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Abstract

The eukaryotic Puf proteins function to regulate gene expression by altering mRNA stability. Specifically, Puf proteins bind the 3' untranslated region (UTR) of mRNA targets to stimulate their turnover. In the yeast *S. cerevisiae*, six Puf proteins have been identified, in which Puf1p-Puf5p share a well conserved RNA-binding domain. The yeast Pufs regulate mRNA stability on a transcript-specific basis, though only a few mRNA targets have been experimentally verified. Yeast Puf3p was originally found to mediate rapid turnover of *COX17* mRNA, which encodes a mitochondrial copper shuttle. More recently, microarray and computational analyses revealed that Puf3p physically associates with >100 nuclear-transcribed mRNAs that encode mitochondrial proteins. Most of these mRNAs contain one or more conserved putative Puf3p binding site(s) within their 3'UTRs, and it is predicted that their steady-state expression levels are altered by different growth conditions. In addition, Puf3p has been shown to localize to the cytoplasmic face of the mitochondrial membrane and affect its motility, suggesting that Puf3p may play an important role in regulating mitochondrial function.

In this work, I have experimentally validated several new mRNAs that are targeted for turnover by Puf3p. I have also determined that these targets are regulated by Puf3p in a condition-specific manner. Specifically, I have analyzed the decay of fifteen putative Puf3p mRNA targets in the absence and presence of Puf3p, identifying

transcripts that represent true targets of Puf3p-mediated mRNA decay. These transcripts are rapidly degraded in the presence of Puf3p and are stabilized in a *puf3Δ* strain. Transcriptional pulse-chase experiments with one of the identified targets, *CYT2,* revealed that Puf3p destabilizes this target by stimulating deadenylation of the transcript and subsequent steps of decay, and this regulation only requires the *CYT2* 3'UTR. I have also monitored the stabilities of *COX17*, *CYT2,* and *TUF1* mRNAs as reporters of Puf3p activity, and found that these RNAs are stabilized in ethanol, galactose, and raffinose conditions, suggesting that Puf3p activity is severely inhibited. Interestingly, Puf3p is rapidly activated or inactivated upon changing carbon sources to or from dextrose, respectively, as measured by *CYT2* and *TUF1* decay phenotypes. *PUF3* mRNA and protein levels are not decreased in conditions that inhibit Puf3p activity, which suggests that Puf3p activity may be regulated post-translationally. Together, my work provides a greater understanding of the role of Puf3p in mRNA decay regulation, and provides insight into the mechanism of Puf3p activity. The significant structural and functional homology between Pufs suggests that the knowledge gained from my work on Puf3p in yeast will make contributions to understanding Puf protein regulation in higher eukaryotes as well.

Acknowledgements

I would like to thank my advisor, Dr. Wendy Olivas, for her guidance and support throughout the entire process of developing my thesis work. I would also like to thank my committee members, Dr. Marc Spingola and Dr. Teresa Thiel, for their support, expertise, and constructive criticism regarding this work. I extend sincere gratitude to all members of the Olivas Lab, past and present, including Florencia, Randi, and Sean for their helpful suggestions and support.

Completion of this project would not have been possible without the continuous support from all of my friends and family. I would like to give another special thanks to my friend and labmate Florencia, a very selfless individual who has been very supportive of me and my work, and has assisted me greatly in the laboratory. Words cannot adequately express my gratitude to my mother, who has been my biggest supporter throughout this process. Thank you for the countless number of times that you have sacrificed your time to transport me to the lab! Lastly, I would like to give special thanks to all of my friends who have lent an ear to allow me to express my concerns regarding this work.

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Chapter I: Introduction

Eukaryotic mRNA Life Cycle

Post-transcriptional gene regulation is mediated in part by translation and mRNA decay events, which compete to effectively control protein expression. Nascent pre-messenger RNAs (mRNAs) are transcribed by the RNA Polymerase II (RNAP II) enzyme in the nucleus, where they simultaneously undergo several modifications such as capping, polyadenylation, and splicing. These RNA processing events are coordinated by the carboxyl-terminal domain (CTD) of RNAP II, which alters phosphorylation status to recruit capping, splicing, and cleavage and polyadenylation complexes to the nascent pre-mRNA (Figure 1; Weaver, 2005). The 5'end of the pre-mRNA is modified by the addition of a 7-methylguanosine triphosphate cap, while the 3'end of the pre-mRNA is polyadenylated by the poly(A) polymerase and poly(A)-binding protein II (Figure 2). The 5'cap enhances proper splicing of the pre-mRNA, and also offers protection from degradation and promotes translation of mature mRNAs. The poly(A) tail also functions to protect the mRNA from nucleases and enhance translation. Furthermore, it promotes efficient transport of mRNAs from the nucleus to the cytoplasm (Coller and Parker, 2004; Tucker and Parker, 2000; Shaktin and Manley, 2000; Weaver, 2005).

Upon export to the cytoplasm, the expression and fate of a mature mRNA is dependent on a highly regulated competition between translation and degradation events. For example, when a cell experiences environmental stresses, such as nutrient starvation, the cell will upregulate the expression of genes that allow the cell to adapt and survive, resulting in increased production of this key protein. When expression of this protein is no longer required by the cell, one mechanism for repressing expression is by degrading the mRNA that encodes the protein (Figure 2). In addition, eukaryotic cells have

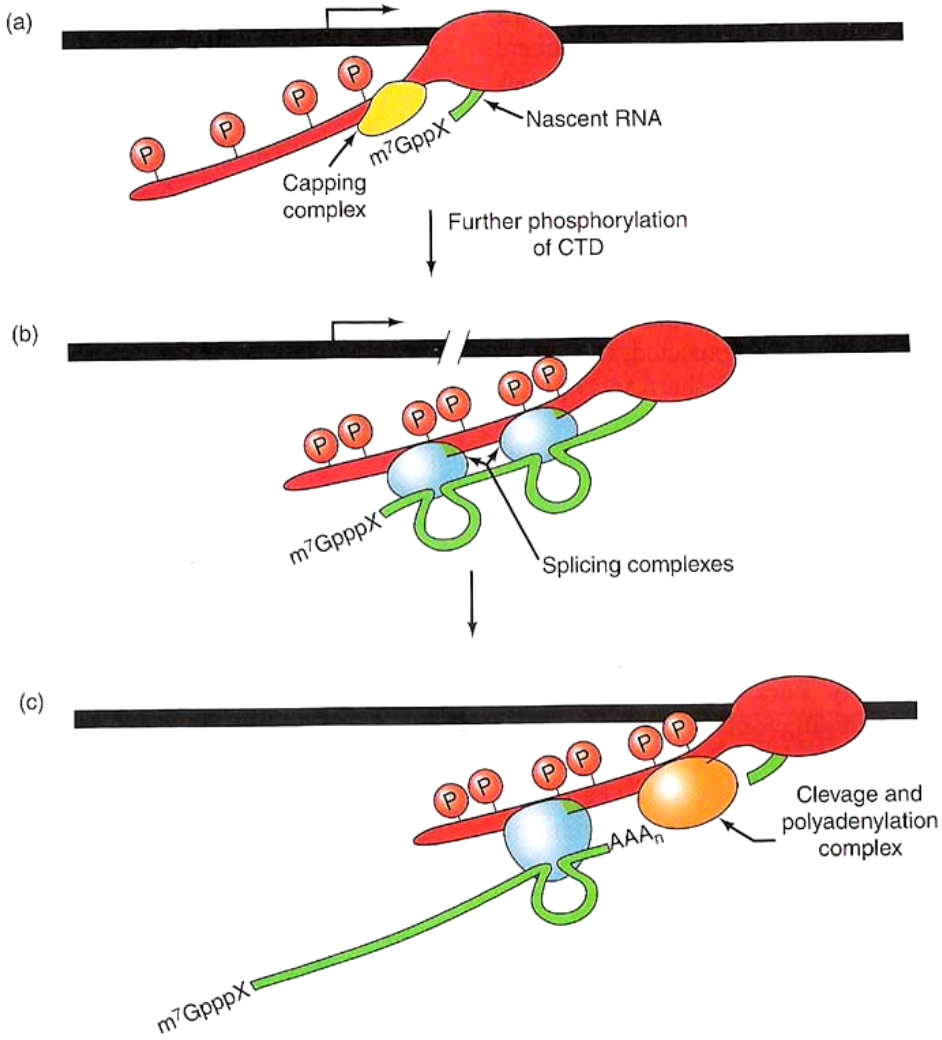


Figure 1. Pre-mRNA processing events. (a) RNA polymerase II (red) is synthesizing a nascent RNA, while the partially phosphorylated CTD has recruited the capping complex (yellow) to cap the RNA. (b) The phosphorylation status of the CTD has changed, as it has become further phosphorylated to recruit the splicing complex (blue), which recognizes and splices out introns from the RNA as they are encountered. (c) The CTD interacts with the cleavage and polyadenylation complex (orange), which synthesizes the poly (A) tail. (Figure 15.39 from Weaver, 2005).

developed decay pathways solely devoted to aberrant mRNAs to prevent translation of truncated proteins (Tucker and Parker, 2000).

The stability and fate of the mRNA transcript is determined by the presence or absence of cap-binding complexes and poly (A) binding proteins. The cap binding complex, eIF-4F, consists of several eukaryotic translation initiation factors, including the cap binding protein eIF-4E, and eIF4G, as well as eIF-3, which recruits the 40S ribosomal subunit to the RNA (Schwartz and Parker, 1999). The poly(A) tail is bound by several poly(A) binding proteins (PABs), which interact with the cap binding complex to promote the circularization of the transcript, thus providing stabilization, protection from decapping enzymes and deadenlyases, and optimal conditions for translation. Specifically, PABs have been shown to interact with the eukaryotic translation initiation complex subunit eIF-4G and stabilize eIF-4E on the mRNA cap (Coller and Parker, 2004; Wilusz et al, 2001). Research also suggests that the poly(A) tails alone may be sufficient to act as inhibitors of decapping, as polyadenylated RNAs were decapped at a 4-5-fold slower rate than transcripts lacking the poly(A) tail (Wilusz et al, 2001). However, proteins involved in the degradation of the mRNA, such as decapping enzymes that remove the 5'cap, constantly vie for access to the cap. Similarly, deadenylases compete with PABs to interact with the poly(A) tails.

Decapping activity is dependent on the stability of the translation initiation complex to remain bound to the cap, and upon destabilization, decapping enzymes can gain access to the cap. Furthermore, this destabilization can disrupt interactions with PABs, causing them to dissociate from the poly(A) tail, which provides deadenylases access to the tail. Yeast strains expressing mutations in the factors that constitute the

translation initiation complex exhibit increased rates of decapping and deadenlyation (Schwartz and Parker, 1999). These deadenlyation and decapping events are an integral part of the degradation of mRNAs.

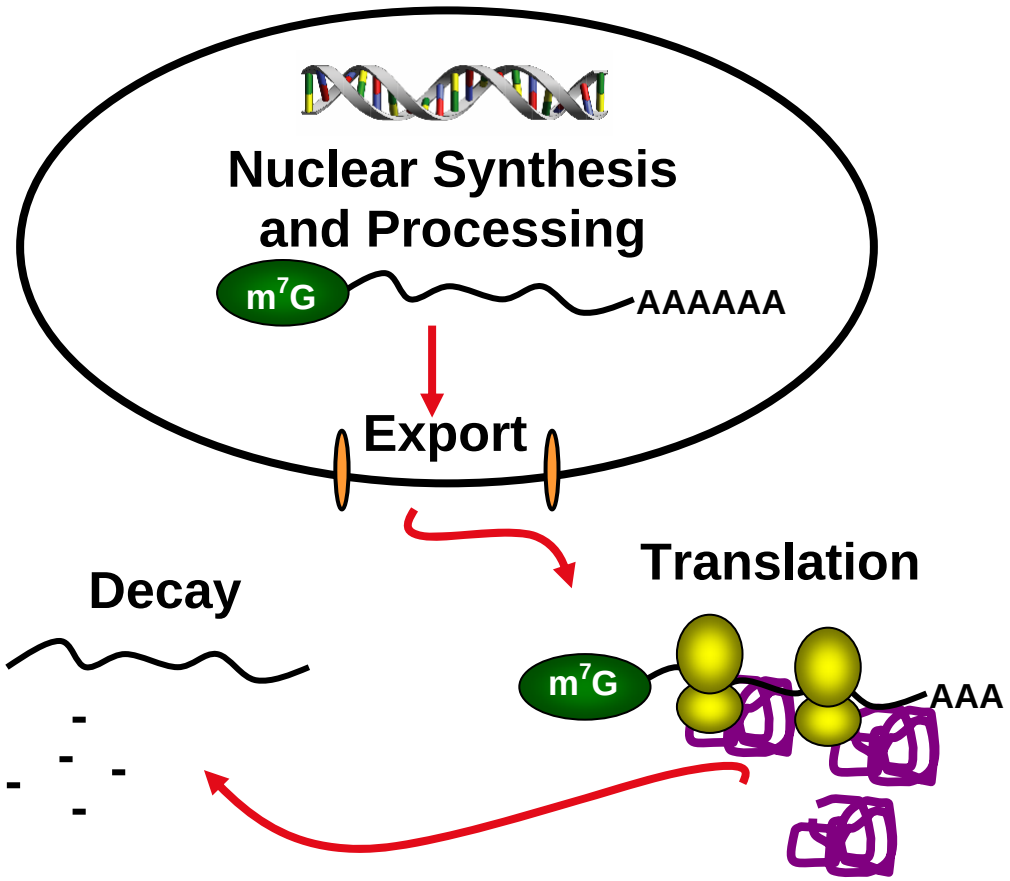


Figure 2. Eukaryotic mRNA life cycle. RNAs are synthesized in the nucleus, where they are capped (green) and polyadenlyated. The mature mRNA is exported to the cytoplasm where it may be translated on ribosomes (yellow) to produce proteins (purple). When the protein is no longer needed by the cell, the mRNA may be degraded by exonucleases (blue) to repress protein expression.

Eukaryotic mRNA Decay in *S. cerevisiae*

While the presence of the cap and tail structures of mRNAs is linked to the upregulation of protein expression, their removal is necessary for subsequent turnover. The major mRNA decay pathway in yeast is described as the deadenylation-dependent decapping pathway. Deadenylation of the poly(A) tail is performed by the Ccr4p-Pop2p-Not deadenylase complex, in which Ccr4p has the predominate catalytic activity in the complex. This protein complex also contains various accessory proteins such as Not1p-Not5p, Caf4p, Caf16p, Caf40p, and Caf130p, whose activities are not well characterized (Parker and Song, 2004; Tucker et al, 2001).

Pop2p is required for the deadenylation of short-lived transcripts *in vivo*, such as yeast *HO* mRNA, but its deadenylase activity is not required. Ccr4p acts as the major deadenylase that is required for the deadenlyation of *HO* mRNA. A current model suggests that Pop2p and the Ccr4-Not complex is recruited to the mRNA, possibly by a trans-acting factor that binds the 3' untranslated region (UTR) of the transcript (Figure 3). Pop2p directly interacts with this factor and simultaneously interacts with the Ccr4-Not complex. Furthermore, Pop2p acts as a bridge to bring Ccr4p in close proximity with the poly(A)tail, this stimulating deadenylation of the transcript (Goldstrohm et al, 2006). This model proposes the means by which the deadenylase complex is recruited to a short-lived mRNA. This process may vary for many other mRNAs, such as highly stable transcripts.

The process of deadenylation occurs in two phases, in which the first phase is characterized by a slow rate of deadenylation. The second phase, referred to as poly(A) shortening, occurs more rapidly, and the poly(A) tail is shortened to an oligo(A) length

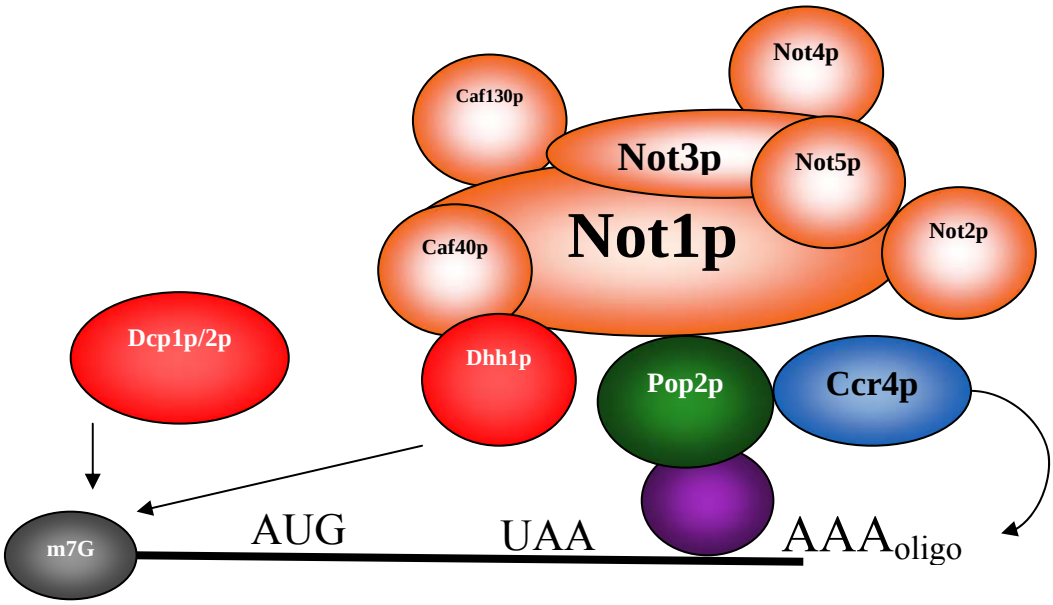


Figure 3. Model for recruitment of the deadenylase complex to a short-lived mRNA. A trans-acting regulatory protein (purple) binds to the 3'UTR of the mRNA,binds Pop2p (green), and recruits Ccr4p (blue) and other accessory proteins (light orange) that constitute the Pop2p-Ccr4-Not complex. These protein interactions bring Ccr4p, the catalytic subunit of the deadenlyase complex, in close proximity to the poly(A) tail to achieve poly(A) shortening. The Pop2p-Ccr4p-Not complex may also associate with the decapping enzyme Dcp1p-2p (red) and associated factors required for decapping (red), which associate with the cap structure.

that averages between 7 to 16 nucleotides in length. This phase appears to be a regulated step of mRNA decay, as populations of different RNAs are deadenylated at varying rates and degrees of uniformity. For example, the yeast *PGK1* mRNA is uniformly deadenylated, while other populations of RNAs exhibit heterogeneous poly(A) tail lengths during this phase (Decker and Parker, 1993).

After deadenlyation, a decapping holoenzyme composed of Dcp1p and Dcp2p catalyzes the removal of the 5' cap (Figure 4). Decapping events are particularly important in controlling the rate of decay and are rate-limiting, similarly as with deadenlyation, which is supported by the observation that mRNAs are decapped at different rates. The Dcp2p subunit exhibits catalytic decapping activity and cleaves within the m^7GTP cap structure, thus releasing a m^7GDP cleavage product (Coller and Parker, 2004; Parker and Song, 2004). Dcp2p contains a MutT or Nudix motif that is present in a class of phosphatases (Dunckley and Parker, 1999). The specificity of Dcp2p interactions with its substrate is dependent on the presence of a 7-methyl group on the cap and the RNA substrate; these interactions are key for efficient decapping activity. Dcp1p, in addition to Dcp2p, is required for decapping. Dcp1p co-immunoprecipitates and physically interacts with Dcp2p, although Dcp1p lacks decapping activity in vitro. However, the catalytic activity of Dcp2p requires the presence of Dcp1p, which likely allows Dcp1p, Dcp2p and the mRNA to form a ternary complex. Therefore, Dcp1p and Dcp2p act together to remove the cap structure (Coller and Parker, 2004; Parker and Song, 2004). Following the decapping event, the mRNA is rapidly degraded 5' to 3' by the Xrn1p exonuclease. This exonuclease activity occurs rapidly and is not a control point in the rate of mRNA turnover.

In short, the deadenylation-dependent decay pathway in yeast begins with deadenylation and poly(A) shortening of the transcript by the Pop2p-Ccr4p-Not complex. Next, the decapping holoenzyme Dcp1p-2p cleaves the m^7GTP cap. Both the deadenylation and decapping steps are regulated and control the rate of mRNA decay. Finally, the mRNA is rapidly degraded by Xrn1p, the 5' to 3' exonuclease (Figure 4; Decker and Parker, 1993; Muhlrad, Decker and Parker, 1995).

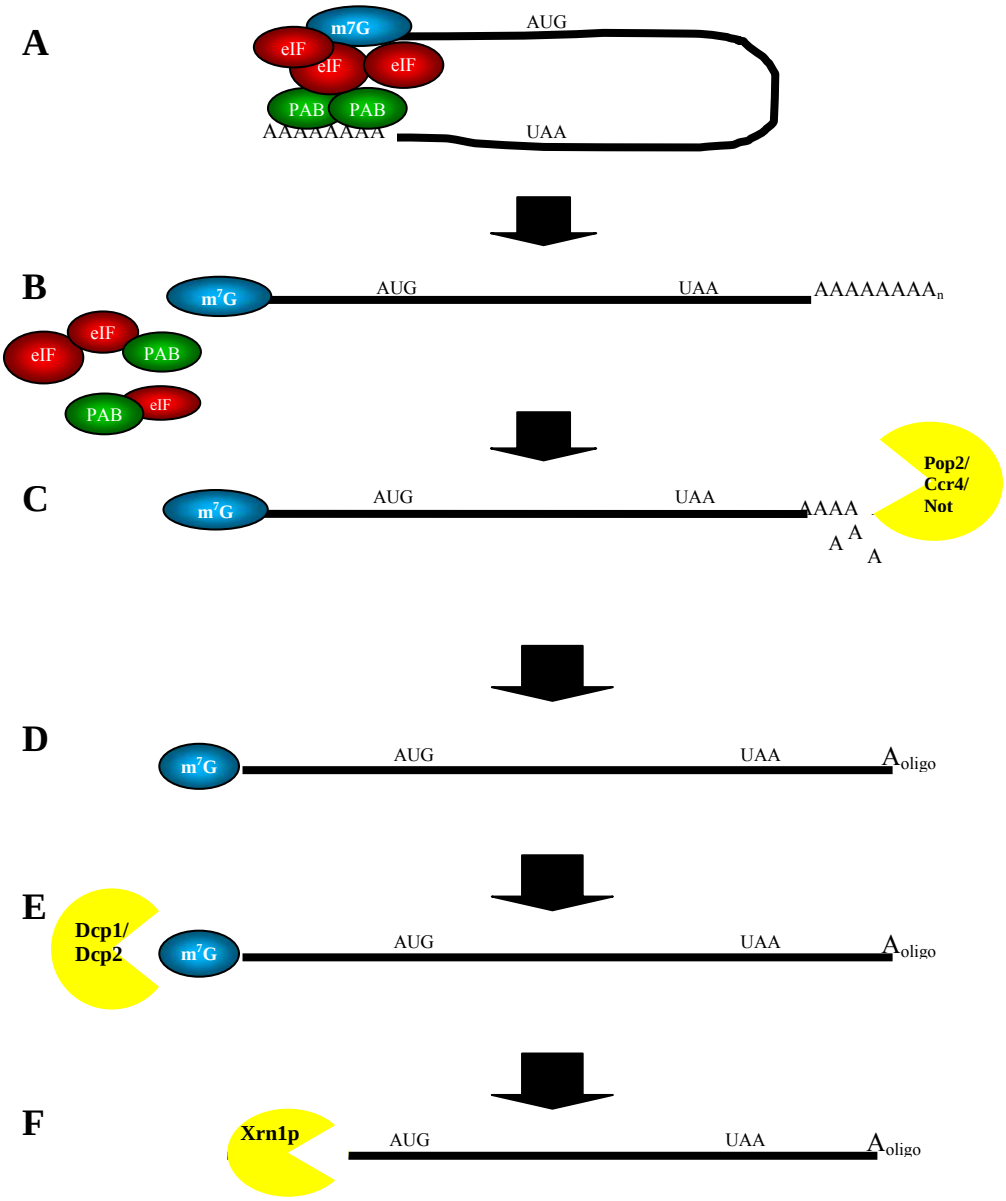


Figure 4. Major mRNA decay pathway in yeast. **A**. The cap binding complex (red) consists of eukaryotic translation initiation factors that interact with the 5' cap. Poly(A) binding proteins (green) associate with the poly(A) tail and interact with the cap binding complex, allowing the mRNA to adopt a circular confirmation that protects it from decay. **B**. Interactions between the cap binding complex and PABs are disrupted, which inhibits translation initiation and leaves the transcript susceptible to decay. **C**. The Pop2p-Ccr4-Not deadenylase complex shortens the poly(A) tail to an oligo length, about 7-16 nucleotides long, as shown in **D**. **E**. The decapping enzyme cleaves the cap structure. Steps **C** and **E** are highly regulated, as different RNAs are deadenylated and decapped at different rates. **F**. The transcript is rapidly degraded by the exonuclease Xrn1p, 5' to 3'.

The degradation of mRNA transcripts is mediated by several trans-acting factors, but is also influenced by the presence of cis-acting factors located within mRNA sequence. An example of these cis-acting factors includes instability elements which may be located in the coding or untranslated regions of transcripts. Adenylate and uridylate rich elements, referred to as AU-rich elements, were first identified in the 3'UTRs of unstable mammalian mRNAs such as c-fos. The AU-rich elements promote turnover by binding AU-rich binding proteins that cause deadenylation followed by decay of the transcript. Three classes of AU-rich elements have been identified in mammalian systems: Class I and II contain an AUUUA motif in the 3'UTR and are distinguished by the types of RNAs in each class; class III lack the AUUUA motif and contain U-rich sequences in the 3'UTR (Chen and Shyu, 1995; Xu et al, 1997; Fan et al, 1997).

In the yeast model system, two classes of AU-rich elements have been identified, represented by *MFA2* and *TIF51a* transcripts, which contain the AUUUA motif surrounded by an AU-rich region in their 3'UTRs. *TIF51a* contains an AU-rich element that promotes decay in the presence of glycerol, and is stable in the presence of glucose. Thus it represents a AU-rich element class of yeast RNAs that is regulated by carbon source. *MFA2*, however, is destabilized in all conditions tested, and does not have a carbon source regulatory mechanism. The transcript is rapidly degraded with a half-life of 4 minutes. (Vasudevan and Peltz, 2001; Duttagupta et al, 2003). Alternatively, the stable *PGK1* mRNA contains stability elements within the coding region, as exhibited by a half-life of 45 minutes. Deletion of 82% of the coding region destabilizes *PGK1*, and

the RNA is degraded at a faster rate, with a half-life of 27 minutes (Heaton et al, 1992). Therefore, cis-acting elements contribute greatly to the regulation of mRNA degradation.

RNA degradation appears to occur at special sites within the cytoplasm, as pools of non-translating RNAs that are in the process of being decapped and degraded are spatially separated from translating RNAs. These degrading transcripts form aggregates into cytoplasmic foci termed processing bodies (P-bodies) (Sheth and Parker, 2003). The concept of two distinct RNA pools within the cell (translating vs. degrading) is supported by the observation that *dcp1*Δ and *xrn1*Δ cells treated with cyclohexamide to trap RNAs on polysomes during translation exhibited a decline in the number of P-bodies. Also, translation factors are not found within the foci, suggesting that translation factors and ribosomes must dissociate from RNAs prior to their movement to P-bodies (Sheth and Parker, 2003; Brengues et al, 2005).

P-bodies contain the decapping enzymes, Dcp1p and Dcp2p, along with other factors that stimulate their activity such as Dhh1p and Lsm1p, and the Xrn1p exonuclease (Sheth and Parker, 2003). The Ccr4p deadenylase does not localize to the processing bodies, suggesting that deadenylation occurs in the cytoplasm, and the decapping and exonucleolytic digestion of the transcript occurs within the P-bodies. This is supported by the observation that deletion of *CCR4*, which blocks the RNA decay pathway at a step prior to decapping, reduces the number of P-bodies in yeast cells when compared to wild-type. Also, deletion of *DCP1* or *XRN1*, which blocks the decapping and digestion steps of decay, results in an increase in the size and number of P-bodies within the cell (Sheth and Parker, 2003). In addition to decapping and decay factors, P-bodies are RNA-

dependent for their formation, as RNaseA treatment disrupts their formation (Teixeira et al, 2005).

A current model suggests that P-bodies form as RNAs exit translation, dissociate from translation factors and ribosomes, and form mRNP structures with decapping and exonuclease factors that aggregate into larger complexes However, not all RNAs that move into P-bodies are destined for degredation. P-bodies may also serves as sites of mRNA storage during stress, as RNAs may exit the foci and return to translation (Brengues et al, 2005; Teixiera et al, 2005).

The Puf Family of Proteins

The Puf family of RNA-binding proteins represents a class of factors that affect mRNA stability and decay by interacting with transcripts that contain instability elements within the 3'UTR. This family was appropriately named Puf based on its founding members, Pumilio and FBF proteins, which were first discovered in *Drosophila* and *C. elegans*, respectively (Wickens et al, 2002). The Puf proteins are eukaryotically conserved regulatory proteins that were first identified as early development determinants in *C. elegans* and *Drosophila*. To date, Puf proteins have been characterized in many other eukaryotes such as *Dictostelium, Xenopus, S. cerevisiae*, mice and humans.

Pumilio and FBF repress translation of their target mRNAs in conjunction with protein partners to recruit the cellular decay machinery. In *Drosophila*, Pumilio regulates posterior patterning of embryos. Pumilio binds Nanos response elements (NRE) in the 3'UTR of *hunchback* mRNA to repress its translation, which is necessary for abdominal segmentation of the embryo (Sonoda and Wharton, 1999; 2001; Wreden et al. 1997). The Nanos protein is also a repressor of *hunchback* that binds nonspecifically to the RNA in the absence of Pumilio. When Pumilio binds the NRE of *hunchback*, it recruits Nanos and engages in protein-protein interactions. In addition, the Brat protein is recruited to the *hunchback*-Pumilio-Nanos ternary complex. Both Nanos and Brat are required to interact with Pumilio to stimulate its activity (Sonoda and Wharton, 1999; 2001). Pumilio also regulates anterior patterning of the embryo, regulates germline stem cell development and division by repressing *cyclin B* mRNA, and plays a role in dendrite morphogenesis in peripheral neurons (Gamberi et al. 2002; Lin and Spradling, 1997;

Forbes and Lehmann, 1998; Asaoka-Taguchi et al. 1999; Parisi and Lin, 1999; Ye et al. 2004).

In *C. elegans*, the fem-3 binding factor proteins FBF-1 and FBF-2 regulate germline sex determination, specifically the switch of spermatogenesis to oogenesis in hermaphodites. FBF along with NOS-3, a Nanos homolog, binds to the 3' UTR of fem-3 mRNA to repress translation. FBF-1 and 2 also regulate germline stem cell maintenance by repressing *gld-1* mRNA expression (Crittenden et al, 2002; Zhang et al, 1997).

In mammals, there are two Pumilio homologs that have been shown to play a role in the cellular response to environmental stimuli and development. In mammals, Pumilio2 has been implicated in the formation of dendritic stress granules in response to stress, which act as temporary sites of storage for mRNAs that are temporarily translationally repressed, which suggests that Puf proteins are important for stimulating the cell response to environmental changes (Vessey et al. 2006). In humans, Pumilio2 is expressed in embryonic stem cells and germ cells and can interact with BOL or DAZL proteins to form RNA-binding complexes that may regulate germ cell development. Specifically, human PUM2 along with DAZL physically interact with human SDAD1 mRNA. PUM2 has also been shown to bind the 3'UTR of the human testis-specific CEP3 transcript, thus supporting its role in human germ cell development (Moore et al. 2003; Fox et al. 2005; Urano et al. 2005; Spik et al. 2006).

PufA has been implicated in promoting vegetative cell division in the slime mold *Dictyostelium*. During nutrient starvation, the YakA kinase pathway is activated, resulting in upregulation of cAMP-dependent protein kinase (PKA-C) expression. PKA-C promotes cell differentiation and the formation of fruiting bodies. PufA antagonizes

this event by repressing PKA-C mRNA, thus promoting vegetative growth. PufA shares similarity with the Pumilio and FBF-1 protiens, as it is 56% identical to Pumilio in its characteristic homology domain, and is 24% identical to FBF1. However, unlike Pumilio and FBF-1, Nanos homologs or other protein partners required for PufA regulation have not been identified (Souza et al, 1999).

The structure of the Puf protein is well conserved in eukaryotes such as *C. elegans, Drosophila, S. cerevisiae*, and humans. The protein is defined by a repeat domain that is composed of eight imperfect repeats, each containing thirty-six amino acids, located near the C-terminus. Each Puf repeat contains three α helicies, termed H1-H3. The three helicies of a single repeat adopt a folded structure that aligns with adjacent repeats, thus producing the curved structure of the Puf homology domain, also called the repeat domain (RD) (Figure 5A). The H1 helicies of each repeat form a layer that covers the outer convex surface, where protein-protein interactions occur, while the H3 helicies form a layer that covers the inner concave surface, which binds a targeted RNA. Amino acid substitutions made to the concave surface of the Pumilio RD disrupt RNA binding. Also, amino acid substitutions, mutations or insertions made to the convex surface of the RD disrupt Nanos and Brat recruitment and binding (Figure 5B and 6A; Edwards et al, 2001). The PufRD is flanked by two sequences that form repeat-like structures called Csp1 and Csp2, which were first identified in *C. elegans* FBF (Zhang et al, 1997). Similarly, the human Puf protein contains two imperfect repeats that flank the RD, termed repeats1' and 8' that contain only one or two helicies (Figure 5B; Wang et al, 2001).

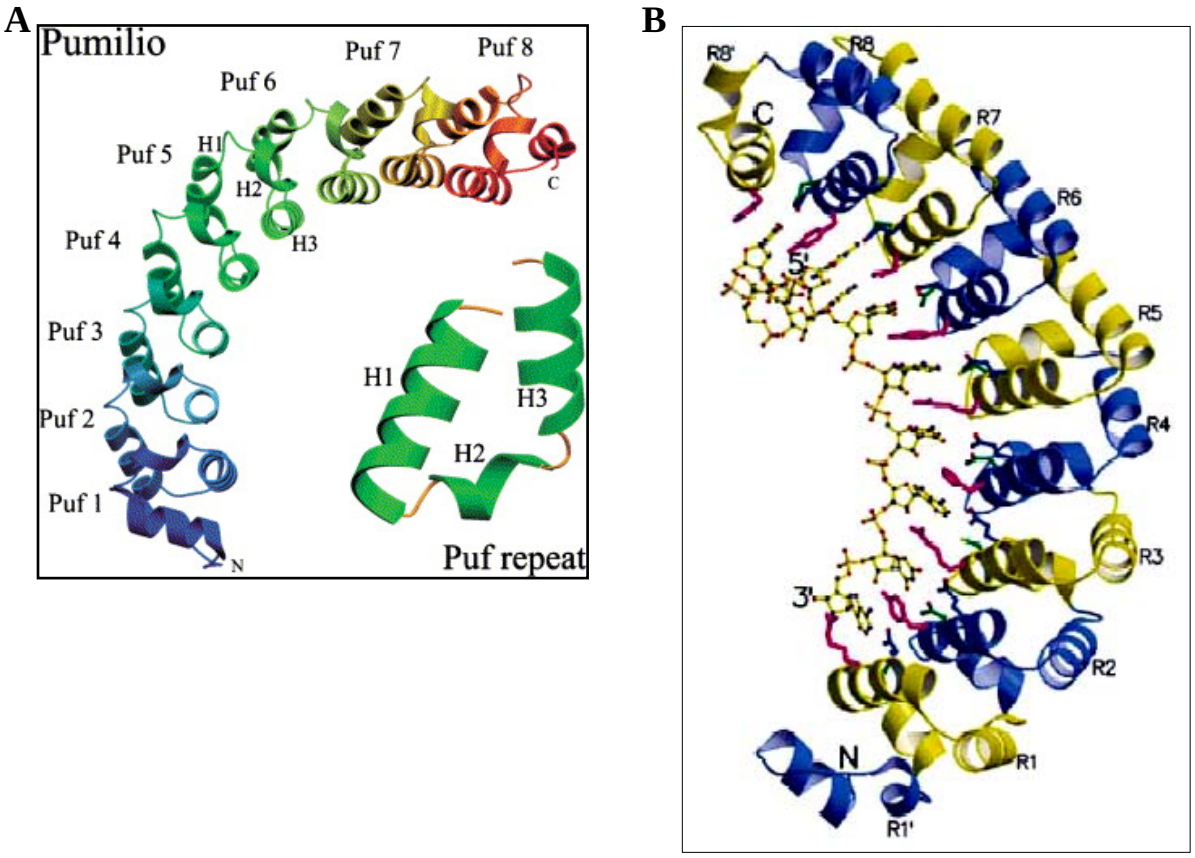


Figure 5. Structure of Pumilio RD and interactions with mRNA targets. **A**. The Pumilio RD is composed of eight repeats that are composed of three alpha-helicies, H1, H2, and H3. The helicies fold into a precise confirmation, as H1 is located on the convex surface of the repeat, where protein-protein interactions occur with Nanos and Brat, and other proteins involved in decay. The H3 helicies orient themselves to form the concave surface of Pumilio, which binds the 3'UTR of the mRNA target, as labeled in repeat 6 "Puf 6" (Figure from Edwards et al, 2001). **B**. Crystal structure of a human Puf RD bound to a RNA. The human Puf RD contains eight conserved repeats, as well as conserved flanking Csp1 and Csp2-like sequences termed R1' and R2'. The concave surface of the RD binds RNA primarily through hydrogen bonding and van der Waals interactions. Additionally, base stacking interactions between the amino acid side chains of arginine and lysine and the phosphate backbone and 2'OH group of the RNA promote binding (Figures are modified from Edwards et al, 2001).

A variety of interactions occur between the RNA and the concave surface of the PufRD to mediate RNA-PufRD binding. Structural analysis of the human and *Drosophila* PufRD reveals that the concave surface has a concentration of basic, positively charged amino acid residues such as arginine and lysine on the H3 helical layer (Figure 5B). These residues may facilitate contacts with the phosphates in the RNA backbone (Edwards et al, 2001; Wang et al, 2001). In addition, the human PufRD makes a few direct interactions with the 2' OH of the RNA, and stacking interactions between RNA bases and amino acid side chains also appear to facilitate binding. However, hydrogen bonds and van der Waals interactions appear to be the most important in facilitating binding (Wang et al, 2002).

The outer convex surface of the PufRD interacts with protein partners that are required for Puf-mediated decay of a bound RNA. Specifically, outer loop regions which protrude between the Puf repeats mediate protein-protein interactions, and the location and number of outer loops may vary in each Puf homolog. In *Drosophila* Pumilio, the degradation of hunchback mRNA is dependent, in part, on interactions between the RD and the Nanos and Brat proteins (Figure 6). An outer loop between repeats 7 and 8 is required for Nanos recruitment, as amino acid insertions in this loop eliminate its recruitment (Sonoda and Wharton, 1999). In *S. cerevisiae*, the Puf3RD also contains an outer loop between repeats 7 and 8, which is important for mediating the turnover of its RNA target (Houshmandi and Olivas, 2005).

The PufRD binds targeted mRNAs in a sequence specific manner. Puf protein RNA targets contain a conserved UGU consensus sequence, which is required for binding (Wickens et al, 2002). For example, both *Drosophila* and human PufRDs require

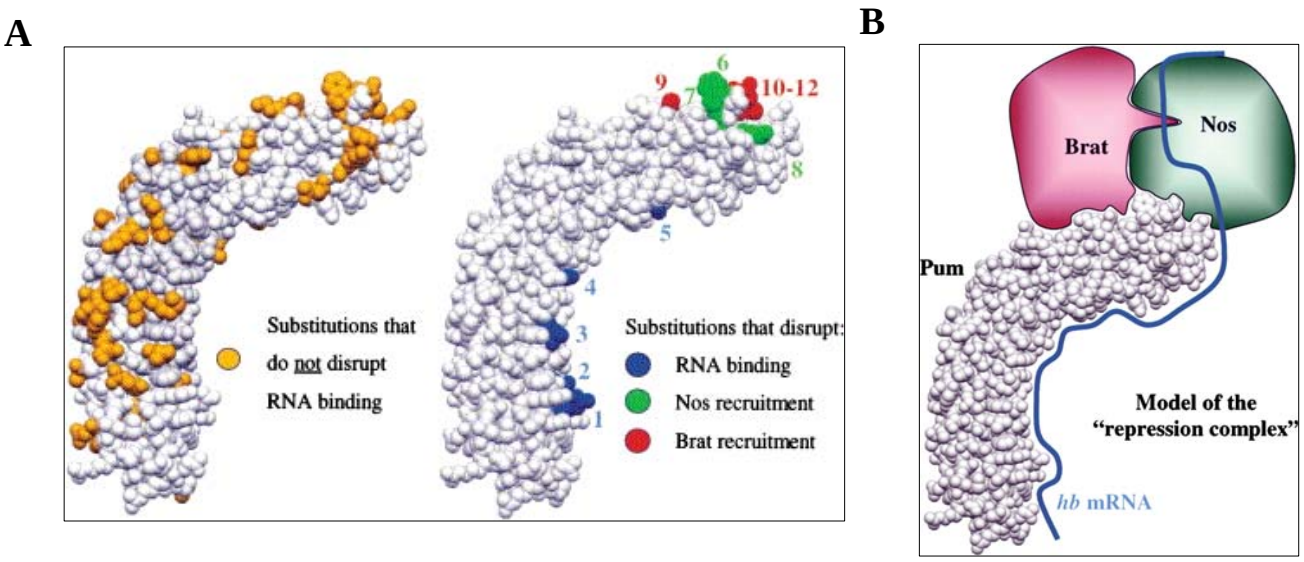


Figure 6. A map of mutations made to the Pumilio RD that disrupt RNA binding and protein recruitment is used to predict the conformation of the hunchback-Pumilio-Nanos-Brat quaternary complex. **A**. A space fill model of the Pumilio RD was used to map the amino acid sites that are important for RNA and protein interactions. The concave surface of the RD is important for RNA binding as Pumilio-RNA actions are inhibited by mutations made to this region (blue). The convex surface of the RD is important for interactions with Nanos and Brat, as mutation to this region disrupts recruitment (green and red). **B**. A model of the hunchback-Pumilio-Nanos-Brat complex is based upon the observations made in **A** along with knowledge about the Nanos and Brat proteins (Figures are modified from Edwards et al, 2001).

the UGU trinucleotide for high affinity binding. Mutations in this minimal sequence reduce the affinity for PufRD-RNA binding. In addition, the RNA targets of *Drosophila* and human Pufs contain two UGU sites, termed Box A and Box B. Box B contains a UGUA sequence, which when mutated reduces the binding affinity of the human PufRD more than 10-fold. This suggests that the UGUA sequence may be required for Puf binding and its associated activity (Zamore et al, 1997). The *C. elegans* Puf homolog, FBF-1, has also been shown to require the UGUA sequence for RNA binding (Bernstein et al, 2005). The UGUA core consensus sequence required for PufRD binding may be further extended to include flanking sequences, as PUM2, a murine Puf protein, requires a UGUANAUA sequence for RNA binding (White et al, 2001). Also, the yeast Puf protein, Puf3p, binds two CNUGUANAUA sites found within its target (Jackson et al, 2004). However, work in *C. elegans* has shown that sequences surrounding the consensus 11nt FBF binding sites are also critical for binding the FBF proteins, suggesting an expanded 22nt binding element (Bernstein, et al, 2005). Therefore, sequences that flank 5' and 3' of the conserved UGU trinucleotide are the determinants that confer the specificity of Puf protein binding.

In *S. cerevisiae*, six Puf proteins have been identified, with Puf1p-Puf5p having the most well conserved RDs, in which each contains eight copies of the repeat. The RD appears to be the most highly conserved region of the protein, as other areas of the protein are far less conserved. To date, the RD is the only region of the protein that has been implicated in regulating decay. However, there is some similarity between the yeast Pufs outside of the RD, as the entire length of the Puf1p and Puf2p are 35% identical and 50% similar. Puf2p and Puf5p share similar sequences within the C-terminus that lie

outside of the RD (Figure 7). The repeat domains of Puf3p, Puf4p, and Puf5p are most highly conserved with the Pumilio RD (Olivas and Parker, 2000). Unlike Puf1p-Puf5p, Puf6p only contains seven repeats in the RD (Figure 7). In addition, Puf6p contains a nuclear localization signal in the N-terminus, and the protein is mainly localized to the nucleus. However, it can be found in the cytoplasm similarly as with other Pufs, although to a lesser extent (Gu et al, 2004).

The yeast Puf proteins appear to regulate distinct groups of RNAs, and each Puf shows a bias to mRNAs encoding proteins that localize to different cellular components. For example, Puf1p and Puf2p physically associate with mRNAs that encode membrane associated proteins, while Puf3p binds nuclear-encoded mRNAs that encode mitochondrial proteins. Puf4p and Puf5p prefer to bind mRNAs that encode nuclear components (Gerber et al, 2004).

Puf5p represses translation of *HO* mRNA, which encodes an endonuclease that is asymmetrically expressed in budding mother cells and stimulates mating type switching. This asymmetric expression of *HO* in the mother cell is due to expression of Ash1p in the daughter cell, which inhibits *HO* transcription in the daughter. The 3'UTR of *HO* is required for Puf5p-mediated repression. Furthermore, a UUGU tetranucleotide within the 3'UTR is required for repression, as mutation to UACU inhibits Puf5p activity (Tadauchi et al, 2001).

Puf6p represses translation of *ASH1* mRNA in the budding mother cell for asymmetric expression of Ash1p in the daughter cell. Puf6p associates with *ASH1* in the nucleus to render the RNA translationally inactive as it is exported to the cytoplasm and transported to the bud tip of the daughter cell. Subsequently, Puf6p dissociates from

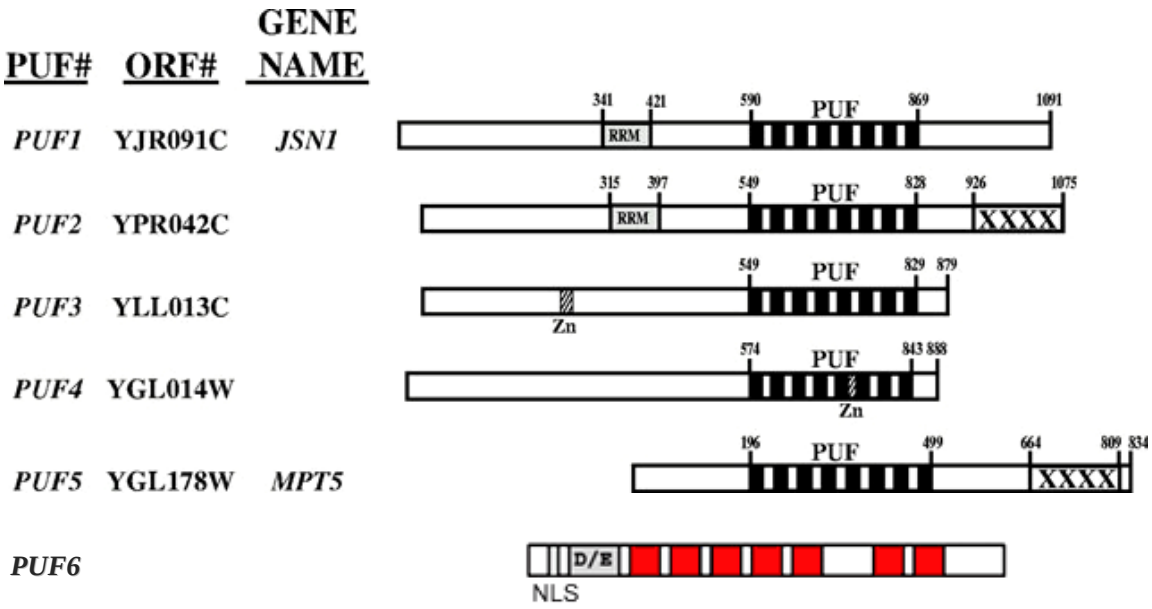


Figure 7. Alignment of the six yeast Puf proteins. Puf1-Puf5 proteins are drawn to scale and are aligned by the RD (black rectangles). Puf1p-Puf5p have eight Pumilio-like repeats. Note that the Puf6 protein is not drawn to scale relative to Puf proteins 1-5. Puf6p only contains seven Pumilio like repeats (red rectangles). Puf1p (also called Jsn1) and Puf2p have putative RRM RNA binding domains, and Puf2p and Puf5p (also called Mpt5) share sequences similarity within their C-termini (XXXX). Puf3p and Puf4p contain putative zinc finger domains (Zn). Puf6p is less conserved within the RD, and contains a nuclear localization signal in the N-terminus. It also has a region that is rich in glutamic and aspartic acid residues (D/E in gray box) (Figures are modified from Olivas and Parker, 2000; Gu et al, 2004).

ASH1 at the bud tip to allow for translation (Gu et al, 2006).

Multiple Puf proteins can act together to repress one mRNA. Recently, Puf4p (along with Puf5p) has been implicated in repressing *HO* expression. Puf4p and Puf5p act in an additive manner to stimulate *HO* repression, but appear to work via different mechanisms as Puf4p requires both Pop2p and Ccr4p, while Puf5p only requires Pop2p. Puf4p and Puf5p bind two different UGU sites within the 3'UTR. The Puf4p binding site contains a UGUAUAUUA sequence. The Puf5p binding site is considerably different as it contains two tandem repeats of the UGUA tetranucleotide: UGUAUGUAAU (Hook et al, 2007). Two additional RNAs have been reported which are subject to repression mediated by multiple Pufs. Puf1p and Puf5p work together to stimulate the decay of the *TIF1* transcript, and *HXK1* decay is regulated by Puf1p, Puf4p and Puf5p (Ulbricht and Olivas, in preparation).

Yeast Puf3 Protein

Puf3p, which is primarily located within the cytoplasm, can localize to the mitochondria. It appears that the protein plays a role in stimulating mitochondrial motility during its inheritance in the daughter yeast cell. Puf3p may also act as a peripheral membrane protein that is localized on the cytoplasmic face of mitochondria. Specifically, Puf3p acts as an adaptor to link the Arp2/3 complex to the mitochore, which is required to link the mitochondria to the cytoskeleton for transport (Garcia-Rodriguez et al., 2007). Therefore, the yeast Puf3p protein plays an important role in regulating mitochondrial function and inheritance.

Puf3p represses *COX17* mRNA expression by stimulating decay of the transcript. (Olivas and Parker, 2000). The *COX17* protein functions as a copper shuttle in the mitochondria, and is nuclearly expressed in both yeast and mammalian cells (Glerum et al, 1996; Beers et al, 1997). Although COX17p is localized in the cell cytoplasm, it transports copper from the cytoplasm into the mitochondria, and indirectly is required for the function of mitochondrial respiratory chains. Specifically, COX17p shuttles copper ions to subunits of the cytochrome oxidase holoenzyme. The requirement of COX17p for mitochondrial function and cell viability is supported by growth assays. *COX17* null mutants, which are not viable when plated on media containing non-fermentable carbon sources, were rescued when copper was added to the growth media (Glerum et al, 1996; Beers et al, 1997). This information is consistent with the observation that Puf proteins display preferences for types of RNAs that function within a particular cellular compartment, as Puf3p physically associates with proteins that have mitochondrial function (Gerber et al, 2004; Garcia-Rodriguez et al., 2007).

COX17 has been shown to be regulated by Puf3p alone, and does not physically interact with Puf5p in *in vitro* binding reactions. Specifically, Puf3p binds two UGUA elements in the 3'UTR of *COX17* to promote rapid deadenylation and subsequent decay. The *COX17* 3'UTR contains two CNUGUANAUA sites, where UGUANAUA is also conserved in targets of Pumilio, *Xenopus* Puf proteins, and mammalian homologs as well (Olivas and Parker, 2000; Jackson et al, 2004). The 3'UTR of *COX17* is sufficient to stimulate Puf3p-mediated decay, suggesting that the coding region of *COX17* does not contain additional elements that are required for Puf3p activity after subsequent recruitment to the 3'UTR. Furthermore, *in vitro* binding assays demonstrate that yeast Puf3p can bind either CNUGUANAUA site in the *COX17* 3'UTR if the other site is deleted. Furthermore, deletion of both sites inhibits Puf3p binding. Although Puf3p can destabilize *COX17* by binding a single CNUGUANAUA site in the 3'UTR , both of these sites are required for full Puf3p-dependent regulation, and mutation of both Puf3p binding sites results in a decay phenotype that is identical to that of WT *COX17* mRNA expressed in a *puf3Δ* strain. Transcriptional pulse-chase experiments, which can be used to monitor the removal of the *COX17* poly(A) tail, reveal that Puf3p destabilizes *COX17* by stimulating the deadenylation phase of mRNA decay, as well as subsequent degradation of the transcript (Jackson et al, 2004).

Thesis Overview

The regulation of mRNA decay by the six Puf proteins in yeast appears very complex, as one or more Puf proteins may cooperatively destabilize a single RNA. Puf4p and Puf5p together have been shown to stimulate the decay of *HO* mRNA (Hook et al, 2007), while Puf1p, Puf4p and Puf5p work together to stimulate the decay of the *HXK1* transcript (Ulbricht and Olivas, in preparation). Alternatively, Puf3p alone regulates the decay of *COX17* mRNA, which encodes a mitochondrial copper transporter (Olivas and Parker, 2000). Coimmunoprecipitation and microarray analysis has revealed 154 mRNAs that physically associate with Puf3p and encode mitochondrial proteins (Gerber et al, 2004). Furthermore, another microarray study identified a pool of RNAs that were coordinately regulated under different environmental conditions. These transcripts contained conserved Puf3p binding elements in the 3'UTR that displayed similarity to the Puf3p binding elements found within the *COX17* 3'UTR (Foat et al, 2005). The goal of my thesis work was to identify new RNAs that are targeted by Puf3p for decay, and characterize the structural or functional requirements of RNA targets such as the presence of conserved Puf3p binding sites in the 3'UTR, which may provide insight into the specificity of Puf3p-mediated RNA regulation. Additionally, I wanted to determine how Puf3p activity is affected by different conditions, and define a mechanism for Puf3p condition-specific activity. I have utilized data from independent microarray studies, information about Puf3p-mediated decay of *COX17* mRNA, and the *Saccharomyces* Genome Database to select 15 putative Puf3p targets. I have experimentally confirmed several new targets of Puf3p-mediated decay, and have shown that Puf3p is required for the destabilization of the transcripts. In this thesis work, I have focused on the analysis of

two of these transcripts, *CYT2* and *TUF1*. Furthermore, I have shown that Puf3p affects the stability of one of the transcripts by stimulating deadenylation of the transcript. I have also identified binding sites within the 3'UTRs of the mRNAs that are required for Puf3p regulation. Finally I have shown that the activity of Puf3p is dependent on carbon source, and that the activation or inactivation of Puf3p is very rapid and likely due to a post-translational mechanism.

Chapter II: Results

In an effort to identify new mRNAs that are regulated by Puf3p, I have selected targets based upon computational and microarray data of yeast mRNAs and compared them to data for *COX17* mRNA, the only confirmed Puf3p target. The putative Puf3p targets have been experimentally confirmed by RNA decay analysis using both steady-state transcriptional shut off methods and pulse-chase methods in both wild type and *puf3*Δ strains. These methods were also applied to observe condition-specific regulation of the targets by Puf3p.

Identification of Putative mRNA Targets Regulated by Puf3p

I have utilized data from two independent microarray studies, which have identified hundreds of RNAs that may be regulated by Puf3p. Gerber et al. purified TAP-tagged Puf proteins from *S. cerevisiae* cell extracts using IgG-Sepharose beads, and tagged Pufs were immunoprecipitated. RNAs that associated with the Pufs and, therefore, co-immunoprecipitated, were used to create cDNAs that were hybridized with DNA microarrays. 220 mRNAs that physically associated with Puf3p were identified by this microarray analysis, in which 154 of the nuclearly-transcribed mRNAs encode mitochondrial proteins. *COX17* mRNA, which physically associates with Puf3p and is rapidly degraded in the presence of the protein, was used as a positive control in the microarray analysis. In addition, the study identified the presence of a conserved putative Puf3p binding sequence (C/U)(A/C/U)UGUA(A/U/C)AUA in the 3'UTRs of 79% of the 220 transcripts, including *COX17* (Gerber et al, 2004).

Foat et al. utilized microarray datasets from the *S. cerevisiae* genome subjected to ~750 conditions and the 200nt downsteam of each yeast gene ORF (3'UTR) and applied

the data to an algorithm termed MatrixREDUCE (regulatory element detection using correlation with expression). MatrixREDUCE identified a subset of RNAs that were coordinately regulated under the different environmental conditions, such as glucose, sucrose, and ethanol (Figure 8). Analysis of sequences within the 3'UTRs of these coordinately regulated RNAs revealed that many shared a common putative Puf3p binding element within the 3'UTR: (C/U)(C/U)UGUAAAUA.

Furthermore, the algorithm predicted that mRNAs containing a conserved Puf3p binding site in the 3'UTR, including the experimentally validated Puf3p target *COX17*, are downregulated in the presence of fermentable carbon sources, such as glucose. On the contrary, transcripts containing the putative cis-regulatory binding site for Puf3p are upregulated in the presence of a non-fermentable carbon source, such as ethanol or during diauxic shift, where the cells shift from anaerobic respiration to aerobic respiration. (Foat et al, 2005). This condition-specific regulation is supported by both experimental and microarray data that suggests that Puf3p targets, such as *COX17*, encode mitochondrial proteins (Olivas and Parker, 2000; Glerum et al, 1996; Beers et al, 1997). Moreover, it is expected that Puf3p targets would be downregulated in the presence of glucose, because the mitochondria would not be needed for fermentation. Alternatively, expression of Puf3p targets would most likely be upregulated in the presence of ethanol, which cannot be fermented, where the mitochondrial proteins (such as *COX17*p) would be needed for proper mitochondrial function.

It is important to note that this UGUANAUA sequence, which appears to be highly conserved in putative Puf3p RNA targets, is also conserved in targets of *Drosophila* Pumilio, *Xenopus* Pumilio, and mammalian homologs. In *C. elegans*, the

sequences surrounding the consensus 11nt FBF binding sites are also critical for binding the FBF Puf proteins, which suggest there is an expanded 22nt binding element (Bernstein, et al, 2005). From this information, it is inferred that the sequences that flank the 5' and 3' regions of the expanded UGUANAUA core sequence are the determinants that confer the specificity of Puf protein binding in different eukaryotes. Therefore, it is likely that the two (C/U)(A/C/U) that are 5' of the UGUANAUA sequence are the determinants that confer specificity of Puf3p binding to *COX17* and other targets. For the purpose of my thesis work, I have recognized the consensus Puf3p-binding element as (C/U)(A/C/U)UGUA(A/U)AUA based upon the consensus sequences identified by two independent microarray studies and the two consensus sequences located in the *COX17* mRNA 3'UTR.

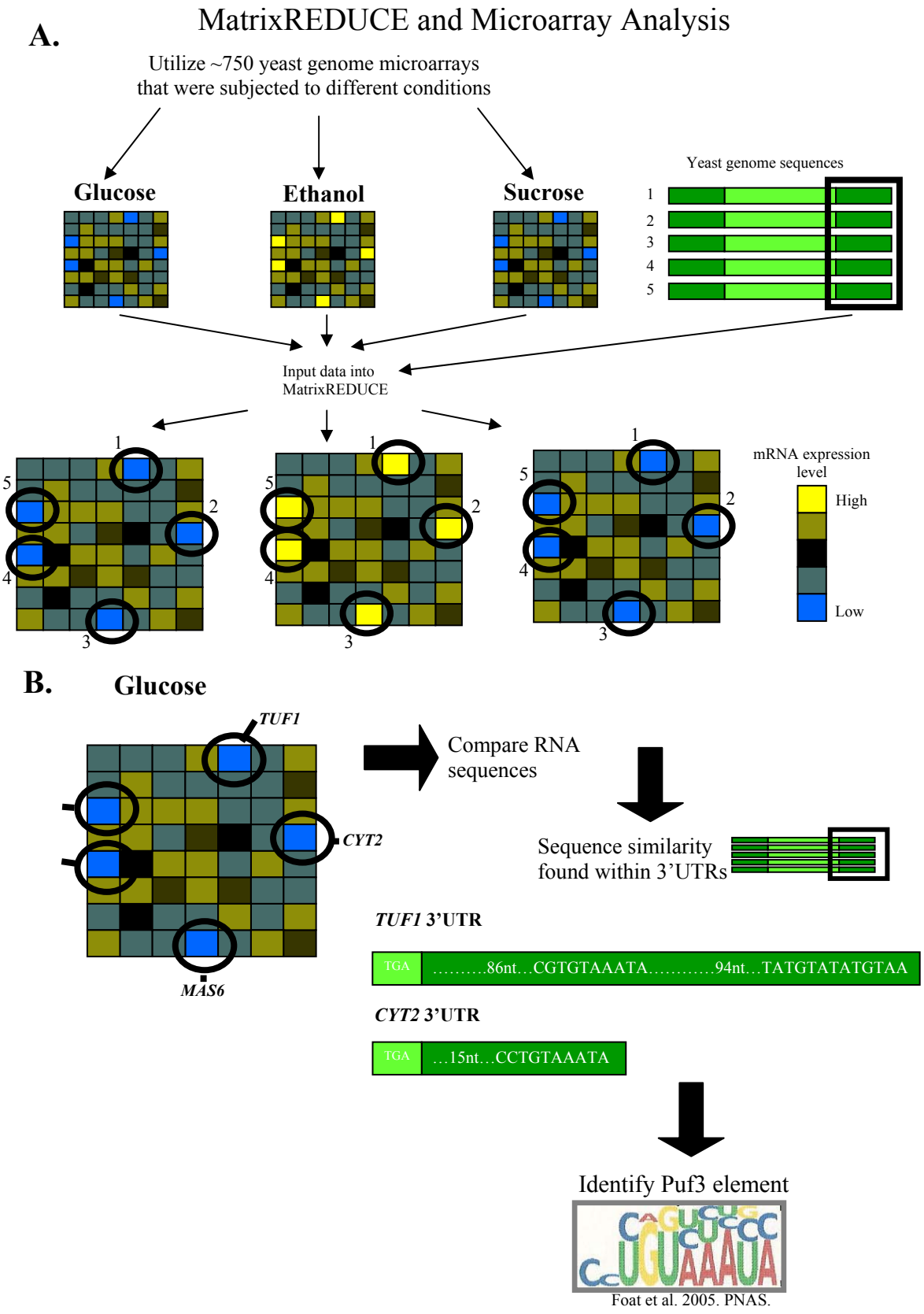
While the microrarray data provides key information about potential Puf3p binding sites, it is important to understand that it only provides information about the steady state levels of RNAs under different conditions, and does not provide information about the effect of Puf3p on the levels of transcripts. Therefore, it was imperative that I experimentally validate the candidate RNAs. Since it would be a daunting task to experimentally analyze hundreds of RNAs, I wanted to refine my selection of candidate RNAs that may be targets of Puf3p regulation.

In an effort to narrow down my search for potential Puf3p candidates, I selected 14 RNAs that were ranked most highly as probable Puf3p targets in both of the independent microarray studies. I focused on RNAs identified by Gerber et al. that co-precipitated most frequently with Puf3p and were expressed at greater than one copy numbers within the yeast cell. I cross-referenced my list of RNAs with those identified

by Foat et al, and utilized the *Saccharomyces* Genome Database to identify the localization and function of the encoded protein. I also wanted to focus on RNAs that had similar characteristics as *COX17* mRNA, such that the selected RNAs encoded mitochondrial proteins and contained the conserved (C/U)(A/C/U)UGUA(A/U)AUA element. In an effort to identify all of the possible Puf3p binding sites located within the 3'UTRs of my candidate RNAs, I searched for conserved sequences located within 300bp 3' of the translational stop codon. Since it is virtually impossible to predict the exact length of a 3'UTR without experimental determination, I wanted to include a region that was significantly longer than the length of an average 3'UTR.

Fourteen of the transcripts were nuclearly-transcribed RNAs that encoded mitochondrial proteins and included: *ATP11*, *CYT2*, *MAS6*, *MRF1*, *MRP1*, *MRP21*, *MRPL6*, *MRPL7*, *MRPL39*, *MSF1*, *PET123*, *RSM10*, *RSM19*, and *TUF1*. Recently, Garcia-Rodriguez et al. observed that the cytoplasmic yeast Puf3p protein can localize to the cytoplasmic face of the mitochondria and function as a peripheral membrane protein, which supports the hypothesis that Puf3p regulates the stability of RNAs that encode mitochondrial proteins. In addition, Pet123 protein expression levels are affected by Puf3p, as deletion of Puf3p results in an increase in Pet123p levels. Conversely, Pet123p levels decrease when Puf3p is overexpressed (Garcia-Rodriguez et al., 2007). Considering that *COX17* mRNA encodes a mitochondrial copper transporter, it is possible that Puf3p has an affinity for proteins that have a generalized copper transporter function. In order to assess this possibility, I selected *CTR1* mRNA for experimental analysis as well, as this transcript encodes a copper transporter that localizes to the

plasma membrane. The next section will discuss my analysis of these RNAs to identify bona fide targets of Puf3p-mediated decay.



Foat et al. 2005. PNAS.

Figure 8. Diagram of the identification of RNAs containing Puf3p binding elements using MatrixREDUCE. **A**. About 750 yeast DNA microarrays, each of which was subjected to a different environmental condition, such as glucose, sucrose, or ethanol, along with sequences from the 3'UTR of each yeast ORF was applied to the algorithm MatrixREDUCE. In glucose conditions (microarray on left), 5 RNAs coordinately have low expression (circled blue squares). In sucrose conditions (microarray on right), these same RNAs also exhibit low expression (circled blue squares). In ethanol conditions (microarray in middle) these RNAs have high levels of expression (circled yellow squares). **B**. The five RNAs that were coordinately regulated were identified and the sequences within the 3'UTR were examined to determine an element that was conserved between them. A conserved Puf3p-binding element was found in many of the transcripts and was also conserved in *COX17* mRNA, the confirmed target of Puf3p. This can be used to predict new targets of Puf3p.

Confirmation of New Puf3p Targets

In order to identify genuine RNA targets of Puf3p-mediated decay, I subjected my 15 putative targets to experimental analyses performed in wild-type (WT) and *puf3Δ* strains. Specifically, I wanted to determine if the stabilities of these RNAs were affected by the deletion of *PUF3*. The results of these experiments are described in the following section.

Steady-State Transcriptional Decay Analysis

In an effort to identify bona fide targets of Puf3p, I wanted to examine how Puf3p affected the stability of the 15 candidate RNAs. Steady-state transcriptional decay analysis was performed *in vivo* to monitor the decay rate of mRNAs that are putatively regulated by Puf3p in both wild-type and *puf3Δ* background strains. RNAs that are regulated by Puf3p-mediated decay should exhibit differential decay rates in wild-type and *puf3*Δ strains. Therefore, targeted RNAs are expected to decay rapidly in the presence of Puf3p, and should be relatively stabilized in a *puf3*Δ strain.

This experimental procedure was modified from that previously described (Caponigro et al, 1993; Olivas and Parker, 2000). Wild-type and *puf3Δ* steady-state cultures were grown overnight at 24°C in rich yeast extract-peptone (YEP) media supplemented with glucose, and cells were harvested at an OD_{600}=0.4, during which cells are in the log growth phase. The strains, which harbor an *rpb1-1* temperature sensitive mutation in RNA polymerase II, transcribe mRNAs at the permissive temperature of 24°C. Transcription can be shut off by shifting the cultures to the restrictive temperature for the mutation at 37°C (Figure 9). At time 0, transcription of RNAs by RNA

polymerase II is repressed, and samples of the cultures were taken 2 minutes, 4 minutes, 6 minutes, etc. after the initial shut off of transcription. Total RNA was prepared from each sample by phenol-chloroform extraction and was separated on an agarose gel. The RNA was transferred to nylon membrane for probing with radiolabled oligos.

The ORF sequence, as well as the 3'UTR sequence, of each candidate gene was retrieved from the *Saccharomyces* Genome Database (www.yeastgenome.org), and a DNA oligo complementary to a ~25nt region near or within the 3' UTR was designed and end labeled using γ^{32}P ATP to detect each endogenous mRNA. The Northern blots were normalized for total RNA loading using 7s RNA, an RNA polymerase III transcript. Transcription of 7s RNA is not affected by the shift to high temperature, as it is not transcribed by RNA polymerase II.

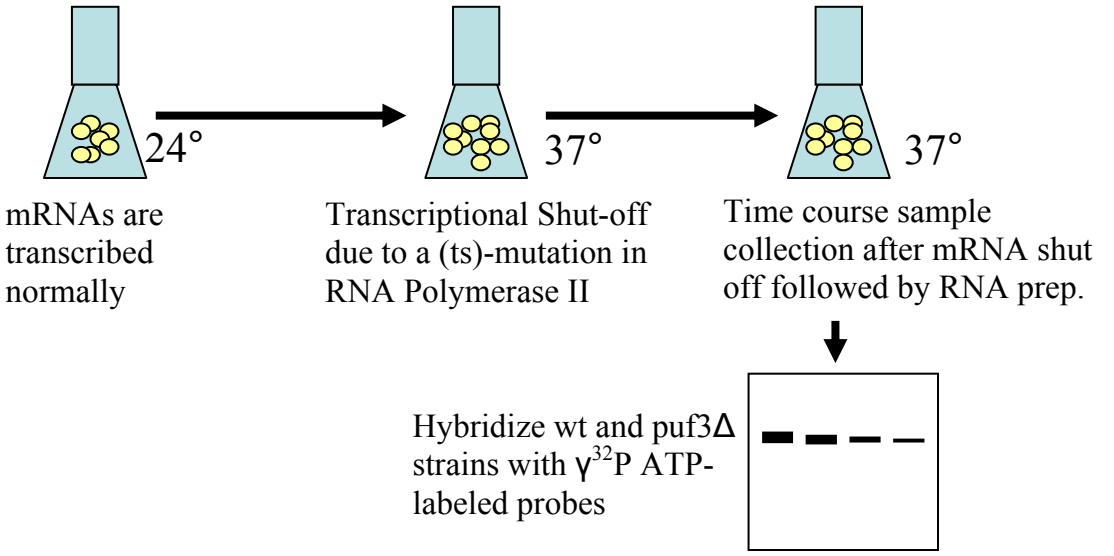


Figure 9. Schematic of steady-state transcriptional shut off analysis. The experiment is performed using yeast strains that contain the *rpb1-1* temperature sensitive mutation in RNA polymerase II. Cultures are grown overnight in which RNAs are transcribed at 24ºC. When the culture is shifted to 37ºC, transcription of new RNAs is halted. Decay of transcripts is monitored by taking an initial sample at time 0, when transcription is first halted, and additional samples as time progresses.

Puf3p Stimulates Degradation of Several RNAs

The results of the steady-state transcriptional shut off experiments for 13 of the 15 RNA candidates are shown in Figure 10. One transcript, *MRPL7*, could not be detected using two different oligos complementary to the 3'UTR region. Another transcript, *MSF1*, was visibly detected, but could not be analyzed due to a weak signal and high background noise. Table 1 presents a summary of the function of each encoded protein, the presence of the conserved Puf3p binding element in the 3'UTR of each RNA, including *COX17* mRNA for comparison, as well as the fold difference in decay rate between the *puf3Δ* and wild-type strain.

ATP11 mRNA, which encodes a mitochondrial protein, contains a Puf3p-binding element in the 3'UTR that is identical to the second binding site in the 3'UTR of *COX17* (Table 1), and is rapidly degraded in the WT strain, with a half-life of 3.8 ± 0.2 minutes. The transcript is stabilized in the *puf3Δ* with a half-life of 10.2 ± 2.6 minutes (Figure 10A; Table 1). Unfortunately, the large deviation in the half-life obtained for *ATP11* in the absence of Puf3p was a consequence of inconsistent results. In the *puf3Δ* strain, the half-life of *ATP11* among individual experiments varied greatly, ranging from 5.4 minutes to 16.0 minutes. It is possible that this large degree of variance among these experiments could be an artifact of changes in transcriptional regulation under these conditions. Despite the variance among these experiments, the stability of this transcript is affected by Puf3p, thus confirming *ATP11* as a new target of Puf3p-mediated decay.

CYT2 mRNA, which encodes a mitochondrial protein involved in the maturation of cytochrome C1, also contains one Puf3p-binding element identical to the second Puf3p binding site in *COX17***.** This transcript is degraded rapidly with a half-life of 1.7 ± 0.1

minutes in the WT strain. The half-life of *CYT2* is increased 2.7-fold (T1/2 = 4.6 ± 0.5) in the absence of Puf3p, suggesting that Puf3p is affecting the stability of this RNA (Figure 10B; Table 1).

MAS6 mRNA encodes a mitochondrial protein transporter and contains 6 core UGUA Puf binding elements, in which 3 of the UGUA elements are in close proximity, with only 2 or 4 nucleotides separating them. Five of the UGUA elements are in the context of the (C/U)(A/C/U)UGUA(A/U)AUA element, with many of the sites lacking conservation (Table 1). *MAS6* visually displays differential rates of decay in the WT versus *puf3Δ* strains (Figure 10C), with a 2.8-fold difference in half-life. Although *MAS6* has a standard error of the mean of 2.4 minutes in the absence of Puf3p, this mRNA stabilized at the very least 1.9-fold greater in the *puf3Δ* strain, suggesting that this mRNA is a bona fide target of Puf3p regulation.

MRF1, which encodes a protein involved in mitochondrial translation, contains 3 well conserved putative Puf3p binding sites (Table 1). Unfortunately, *MRF1* was particularly difficult to detect on blots containing RNA from the WT strain. Furthermore, only one blot could be statistically analyzed. The image presented in Figure 10D is representative of a number of other experiments using the WT strain. As observed in multiple experiments, this transcript was destabilized in the presence of Puf3, given a half-life of 2 minutes (Figure 10D). In the *puf3Δ* strain, the half-life increased to 8.4 ± 0.4 minutes. Given the differential rate of decay between the WT and *puf3Δ* strains, these results indicate that Puf3p destabilizes and promotes the rapid decay of *MRF1*.

MRP1 RNA encodes a constituent of the mitochondrial ribosome, and contains 2 putative Puf3p binding elements, in which one of the elements is highly conserved and

the other site lacks conservation in the sequences flanking the core UGUA tetranucleotide (Table 1). This RNA was quickly destabilized in the WT strain, with a half-life of 3.2 ± 0.4 minutes. In the *puf3Δ* strain, *MRP1* decay occurred quickly as well, with a half-life of 5.1 ± 0.4 minutes. Although the transcript exhibited a half-life similar to that in the WT strain, it appeared to undergo a second, slow phase of decay in which its abundance diminished extremely slowly when compared to WT (Figure 10E). In the WT strain, only 11% of the initial pool of *MRP1* remained 10 minutes after transcriptional shut off. On the contrary, in the *puf3Δ* strain, 10% of the initial pool of RNA remained 20 minutes after decay. This transcript has a 1.6-fold difference between the half-lives in WT and *puf3Δ* strains, suggesting that Puf3p is regulating the stability of this transcript to a small degree.

MRP21 also represents a class of proteins that constitute the mitochondrial ribosome, and contains one conserved potential Puf3p binding element (Table 1). In the presence of Puf3p, the transcript was degraded swiftly, with a half-life of 2.3 ± 0.3 minutes (Figure 10F). In the absence of Puf3p, the transcript appeared more stabile, given a half-life of 4.7 ± 0.3 minutes. From these results, the destabilization and degradation of *MRP21* is dependent on Puf3p.

MRPL6, another mitochondrial ribosome protein, contained 2 putative Puf3 binding sites within 300bp downstream of the translational stop codon, in which the first site lacked conservation in the last 3nt that flanked the 3' end of the consensus sequence. The second element was highly conserved and identical to the first Puf3p binding site found within *COX17* (Table 1). The half-life of this RNA was 3.0 ± 0.3 minutes and 4.8 ± 0.2 minutes in the WT and *puf3Δ* strains, respectively (Figure 10G). Thus, Puf3p has a

small effect on the stability of *MRPL6*, despite the presence of a well conserved consensus sequence within the 3'UTR.

MRPL39, which encodes a protein of the large subunit of the mitochondrial ribosome, contains two potential Puf3p binding sites (Table 1). The first element is highly conserved, and identical to the second confirmed Puf3p binding site located in the *COX17* 3'UTR. The second site is well conserved, although it lacks conservation in the last two nucleotides. The half-life of this RNA was 2.9 ± 0.2 minutes and 6.7 ± 1.1 minutes in WT and *puf3Δ* strains, respectively (Figure 10H). *MRPL39* displayed large variance in the rate of decay in the absence of Puf3p, as previously demonstrated with *ATP11* under the same conditions. Due to these results, I cannot positively conclude that the stability of *MRPL39* is affected by Puf3p. Similarly as for *ATP11*, the stability of *MRPL39* may be affected by other cellular factors such as variance in transcriptional regulation, for example.

PET123 mRNA, which encodes a constituent of the mitochondrial ribosome, contains two putative Puf3p binding sites, in which the second site is highly conserved (Table 1). Pet123 protein levels have been observed to be affected inversely by Puf3p levels (Garcia-Rodriguez et al, 2007). Decay analysis of the mRNA reveals that the transcript is rapidly degraded in the WT strain with a half-life of 1.7 ± 0.2. In the *puf3Δ* strain, the transcript was comparatively stabilized and exhibited a half-life of 7.2 ± 0.7 minutes (Figure 10I). Considering that there is a 4.2-fold difference between the average half-lives in the WT and *puf3Δ* strains, it is clear that Puf3p destabilizes and promotes rapid decay of *PET123* mRNA.

RSM10 mRNA contains two possible Puf3p binding elements in its 3'UTR, in which the first consensus sequence is identical to the first binding site within the *COX17* 3'UTR (Table 1). This transcript was rapidly degraded in both the presence and absence of Puf3p. It exhibited half-lives of 3.2 ± 0.1 and 3.0 ± 0.5 in the WT and *puf3Δ* strains, respectively (Figure 10J). Therefore, there is no difference between the decay rate of this transcript when *PUF3* is deleted, and *RSM10* decay is not regulated by Puf3p.

RSM19 contains three conserved consensus sequences in its 3'UTR, in which two of the sites only deviate from the sequence by 1nt located in the first position. This RNA is destabilized and quickly degraded in the WT strain, given a half-life of 4.2 ± 0.4 minutes. In the *puf3Δ* strain, *RSM19* had an average half-life of 8.5 ± 0.8 minutes. Although *RSM19* contains three conserved Puf3p binding sites, this transcript only displays a 2-fold difference in the average half-lives among the WT and *puf3Δ* strains. Nonetheless, *RSM19* represents yet another target of yeast Puf3p.

TUF1 mRNA encodes the mitochondrial translation elongation factor Tu and contains 3 core UGUA elements within the 3'UTR. The first UGUA tetranucleotide is placed in the context of the 10nt consensus sequence, and only lacks conservation at the second position. The other two UGUA elements are separated by 2nt, in which the second UGUA lacks conservation only in the ninth position (Table 1). In the presence of Puf3p, *TUF1* was degraded fairly rapidly with a half-life of 5.5 ± 0.6 minutes. The RNA was significantly more stabilized in the *puf3Δ* strain, with a half-life of 16.7 ± 0.7 minutes (Figure 10L). Therefore, Puf3p is affecting the stability of this transcript.

CTR1 mRNA represents the only candidate that does not encode a mitochondrial protein. However, this transcript encodes a copper transporter that functions at the plasma

membrane. The transcript contains one highly conserved Puf3p binding element within its 3'UTR that is identical to the first Puf3p binding site of *COX17*. This RNA displayed a large difference in the rate of decay between the WT and *puf3Δ* strains, in which the half-life of this transcript was 2.7 ± 0.7 versus 17.5 minutes, respectively (Figure 10M). Although these results in the *puf3Δ* strain were based upon a single experiment, these results suggest that the stability of *CTR1* mRNA is greatly affected by Puf3p. Moreover, since *COX17* is also a copper transporter, this may indicate that Puf3p has an affinity for transcripts that encode proteins with general copper transporter activities.

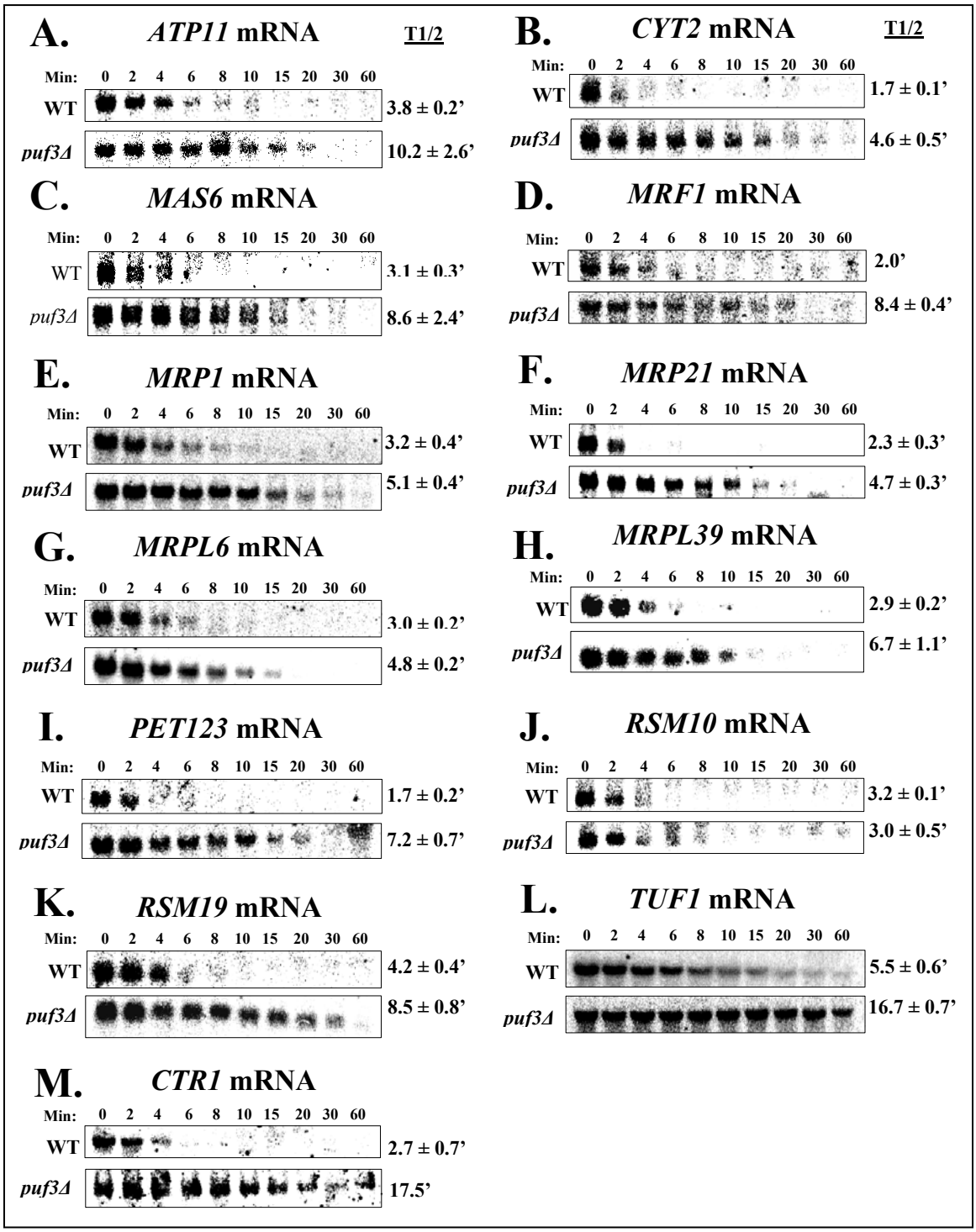


Figure 10. Decay Rates of Putative Puf3p RNA Targets. Northern blot analyses of the decay of 13 candidate RNAs are shown. The decay rates of endogenous RNAs expressed in a WT or *puf3Δ* strain are expressed as average half-lives (T1/2) with standard error of the mean as calculated from ≥3 steady-state transcriptional shut off experiments. The minutes following transcriptional repression are listed above each set of blots (WT and *puf3Δ*).

mRNA	Function/Location of Encoded Protein	(C/U)(A/C/U)UGUA(A/U)AUA elements located within 300bp downstream of stop codon	Ratio of T1/2 *puf3Δ* T1/2 *WT*
ATP11	Chaperone for assembly of mitochondrial ATP synthase	CCUGUAAAUA	2.7
COX17	Mitochondrial copper transporter	CUUGUAUAUA CCUGUAAAUA	5.7
CYT2	Involved in maturation of cytochrome C1 and located in mitochondrial intermembrane space	CCUGUAAAUA	2.7
CTR1	Copper transporter located in plasma membrane	CUUGUAUAUA	6.5
MAS6	Protein transporter located in mitochondrial inner membrane	CUUGUAUAUA CAUGUAUGUGUAGAUAUGUACAUA CUUGUAUGUU UUUGUACUGU	2.8
MRF1	Mitochondrial polypeptide chain release factor involved in mitrochondrial translation	CUUGUAAAUA UCUGUAAAUC UAUGUAAUUA	4.2
MRP1	Protein of the small subunit of the mitochondrial ribosome	UCUGUAAAUA AUUGUAGCGC	1.6
MRP21	Protein of the mitochondrial ribosome	UUUGUAUAUU	2.0
MRPL6	Protein of the large subunit of the mitochondrial ribosome	CAUGUAUCCU CUUGUAAAUA	1.6
MRPL7	Protein of the large subunit of the mitochondrial ribosome	CAUGUAAAUA ACUGUACAAG UCUGUAUAAA AAUGUACAUA	RNA not detected
MRPL39	Protein of the large subunit of the mitochondrial ribosome	CCUGUAAAUA UUUGUAUACG	2.3
MSF1	Mitochondrial phenylalanyl-tRNA synthetase α subunit	CUUGUAAAUA	Visible difference in T1/2
PET123	Protein of the small subunit of the mitochondrial ribosome	CAUGUAUUCG CAUGUAUAUA	4.2
RSM10	Protein of the small subunit of the mitochondrial ribosome	CUUGUAAAUA UUUGUACUAA	0.9
RSM19	Protein of the small subunit of the mitochondrial ribosome	AGUGUAUAUA CAUGUAAAUA GCUGUAUAUA	2.0
TUF1	Mitochondrial translation elongation factor Tu	CGUGUAAAUA UAUGUAUAUGUAAAGA	3.0

Table 1. Candidate Puf3p Targets, Conserved Elements Within Their 3'UTRs, and Results of Decay Analysis. This table presents each of the candidate mRNAs and the function of their encoded proteins in the first two columns. The third column from the left lists the conserved putative Puf3p binding sites located within the 3'UTR of each RNA. The core UGUA element is underlined in each element, and nucleotides which are conserved with the 10 nucleotide (C/U)(A/C/U)UGUA(A/U)AUA element are colored black, while nucleotides that lack conservation with the sequence are light purple. The Ratio of $\frac{T1/2\ puf3\Delta}{T1/2WT}$ displays the results of the steady-state transcriptional shut off experiments as a fold-difference in the half-lives (T1/2) between the *puf3Δ* and WT strains.

The results of the steady-state transcriptional shut off experiments were effective in identifying a number of transcripts whose stability was affected by Puf3p including: *ATP11, CYT2, MAS6, MRF1, MRP1, MRP21, MRPL6, MRPL39, PET123, RSM19* and *TUF1*. Four of these transcripts, *CYT2*, *MRF1*, *PET123*, and *TUF1*, each contained at least one highly conserved (C/U)(A/C/U)UGUA(A/U)AUA element within their 3'UTRs and displayed a ≥2.7-fold difference in the half-lives between the WT and *puf3Δ* strains with acceptable standard error of the mean values. Therefore, these endogenous transcripts appear to be regulated by Puf3p. On the contrary, I also identified one transcripts that was definitively not affected by Puf3p, *RSM10*. Interestingly, this transcript encoded a mitochondrial protein and contained at least one highly conserved putative Puf3p binding element as well. Considering that *C. elegans* FBF can recognize an expanded 22nt consensus binding sequence (Bernstein et al, 2005), it is also likely that yeast Puf3p may also recognize sequences flanking the 10nt consensus sequence that may be required for regulation.

While many candidate mRNAs displayed a large degree of regulation by Puf3p, as exhibited by a 2-fold or greater difference in half-lives among the WT and *puf3Δ* strains, the stabilities of two transcripts, *MRP1* and *MRPL6*, were only affected by Puf3p to a relatively small degree, when compared to *COX17*, for example. These two transcripts each contained two Puf3p binding sites, in which one of these sites is highly conserved (Table 1). This indicates that are other factors that determine the extent by which Puf3p destabilizes its targets. The data from these experiments highlights the large number of false positives that may exist in datasets from the microarray studies used to

determine Puf3p targets. This also supports the necessity to experimentally validate RNA targets from such large scale analyses.

Determining the Sufficiency of the 3'UTR in Stimulating Puf3p-mediated Decay

In an effort to further understand Puf3p-mediated decay of mRNAs, it is important to identify the regions of the *CYT2* and *TUF1* transcripts that are necessary for stimulating decay. Considering that one of the candidate transcripts containing the consensus Puf3p binding element in its 3'UTR was not affected by Puf3p, there exists two possibilities that may account for the apparent regulation of the *CYT2* and *TUF1* transcripts. The first explanation takes into account that the consensus Puf3p binding site may be longer than 10nt long, as observed with the 22nt binding site observed with FBF in *C. elegans* (Bernstein et al, 2005). Also, surrounding sequences, such as an AU-rich region near the consensus sequence may influence Puf3p binding, and therefore, regulation. The two Puf3p binding sites found within the *COX17* 3'UTR are in the context of either AU-rich or U-rich regions (Jackson et al, 2004). The second explanation for these results may be that there are additional regions that lie outside of the 3'UTR that may be required for Puf3p-mediated decay. In the following section, I will determine if the 3'UTRs of *CYT2* and *TUF1* are sufficient to stimulate Puf3p regulation.

In order to determine whether Puf3p regulates *CYT2* and *TUF1* mRNAs by associating with their 3'UTRs, I created hybrid transcripts in which the 3'UTR of *TUF1* or *CYT2* RNA was fused to the coding region of another mRNA that is not affected by Puf3p. Jackson et al. successfully cloned the 3'UTR of *COX17* behind the coding region

of plasmid-expressed *MFA2*, which is not affected by Puf3p, and demonstrated that Puf3p interacted with elements located within the 3'UTR to stimulate the decay of this fusion *MFA2-COX17* 3'UTR mRNA (Jackson et al, 2004). I designed a construct such that the *CYT2* 3'UTR replaced the 3'UTR of *MFA2*, which is not regulated by Puf3p. The *MFA2-CYT2* 3'UTR fusion transcript was expressed from the plasmid under the control of the inducible/repressible *GAL* upstream activating sequence, in which transcription of this hybrid transcript could be induced by the addition of galactose to the media and subsequently repressed by the addition of glucose.

Unfortunately, the *MFA2-TUF1* 3'UTR transcript failed to display differences in half-lives between WT and *puf3*Δ strains. Considering that *MFA2* mRNA is rapidly degraded and only has a half-life of 4 minutes (Jackson et al, 2004), it is possible that the innate instability of the 5'UTR and coding region of this transcript overpowers the stabilizing effect of the *TUF1* 3'UTR in the absence of Puf3p. To test this theory, I wanted to fuse the *TUF1* 3'UTR to the coding region of another RNA that was not affected by Puf3p, but was innately more stable than *MFA2*. *PGK1* mRNA is a very stable transcript with a half-life of 45 minutes. The coding region of this mRNA is so stable that replacement of the *PGK1* 3'UTR with the 3'UTR of the unstable *STE3* mRNA has no destabilizing effect on this RNA. However, Heaton et al. demonstrated that increasing deletions of the *PGK1* coding region confer instability of the RNA in transcriptional shut off experiments. The plasmid-expressed transcript *PGK1Δ82* was destabilized upon deletion of 82% of the coding region to a half-life of 27 minutes. Moreover, fusion of the 3'UTR of the unstable *STE3* transcript to the coding region of *PGK1Δ82* now promoted rapid decay with a half-life of 5 minutes, suggesting that the

STE3 3'UTR was sufficient to control decay activity of this fusion mRNA (Heaton et al, 1992). Based on these results, I cloned the *TUF1* 3'UTR behind the coding region of the *PGK1Δ82* transcript. Expression of this transcript was controlled by the GAL UAS as well.

Both of the *MFA2-CYT2* 3'UTR and *PGK1Δ82-TUF1* 3'UTR plasmids were used to transform wt and *puf3*Δ strains containing the *rpb1-1* allele. Steady-state cultures were grown at 24ºC, the permissive temperature for RNA polymerase II, in galactose to induce transcription of *MFA2-CYT2* 3'UTR. Transcription was shut off simultaneously by increasing temperature and the addition of glucose. All shut off experiments were performed as previously described, and the *MFA2-CYT2* 3'UTR and *PGK1Δ82-TUF1* 3'UTR transcripts were detected using radiolabeled oligos that were complementary to the *MFA2-CYT2* 3'UTR junction and a 32 nt insertion located in the coding region of *PGK1Δ82*, respectively. If the 3'UTRs of *CYT2* and *TUF1* contain the elements required for Puf3p regulation, then the hybrid transcripts should exhibit differential decay rates in WT and *puf3*Δ yeast strains.

***CYT2* and *TUF1* 3'UTRs Promote Puf3p Regulation**

Figure 11 illustrates the results of the shut off experiments performed with the plasmid-expressed *CYT2* and *TUF1* 3'UTRs. Shut off experiments performed with plasmid expressed *MFA2* demonstrate that this RNA is not affected by Puf3p, as this transcript is rapidly degraded in WT and *puf3Δ* strains with half-lives of 4.0 minutes in both strains. The results of this control experiment allow me to attribute the results of the *MFA2-CYT2* 3'UTR decay analysis solely to the interaction between Puf3p and the

3'UTR. The *MFA2-CYT2* 3'UTR transcript was rapidly degraded in the presence of Puf3p, as this RNA had a half-life of 3.2 ± 0.4 minutes. In the *puf3Δ* strain, the half-life of this chimeric RNA was increased to 6.4 ± 0.9 minutes (Figure 11A). The deviation in half-life among experiments in the *puf3Δ* strain was attributed to an outlying dataset from a single experiment, as exclusion of this single experiment from statistical analysis would result in an average half-life of 7.3 ± 0.4 minutes. From these results, it is clear that the *CYT2* 3'UTR is sufficient to promote Puf3p-mediated decay.

The results for the shut off analysis of the *PGK1Δ82-TUF1* 3'UTR transcript yielded similar results, in which this RNA was destabilized in the presence of Puf3p and had a half-life of 6.7 ± 1.2 minutes. The half-life of this transcript was increased 2-fold to a half-life of 12.7 ± 3.2 minutes in the *puf3Δ* strain, suggesting that the *TUF1* 3'UTR is also sufficient to stimulate the recruitment of Puf3p and subsequent decay of the RNA (Figure 11B). Shut off experiments performed with plasmid expressed *PGK1Δ82* demonstrate that this RNA is not affected by Puf3p, as this transcript is slowly degraded in WT and *puf3Δ* strains with half-lives of 27.0 minutes in both strains. Therefore, the fusion of the *TUF1* 3'UTR to the *PGK1Δ82* coding region was sufficient to stimulate Puf3p activity, confer Puf3p regulation onto *PGK* and further destabilize the transcript. Together, the data from these experiments suggest that the 5' UTR or coding region of *CYT2* and *TUF1* mRNAs does not contain additional elements that are required for Puf3p activity after subsequent recruitment to the 3'UTR. Furthermore, the Puf3p targets *COX17*, *CYT2*, and *TUF1* collectively appear to be regulated by this protein solely through their 3'UTRs.

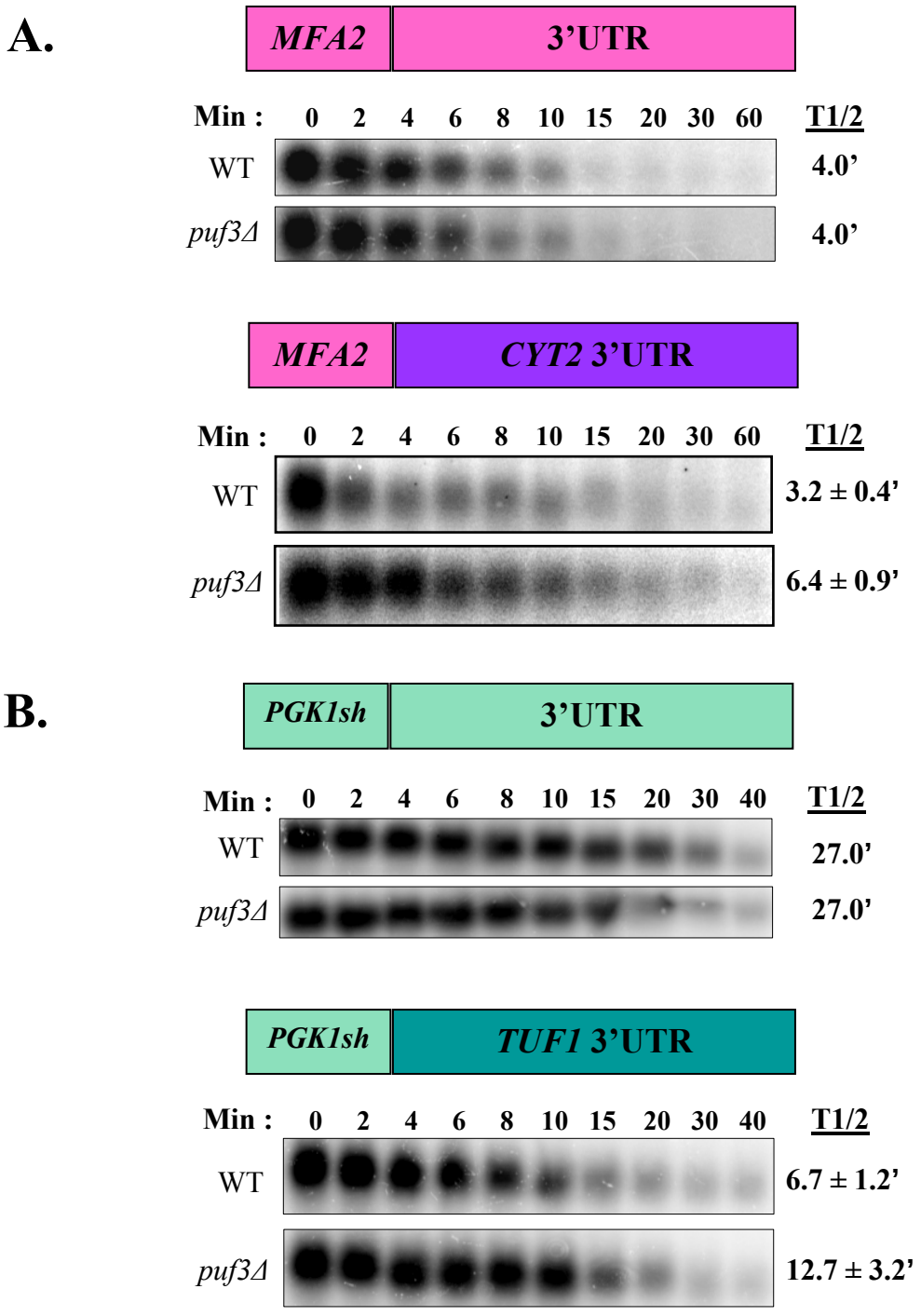


Figure 11. Decay analysis of *MFA2-CYT2* 3'UTR and *PGK1Δ82-TUF1* 3'UTR mRNAs. **A.** Shown are Northern blot analyses of the decay of the MFA2-*CYT2* 3'UTR transcript in WT and *puf3Δ* strains. **B.** Shown are Northern blot analyses of the decay of the *PGK1Δ82-TUF1* 3'UTR transcript in WT and *puf3Δ* strains. The average half-lives (T1/2) with the standard error of the mean are determined from multiple experiments.

Determining the Requirement of UGUA Elements for Puf3p Regulation

Based on the results of the transcriptional shut off experiments from the previous section, I can conclude that the 3'UTRs of *CYT2* and *TUF1* are sufficient to control full regulation by Puf3p. The most striking feature of the *COX17, CYT2,* and *TUF1* 3'UTRs is the presence of one or more conserved (C/U)(A/C/U)UGUA(A/U)AUA elements. Puf3p requires the UGUA core tetranucleotide for binding to the *COX17* 3'UTR, as mutation of this site to ACAC eliminates binding (Jackson et al, 2004). Considering that the UGUA tetranucleotide is conserved in many Puf protein targets, it is logical to hypothesize that this consensus sequence is important for the recruitment of Puf3p and subsequent degradation of the *CYT2* and *TUF1* RNAs as well. For the purpose of this study due to time constraints, I focused on examining the requirement of the UGUA sequence located in the 3'UTR of *CYT2* since this RNA contained only one CCUGUAAAUA site. I mutated the UGUA sequence in the *MFA2-CYT2* 3'UTR transcript to ACAC using site-directed mutagenesis techniques to produce *MFA2-CYT2mut* 3'UTR, and expressed this plasmid in WT and *puf3Δ* strains (Figure 12A). If the UGUA site is required for full Puf3p regulation, then I would expect this mutant chimeric transcript to be stabilized both in the presence and absence of Puf3p. Furthermore, this transcript would likely display a decay phenotype that was similar to the wild-type *MFA2-CYT2* 3'UTR RNA expressed in a *puf3Δ* strain.

Puf3 Regulation of *CYT2* Requires UGUA Element

The *MFA2-CYT2mut* 3'UTR transcript, when expressed in a WT strain, was stabilized 2-fold to a half life of 7.2 ± 0.7 minutes versus the *MFA2-CYT2* 3'UTR half-

life of 3.2 ± 0.4 minutes (Figure 12C, compare black and red lines). Moreover, the *MFA2-CYT2mut* 3'UTR RNA in the WT strain displayed a decay phenotype similar to that of *MFA2-CYT2* 3'UTR expressed in a *puf3Δ* strain (Figure 12C, compare black and blue lines). These results suggest that the UGUA sequence is required for Puf3p binding and subsequent destabilization and decay, as mutation of the UGUA tetranucleotide to ACAC results in a more-stable transcript that mimics the slow rate of decay exhibited by the wild-type *MFA2-CYT2* 3'UTR transcript in the absence of Puf3p. As a control, *MFA2-CYT2mut* 3'UTR was also expressed in a *puf3Δ* strain (Figure 12B; C, pink line), in which the transcript was degraded slowly in a manner similar to the *MFA2-CYT2 3'UTR* in the absence of Puf3p (Figure 12C, blue line) and *MFA2-CYT2mut* 3'UTR in the presence of Puf3p (Figure 12C, black line). Together, these results suggest that the UGUA tetranucleotide located within the 3'UTR of *CYT2* is required for Puf3p binding and regulation.

A.

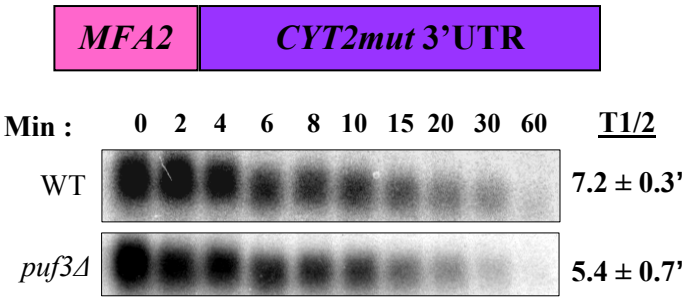
***CYT2* 3'UTR**

UGAAUUACUACAACUAUUCC**UGUA**AAUAAAAACAU
ACCCUCCCUAGUU…

***CYT2mut* 3'UTR**

UGAAUUACUACAACUAUUCC**ACAC**AAUAAAAACAU
ACCCUCCCUAGUU…

B.



C.

***MFA2-CYT2* 3'UTR mRNA Decay**

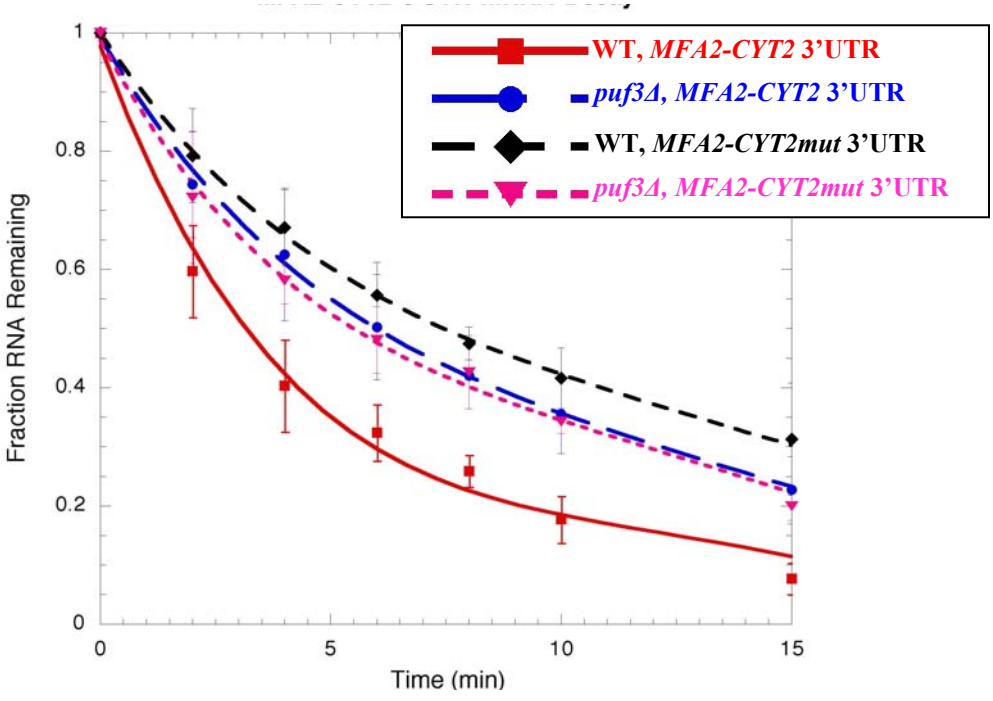


Figure 12. Decay analysis of *MFA2-CYT2mut* 3'UTR. **A.** Sequences of the wild-type and mutant *CYT2* 3'UTRs are illustrated, with the 10nt Puf3p binding element underlined. The core UGUA element in the wild-type 3'UTR is in bold font. Site-directed mutagenesis was used to change the UGUA element to ACAC to produce the mutant 3'UTR. In the mutant 3'UTR, the 10nt Puf3p element is underlined and the mutated sequence is highlighted yellow and in bold font. **B.** Shown are Northern blot analyses of the decay of *MFA2-CYT2mut* 3'UTR in WT and *puf3Δ* strains. The average half-lives (T1/2) with the standard error of the mean are determined from 3 or more experiments. **C.** Shown is a graphical representation of the decay of the *MFA2-CYT2* 3'UTR and *MFA2-CYT2mut* 3'UTR in WT and *puf3Δ* strains. *MFA2-CYT2* 3'UTR expressed in a WT strain (red line), *MFA2-CYT2* 3'UTR expressed in a *puf3Δ* strain (blue line), *MFA2-CYT2mut* 3'UTR expressed in a WT strain (black line), and *MFA2-CYT2mut* 3'UTR expressed in a *puf3Δ* strain (pink line) are shown, representing the average of 3 or more experiments with standard error of the mean.

Puf3p-Regulation of *CYT2* and the mRNA Decay Pathway

My and previous studies indicate that the Puf3 promotes rapid decay of its targets, *COX17*, *CYT2*, and *TUF1*, by interacting with cis-elements in the 3'UTR. Specifically, Puf3p-mediated degradation of these targets requires interaction with UGUA elements in *COX17* and *CYT2*. Mutations made to either of the UGUA elements within the *COX17* 3'UTR eliminate Puf3p-binding (Jackson et al, 2004), and also result in stabilization of *COX17* 3'UTR and *CYT2* 3'UTR hybrid transcripts. Olivas and Parker determined that Puf3p promotes the rapid decay of *COX17* mRNA by stimulating the deadenylation-dependent decay pathway, and specifically promotes both the deadenylation phase of decay as well as subsequent decapping steps (Olivas and Parker, 2000). Based on these results, I wanted to determine if Puf3p systematically promotes the decay of its targets in a deadenylation-dependent manner.

The *COX17* 3'UTR contains two Puf3p binding sites which are required for rapid deadenylation and subsequent decay of the *COX17* transcript. Based on this knowledge, one Puf3 protein may stimulate deadenylation upon binding one site, while another Puf3 protein may stimulate the decapping step of mRNA decay. This model suggests that a single Puf3 protein may only stimulate one decay factor complex at a time. Alternatively, it is possible that a single Puf3 protein can stimulate both deadenylation and decapping by interacting with multiple decay factor complexes. I hypothesized that the later model reflected the mechanism by which Puf3p stimulated decapping, as Puf3p target *CYT2* mRNA contains only one Puf3p binding site.

In an effort to examine if the degradation of the newly identified mRNA target is regulated by Puf3p at the deadenylation and/or decapping step of decay, I examined the

decay of the *MFA2-CYT2* 3'UTR and *MFA2-CYT2mut* 3'UTR transcripts in WT and *puf3Δ* strains using transcriptional pulse-chase experiments. I focused on the decay of *CYT2* mRNA for simplicity, since this transcript only contains one UGUA element that has been shown to control Puf3p-regulation. Moreover, the use of this transcript allowed me to distinguish between the two proposed models for the means by which Puf3p stimulates deadenylation and decapping.

The transcriptional pulse-chase experiments utilize a construct in which the *MFA2-CYT2* 3'UTR hybrid gene is placed downstream of the inducible/repressible *GAL10* promoter. An initial pulse of *MFA2-CYT2* 3'UTR transcription is induced by adding galactose to cell cultures, followed by incubation for 8 minutes. Transcription is subsequently repressed by the addition of glucose (Figure 13A). This pulse-chase creates a small pool of newly transcribed RNAs, which allows for monitoring of both deadenylation and decay over time. A time course sample collection is performed, followed by total RNA extraction from cell samples.

In order to monitor the deadenylation of the *CYT2* 3'UTR, a DNA oligo complementary to a region just upstream of the *MFA2-CYT2* 3'UTR junction is hybridized to this region (Figure 13B). The RNase H enzyme is added to cleave the DNA-RNA hybrid, thus cleaving the polyadenylated *CYT2* 3'UTR fragment from *MFA2*. In addition, RNA from yeast cells collected a time 0 are hybridized with a poly(dT) oligo followed by RNaseH cleavage to produce a deadenlyated *CYT2* 3'UTR. The RNA is separated on a denaturing polyacrylamide gel to allow visualization of poly(A) tail lengths. The *CYT2* 3'UTR is detected on Northern blots using a radiolabeled oligo complementary to the *MFA2-CYT2* 3'UTR junction.

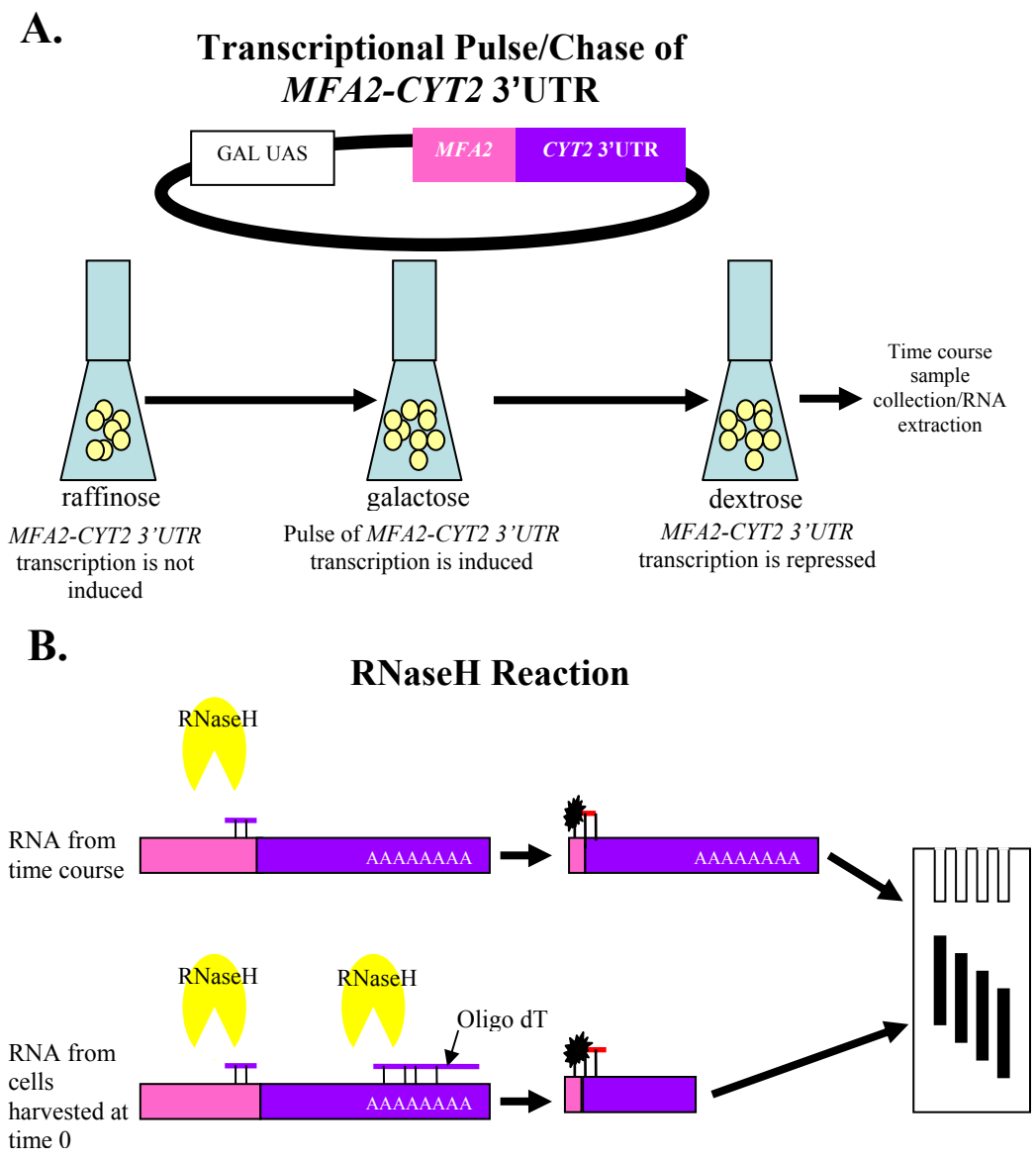


Figure 13. Diagram of a transcriptional pulse-chase experiment. **A.** The *MFA2-CYT2* 3'UTR fusion transcript (*MFA2* coding region illustrated as pink bar; *CYT2* 3'UTR illustrated as purple bar) is expressed from a plasmid containing the inducible/repressible GAL UAS. WT and puf3Δ yeast strains are transformed with the construct and grown in raffinose, which stimulates cell growth and budding, but does not induce *MFA2-CYT2* 3'UTR expression. Cells are harvested in the log growth phase and are supplemented with galactose to induce transcription of the hybrid RNA. Transcription is then repressed by the addition of dextrose, thus producing a pulse of newly transcribed RNAs that are polyadenylated. Samples of the cells are collected in a time course and total RNA is extracted from the cells. **B.** A DNA oligo complementary to the 3' end of *MFA2* (short purple and black lines) is hybridized to the chimeric transcript. For RNA from the 0 time point, an additional oligo(dT) is hybridized to the poly(A) tail (long purple and black lines). RNaseH is added to cleave the DNA-RNA hybrids to produce a polyadenylated and deadenylated *CYT2* 3'UTR fragment. The *CYT2* 3'UTRs are separated on denaturing polyacrylamide gels and transferred to nylon membranes. The *CYT2* 3'UTRs are detected using a radiolabeled probe (red and black lines with star) that is complementary to the *MFA2-CYT2* 3'UTR junction.

Puf3p Stimulates Deadenylation and Decay of *CYT2*

In the WT strain, *CYT2* mRNA initially displayed a heterogeneous population of poly(A) tail lengths ranging from ~17.5-43 residues following the induction of transcription (Figure 14A, 0 lane). This very broad range of poly(A) tail lengths may indicate that many of the newly transcribed *CYT2* RNAs undergo partial deadenylation during the 8 minute pulse of transcription. The poly(A) tails are shortened further between 1 and 2 minutes, at which time a small pool of the heterogeneous *CYT2* RNA population appears to be fully deadenylated (Figure 14A, 1 and 2 lanes). The *CYT2* persists in this state until 4 minutes post transcriptional repression, and the transcripts are almost fully degraded after 5 minutes after transcriptional repression (Figure 14, 5 lane). The levels of *CYT2* transcripts were virtually undetectable at 8 minutes. The main pool of RNA does not appear to be fully deadenylated prior to degradation, as decapping can occur once the poly(A) tail length has been shortened to A_{10}.

Although both *COX17* and *CYT2* appear to be deadenylated heterogeneously, *CYT2* mRNA appears to be far less stable, as a subset of the *CYT2* population is almost fully deadenylated at the time of transcriptional repression. Deadenylation of this transcript occurs too quickly to observe whether this process occurs in a slow phase, followed by rapid poly(A) shortening, as observed with *COX17* mRNA. On the contrary, *COX17* mRNA is deadenylated slowly for the first 2 minutes post transcription repression, which is followed by rapid poly(A) shortening which occurs between 2 to 4 minutes post transcriptional repression and subsequent decapping and decay. The decapping step proceeds relatively slowly for *COX17* transcripts, as full deadenylation of the transcript was observed prior to decay (Olivas and Parker, 2000). For *CYT2* mRNA,

the decapping step occurs rapidly, as I did not observe full deadenylation of *CYT2* transcripts prior to decay of the transcript.

In the *puf3Δ* strain, *CYT2* initially displayed similar poly(A) tail lengths that ranged from ~20-40 residues following the induction of transcription (Figure 14B, 0 lane). *CYT2* is deadenylated more slowly, with fully deadenylated transcripts beginning to appear at 3 minutes, and the main pool of the transcripts are not fully deadenylated until 6 minutes after transcriptional repression (Figure 14B). Furthermore, RNAs containing poly(A) oligo lengths persist to 10 minutes after repression of transcription, indicating that the decay step following deadenylation is also slowed. Together, the results of *CYT2* deadenylation and decay in WT and *puf3Δ* strains suggest that Puf3p stimulates the deadenylation and subsequent decapping of *CYT2* mRNA. In addition, this indicates that a single Puf3p site is sufficient for stimulating deadenylation and decapping of an mRNA target.

After determining that Puf3p mediates the turnover of *CYT2* via stimulation of deadenylation and decapping, I wanted to examine the effect of eliminating the *CYT2* 3'UTR UGUA element on the deadenylation of *CYT2*. Previous experiments reveal that the UGUA element is required for Puf3p-mediated decay of *CYT2* mRNA, as mutation of this sequence to ACAC eliminates Puf3p regulation. Therefore, I expect that mutation of this site will disrupt Puf3p binding, and in turn, prevent Puf3p from recruiting the decay machinery.

Transcriptional pulse chase experiments were performed in a WT strain containing the inducible/repressible *MFA2-CYT2*mut 3'UTR construct. After the initial pulse of newly transcribed mutant *CYT2* RNAs, these transcripts contained poly(A) tail

lengths of ~15-36 residues (Figure 14C), which were similar to the observed poly(A) tail lengths of the wild-type *CYT2* 3'UTR expressed in WT and *puf3Δ* strains. This mutant transcript displays defects in deadenylation and decay, as this heterogenous population of poly(A) tail lengths persists even after 10 minutes. It appears that only a small pool of RNAs with the shortest poly(A) tail lengths are terminally deadenylated, while the majority of the *CYT2* population resists deadenylation. Furthermore, the deadenylation rate of the mutant *CYT2* RNAs is slower than that of the wild-type *CYT2* 3'UTR in the absence of Puf3p. These results suggest that Puf3p must bind the UGUA sequence within the *CYT2* 3'UTR to promote deadenylation and decay.

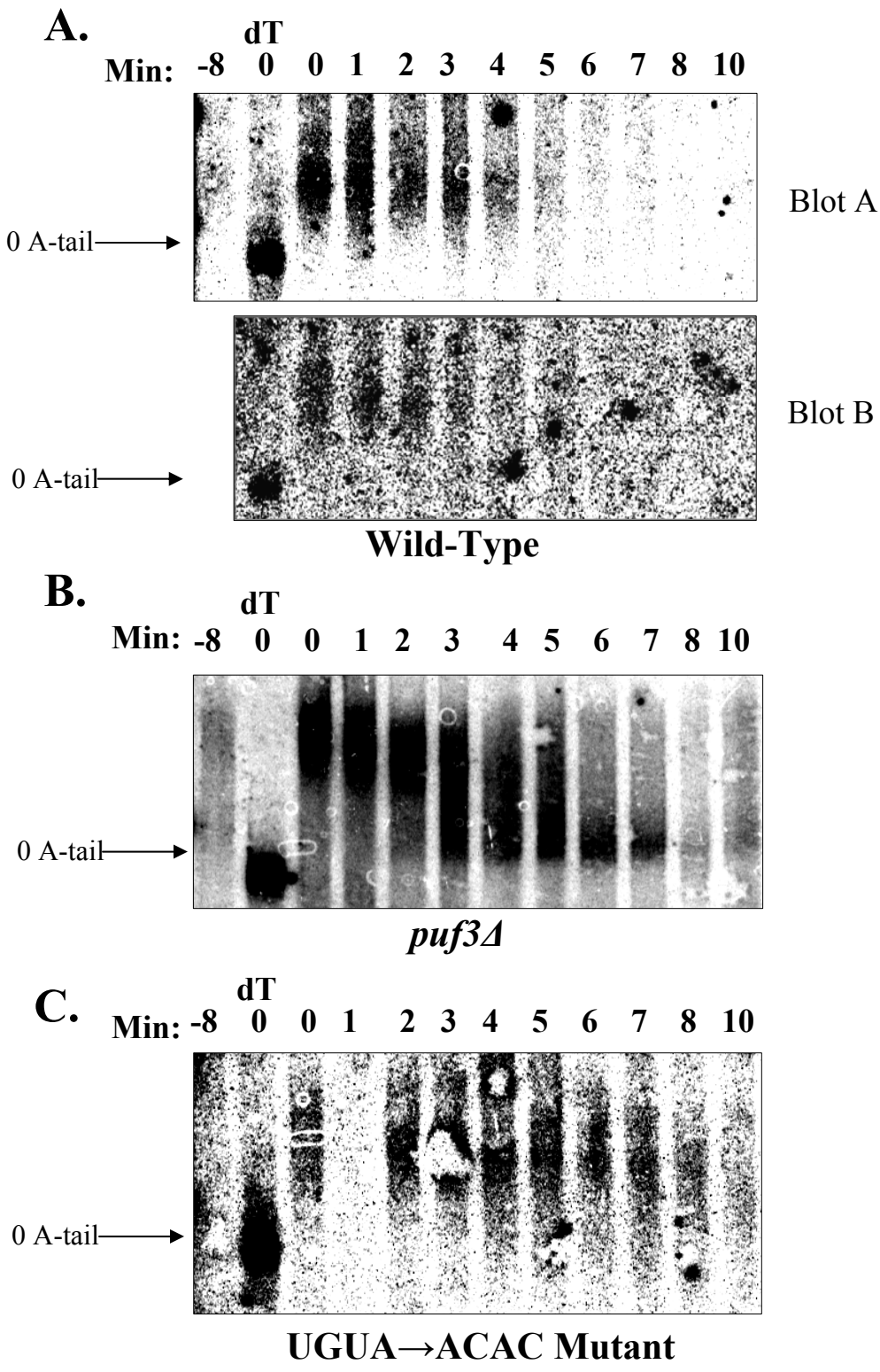

A.
dT
Min: -8 0 0 1 2 3 4 5 6 7 8 10
0 A-tail
Blot A
0 A-tail
Blot B
Wild-Type
B.
dT
Min: -8 0 0 1 2 3 4 5 6 7 8 10
0 A-tail
puf3Δ
C.
dT
Min: -8 0 0 1 2 3 4 5 6 7 8 10
0 A-tail
UGUA→ACAC Mutant

Figure 14. Puf3p promotes deadenylation and decay of *CYT2* mRNA. Shown are Northern blot analyses from transcriptional pulse chase experiments analyzing the decay of *CYT2*. Experiments were performed in (**A**) WT and (**B**) *puf3Δ* strains to detect the wild-type *CYT2* 3'UTR. **(A)** Shown are two blots (BlotA and BlotB) from independent experiments, in which *CYT2* mRNA is rapidly deadenylated and degraded in the WT strain. (**C**) Experiments were also performed in WT strains to detect the *CYT2mut* 3'UTR in which the UGUA element is mutated to ACAC. The minutes after transcriptional repression are indicated. The -8 lane refers to background levels of *CYT2* expression prior to the addition of galactose. The 0(dT) lane refers to RNA from the 0 time point that has the poly(A) tail removed. The arrow marks the deadenylated *CYT2* RNAs.

Condition-Specific Activity of Puf3p

Yeast must be able to regulate global gene expression in response to different environmental changes and stresses. Furthermore, they must quickly adapt to the slightest changes in temperature, osmolarity, nutrient availability, and exposure to chemicals. In fact, yeast may alter the expression of thousands of genes to adapt to these changes (Gasch et al, 2000).

Foat et al. used the MatrixREDUCE algorithm in conjunction with experimental microarray data from the *S. cerevisiae* genome to identify potential Puf3p targets, including *CYT2* and *TUF1* mRNAs, which I have experimentally confirmed as true targets of Puf3p regulation. In addition, this mathematical analysis also predicted that Puf3p target expression may be altered by different environmental stresses and growth conditions (Figure 8).

The algorithm predicts that the expression of transcripts containing the cis-regulatory binding site for Puf3p is upregulated in the presence of a non-fermentable carbon source, such as ethanol. This condition-specific regulation is supported by both experimental and microarray data that suggest that Puf3p targets, such as *COX17*, encode mitochondrial proteins (Foat et al, 2005). On the contrary, predictions for the expression of transcripts containing the Puf3p element in fermentable carbon source conditions are not straightforward. It is logical that Puf3p target expression would be downregulated in the presence of sugars, since the mitochondria would not be needed for fermentation. In glucose conditions, this prediction is true, as *COX17*, *CYT2* and *TUF1* transcripts are rapidly degraded in the presence of Puf3p. Alternatively, MatrixREDUCE predicts that

the expression of RNAs containing the Puf3p binding site will be upregulated in the presence of the sugars galactose and raffinose (Foat et al, 2005).

On the same accord, Puf3p expression or activity may be upregulated or downregulated during varying environmental conditions and stresses. Subjecting yeast cells expressing Puf3p to different growth conditions and stresses such as ethanol, galactose, and raffinose, provides insight into changes in cellular conditions that may regulate Puf3p activity. MatrixREDUCE provides information about steady-state expression of mRNAs containing Puf3p elements under different environmental conditions. However, this analysis does not demonstrate the relationship between Puf3p expression and the stability of these RNAs under these conditions. Decay analysis of these RNAs must be performed to confirm these predictions about differential Puf3p regulation of decay under these conditions.

In an effort to analyze the condition specific activity of Puf3p, I have used the bona fide Puf3p targets *CYT2* and *TUF1* mRNAs as reporters to monitor Puf3p activity. I have experimentally analyzed the decay of these transcripts in ethanol, galactose, and raffinose conditions to test the predictions made using MatrixREDUCE. In dextrose conditions, the algorithm predicts that the expression of Puf3p targets is downregulated (Foat et al, 2005). This prediction is confirmed by steady-state transcriptional shut off experiments in which the *COX17*, *CYT2*, and *TUF1* transcripts are rapidly degraded in the presence of Puf3p. These results suggest that yeast Puf3p is highly active in the preferred carbon source dextrose. Alternatively, *COX17* mRNA is stabilized when subjected to a non-fermentable carbon source such as ethanol in the presence of Puf3p, exhibiting a decay phenotype identical to that observed in a *puf3Δ* strain in dextrose

conditions (Foat et al, 2005). Together, these results suggest that Puf3p is inactive in ethanol conditions. It is possible that Puf3p may not be inactivated by all of the conditions that are predicted to impair its activity. Alternatively, if Puf3p activity is inactivated in all of the predicted conditions, it is possible that Puf3p activity may not be affected equally by all conditions. Moreover, Puf3p may only be partially inactivated in one condition, but completely inactivated in another. Finally, the degree of Puf3p regulation may not be the same for all RNA targets under these different environmental conditions. To distinguish between these possibilities, the decay of *TUF1* and *CYT2* mRNAs in ethanol, galactose, and raffinose conditions were determined to see if they exhibited similar decay phenotypes exhibited by *COX17* in the presence of Puf3p. Furthermore, I wanted to determine if these Puf3p targets were coordinately regulated by Puf3p in these varying conditions.

Steady-state transcriptional shut off experiments were performed with WT and *puf3Δ* yeast strains bearing the *rpb1-1* ts allele. Cells were grown in YEP media supplemented with 2% ethanol, galactose, or raffinose, and harvested at OD_{600}=0.4, when cells are in the log growth phase. The shut off, sample collection, RNA extraction, and Northern blot analyses were performed as previously described.

Coordinate Regulation of Puf3p Targets is Condition-Specific

The decay analysis of *CYT2* and *TUF1* RNAs in ethanol conditions yielded results that were similar to those observed for *COX17* mRNA, in which the transcripts were stabilized even in the presence of Puf3p. For *CYT2* mRNA, the results were particularly interesting. In the presence of Puf3p, *CYT2* was rapidly degraded in dextrose

conditions with a half-life of 1.7 ± 0.1 minutes. In ethanol conditions, this transcript was stabilized with a half-life of 11.9 ± 1.0 minutes (Figure 15A). Furthermore, *CYT2* was more stable in ethanol conditions than in *puf3Δ*-dextrose conditions, in which the RNA has a half-life of 4.6 ± 0.5 minutes. Shut off experiments were also performed in *puf3Δ*-ethanol conditions to eliminate the possibility that these observations were simply due to the media containing ethanol. In these conditions, *CYT2* was stabilized similarly as in WT-ethanol conditions, with a half-life of 10.1 ± 0.8 minutes. Thus, while ethanol may have some general effect on *CYT2* stability, the fact that the presence or absence of Puf3p has no effect on *CYT2* decay suggests that Puf3p activity is severely reduced or inhibited in ethanol

Analysis of *TUF1* revealed that this mRNA was rapidly degraded with a half-life of 5.5 ± 0.6 minutes in WT-dextrose conditions. While in ethanol conditions, *TUF1* was stabilized 3-fold to a half-life of 17.9 ± 0.9 minutes (Figure 15B). This result is similar to the observed stabilization of the transcript in *puf3Δ*-dextrose conditions, in which *TUF1* decayed with a half-life of 16.7 ± 0.7 minutes. The ethanol present in the media did not appear to be responsible for the observed decay phenotype, as the half-life of *TUF1* in *puf3Δ*-ethanol conditions was 18.0 ± 0.7 minutes. Together, the results of the decay analysis of *COX17*, *CYT2*, and *TUF1* mRNAs demonstrate that ethanol is a severe inhibitor of Puf3p activity. As a consequence of the inhibition of Puf3p activity, ethanol causes a stabilizing effect on these transcripts, in which *CYT2* mRNA is most greatly affected.

In contrast to ethanol growth conditions, the stabilities of *CYT2*, *TUF1*, and *COX17* mRNAs were not equally affected by galactose, suggesting that the extent of

Puf3p activity in galactose is transcript-dependent. In WT-galactose conditions, *COX17* mRNA was stabilized to a half-life of 10.5 minutes, suggesting that Puf3p activity is inhibited by galactose (Lopez Leban, personal communication). Similarly, *CYT2* mRNA was stabilized ~3.5-fold to a half-life of 6.1 ± 0.6 minutes in WT-galactose media versus a half-life of 1.7 ± 0.1 minutes in WT-dextrose (Figure 15C). This transcript had a half-life of 6.6 ± 0.9 minutes in *puf3Δ*-galactose conditions, suggesting that the changes in decay were due to Puf3p activity. *TUF1* mRNA was previously shown to have a half-life of 5.5 ± 0.6 minutes in WT-dextrose conditions. In galactose conditions, the transcript was stabilized 2.4-fold to a half-life of 13.2 ± 0.7 minutes (Figure 15D). However, in *puf3Δ*-dextrose and *puf3Δ*-galactose conditions, the transcript was even further stabilized, given half-lives of 16.7 ± 0.7 and 20.2 ± 1.5 minutes, respectively. These results suggest that there is residual Puf3p activity in galactose conditions that can at least partially function on the *TUF1* mRNA. Because the tested mRNAs are not regulated by Puf3p to the same extent in galactose conditions, these results emphasize the transcript-specific nature of Puf3p activity even between verified targets.

In raffinose conditions, *COX17* decayed slowly, with a half-life of 10.5 minutes, which indicates that Puf3p activity is inhibited (Lopez Leban, personal communication). *CYT2* also decayed slowly given a half-life of 6.5 ± 0.5 minutes versus a half-life of 1.7 ± 0.1 minutes in dextrose conditions (Figure 15E). In *puf3Δ*-dextrose conditions, this transcript had a half-life of 4.6 ± 0.5 minutes, suggesting that Puf3p activity is severely inhibited in the presence of raffinose. Surprisingly, *CYT2* mRNA was extremely stable in *puf3Δ*-raffinose conditions. As observed from multiple independent experiments, this transcript had a half-life greater than 60 minutes. Analysis of *TUF1* mRNA yielded more

expected results, in which this transcript was stabilized 2.8-fold to a half-life of 15.7 ± 1.2 minutes in raffinose versus WT-dextrose conditions. This transcript was similarly stabilized in *puf3Δ*-dextrose and *puf3Δ*-raffinose conditions, as *TUF1* had half-lives of 16.7 ± 0.7 and 15.9 ± 0.9 minutes, respectively. These results strongly suggest that the altered decay rate of *TUF1* in raffinose conditions is due to Puf3p activity. Together, the decay analysis of *COX17*, *CYT2*, and *TUF1* mRNA in different environmental conditions suggest that these conditions inhibit Puf3p activity, albeit to different extents for different target mRNAs.

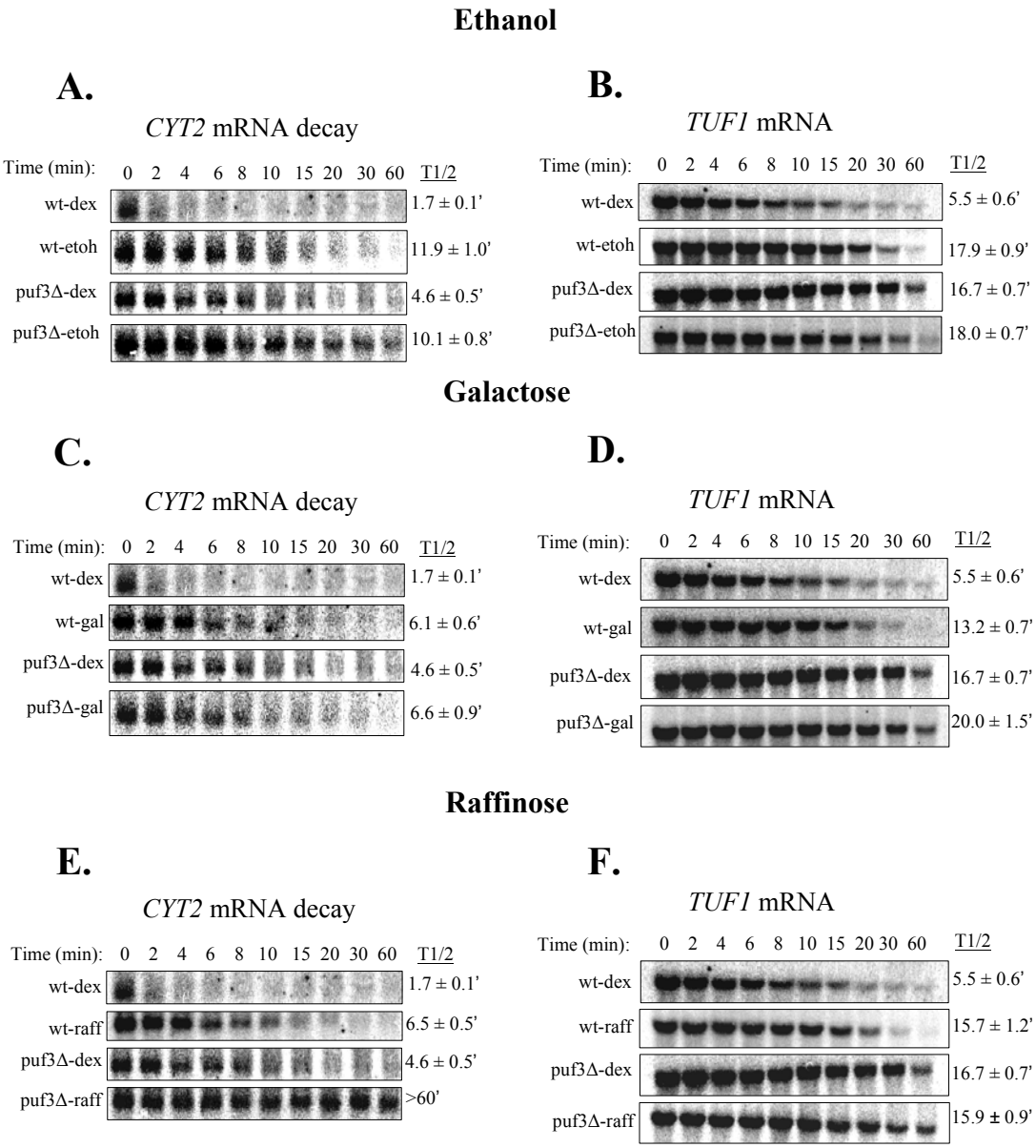


Figure 15. Decay analysis of TUF1and CYT2 mRNAs in different carbon sources. Shown are Northern blots of the decay of CYT2 and TUF1 in **A, B** ethanol **C, D** galactose and **E, F** raffinose. The average half-lives (T1/2) with the standard error of the mean are determined from multiple experiments.

Dynamics of Puf3p Condition-Specific Activity

After assessing the condition-specific activity of Puf3p in different cellular environments, I wanted to determine how quickly Puf3p could be activated or inactivated by changing the available carbon source. In the previous section, I determined that Puf3p-mediated decay activity was partially or severely inhibited in galactose, raffinose, or ethanol conditions. The method by which I performed transcriptional pulse-chase assays required me to grow cells overnight in raffinose, and then incubate cells in the presence of galactose for 8 minutes to create a pulse of newly transcribed RNAs. Both the raffinose and galactose conditions would be expected to inhibit Puf3p activity. Subsequently, the carbon source was changed to dextrose, at which point a time course sample collection was immediately performed to analyze the decay of these RNAs. Data from the transcriptional pulse-chase experiment showed that RNAs were rapidly deadenylated and decayed after the addition of dextrose, suggesting that Puf3p was rapidly activated upon dextrose addition. Based on this observation, I wanted to study the dynamics of Puf3p activity by determining how quickly Puf3p could be activated or deactivated by altering the available carbon source.

In an effort to assess these dynamics, I chose to use *CYT2* and *TUF1* mRNAs as reporters of Puf3p activity. I performed modified steady-state transcriptional shut off experiments with WT and *puf3Δ* yeast strains bearing the *rpb1-1* ts allele. In order to determine how quickly Puf3p could be activated by a change in carbon source, cells were first grown overnight in YEP media supplemented with 2% raffinose, which inhibit Puf3p activity, and harvested at OD_{600}=0.4. Cells were then quickly resuspended in YEP media containing 2% dextrose and were incubated for 10, 5, or 2 minutes prior to

transcriptional repression and time course RNA sample collection. Alternatively, to determine how quickly Puf3p could be inactivated by a change in carbon source, cells were grown in YEP media supplemented with 2% dextrose and harvested at OD_{600}=0.4. Cells were quickly resuspended in media containing 2% galactose or raffinose and were incubated for 10, 5, or 2 minutes, prior to transcriptional repression. The transcriptional shut off, sample collection, RNA extraction, and Northern blot analyses were performed as previously described.

The Status of Puf3p's Activity May be Altered in as Little as Two Minutes

I first analyzed the dynamics of Puf3p activity using the *TUF1* transcript as a reporter. Figure 16A shows the results of *TUF1* decay analysis in conditions that activated Puf3p. For cells continuously grown in WT-dextrose conditions, *TUF1* decays rapidly with a half-life of 5.5 ± 0.6 minutes while in cell grown continuously in WT-raffinose, *TUF1* decays with a half-life of 15.7 ± 1.2 minutes. When cells were grown in raffinose to log phase, and then incubated with dextrose for 10 minutes, *TUF1* decayed as quickly as transcripts grown continuously in dextrose conditions with a half-life of 6.5 ± 0.5 minutes. When cells were briefly incubated with dextrose for 2 minutes, these *TUF1* transcripts still decayed rapidly with a half-life of 9.0 ± 0.6 minutes. To ensure that these results were due to the incubation with dextrose, and not simply the process of collecting and resuspending cells in fresh media, cells were grown in raffinose to log phase and then collected and incubated in fresh media containing raffinose for 2 minutes. *TUF1* mRNAs decayed slowly with a half-life of 14.2 ± 1.2 minutes, which was nearly identical to the half-life of transcripts grown continuously in WT-raffinose conditions (T1/2 = 15.7 ± 1.2 minutes). The results of these experiments indicate that shift of the cells to new media

did not affect the stability of the transcripts, but that Puf3p can be activated within 2 minutes of changing the available carbon source to dextrose.

In Figure 16B, cells were continuously grown in dextrose to log phase and then briefly incubated with raffinose to inactivate Puf3p. For cells that were incubated with raffinose for 10 minutes prior to transcriptional repression, *TUF1* transcripts were stabilized 2.5-fold to a half-life of 13.8 ± 3.1 minutes versus 5.5 ± 0.6 minutes in WT-dextrose conditions. This stabilization was similar to that seen as for *TUF1* from yeast grown continuously in WT-raff conditions, which had a half-life of 15.7 ± 1.2 minutes. When cells were incubated with raffinose for 5 minutes, *TUF1* was stabilized similarly as for transcripts isolated from cells that were incubated with raffinose for 10 minutes with a half-life of 18.0 ± 4.0 minutes. When cells were incubated with raffinose for only 2 minutes, *TUF1* transcripts were stabilized with a half-life of 12.0 ± 4.0 minutes, indicating that Puf3p can also be inactivated in as little as 2 minutes. I cannot account for the large degree of variation among the half-lives of *TUF1* in individual experiments. However, this variation does not appear to be a direct result of changing the media, as *TUF1* from cells grown continuously in WT-dextrose conditions or in the WT-dextrose to dextrose control conditions decay rapidly with identical half-lives.

Next I wanted to analyze the dynamics of Puf3p activity using *CYT2* mRNA as a reporter. Figure 16C demonstrates how quickly Puf3p can be activated. *CYT2* decays rapidly in WT-dextrose conditions, when Puf3p is highly active, with a half-life of 1.7 ± 0.1 minutes. When cells were grown in raffinose to log phase and then incubated with dextrose for 10 or even 2 minutes, *CYT2* decayed rapidly with a half-life of 3.0 minutes versus 4.6 ± 0.6 minutes in *puf3Δ*-dextrose conditions, again supporting that Puf3p can be

activated in as little as 2 minutes. These results were due to changes in Puf3p activity and not the change in media, as *CYT2* from cells subjected to WT-raffinose to raffinose conditions was stabilized similarly as for *CYT2* from cells grown in WT-raffinose conditions with half-lives of 5.0 minutes and 6.5 ± 0.8 minutes, respectively.

In Figure 16D, I determined how quickly Puf3p could be inactivated using *CYT2* as a reporter. Again, this transcript is rapidly degraded in the presence of dextrose and Puf3p and has a half-life of 1.7 ± 0.1 minutes. However, when cells were grown in dextrose to log phase and were then incubated with raffinose for 10 or 2 minutes, these *CYT2* transcripts were stabilized similarly with half-lives of 9.3 ± 1.2 and 8.7 ± 1.3 minutes, respectively. In addition, these transcripts were stabilized similarly as for *CYT2* from cells grown continuously in raffinose, which had a half-life of 6.5 ± 0.8 minutes. *CYT2* from cells grown in WT-dextrose to dextrose control conditions decayed rapidly with a half-life of 2.0 minutes, indicating that these results were due to inhibition of Puf3p activity.

In summary, Puf3p's mRNA destabilizing function can be rapidly activated within 2 minutes after incubation with the carbon source dextrose, as determined by monitoring the stabilities of *TUF1* and *CYT2* mRNAs. Puf3p is also rapidly inactivated in as little as 2 minutes upon incubation with raffinose. However, when Puf3p activity changes from an active state to an inactive state, this process does not appear to be as tightly regulated. The decay of *CYT2* and *TUF1* in dextrose to raffinose shut off experiments varied greatly, as exhibited by large standard error of the mean values which ranged from 3 to 4 minutes for *TUF1*, and 1.2 to 1.3 minutes for *CYT2*. In contrast, *TUF1* and *CYT2* had very small standard error of the mean values in raffinose to dextrose

shut off experiments. Despite the variance among these experiments, the activity status of Puf3p can be altered quickly, which is indicative of a post-translational mechanism of condition-specific regulation.

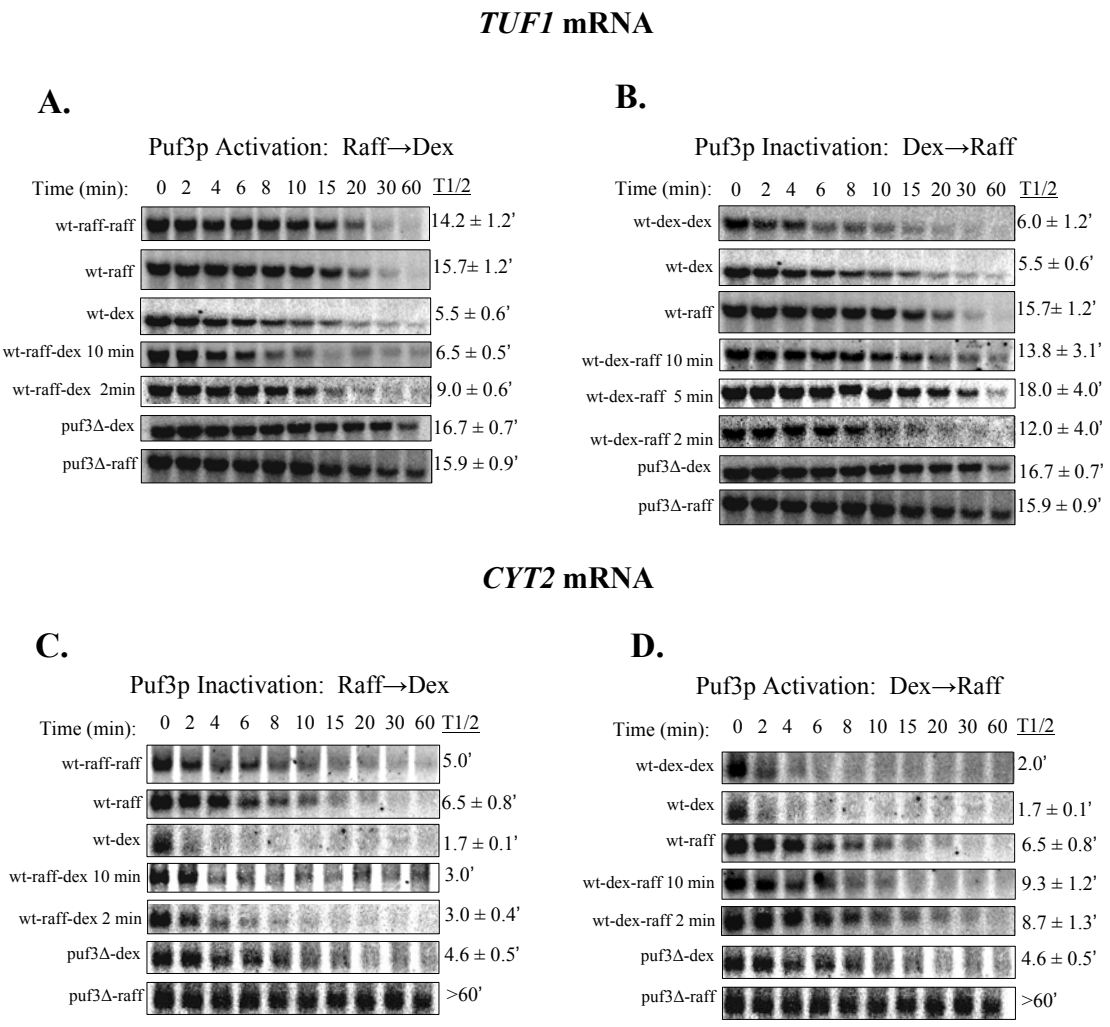


Figure 16. Decay analysis of *TUF1*and *CYT2* mRNAs in altered carbon sources. Cells were grown to log phase in media containing the first indicated carbon source. The cells were then shifted to the second carbon source (indicated to the right of the arrow) and incubated for 10, 5, or 2 minutes prior to transcriptional repression. **A.** Shown are Northern blots of the decay of *TUF1*mRNA for cells initially grown in raffinose and then shifted to dextrose-containing media. **B.** Shown are Northern blots of the decay of *TUF1*mRNA for cells initially grown in dextrose and then shifted to raffinose-containing media. **C.** Shown are Northern blots of the decay of *CYT2* mRNA for cells initially grown in raffinose and then shifted to dextrose-containing media. **D.** Shown are Northern blots of the decay of *CYT2* mRNA for cells initially grown in dextrose and then shifted to raffinose-containing media. The average half-lives (T1/2) with the standard error of the mean are determined from multiple experiments.

Chapter III: Discussion

In this work, I have experimentally analyzed several mRNAs that were predicted to be regulated by Puf3p based on computational and microarray studies. Decay analysis of these transcripts has provided a wealth of information about key features of mRNAs that are subjected to Puf3p-mediated decay. In addition, I have identified several bona fide mRNA targets of Puf3p that are destabilized in the presence of this protein, and I have further analyzed two of these transcripts, *CYT2* and *TUF1*. Condition-specific regulation of these mRNAs as a consequence of Puf3p activation versus inhibition has also been observed. Together, this work provides knowledge about the means by which Puf3p selects and regulates target mRNAs, as well as the condition-specific regulation of Puf3p activity.

Degree of Puf3p Regulation is Not Correlated with Number of Binding Sites

I initially performed in vivo decay analyses of fourteen putative Puf3p mRNA targets that encoded mitochondrial proteins and contained one or more conserved Puf3p binding sites similarly as for *COX17* mRNA. Considering that Cox17p functions as a mitochondrial copper transporter, I also selected one mRNA that encoded a plasma membrane copper transporter to examine the possibility that Puf3p selectively regulates mRNAs that encode proteins with a generalized copper transporter function.

As shown in table 1, decay analysis of *COX17* mRNA, which contains two conserved Puf3p binding sites, demonstrated a greater than 5-fold difference in the half-life between *puf3Δ* and WT strains. One might speculate that there is a correlation between the number of Puf3p binding sites within the 3'UTR and the degree of Puf3p regulation, predicting that transcripts containing multiple Puf3p binding sites would be

more greatly stabilized in the absence of Puf3p as demonstrated by a very long half-life versus transcripts with only one Puf3p site. On the contrary, the results summarized in Table 1 illustrate that several mRNAs containing two or more Puf3p binding sites, such as *MAS6*, *MRP1, MRPL6, MRPL39* and *RSM19,* did not display nearly as large a degree of regulation by Puf3p in comparison with *COX17*. These five transcripts had a 1.6-2.8-fold differences in their half-lives between *puf3Δ* and WT strains versus 5-fold with *COX17*. Even a single highly conserved binding site can stimulate regulation by Puf3p to a similar extent as seen for *MAS6* and *RSM19*, as *CYT2* had a 2.7-fold difference in half-life between the WT and *puf3Δ* strains. *MAS6* mRNA contains six conserved UGUA sites within the 3'UTR, in which five of these sites were in the context of the 10nt Puf3p binding site. Although this transcript has numerous binding sites, there was only a 2.8-fold difference in its half-life between *puf3Δ* and WT strains. Three of the transcripts analyzed, *MRP1*, *MRPL6*, and *RSM10*, each contained two binding sites, in which one of the sites was highly conserved. Despite the presence of these sites, *MRP1* and *MRPL6* only displayed a small degree of regulation by Puf3p (1.6-fold), and the stability of *RSM10* was not affected by Puf3p. Interestingly, *CTR1* mRNA, which encodes a plasma membrane copper transporter, displayed a very large degree of regulation by Puf3p (6.5-fold). The *CTR1* 3'UTR contains a single conserved Puf3p element within the 3'UTR, which appears to be sufficient to simulate a large degree of regulation by Puf3p. In contrast, complete Puf3p-mediated stability regulation of *COX17* required two conserved binding sites. Further analysis of *CTR1* must be conducted to determine the means by which Puf3p is regulating this transcript. With both *CTR1* and *COX17* encoding copper transporters, these observations suggest that Puf3p may have a specific role in regulating

copper transport, although additional transcripts should be experimentally analyzed to further support this prediction. Moreover, the results from the preceding experiments indicate that there is not a clear correlation between the number of Puf3p binding sites and the degree of Puf3p regulation.

Conservation of Puf3p Binding Sites Is Important for Stimulating Puf3p Regulation

Gel mobility shift assays performed with the Puf3RD and short *COX17* RNA sequences that encompass a single Puf3p binding site suggest that a minimal UGUA element as well as short sequences downstream of this site are required for Puf3p to bind *COX17*. In addition, mutation of a single Puf3p binding site in the full-length *COX17* 3'UTR results in an intermediate decay phenotype in which the transcript decays more slowly than the wild-type *COX17* 3'UTR, but decays faster than the wild-type *COX17* 3'UTR in the absence of Puf3p. If both Puf3p binding sites in the *COX17* 3'UTR are mutated, this RNA displays a decay phenotype essentially identical to the wild-type *COX17* 3'UTR in a *puf3Δ* strain (Jackson et al, 2004).

Based on this observation with *COX17*, I hypothesize that the conserved 10nt binding site is required for the full degree of Puf3p regulation. For example, *MAS6* mRNA contains five 10nt Puf3p binding sites, in which three of the 10nt core binding sites lack some conservation in two or more nucleotide positions, and the mRNA only has a 2.8-fold difference in half-life between WT and *puf3Δ* strains (Table 1). In addition, *PET123* mRNA has two binding sites, yet nucleotides in positions 7-10 of the first site are not conserved, and the mRNA exhibits a 4.2-fold difference in half-life between WT and *puf3Δ* strains. In comparison, *COX17* mRNA has two highly conserved

Puf3p binding sites and has a 5.7-fold difference in its half-life. After assessing the decay of the fourteen candidate mRNAs, I hypothesize that RNAs with multiple Puf3p binding sites such as MAS6 display a relatively smaller degree of regulation by Puf3p versus *COX17*, because Puf3p likely has a lower affinity to bind Puf3p binding sites that lack full conservation. Most of the RNAs analyzed contain a single highly conserved Puf3p binding site, along with at least one site that lacks conservation in the sequence found downstream of the UGUA tetranucleotide, with the exception of *CYT2* and *COX17*. *CYT2* contains one highly conserved binding site and a 2.7-fold difference in decay rates. *COX17* contains two highly conserved binding sites and has 5.7-fold difference in decay rates, which is 2 times greater than *CYT2* containing only one site. This general trend may explain why many of these multi-site transcripts display a smaller degree of regulation by Puf3p versus *COX17*. On the contrary, if *MAS6* contained five highly conserved Puf3p binding sites, I might expect the degree of Puf3p regulation to be greater than that of *COX17*. In this hypothetical instance, I would expect that the presence of multiple conserved binding sites would produce an additive effect in terms of the degree of Puf3p regulation. However, it is possible that there is a kinetic limit to how much decay stimulation can be mediated by additive Puf3p effects, especially when half-lives are short. In other words, can three or more Puf3 proteins stimulate decay significantly more than two? In future studies, I will analyze the three Puf3p binding sites in the *TUF1* 3'UTR to determine if Puf3p normally regulates these sites in an additive manner, and/or if mutation of these sites to more conserved sequences allows additive Puf3p regulation.

Several factors appear to influence Puf3p's selection of target mRNAs, as the presence of one or more conserved binding sites alone does not necessarily confer Puf3p regulation upon RNA targets. The degree of conservation in the Puf3p binding site may also be a determinant of how well Puf3p is able to regulate a transcript. Additionally, while the function or mitochondrial localization of the protein encoded by a transcript may be used to initially identify a pool of mRNAs that are likely regulated by Puf3p, these transcripts must be experimentally validated. I have identified several transcripts from this pool as bona fide Puf3p targets, including *CYT2* and *TUF1*. Interestingly, there are not any apparent differences between conserved cis-elements located within *CYT2* and *TUF1* versus those found in mRNAs that are not affected by Puf3p or only display a small degree of Puf3p regulation. This suggests that the 3'UTRs of Puf targets may contain additional determinants that are required for Puf3p regulation. This is consistent with the observation that *S. cerevisiae* Puf3p, human PUM1, murine PUM2, *Drosophila* Pum, and *Xenopus* Pum share conserved UGUANAUA binding sequences (Jackson et al, 2004; Zamore et al, 1997; Wang et al, 2002; White et al, 2001; Nakahata et al, 2001; Murata and Wharton, 1995). Therefore, yeast Puf3p must recognize additional 3'UTR determinants outside of the 10nt binding site which determine the specificity for Puf3p target binding.

***CYT2* and *TUF1* are Regulated by Puf3p**

Decay analysis with only the *CYT2* and *TUF1* 3'UTRs was performed to assess whether there were any determinants in the 5'UTR or coding region of these transcripts that were required for stimulating Puf3p-mediated decay (Figure 11). The *COX17*

3'UTR had previously been fused to the coding region of *MFA2* mRNA, which is not regulated by Puf3p, and the 3'UTR region alone was sufficient to destabilize this chimeric transcript in the presence of Puf3p. Fusion of the *CYT2* 3'UTR to *MFA2* yielded similar results in which the transcript was rapidly degraded in the presence of Puf3p, but stabilized in the absence of this protein. I also fused the *TUF1* 3'UTR to the *MFA2* coding region, but this failed to stabilize the transcript in the absence of Puf3p. I attribute this inability of the *TUF1* 3'UTR to induce differential decay in the presence and absence of Puf3p to the fact that *MFA2* itself is highly unstable. Specifically, the decay of *MFA2* alone is nearly as rapid as Puf3p-induced decay of *TUF1*. It is likely that the determinants within the 5'UTR and coding region of *MFA2* that confer its innate instability can overpower any stabilizing effect attributed to the *TUF1* 3'UTR even in the absence of Puf3p. As a result, I was unable to visualize differences in the decay rate of *MFA2-TUF1* 3'UTR between WT and *puf3Δ* strains. In contrast, both the *COX17* and *CYT2* 3'UTRs could further destabilize *MFA2* in the presence of Puf3p, since their native Puf-induced decay rates are faster than *MFA2* alone.

On the other hand, the *PGK1* transcript is innately stable due to determinates located in its coding region. Deletion of 82% of the coding region decreases the stability of this transcript and reduces the half-live from 45 minutes for the full-length transcript to 27 minutes. Fusion of the 3'UTR of the unstable *STE3* mRNA to the shortened *PGK1* coding region further destabilizes the transcript, giving a half-life of 5 minutes (Heaton et al, 1992). Based on these results, I then fused the *TUF1* 3'UTR to the shortened *PGK1* coding region, which was significantly more stable than *MFA2*, but could still be controlled by the presence of other destabilizing elements or potentially, trans-acting

factors. The *PGK1Δ82-TUF1* 3'UTR fusion transcript was rapidly degraded in a WT strain, and was stabilized 2-fold in a *puf3Δ* strain. Together, the results of the *MFA2-CYT2* 3'UTR and *PGK1Δ82-TUF1* 3'UTR decay analysis demonstrated that the 3'UTRs of *CYT2* and *TUF1* alone were sufficient to stimulate Puf3p-mediated turnover similarly as previously shown for *COX17*. This also suggests that the unknown determinants that may surround the 10nt conserved Puf3p binding site to affect stimulation of Puf3p-destabilizing activity are also located in the 3'UTR.

UGU elements located within the 3'UTRs of eukaryotic transcripts that are targeted by Puf proteins are required for decay regulation. Moreover, UGUA elements have been shown to be important for Puf3p binding and regulation of *COX17* mRNA. Further analysis of the 3'UTR of *CYT2* mRNA revealed that the UGUA tetranucleotide was also required for regulation by Puf3p. Mutation of this sequence to ACAC resulted in a decay phenotype that was similar to that of a wild-type *CYT2* 3'UTR in the absence of Puf3p, suggesting that the UGUA element may be important for recognition and regulation of all Puf3p mRNA targets. In the future, I want to analyze the decay of the *TUF1* 3'UTR, containing three UGUA elements, in which two of these elements are in the context of the 10nt binding site. I wish to mutate each of these elements separately, and then create a triple mutant to determine if Puf3p regulates these sites in an additive manner similar to that previously shown with *COX17*.

A Single Puf3p Binding Site is Sufficient to Promote Deadenylation and Decapping

Analysis of the poly(A) tail lengths of *COX17* during turnover in the presence and absence of Puf3p revealed that *COX17* stimulated deadenylation and subsequent

decapping steps in the decay pathway (Olivas and Parker, 2000). The *COX17* 3'UTR contains two Puf3p binding sites, suggesting the possibility that one Puf3 protein can bind one site to stimulate deadenylation, while another Puf3 protein binds the other site to stimulate decapping for the most efficient turnover of *COX17*. Therefore, in this model, a single Puf protein can stimulate only one step of decay. This may be supported by the observation that many of the transcripts that I identified as targets of Puf3p contain multiple binding sites within their 3'UTRs. Mutation of either Puf3p binding site results in partial regulation by Puf3p, as demonstrated by less efficient turnover and a slower decay rate (Jackson et al, 2004). Alternatively, I hypothesized that the single Puf3p binding site of *CYT2* can bind one Puf3 protein that will stimulate both deadenylation and decapping of this transcript.

Transcriptional pulse-chase analysis of the *CYT2* 3'UTR revealed that *CYT2* mRNAs are deadenylated rapidly to an oligo(A) length between 1 to 2 minutes, and the transcripts are almost fully degraded 5 minutes post transcriptional repression (Figure 14A). Decapping of *CYT2* transcripts occurs rapidly, as these RNAs are not fully deadenylated prior to degradation. The deadenylation and decapping steps of decay for *CYT2* are not distinct. On the contrary, *COX17* mRNA deadenylation occurs in two distinct phases. In the first phase *COX17* is deadenylated slowly for 2 minutes post transcriptional repression, which is followed by a fast phase of deadenylation where poly(A) shortening occurs and *COX17* is fully deadenylated between 2 and 4 minutes after transcriptional repression (Olivas and Parker, 2000). However, in the absence of Puf3p, *CYT2* and COX17 display similar decay phenotypes in which the pool of transcripts is deadenylated at a slow rate. Once the main pool of transcripts become

shortened to an oligo(A) length or are fully deadenylated, they accumulate and persist in this state. Oligo(A) and fully deadenylated *CYT2* species specifically persist to 10 minutes after transcriptional repression, suggesting that the decay steps following deadenylation, such as decapping, is also slowed. The comparison of the deadenylation rates for *CYT2* and *COX17* highlights the fact that deadenylation is a rate limiting step in the turnover of mRNAs. Each Puf3p target mRNA is deadenylated at different rates. More importantly, these results indicate that a single Puf3p protein may interact with decay factor complexes to stimulate deadenylation and decapping of its target mRNA. This also indicates that Puf3p promotes the turnover of its RNA targets via a conserved mechanism.

Puf3p Activity is Regulated in a Condition-Specific Manner

The use of global genome expression data is a useful resource to help identify key factors that regulate gene expression. The use of microarray datasets along with the algorithm MatrixREDUCE predicted that Puf3p is a condition-specific regulator of mRNAs that encode mitochondrial proteins. In ethanol conditions, Puf3p was inactivated, as the half-lives of *CYT2* and *TUF1* mRNAs were similar to their half-lives in *puf3Δ*-dextrose conditions (Figure 15), which is consistent with previous observations for *COX17* mRNA. *CYT2* mRNA was affected by ethanol to a greater extent than *TUF1* and *COX17*, as this transcript was stabilized ~2.5-fold greater in WT-ethanol conditions versus *puf3Δ*-dextrose conditions. Ethanol appears to have a general effect on *CYT2* stability, but the increased stabilization may be attributed to inhibition of Puf3p activity, as the presence or absence of Puf3p did not affect the turnover of this transcript in

ethanol. Together, these results suggest that ethanol is an inhibitor of Puf3p activity, and Puf3p's activity under these conditions is not RNA-specific.

In raffinose, Puf3p activity is inhibited as assessed by the stabilization of the *CYT2* and *TUF1* mRNA reporters. Both of these transcripts have half-lives that are nearly identical to those seen in *puf3Δ*-dextrose conditions. However, raffinose appears to have a general effect on the stability of *CYT2,* and strangely, this transcript is stabilized to a half-live greater than 60 minutes in the absence of Puf3p as determined from multiple experiments. I cannot account for this extremely large stabilizing effect, but it seems that *CYT2* appears to be more sensitive to ethanol and raffinose.

In galactose, Puf3p activity is only partially inhibited for decay stimulation of *TUF1* mRNA, as it has a half-life of 13.2 ± 0.7 minutes versus 16.7 ± 0.7 minutes in *puf3Δ*-dextrose conditions and 20.2 ± 1.5 minutes in *puf3Δ*-galactose conditions (Figure 15D). However, *CYT2* appears to be differentially regulated by Puf3p under these conditions, as Puf3p activity is inhibited to a greater extent for function with this mRNA. Galactose, unlike ethanol and raffinose, appears to inhibit Puf3p activity in an mRNA-dependent manner. Together these results suggest that not all Puf3p mRNA targets are affected equally by different conditions. Additionally, not all conditions affect the activity of Puf3p equally, as its residual activity appears to be transcript-dependent.

Changes in Puf3p Activity Status May be Regulated Post-Translationally

I have determined that Puf3p activity status is dependent on the available carbon source and, in some cases, the transcript being regulated. Puf3p must be able to quickly alter its level of mRNA decay-stimulating activities in situations where there are rapid

environmental changes. For example, in transcriptional pulse-chase assays, *COX17* mRNAs were transcribed in the presence of galactose for 8 minutes, in which Puf3p activity is inhibited. Subsequently, the carbon source was changed to dextrose, at which time a time course sample collection was performed to analyze *COX17* decay. In dextrose conditions, Puf3p is highly active, which was demonstrated by rapid deadenylation of *COX17* after the addition of dextrose. Based on these observations in which Puf3p must quickly alter its activity status from "repressed" in galactose to "highly active" in dextrose conditions, there are a few mechanisms that may account for this.

One possibility was that *PUF3* expression is altered among these conditions. For example, *PUF3* expression would be downregulated in conditions that inhibited its activity, such as galactose, raffinose, and ethanol. Alternatively, it is possible that Puf3 proteins are forming aggregates in inhibitory conditions, and that these protein aggregates may localize to a part of the cell that is inaccessible to the targeted mRNA and/or the decay machinery. A final hypothesis is that Puf3p may be post-translationally modified in different environmental conditions that affect its activity. This final hypothesis is supported by the observation that when cells are grown to log phase in conditions that inhibit Puf3p activity, such as galactose or raffinose, and are then incubated with dextrose for two minutes prior to transcriptional repression, *CYT2* and *TUF1* mRNA display decay phenotypes similar to RNAs extracted from yeast cells that were continuously grown in dextrose conditions. Alternatively, when yeast were grown in dextrose to log phase, and were then incubated with raffinose for two minutes, *CYT2* and *TUF1* displayed slow decay phenotypes similar to RNAs extracted from yeast cells that were continuously grown in raffinose conditions.

Northern and Western analyses of *PUF3* mRNA and protein levels in dextrose, galactose, raffinose and ethanol conditions revealed that *PUF3* expression levels in galactose, raffinose, and ethanol was equal to or greater than mRNA and protein levels in dextrose conditions (Lopez Leban, personal communication). The fact that PUF3 mRNA and protein levels were not decreased in conditions that inhibited Puf3p activity supported the hypothesis that Puf3p may be differentally post-transcriptionally modified in activating versus inactivating conditions. To analyze this possibility, FLAG-tagged Puf3RDp was FLAG-affinity purified from yeast grown in dextrose and ethanol conditions. Phosphorylated Puf3RDp was detected both from samples purified from cells grown in dextrose and ethanol (Lopez Leban, personal communication). Although this data does not provide any information about the location of phosphorylation sites or the degree of phosphorylation in these different conditions, it demonstrates that Puf3p is phosphorylated, and supports the hypothesis that the condition-specific activity of Puf3p is likely regulated post-translationally.

In future studies, I would like to isolate Puf3RDp from cells grown in dextrose, galactose, raffinose, and ethanol conditions and analyze the location and number of phosphorylation sites using mass spectrometry. Also, I would like to identify kinases that phosphorylate Puf3p in response to nutrient status. In an effort to support or dispel the "Puf3p aggregate" hypothesis, I would like to analyze a Puf3p to GFP fusion to visualize the localization of Puf3p in cells grown in different conditions. Finally, I would like to analyze Puf3p's ability to interact with decay factors in different conditions using co-immunoprecipitation assays. Together, these experiments should help elucidate the mechanism by which Puf3p activity is regulated in a condition-specific manner.

Summary

In this thesis work, I have experimentally validated several new targets of the yeast Puf3 protein. Regulation of an mRNA by Puf3p appears to be dependent on several factors. Puf3p has been shown to regulate nuclear-transcribed mRNAs that encode mitochondrial proteins. Also, there does not appear to be a direct correlation between the number of Puf3p binding sites and the degree of regulation by Puf3p. However, conservation of a 10nt binding site appears to be important for full regulation by Puf3p, as mRNAs that contain highly conserved binding sites display a greater degree of regulation by Puf3p than RNAs that contain a similar number of binding sites that lack conservation. Moreover, additional elements in the 3'UTRs of targeted mRNAs appear to be required for Puf3p regulation, as mRNAs that are not regulated by Puf3p in this screen appear to have conserved cis-elements that are identical to true Puf3p targets. Puf3p appears to stimulate the turnover of its targets via a conserved mechanism, by which Puf3p stimulates deadenylation and decapping steps of decay. In addition, a single Puf protein is sufficient to interact with both deadenylation and decapping complexes to stimulate decay. Puf3p activity appears to be regulated in a condition-specific manner, in which Puf3p may be modified post-translationally to quickly adapt to changes in the available carbon source. My work on Puf3p in yeast will provide a greater understanding of general 3'UTR regulatory mechanisms. More importantly, since Puf proteins are highly conserved in eukaryotes and play an important role in both stem-cell and germ cell development, the knowledge from this work may be applied to promote a greater understanding of how Puf proteins affect gene regulation in humans, and other higher eukaryotes, as well.

Chapter IV: Materials and Methods

Yeast Strains

The genotypes of all *S. cerevisiae* strains used in the study are as follows: ywo7 MATα, *leu2-3, 112, ura 3-52, rpb1-1* (yRP693) (Olivas and Parker, 2000); ywo43 MATα, *his4-539, leu2-3, 112, ura3-52,* cup1::LEU2/PM, *rpb1-1*, puf3::Neo^r (yRP1360) (Olivas and Parker, 2000).

***In vivo* Decay Analysis**

Steady-state transcriptional shut off experiments were performed essentially as described (Caponigro et al, 1993) on yeast strains ywo7 (wild-type) and ywo43 (*puf3Δ*), which contain the temperature-sensitive *rpb1-1* allele for RNA polymerase II. The strains were grown in standard yeast extract/peptone (YEP) containing 2% glucose, galactose, raffinose, or ethanol at 24ºC to an OD_{600} of 0.4. Transcription was quickly repressed by shifting the culture to 37ºC. Total RNA was separated on 1% agarose gels and transferred to nylon membrane for probing with radiolabeled oligos complementary to the *CYT2* and *TUF1* mRNAs, o*CYT2*-P, GTATGTGGAAACTAGGGAGGGTATG and o*TUF1*-P, CGTGTGATTAGACCGGTACCAACAGTTC, respectively. Northern blots were normalized for loading by probing for 7S RNA, an RNA polymerase III transcript (Felici et al, 1989).

Steady-state transcriptional shut off experiments were also performed using strains ywo7 and ywo43, each transformed with plasmids expressing *MFA2* RNA (pRP485), a shortened *PGK1Δ82* transcript (pRP227) (Heaton et al, 1992), *MFA2-CYT2* 3'UTR RNA (pWO97), *MFA2-CYT2mut* 3'UTR (pWO98), or *PGK1 Δ82-TUF1* 3'UTR

RNA (pWO99). pRP485 was created as described (Decker and Parker, 1993), with the *MFA2* RNA expressed under the control of the GAL1 UAS.

The *MFA2* 3'UTR was replaced by the *CYT2* 3'UTR to produce pWO97 as follows: A 464 bp PCR product containing 448 bp of *CYT2* sequence 3' of the stop codon and BglII and HindIII restriction sites was inserted into pRP485 betweeen BglII and HindIII, creating pWO97. A mutant version of this plasmid, p*MFA2-CYT2mut* (pWO98) was generated by site-directed mutagenesis of pWO97 using the QuickChange XL Kit (Stratagene) and was verified by sequencing. The shortened *PGK1Δ82* transcript (pRP227) was created as described in Heaton et al, 1992, with the shortened RNA expressed under the control of the GAL1 UAS.

The *PGK1* 3'UTR of pRP227 was replaced by the *TUF1* 3'UTR to produce pWO99 as follows: A 490 bp PCR product containing 474 bp of *TUF1* sequence 3' of the stop codon and ClaI and HindIII restriction sites was inserted into pRP227 between ClaI and HindIII to create pWO99. Yeast strains transformed with pWO97, pWO98, and pWO99 were grown in standard YEP media containing 2% galactose to an OD_{600} of 0.4. Transcription was rapidly repressed by simultaneous shifting of the culture to 37 ºC and adding 4% glucose. Total RNA was separated on 1.25% agarose gels and transferred to nylon membrane for probing with radiolabeled oligos complementary to the *MFA2-CYT2* 3'UTR hybrid RNA at the *MFA2-CYT2* junction (o*CYT2*jct. GTAATTCACATCTCTTGGTTGTCGTC) (owo433) or the *PGK1Δ82-TUF1* 3'UTR hybrid RNA at the *PGK-TUF1* junction (o*TUF1*jct. CCAGTTGCACCAATAAGTCATCGATTTCAATTC) (owo452). Northern blots were normalized for loading using 7s RNA (Felici et al, 1989).

Modified steady-state transcriptional shut off experiments were also performed in which yeast strains ywo7 (wild-type, *rbp1-1*) and ywo43 (*puf3Δ, rpb1-1)* were grown in standard YEP media containing 2% galactose, raffinose, or ethanol to an OD_{600} of 0.4. The cultures were then centrifuged at 4000 rpm to pellet cells, and the cells were resuspended in YEP media containing a different carbon source with a final concentration of 2%. Cultures were incubated at 24ºC for 10, 5, or 2 minutes and promptly centrifuged at 4000 rpm to pellet cells. The cells were immediately shifted to YEP media heated to 37ºC. Northern analysis was performed using radiolabeled probes for *CYT2* and *TUF1* as previously described.

Transcriptional pulse chase experiments were performed essentially as described (Decker and Parker, 1993) on strains ywo7 (wild-type, *rbp1-1*) and ywo43 (*puf3Δ, rpb1-1).* Regulated expression of wild-type or mutant *MFA2-CYT2* 3'UTR hybrid RNA was accomplished by transformation of ywo7 or ywo43 with pWO97 or pWO98, in which the expression of the chimeric RNA is under the control of the GAL1 UAS. Cultures were grown in raffinose, which does not induce transcription of the fusion RNA, to an OD_{600} of 0.4. Cultures were then incubated with galactose to induce transcription of this RNA for 8 minutes to create a pulse of newly transcribed RNAs. Finally, dextrose was added to the media to repress transcription of this RNAs. Poly(A) tail lengths were monitored using RNaseH reactions as previously described (Muhlrad and Parker, 1992) with an oligo complementary to a sequence just upstream of the *MFA2* stop codon (o*CYT2*-C, CGATAACACAGGCGGGATCC) (owo432). Total RNA was separated on 6% denaturing polyacrylamide gels at 300V for 6h and then transferred to nylon membrane for probing with a radiolabeled oligo complementary to a sequence that spanned the

MFA2-*CYT2* junction (oCYT2-P, GTAGTAATTCAGATCTCTTGGTTGTCG) (owo433).

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