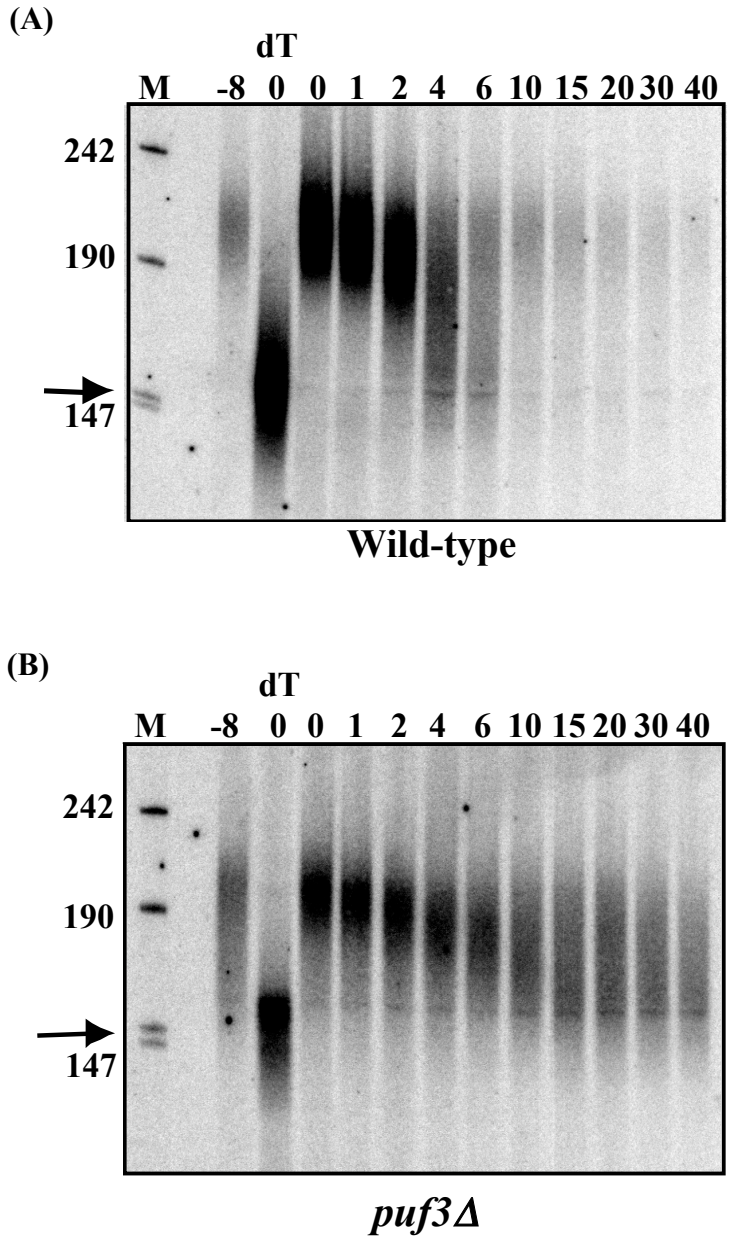


Figure – 10: Puf3p promotes rapid deadenylation and decapping of *COX17* mRNA. Northern blot analyses of transcriptional pulse-chase experiments examining decay of the *COX17* mRNA transcript from wild-type (A) and *puf3Δ* (B) strains. Minutes following transcriptional repression are indicated above each blot. The OdT lane in each blot corresponds to RNA from the 0-min time point in which the poly(A) tail was removed by RNaseH cleavage with oligo(dT). The –8 lane in each blot corresponds to background levels of RNA expression prior to galactose induction of the *COX17* transcript. Size markers (M lane) are given in nucleotides. Arrows denote the position of the deadenylated 3'UTR species (Olivas and Parker, 2000).

In the *puf3Δ* strain, *COX17* mRNA is initially produced with a poly(A) tail of approximately the same initial length as in wild-type (Figure-10B, 0 lane). However, the *COX17* transcript then deadenylates at a slower rate, such that the main pool of mRNA is not fully deadenylated until 15 minutes after glucose addition (Figure-10B). This finding is significant in that it clearly indicates that Puf3p promotes the decay of *COX17* mRNA via rapid deadenylation (Olivas and Parker, 2000). Moreover, the observation that *COX17* mRNAs with short poly(A) tails persist at 40 minutes in the *puf3Δ* strain suggests that the subsequent decapping step is also slowed, and therefore Puf3p also stimulates decapping (Olivas and Parker, 2000).

Dissertation Overview

In this research a combination of genetic and biochemical approaches will be used to further understand the role of Puf proteins in mRNA decay though analysis of the Puf3p interactions with *COX17* mRNA. Chapter II explains the general experimental techniques used in this body of work. Chapter III focuses on the repeat domain of the Puf3p and will demonstrate that the repeat domain of Puf3p is sufficient for both binding to the *COX17* mRNA and signaling to the decay machinery, supporting a conserved role of the Puf repeat domain as an independent regulator of mRNA metabolism. Chapter IV will focus on understanding how yeast Puf3p attains specificity to its RNA target by determining the elements involved in RNA binding and decay regulation. Comparative mutational analyses will show that a single amino acid change of the Puf3p repeat domain prevents binding of the protein to its *COX17* mRNA target. Also identified is a loop region on the outer surface of Puf3p that is required for its ability to promote both deadenylation and subsequent decapping of the *COX17* mRNA, indicating that regulation of these processes is linked by a single interaction point on Puf3p. Chapter V will focus on the protein-protein interactions of Puf3p and will demonstrate that Puf3p interacts with other known decay factors via its repeat domain. Chapter VI will show a collaborative study into the condition-specific regulation of mRNA decay by the Puf3 protein and will demonstrate that carbon source as well as the target of rapamycin signaling pathway regulate the activity of Puf3 protein. Together the results of these studies will further our understanding of the nature of 3'UTR-dependant mRNA regulation by Puf proteins in yeast as well as other eukaryotes.

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CHAPTER II:

General Methodology

PufRD protein expression and purification

The GST-*PUF3* fusion construct was previously created (Olivas and Parker 2000) in pGEX-6P-1 (Amersham Biosciences). To create the GST-*PUF3* Repeat Domain fusion construct, a fragment containing the *PUF3RD* was isolated then inserted into a pGEX-3X plasmid. The *PUF3RD* was then isolated from pGEX-3X plasmid and inserted into the pG-1 yeast expression plasmid (Schena et al. 1991). The *PUF3RD* was placed just downstream from an inserted FLAG tag sequence.

Mutant *PUF3RD* constructs in the yeast plasmid were created using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). All mutations were verified by sequencing. Mutant GST-PUF3RD fusion constructs were created by restriction digestion of the above yeast plasmids and insertion into a modified pGEX-3X plasmid.

The GST-PUF5 fusion construct was created by PCR amplifying the complete *PUF5* ORF and inserting it into pBluescript (Stratagene). To create the GST-PUF5RD fusion construct, a fragment containing the *PUF5RD* was isolated and first ligated into pBluescript. Then, the *PUF5RD* was isolated from pBluescript using vector restriction sites and inserted into a pGEX-6P plasmid to make the GST-Puf5RD expression vector. All constructs were verified by sequence analysis.

The GST fusion constructs were transformed into the protease-deficient BL-21 *E. coli* strain, and GST fusion proteins were purified as recommended by the manufacturer. The expression of each protein product was verified by western analysis with anti-GST antibodies.

In vitro mRNA Binding Analysis

Crosslinking Analysis (Figure-1) - The pBluescript (pBS) plasmid containing the *COX17* 3'-UTR sequence (pBS-COX17 3'UTR) was linearized at the end of the *COX17* 3'UTR and used to transcribe *COX17* 3'-UTR RNA by the T7 RNA polymerase in the presence or absence of radioactive nucleotides *in vitro*. A control pBS plasmid was also digested and transcribed to produce a similarly-sized RNA to be used as a negative control. Transcripts were purified by separation on denaturing polyacrylamide gels, elution from gel slices, and ethanol precipitation. RNA-protein binding reactions included radiolabled RNA in binding buffer, with the presence or absence of GST-Puf3RD, and with the presence or absence of ~10-fold excess unlabeled transcript. Reactions were then subjected to UV cross-linking. Cross-linked reactions were treated with RNase to remove unbound RNA prior to loading on SDS-polyacrylamide gels. For more details refer to Chapter III (pages 58-59).

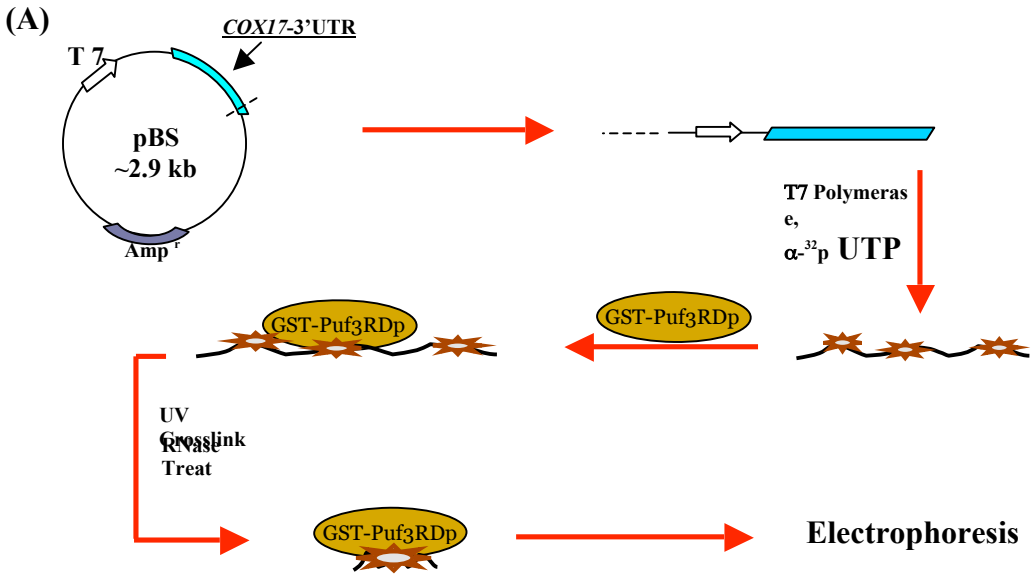

Figure – 1: Crosslinking analysis.

Gel Mobility Shift Analysis (Figure-2) - Short *COX17* and *HO* RNAs containing Puf binding sites were transcribed from single-stranded oligonucleotide templates containing the T7 RNA polymerase promoter annealed to a complementary primer. Radiolabeled transcripts were purified via separation on denaturing polyacrylamide gel, elution from gel slices, and ethanol precipitation. Unlabeled transcripts were also prepared and purified to be used as specific and non-specific competitors. Each RNA-protein binding reaction contained radiolabeled RNA and binding buffer in the presence or absence of wild-type GST-Puf3RDp, mutant GST-Puf3RDp or wild-type GST-Puf5RDp, as well as in the presence or absence of ~10-fold excess unlabeled transcript. Reactions were electrophoresed on native PAGE. For more details refer to Chapter III (pages 59-60) and Chapter IV (pages 79-80).

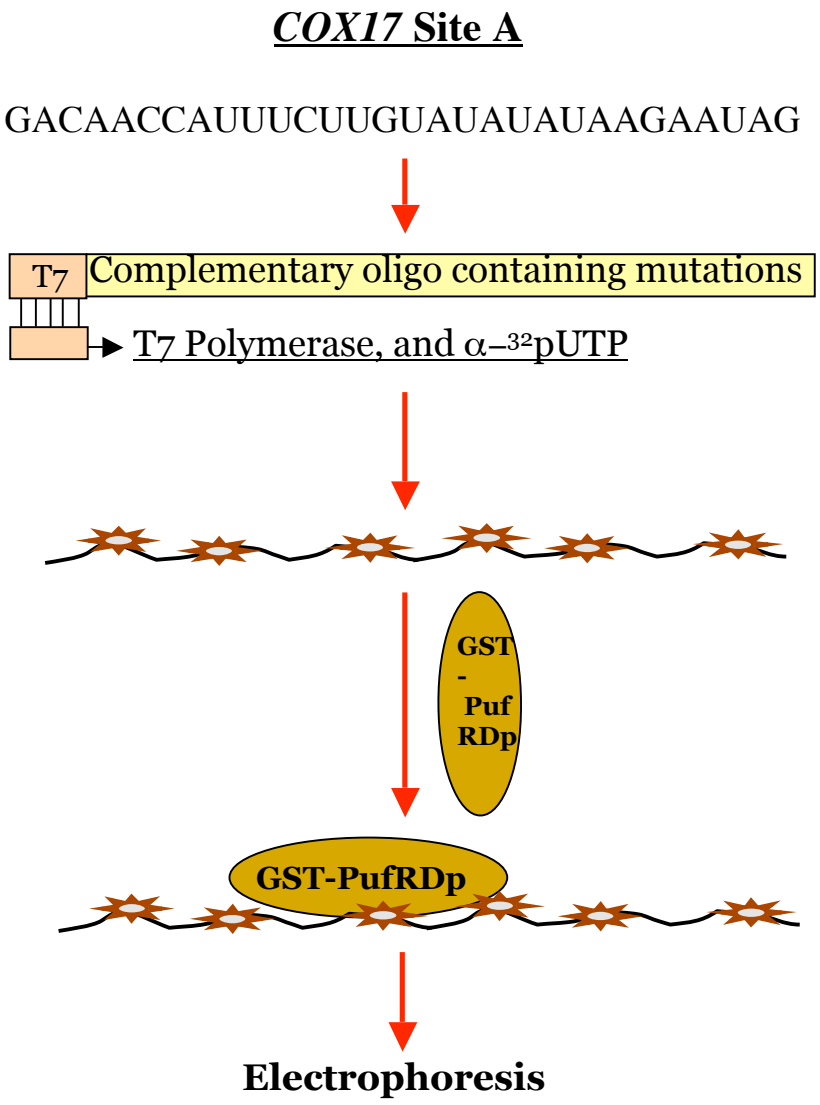

Figure – 2: Gel mobility shift analysis.

***In vivo* mRNA decay analysis**

Steady-state transcriptional shut-off analysis (Figure-3) – Shut-off experiments were performed assentially as described (Caponigro et al., 1993) using wild-type and PUF3 deletion (*puf3Δ*) strains that contain the *rpb1-1* temperature-sensitive allele for RNA polymerase II. The *puf3Δ* strains were transformed with plasmids expressing full-length Puf3p or the Puf3RDp. Deletion strains were also analyzed after transformation with each of the mutant Puf3RD plasmids. Strains were grown to mid-log phase at 24^0C to express all RNA polymerase II transcripts. The cells were pelleted and then resuspended in media pre-heated to 37^0C to immediately shut off transcription of all RNA polymerase II transcripts, including *COX17*. The resuspended cells were then incubated at 37^0C and sample aliquots were taken at increasing time intervals. RNA was prepared from the cells at each time interval, and equal concentrations of RNA were loaded into formaldehyde RNA gels. Remaining mRNA levels at each time interval were determined by northern blot analysis, using radiolabled oligonucleotide probes specific to the mRNA. All Northern blots were normalized for loading to the stable *scRI* RNA, an RNA polymerase III transcript (Felici et al., 1989). For more details refer to Chapter III (page 60) and Chapter IV (page 80).

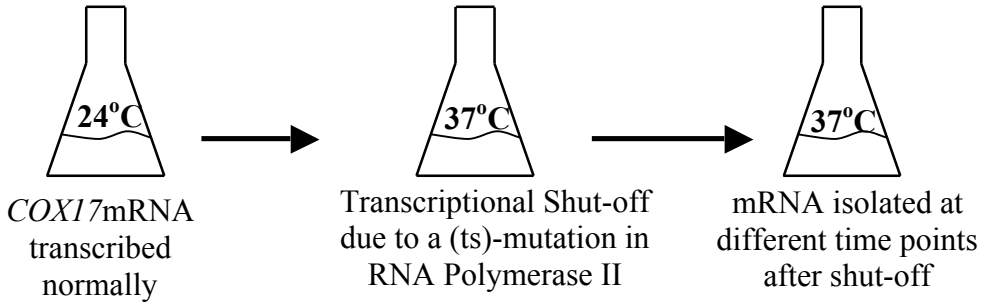

Figure – 3: Transcriptional shut-off analysis.

Transcriptional pulse-chase analysis (Figure-4) – Pulse-chase experiments was performed essentially as described (Decker and Parker, 1993) on strains that contain the temperature-sensitive *rbp1-1* allele for RNA polymerase II. Regulated expression of *COX17* mRNA was accomplished by transformation of *puf3Δ*, *cox17Δ* yeast strains with a plasmid in which the *COX17* gene is under the control of the *GAL10* promoter, as well as with plasmids containing *PUF3RD* or each of the *PUF3RD* mutants. Strains were grown to mid-log phase at 24^{o}C in raffinose, where there is no induction of *COX17* transcription. Then, cells were isolated and resuspended in galactose-containing media to induce a pulse of *COX17* mRNA transcripts. After 8 minutes cells were isolated and resuspended in glucose-containing media at 37^{o}C to shut off transcription. To monitor poly(A) tail lengths, *COX17* mRNA was cleaved just upstream of the stop codon using RNase H reactions essentially as described (Olivas and Parker, 2000). RNA was separated on denaturing polyacrylamide gels and transferred to nylon membranes for probing. For more details refer to Chapter IV (page 80).

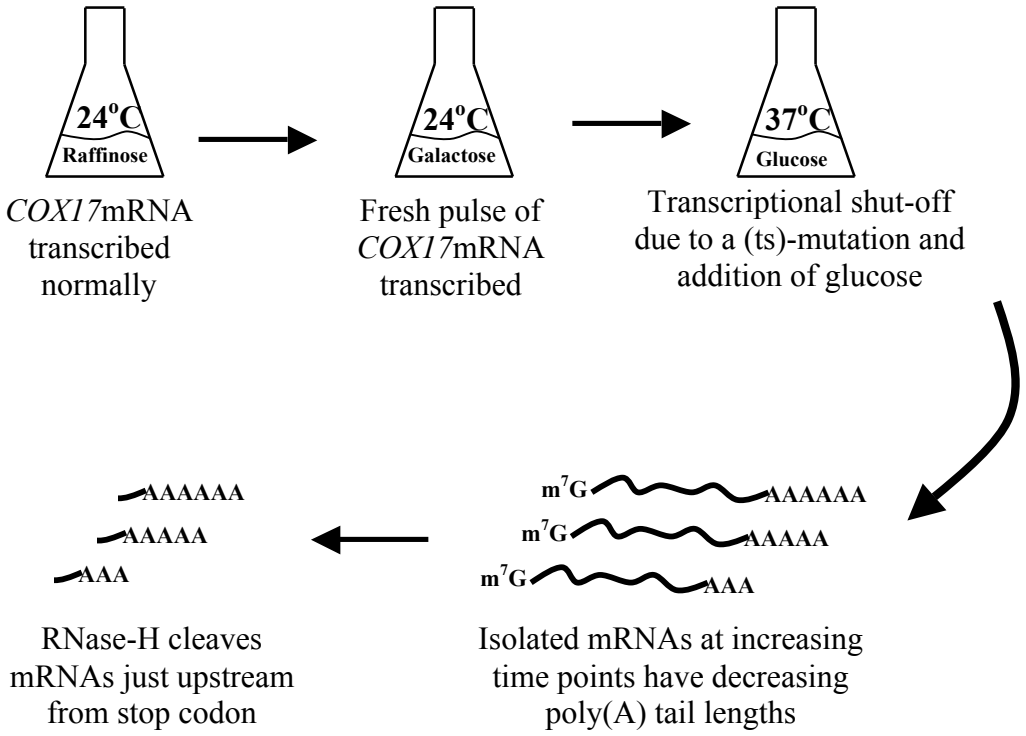

Figure – 4: Transcriptional pulse-chase analysis.

Carbon source and rapamycin analyses (Figure-3) - The physiological state analysis utilized steady-state transcriptional shut-off experiments as previously described, where appropriate strains were transformed with plasmids expressing *MFA2* RNA or the hybrid *MFA2/COX17* 3'-UTR RNA. Transformed strains were grown in selective media with the appropriate carbon source. Rapamycin, when used, was added to a final concentration of 0.2 μg/mL when the culture reached an OD_{600} of 0.3, then the cells were incubated a further 60 minutes prior to the temperature shift. Northern blots were probed for *MFA2* mRNA or *MFA2/COX17* hybrid mRNA. All Northern blots were normalized for loading to the stable *scRI* RNA, an RNA polymerase III transcript (Felici et al, 1989). For more details refer to Chapter VI (pages 133-134).

Co-Immunoprecipitation Analysis

Epitope tagging (Figure-5) – Several decay factors were epitope tagged by homologous recombination using the Myc-9 epitope. Upstream PCR amplification primers containing an upstream region homologous to the decay factor gene to be tagged just before the stop codon, as well as a homologous region to the 5' end of the MYC gene on the PCH905-Myc9 plasmid (Zhang Lab) were designed. Downstream primers containing a region homologous to the TRP gene (used as a marker) on the PCH905-Myc9 plasmid and a region homologous to the gene to be tagged downstream of the stop codon were also designed. The desired region of the Myc-9 plasmid was PCR amplified with each set of decay factor-specific primers to amplify the regions required for homologous recombination. Entire PCR products were transformed into *puf3Δ* (trp-) strains. Genomic DNA from candidates was then extracted, PCR amplified, and sequenced to verify proper tagging. For more details refer to Chapter V (pages 119-120).

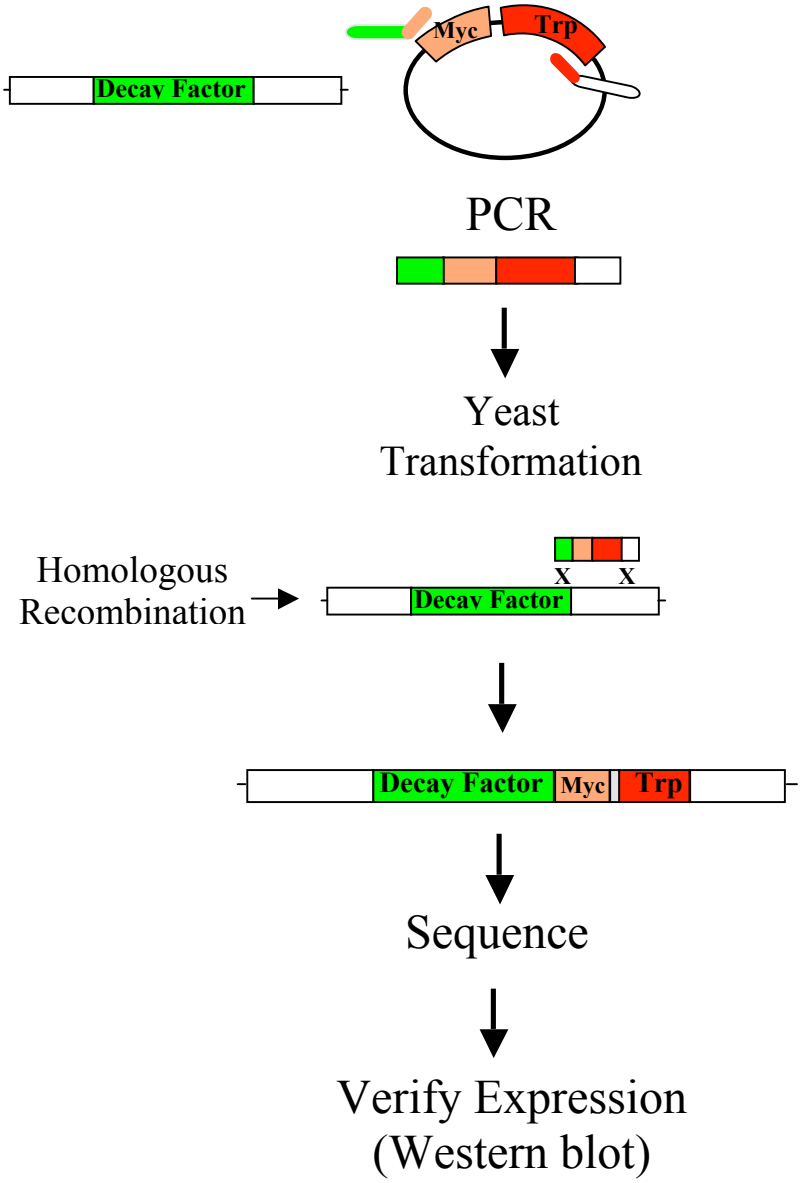

Figure – 5: Epitope-tagging process. Schematic representation of the myc-tagging procedure used in this study for endogenous tagging of decay factor genes.

Co-immunoprecipitation assay (Figure – 6) - Protein boil prep extracts were prepared from the verified candidates. The resulting supernatants and pellets were loaded onto a SDS-PAGE gel and then blotted onto a nitrocellulose membrane. The blots were then probed with an anti-Myc antibody to verify the expression of each epitope tagged protein in a *puf3Δ* strain. Once *puf3Δ* deletion strains that contained the appropriate tagged protein were verified, each strain was transformed with a plasmid expressing the PUF3RD fused to an N-terminal FLAG-tag sequence. The transformants were then grown to mid-log phase, the cells lysed, and lysates immunoprecipitated using anti-FLAG antibody agarose (Sigma). To study the RNA dependence of any interactions, lysates were treated with or without RNaseA prior to FLAG agarose incubation. Immunoprecipitated proteins were extracted by boiling in SDS sample buffer and analyzed by western blots using 9E10Anti-Myc antibody. For more details refer to Chapter V (pages 120-121).

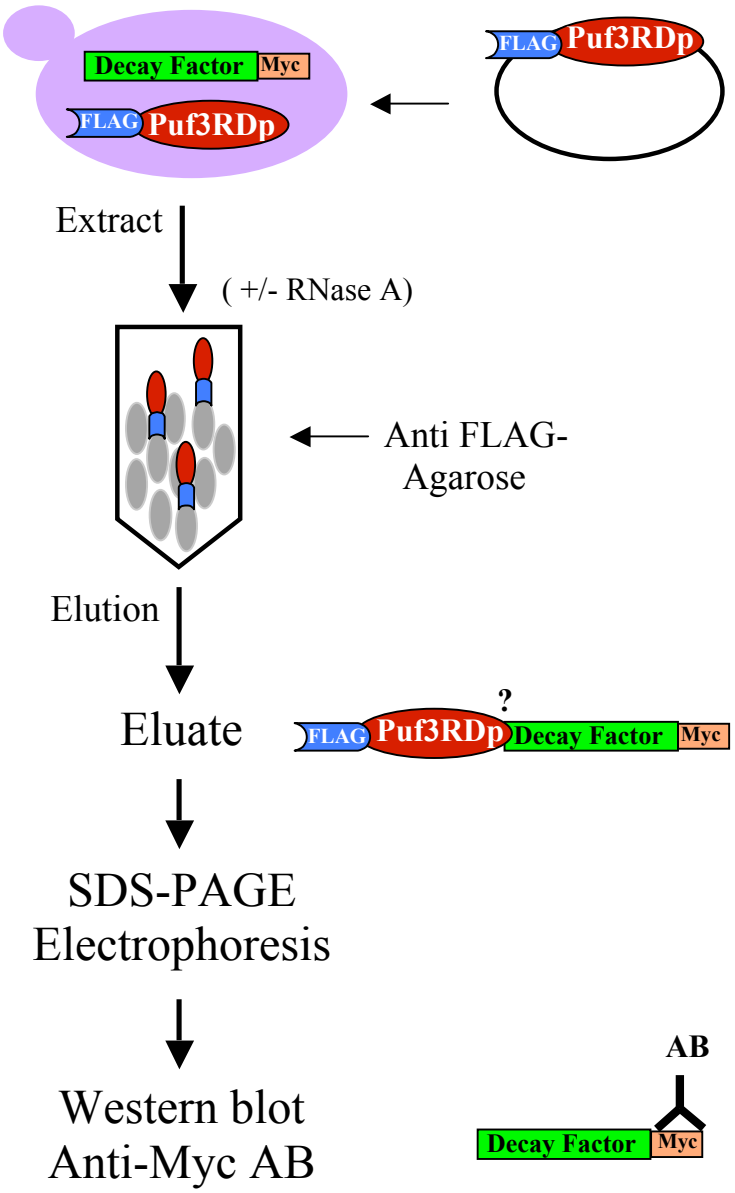

Figure – 6: Co-immunoprecipitation assay.

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CHAPTER III:

Regulation of mRNA Decay by the Puf3 Repeat Domain

Introduction

Previous studies had shown that Puf3 protein regulation of *COX17* mRNA occurs through the 3'UTR of the transcript (Olivas and Parker, 2000). All Puf proteins studied so far have been shown to bind to RNA targets with a conserved UGU sequence, with specificity conferred by flanking sequences. For example, human PUM1 (Zamore et al. 1997; Wang et al. 2002), murine PUM2 (White et al. 2001), and *Xenopus* Pum (Nakahata et al. 2001) proteins bind to sequences containing UGUANAUA, which is also found in box B of the bipartite NRE target of DmPUM (Murata and Wharton 1995). In contrast, *C. elegans* FBF binds a target sequence containing UCUUGUGU (Zhang et al. 1997), while yeast Puf5 binds a target sequence containing AGUUGUGU (Tadauchi et al. 2001), where the underlined nucleotides have been shown to be important for binding. However the details of the interaction between Puf3 protein and *COX17* mRNA were still unknown. In addition, the repeat domain of Puf proteins, which is involved in Puf/RNA interactions, has been reported sufficient to interact with RNA targets in other organisms (Zamore et al., 1997; Zhang et al., 1997). Moreover, the repeat domain of the *Drosophila* Dm-Pum has also been shown to be sufficient to also regulate the *hunchback* mRNA (Wharton et al., 1998). Therefore, the focus of this chapter is to determine the 3'UTR sequences bound by Puf3p, determine if the repeat domain of Puf3p is sufficient for interaction with *COX17* mRNA, and determine if the repeat domain of Puf3p is sufficient to regulate the decay of *COX17* mRNA.

Earlier studies had shown that Puf3p interacts with two UGUA containing sequences in the *COX17* mRNA 3'UTR. Studies in this chapter demonstrate that the

Puf3RDp interacts with the same UGUA sequences, and that mutations in these UGUA sequences as well as mutations immediately downstream inhibit the interaction. The results also demonstrate that the repeat domain of Puf3p is sufficient for both binding to the *COX17* mRNA and signaling to the decay machinery, supporting a conserved role of the Puf repeat domain as an independent regulator of mRNA metabolism. This work contributed to the following publication:

> Jackson, J. Houshmandi, S.S., Lopez Leban, F., Olivas W.M. (2004) Recruitment of the Puf3 protein to its mRNA target for regulation of mRNA decay in yeast. *RNA* **10:** 125-1636.

Experimental Procedures

Yeast strains - The genotypes of the *S. cerevisiae* strains are as follows: yWO7: MATα, *leu2-3,112, ura3-52, rpb1-1* (Olivas and Parker, 2000, yRP693). yWO43: MATα, *his4-539, leu2-3,112, trp1-1, ura3-52, cup1::LEU2/PM, rpb1-1, puf3::Neo*^{r} (Olivas and Parker, 2000, yRP1360)

Protein expression and purification - The GST-*PUF3* fusion construct pWO3 was previously created (pRP1020, Olivas and Parker 2000) in pGEX-6P-1 (Amersham Biosciences). To create the GST-*PUF3* Repeat Domain fusion construct, a fragment containing the *PUF3* Repeat Domain (amino acids 465-879) was isolated from pWO3 by digesting with *Xba*I (filled in by Klenow Fragment) and *Not*I, then inserted into a derivative of pGEX-3X (Amersham Biosciences) to yield pWO12. The GST fusion constructs were transformed into the protease-deficient *E. coli* strain BL-21, and GST fusion proteins were purified as recommended by the manufacturer. Protein eluates were dialyzed into 50 mM Tris-HCL pH 8.0, and expression products were verified by western analysis with anti-GST antibodies.

In vitro binding analyses - *In vitro*-transcribed RNA containing the *COX17* 3'-UTR sequence was made from pWO6 (Olivas and Parker 2000, pRP1019). After digestion of pBS or pWO6 with *Mse*I, RNA was transcribed using T7 RNA polymerase in the presence or absence of α-^{32}P UTP to produce 145 and 147 nt transcripts, respectively. Transcription reactions were treated with DNase I. Radiolabeled

transcripts were purified by separation on denaturing polyacrylamide gels, elution from gel slices and ethanol precipitation.

Binding reactions with RNA transcribed from pWO6 or pBS included radiolabeled RNA (500,000 c.p.m.) and 1X binding buffer [10 mM HEPES pH 7.5, 50 mM KCl, 1 mM EDTA, 2 mM DTT, 200 U/ml RNasin, 0.1 mg/ml bovine serum albumin, 0.01% Tween-20, 0.1 mg/ml poly(rU) and 10 µg/ml yeast tRNA] in the presence or absence of GST-Puf3p (0.2 µM) or GST-Puf3RD (0.5 µM), and in the presence or absence of ~10-fold excess unlabeled transcript in a total of 15 µl. Reactions were incubated for 30 min at 24˚C, then subjected to UV cross-linking (energy mode 8000 x100µJ/cm^2). Cross-linked reactions were treated with 100 U of RNase T_1 for 30 min prior to loading on SDS-7.5% polyacrylamide (29:1 acrylamide:bis-acrylamide) gels.

Short RNAs of sites A and B (29-30 nt) were transcribed from single-stranded oligonucleotide templates containing the 18 nt T7 RNA polymerase promoter annealed to a complementary primer. RNAs were transcribed using the T7-MEGAshortscript kit (Ambion) as recommended by the manufacturer with the following changes: the reaction contained 500 μM each of ATP, CTP and GTP, 50 μM of UTP, 40 μCi of α-^{32}P UTP (800 Ci/mmol) for labeled reactions, and 20 U RNasin. Radiolabeled transcripts were purified by separation on denaturing polyacrylamide gels, elution from gel slices and ethanol precipitation. Unlabeled transcripts were purified using a Nucleotide Removal Kit (Qiagen).

Binding reactions with short RNAs included radiolabeled RNA (20,000 c.p.m.) and 1X binding buffer in the presence or absence of GST-Puf3p or GST-Puf3RD, and in the presence or absence of ~10-fold excess unlabeled transcript in a total of 30 µl.

Reactions were incubated for 30 min at 24˚C, 5 µg of heparin was added and reactions incubated for a further 10 min at 24˚C, then reactions were electrophoresed on 8% non-denaturing polyacrylamide gels for 2.5 h at 200V at 4˚C. Best-fit curves were obtained for the binding data using KaleidaGraph software.

In vivo COX17 mRNA decay analysis - Steady-state transcriptional shut-off experiments were performed essentially as described (Caponigro et al., 1993) on strains yWO7 (wild-type) and yWO43 (*puf3Δ*) that contain the *rpb1-1* allele. yWO43 was also analyzed after transformation with plasmids expressing full-length Puf3p (pWO13) or the Puf3RD (pWO14) under the control of the constitutive *GPD* promoter. pWO13 was created by insertion of the *PUF3* ORF into a derivative of pG-1 as previously described (Olivas and Parker 2000, pRP1021). pWO14 was created by digestion of pWO12 with *Bam*HI and *Not*I (filled in with Klenow fragment) to isolate the *PUF3RD*, and insertion of this fragment into a derivative of pG-1 between *Bam*HI and *Nco*I (filled in with Klenow fragment) to place the *PUF3RD* ORF just downstream of an inserted FLAG tag sequence and the *GPD* promoter. Northern blots were normalized for loading to the stable *scRI* RNA, an RNA polymerase III transcript (Felici et al., 1989).

Results

The repeat domain of Puf3p is sufficient and specific for binding COX17 mRNA - Previous work identified yeast *COX17* mRNA as a target of Puf protein regulation, with Puf3p directly binding the 3'-UTR of *COX17* mRNA and promoting rapid deadenylation and decay of this transcript, while deletions of the other four Puf genes in yeast have no effect on *COX17* mRNA decay *in vivo* (Olivas and Parker 2000). In this work, the focus was to determine how the specificity of binding and regulation of *COX17* mRNA is attained by Puf3p. First, the binding of the *COX17* 3'-UTR by Puf3p was examined to see if the binding is mediated by the Puf repeat domain, as has been shown in other organisms (Zamore et al., 1997; Zhang et al., 1997). For this experiment, *in vitro* binding was assayed using glutathione *S*-transferase (GST)-tagged proteins purified from *E. coli* encompassing only the Puf3 repeat domain (Puf3RD) sequence. The purified protein was then incubated with *in vitro* transcribed, uniformly radiolabeled RNA of the *COX17* 3'UTR sequence or a non-specific vector RNA sequence. The reactions were UV cross-linked to attach the radiolabel of any bound RNA to the protein, then treated with RNase T_1 to degrade unbound RNA. As shown in Figure 1, Puf3RD becomes radiolabeled when incubated with the *COX17* 3'UTR (lane 4). Puf3RD is therefore sufficient for binding to *COX17* mRNA. The results also show that in the presence of excess unlabeled specific competitor RNA, the interaction is inhibited, whereas in the presence of excess unlabeled non-specific competitor RNA, the interaction is unaffected (lanes 5 and 6). In Addition there is no interaction between Puf3RDp and the non-specific vector RNA (lane 2). Thus, interaction between Puf3RDp and the *COX17*-3'UTR is indeed specific.

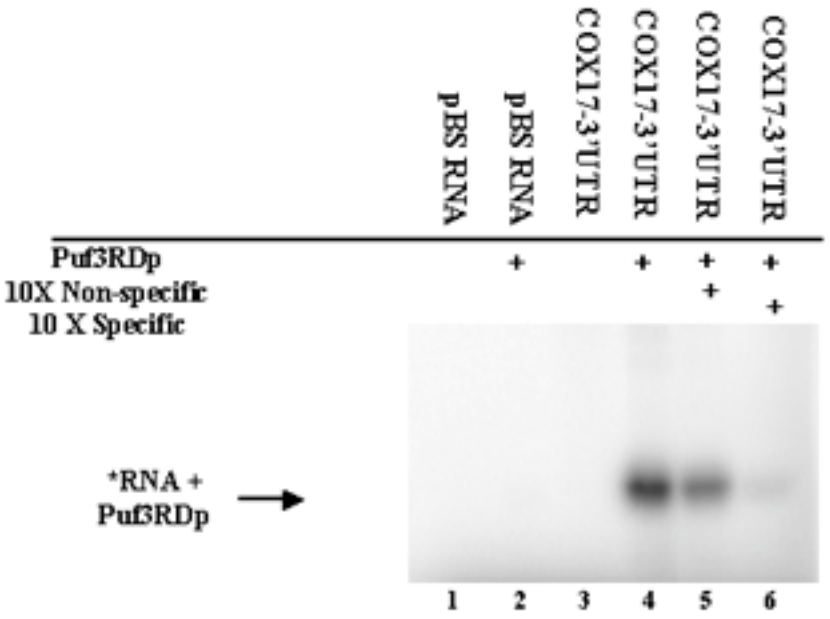

Figure – 1: Puf3RDp is sufficient to bind to the *COX17* 3'UTR. In vitro binding reactions of uniformly radiolabeled transcripts (pBS vector or *COX17* 3'UTR) in the presence or absence of GST-Puf3RDp were UV-crosslinked and digested of unbound RNA. The position of the Puf3RDp (74 kDa) in the SDS polyacrylamide gel is shown by the arrow. The (+) sign indicates the presence of the Puf3RDp and/or the denoted unlabeled competitor RNA (pBS vector and *COX17* 3'UTR as non-specific and specific competitors, respectively).

Puf3 protein binding requires UGUA as well as specific surrounding sequences - Earlier studies had shown that Puf3p interacts with both UGUA sequences in the 3'UTR of *COX17* mRNA. To Examine the importance of individual nucleotides within the UGUA sequence, as well as the role of flanking sequences, the binding of Puf3RDp to a series of mutant target sequences was analyzed (Figure-2A). For these experiments, the Puf3RDp was used because it was shown to be sufficient for specific binding (Figure-1), and it was much easier to purify in the stable form than the full-length protein. As expected, the Puf3RD protein interacts with both Site A and Site B UGUA sequences, specifically (Figure-2B, lanes 2 and 3 as well as 18 and 19, respectively). Other work has shown that while binding of Puf3RDp to Site B is specific the affinity is at least 4-fold weaker (Jackson et al., 2004). A single 0.45 μM concentration was used, as it is concentration equal to its apparent K_D value with the Site A target (Jackson et al., 2004). The Puf3RDp was unable to bind either the Site A or Site B target sequence when the UGUA was mutated to ACAC (Figure-2B, lane 6 and 21, respectively). The interaction of Puf3RDp with the wild-type target is specific as it cannot be competed with excess unlabeled RNA of the UGUA→ACAC Site A mutant, but can be competed with excess unlabeled wild-type Site A RNA (Figure-2B, lanes 3 and 4, respectively). Also created were mutant RNAs with **C**GUA, U**A**UA, and UGU**C** sequences in place of the wild-type UGUA of Site A (where the bold, underlined nucleotide is mutant). Binding of Puf3RDp to the U**A**UA mutation was undetectable (Figure-2B, lane 14), while binding to RNAs with the **C**GUA and UGU**C** mutations was very weak. (Figure-2B, lanes 12 and 16). This clearly demonstrates the significant role that each of these nucleotides play in binding to Puf3RDp.

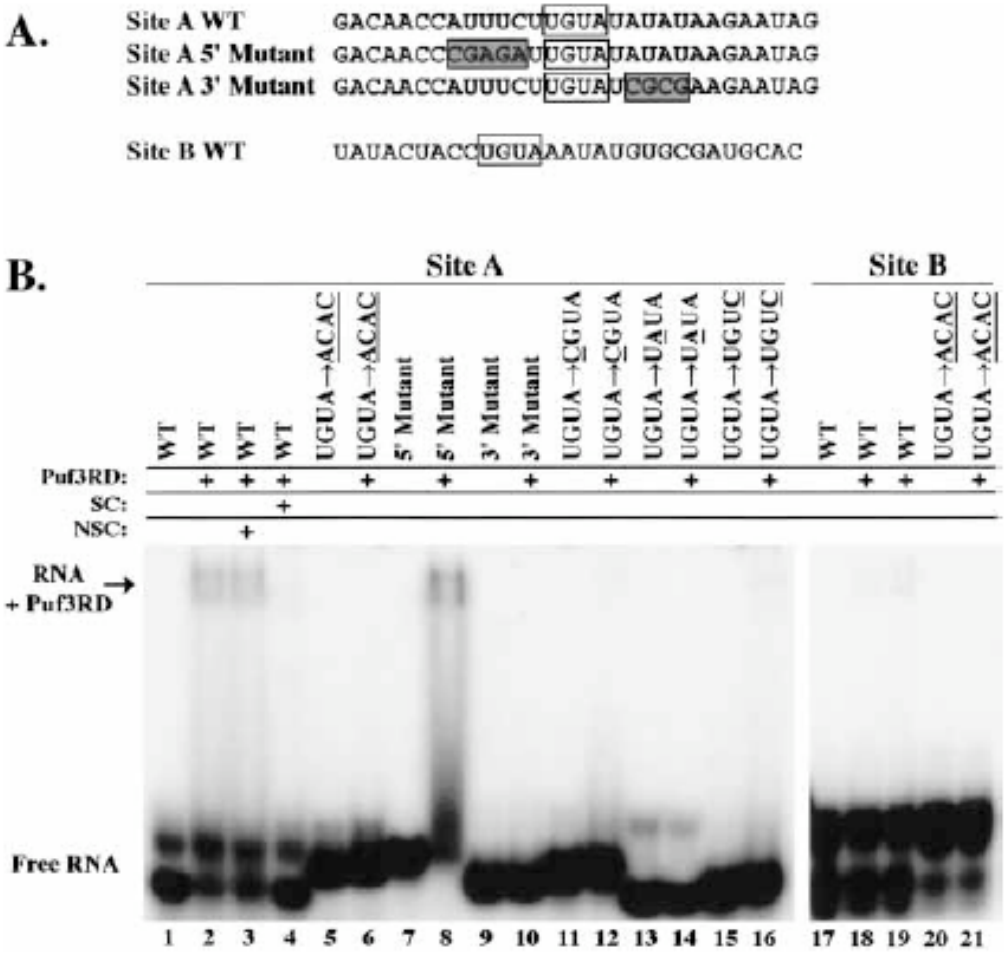

Figure – 2: Puf3RD binding requires additional sequences flanking the conserved UGUA regions. (A) Sequences of wild-type (WT) and mutant Site A and Site B transcripts used in binding reactions are shown. UGUA regions are boxed. Sequences altered in the mutant transcripts are indicated by shaded boxes. (B) *In vitro* binding reactions of radiolabeled transcripts in the absence or presence of 0.45 μM Puf3RD were separated on a native polyacrylamide gel. Base substitutions in UGUA mutants of Site A and Site B transcripts are given, with altered bases underlined. Excess unlabeled wild-type Site A RNA was used as specific competitor (SC, lane 4), and excess unlabeled mutant Site A RNA containing a UGUA→ACAC alteration was used as nonspecific competitor (NSC, lane 3). Lane 19 contains 0.9 μM Puf3RD. Similar results were also obtained with full-length Puf3p (data not shown). Positions of free radiolabeled RNA (Free RNA) and RNA bound to Puf3RD (RNA + Puf3RD) are indicated.

To determine whether specific sequences upstream or downstream of the UGUA are important for binding, five nucleotides 5' of the UGUA, or four nucleotides 3' of the UGUA were mutated (Figure-2A, Site A 5' and 3' Mutants, respectively). The 5' mutation had no detrimental effect on binding (Figure-2B, lane 8), but replacing the 3' AUAU with CGCG completely inhibited detectable binding (Figure-2B, lane 10). A downstream

AUA region is also involved in binding human PUM-HD in crystal structures (Wang et al., 2002), murine PUM2 in RNA selection experiments (White et al., 2001), and is present downstream of the box B region of the Drosophila NRE (Murata and Wharton 1995). Thus, an expanded recognition sequence of UGUANAUA is conserved across several Puf proteins.

The Puf3 Repeat Domain is sufficient for COX17 decay regulation - The repeat domains of Puf proteins typically compose less than one-half of the total protein. For example, the repeat domain of Puf3p occupies only one-third of the protein, and the repeat domain of Pumilio occupies only one-fourth of the protein. Yet expression of just the repeat domain of Dm-Pum is sufficient for nearly complete rescue of Dm-Pum's activity of translational repression of *hunchback* mRNA *in vivo* in a *dm-pum* deletion background (Wharton et al., 1998). It is possible that Puf3p is similar to Dm-Pum in acting solely through its repeat domain, or it may stimulate decay by a different mechanism that requires additional protein sequences outside of its repeat domain. To test these possibilities, yeast lacking endogenous *PUF3* were transformed with plasmids

expressing either full-length Puf3p or just the Puf3RDp. The decay rates of *COX17* mRNA in the wild-type, *puf3*Δ and transformed *puf3*Δ strains were then compared by using the *rpb1-1* lesion in the RNA polymerase II to shut off transcription following a shift to high temperature. As shown in Figure 3 and in previous work (Olivas and Parker, 2000), *COX17* mRNA in wild-type *PUF3* cells decays with a half-life of ~3 minutes, while in the *puf3*Δ strain the half-life is dramatically increased to 22 minutes. In comparison, expression of either the full-length Puf3p or the Puf3RDp in the *puf3*Δ strain rescues the rapid decay of *COX17*, with half-lives of 9 minutes and 11 minutes, respectively (Figure-3). It is unclear why even full-length Puf3p does not completely rescue the *COX17* decay rate to wild-type levels, but it might be due to the different level of expression achieved from the high-copy 2μm plasmid versus endogenous Puf3p levels. On the plasmid, *PUF3* expression is under the control of the high-level, constitutive *GPD* promoter. Nonetheless, the important finding is that expression of Puf3RDp rescues decay of the *COX17* mRNA to nearly the same level as full-length Puf3p, indicating that the repeat domain contains the minimal elements necessary for both binding to the mRNA as well as signaling for rapid decay.

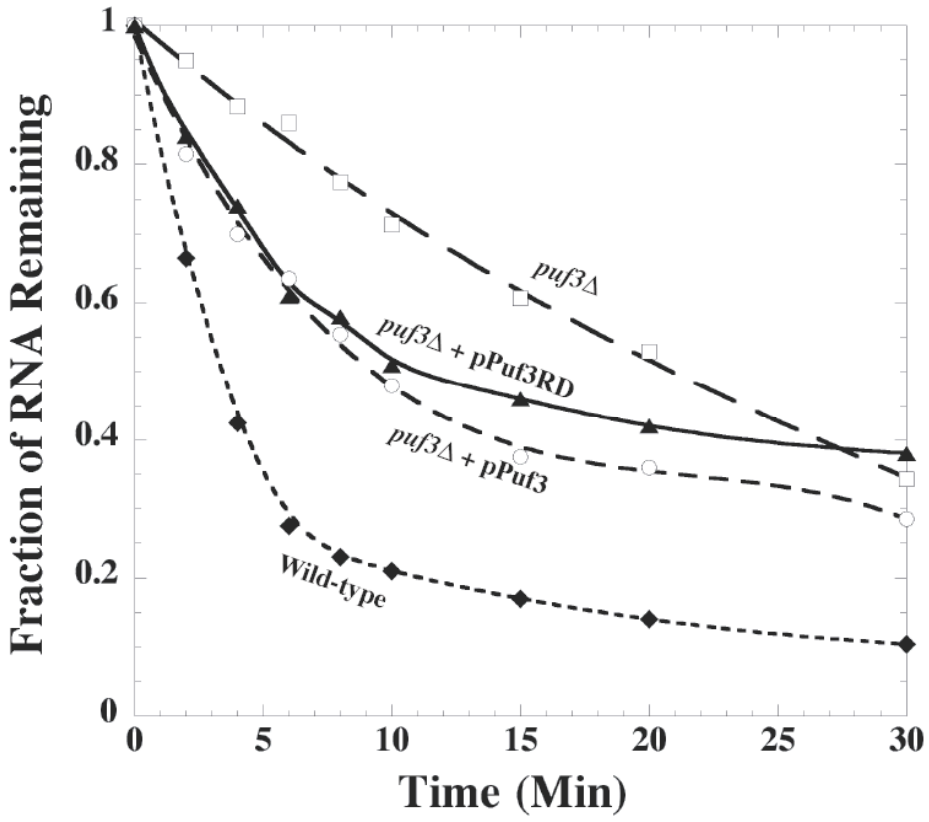

Figure – 3: The Puf3RD rescues decay of *COX17* mRNA in a *puf3*Δ strain. Data from northern blot analyses of *COX17* decay are plotted, with minutes following transcriptional repression on the x-axis and the fraction of RNA remaining as compared to the steady-state RNA level at time 0 on the y-axis. Decay was monitored in the following strains: wild-type (closed diamond), *puf3*Δ (open square), *puf3*Δ transformed with a plasmid expressing Puf3p (open circle), and *puf3*Δ transformed with plasmid expressing Puf3RD (closed triangle). Data points are averages of multiple experiments.

Discussion

Puf proteins play important roles in regulating mRNA metabolism in eukaryotes. In yeast, Puf3p promotes deadenylation and degradation of *COX17* mRNA. In this report evidence is provided to show that the Puf3 repeat domain is sufficient to specifically interact with the *COX17* 3'UTR (Figure-1). Additional evidence is provided to show that Puf3RDp specifically interacts with two UGUA sequences in the 3'UTR (Figure-2). The importance of the UGUA sequence, as well as a downstream AU-rich element in the binding of Puf3RDp was also revealed (Figure-2). It is possible that each binding site within the *COX17* 3'-UTR recruits a Puf3 protein, which can individually stimulate decay. The two sites in the *COX17* 3'-UTR thus would allow two Puf3 proteins to be recruited for increased stimulation of decay. A similar situation is seen in *Drosophila*, where the *hunchback* mRNA contains two NRE binding sequences that are both required for full translational repression, though one site contributes more activity than the other site (Wharton and Struhl 1991; Curtis et al., 1997).

The two Puf3p binding sites in *COX17* both contain a UGUANAUA sequence, which is also conserved in the binding sites of Dm-Pum, Hs-Pum, murine Pum2, and *Xenopus* Pum. This conservation of binding sequences correlates to the similarity of the amino acid sequences between the repeat domains of these Puf proteins, which all group to the same branch of the unrooted phylogenetic tree (Chapter I, Figure-4; Wickens et al., 2002). In contrast, the other yeast Pufs fall on other branches of the tree, which suggests that they might indeed have altered target specificities versus Puf3p. Additional support for this hypothesis comes from a microarray analysis that identified mRNAs associated

with each of the five yeast Puf proteins (Gerber et al., 2004). Specifically, each Puf protein was found to interact with a discrete set of mRNAs, and similar yet distinct conserved sequence motifs were identified in the 3'-UTRs of the mRNAs targeted by Puf3p, Puf4p and Puf5p. All the sequence motifs contain UGUR followed by UA located two, three, or four nucleotides downstream. The UGUA(U/A)AUA binding sequence contained within both *COX17* binding sites matches the conserved Puf3p target sequence motif identified in the microarray analysis: (U/C)(A/C/U)UGUA(U/A)AUA (Gerber et al., 2004). Because of the similarity in these sequence motifs, it is possible that the yeast Puf proteins could have overlapping target specificities. In fact, 12% of the mRNAs identified in the microarray screen bound to more than one Puf protein. In such cases, functional specificity could still occur through variations in protein partners or signals. Closer examination of the mRNA targets of the five yeast Puf proteins will address these issues in the future. Together, the characterization of the Puf3p binding site will allow a better evaluation of novel mRNA targets of Puf protein binding and regulation.

This work also demonstrated that the expression of just the repeat domain of Puf3p rescues rapid *COX17* mRNA decay in a *puf3Δ* strain (Figure-3). Thus, sequences necessary for both mRNA binding and decay regulation are contained within this region. The repeat domain of Pumilio is also sufficient to regulate translation in Drosophila (Wharton et al., 1998). Pumilio function requires interactions with Nanos and Brat, and their sites of interaction have been mapped to the outer surface of the rainbow-shaped Puf repeat domain (Edwards et al., 2001). Though no Puf3p interacting partners required for mRNA decay have yet been identified in yeast, these results would argue that any such interactions would also map to the repeat domain, and this possibility is further studied in

future chapters. Furthermore, the ability of the conserved repeat domain to regulate both translation and decay supports a model in which the Puf-mediated signal affects both processes through a similar mechanism.

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CHAPTER IV:

Puf3 Protein Elements Required for Binding and Regulation of mRNA Decay in Yeast

Puf3 Protein Elements Required for Binding and Regulation of mRNA Decay in Yeast

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Summary

The eukaryotic Puf proteins regulate mRNA translation and degradation by binding the 3' untranslated regions of target mRNAs. Crystal structure analysis of a human Puf bound to RNA suggested a modular mode of binding, with specific amino acids within each of eight repeat domains contacting a single nucleotide of the target RNA. Here we study the mechanism by which the yeast Puf3p binds and stimulates the degradation of *COX17* mRNA. Mutation of the predicted RNA-binding positions of Puf3p to those found in Puf5p demonstrated that a single amino acid change in Puf3p abolishes detectable binding to *COX17*. Since this amino acid position in both Puf3p and Puf5p is predicted to contact an adenine in the respective target RNAs, the amino acid in Puf3p must play a more critical role in promoting *COX17* interaction. In contrast, an amino acid change in the third repeat of Puf3p, which interacts with the only divergent nucleotide between the Puf3p and Puf5p targets, has no effect on binding *COX17*. These results argue that a simple set of rules cannot reliably link specific amino acid positions with target specificity. We also found that each of these amino acid changes in Puf3p enhances binding to the Puf5p target *HO* RNA, suggesting a different mode of binding to this target. Finally, we identified an outer surface loop that is dispensible for binding, but is required to promote both rapid deadenylation and subsequent decapping of the *COX17* mRNA, most likely as a point of protein-protein interactions.

Introduction

Regulation of mRNA-specific rates of translation and degradation is essential for proper control of gene expression. This type of regulation is especially apparent during early development, but is also important in somatic cells and germline sex determination (1, 2). Such post-transcriptional control is commonly mediated by proteins that bind in a sequence specific fashion to regulatory elements located in the 3' untranslated regions (UTRs) of mRNAs (3-5). However, the mechanisms by which these proteins either promote or inhibit translation and/or degradation of the bound mRNAs remain largely unclear.

The Puf family of proteins is one group of 3' UTR-binding proteins that has been found to regulate both translation and mRNA degradation in diverse eukaryotic organisms (6). Pumilio from *Drosophila melanogaster* (DmPum) and FBF from *Caenorhabditis elegans* were the founding members of this group, thus providing the Puf family name. DmPum represses translation and stimulates deadenylation of the *hunchback* mRNA, thereby promoting abdominal segmentation in the early embryo (7, 8). DmPum also plays roles in the translational repression of *cyclin B* mRNA for germline stem cell development (9-11), and in anterior patterning (12). FBF regulates the sperm/oocyte switch by repressing the expression of the *fem-3* mRNA (13), and controls germline stem cell maintenance by repressing *gld-1* mRNA expression (14).

Both DmPum and FBF require interactions with other proteins to regulate mRNA expression. For example, DmPum must form a complex with Nanos and Brat proteins to regulate *hunchback* mRNA (15, 16), whereas Nanos but not Brat is recruited for regulation of *cyclin B* mRNA (16). Similarly, FBF interacts with a Nanos-like protein to

regulate *fem-3* mRNA (17), as well as a CPEB (cytoplasmic polyadenylation element-binding protein) homolog for a possible role in spermatogenesis (18). While the requirement for protein partners is likely true of all Puf-mediated mRNA regulation, Puf partners have yet to be identified in unicellular eukaryotes.

All Puf proteins have a domain containing eight imperfect repeats of a 36 amino acid sequence plus short flanking regions. This Puf repeat domain is not only sufficient for mRNA binding (13, 19), but also for interacting with protein partners (15-18), and at least in DmPum and the yeast Puf3p, for regulating mRNA metabolism (20, 21). The crystal structures of the repeat domains of DmPum (22) and a human Puf protein (HsPum) (23) are similar. In both, each repeat folds into three α helices that stack on the helices of neighboring repeats to form an extended crescent shaped structure. The core consensus sequences of each repeat are arranged on parallel helices located on the inner concave surface (22, 23). The crystal structure of HsPum bound to an RNA ligand confirmed that this inner surface binds RNA, and the binding was predicted to be modular, with each repeat recognizing a successive base along the RNA (24). Conversely, mutational analysis of DmPum indicates that amino acids on the outer convex surface of the repeat domain contact the Nanos and Brat proteins (22).

The binding sequences of all RNA targets analyzed to date contain a shared UGUR motif required for Puf binding, with flanking sequences providing specificity (6). Inspection of the RNA target sequences of DmPum and its most closely related Puf proteins, including yeast Puf3p and Pufs in human, mouse and *Xenopus*, reveals an expanded shared binding motif of UGUANAUA (7, 19, 21, 25, 26). The crystal structure

of HsPum shows that nucleotides 1 through 8 of this RNA motif are contacted by protein repeats 8 through 1, respectively (24).

Saccharomyces cerevisiae contains six members of the Puf protein family (Puf1p-Puf6p). To date, only three of the yeast Pufs have verified roles in regulating specific RNA targets. Puf3p binds the 3' UTR of *COX17* mRNA and promotes its deadenylation and subsequent decay (27), Puf5p binds the 3' UTR of the *HO* mRNA, repressing its expression and stimulating its decay (28), and Puf6p binds the 3' UTR of the *ASH1* mRNA to regulate its translation and localization (29). In addition to these studied targets, a microarray analysis has identified several hundred candidate RNA targets that interact with one or more of the yeast Pufs 1-5 (30). Moreover, consensus sequence motifs containing UGUR were identified in many of the RNAs associated with Pufs 3, 4 and 5, with RNAs bound by each Puf protein having distinct sequences following the UGUR. However, it is still unclear how each of the yeast Pufs recognizes its unique target RNA sequence, or how the bound Pufs promote functional changes of the mRNAs.

In this work we have focused on understanding how yeast Puf3p attains specificity to its mRNA target. By mutating predicted RNA-binding residues of Puf3p to those found in Puf5p, we show that a single amino acid change is sufficient to prevent detectable binding of the protein to its *COX17* mRNA target, whereas replacing other residues has no effect on binding. Conversely, any combination of these amino acid changes in Puf3p enhance binding of the protein to the Puf5p target *HO* mRNA, with each amino acid playing a small but equal role in binding affinity. These results suggest that corresponding amino acid positions in Puf3p and Puf5p have divergent importance in determining target specificity, and therefore, the mode of binding of these Pufs to their

target RNAs is likely different. We also identify a loop region on the outer surface of Puf3p that is required for promoting both deadenylation and subsequent decapping of the *COX17* mRNA, indicating that regulation of these processes is linked by a single interaction point on Puf3p.

Experimental Procedures

Yeast Strains

The genotypes of all *S. cerevisiae* strains used in the study are as follows: yWO43 *MAT*α, *his4-539, leu2-3,112, trp1-1, ura3-52, cup1::LEU2/PM, rpb1-1, puf3::Neo*^{r} (yRP1360, 27); yWO51 *MAT***a**, *his4-539, leu2-3,112, trp1-1, ura3, rpb1-1, cox17::TRP1, puf3::Neo*^{r} (yRP1547, 27).

Plasmids

The GST-PUF3 Repeat Domain (amino acids 465-879) fusion construct (pWO12) was created in a derivative of pGEX-3X (Amersham Biosciences) as previously described (21). To create pWO14, the *PUF3* Repeat Domain (*PUF3RD*) was isolated from pWO12 and inserted into a derivative of pG-1 (31), placing the *PUF3RD* just downstream from an inserted FLAG tag sequence and the GPD promoter as previously described (21). Mutant *PUF3RD* constructs pWO29 - pWO38 (Table 1) were created from pWO14 using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). All mutations were verified by sequencing. Mutant GST-*PUF3RD* fusion constructs pWO39 – pWO49 (Table 1) were created by BamHI and SalI digestion of pWO29 - pWO38 and insertion into a derivative of pGEX-3X using the same restriction sites. The pWO18 GST-*PUF5RD* fusion construct was created in pGEX-6p-3 (Amersham Biosciences) as previously described (21).

Protein Expression and Purification

All GST fusion constructs were transformed into the protease deficient *E. coli* strain BL-21, and GST fusion proteins were purified as recommended by the manufacturer. Protein eluates were dialyzed into 50 mM Tris-HCl pH 8.0, and expression products were verified by western analysis with anti-GST antibodies.

***In Vitro* Binding Analyses**

Short *COX17* Site A and *HO* RNAs (29-30nt) were transcribed from single-stranded oligonucleotide templates containing the 18 nt T7 RNA polymerase promoter annealed to a complementary primer. The T7-Megashortscript Kit (Ambion) was used to transcribe the RNAs with the following changes: the reactions contained 500 μM each of ATP, CTP, and GTP, 50 μM of UTP, 40 μCi of α-^{32}P UTP (800 Ci/mmol) for labeled reactions, and 20 U RNasin. Radiolabeled transcripts were purified via separation on denaturing polyacrylamide gels, elution from gel slices, and ethanol precipitation. Unlabeled transcripts were purified using the Nucleotide Removal Kit (Qiagen).

Each 20 μl RNA-protein binding reaction contained radiolabeled RNA (20,000 c.p.m) and 1X binding buffer (10 mM HEPES pH 7.5, 50 mM KCl, 1 mM EDTA, 2 mM DTT, 200 U/ml RNasin, 0.1 mg/ml bovine serum albumin, 0.01% Tween-20, 0.1 mg/ml poly(rU) and 10 μg/ml yeast tRNA) in the presence or absence of wild-type GST-Puf3RDp, mutant GST-Puf3RDp or wild-type GST-Puf5RDp, and in the presence or absence of ~10-fold excess unlabeled transcript. Reactions were incubated at 24^{o}C for 30 min, 5 μg of heparin was added, then reactions incubated a further 10 min at 24^{o}C. Reactions were electrophoresed on 8% nondenaturing polyacrylamide gels at 200 V for

2.5 h at 4^{O}C. Apparent K_D values were determined using the KaleidaGraph software by fitting the binding data to the Langmuir Isotherm: Fraction of RNA Bound = [Protein] / (K_D + [Protein]). The determined K_D values are averages of multiple experiments with the errors representing the standard deviation of the experiments. The fraction of RNA bound was calculated using the following definition: Fraction Bound = Shifted RNA / (Shifted RNA + Free RNA), where Shifted RNA and Free RNA represent the storage phosphor signal of all shifted complexes located over the entire lane above the free RNA species, or the free RNA species alone, respectively. Binding-incompetent RNAs (BI-RNA), when present, had a different mobility than the expected free binding-competent RNA. Since the concentration change in these RNAs did reflect the increase in protein concentrations, these RNAs were likely to be a product of changes in the structure of the radiolabeled RNA; therefore, they were considered to be aberrant and were not included in the calculations.

***In Vivo* Decay Analysis**

Steady state transcriptional shut-off experiments were performed essentially as described (32) on yWO43 (*puf3*Δ), which contains the *rbp1-1* temperature-sensitive allele for RNA polymerase II. yWO43 was also analyzed after transformation with plasmids expressing the wild-type Puf3RDp (pWO14) as well as the mutant Puf3RDp's (pWO29-38). Northern Blots were normalized for loading using the stable RNA polymerase III transcript, *scRI* RNA (33).

Transcriptional pulse-chase experiments were performed essentially as described (34) on yWO51 (*cox17*Δ*, puf3*Δ*,rbp1-1*). Regulated expression of *COX17* RNA was accomplished by transformation of yWO51 with pWO5 (pG74/ST30, 35), in which the *COX17* gene is under the control of the *GAL10* promoter. In addition, yWO51 was transformed with pWO14 (pPuf3RD-WT) or pWO29-38 (pPuf3RD-Mutants). Poly(A) tail lengths were monitored by the cleavage of *COX17* mRNA just upstream of the stop codon using RNaseH reactions with oWO1 as described (oCOX17-C, 27). RNA was separated on 6% denaturing polyacrylamide gels at 300 V for 4 h, then transferred to nylon membrane for probing with radiolabeled oWO2 (oCOX17-P, 27).

Results

Creation of Puf3RDp mutants based on sequence and structural alignments

We have previously shown that the repeat domain of Puf3 protein (Puf3RDp) is not only sufficient for *in vitro* binding to the *COX17* mRNA, but also for *in vivo* regulation of this transcript's decay (21). In this work we wished to characterize the specific interactions of the Puf3RDp involved in the binding and regulation of the *COX17* mRNA. The crystal structure of HsPum bound to RNA shows that each base is recognized by amino acids located at three conserved positions within an individual Puf repeat domain (24). Alignment of the repeat domains of HsPum, DmPum, and Puf3p reveals that the amino acids of HsPum that interact with RNA bases are absolutely conserved with those of DmPum and Puf3p (24). Further support that these conserved amino acid positions within Puf3p are involved in specific RNA interactions comes from the finding that the optimal RNA target sequence of Puf3p is identical to that of the NRE1 boxB bound by DmPum, and the sequence used in crystal structures with HsPum (21, 24, 36).

To determine which amino acids of Puf3p promoted specificity of binding to the *COX17* mRNA, we analyzed a sequence alignment (24) of Puf3p with another yeast Puf protein, Puf5p (Figure 1A). Puf5p has previously been shown to bind and regulate the *HO* mRNA (28). The likely Puf5p target region within the *HO* 3'UTR is very similar to the Puf3p target (Figure 1D), yet Puf5p cannot bind the *COX17* target sequence (21), and Puf3p binds only weakly to the *HO* target sequence (see below, Figure 7). A comparison of the amino acids located at the predicted RNA-interacting positions of Puf3p and Puf5p reveals only three differences between the two proteins, one each in repeats 1, 3, and 5

(boxed in green, olive, and magenta, respectively, Figure 1A). The amino acid difference in repeat 3 was especially intriguing, since in the HsPum-RNA complex, the third repeat interacts with the seventh nucleotide position of the RNA sequence (UUGUAUAUA). This position is the only difference between the Puf3p target sequence and the predicted Puf5p target sequence, where there is a G at that position (Figure 1D).

To determine whether any of these amino acid differences are critical for the binding specificities of Puf3p and Puf5p, we exchanged the three differential amino acids of Puf3p with the respective amino acids of Puf5p. In repeat 1, serine 553 was changed to cysteine to create the mutant Puf3RDp-R1; in repeat 3, cysteine 625 was changed to threonine to create the mutant Puf3RDp-R3; and in repeat 5, arginine 698 was changed to cysteine to create the mutant Puf3RDp-R5. Combinations of these point mutations (R1/R3, R1/R5, and R3/R5) were also made to test whether multiple amino acid changes were necessary to alter binding specificity. A prediction of where these amino acid positions might be located on a theoretical structure of Puf3RDp is shown in Figure 1C (color coded as in Figure 1A). This structure was created by Swiss-Model, a protein homology-modeling server, by utilizing a sequence alignment of Puf3RDp with Puf proteins of known structure to model the Puf3RDp sequence on those structures (37-39).

In addition to testing Puf3RDp interactions involved in specificity of RNA binding, we also wished to characterize Puf3RDp amino acids involved in regulation of *COX17* mRNA decay. In the case of DmPum, interactions with Nanos and Brat map to the loop structures on the outer convex surface of DmPum between repeats 6, 7, and 8 (22). Though Puf protein partners have yet to be identified in yeast, we hypothesize that the ability of Puf3p to regulate *COX17* mRNA decay requires protein-protein interactions

with Puf3p. Moreover, since the repeat domain of Puf3p is sufficient for mRNA decay regulation, such interactions would have to map within the Puf3RDp. Analysis of our modeled Puf3RDp structure shows two outer surface loops in the same regions between repeats 6, 7, and 8 as found in DmPum (Figure 1B). We therefore focused our efforts of identifying amino acids required for mRNA decay regulation to these loop regions. The extended loop located between repeats 7 and 8 encompasses 16 amino acids, 11 amino acids larger than the equivalent loop in DmPum. To analyze whether any part of this loop is involved in RNA decay regulation, two different deletion mutants were made. In the Puf3RDp-R7A mutant, amino acids 3-6 of the loop were deleted (Figure 1C), corresponding to arginine 800 through asparagine 803 (Figure 1A, boxed in red). In the Puf3RDp-R7B mutant, amino acids 10-15 of the loop were deleted (Figure 1C), corresponding to asparagine 807 through serine 812 (Figure 1A, boxed in azure). The outer surface loop between repeats 6 and 7 is more ambiguous in terms of structure. A sequence alignment of Puf3RDp with several other Pufs, including HsPum and DmPum (24), showed that Puf3RDp contains an extra six amino acid region located between the latter two α-helix domains of repeat 6 (Figure 1A, boxed in yellow). These six amino acids could form an outer surface loop unique to the Puf3 protein that might be involved in a novel protein interaction involved in mRNA decay regulation. To test this prediction, the Puf3RDp-R6A mutant was created by deleting these six amino acids (Figure 1C), corresponding to phenylalanine 758 through methionine 763 (Figure 1A, boxed in yellow). In contrast, the Swiss-Model prediction of the Puf3RDp structure places these six amino acids into the third helix of repeat 6 (compare the location of R6A in Figure 1B and C). Moreover, downstream amino acids that were originally proposed

by the Puf sequence alignment to be in the third helix of repeat 6 (Figure 1A boxed in violet) are placed in a loop region between repeats 6 and 7 in the Swiss-Model structure (Figure 1B). Therefore, to determine whether the six downstream amino acids might really be in a loop involved in mRNA decay regulation, the Puf3RDp-R6B mutant was created by deleting these six amino acids (Figure 1C), corresponding to isoleucine 771 through aspartate 776 (Figure 1A, boxed in violet).

Analysis of Puf3RDp sequences required for *COX17* mRNA interaction

To test whether any of the mutations or deletions created in the Puf3RDp affect the protein's ability to bind the *COX17* 3'UTR target sequence, *in vitro* binding assays were performed with glutathione-*S*-transferase (GST)-tagged wild-type and mutant proteins purified from *E. coli*. The purified proteins were incubated with *in vitro* transcribed and radiolabled RNA encompassing the *COX17* Site A sequence (Figure 2A), which we have previously shown to be the higher affinity target of two Puf3p binding sequences in the *COX17* 3'UTR (21). The resulting complexes were analyzed by gel mobility shift experiments. As shown in Figure 2B, wild-type Puf3RDp binds to the Site A RNA (lane 3), while GST protein alone does not bind (lane 2). Analysis of the mutant proteins in Figure 2B shows that point mutations in R3 (lane 7), R5 (lane 9), or both R3/R5 (lane 8) do not disrupt binding. In contrast, a point mutation in R1 (lane 4), or any double mutation with R1 (R1/R3 in lane 5 or R1/R5 in lane 6) completely inhibit detectable RNA binding. This indicates that of these three amino acid differences between Puf3p and Puf5p, only the S553C change in the R1 mutant alters binding specificity to the Puf3p target.

We hypothesized that deletions in the predicted outer surface loops of Puf3RDp should not affect RNA binding to the inner surface of the protein unless a deletion alters the overall structure of the protein. As expected, the R6A and the R7A deletions (Figure 2B, lanes 10 and 12) do not disrupt RNA binding. However, the R6B and R7B deletions (Figure 2B, lanes 11 and 13) completely inhibit binding, most likely due to an altered protein structure. The results of the R6A and R6B deletion mutants support the sequence alignment of these amino acids in Figure 1A versus the Swiss-Model structural prediction of these amino acids in Figure 1B. Specifically the R6A region that is dispensable for binding is more likely to be in an outer surface loop than part of the integral helix structure of repeat 6. Moreover, the R6B region that is required for binding is more likely to be located in the third helix of repeat 6 that is presumably important for the overall protein structure versus an accessory outer surface loop.

To verify the specificity of interactions between the binding-competent mutant proteins and the *COX17* Site A RNA, gel mobility shift assays were performed in the presence of excess nonspecific or specific unlabeled competitor RNA (Figure 2C). All interactions of the wild-type and mutant Puf3RD proteins with the Site A RNA are specific. Excess nonspecific vector RNA had no effect on binding (Figure 2C, lanes 3, 6, 9, 12, 15, and 18), while excess *COX17* 3'UTR RNA abolished the signal from the bound complex (Figure 2C, lanes 4, 7, 10, 13, 16, and 19).

Though point mutations R3 and R5, and deletion mutations R6A and R7A do not inhibit binding to the Puf3p target RNA, it was possible that these mutations still cause altered binding affinity. Therefore, to estimate mutant protein binding affinities toward the Site A target RNA, increasing concentrations of wild-type or each mutant protein

(0.13 to 1.3 μM) were incubated with 200 pM of radiolabled Site A RNA, then complexes were analyzed by gel mobility shift assays. Figure 3A shows representatives of these assays, while the binding data from all gel mobility shift assays are plotted in Figure 3B. We found no significant difference in the binding curves of the binding-competent mutant proteins from that of the wild-type protein (Figure 3B). Furthermore, the equilibrium dissociation constant (K_D) of WT-Puf3RD was determined to be 0.5 μM $\pm$ .006. Similar K_D values were also calculated for the other binding-competent mutant Puf3RD proteins (Table 2). We have previously shown the specific activity of our WT-Puf3RDp to be at 5-10% of total protein concentration (21). Since all proteins were purified in the same manner, the specific activity of all mutant proteins is predicted to be similar to that of the WT-Puf3RDp. This prediction is supported by the fact that all proteins tested in Figure 3B display similar K_D values. Together, these results indicate that unlike the R1 mutation that completely inhibits detectable binding, the amino acid differences in R3 and R5 between Puf3p and Puf5p play no role in the differential binding affinities of the two proteins toward the Puf3p target RNA. Moreover, the amino acids of the R6A and R7A regions that are likely located in outer surface loop structures have no role in Puf3RDp RNA binding affinity.

An outer surface loop of the Puf3RDp is required for RNA decay regulation

We showed that only serine 553 was involved in specific binding of Puf3p to its mRNA target *in vitro*, but we expected that other amino acids were involved in mRNA decay regulation *in vivo*. In particular, amino acids in the outer surface loops are prime candidates for interacting with other proteins involved in mRNA decay. We also wanted

to verify that the inner surface point mutants that did not disrupt mRNA binding are still competent for mRNA decay regulation, and that the mutants that disrupt mRNA binding are unable to regulate mRNA decay. Therefore, *in vivo* transcriptional shut-off assays were performed to test for the functional rescue of a yeast *puf3*Δ strain by each mutant protein by monitoring the decay of steady-state *COX17* mRNA. For this experiment, plasmids encoding the wild-type or each mutant protein were transformed into a *puf3*Δ strain containing a temperature-sensitive lesion in RNA polymerase II (*rpb1-1*), in which transcription is rapidly repressed following a shift to the non-permissive temperature.

In the *puf3*Δ strain, *COX17* mRNA decays with a half-life of 27 minutes, while expression of the wild-type Puf3RDp in the *puf3*Δ strain rescues rapid decay of *COX17* mRNA to a half-life of 15 minutes (21, Figure 4A and B). When mutant proteins that cannot bind the *COX17* mRNA (Puf3RDp-R1, -R1/R3, -R1/R5, -R6B, and –R7B) were expressed in the *puf3*Δ strain, *COX17* decayed with a half-life of 28-30 minutes, nearly identical to that of the *puf3*Δ alone (Figure 4C, D, E, J, and L). This indicates that RNA binding is required for the Puf3RDp to mediate rapid *COX17* mRNA decay. Conversely, when proteins containing inner surface point mutations that do not inhibit RNA binding (Puf3RDp-R3, -R3/R5, and –R5) were expressed in the *puf3*Δ strain, *COX17* decayed with a half-life of 16 minutes, identical to wild-type Puf3RDp (Figure 4F, G, and H). This demonstrates that these mutations have no detrimental effect on mRNA decay regulation. Most interesting were our results with the outer surface loop deletions that did not disrupt RNA binding. Expression of Puf3RDp-R6A in the *puf3*Δ strain rescued *COX17* mRNA decay to wild-type levels, with a half-life of 16 minutes (Figure 4I). Thus, this outer loop region plays no role in protein-protein interactions nor any other

signaling for the decay of *COX17* mRNA. However, expression of Puf3RDp-R7A in the *puf3*Δ strain did not rescue rapid decay of *COX17* mRNA, with a half-life of 28 minutes, similar to the *puf3*Δ (Figure 4K). Therefore, the amino acids of the R7A loop are absolutely essential for the Puf3RDp to mediate rapid mRNA decay. We predict that this region may be involved in protein-protein interactions that signal to the decay machinery.

The regulation of *COX17* mRNA by the mutant Puf3RD proteins is mediated through deadenylation and decapping

Because Puf3p regulates *COX17* mRNA decay by promoting both rapid deadenylation and rapid decapping (27), we wished to determine whether the differences in the half-life of *COX17* mRNA were due to differences in the rate of deadenylation, decapping, or both. To examine these steps of decay, *in vivo* transcriptional pulse-chase assays were performed. For these experiments, a plasmid expressing *COX17* under the control of the regulatable *GAL10* promoter was transformed into the *puf3*Δ strain along with or without plasmids expressing either wild-type or mutant Puf3RD proteins. The transcription of *COX17* mRNA is induced by the addition of galactose to the growth medium, and then rapidly repressed by the addition of glucose (34). This produces a pulse of newly synthesized transcripts whose deadenylation and subsequent decay can be monitored over time.

In confirmation of previous work (21), we found that a pulse of *COX17* transcripts expressed in a *puf3*Δ strain is synthesized with poly(A) tails of 35-60 residues (Figure 5A, lane 0). The poly(A) tails then deadenylate slowly such that fully deadenylated species do not appear until 15 minutes after transcriptional repression, and

transcripts with short poly(A) tails persist to 40 minutes, indicative of a slow decapping step (Figure 5A). In contrast, expression of the wild-type Puf3RDp in the *puf3*Δ strain rescued rapid deadenylation, with transcripts that start out with similar poly(A) tails of 45-60 residues (Figure 5B, lane 0) reaching a deadenylated state within 4 minutes. Moreover, there is no buildup of transcripts with short poly(A) tails, and all transcripts are nearly completely degraded by 6 minutes (Figure 5B). This indicates that Puf3RDp is sufficient to promote both rapid deadenylation and rapid decapping of *COX17* mRNA.

Next, the expression of each mutant Puf3RD protein in the *puf3*Δ strain was analyzed for the ability to promote rapid *COX17* deadenylation and decapping. As predicted, any mutant protein that failed to bind *COX17* mRNA also failed to promote rapid deadenylation and decapping. Figure 5C shows a representative Northern blot analysis for such a mutant protein, Puf3RDp-R1. The slow deadenylation and persistence of transcripts with short poly(A) tails in this and the other binding-incompetent mutants look identical to that seen in the *puf3*Δ strain (compare Figure 5C with 5A). For the mutant proteins that could bind *COX17* mRNA, all but one promoted rapid deadenylation and decapping. Representative Northern blot analyses of two such proteins, Puf3RDp-R3/R5 andPuf3RDp-R6A, are shown in Figures 5D and 5F, respectively. Deadenylation proceeds rapidly in the binding-competent mutants, with no buildup of transcripts with short poly(A) tails and nearly complete degradation by 6 minutes. This is identical to the pattern seen with the wild-type Puf3RDp (compare Figure 5D and 5F with 5B). In contrast, our analysis of the Puf3RDp-R7A mutant shows that this protein fails to promote both rapid deadenylation and decapping (Figure 5E). Transcripts do not reach a deadenylated state until 15 minutes, then persist with short poly(A) tails to 40 minutes, a

pattern identical to that seen in the *puf3*Δ strain (compare Figure 5E with 5A). This result suggests that while the R7A loop region has no role in binding to the *COX17* mRNA, it is absolutely required for interactions that mediate signals to both the deadenylation and decapping machinery.

Point mutations in Puf3RDp enhance affinity to a Puf5p target mRNA

We next wished to determine whether the point mutations made on the inner surface of the Puf3RDp altered the binding affinity of the protein to a known Puf5p target, the *HO* mRNA. Since each of the three mutations replaced an amino acid of the Puf3RDp that was predicted to bind RNA with the respective amino acid found in the Puf5 protein, we hypothesized that these mutations might enhance binding to the *HO* mRNA target. We also wanted to determine whether the outer surface deletion mutations had any effect on binding to the *HO* mRNA. Therefore, to examine RNA-protein complex formation, each of the wild-type and mutant Puf3RD proteins purified as GST-fusions, or GST protein alone, was incubated with uniformly radiolabeled RNA encompassing the target binding sequence within the *HO* 3'UTR (Figure 6A). The resulting complexes were analyzed by a gel mobility shift assay (Figure 6B). As shown, GST protein alone does not bind to the *HO* RNA (lane 2). As expected, the wild-type Puf5RDp binds the *HO* target sequence (lane 3), while the wild-type Puf3RDp binds the *HO* RNA less well (lane 4). Evaluation of complex formation with each of the mutant proteins shows that all single and double point mutations on the RNA binding surface of Puf3RDp allow binding to the *HO* RNA (lanes 5-10). In addition, the R6A and R7A outer surface loop deletions that have no detrimental effect on binding to the *COX17*

RNA also have no detrimental effect on binding to the *HO* RNA (lanes 11 and 13, respectively). In contrast, the R6B and R7B outer surface loop deletions prevent binding of the protein to the *HO* RNA (lanes 12 and 14, respectively). Since these deletions also prevented binding to the *COX17* RNA, these results provide additional evidence that the R6B and R7B deletions cause overall structural changes to the Puf3RDp that block its ability to bind RNA.

To verify that all of the complexes formed with the *HO* RNA are sequence specific, each protein was incubated with the radiolabeled *HO* target RNA in the presence or absence of excess unlabeled *HO* target RNA as a specific competitor or unlabeled vector RNA as a nonspecific competitor. As shown in the gel mobility shift assay in Figure 6B, all interactions are specific since they can be competed with excess specific competitor (lanes 4, 7, 10, 13, 16, 19, 22, 25, 28, and 31), but not with excess nonspecific competitor (lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30).

Finally, to determine whether the point mutations made on the inner surface increase the affinity of these mutant Puf3RD proteins to the *HO* RNA versus the wild-type Puf3RDp, increasing concentrations of each protein (0 to 1.3 μM) were incubated with 200 pM of radiolabled *HO* RNA. Complexes were analyzed by gel mobility shift assays. Three representative gel mobility shift assays are shown in Figure 7A, while the data from all gel mobility shift analyses are plotted in the graph of Figure 7B. For the wild-type Puf5RDp, the apparent K_D value was calculated to be 0.27 ± 0.01 μM (Figure 7A, B). In contrast, the interaction of Puf3RDp with *HO* RNA was determined to be very weak, with only $28.9 \pm 0.5\%$ of the RNA shifted into a complex at the highest protein concentration tested (Figure 7A, B) and an apparent K_D value of 1.02 ± 0.02 μM.

Interestingly, analyses of the R1, R3, and R5 single point mutations showed that most promote a slight increase in affinity toward the *HO* RNA versus the wild-type Puf3RDp, with 33.8 $\pm$ 1.3%, 31.8 $\pm$ 0.8%, and 32.8 $\pm$ 0.9% of the RNA shifted into complexes, respectively, at the highest concentration of protein added (Figure 7A, B). The apparent K_D values for R1, R3, and R5 single point mutant proteins were determined to be 0.88 $\pm$ 0.08 μM, 0.81 $\pm$ 0.17 μM, and 1.11 $\pm$ 0.05 μM, respectively. Furthermore, any combination of two point mutations (R1/R3, R1/R5, or R3/R5) promotes a larger increase in affinity of the protein to the *HO* RNA, with 40.3 $\pm$ 1.3%, 39.8 $\pm$ 4.5%, or 38.6 $\pm$ 1.8% of the RNA shifted into a complex, respectively, at the highest concentration of protein added (Figure7B). The apparent K_D values for these double mutant proteins were determined to be 0.75 $\pm$ 0.09 μM, 0.92 $\pm$ 0.19 μM, and 0.72 $\pm$ 0.03 μM, respectively. Since all combinations of double mutants promote a general enhanced affinity toward the *HO* RNA, these results suggested that the three amino acid positions tested play similar roles in RNA binding, and the small contribution of each amino acid toward binding affinity may be additive. Upon this observation, the Puf3RDp-R1/R3/R5 triple mutant was created. The binding of the triple mutant to the *HO* RNA and its interaction specificity was confirmed by gel mobility shift analysis (data not shown). The affinity analysis of the triple mutant (R1/R3/R5) shows that point mutations of all three amino acids promotes a significantly greater increase in affinity of the protein to *HO* RNA, with 42.5 $\pm$ 1.6 % RNA shifted into a complex at the highest concentration of protein added (Figure 7A, B), and an apparent K_D value of 0.56 + 0.01 μM. This further verifies the previous observation regarding the small contribution of each amino acid toward binding

affinity. However, this apparent K_D for the triple mutant is still two-fold weaker than wild-type Puf5RDp.

Discussion

The conserved repeat domain of Puf proteins serves as the RNA binding surface as well as the protein docking point for interactions required for regulation of mRNA translation and degradation. In this work we have characterized the interactions involved in RNA target specificity and mRNA decay regulation by the yeast Puf3 protein. First, we provide evidence that a single serine to cysteine point mutation in the first repeat (R1) abolishes binding and regulation of the *COX17* mRNA by Puf3RDp. *In vitro* gel mobility shifts assays show no detectable binding of this R1 mutant to a target binding site in the *COX17* 3' UTR (Figure 2), and expression of the R1 mutant protein in a *puf3*Δ strain fails to rescue rapid decay of the *COX17* mRNA (Figure 4), with deadenylation and decapping slowed to the same extent as in a *puf3*Δ strain (Figure 5). In contrast, neither a cysteine to threonine point mutation in repeat 3, nor an arginine to cysteine point mutation in repeat 5 have any detrimental effects on binding or regulation of *COX17* mRNA. Both the R3 and R5 mutant proteins show wild-type binding affinities *in vitro* (Figure 3), and both rescue wild-type decay of *COX17* mRNA (Figure 4) through rapid deadenylation and decapping (Figure 5).

The results with the inner surface point mutations of the Puf3p repeat domain are surprising, given the predicted roles of these amino acids in binding to a target RNA. Based on the alignment with the HsPum crystal structure (24), the serine in the first repeat of Puf3p was predicted to make a specific van der Waals interaction with the final adenine of the *COX17* RNA target sequence (Figure 1C). In Puf5p, a cysteine is located at this position, but its predicted role is to still make a van der Waals interaction with a final adenine of the *HO* RNA target sequence (Figure 1C). Therefore, the serine to

cysteine mutation in R1 of Puf3RDp was not expected to cause a significant change in the ability of Puf3RDp to bind the *COX17* target RNA. In fact, Puf3p utilizes a cysteine in Repeat 3 to make a predicted van der Waals interaction with another adenine in the *COX17* target sequence (Figure 1C). However, since the serine to cysteine mutation in R1 completely abolishes detectable RNA binding, this result demonstrates that the role of the R1 serine cannot be to simply make a low energy van der Waals interaction with the adenine. It is actually quite remarkable that a single amino acid change would have such a drastic effect on a protein that utilizes 24 predicted RNA contacts over its extended 8-repeat structure. Therefore, we hypothesize that this serine is critical for other intra-protein interactions, such as with other side chains, to promote the precise architecture and chemical surface necessary to bind the *COX17* RNA target. Moreover, we show that the serine to cysteine mutation does not interrupt binding to the *HO* target sequence (Figure 7), demonstrating that the R1 mutant protein is still active, and suggesting that Puf protein binding to this *HO* target utilizes a different architecture of the Puf binding surface.

Our studies with the mutation in Repeat 3 are also intriguing. Alignment with the HsPum crystal structure (24) places the cysteine of the Puf3p Repeat 3 in a van der Waals contact with an adenine (Figure 1C), while Puf5p utilizes a threonine at this position to contact a guanine (Figure 1C). Since the adenine versus guanine contact is the only difference between the Puf3p and Puf5p RNA target sequences, we had originally predicted this amino acid position to be a point of target specificity. Previous work with the HsPum supported a modular role of each repeat, with specificity determined by three amino acids located at predicted RNA-binding positions of each repeat. In particular,

directed alteration of the three RNA-binding amino acids within repeat 6 of the HsPum could alter target specificity (24). However, our results show that binding specificity is not necessarily modular. Since the cysteine versus threonine change is the only difference in the three predicted RNA-binding positions of Repeat 3 between Puf3p and Puf5p, yet binding of Puf5p to the *COX17* target sequence cannot be detected, our finding that a cysteine to threonine mutation in R3 of Puf3RDp does not reduce binding affinity to the *COX17* target RNA (Figure 3) implies that other amino acids outside the three predicted positions of this repeat are promoting RNA binding specificity. This result also supports our hypothesis that RNA target specificity may be dependent on changes in protein surface architecture that are promoted by intra-protein interactions between amino acids that are not necessarily critical for direct contact with the RNA. For example, the serine in Repeat 1 of Puf3p may be promoting specific intra-protein interactions in a cascading effect that causes the cysteine of Repeat 3 to be in the precise orientation for contacting adenine, but not for contacting guanine. Thus, it may be the specific orientation of this amino acid that is important for contact versus its identity, with a threonine able to work just as well in its place when in the correct orientation. Precedence for nucleic acid binding proteins that appear to act in a modular fashion but are, in fact, much more complex are the zinc-finger proteins. These proteins, like the Puf proteins, were originally thought to attain specificity to target sequences by the identity of amino acids at particular base-interacting positions within each zinc-finger module. However, closer inspection has shown that intra-protein interactions such as side chain-side chain contacts and interactions with ordered water molecules are critical for

determining binding specificity (40). We believe this may be what is occurring in the Puf proteins as well.

In contrast to the *COX17* binding studies, our analysis of the Puf3RD mutant proteins binding to the *HO* RNA target shows that the identities of the amino acids at all three mutagenized RNA-interacting positions are important for binding to this Puf5p target RNA. In addition, amino acids outside these predicted positions are also critical for promoting binding affinity. We first determined that unlike the inability of Puf5p to bind the *COX17* RNA target, Puf3p can weakly bind the *HO* target (Figure 7), suggesting that the architecture of the Puf3p binding surface is flexible enough to accommodate binding to this RNA. Any single mutation that replaces a Puf3p amino acid at a predicted RNA-interacting position with that found in Puf5p promotes a small but detectable increase in affinity to the *HO* target RNA. Since Puf3RDp along with the single point mutant proteins have relatively weak interactions with *HO* RNA that were difficult to quantitate from gel-shifts, additional experiments may be necessary to obtain better fit binding curves for more accurate affinity comparisons. Interestingly, any combination of double mutations promotes a bigger increase in affinity, while a triple mutation promotes an even larger increase in affinity (Figure 7). This supports a model in which each *HO* RNA-protein contact makes a small but equal contribution to the binding energy, with the identity of the amino acid contact making a significant difference in the energy. Interestingly, the Puf3RDp triple mutant effectively mimics 24 out of 24 RNA-interacting amino acids of Puf5p, yet the affinity of the triple mutant is still two-fold less than that of wild-type Puf5RDp toward the *HO* target (Figure 7). This suggests that there are other amino acids unique to Puf5RDp that play significant roles in RNA binding, further

supporting our hypothesis that RNA target specificity may be dependent on protein surface architecture that is promoted by intra-protein interactions between amino acids not involved in direct RNA contacts.

In addition to characterizing the interactions involved in RNA target specificity, our results provide key information regarding the Puf3p interactions required for mRNA decay regulation. Most interestingly, we show that the R7A deletion of amino acids RDKN, which are predicted to be located at one end of a 16 amino acid loop structure on the outer surface of the protein between repeats 7 and 8, completely abolishes *COX17* decay regulation (Figure 4), with deadenylation and decapping slowed to the same extent as in a *puf3*Δ strain (Figure 5), while having no effect on binding of the protein to the mRNA (Figure 3). We therefore hypothesize that one or more of these amino acids are involved in protein-protein interactions that provide a signal to the decay machinery to rapidly degrade the bound mRNA. This signal could be involved in recruiting the decay machinery to the mRNA, or alternatively the signal could enhance the activity of the decay machinery, perhaps by altering the mRNP structure. The fact that the RDKN deletion disrupts both rapid deadenylation and rapid decapping suggests that regulation of these processes is linked by protein interactions at this site. Such interactions could involve direct contacts with regulators or components of both the deadenylation and decapping machineries, or these interactions could simply be altering the mRNP structure to allow more rapid access of the mRNA to both decay machineries. A corresponding loop region between repeats 7 and 8 of DmPum is required for protein interactions with Nanos and Brat, which are necessary for *hunchback* mRNA repression (22). However, it is unknown how these interactions repress hunchback mRNA. While this site of protein

interaction appears to be conserved between Puf proteins, it is intriguing that the characteristics of the loops in DmPum and Puf3p are quite different, with no conservation of sequence, and the Puf3p loop being eleven amino acids longer. Since there are no obvious homologs of Nanos or Brat in yeast, and the loop regions between DmPum and Puf3p are dissimilar, Puf3p may be utilizing a novel protein interaction mechanism at this site to promote mRNA decay. Efforts are underway to study the role of this loop in potential Puf3p protein interactions.

In contrast to the R7A deletion, the R6A deletion of amino acids FTNKEM, which our results suggest are located in an outer surface loop of Puf3p within repeat 6, has no deleterious effects on either binding (Figure 3) or *COX17* decay regulation (Figure 4), with deadenylation and decapping occurring as rapidly as with wild-type Puf3RDp (Figure 5). While these results show that this amino acid region plays no role in the decay of *COX17* mRNA, it is possible that this region is important for regulation of other Puf3p target mRNAs. Precedence for a single Puf protein recruiting different sets of protein partners on different mRNA targets comes from Drosophila, where DmPum recruits both Nanos and Brat when bound to *hunchback* mRNA, but only recruits Nanos when bound to *cyclinB* mRNA (16). In addition, since no other Puf protein contains this unique loop sequence, we speculate that the yeast Puf3 protein may have acquired and maintained this domain for a novel regulatory function. It will be interesting to determine what roles the R7A and R6A loop domains play in decay regulation of other Puf3p target mRNAs.

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Figure Legends

Figure 1. The Puf3p Repeat Domain. (A) Amino acid sequence alignment of the Puf3p and Puf5p Repeat Domains (24). Each repeat is numbered R1-R8, with thick lines above each repeat indicating the predicted regions of the RNA-interacting helices. Individual amino acids predicted to make RNA contacts are underlined. The region predicted to form the third helix of repeat 6 is marked over the sequence with a thin line. Amino acids mutated in this study are as follows: amino acids predicted to make van der Waals interactions with the RNA are in green (R1) and olive (R3) boxes, and an amino acid predicted to make a stacking interaction with the RNA is in a magenta (R5) box. Regions of amino acids deleted in this study are as follows: regions predicted to be positioned in an outer surface loop within repeat 6 are in yellow (R6A) and violet (R6B) boxes, and regions predicted to be positioned in an outer surface loop following repeat 7 are in red (R7A) and azure (R7B) boxes. (B) Predicted Puf3RDp structure created by Swiss-Model (37-39). (C) The locations of all mutated and deleted amino acids on the Puf3RDp structure are indicated with colors corresponding to the respective boxes in part (A). (D) Alignment of target RNA sequences from the *COX17* and *HO* mRNAs bound by the Puf3p and Puf5p proteins, respectively. The Puf repeat predicted to interact with each nucleotide is indicated above the sequences (24).

Figure 2. *In Vitro* binding of wild-type and mutant Puf3RD proteins to *COX17* Site A RNA. (A) Sequence of the 30 nucleotide *COX17* Site A transcript used in binding reactions is shown. The UGUA core binding element is underlined. (B) and (C) *In vitro*

binding reactions of radiolabeled *COX17* Site A RNA in the absence or presence of 0.65 μM protein were separated on native polyacrylamide gels. Positions of unbound RNA (Free RNA) as well as RNA-Puf3RDp complexes (RNA+Puf3RDp) are indicated. (B) Reactions were performed in the presence of GST alone (lane 2) wild-type Puf3RDp (lane 3) or mutant Puf3RD proteins (lanes 4-13). (C) The specificity of RNA-protein interactions was analyzed using excess unlabeled vector RNA or full length *COX17* 3'-UTR RNA as non-specific (NSC) or specific (SC) competitors, respectively, in binding reactions with wild-type Puf3RDp (lanes 2-4), or mutant Puf3RD proteins (lanes 5-19). The presence of either competitor is marked (+).

Figure 3. Comparison of binding affinities of wild-type and mutant Puf3RD proteins to *COX17* Site A RNA *in vitro*. (A) Gel mobility shift assays of *in vitro* binding reactions of radiolabeled *COX17* Site A RNA in the absence or presence of increasing concentrations of Puf3RDp-WT, Puf3RDp-R3, and Puf3RDp-R7A are shown as representatives of similar assays performed on all mutant proteins. Concentrations of protein used in binding reactions were 0, 0.13, 0.26, 0.39, 0.52, 0.65, and 1.3μM in lanes 1-7, respectively. Positions of unbound RNA (Free RNA) as well as RNA-Puf3RDp complexes (RNA+Put3RDp) are indicated on each gel. The panel below each gel reflects a lighter exposure of the binding-incompetent RNA (BI-RNA) as well as the unbound (binding competent) RNA (Free RNA) from the same gel, where the position of each RNA species is indicated. (B) Data from the gel mobility shift assays performed with Puf3RDp-WT and all mutant Puf3RD proteins capable of binding *COX17* Site A RNA are plotted with the μM concentration of protein used in the binding reaction on the x-

axis and fraction of RNA shifted from free RNA to bound RNA on the y-axis. Binding curves are shown for Puf3RDp-WT (filled circle), Puf3RDp-R3 (filled inverted triangle), Puf3RDp-R3/R5 (filled square), Puf3RDp-R5 (filled diamond), Puf3RDp-R6A (open triangle), and Puf3RDp-R7A (open crossed square). Data points are averages of multiple experiments.

Figure 4. *COX17* mRNA decay rates in the presence of wild-type or mutant Puf3RD proteins *in vivo*. (A) Shown are Northern blot analyses of the decay of *COX17* mRNA from a *puf3*Δ strain transformed with or without constructs expressing either Puf3RDp-WT or each of the mutant Puf3RD proteins. Minutes following transcriptional repression are indicated above the sets of blots, with the half-lives ($t_{1/2}$) as determined from multiple experiments.

Figure 5. Deadenylation rate of *COX17* mRNA in the presence of wild-type or mutant Puf3RD proteins *in vivo*. Shown are northern blot analyses of transcriptional pulse-chase experiments examining decay of *COX17* mRNA from a *puf3*Δ strain (A), and *puf3*Δ strains transformed with constructs expressing either wild-type Puf3RDp (B), Puf3RDp-R1 (C), Puf3RDp-R3/R5 (D), Puf3RDp-R6A (E), or Puf3RDp-R7A (F). Minutes following transcriptional repression are indicated above each blot. Size markers (M) are given in nucleotides. The –8 lane in each blot corresponds to background levels of RNA expression before galactose induction of *COX17* RNA transcription. The 0dT lanes in (A) and (B) correspond to RNA from the 0 min time point in which the poly(A)

tail was removed by RNase H cleavage with oligo(dT). Arrows denote the position of deadenylated 3'UTR species.

Figure 6. *In Vitro* Binding of wild-type and mutant Puf3RD proteins to *HO* RNA. (A) Sequence of the 35 nucleotide *HO* RNA transcript used in binding reactions is shown. The UGU core binding element is underlined. (B) and (C) *In vitro* binding reactions of radiolabeled *HO* RNA in the absence or presence of 0.65 μM protein were separated on native polyacrylamide gels. Positions of unbound RNA (Free RNA) as well as RNA-Puf5RDp or RNA-Puf3RDp complexes (RNA+PufRDp) are indicated. (B) Reactions were performed in the presence of GST alone (lane 2), wild-type Puf5RDp (lane 3), wild-type Puf3RDp (lane 4), or mutant Puf3RD proteins (lanes 5-14). (C) The specificity of RNA-protein interactions was analyzed using excess unlabeled vector RNA or full length *COX17* 3'UTR RNA as non-specific (NSC) and specific competitors (SC), respectively, in binding reactions with wild-type Puf5RDp (lanes 2-4), wild-type Puf3RDp (lanes 5-7), or mutant Puf3RD proteins (lanes 8-31). The presence of either competitor is marked (+).

Figure 7. Comparison of binding affinities of wild-type and mutant Puf3RD proteins to *HO* RNA *in vitro*. (A) Gel mobility shift assays of *in vitro* binding reactions with radiolabeled *HO* RNA in the absence or presence of increasing concentrations of Puf5RDp-WT, Puf3RDp-WT, and Puf3RDp-R1/R3/R5 are shown as representatives of similar assays performed on all mutant proteins. Concentrations of protein used in the Puf5RDp-WT binding reactions were 0, 0.12, 0.24, 0.37, 0.49, 0.61, and 1.2 μM in lanes

1-7, respectively. Concentrations of protein used in all Puf3RDp binding reactions were 0, 0.13, 0.26, 0.39, 0.52, 0.65, and 1.3μM in lanes 1-7, respectively. Positions of unbound RNA (Free RNA) as well as RNA-Puf5RDp and RNA-Puf3RDp complexes (RNA+PufRDp) are indicated on each gel. The panel below each gel reflects a lighter exposure of the binding-incompetent RNA (BI-RNA) as well as the unbound (binding-competent) RNA (Free RNA) from the same gel, where the position of each RNA species is indicated. (B) Data from the gel mobility shift assays performed with Puf5RDp-WT, Puf3RDp-WT, and all mutant Puf3RD proteins capable of binding *HO* RNA are plotted with the μM concentration of protein on the x –axis and the fraction of RNA shifted from free RNA to bound RNA on the y-axis. Data points are averages of multiple experiments.

Table 1. Puf protein mutations and respective expression plasmids used in this study.

Protein	Mutation	GST-Plasmid	Yeast Plasmid
Puf3RDp	WT	pWO 12	pWO 14
Puf5RDp	WT	pWO 18	-
Puf3RDp-R1	S553C	pWO 39	pWO 29
Puf3RDp-R1/R3	S553C and C625T	pWO 40	pWO 30
Puf3RDp-R1/R5	S553C and R698C	pWO 41	pWO 31
Puf3RDp-R3	C625T	pWO 42	pWO 32
Puf3RDp-R3/R5	C625T and R698C	pWO 43	pWO 33
Puf5RDp-R5	R698C	pWO 44	pWO 34
Puf3RDp-R6A	Δ F758-M763	pWO 45	pWO 35
Puf3RDp-R6B	Δ I771-D776	pWO 46	pWO 36
Puf3RDp-R7A	Δ R800-N803	pWO 47	pWO 37
Puf3RDp-R7B	Δ N807-S812	pWO 48	pWO 38
Puf3RDp-R1/R3/R5	S553C, C625T, and R698C	pWO 49	-

Table 2. Calculated K_D values for interacting Puf3RD proteins.

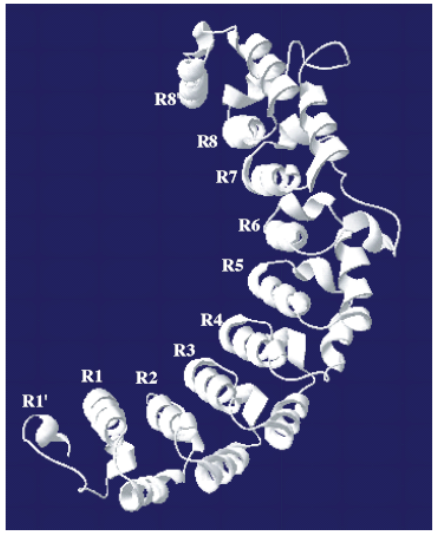
Protein	$\boldsymbol{K_D}$
Puf3RDp-WT	0.50 ± 0.01
Puf3RDp-R3	0.52 ± 0.02
Puf3RDp-R3/R5	0.53 ± 0.04
Puf5RDp-R5	0.54 ± 0.01
Puf3RDp-R6A	0.52 ± 0.02
Puf3RDp-R7A	$0.52 + 0.07$

Figure 1

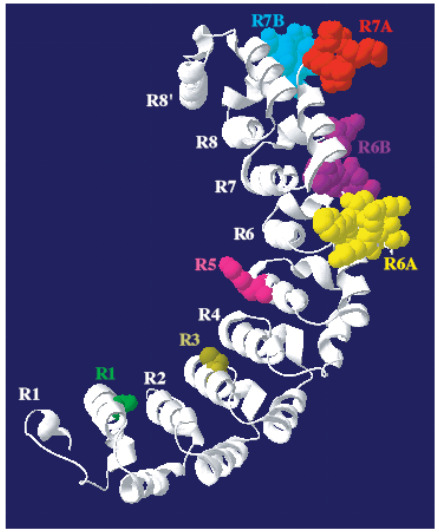
(A)

```
                  R1                                                                                R2
Puf3p   HSLEFCKDQHGSRFIQRELATSP--ASEKEVIFNEIRD                                    DAIELSNDVFGNYVIQKFFEFGSKIQKNTLVDQFKG
Puf5p   DYIKLATDQFGCRFLQKKLETPSESNMVRDLMYEQIKP                                    FFLDLILDPFGNYLVQKLCDYLTAEQKTLLIQTIYP
                  R3                                                                                R4
Puf3P   NMKQLSLQMYACRVIQKALEYIDSNQR-----IELVLELSD--                              SVLQMIKDQNGNHVIQKAIETIPIEKLPFILSSL--TG
Puf5p   NVFQISINQYGTRSLQKIIDTVDNEVQIDLIIKGFSQEFTSIE                               QVVTLINDLNGNHVIQKCIFKFSPSKFGFIIDAIVEQN
                  R5                                                                                R6
Puf3p   HIYHLSTHSYGCRVIQRLLEFGSSEDQESILNELKD                                      FIPYLIQDQYGNYVIQYVLQQDQFTNKEMVDIKQEIIETVAN
Puf5p   NIITISTHKHGCCVLQKLLSVCTLQQIFKISVKIVQ                                      FLPGLINDQFGNYIIQFLLDIKELD----FYLLAELFNRLSN
                  R7                                                                                R8
Puf3P   NVVEYSKHKFASNVVEKSILYGSKNQKDLIISKILPRDKNHALNLED----------------DSPMILMIKDQFANYVIQKLVNVSE-------GEGKKLIVIAIRA
Puf5p   ELCQLSCLKFSSNVVEKFIKKLFRIITGFIVNNNGGASQRTAVASDDVINASMNILLTTIDIFTVNLNVLIRDNFGNYALQTLLDVKNYSPLLAYNKNSNAIGQNSSS
```

(B)



(C)



(D)

Puf Repeat	87654321
Puf3p/*COX17*	UGUAUAUA
Puf5p/*HO*	UGUAUGUA

Figure 2

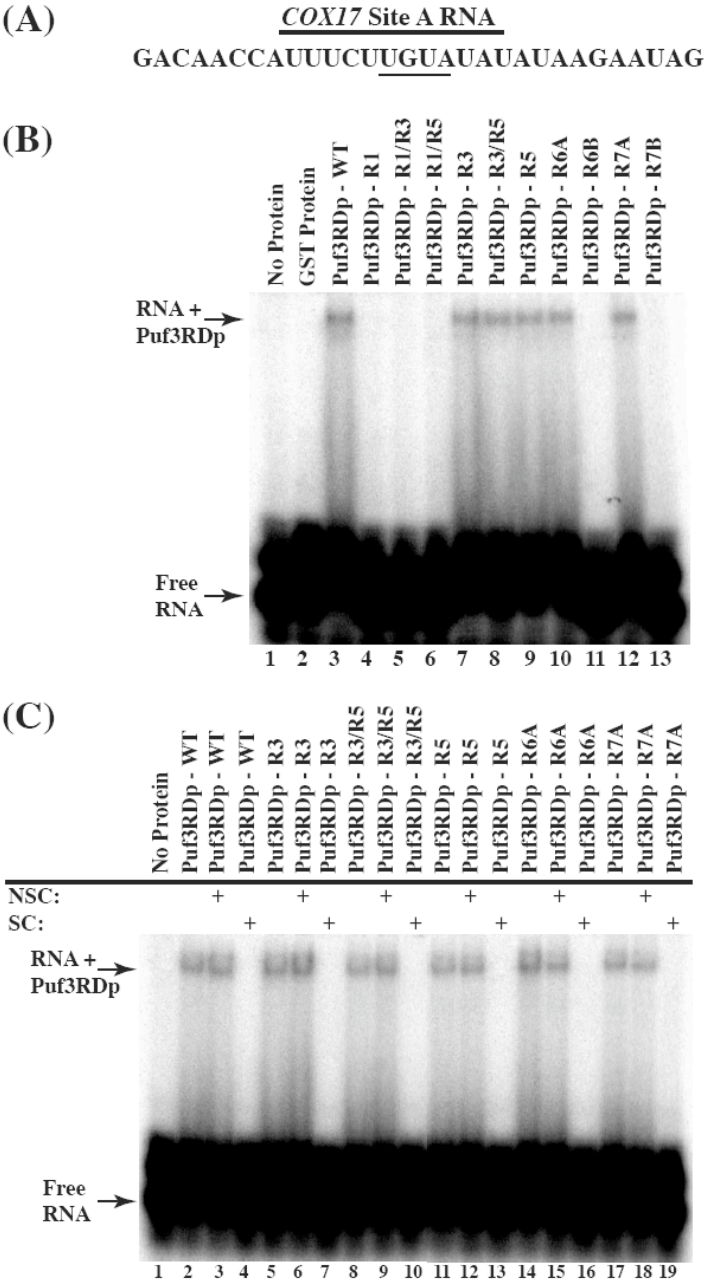
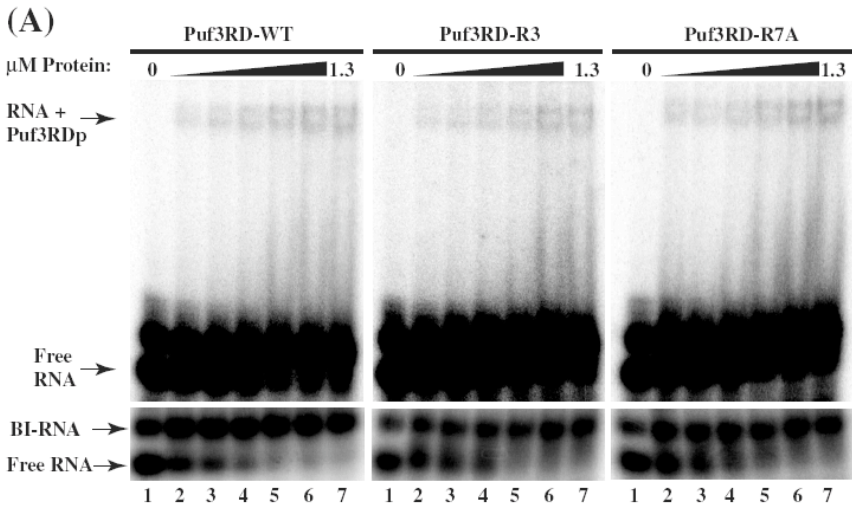

(A)
COX17 Site A RNA
GACAACCAUUUCUUGUAUAUAUAAGAAUAG
(B)
No Protein
GST Protein
Puf3RDp - WT
Puf3RDp - R1
Puf3RDp - R1/R3
Puf3RDp - R1/R5
Puf3RDp - R3
Puf3RDp - R3/R5
Puf3RDp - R5
Puf3RDp - R6A
Puf3RDp - R6B
Puf3RDp - R7A
Puf3RDp - R7B
RNA + Puf3RDp
Free RNA
1 2 3 4 5 6 7 8 9 10 11 12 13
(C)
No Protein
Puf3RDp - WT
Puf3RDp - R3
Puf3RDp - R3/R5
Puf3RDp - R5
Puf3RDp - R6A
Puf3RDp - R7A
NSC:
SC:
RNA + Puf3RDp
Free RNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 3


(A)
Puf3RD-WT
Puf3RD-R3
Puf3RD-R7A
µM Protein:
0
1.3
RNA + Puf3RDp
Free RNA
BI-RNA
Free RNA
1 2 3 4 5 6 7

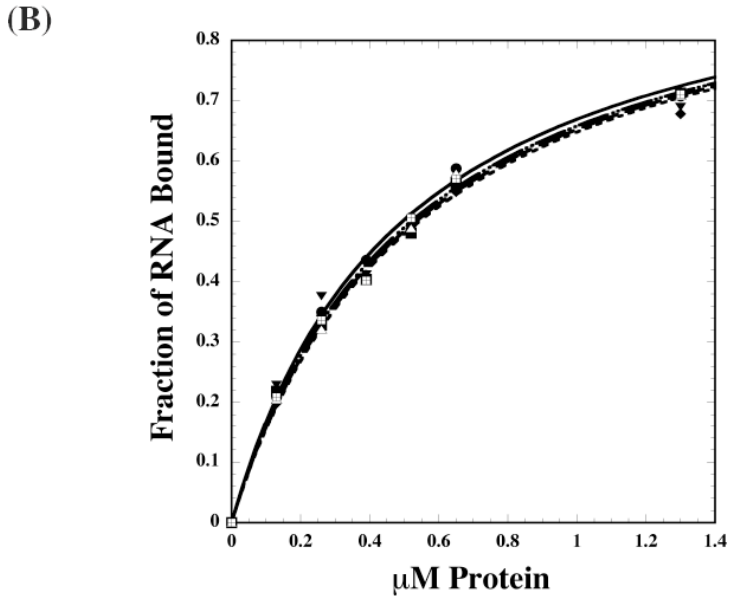

(B)
Fraction of RNA Bound
µM Protein
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.2
0.4
0.6
0.8
1
1.2
1.4

Figure 4

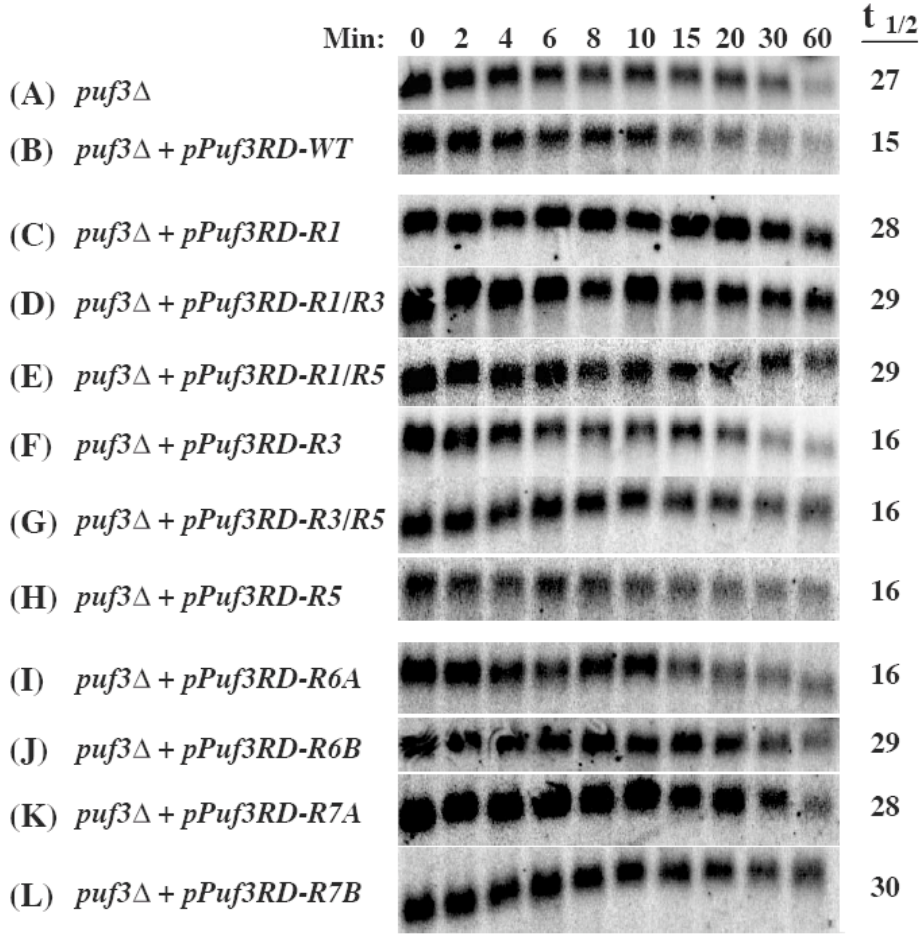

Min: 0 2 4 6 8 10 15 20 30 60
t 1/2
(A) puf3Δ
27
(B) puf3Δ + pPuf3RD-WT
15
(C) puf3Δ + pPuf3RD-R1
28
(D) puf3Δ + pPuf3RD-R1/R3
29
(E) puf3Δ + pPuf3RD-R1/R5
29
(F) puf3Δ + pPuf3RD-R3
16
(G) puf3Δ + pPuf3RD-R3/R5
16
(H) puf3Δ + pPuf3RD-R5
16
(I) puf3Δ + pPuf3RD-R6A
16
(J) puf3Δ + pPuf3RD-R6B
29
(K) puf3Δ + pPuf3RD-R7A
28
(L) puf3Δ + pPuf3RD-R7B
30

Figure 5

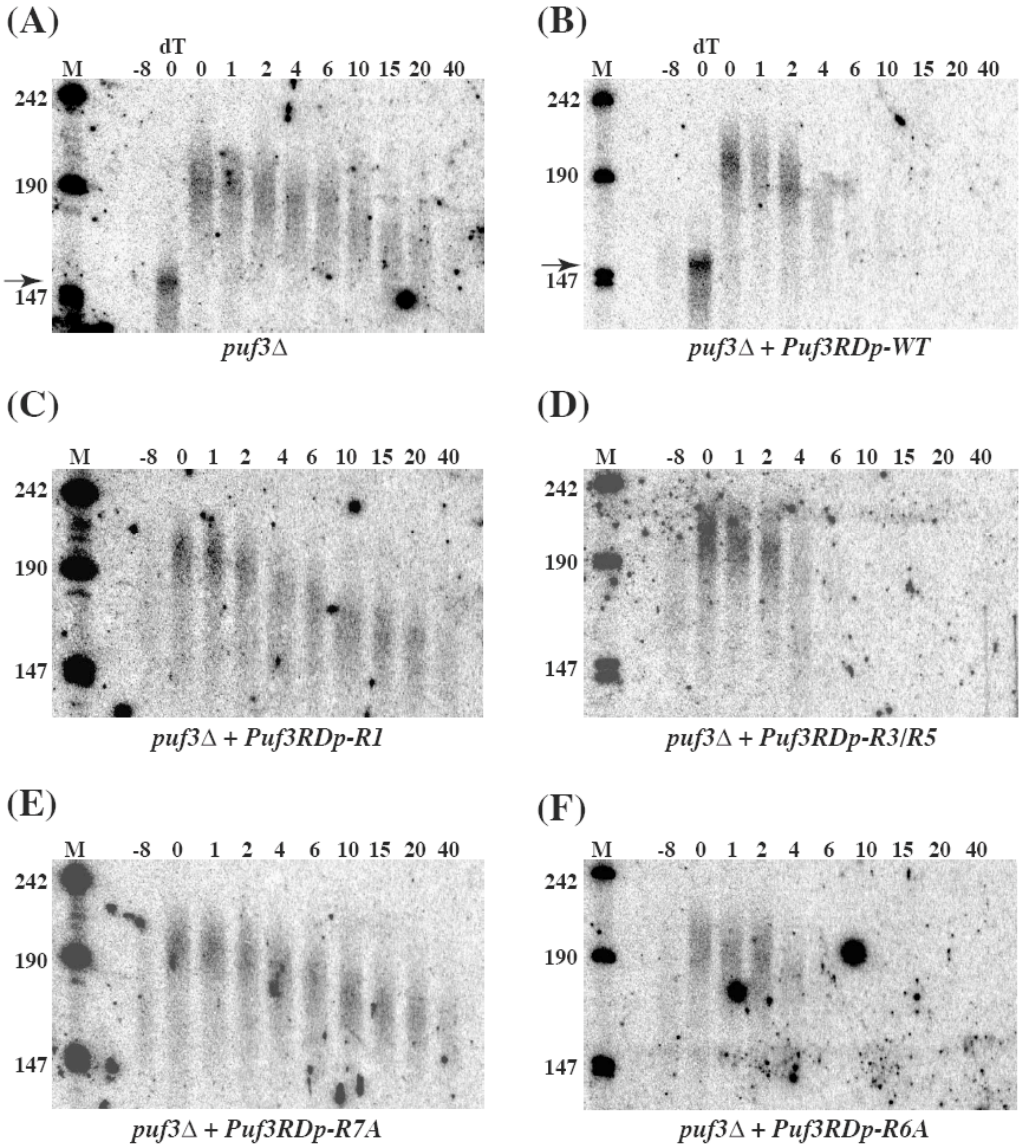

(A)
dT
M -8 0 0 1 2 4 6 10 15 20 40
242
190
147
puf3Δ
(B)
dT
M -8 0 0 1 2 4 6 10 15 20 40
242
190
147
puf3Δ + Puf3RDp-WT
(C)
M -8 0 1 2 4 6 10 15 20 40
242
190
147
puf3Δ + Puf3RDp-R1
(D)
M -8 0 1 2 4 6 10 15 20 40
242
190
147
puf3Δ + Puf3RDp-R3/R5
(E)
M -8 0 1 2 4 6 10 15 20 40
242
190
147
puf3Δ + Puf3RDp-R7A
(F)
M -8 0 1 2 4 6 10 15 20 40
242
190
147
puf3Δ + Puf3RDp-R6A

Figure 6

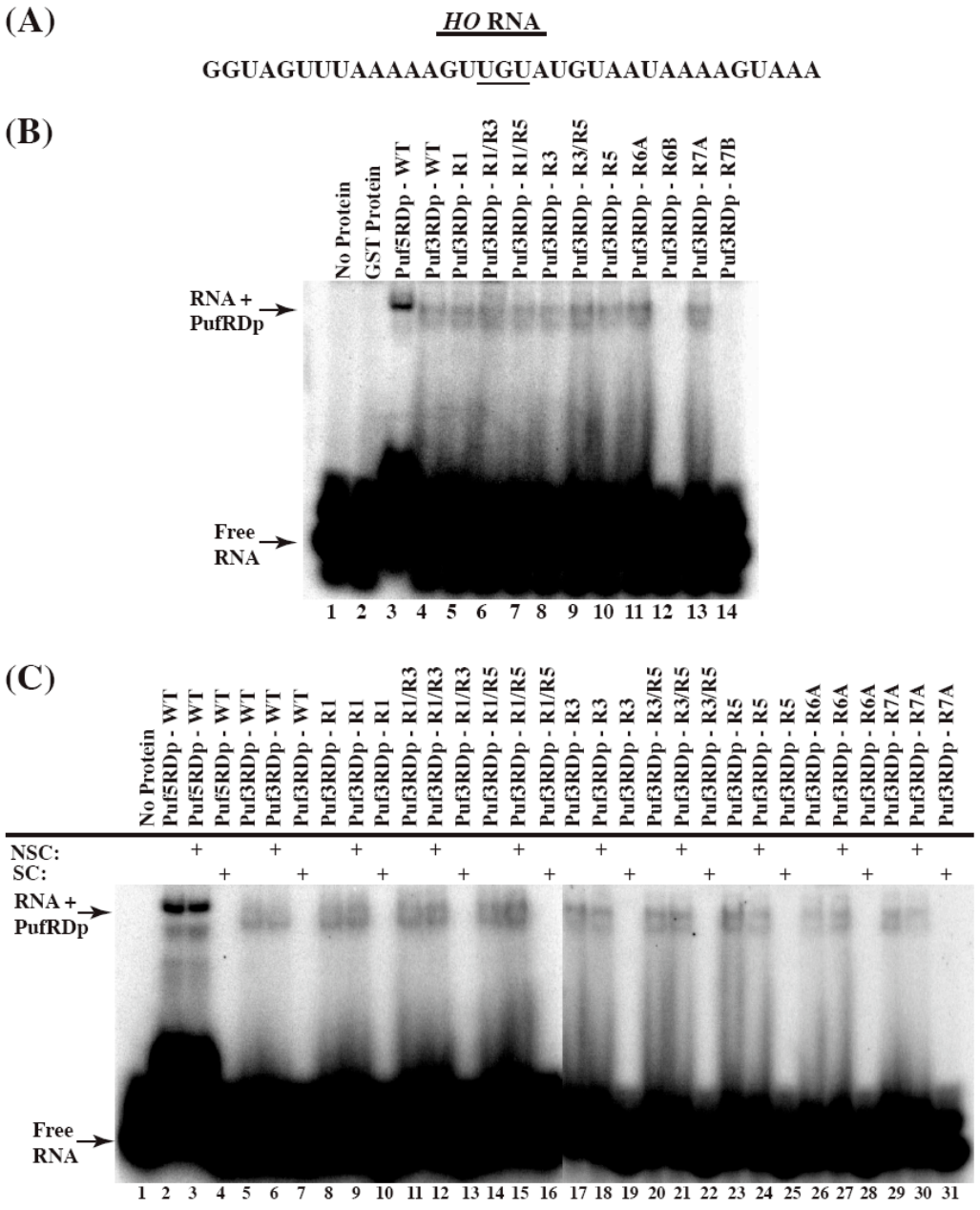
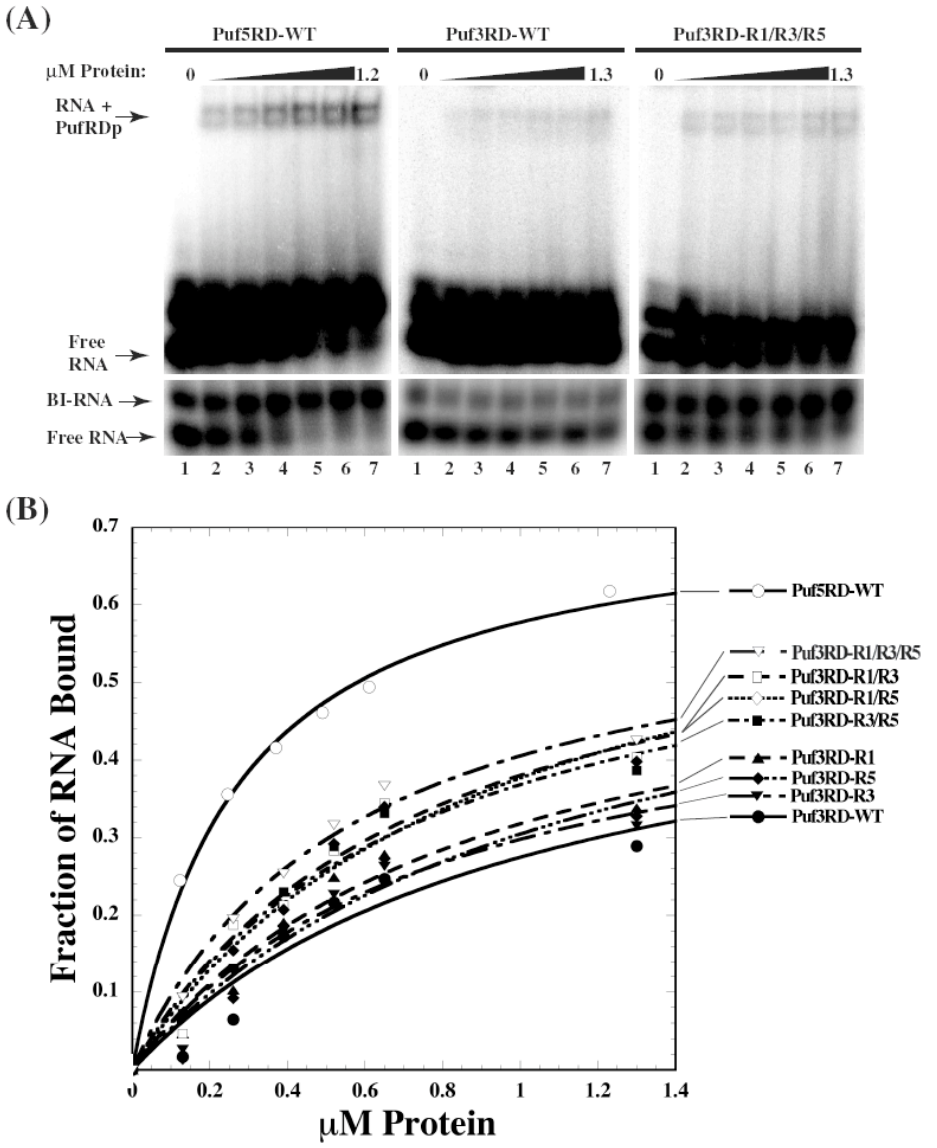

(A)
HO RNA
GGUAGUUUAAAAAGUUGUAUGUAAUAAAAGUAAA
(B)
No Protein
GST Protein
Puf5RDp - WT
Puf3RDp - WT
Puf3RDp - R1
Puf3RDp - R1/R3
Puf3RDp - R1/R5
Puf3RDp - R3
Puf3RDp - R3/R5
Puf3RDp - R5
Puf3RDp - R6A
Puf3RDp - R6B
Puf3RDp - R7A
Puf3RDp - R7B
RNA + PufRDp
Free RNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
(C)
No Protein
Puf5RDp - WT
Puf5RDp - WT
Puf5RDp - WT
Puf3RDp - WT
Puf3RDp - WT
Puf3RDp - WT
Puf3RDp - R1
Puf3RDp - R1
Puf3RDp - R1
Puf3RDp - R1/R3
Puf3RDp - R1/R3
Puf3RDp - R1/R3
Puf3RDp - R1/R5
Puf3RDp - R1/R5
Puf3RDp - R1/R5
Puf3RDp - R3
Puf3RDp - R3
Puf3RDp - R3
Puf3RDp - R3/R5
Puf3RDp - R3/R5
Puf3RDp - R3/R5
Puf3RDp - R5
Puf3RDp - R5
Puf3RDp - R5
Puf3RDp - R6A
Puf3RDp - R6A
Puf3RDp - R6A
Puf3RDp - R7A
Puf3RDp - R7A
Puf3RDp - R7A
NSC:
SC:
RNA + PufRDp
Free RNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31

Figure 7


(A)
Puf5RD-WT
Puf3RD-WT
Puf3RD-R1/R3/R5
μM Protein:
0
1.2
0
1.3
0
1.3
RNA + PufRDp
Free RNA
BI-RNA
Free RNA
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
(B)
Fraction of RNA Bound
μM Protein
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Puf5RD-WT
Puf3RD-R1/R3/R5
Puf3RD-R1/R3
Puf3RD-R1/R5
Puf3RD-R3/R5
Puf3RD-R1
Puf3RD-R5
Puf3RD-R3
Puf3RD-WT

CHAPTER V:

Puf3 Protein Interactors

Introduction

Protein-protein interactions have always played a major part in the proper function of various regulatory systems in all organisms. The mRNA decay pathway is no exception, and this is evident from the first step of eukaryotic mRNA decay, deadenylation. The removal of the poly(A) tail of an mRNA transcript requires a series of steps that involves two primary and many accessory factors that interact with one another. Ccr4 and Pop2 are the two primary exonuclease proteins that make up the deadenylase complex. There are also other accessory proteins that are included in the deadenylase complex. Ccr4 is believed to carryout the catalytic function of the deadenylase complex. Although under certain circumstances Pop2 protein can be responsible for deadenylation of mRNA, the primary role of the Pop2 protein is thought to be the enhancement of the function of the Ccr4 deadenylase through the stabilization of the deadenylase complex. Pop2 is also thought to provide interaction points for the other accessory proteins such as Dhh1 (Hata et al., 2001). This was based on the observation that in yeast strains lacking Pop2, there is a defect in deadenylation, whereas in strains that contain a catalytically inactive Pop2, deadenylation occurs normally (Thore et al., 2003; Tucker et al., 2002; Chen et al., 2002). Therefore protein-protein interactions between Ccr4, Pop2 and the other accessory proteins are essential for proper activity of the deadenylase complex in the process of deadenylation.

Protein-protein interactions have also been shown to play an important role in the decapping step of the mRNA decay pathway. The decapping enzyme is made up of two distinct proteins, Dcp1 and Dcp2. Dcp1 is believed to stimulate the activity of Dcp2.

Based on structural analyses, Dcp1 is said to be a member of the EVH1/WH1-domain proteins (Callebaut, 2002). These domains are components for interactions with proline-rich ligands used in protein-protein association by complex assembly and linkage (Ball et al., 2002). There are two such conserved domains on the molecular surface of Dcp1, and both are required for the proper function of the Dcp1-Dcp2 enzyme. It is hypothesized that Dcp1 may enhance the function of Dcp2 by increasing substrate interaction or metal binding. In addition, since the EVH1/WH1-domain proteins often connect protein partners, Dcp1 may play a role in linking Dcp2 to other proteins that may be involved in decapping (Parker and Song, 2004). This possibility is further supported by the observation that among the several secondary proteins that have been shown to play a role in the decapping process, there are proteins that make direct interactions with Dcp1, including Dhh1 and Lsm1 of the Lsm1-7 complex (Coller et al., 2001; Uetz et al., 2000; Tharun et al., 2000).

Similar protein-protein interactions play a role in the proper function of Puf proteins as well. The Dm-Pum has been shown to interact with the *Drosophila* Nanos (Sonada et al., 1999); the FBF has been shown to interact with Nanos-3 of *C. elegans* (Kraemer et al., 1999) and the *Xenopus* Puf binds to XCAT-2, a Nanos homolog in frog oocytes (Nakahata et al., 2001). Nanos proteins are characterized by two distinct CCHC zinc finger motifs. The *Xenopus* Puf and FBF have also been shown to interact with cytoplasmic polyadenylation element-binding proteins in frog oocytes and in *C. elegans* (Nakahata et al., 2001; Luitjens et al., 2000). In another case, the *Drosophila* Brat, a member of the NHL family of proteins characterized by the presence of sequences rich in glycine, hydrophobic, and basic residues, has also been shown to interact with the Dm-

Pum (Sonada et al., 2001). The association between Dm-Pum, Nanos and Brat, as well as the interaction between FBF and Nanos-3, require the presence of the mRNA target in the interacting complex (Sonada et al., 1999; Kraemer et al., 1999). Furthermore, in *Drosohila* and *C. elegans* it is the repeat domains of the Puf proteins that have been shown to be sufficient for interaction with their respective protein partners (Sonada et al., 1999; Kraemer et al., 1999). Moreover, the interaction between Dm-Pum and Nanos has been mapped to a specific region between repeats seven and eight of the Puf repeat domain (Sonada et al., 1999).

Although there are no obvious homologs of Nanos or Brat in yeast, it is logical to assume that yeast Puf proteins may also have unknown protein partners. Additional support for this assumption is provided in the previous chapter (Chapter IV) where the R7A deletion of the yeast Puf3 repeat domain, corresponding to the same region of the Dm-Pum that interacts with Nanos, inhibits the ability to regulate *COX17* mRNA degradation. Therefore, in this chapter of the research, attempts are made to identify protein partners for the yeast Puf3 protein.

Experimental Procedures

Epitope tagging - Tagging was conducted as explained previously (Chapter II, Figure-5). Several decay factors were epitope tagged by homologous recombination using the Myc-9 epitope. The PCH905-Myc9 plasmid along with myc oligo-primers for CCr4, Dhh1, Lsm1, and Pop2 decay factors, were generously donated by the Zhang Lab (Washington University School of Medicine). Dcp1 myc oligo-primers were also designed. As previously described, each primer contained a region homologous to a corresponding gene to be tagged, as well as a region on the Myc9-plasmid. These primers along with the myc-plasmid were used in the Expand High Fidelity PCR System (Roche) to PCR amplify the regions required for homologous recombination. Each amplification product was then transformed into yWO18 *puf3Δ* strain individually (yWO18: *MAT* **a**, trp1, ura3-52. leu2-3, 112, his4-539, CUP1::LEU (PM) PUF3::NEO). Genomic DNA from transformed candidates was then extracted using the Puregene DNA Isolation Kit (Gentra). Additional appropriate PCR primers for each gene were designed to amplify the genomic DNA surrounding each recombination site, then these PCR products were sequenced to verify proper tagging.

Verification of the expression of tagged proteins - To verify the expression of each myc-tagged decay factor, a protein boil prep extract of each positively identified tagged strain was prepared. 10 mL cultures were grown to mid-log phase. Cells were pelleted and resuspended in 100 μL sample buffer (125 mM Tris pH 6.8, 1% SDS, 2% Glycerol) with 10% β-Mercaptoethanol. Acid-washed glass beads were added to the resuspended cells and samples were boiled and vortexed in three and one minute

intervals, respectively. The resulting supernatants and pellets were loaded onto a 7% bottom-3% top stack SDS-PAGE gel and then blotted onto a Trans-Blot nitrocellulose membrane (BioRad). The blots were then probed to verify the expression of each epitope tagged protein. The blot was probed with the 9E10 Anti-c-myc antibody (Covance) at a dilution ratio of 1:1000 as the primary antibody, and the Anti-mouse IgG bound to horseradish peroxidase (Sigma) at a dilution ratio of 1:10000 as the secondary antibody. The Super Signal West Dura Substrate (Pierce) was used to image the blot.

FLAG-PUF3RD plasmid construction – To design a Puf3RDp expression plasmid with the correct auxotrophic markers, the FLAG-PUF3RD region from pWO14 (TRP) was removed using the *Cla*I restriction enzyme. pWO16 (Pav72-URA) was also digested with the *Cla*I enzyme. The appropriate fragments were then gel purified (Qiagen). The FLAG-PUF3RD fragment was then ligated into pWO16 creating pWO17 (FLAG-Puf3RD). Proper insertion was confirmed by sequencing.

Co-immunoprecipitation – pWO17 was transformed into each *puf3Δ* strain previously verified to contain each tagged decay factor. The expression of the FLAG-Puf3RDp was verified using western analysis and immunoprecipitation with anti-FLAG antibody (Sigma). Co-immunoprecipitations were then performed essentially as described (Coller et al., 2001) and depicted (Chapter II, Figure-6). 200 mL cultures of each transformed strain was grown to mid-log phase. Cells were pelleted and resuspended in IP buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 2 mM $MgCL_2$, 1 mM β-Mercaptoethanol, 1X protease inhibitor, 0.1% Igepal CA-360). Acid-washed glass beads (Sigma) were added to each sample and samples were boiled and vortexed to prepare extracts. Each extract was then centrifuged and the supernatant collected. The

supernatants were incubated with Anti-FLAG agarose (Sigma). Where indicated, samples were treated with 0.5 μg/μL RNAse A (Sigma) prior to incubation with anti-FLAG agarose. Samples were then pelleted and washed with IP wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCL_2$, 0.1% Igepal CA-360). Bound complexes were eluted by boiling samples in SDS-loading buffer. Bound complexes were also eluted by the FLAG-elution buffer (Sigma) to reduce background levels. For western analysis, all samples were electrophoresed SDS-PAGE gels, blotted, and probed with anti-myc antibody as previously described.

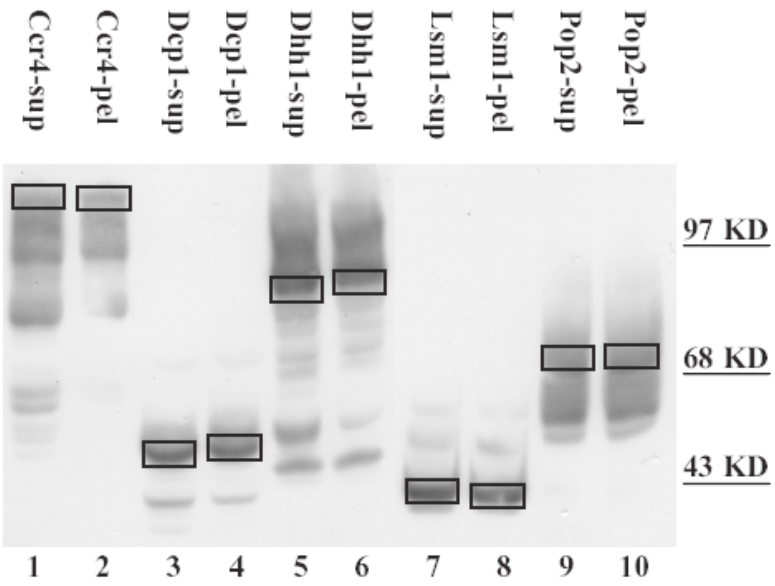
Results

The myc-tagged decay factors and the FLAG-tagged Puf3RDp are expressed properly – Before the co-immunoprecipitations were conducted, the expression of the myc-tagged Ccr4, Dcp1, Dhh1, Lsm1, and Pop2 proteins were verified by western analysis as described in experimental procedures. Proteins were found in both the supernatants and the pellets in what appeared to be equal levels (Figure-1A, compare lanes 1& 2, 3&4, 5&6, 7&8, and 8&9).

Expression of the FLAG-tagged Puf3RDp as well as the proper pull-down of the FLAG-Puf3RDp by the anti-FLAG agarose was also verified. After transformation of the tagged strains with FLAG-PUF3RD plasmid, protein boil prep extracts were made from each strain and samples were incubated with the anti-FLAG agarose as described in the experimental procedures. Bound proteins were eluted and electrophoresed on an SDS-PAGE gel. Western blot analysis with anti-FLAG antibody confirmed the expression of the FLAG-tagged Puf3RDp in each strain (Figure-1B). This also confirmed that FLAG-Puf3RDp could be captured from the extract on the anti-FLAG agarose. In addition, previous *in vivo* experiments verified that FLAG-Puf3RD is functional, since it rescued a *puf3Δ* strain (data not shown).

Puf3RDp interacts with mRNA decay factors – Once the expression of each epitope-tagged decay factor was established and the FLAG-Puf3RDp could be captured on the anti-FLAG agarose, co-immunoprecipitation pull-downs were performed using anti-FLAG agarose as described in the experimental procedures. The bound proteins

(A)



(B)

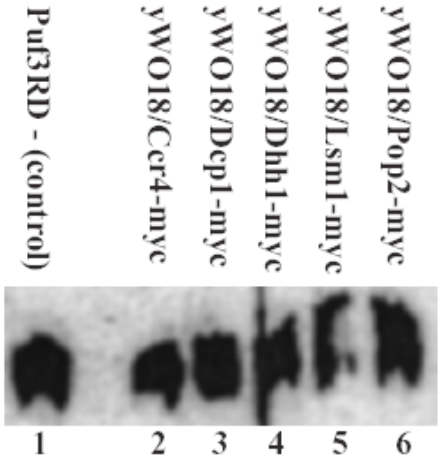


Figure – 1: (A) Western blot analysis of extracts from strains with tagged decay factors using anti-myc antibodies. Boxes indicate the expected positions of the tagged proteins. (B) Western blot analysis of tagged strains transformed with the FLAG-PUF3RD plasmid. Extracts were incubated with Anti-FLAG agarose, then bound proteins were eluted, electrophoresed and blotted. FLAG-Puf3RDp was detected using anti-FLAG antibodies.

were eluted and samples were electrophoresed an SDS-PAGE gel. Proteins were blotted on nitrocellulose membrane and probed with anti-myc antibody as previously described.

As shown in Figure 2, all decay factors appear to co-immunoprecipitate with the FLAG-Puf3RDp (Figure-2A). It is possible that these interactions could be direct or indirect, RNA-dependent, interactions. Therefore, the co-immunoprecipitation assays were repeated such that prior to incubation of extracts with the anti-FLAG agarose, a portion of each sample was treated with RNase A to disrupt RNA-dependent interactions. The results show that only three decay factors appear to have direct interactions with Puf3RDp (Figure 2B). The interaction of Ccr4 with Puf3RDp, as observed previously, was eliminated with RNase treatment (compare lanes 1 and 2), indicating its dependency on the presence of RNA. The interaction of Dhh1 with the Puf3RDp appears to be weak and also RNA dependent (lanes 5 and 6). In contrast, the interaction of the Dcp1 protein (compare lanes 3 and 4), the Lsm1 protein (compare lanes 7 and 8), and the Pop2 protein (compare lanes 9 and 10) with the Puf3RDp are RNA-independent interactions, which establishes a direct interaction between Puf3RDp and Dcp1, Lsm1, and Pop2 proteins.

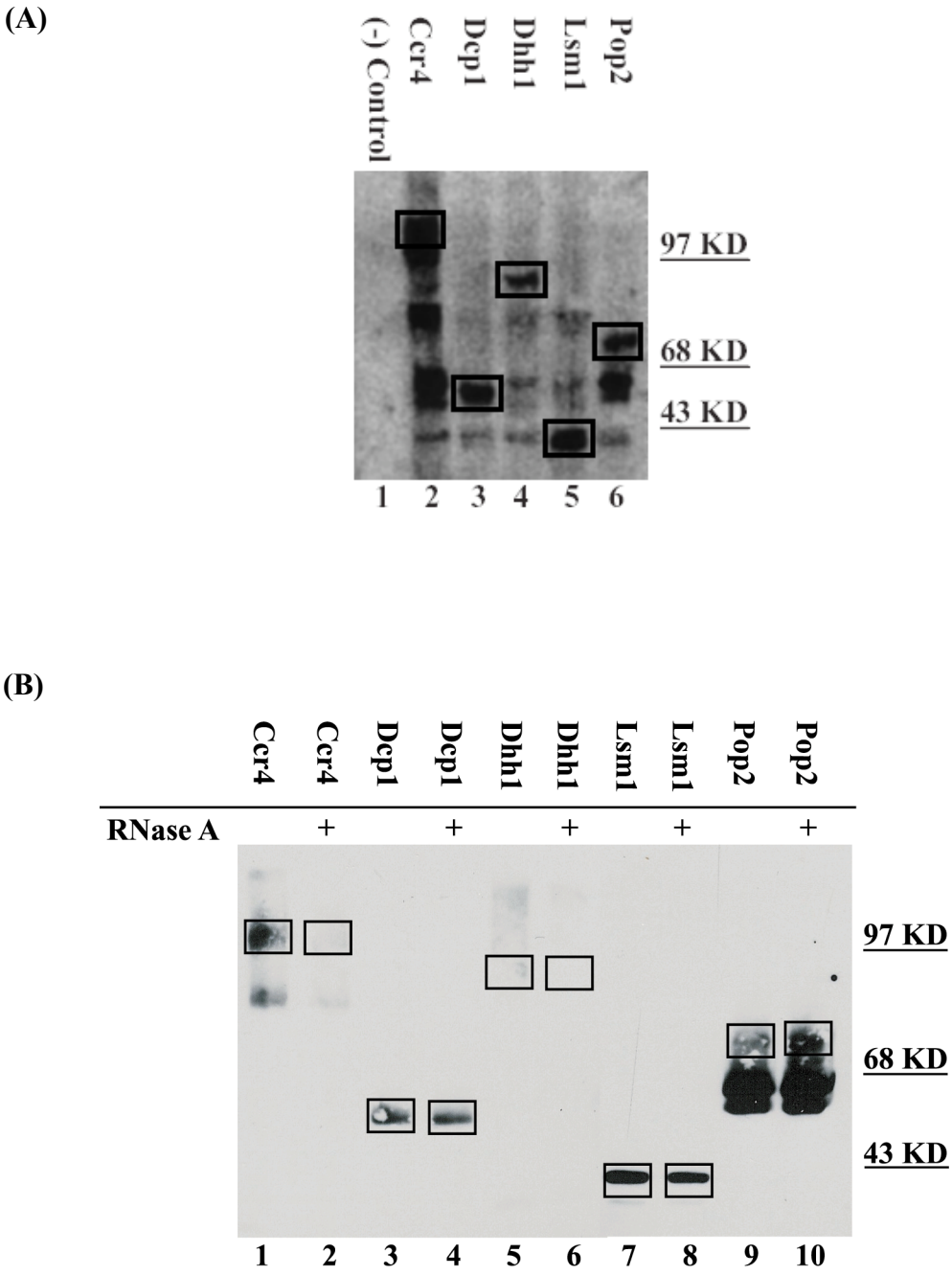


Figure – 2: (A) Western blot analysis of anti-FLAG resin pull-downs with the FLAG-Puf3RDp. The blot was probed with anti-myc antibody. A pull-down sample from a *puf3Δ* strain was used as the negative control. (B) Western blot analysis of anti-FLAG agarose pull-downs with the FLAG-Puf3RDp. The blot was probed with anti-myc antibody. A portion of each sample was treated with RNase A as indicated (+). Boxes indicate the approximate positions of the tagged proteins.

Discussion

Although protein partners for the *Drosophila* Dm-Pum and the *C. elegans* FBF have been previously identified, until this study no such partners for the yeast Puf3 protein had been uncovered. The Puf3 protein has been shown to regulate *COX17* mRNA decay through promotion of deadenylation and decapping (Olivas and Parker, 2000). Moreover, as shown in Chapter III, the Puf3RD protein is sufficient for binding and regulation of the *COX17* mRNA. Therefore, in this chapter several decay factors specifically involved in deadenylation and decapping were tested for their ability to interact with the Puf3RDp. Co-immunoprecipitation assays were performed to determine the existence of previously unknown interactions between these decay factors and the Puf3RD protein. The decay factors involved in deadenylation were Ccr4 and Pop2, which are parts of the deadenylase complex (Tucker et al., 2002; Chen et al., 2002; Tucker et al., 2001). Tagged decay factors involved in decapping were Dcp1, an essential part of the decapping enzyme (Dunckley and Parker, 1999; Beelman et al., 1996), and Lsm1, which has been shown to be involved in decapping and also associate with the Dcp1 protein (Tharun et al., 2000). Another accessory protein, Dhh1, was also tested. This protein has been shown to associate with the Pop2 of the deadenylase complex (Hata et al., 2001), the Dcp1 of the decapping enzyme (Coller et al., 2001; Uetz et al., 2000), as well as the Xrn1 exonuclease (Fischer and Weis, 2002).

Once the expression of these myc-tagged proteins in a *puf3Δ* strain was verified (Figure-1A), a plasmid encoding a FLAG-tagged Puf3RDp was transformed into each strain. The expression of the FLAG-Puf3RDp was also verified (Figure-1B). The co-immunoprecipitation pull-downs with anti-FLAG agarose and subsequent western blot

analysis with anti-myc antibody established interaction with all tagged decay factors (Figure-2A). Upon treatment with RNase A to confirm RNA-independent interaction between decay factors and the Puf3RDp, only interactions with Dcp1, Lsm1, and Pop2 were determined to be direct protein-protein interactions (Figure-2B). The mode of interaction between these proteins, or whether these decay factors interact as a complex, is yet to be determined. In addition, these interactions may require other unknown factors for recruitment as well as stabilization of the interacting complexes. Further analysis is needed to look into such possibilities.

It is interesting that these interactions occur with only the repeat domain of Puf3p, as previously observed in the *Drosophila* Dm-Pum. In the previous chapter, studies had shown that the R7A outer loop region of the Puf3RDp was essential in the regulation of *COX17* mRNA decay by the Puf3 protein. This region was also implicated as the region of interaction of Dm-Pum with its protein partners. It would be logical to hypothesize that this region might be involved in the protein-protein interactions between Puf3p and its partners. Therefore, future studies will examine whether the PufRDp-R7A mutant protein can still interact with the Dcp1, Lsm1, and Pop2 decay factors.

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CHAPTER VI:

Condition Specific Activity of Puf3 Protein

Introduction

The computational studies on the condition-specific regulation of mRNA stability in yeast were initiated by Dr. Harmon Bussemaker and Barret Foat at Columbia University, New York. The Bussemaker Lab had designed a modified algorithm (Bussemaker et al., 2001) for regulatory element detection using correlation with expression (REDUCE). To identify regulatory 3'UTR elements, this algorithm was applied to 3'UTR regions of every ORF in yeast and also to a library of microarray data corresponding to yeast genes expressed under approximately seven hundred different experimental conditions. Genes that were coordinately regulated under the same conditions that had similar 3'UTR elements were then ranked and corroborated with protein activity profiles created from previously determined Puf binding data (Gerber et al., 2004). These results enabled the prediction of the physiological states in which mRNA stability regulators (de) stabilize their targets.

For example, computational results indicated a strong correlation between the Puf3-3'UTR element and the destabilization of mitochondrion mRNA transcripts in the presence of a fermentable carbon source, and the stabilization of these transcripts in the presence of a non-fermentable carbon source. This suggested that Puf3p activity is regulated by carbon source availability. Additionally, strong correlation was also observed with this Puf3 element and the stabilization of transcripts after rapamycin treatment, leading to the likelihood of the involvement of the target of rapamycin (TOR) pathway in the regulation of Puf3 protein. The TOR pathway is an important and conserved signaling cascade that is essential for cellular proliferation, for it senses

external nutrient availability and mediates changes necessary for proper gene expression under stressful conditions (Culter et al., 1996). TOR proteins are the essential part of this regulation. Yeast *Saccharomyces cerevisiae* has two TOR proteins which rapamycin has been shown to bind and repress (Heitman et al., 1991). The TOR proteins themselves are evolutionary conserved signaling kinases that are essential for viability, cell cycle progression and gene expression (Heitman et al., 1991; Barbet et al., 1996; Cardenas et al., 1999).

In this chapter, the above computational results were experimentally verified. Specifically the decay of *COX17* mRNA, which is the known target of Puf3p regulation, was monitored under fermentable and non-fermentable carbon source conditions, as well as normal and rapamycin-treated conditions. The results of these studies confirmed that the activity of the Puf3p is indeed dependent on both carbon source and rapamycin, and supports the use of REDUCE algorithm to identify mRNA stability factors and the conditions in which those factors regulate stability. This work contributed to the following manuscript submitted for publication:

> Foat, B. C., Houshmandi, S. S., Olivas W. M., Bussemaker, H. J. (2005) Profiling condition-specific regulation of mRNA stability in yeast.

Experimental Procedures

Yeast Strains - The genotypes of the three *S. cerevisiae* strains used in the experimental studies are as follows: yWO7 (Olivas and Parker, 2000 yRP693) *MATα*, *leu2-3,112, ura3-52, rpb1-1*; yWO43 (Olivas and Parker, 2000-yRP1360) *MATa, his4-539*, *leu2-3,112, trp1-1*, *ura3-52*, *cup1*::*LEU2/PM*, *rpb1-1*, *puf3*::*Neo*^{r}; yWO50 (Olivas and Parker, 2000-yRP1546) *MAT***a**, *his3-1,15, his4-539, leu2-3,112, trp1-1, ura3, rpb1-1, cox17::TRP1*.

In vivo mRNA decay analysis - Steady-state transcriptional shut-off experiments were performed essentially as described (Caponigro et al., 1993) on strains yWO7, yWO43, and yWO50 that contain the temperature-sensitive *rpb1-1* allele for RNA polymerase II (Herrick et al., 1990). For carbon source analysis, yWO7 or yWO43 was transformed with plasmids expressing *MFA2* RNA (pWO68) or the hybrid *MFA2/COX17* 3'-UTR RNA (pWO69) under the control of the constitutive *GPD* promoter. pWO68 and pWO69 were created by inserting *Sac*I-*Hind*III fragments from either pRP485 containing *MFA2* (Decker and Parker, 1993), or from pWO25 containing *MFA2/COX17* 3'-UTR (Jackson et al., 2004), respectively, into pWO67. A 662 bp PCR product containing the *GPD* promoter was inserted between the *EcoR*I sites on pRP22 (Decker and Parker, 1993) to create pWO67. Transformed strains were grown in YP media supplemented with 2% glucose or 2% ethanol as a carbon source. A transcriptional shut-off was performed

by expressing the *MFA2* or *MFA2/COX17* mRNAs to steady-state levels under the control of the constitutive *GPD* promoter, then transcription was rapidly repressed by a shift to high temperature. Northern blots were probed for the plasmid-derived *MFA2* mRNA using oRP140 (Caponigro and Parker, 1995) or the *MFA2/COX17* hybrid mRNA using oWO303 (5'GTCAGTAAGATCGATCTAGAGGATCTCTTGGTTGTCG). For rapamycin treatment analysis, strain yWO50, which is deleted for the endogenous *COX17* gene, was transformed with plasmids expressing *MFA2* RNA (pRP485) or the hybrid *MFA2*/*COX17* 3'-UTR RNA (pWO25) under the control of the *GAL1* UAS. Transformed strains were grown in selective media with 2% galactose. Rapamycin (Sigma), when used, was added to a final concentration of 0.2 μg/mL when the culture reached an OD_{600} of 0.3, then the cells were incubated a further 60 minutes prior to the temperature shift. Northern blots were probed for *MFA2* mRNA using oRP140 or *MFA2/COX17* hybrid mRNA using oWO2 (Olivas and Parker, 2000-oCOX17-P). All Northern blots were quantified with a Molecular Dynamics PhosphorImager, and the signal for each RNA normalized for loading to the stable scRI RNA, an RNA polymerase III transcript (Felici et al., 1989).

Results

Destabilizing activity of Puf3RD is dependent on the available carbon source – First the ability of Puf3p to destabilize mitochondrion-related transcripts was tested under fermentable versus non-fermentable carbon source conditions. Previous studies had shown that *COX17* is a target of Puf3p regulation, with Puf3p binding directly to the *COX17* 3'-UTR and promoting rapid deadenylation and decay of this transcript (Olivas and Parker, 2000). Also, the *COX17* 3'-UTR was previously shown to be sufficient to direct Puf3p decay regulation when attached to the ORF of *MFA2* (Jackson et al., 2004). Thus, transformed strains expressing either wild-type *MFA2* mRNA or the *MFA2/COX17* 3'-UTR hybrid mRNA were grown in media containing either glucose (fermentable) or ethanol (non-fermentable) as the carbon source. A transcriptional shut-off was performed, and as expected, wild-type *MFA2* mRNA decays with a half-life of 4 minutes in both growth conditions (Figure-1). An AU-rich element in the 3'UTR of *MFA2* mRNA mediates rapid decay of this transcript (Duttagupta et al., 2003), which not regulated by Puf proteins. In contrast, the Puf3p-regulated *MFA2/COX17* mRNA decays rapidly with a half-life of 2.5 minutes in the glucose media, but is stabilized ~4-fold in the ethanol media to a half-life of 10.5 minutes (Figure-1). To be certain that it was indeed Puf3p that was mediating the altered stability between the two media conditions, the above experiment was repeated in a *puf3* deletion strain (*puf3Δ*). As expected, in the absence of Puf3p, the *MFA2/COX17* mRNA is stable, and the half-life is unaltered by media conditions (Figure-1). These results indicate that the destabilizing activity of Puf3p is dependent on the presence of a fermentable carbon source.

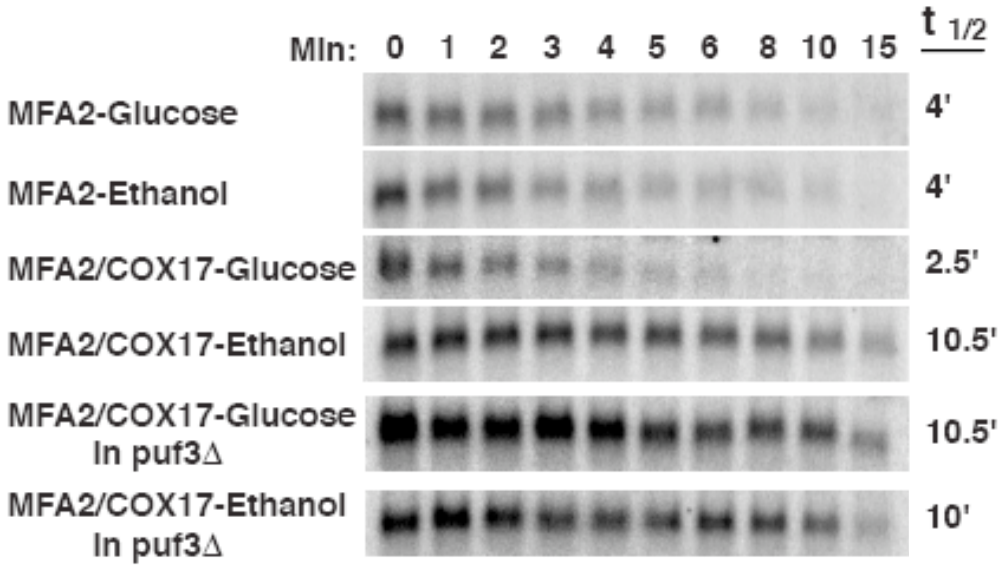

Figure -1: Regulation of Puf3p by carbon source. Shown are Northern blot analyses of the decay of *MFA2* mRNA or the hybrid *MFA2/COX17* mRNA expressed from wild-type of *puf3Δ* yeast grown in media containing 2% glucose or 2% ethanol. Minutes following transcriptional repression are indicated above the set of blots, with the half-lives ($t_{1/2}$) as determined from multiple experiments.

Rapamycin reduces the ability of Puf3p to destabilize target mRNAs – The effect of rapamycin on Puf3p activity was also experimentally detemined. As shown in Figure-2 and in previous work (Albig and Decker, 2001), *MFA2* mRNA decays rapidly with a half-life of 3.5 minutes with or without rapamycin treatment. It is important to note that these experiments were done in a different strain background than the carbon source experiments, which likely accounts for sloght differences in half-lives between these two sets of experiments. In contrast, rapamycin treatment stabilizes the *MFA2/COX17* mRNA by 2-fold, with the half-life increased from 2 minutes in the non-treated strain to 4 minutes in the rapamycin-treated strain (Figure-2). These results provide evidence that rapamycin treatment reduces the ability of Puf3p to destabilize target mRNAs, and support the prediction that Puf3p is downstream of the TOR regulatory pathway.

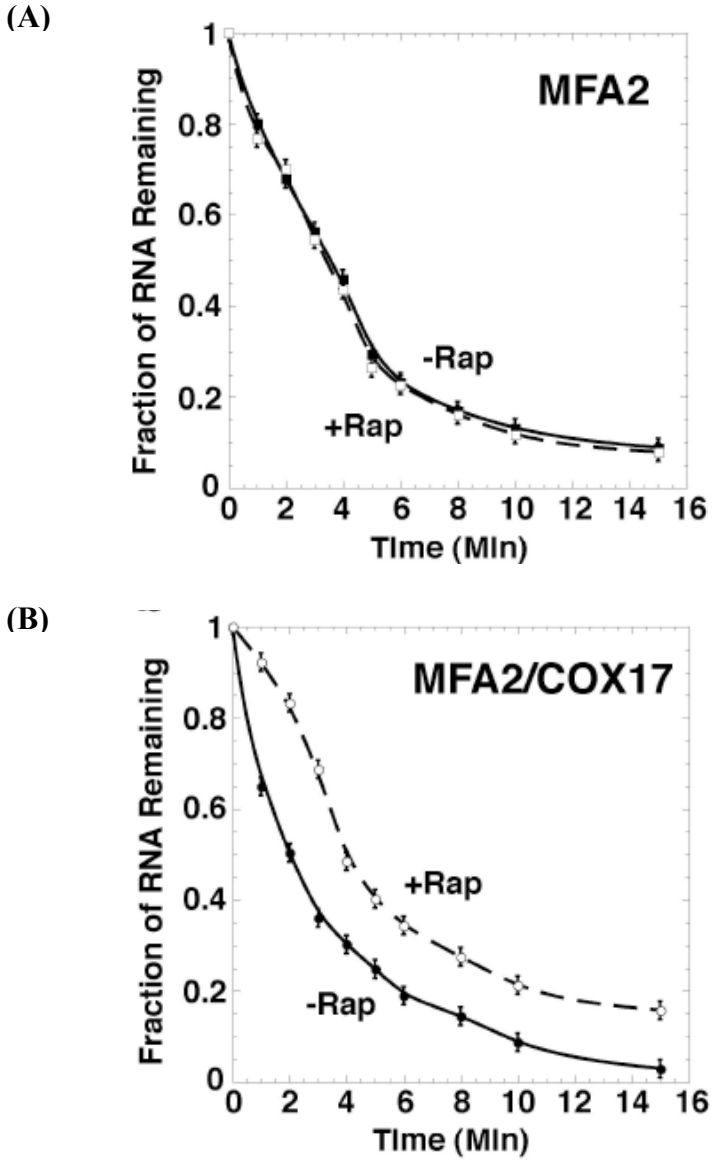

Figure - 2: Inhibition of Puf3p by rapamycin. Data from Northern blot analyses of *MFA2* (A) or the hybrid *MFA2/COX17* (B) mRNA decay are plotted, with minutes following transcriptional repression on the x-axis and the fraction of RNA remaining as compared to the steady-state RNA level at time 0 on the y-axis. Decay was monitored with or without rapamycin treatment for 60 minutes prior to transcriptional repression as follows: (A) *MFA2* without rapamycin (closed square), *MFA2* with rapamycin (open square), (B) *MFA2/COX17* without rapamycin (closed circle), and *MFA2/COX17* with rapamycin (open circle). Data points are averages of multiple

Discussion

The Bussemaker Lab's REDUCE algorithm discovered position-specific RNA-binding factors and inferred their condition-specific activities. The binding sites for several mRNA stability regulators, including Puf3p, were identified and computationally characterized. Computational analysis suggested that regulation of mRNA stability by these factors, including Puf3p, is dynamic and responds to a variety of environmental stimuli. Here, this work has provided experimental evidence that Puf3p promotes condition-specific changes in gene expression via the control of mRNA stability. Ethanol and rapamycin both cause stressful growth condition for yeast cells. Based on this work, the activity of Puf3p is negatively regulated under stressful conditions. This observation is consistent with the function of Puf3p, which is the regulation of *COX17* mRNA. It is predicted from previous microarray experiments that Puf3p also regulates many other transcripts that encode mitochondrial proteins (Gerber et al., 2004). Cox17 protein is a cytochrome oxidase necessary for copper transport that is essential for energy production in the mitochondria. Under stressful conditions, the need for available energy increases; therefore Cox17 and other mitochondrial proteins must be readily available. Hence it is logical that the activity of Puf3p would decrease to allow a more stable *COX17* mRNA, and thus more Cox17 protein production. Whereas in non-stressful conditions, Puf3p must be active to down-regulate these transcripts when not needed to decrease energy waste. The results from this work validate the use of computational methods to determine activity profiles across different conditions for RNA binding proteins, including Pufs, which will contribute to the understanding of these proteins.

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CHAPTER VII

Summary and Future Directions

While the role of the Puf3 protein in regulation of *COX17* mRNA decay had been previously determined (Olivas and Parker, 2000), the precise mode of the interaction of this protein with its target and the mechanism by which the protein promotes decay were unknown. In this research, a variety of studies have contributed to the further understanding of the specific interactions made by the Puf3 protein, and the roles these interactions play in binding and regulation of *COX17* mRNA.

So far, the results from this work have demonstrated that the repeat domain of the Puf3 protein alone is sufficient and specific for binding *COX17* mRNA, and also rescues rapid *COX17* mRNA decay in a *puf3Δ* strain, indicating that sequences that are required for both binding and regulation of *COX17* mRNA reside within the repeat domain of the Puf3 protein.

The above observations led to additional studies focusing on the analyses of the Puf3 repeat domain. A Puf3RDp and Puf5RDp comparative alignment indicated differences in only three out of the twenty-four previously predicted binding residues, though these proteins regulate different mRNAs. Additionally, seven out of the eight mRNA bases that these proteins were predicted to bind are identical, with the only difference at the base where the 3rd Puf repeat (R3) was predicted to interact with the RNA. The three different amino acids of the Puf3RDp were therefore mutagenized to mimic those of the Puf5RDp to determine whether these positions were sufficient to alter binding specificity. This work demonstrated that a single mutation in the 1st Puf repeat (R1) of the Puf3RDp inhibits detectable binding of the protein. Mutations in other repeats, including the 3rd repeat (R3), had no effect on binding. Further affinity studies

compared the interactions of Puf3p and Puf5p with the Puf5 target, the *HO* mRNA. The results show that single, double, and triple mutations enhance the binding affinity of Puf3RDp to *HO* mRNA, but they do not rescue the affinity to the same level seen with Puf5p. This suggests that these two Puf proteins, although very similar, may have different modes of interaction with the RNA. Importantly, since even the triple mutant fails to rescue full binding affinity to *HO* RNA, though it mimics all 24 amino acid binding positions, there have to be other amino acids involved in binding. One possibility is that such amino acids could be involved in intraprotein interactions that may be important in the proper positioning of the interacting amino acids in the correct orientation for interaction. It had been previously predicted that target specificity could be defined in a modular fashion, where each nucleotide of the target was specified by three distinct amino acids of a Puf repeat. However, this work demonstrates that the "modular mode" of interaction for Puf proteins may be an oversimplification of the mechanism by which Pufs attain specificity to their targets.

Although RNA/protein interactions are complex in nature, the possibility of determining and altering the specificity of interactions is very fascinating. If the specificity of an interaction could be defined, then proteins could be modified to bind to specific mRNAs. For example, this work has shown that three mutations of Puf3RD do increase the affinity of this protein for the *HO* mRNA. Ultimately, it might be possible to modify the Puf3RD to bind various other targets with considerable affinity. Future work could focus on identifying other amino acids of the repeat domain that are required for target binding specificity.

The outer regions of the Puf repeat domains had been predicted to interact with other proteins. Based on the crystal structures of Dm-Pum and Hs-Pum (Edwards et al., 2001; Wang et al., 2001) there are several outer loops between the repeats that were believed to be involved in protein-protein interactions in Puf proteins. In fact, such a region in Dm-Pum, between repeats seven and eight, was shown to be the interacting point with its protein partners (Edwards et al., 2001). In this work, two outer loops of the Puf3RDp were deleted to examine their role in mRNA regulation. Surprisingly, deletion of part of a loop region between repeats seven and eight led to inhibition of detectable binding, indicating a possible role for this region in the establishment of a proper structural conformation necessary for binding. Interestingly, another portion of this loop is not required for RNA binding, but is necessary for the regulatory function of Puf3RDp in the decay of *COX17* mRNA. Based on these results, this region is hypothesized to be a candidate site for interactions between Puf3RD and its partners.

The outer loop regions of the Puf proteins have been discovered to be quite important in the recruitment of other proteins necessary for proper regulation of mRNA decay, although the mechanism of those interactions is yet unknown. It has been shown that modification of an outer loop allows one Puf to interact with a protein partner of another Puf. The outer loop between repeat seven and eight of Dm-Pum has been determined to interact with Nanos (Edwards et al., 2001). This outer loop in Hs-Pum differs from that of Dm-Pum by an extra three amino acids. Interestingly, insertion of these amino acids into Dm-Pum has been shown to inhibit the interaction of Dm-Pum and Nanos (Sonoda and Wharton, 1999). Conversely, deletion of these amino acids in Hs-Pum enables it to interact with Nanos (Sonoda and Wharton, 1999). Therefore,

although wild-type Puf3RD does not appear to regulate the *HO* mRNA, similar experiments could be performed on the outer surface of Puf3RDp, swapping Puf5RD amino acid loops in the place of Puf3RD loops. Such modifications of the Puf3RD might then enable this protein to also regulate the *HO* mRNA. By understanding the role of these loops and how they stimulate decay, creation of modified Puf proteins might be possible for the function of down-regulating specific mRNAs that code for proteins whose overproduction could lead to severe abnormalities.

It is also noteworthy that the regions outside the Puf repeat domain have no known function, even though they usually comprise well over half of the total protein. While these regions may enhance the activity of the repeat domain (Wharton et al. 1998), the conservation of such large N-terminal regions among Puf proteins suggests that these regions are important for other unknown activities. Systematic screens to identify protein-protein interactions have identified eight candidate Puf3p interactors (Ito et al. 2001; Gavin et al. 2002; Ho et al. 2002). However, none of these candidates have known functions in mRNA metabolism, none bind directly to the Puf3RD in yeast two-hybrid experiments, and deletions of several of the candidates have no effect on *COX17* mRNA decay (Wendy Olivas, personal communication). Therefore, if these candidate proteins do function with Puf3p, they may be acting through the N-terminal domain, and may be involved in processes other than mRNA metabolism. Further studies are needed to unveil the roles the Puf N-terminal domains might play.

In this work, identification of Puf3RD protein interactors was also studied. As there are no homologs of the *Drosophila* Nanos or Brat proteins in yeast, co-immunoprecipitation analyses were conducted to identify possible protein partners for the

yeast Puf3RD protein. It was hypothesized that Puf3RD might stimulate decay by interacting with RNA decay factors. For this reason, several known decay factors, Ccr4, Dcp1, Dhh1, Lsm1 and Pop2, were epitope-tagged endogenously. These factors have been shown to be involved in the decapping and deadenylation processes of mRNA decay. Co-immunoprecipitations with FLAG-tagged Puf3RDp indicated interactions with the Dcp1, Lsm1, and Pop2 decay factors. These interactions were shown to be direct interactions, as the elimination of mRNA by RNAse treatment had no effect on the associations.

The results from this interaction study will guide future work in verification of the interactions through a reverse co-immunoprecipitation with the tagged decay factors. It would be fascinating to further analyze the outer loop region that was shown to be essential for mRNA decay regulation by Puf3 in an effort to determine if this region plays a role in the interaction of decay factors with the Puf3 protein. Another exciting inquiry would be to use the current Puf3RD construct for additional co-immunoprecipitations to look for other novel proteins that might interact with this protein. Such analysis could be done through trypsin digest mass spectrometric analysis of co-immunoprecipitants.

If the above interactions are further verified, they will shed light onto the mechanism of Puf3-mediated regulation of *COX17* mRNA decay. Based on the results so far, there are several possible ways for this regulation to occur. It is possible that Puf3RD recruits the interacting decay factors Dcp1, Lsm1, and Pop2 to the RNA (Figure-1A). Although the results indicate that the aforementioned are RNA-independent interactions, it is yet unknown whether these proteins interact as a complex or if other proteins are required for their interaction. Previous studies have shown that Lsm1

associates with Dcp1. Therefore, it would be logical to hypothesize that of the three interacting proteins, Lsm1 and Dcp1 could be candidates for recruitment as a complex. Another possibility of Puf3-mediated regulation could involve stabilization of the interacting factors on the RNA by Puf3. Due to the unique structure of the repeat domain and the fact that its inner concave surface interacts with the RNA while the outer concave surface interacts with other proteins, it is entirely possible that Puf proteins act as stability anchors for the interaction of other necessary proteins, as is the case with Dm-Pum and its relationship with Nanos and Brat. In this case, Puf3RD might also act as an anchor for the interacting decay factors (Figure-1B). Furthermore, these possibilities may not necessarily be mutually exclusive, for it may be possible for the Puf3 to not only recruit the decay factors, but also stabilize them on the RNA. In addition, the RNA-dependent interactions between Puf3RD and Ccr4 and Dhh1 cannot be ignored either. The presence of the *hunchback* mRNA has been shown to be essential for Dm-Pum to interact with Nanos and Brat. Upon binding to the *COX17* mRNA, Puf3RD can undergo a structural change enabling it to interact with other decay factors in an RNA-dependent manner (Figure-1C). Therefore, even though Puf3RD may not interact independently with these two decay factors, Puf3RD could still play an important role in their recruitment and/or stabilization when bound to the RNA target.

In the collaborative portion of this work, condition-specific studies confirmed the dependence of Puf3 protein activity on the available carbon source. Specifically, in non-fermentable growth conditions (ethanol) the decay of the *COX17* mRNA was prolonged by over four-fold, indicating a reduction of Puf3 destabilizing activity. Rapamycin was also shown to decrease the activity of Puf3 protein, as cultures treated with rapamycin

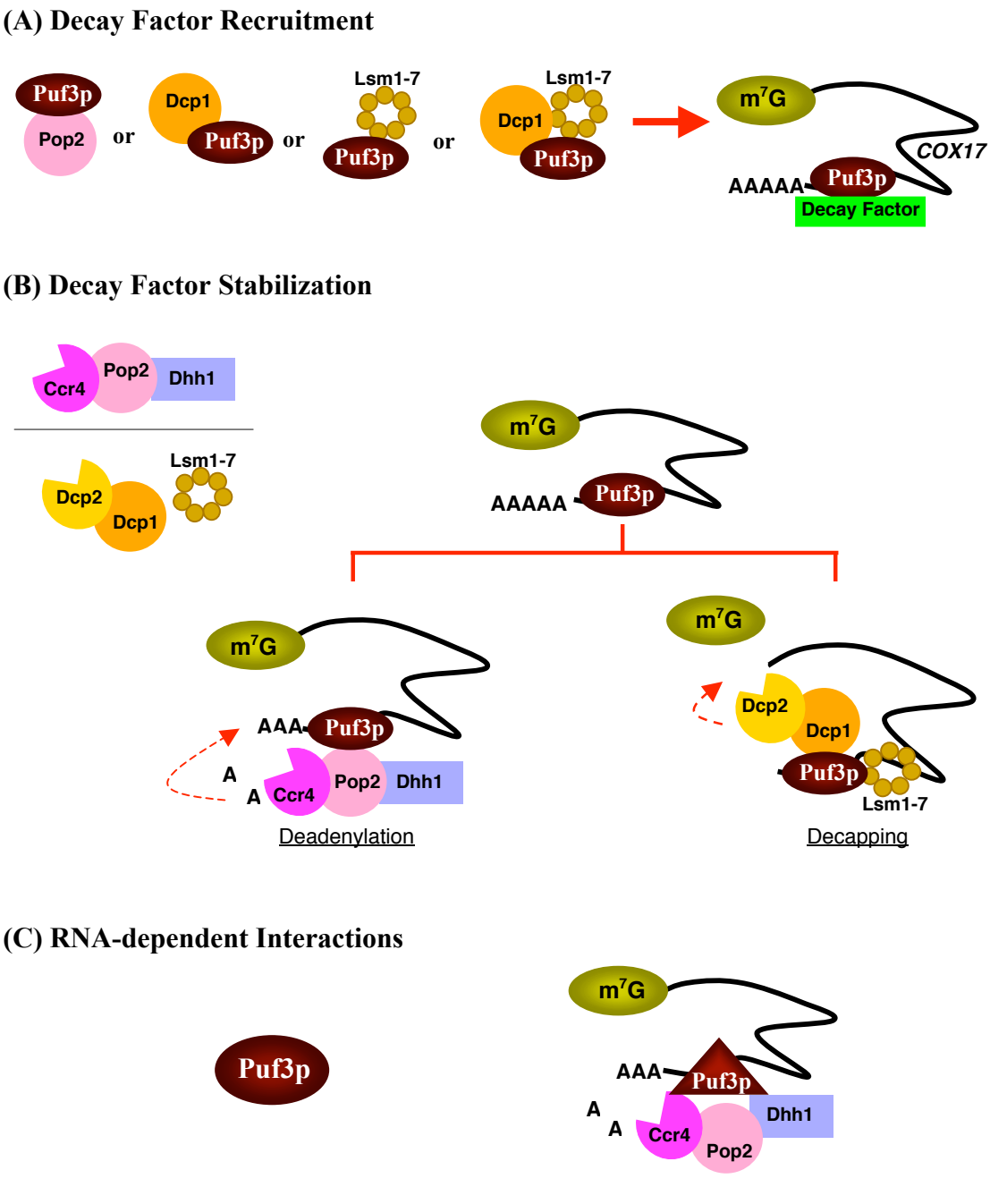

Figure – 1: Possible models for Puf3p and mRNA decay factor protein-protein interactions. (A) Decay factor recruitment: Puf3p binds the decay factor alone or within a complex and recruits it to the *COX17* mRNA. (B) Decay factor stabilization: Puf3p bound to the *COX17* mRNA anchors the interacting decay factor on the mRNA, allowing the factor to remain in close proximity to the mRNA during the course of deadenylation and/or decapping. (C) RNA-dependent interactions: Puf3p interaction with *COX17* mRNA results in a structural change, enabling it to interact with decay factors.

showed a two-fold increase in the *COX17* mRNA half-life. This suggests that Puf3p is downstream from the TOR regulatory pathway. In the same collaboration study, several other conditions were also identified that may be involved in regulating Puf3p activity. It would be appealing to experimentally investigate the effect of those particular conditions on the activity of Puf3p.

The role of the TOR pathway in the regulation of Puf3p may be intriguing in itself. The results from this work suggest that the activity of Puf3p is negatively regulated under stressful conditions. The TOR signaling pathway is required for the regulation of cell proliferation through the adaptation of growth by regulation of TOR signaling kinases under stressful conditions (Culter et al., 1999). It is likely that the TOR pathway may regulate the activity of Puf3 protein, since rapamycin inhibits the TOR pathway, and rapamycin treatment decreases Puf3p activity. The mechanism of this regulation, however, is yet unknown. One possibility is the role of the essential TOR proteins. Since TOR proteins are kinases, it is possible that the activity of Puf3p may be regulated by the TOR proteins through the process of phosphorylation (Figure-2). Therefore, it would be interesting to determine if phosphorylation by the two yeast TOR proteins plays a role in the activity of the Puf3p.

In conclusion, the results from the research conducted in this body of work provide insight into the mode of interactions between Puf proteins and their target mRNAs in yeast. The results from this work also contribute to the identification of other protein factors that may interact with Puf proteins and regulate the mRNA decay in yeast. In addition, this work has also contributed to the finding that physiological conditions play a role in the activity of Puf proteins and affect mRNA decay. Given the general

structural and functional similarities between the Puf proteins, together these results significantly increase our understanding of the role of Puf protein interactions, as well as physiological conditions, in the regulation of mRNA decay in yeast and other eukaryotes.

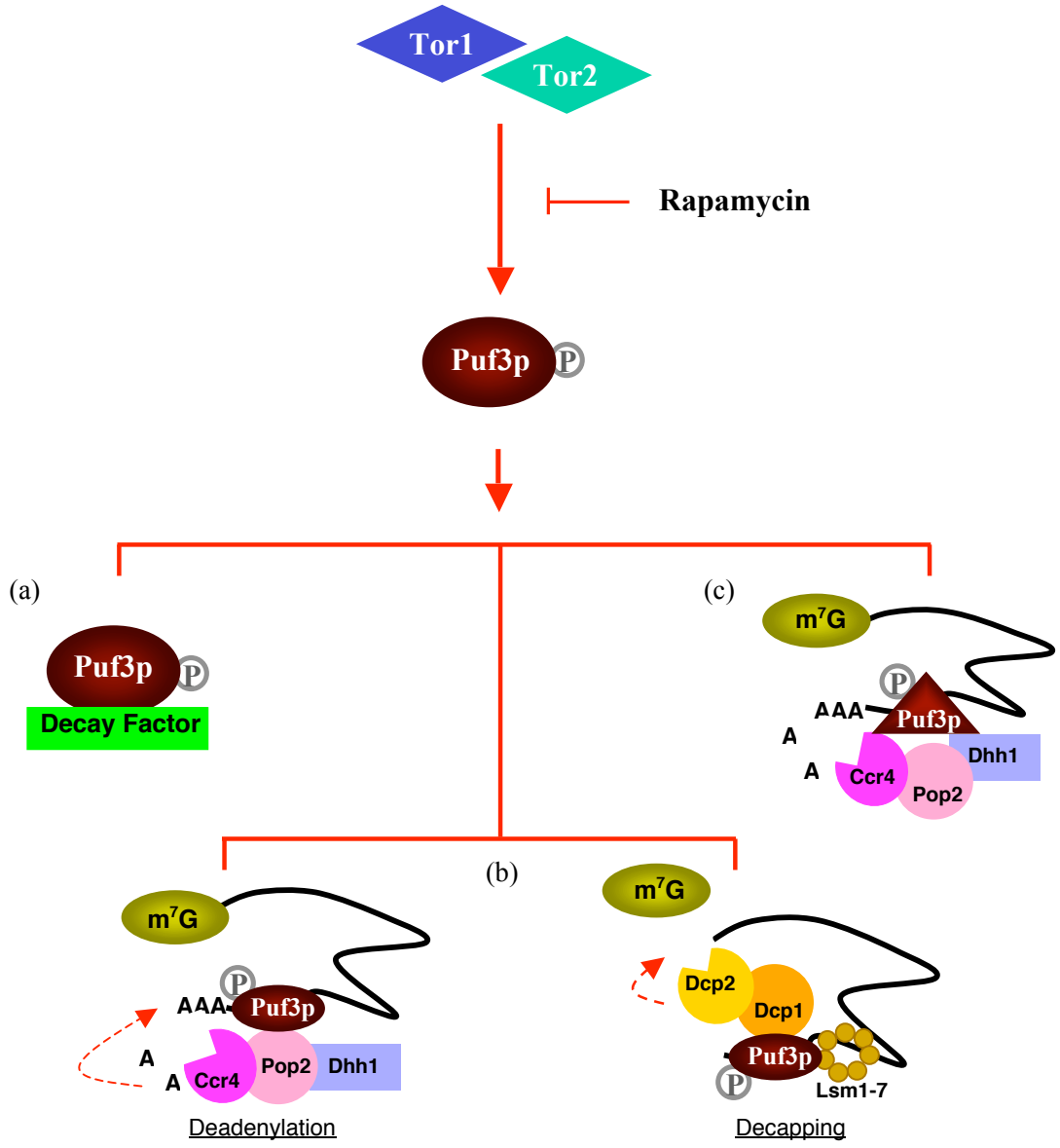

Figure – 2: Possible model for the regulation of Puf3p activity by the TOR pathway in yeast. Rapamycin deactivates both of the yeast Tor proteins. The kinase activity of the Tor proteins may be necessary for Puf3p phosphorylation, which may be required for decay factor recruitment (a), decay factor stabilization for deadenylation and/or decapping (b), as well as RNA-dependent interactions (c).

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