Puf Proteins: Regulation of Condition-Specific mRNA Decay and Contributions to Ribosome Biogenesis in Yeast

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Puf Proteins: Regulation of Condition-Specific mRNA Decay and Contributions to Ribosome Biogenesis in Yeast

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B.S. Biology, University of Missouri-St. Louis, 2005

A Dissertation Submitted to The Graduate School
at the University of Missouri-St. Louis
in partial fulfillment of the requirements for the degree
Doctor of Philosophy in Biology with an emphasis in Cell and Molecular Biology

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Dedication

I dedicate this work to my wife Amanda. She has been a constant source of love and support in my life, which I truly believe is the major factor in why I am not on a grocery list of antidepressants. I could not have accomplished this without your dedication to me and our family, and I am thankful every day you are in my life.
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I would first like to thank my advisor Dr. Wendy Olivas for the opportunity to work in her lab. It has been an enriching experience. Dr. Olivas is always open to suggestions from her students about how to proceed during an experiment, and is dedicated to their success, both inside and outside the lab. She has amassed a great deal of knowledge and experience not only concerning the key work in her field but also how to be a successful paper and grant writer and presenter, and always makes sure her students shine at conferences and network with our colleagues. I would also like to thank Dr. Randi Ulbricht who was my mentor when I was first starting out in the lab, and after our discussions made me realize I wanted to earn my PhD in molecular biology. I would also like to thank Dr. Florencia Lopez-Leban, whom I inherited the Puf4/Puf5 work from, for her initial mentoring.

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while their constant questions of “When are you graduating?” are commonly considered one the banes of graduate students, I’m sure it contributed to my motivation. They were also a source of support and love that has been present since I’ve known them.

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stay on in the Olivas lab, and I therefore feel the lab will be in good hands with these highly motivated people leading it into the next chapter.
Abstract

Regulation of protein expression is critical to the survival of an organism. Multiple disease states have arisen from aberrant accumulation/aggregation of proteins or by reduced production of key enzymes. The cell has many ways to manipulate protein levels, including transcription factors, chromatin modification, modification of messenger RNA (mRNA), and manipulation of proteolytic protein decay. Cells can indirectly control protein volume by controlling mRNA lifespan, which is directly correlated with protein output. Often, sequence-specific elements in mRNA contribute to this lifespan. The Puf family of RNA-binding proteins is ubiquitous throughout eukarya, and plays important cellular and developmental roles through regulation of mRNA lifespans. Puf proteins contain a C-terminal conserved domain that is primarily responsible for binding target mRNA, which they recognize via conserved sequences in the 3’ untranslated region of the mRNA, and protein cofactors. Puf proteins recruit decay machinery to stimulate degradation of the mRNA. The mechanisms that regulate Puf protein stimulation of mRNA decay, especially the underlying signal transduction pathways from an environmental stimulus, are poorly understood. Here, I demonstrate the means by which the activity of Puf3p in the yeast *Saccharomyces cerevisiae* is regulated through alteration of Pop2p binding and activity, which is in turn influenced by the protein kinase Yak1p. Pop2p is a scaffolding protein, allowing other decay factor proteins to bind Puf3p and act on the bound mRNA. In carbon sources that inhibit Puf3-mediated decay stimulation, Puf3p binds Yak1p, which phosphorylates Pop2p in the vicinity of the bound mRNA. Phosphorylation of Pop2p is detrimental to decay
stimulation by Pop2p, not only for Puf3p targets but also non-targets, indicating a general cellular mechanism for modulating Pop2p activity.

Separately, I show that both combinatorial and opposing regulation of mRNA targets encoding ribosome biogenesis factors by Puf proteins 1 through 5 of S. cerevisiae is critical for proper ribosome biogenesis and trafficking. Specifically, while Puf proteins 2, 4 and 5 stimulate decay of the target mRNAs, this work provides the first evidence in yeast for the role of Puf proteins 1 and 3 in stabilizing mRNAs, a phenomenon seen rarely in other organisms. Control of Puf protein levels is critical for this process, as overexpression of Puf4p leads to deficiencies in ribosome biogenesis and trafficking. Together, these two bodies of work expand our knowledge of the plasticity with which Puf proteins regulate their mRNA targets, reveal new roles for Puf proteins in mRNA stability and ribosome biogenesis, and reveal a mechanism by which Puf protein activity can be modulated by environmental conditions.
Chapter 1: Introduction
Introduction

Control of protein production in the cell is extremely important. Multiple problems can arise if too little or too much protein is made. The cell has several mechanisms to control proper protein expression, including transcriptional and translational regulation. Altering chromatin structure and recruitment of transcription activation/repression factors control expression from the gene to mRNA level, while mechanisms such as alteration of the rate of translation initiation control expression from the mRNA to protein level. Another key mechanism the cell has to control expression is post-transcriptionally by altering the lifespan of mRNAs; the lifespan of an mRNA is directly correlated with the amount of protein that can be made from a given transcript. The longer an mRNA survives, the more opportunity it has to be translated by circulating ribosomes. Therefore, careful control of mRNA lifespan is critical for proper protein expression.

Cytoplasmic mRNA Decay in Eukaryotes

After a transcript has been successfully made (and spliced, if necessary), it is capped at the 5’ end with a 7-methylguanylate (m7G) cap. It is then cut at specific sequences in the 3’ untranslated region (UTR) and a polyadenosine (poly(A)) tail is added. These two steps help protect the mRNA from 5’ and 3’ exonucleases, respectively, although there are a few mRNA transcripts that have an alternative 3’ structure instead of a polyA tail for protection. Barring any defects in the mRNA that may target it for nuclear degradation exonucleolytically 3’ to 5’ by the nuclear exosome, the mRNA is exported to the cytoplasm where there are a number of factors that
determine how long the mRNA exists before degradation takes place. Functionally related mRNAs often have similar lifespans, giving rise to the idea of “decay regulons” [reviewed in (Keene, 2007)].

While specific regulatory factors control the rate at which mRNAs are degraded, most mRNAs move through the same general decay pathway (Figure 1.1). Decay usually begins with stimulation of deadenylation, or poly(A) tail removal. This is mediated by a suite of proteins acting at different times during deadenylation, specifically Caf1p (Pop2p), Ccr4p, Not1p-Not5p, Pan2p, Pan3p, Caf40p, and Caf130p (Brown and Sachs, 1998; Tucker et al., 2001; Denis and Chen, 2003). Pan2p and Pan3p are involved in the initial trimming of the poly(A) tail, after which Ccr4p-NOT-Pop2p, the major deadenylase complex, rapidly removes the rest of the tail (Tucker et al., 2001); Ccr4p is the major player in this activity. Poly(A) binding protein Pab1p is a member of the messenger ribonucleotide protein (mRNP) complex bound to actively translating transcripts, and it interacts with 5’ cap-binding proteins to hold the mRNA in a protected circular structure. Pab1p has been found to stimulate the initial trimming by Pan2p (Boeck et al., 1996), but its presence inhibits Ccr4p and must be removed before full poly(A) tail removal occurs (Tucker et al., 2002). Ccr4p-NOT proteins and Pop2p homologs are extremely well conserved between unicellular eukaryotes, such as Saccharomyces cerevisiae, and higher organisms. The rate at which the poly(A) tail is removed is specific for each individual mRNA, and several sequence-specific elements, especially in the 5’ and 3’ UTRs, can contribute to mRNA lifespan determination, often through interactions with specific regulatory proteins (Cao and Parker, 2001). As the shortening
Figure 1.1 mRNA Decay in Eukaryotes

Shown is the normal degradation pathway for mRNA in eukaryotes. The proteins described are from *S. cerevisiae*. The mRNA is held in a closed loop by proteins including Pab1 and initiation factors. After initial trimming of the polyadenosine tail by the Pan proteins, the Ccr4-Not complex rapidly deadenylates the transcript. Dcp1/Dcp2 form the decapping complex and remove the methyl-7-guanosine cap, assisted by the Lsm1-7 proteins, the Edc1-3 proteins, and Dhh1. Following removal of the cap, the transcript is degraded slowly in the 5’ to 3’ direction by the exosome and rapidly in the 5’ to 3’ direction by the exonuclease Xrn1.
of the poly(A) tail is usually the first step in cytoplasmic mRNA decay, it is often the rate limiting step. The length of the poly(A) tail can differ between mRNAs, and this size is a major contributor to the time required to deadenylate the transcript (Beilharz and Preiss, 2007).

After removal of the poly(A) tail, decay can proceed with the 3’ exonucleolytic decay by the exosome (Anderson and Parker, 1998), although this process is relatively slow as compared to subsequent 5’ decay. More commonly, the next step is the removal of the m7G 5’ cap by Dcp1p/Dcp2p. Decapping occurs via the decapping complex that is recruited to the mRNA, specifically by the catalytic pyrophosphatase subunit Dcp2p, in a process that removes the m7G to leave a 5’ monophosphate mRNA. This activity is enhanced by Dcp1p, another member of the complex (She et al., 2008). Decapping factors compete with translation initiation factors such as eIF4E and eIF4G, whose presence inhibits decapping (Schwartz and Parker, 2000). Decapping is enhanced by several other proteins including the DEAD-box helicase Dhh1p, (Coller et al., 2001), the scaffolding protein Pat1p (Bouveret et al., 2000; Tharun et al., 2000), Lsm1-7p (Bouveret et al., 2000), the eIF4G inhibitors Scd6p and Sbp1p (Nissan et al., 2010; Rajyaguru et al., 2012), and enhancers of decapping Edc1p and Edc2p (Neef and Thiele, 2009). After the decapping step, the exonuclease Xrn1p rapidly degrades the mRNA in a 5’ to 3’ direction (Hsu and Stevens, 1993; Muhlrad et al., 1994).

In addition to the pathway of mRNA decay for normal mRNAs, there are quality control mechanisms in place in the cytoplasm to deal with degradation of aberrant mRNAs. Nonsense-mediated decay (NMD) occurs when a transcript has a premature
stop codon or translation is abnormally terminated. This can occur when alternative
translation initiation sites are present, introns are mistakenly included that contain stop
codons, or frameshift mutations occur. The Upf1, 2, and 3 proteins target an mRNA for
NMD. Another control mechanism is No-Go decay (NGD), which occurs on transcripts
that have unusually long stalls in translation elongation. Mediated by the Dom34 and
Hbs1 proteins, the transcript is targeted for endonucleolytic cleavage and subsequent
degradation. A third mechanism is Nonstop decay (NSD), which occurs on transcripts
lacking stop codons. Such transcripts can occur because of events such as alternative
polyadenylation, endonucleolytic cleavage upstream of the stop codon, or point
mutations in the stop codon. Translating ribosomes reach the 3’ end without being able
to terminate translation, which signals for the mRNA to be rapidly degraded in a 3’ to 5’
direction by the exosome in a manner that is different from exosomal degradation of
normal mRNAs (Parker, 2012).

Sequence elements within mRNAs have a profound impact on their lifespans,
especially elements within the 5’ and 3’ UTRs. 3’ UTR regulatory sequences include AU-
rich elements (AREs), cytoplasmic polyadenylation elements (CPEs), and GU-rich
elements. Frequently, these elements are targeted by RNA binding proteins (RBPs) as a
means to regulate target mRNA lifespans. It is sequences like these that the Puf family
of proteins utilizes to exert their control of mRNA degradation.

**Puf Proteins: A Diverse Family of Eukaryotic RNA Binding Proteins**

Puf proteins are ubiquitous throughout eukarya and comprise a large class of
mRNA binding proteins. One of their primary roles in the cell is to stimulate rapid
degradation of the specific transcripts they bind, mainly through recruitment of decay factors such as the deadenylase and decapping complexes, although other means of translational repression can occur in a non-decay manner. As such, Puf proteins are mainly found in the cytoplasm, although occasionally they are found in the nucleus (Gu et al., 2004; Droll et al., 2010). The vast majority of mRNAs that are bound by Puf proteins are bound in their 3’ UTRs (Zamore et al., 1997; Wharton et al., 1998; Zamore et al., 1999; Gu et al., 2004; Jackson et al., 2004; Houshmandi and Olivas, 2005; Ulbricht and Olivas, 2008; Menon et al., 2009), although some mRNAs are bound by Pufs in their ORFs and 5’ UTRs (Muraro et al., 2008; Miao et al., 2013; Wilinski et al., 2015). Regardless of the region bound, Puf proteins share a common binding site sequence composed of a UGU trinucleotide followed by an AU-rich downstream region. Each Puf protein prefers a specific variation of the binding site, though Puf-specific consensus sequences are sometimes overlapping within and between organisms (Murata and Wharton, 1995; Zamore et al., 1997; Asaoka-Taguchi et al., 1999; Zamore et al., 1999; White et al., 2001; Crittenden et al., 2002; Gamberi et al., 2002; Gu et al., 2004; Jackson et al., 2004; Lamont et al., 2004; Bernstein et al., 2005; Padmanabhan and Richter, 2006; Muraro et al., 2008; Archer et al., 2009). Many of the Puf-specific consensus binding sites were discovered by large scale studies analyzing all mRNAs that bind a particular Puf protein (Gerber et al., 2004; Foat et al., 2005; Gerber et al., 2006; Galgano et al., 2008; Wilinski et al., 2015). Some Puf proteins require additional binding sequences to facilitate Puf regulation, such as Puf3p in yeast requiring a cytosine two bases upstream of the conserved UGU (Zhu et al., 2009) or the C. elegans FBF-1
requiring a 22 nucleotide sequence for optimal binding (Bernstein et al., 2005). While certain Puf proteins have only a highly specific subset of transcripts that they regulate exclusively (known as regulons), other Puf proteins are more promiscuous in the mRNAs they bind. Multiple Puf proteins in yeast can regulate the same mRNA, sometimes through the same site (Hook et al., 2007; Ulbricht and Olivas, 2008; Russo and Olivas, 2015). One way promiscuous Puf proteins can bind large pools of cognate mRNAs is by using “base flipping” mechanisms, whereby one or more bases in the target sequence of the transcript are turned away from the RNA binding surface of the Puf, such that the remaining bases make the correct hydrogen bonding/Van der Waals and stacking interactions with the residues of the Puf repeats (Opperman et al., 2005; Miller et al., 2008; Wang et al., 2009; Valley et al., 2012). Some Puf proteins even change their shape slightly to achieve this effect (Koh et al., 2009).

Puf proteins in all eukaryotes share a highly conserved region near the C-terminus that is largely responsible for binding target mRNAs and other factors. This conserved region is made up of eight imperfect repeats of three alpha helices each, hence the region is often called the Puf repeat domain. Structurally, this region typically adopts a crescent shape, whereby RNA binds on the “inner” concave surface, and protein-protein contacts facilitating regulation occur on the “outer” convex surface [reviewed in (Miller and Olivas, 2011)] (Edwards et al., 2001; Wang et al., 2001; Wang et al., 2002; Miller et al., 2008; Jenkins et al., 2009; Wang et al., 2009; Zhu et al., 2009; Dong et al., 2011; Koh et al., 2011; Lu and Hall, 2011; Qiu et al., 2012; Valley et al., 2012; Wilinski et al., 2015; Weidmann et al., 2016). Figure 1.2 details this structure. Some Puf
Figure 1.2 Puf Proteins and RNA Targets. Shown are crystal structures of some Puf proteins and the different targets they bind (Puf:Target). Arrows on FBF and yeast Puf4p indicate bases that are “flipped” away from the inner concave binding surface. On Puf3p this base is the conserved cytosine that is two nucleotides upstream from the UGU trinucleotide. Figure was adapted from (Miller and Olivas, 2011) with permissions.
or Puf-like proteins, such as human PUM-A, yeast Puf6p, and yeast Nop9p, do not share the same number of repeats nor the similar crescent shape of prototypical Puf proteins (Qiu et al., 2014; Zhang et al., 2016; Wang and Ye, 2017). The structure and function of the Puf repeat domain is usually so well conserved that a Puf protein from one organism can bind Puf target mRNAs from other organisms, provided the mRNA meets the sequence criteria for being a target of regulation for the indicated Puf (Gupta et al., 2008). Often, the Puf repeat domain is sufficient for regulation of target transcripts (Wharton et al., 1998; Jackson et al., 2004; Kadyrova et al., 2007), although studies have also pointed to other regions of Puf proteins as having standalone repression abilities (Weidmann and Goldstrohm, 2012) or being required for optimal or properly localized repression by Pufs (Chritton and Wickens, 2010; Wang et al., 2016). Within the Puf repeat domain, specific amino acid residues in each repeat form both edge-on and stacking interactions with a single base in the mRNA, giving a one repeat per base pattern of binding. The mRNA binds to the repeats in an antiparallel fashion, with the 5’ end base recognizing the C-terminal end repeat (Koh et al., 2011). Mutation of this repeat domain, especially to residues that contact the RNA bases, predictably reduces or eliminates the Puf protein’s ability to bind and/or regulate target mRNAs (Wharton and Struhl, 1991; Murata and Wharton, 1995; Zamore et al., 1997; Zhang et al., 1997; Wharton et al., 1998; Nakahata et al., 2001; Tadauchi et al., 2001; Nakahata et al., 2003; Gu et al., 2004; Jackson et al., 2004; Lamont et al., 2004; Bernstein et al., 2005; Kadyrova et al., 2007; Muraro et al., 2008; Ulbricht and Olivas, 2008; Menon et al., 2009). The modular pattern of binding has led to studies demonstrating that altering
residues in the Puf repeat domain alters nucleotide recognition in a specific manner. For example, replacing the residues within a particular repeat that recognize uracil with residues that recognize a cytosine can alter the mRNA recognition sequence at the respective position from a U to a C (Dong et al., 2011; Filipovska et al., 2011). These studies give rise to the idea that Puf proteins can be designed to recognize any mRNA sequence provided the correct residues within each repeat that recognize a specific base are present (Wang et al., 2002; Cheong and Hall, 2006; Campbell et al., 2014). The specific residues in each repeat that make edge-on contacts and stacking interactions with a particular base in the target site are extremely well conserved; Table 1.1 summarizes a recent study that determined the optimal and sub-optimal tripartite recognition motif (TRM) required to bind the second base (in the 5’ to 3’ direction) of a nine-base target sequence by substituting repeat 7 of *C. elegans* FBF-2 with combinations of residues from fungal Pufs [reviewed in (Hall, 2016)(Campbell et al., 2014)]. Interestingly, residues that recognized a C in human PUMILIO1 recognized either a G or a U when placed in the repeat 7. Another study showed that modular protein units corresponding to single Puf repeats that recognize either a U, C, A, or G could be concatenated to recognize any 8- to 16-mer sequence (Adamala et al., 2016), indicating that synthetic Pufs could be designed to target any number of specific sequences in mRNAs and suggests their use as a therapeutic tool. This programmable targeting by Pumilio proteins has even allowed them to find their way into CRISPR/Cas9 biology as a way to enhance and multiplex the nuclease dead CRISPR system commonly used for targeted transcriptional changes (Cheng et al., 2016).
<table>
<thead>
<tr>
<th>Base</th>
<th>End-On Contact Residues</th>
<th>Stacking Interaction Residue</th>
<th>Overall Specificity</th>
<th>5’U Specificity</th>
<th>+2 Specificity</th>
<th>3’U Specificity</th>
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<td>Ser1Glu5</td>
<td>His2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
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<td>Ser1Glu5</td>
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Table 1.1 Binding Specificities of Contact/Stacking Residues and Cognate Bases. Shown are the FBF-2 repeat 7 replacement residues labeled 1-5 from N to C termini within the region of the Puf repeat that make contact with the indicated bases or form stacking interactions with the base by positioning in between it and the next 3’ base. Residue 1, 2, and 5 of the specific region correspond to residues 12, 13, and 16 of the entire repeat. +2 specificity indicates the likelihood of indicated residues binding the base at the 2nd position. 5’U and 3’U specificities indicate the likelihood of indicated residues binding the base at the 2nd position with a uracil either 5’ or 3’ of the base, respectively. Overall specificity is a function of these three columns. Bolded text indicates residues coming from human Pumilio 1. The G and U bases to the immediate right of the C denote that the residues were shown to bind cytosine in human Pumilio 1, but bound either a guanosine or uracil when FBF-2 repeat 7 was replaced with these key residues. Figure was adapted from (Hall, 2016).
The Function of Puf Proteins in mRNA Decay

The major way Puf proteins promote deadenylation, decapping, and subsequent degradation of their target mRNAs is by recruitment of decay factors, though different organisms have unique subsets of proteins that are involved in this process. Some proteins that are present in more complex (multicellular) eukaryotes, which are known to be recruited by and/or necessary for Puf activity, have no homologs in simpler or unicellular eukaryotes. The recruitment of the CCR4-POP2-NOT deadenylase complex is well conserved in eukaryotes (Goldstrohm et al., 2006; Goldstrohm et al., 2007; Hook et al., 2007; Kadyrova et al., 2007; Lee et al., 2010), while recruitment of other factors to promote Puf regulation is organism specific. Decapping factors are also recruited in a similar manner, and multiple studies have shown that decapping factors are associated with the CCR4-POP2-NOT complex (Goldstrohm et al., 2006; Goldstrohm et al., 2007; Hook et al., 2007; Nishimura et al., 2015; Ozgur et al., 2015). In Drosophila, Nanos is required to mediate Puf repression of hunchback mRNA (Sonoda and Wharton, 1999) and is recruited by Pumilio in an RNA dependent manner. Like Pumilio, Nanos also binds regions in the 3’ UTR region of the transcript and recruits deadenylase machinery to the mRNA (Wharton and Struhl, 1991; Wreden et al., 1997). This regulation is also dependent on Brat, though Brat is not necessary for Pumilio regulation of all mRNAs, such as cyclin B mRNA (Sonoda and Wharton, 2001; Kadyrova et al., 2007). Brat is also not required for regulation of zebrafish cyclin B1, which alternatively utilizes cytoplasmic polyadenylation element binding (CPEB) in addition to Nanos homolog Xcat-2 and Pumilio 2 (Nakahata et al., 2001; Nakahata et al., 2003). Brat is required for sodium
channel regulation in Drosophila motorneurons (partly via regulation of paralytic mRNA), but channel regulation in other neurons does not require Brat (Muraro et al., 2008).

A recent study indicates that in addition to the 3’ UTR Puf binding site, the poly(A) tail of mRNAs is required for proper regulation by Puf proteins. Furthermore, it is the poly(A) binding protein (Pab1p in yeast) that was shown to be indispensable for this regulation (Weidmann et al., 2014). This requirement for the poly(A) tail and for deadenylation to enable efficient regulation by Puf proteins is mirrored in humans (Van Etten et al., 2012). However, these same studies and others found that Puf proteins can still elicit repression without a poly(A) tail, albeit less efficiently (Chagnovich and Lehmann, 2001; Weidmann and Goldstrohm, 2012). In yeast, Puf5p was found to require the eukaryotic translation initiation factor 4E (eIF4E)-binding protein Eap1p for decay of certain mRNAs; the presence of Eap1p was found to stimulate decapping of these mRNAs and associate with decapping factor Dhh1p. Puf4p was found to have no such requirement for Eap1p, and Caf20p, another protein implicated in the mRNP complex, was not required for either Puf4p or Puf5p regulation of decay (Blewett and Goldstrohm, 2012). In animals, Puf proteins commonly function with microRNAs to regulate expression (Nolde et al., 2007; Galgano et al., 2008; Triboulet and Gregory, 2010; Miles et al., 2012; Yi et al., 2015). For example, PUM binding to the p27 3’UTR in humans allows for greater accessibility by miR-221 and miR-222, a mechanism that is enhanced by the phosphorylation of the PUM protein (Kedde et al., 2010).
The Function of Puf Proteins in Translational Inhibition

Puf proteins are not limited to repression of their targets via mRNA decay. Several studies have shown that Puf proteins can elicit translational inhibition independent of mRNA decay. A conserved mechanism is described in humans and *C. elegans* whereby a Puf protein interacts with Argonaute and translation elongation factor 1A (eEF1A) to inhibit translation elongation by interfering with the GTPase activity of eEF1A (Friend *et al.*, 2012). While the presence of a poly(A) tail, poly(A) binding proteins, and deadenylation has been shown to be important for decay regulation by Puf proteins, Pufs were shown to repress translation in a decay-independent manner on transcripts that contained no poly(A) tail (Van Etten *et al.*, 2012). In zebrafish, Pumilio 2 was shown to bind the 5’ cap of *ringo/spy* mRNA, and this interaction prevents the binding of eIF4E to the cap and thus inhibits translation initiation (Cao *et al.*, 2010). In Drosophila, Brat binds d4EHP and competes with eIF4E for binding to the 5’ cap of *hunchback*, and in this manner inhibits translation initiation of *hunchback*, while Pumilio and Nanos stimulate the mRNA’s decay (Cho *et al.*, 2006). The non-canonical Pumilio Puf6p in yeast associates with Fun12p (eIF5b in other eukaryotes) to repress *ASH1* mRNA translation until the mRNP complex is transported to the daughter bud during replication (Deng *et al.*, 2008).

Puf Proteins as mRNA Stabilizers or Activators of Expression

While the main role of Puf proteins is in destabilizing target transcripts, there is emerging evidence pointing towards their role in promoting stabilization or activating translation of some mRNAs (Pique *et al.*, 2008). In *Trypanosoma brucei*, PUF9 was found
to stabilize its bound transcripts (Archer et al., 2009). FBF in C. elegans can achieve
deadenylation or polyadenylation of target gld-1 based on whether it associates with
ccf-1 or gld-2, respectively (Suh et al., 2009), and FBF also enhances translation of the
target egl-4 (Kaye et al., 2009). Recently, expression of the FOXP1 mRNA targeted by
PUM1/PUM2 was found to have two distinct PUM binding elements in its 3’ UTR, and its
expression was positively correlated to PUM levels (Naudin et al., 2017). Levels of the
kinase IK2 transcript, a major factor in the liver infection stage of the malaria parasite
Plasmodium, were shown to be reduced in cells deficient in Puf2, suggesting a stabilizing
role for the Puf protein (Muller et al., 2011). There is also some synthetic evidence to
support the idea that Puf proteins can be translational activators. At their root, Puf
proteins are RNA binding proteins, so tethering translation stimulating factors to Puf
proteins was shown to promote translation of reporter constructs bearing cognate Puf
response elements, while tethering deadenylation factors promoted decay of the
reporter mRNAs (Cooke et al., 2011).

**Cellular Processes Affected by the Actions of Puf Proteins**

There is a dizzying array of cellular processes in eukarya that are affected by the
actions of Puf proteins as a result of the regulation of mRNA lifespan or translation.
Some of the more notable functions of Puf proteins are summarized in Table 1.2. In the
yeast Saccharomyces cerevisiae, the most well studied Puf protein is Puf3p, whose main
role is to regulate decay of nuclear encoded mitochondrial protein transcripts (Olivas
and Parker, 2000; Jackson et al., 2004), and as such contributes to negative regulation of
mitochondrial translation, respiration, and biogenesis (Chatenay-Lapointe and Shadel,
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**Table 1.2 Cellular Processes Affected by Puf Proteins.** Shown are some of the more notable processes that are affected by the actions of Puf proteins. Several functions of Puf proteins that were first discovered in lower eukaryotes were later found to have homologous roles in higher eukaryotes.
Puf3p has also been shown to have a role in positively regulating translation of these mRNAs during mitochondrial biogenesis when fermentable carbon sources are scarce (Lee and Tu, 2015), and is thought to transport transcripts to the mitochondria for localized translation (Garcia-Rodriguez et al., 2007; Saint-Georges et al., 2008). Puf5p was shown to have a role in stress tolerance, regulation of lifespan, maintenance of cell wall integrity (Kaeberlein and Guarente, 2002; Stewart et al., 2007) and localization of peroxisomal protein transcripts (Zipor et al., 2009). Puf4p and Puf5p combinatorially regulate the HO mRNA and thus are involved in control of mating-type switching (Tadauchi et al., 2001; Hook et al., 2007). Puf4p acts together with Puf1p and Puf5p to regulate the YHB1 transcript, which is essential for protection from nitric oxide stress (Russo and Olivas, 2015). These three Puf proteins also regulate the hexokinase HXK1 mRNA, indicating their importance in regulating glycolysis (Ulbracht and Olivas, 2008).

Puf proteins play an important role in gametogenesis. In C. elegans, PUF-8 functions redundantly with GLD-1 to promote proper meiotic progression in spermatogenesis (Priti and Subramaniam, 2015). PUF-3, -5, -6, -7, AND -11 function in oogenesis in C. elegans by regulating distinct sets of mRNAs; PUF-3 AND PUF-11 limit the growth of oocytes, while PUF-5, PUF-6, AND PUF-7 stimulate oocyte formation and organization with the help of RAS-MAPK/ERK pathways (Hubstenberger et al., 2012). PUF-8 also functions redundantly with GAP-3 to repress let60 ras in order to regulate RAS/MAPK signaling in germline cells to ensure correct mitotic/meiotic shift (Vaid et al., 2013), and the presence of PUF-8 is known to inhibit proliferative fates in germline cells
through its negative regulation of GLP-1/Notch signaling (Racher and Hansen, 2012). PUF-8 functions redundantly with MEX-3, albeit at different stages of the germline stem cell life cycle, to repress pal-1 mRNA, a gene which is involved in correct posterior patterning in *C. elegans* (Mainpal *et al.*, 2011). PUF-8 also functions redundantly with TCER-1 in the nucleus to maintain proper splicing and other processing of mRNAs; their deletion leads to decreased germ cell replication and sterility (Pushpa *et al.*, 2013). In *Drosophila*, Pumilio acts with Nanos and Ccr4 to regulate mei-p26 expression, and this regulation is key to maintaining germline stem cell self-renewal (Joly *et al.*, 2013). Also in *Drosophila*, Pumilio negatively regulates several targets within the epidermal growth factor receptor (EGFR) pathway that bear Nanos response element (NRE)-like sequences in their 3’ UTR. Such regulation was shown to be important for development of the proper number of wing veins and bristles (Kim *et al.*, 2012). In humans, PUM1 contributes to spermatogenesis by repressing p53 activation to promote this activity in testes (Chen *et al.*, 2012), and to female mammalian germline cell development before birth via folliculogenesis (Mak *et al.*, 2016). PUM1 and PUM2 were found to promote hematopoietic stem/progenitor cell proliferation and survival, and to promote myeloid leukemia cell growth, both by promoting *FOXP1* expression that leads to downregulation of *p21* and *p27* expression (Naudin *et al.*, 2017). Also in humans, PUM2 was found to be important for proliferation of mesenchymal stem cells (MSC) derived from adipose tissue (Shigunov *et al.*, 2012).

Puf proteins also have a role in ribosome biogenesis (discussed in detail in Chapter 3). Nop9p in yeast is required for 18S rRNA synthesis (Thomson *et al.*, 2007),
and Puf6p in yeast is required for correct localization of pre-ribosomes and rRNA processing (Li et al., 2009; Qiu et al., 2014). In C. elegans, PUF-5, -8, and -9 act coordinately to control rRNA pool size (Yi et al., 2015). *Trypanosoma brucei* PUF7/PUF10 (Droll et al., 2010; Schumann Burkard et al., 2013) and *Arabidopsis thaliana* APUM23 (Abbasi et al., 2010; Zhang and Muench, 2015) are required for proper processing of rRNA.

Some of the Pumilio proteins expressed in *A. thaliana* are thought to have arisen from genome duplication, and certain Pumiliios are expressed only in certain tissues. As a whole, Pumilio proteins in this organism are essential for proper growth patterning and development (Abbasi et al., 2011). In addition to its role in rRNA processing, APUM23 is also specifically responsible for regulating *KANADI* gene transcripts and several other mRNAs that are important for leaf polarity and proper cell division; expression of these genes is upregulated in an *apum23* mutant (Huang et al., 2014). Also in *A. thaliana*, APUM5 was shown to be a negative regulator of abiotic stress genes; overexpression lines were hypersensitive to salt and drought stress during germination, primary root elongation, and vegetative stages (Huh and Paek, 2014). APUM5 was also shown to directly bind Cucumber Mosaic Virus RNA at the 3‘UTR and regulate its translation, indicating its role in response to pathogens (Huh et al., 2013). *T. brucei* PUM2 RNAi knockdown results in loss of mRNAs with long coding regions and enrichment of those with short ORFs (Jha et al., 2014).

Puf proteins have long been known to be involved in proper neuronal development and function. In zebrafish, Pumilio-2 is expressed only in the brain, liver,
kidney, testes, and ovaries, but not in the heart or muscle, indicating that it may be important in the maintenance of germline cells and neuronal function (Wang et al., 2012). PUM2 is important in maintaining membrane excitability homeostasis; RNAi knockdown of PUM2 in rats leads to increased action potential firing, similar to synaptic depolarization deprivation, which correlates with PUM2’s repression of the voltage-gated sodium channel mRNA, Nav1.6 (Driscoll et al., 2013). PUM2 expression is downregulated in patients with Temporal Lobe Epilepsy (TLE) and in rats with artificially induced epilepsy (Wu et al., 2015). PUM2 is also integral to synaptic downscaling during homeostatic synaptic depression (HSD); perturbations to HSD are associated with mental retardation, autism, and schizophrenia (Fiore et al., 2014). PUM2 is found at the neuromuscular synapse where it regulates the acetylcholinesterase (Ache) mRNA (Marrero et al., 2011). In mice, the removal of the binding domain of PUM2 results in several abnormalities, including altered gene expression in the hippocampus, lower body weight, increased seizures, and altered nesting and memory test behaviors (Siemen et al., 2011).

Puf proteins have been shown to have a role in cancer. Puf protein regulation of elongation factor E2F3 in humans promotes miRNA accessibility to this transcript. Several cancer lines display shortened E2F3 3’ UTRs, which results in the removal of the Puf binding site from the mRNA, preventing their actions (Miles et al., 2012). Alternative polyadenylation has also been shown to eliminate Puf binding sites from mRNAs. In humans, breast tumors were found to carry high levels of alternatively
polyadenylated transcripts in which the PRE had been removed, implicating PUMs as tumor suppressors (Miles et al., 2016).

Puf proteins also play a role in response to pathogen invasion. PUM1 and PUM2 were found to localize to Newcastle Disease Virus (NDV)-induced stress granules and increase binding of LGP2 to dsRNA. LGP2 is part of the RIG-1 like receptor (RLR) complex that promotes expression of type I interferon, a crucial component of the host’s innate immune response (Narita et al., 2014). The malaria parasite PfPuf2 protein binds to conserved sequences in the 5’ UTR in order to mediate translation repression of several genes in parasite gametocytes, including pfs25 and 28, which are candidate targets of the transmission-blocking malaria vaccine (Miao et al., 2013). PfPuf2 was also shown in malaria to be important for the parasite’s sporozoite conversion in the liver stage via upregulation of the kinase IK2 transcript. Plasmodium that are deficient in Puf2 failed to initiate the liver infection stage (Gomes-Santos et al., 2011; Muller et al., 2011).

**Regulation of Puf Protein Activity**

Although sequence and structure are conserved between Pufs across kingdoms, there is less similarity in terms of regulation of their activity. Regulation occurs through several mechanisms, both with respect to some alteration of the Puf protein itself (whether post-translational modifications or altered localization), or its target transcripts. Recently, yeast Puf3p has been shown to undergo multiple phosphorylation events largely in the N-terminus upon glucose deprivation, and this phosphorylation positively regulates Puf3p’s ability to promote translation of its target mRNAs (Lee and
Phosphorylation of yeast Puf6p at several serine residues of the N-terminus by kinase CK2 derepresses ASH1 translation by Puf6p (Deng et al., 2008). The long non-coding RNA NORAD has been implicated as a negative regulator of Puf activity by sequestering both PUM1 and PUM2 in humans to promote genomic stability (Lee et al., 2016). Human PUM2 binding affinity to its target mRNAs is weakened by pseudouridine and N6-methyladenosine modification of target transcripts (Vaidyanathan et al., 2017).

Alternative polyadenylation (APA) can remove Pumilio recognition elements (PREs) to repress binding, although this is more of a disease mechanism than a normal escape from Puf regulation, as described above. In Caenorhabditis elegans, dynein light chain 1 (DLC-1) was found to regulate localization of Puf protein FBF-2 to P granules, thereby localizing its activity (Wang et al., 2016). The P granule component PGL-1 was also found to be important for localization of FBF-2 to P granules and for proper binding of FBF-2 to target transcripts, a mechanism which is not required for FBF-1 activity (Voronina et al., 2012). Drosophila Nanos has been shown to enhance Pumilio/RNA binding to a wider array of transcripts through its C-terminal region and zinc finger domains (Weidmann et al., 2016). In flies and in humans, Pumilio exists in a complex feedback loop with the histone demethylase family LSD1, wherein Pumilio levels are inversely correlated with LSD1 levels, and Pumilio posttranscriptionally regulates the LSD1 transcript; this process has implications in cancer biology, as LSD1 is frequently downregulated in tumors (Miles et al., 2015). Phosphorylation of PUM1 is required for optimal binding to the p27 3’ UTR in humans, which results in the unfolding of hairpin structures in the mRNA to expose microRNA binding sites for miR-221 and -222 (Kedde...
et al., 2010). In humans, miR-134 has been shown to spatially downregulate PUM2 expression upon homeostatic synaptic depression (HSD); HSD is part of synaptic plasticity, and PUM2’s association with polo-like kinase 2 (PLK2) is an integral portion of the synaptic downscaling that occurs during HSD (Fiore et al., 2014).

**Contributions to the Field of mRNA Decay**

In this work, I present the findings for two main areas of my research. First, I elucidate a mechanism for how Puf3p activity to stimulate decay may be modulated in different carbon sources. We have established that the decreased Puf3p activity found in carbon sources such as galactose is not due to either a decrease in cellular concentrations of Puf3p, altered localization of the protein, or reduced binding affinity for its mRNA targets (Miller et al., 2014). Instead, we have found that Pop2p acts as a necessary bridging or scaffolding molecule between Puf3p and other decay factors. We show that in galactose, Pop2p binds less well to Puf3p, indicating that in this condition the decay factors would also be less associated with Puf3p and the target mRNA. Deletion of Yak1p, which phosphorylates Pop2p, results in destabilization of Puf3p target mRNAs in galactose conditions, as does mimicking this phosphorylation of Pop2p. Puf3p interacts with Yak1p more strongly in galactose conditions, leading to a model whereby in galactose, Puf3p bound to a target mRNA recruits Yak1p, which would phosphorylate any Pop2p that comes in the vicinity of the mRNA. The phosphorylation of this Pop2p disrupts interactions with Puf3p and/or interactions with other decay factors, leading to reduced decay activity on the mRNA.
Second, I present my published findings on the role of Puf proteins in ribosome biogenesis (Fischer and Olivas, 2018). Several mRNAs that encode proteins involved in ribosome biogenesis are negatively regulated by Puf proteins 2, 4, and 5. Interestingly, these mRNAs are also stabilized by Puf proteins 1 and 3, and this stabilization is a novel role for Puf proteins in yeast. I elucidate the presence of a secondary mechanism regulating decay of these mRNAs that also works through the Puf binding site. Finally, I show that this regulation is necessary for the proper pace of ribosome biogenesis and trafficking, and that cellular concentrations of Puf4p are critical for this regulation. Overexpression of Puf4p results in mislocalization of ribosomal subunits such that they are more nuclearly retained, and also results in slowed processing kinetics of the original 35S ribosomal RNA transcript.

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Chapter 2: Regulation of Puf3p Activity
Introduction

A cell must be able to adapt to external stimuli and nutrient availability, often at a moments notice. One of the most basic responses to an abundance of nutrients, particularly carbohydrates, is glycolysis, which is ubiquitous throughout the kingdoms of life. Organisms have a range of end products of fermentation of simple sugars, but the pathways are very well conserved. Fermentation end products can be further utilized for energy using the citric acid cycle, which includes oxidative phosphorylation of ADP to ATP, which occurs in the mitochondria in eukaryotes.

Many of the mRNAs that encode proteins destined for the mitochondria are transcribed from the cell nucleus instead of from mitochondrial DNA. These nuclear-encoded mitochondrial transcripts are frequently destined for localized translation at the mitochondrial membrane for import. In the yeast *Saccharomyces cerevisiae*, it was shown that Puf3p is responsible for binding many of these transcripts (Gerber *et al.*, 2004), and it is thought that Puf3p is responsible for transporting these mRNAs to the mitochondria for localized translation (Lee *et al.*, 2007; Cooke *et al.*, 2011; Lee and Tu, 2015). Puf3p was earlier described to be a key regulator of the decay of transcripts involved in mitochondrial function (Olivas and Parker, 2000), and repress mitochondrial biogenesis (Lee *et al.*, 2007; Zhu *et al.*, 2009; Cooke *et al.*, 2011). How then can Puf3p be serving dual roles as mRNA chaperone and decay stimulator?

Control of mRNA decay is a key intermediate step between transcription from DNA and translation into protein. mRNA lifespan is directly correlated to the amount of protein produced from an mRNA. Often, sequence specific elements within the mRNA,
particularly those within the 5’ and 3’ UTRs, contribute to an individual transcript’s lifespan. Puf proteins represent a large class of eukaryotic RNA-binding proteins that recognize distinct sequences, overwhelmingly in the 3’UTRs of mRNAs. Puf proteins are responsible for a variety of different cellular and developmental activities in multiple organisms through regulation of mRNA decay and translation. Yeast Puf3p shares the most homology with mammalian Puf proteins. In yeast, under fermentative conditions the need for mitochondrial activity is low, since the cell can derive ATP from fermentation of simple sugars. Under non-fermentative conditions, the cell requires the actions of the mitochondria, sometimes even to modify monosaccharides so that they may enter into the glycolysis cycle. Under these conditions, the Olivas lab demonstrated that Puf3p is inactive for decay stimulation (Miller et al., 2014). The mechanism underlying the control of Puf3p stimulation of decay is unknown. Evidence for regulation of Puf activity is strong but sporadic; the means by which most Puf proteins are regulated remains a mystery (refer to Chapter 1). In yeast, one study in particular suggests that phosphorylation at multiple residues of Puf3p, which occurs when cells are grown in media depleted of glucose, assists in translation of Puf3p target mRNAs (Lee and Tu, 2015).

In this study, we examine the mechanism underlying the control of Puf3p stimulation of decay. We provide evidence that inhibition of Puf3p decay activity is achieved partly through the actions of the Yak1p kinase on Pop2p. Through modulation of Yak1p interactions with Puf3p, we hypothesize that the cell is able to spatially control Thr97 phosphorylation of Pop2p. Specifically, in galactose conditions, Puf3p more
strongly associates with Yak1p, which then phosphorylates Pop2p molecules that come into contact with Puf3p and the bound mRNA. Phosphorylation of Pop2p appears to disrupt interactions with Puf3p and inhibit decay of the mRNA. We show that constitutive mimicked phosphorylation of Pop2p at Thr97 results in constitutively stabilized mRNA, not only of Puf3p targets but all transcripts. This suggests a global mechanism for tuning Pop2p decay activity, which Puf3p is able to harness to achieve the proper stability of its target transcripts.

Results

Sections 1, 2, and 3 of the results detail the work that was published in (Miller et al., 2014) and performed by me and Dr. Joseph Russo (a previous graduate student in the Olivas lab). Sections 5, 6, and 9 were also performed together with Dr. Russo, while sections, 4, 7, 8, 10, and 11 were performed primarily by me with the assistance of fellow graduate student in the Olivas lab, Ariel Bulmash.

1. Puf3p Localization is Not Altered in Non-Fermentative Carbon Sources

Work previously performed in (Miller et al., 2014) indicated that the switching of Puf3p activity was extremely rapid; within the span of two minutes of switching from a non-fermentative carbon source to dextrose, the half-lives of Puf3p mRNA nearly mimicked that seen as if the cells had been grown continuously in dextrose, and after ten minutes the two half-lives were indistinguishable. To explain this phenomenon, several theories were posited. In one theory, we hypothesized that the inhibition of Puf3p’s activity to stimulate decay when cells are grown under non-fermentative carbon sources is due to alteration in the protein’s localization. Indeed, Puf3p has been shown
to localize to the mitochondria, and this localization is important for mitochondrial biogenesis, recruitment of factors, and mitochondrial biogenesis transcript localization (Garcia-Rodriguez et al., 2007; Saint-Georges et al., 2008). To this end, we investigated the subcellular localization of Puf3p under dextrose (fermentative) versus galactose and ethanol (both non-fermentative) conditions using confocal fluorescence microscopy. As shown in figure 2.1, in all three conditions, cells expressing a Puf3p-GFP chimeric protein showed uniform cellular distribution. No punctate Puf3p foci were seen, which would have indicated that Puf3p was differentially localized or sequestered in non-fermentative conditions. Mitochondrial distribution was also observed, and although there was some co-localization of Puf3p and mitochondria (seen in the merged panel as yellow fluorescence signal), Puf3p was not exclusively associated with mitochondria under in any condition. These results indicate that Puf3p’s altered activity in non-fermentative carbon sources is not due to altered localization or sequestering of the protein.

2. Puf3p Protein Levels Do Not Change in Non-Fermentative Carbon Sources

Another possibility for the reduction of Puf3p activity in non-fermentative carbon sources could be that Puf3 protein levels in the cell are reduced in these conditions. This theory would also necessitate rapid reduction of these levels when encountering a non-fermentative carbon source, as Puf3p stimulation of decay activity can be rapidly reduced when changing carbon sources (Miller et al., 2014). We therefore used the existing confocal fluorescence microscopy data to evaluate the levels of Puf3p in dextrose, galactose, and ethanol growth conditions. As shown in figure 2.2, the
Figure 2.1 Puf3p Localization is not altered under non-fermentative carbon sources. Yeast expressing chimeric Puf3p-GFP fusion protein from the endogenous PUF3 locus were subjected to confocal fluorescent microscopy. Mitochondrial localization was observed using the in situ dye Mitotracker Deep Red (see Materials and Methods). Bars represent 5μm distance. Merge panels and image post processing were performed using Photoshop.
relative average levels of Puf3p did not decrease as expected when the cells were grown in non-fermentative carbon source. In fact, levels of Puf3p increased under these conditions. The results indicate that Puf3p’s reduced ability to stimulate decay of mitochondrial biogenesis transcripts in galactose and ethanol is not due to decreased levels of the protein, which is also demonstrated by Western blot analysis of Puf3p (see figure 2.3D) (Miller et al., 2014).

3. Puf3p Ability to Bind Transcripts is Not Reduced in Non-Fermentative Carbon Sources

We next hypothesized that the reason Puf3p is not be able to regulate its target mRNAs in non-fermentative carbon sources may be because of a reduced ability to bind these targets. Carbon source-specific alterations to Puf3p could disrupt these interactions. To investigate this possibility, we performed RNA immunoprecipitations (hereafter RNA-IP) using a Tandem Affinity Purification (hereafter TAP) epitope-Puf3p fusion protein from yeast grown in dextrose and galactose. RNA that immunoprecipitated with the protein was extracted and subjected to RT-qPCR to analyze the relative levels of the Puf3p mRNA target COX17. As shown in figure 2.3, the amount of COX17 mRNA bound to Puf3p was enriched an average of 800 and 200 fold in dextrose and galactose, respectively, over the levels of control non-Puf3p target mitochondrial mRNA CBS1 and control mRNA STE3. In fact, when normalizing for differences in the starting pools of mRNA in the RT-qPCR reactions, we showed that Puf3p-bound COX17 mRNA was enriched over 2 fold in galactose when compared to dextrose. The results indicate that reduced Puf3p stimulation of decay for COX17 and likely other targets is not due to a
Figure 2.2 Levels of Puf3p Protein Do Not Decrease in Galactose or Ethanol. The levels of Puf3p were analyzed by quantitating the relative fluorescence of the Puf3p-GFP complex in each of the carbon sources indicated. Error bars represent SEM of three individual biological growth trials of multiple cells.
Figure 2.3 Puf3p Association with Target mRNA COX17 is Not Reduced in Galactose. Levels of COX17, CBS1, and STE3 were analyzed via qPCR in cells grown in (A) dextrose and (B) galactose. Error bars represent the SEM of three independent biological trials. (C) Galactose COX17 qPCR values were normalized using Cq values in dextrose. (D) Levels of Puf3p-TAP that were isolated in the RNA-IP procedure (lower IP panel) show relatively equal levels of isolated protein.
decreased ability of the protein to bind its target mRNA. If anything, Puf3p binds more COX17 in galactose conditions. This result corroborates Puf3p’s role in localizing these targets to the mitochondria even when Puf3p is not competent to stimulate decay (Saint-Georges et al., 2008).

4. The Deadenylation Step of Puf3p Target COX17 is Inhibited in Galactose

Decay of most mRNAs begins with the removal of the 3’ poly(A) tail before the removal of the 5’ cap (Decker and Parker, 1993). Exonucleolytic 5’ to 3’ decay by Xrn1p proceeds rapidly after cap removal (Muhlrad et al., 1994), such that the removal of the two RNA end modifications are rate limiting to the decay of the mRNA. To investigate which of these two steps was inhibited for Puf3p-mediated decay in galactose, we decided to first focus on removal of the poly(A) tail, as this is the first step of decay. To this end, we employed a deadenylation assay to measure the progressive real-time removal of the poly(A) tail of the COX17 mRNA in dextrose and galactose. As seen in figure 2.4, the poly(A) tail of the transcript in dextrose begins to be deadenylated starting at 2 minutes and continues into 4 and 6 minutes. By 8 minutes, most of the transcript is degraded. In galactose however, we see little shifting of the band corresponding to the transcript with a full poly(A) tail over the entire course of the assay. A pool of fully or nearly fully adenylated species can be seen even at the 40 minute time point. This indicates that in galactose, the deadenylation step of COX17 is inhibited, and suggests that the mechanism whereby the transcript is stabilized is through this inhibition. While the rate of degradation is also slowed (the point at which the transcript is barely visible occurs later in the time course), the reason we do not see
Figure 2.4 The Deadenylation Step for COX17 mRNA is Inhibited in Galactose. Deadenylation assays for reporter construct MFA2-COX17 3’UTR in (A) dextrose and (B) galactose. Time after transcriptional shutoff is denoted. -8 denotes after puling cells with galactose 8 minutes before shift to media containing dextrose or galactose. dT lane shows size of deadenylated transcript.
large amounts of fully deadenylated transcript at these later times is that when a transcript does become deadenylated, it is rapidly degraded. Inhibited deadenylation in galactose indicates altered processing by the deadenylation complex, of which Pop2p is a member.

5. Pop2p Shows Reduced Binding to Puf3p in Galactose

As discussed earlier, one of our hypotheses for the inactivity of Puf3p in non-fermentative carbon sources was that the binding of decay factors to Puf3p may be altered under these conditions. To this end, we analyzed binding of Puf3p to Pop2p, Dcp1p, Dhh1p, and Ccr4p in dextrose as compared to galactose using co-immunoprecipitation (co-IP) studies. We utilized epitope tagged versions of these cofactors, which each bear 8 consecutive c-myc protein tags on their C-terminal ends (see Materials and Methods for construction). Co-IPs were performed in cells with a puf3Δ that had been transformed with a plasmid bearing an N-terminal FLAG epitope tagged version of the Puf3 repeat domain (hereafter Puf3RDp), under the transcriptional control of the yeast GDP promoter and having a 2µ (high copy) origin of replication. Co-IP conditions are described in Materials and Methods. As shown in figure 2.5, Dcp1p, Ccr4p, and Dhh1p showed similar levels of interaction with Puf3RDp between dextrose and galactose conditions. However, the levels of Pop2p were consistently reduced in galactose. This result indicates that Pop2p’s ability to bind Puf3p is reduced in this condition, and this altered interaction may contribute to Puf3p inactivation in this condition. Further, this result points to the importance of Pop2p in the decay complex, given Pop2p’s role in deadenylation.
Figure 2.5 Pop2p Shows Reduced Affinity for Puf3RDp in Galactose. Co-IPs showing binding of c-myc tagged cofactors Dcp1p, Ccr4p, Dhh1p, and Pop2p to FLAG-Puf3RDp. Left panels, Co-IP’d cofactors input and elution in dextrose and galactose. Right panels, IP’d FLAG-Puf3RDp in dextrose and galactose.
6. Decay Factors Bind to Puf3p through Pop2p

Pop2p is an important member of the deadenylation complex. Previous *in vitro* studies have shown the role of Pop2p in proper recruitment and complex formation of decay factors by Puf4p and Puf5p (Goldstrohm *et al.*, 2006). We sought to determine Pop2p’s role *in vivo* in the ability of Puf3p to associate with decay factors. To this end, we deleted *pop2* from the strains with the c-myc tagged Dcp1p, Ccr4p, and Dhh1p proteins and assessed whether deletion of this gene altered their ability to bind Puf3p. Co-IP conditions were the same as in the previous study. As shown in figure 2.6, decay factors Dcp1p, Ccr4p, and Dhh1p were unable to co-IP with the FLAG tagged Puf3RDp in a *pop2Δ* strain. Thus, Pop2p is necessary for proper binding of Puf3p to these three cofactors, and suggests that in addition to its intrinsic deadenylase activity, Pop2p acts as a scaffolding protein for other decay factors. It is interesting to note that although Pop2p is necessary for co-IP of decay factors with Puf3p, no concomitant reduction of the cofactors was seen in the prior study looking at differences in protein levels in dextrose versus galactose as was seen for Pop2p. It may be that although Pop2p interaction with Puf3p is reduced in galactose, enough still binds in order to promote near maximal association of Puf3p with decay factors through its scaffolding attributes.

7. Mimicking Phosphorylation of Ser563/566 of Puf3RDp Results in Reduced Stability in Galactose

We next hypothesized that a mechanism whereby Puf3p’s activity could be rapidly altered may be post-translational modification, either of Puf3p or perhaps one of the cofactors involved in decay. We chose to focus on phosphorylation as the post-
Figure 2.6 Decay Factor Binding to Puf3p Requires Pop2p.
Co-IPs in WT and pop2Δ strains. C-myc tagged cofactors Co-IP’d are shown on the left side, FLAG-Puf3RDp IP is shown on the right. Co-IPs were repeated ≥2 more times with similar results.
translational modification that may be occurring, as phosphorylation is reversible and ubiquitous, and there are over 100 proteins in yeast with confirmed kinase activity. We began by using the bioinformatic tool NetPhosK (Blom et al., 2004) to predict kinase specific sites within Puf3p and Puf5p that were positionally conserved between them based on alignment of the two proteins’ repeat domains. From this analysis, we centered on five potential phosphorylation sites in four locations in the Puf3 repeat domain, including Ser543 in the first Puf repeat, Ser563 and Ser566 in the first Puf repeat, Tyr634 in the third Puf repeat, and Ser834 in the eighth repeat. We mutated these residues on the Puf3RD expression plasmid to aspartate in order to mimic constant phosphorylation at this site and asked whether the individual or combined mutations could rescue the stable decay phenotype of COX17 mRNA seen in galactose. As shown in figure 2.7, mutation of Ser543, Tyr634, and Ser834 to aspartate produced half-lives for COX17 mRNA that were indistinguishable from those seen with the WT protein. When analyzing the double Ser563Asp Ser566Asp mutant, we noticed a significant reduction in the stability of the mRNA, from a half-life of 32 minutes with the WT protein down to 18.6 minutes with this mutant. For comparison, the WT protein produces a half-life of 12 minutes for the mRNA when the cells are grown in dextrose. Not surprisingly, the half-life obtained when all five sites were mutated to aspartate was also lower than WT, with a value of 14 minutes. The results indicate that phosphorylation of Ser563 and Ser566 play a major role in regulating Puf3RD stimulation of decay, as mimicking constitutive phosphorylation at these sites destabilized the COX17 mRNA to an extent similar to the WT protein when the cells
**Figure 2.7 Mimicking Constant Phosphorylation at Ser563 and Ser566 of Puf3RDp Results in Constitutive Puf3p Stimulation of Decay.** Transcriptional shutoff assays with cells containing Puf3 Repeat Domain mutants with the indicated mutations. Except where noted (Dex), assays were performed in galactose. Quintuple mutant incorporates all five mutations. Error bars represent SEM and are the result of ≥2 independent biological trials. Indicated amino acid mutations are respective to their position within the curated full length protein.
were grown in dextrose. This is consistent with earlier work in the lab showing that mutation of these two sites to alanine stabilized COX17 mRNA when cells were grown in dextrose (Lopez-Leban et al., unpublished). We also tested a single S563D mutation, as this had been identified by another kinase-specific phosphorylation prediction software, Musite (Gao et al., 2010), as highly likely to be phosphorylated. However, the decay phenotype was similar to the WT Puf3RDp, indicating that phosphorylation of both sites is likely needed. It is still unclear what roles the other sites have in modulation of Puf3RD activity, but their contribution, if any, is likely small since the quintuple mutant incorporating all five mutations only reduced the half-life to 14 minutes as compared to 18 minutes for the Ser563/566 mutants.

8. Deletion of the Kinase YAK1 Results in Constitutively Rapid COX17 mRNA Decay

To continue our investigation into the posttranslational modifications that could be controlling Puf3p activity, we decided to ascertain which kinase was responsible for the phosphorylation event. We tested deletion of multiple kinases for their ability to alter Puf3p target COX17 mRNA decay through a variety of methods (see Materials and Methods for a detailed list of kinases and their respective decay assay conditions), focusing on kinases that either had been previously shown to bind Puf3p or that changed their kinase activity or subcellular localization based on the carbon source available. Kinases tested included SNF1, FUN31, PSK2, PTK2, SKY1, TOR1, TPK1, TPK2, TPK3, SLT2, FMP43, FMP48, MKT1, PHO85, PTC1, SKS1, and HRR25. Deletion of the genes for these kinases had no effect on decay of COX17 mRNA, or if decay was altered for COX17, it was also altered for a control mRNA, indicating a role for the kinase in
altering general decay mechanisms instead of being specific for Puf3p targets. *YAK1* encodes a kinase that is known to partly shift its subcellular localization during periods of glucose deprivation to phosphorylate Pop2p (Moriya *et al.*, 2001), and its transcript has been shown to bind Puf3p (Freeberg *et al.*, 2013). Given these associations, we decided to analyze *YAK1* for its ability to influence Puf3p activity. We began by analyzing the decay rate of *COX17* mRNA in dextrose and galactose in both WT cells and a *yak1Δ* deletion strain, with both strains containing a temperature sensitive mutation to *rpb1-1* in order to perform transcriptional shut-off assays for RNA half-life analysis (see Materials and Methods for this section). As shown in figure 2.8, the half-life of *COX17* mRNA in WT cells grown in dextrose was 3.25 minutes, whereas it was 14 minutes in galactose, over 4-fold longer. A similar half-life of 2.75 minutes was also seen in the *yak1Δ* strain grown in dextrose; however, the half-life of *COX17* mRNA for this strain when cells were grown in galactose was 4.5 minutes, indicating a destabilization of the mRNA in an normally stabilizing condition. These results indicate the necessity of Yak1p kinase to achieve the normal lifespan of *COX17* in non-fermentative conditions, and suggest that this activity may also be influencing Puf3p stimulation of decay.

9. *Yak1p Association with Puf3p Increases in Galactose*

Previous work has hinted at the importance of the phosphorylation state of Pop2p. Exogenous expression of Pop2p with a T97A mutation is dominant, with cells showing overgrowth after the diauxic shift when glucose has been depleted, and cells failing to arrest at the G₁ cell cycle checkpoint as WT cells do during this shift (Moriya *et al.*
**Figure 2.8 Deletion of YAK1 Results in Constitutive Puf3 Activity.** Transcriptional shutoffs of WT cells or cells in which yak1 had been deleted performed under dextrose or galactose. Cells were grown to early log phase (O.D.600 ~ 0.4) and then shifted to the restrictive temperature. Cells were collected at the indicated times, and RNA was extracted and subjected to Northern blot analysis for COX17 detection. $T_{1/2}$ – Half Life of COX17. Error bars represent SEM of two biological trials.
Given the association of Pop2p with Puf3p, the role of Yak1p in phosphorylation of Pop2p, and the role of Yak1p in decay of Puf3p target mRNAs we asked if Yak1p associated with Puf3p, and furthermore, if that association was increased or decreased in cell grown in different carbon sources. We performed similar co-IPs as described earlier with a c-Myc C-terminal epitope tagged endogenous Yak1p and analyzed the Yak1p bound by FLAG-Puf3RD. As shown in figure 2.9A, increased association of Yak1p with Puf3RDp was present in galactose versus dextrose grown cells. This result suggests that in galactose, Puf3p and Yak1p have a stronger affinity for each other. This also suggests that in galactose-grown cells, a stronger association of Puf3p with Yak1p could place Yak1p in a position to phosphorylate any Pop2p associated with or coming in the vicinity of Puf3. It has been demonstrated that Yak1p does not change its kinase activity based on the carbon source available (Moriya et al., 2001). Also, the interaction of Yak1p with Puf3p is not dependent upon the presence of Pop2p, as was demonstrated earlier for Dcp1p, Dhh1p, and CCr4p. As demonstrated in figure 2.9B, Yak1p still associates with Puf3RD even in the absence of Pop2p. Taken together, these results suggest that in galactose-grown cells, more Puf3p-associated Pop2p may be phosphorylated, possibly altering Pop2p’s catalytic activity or binding to other protein cofactors.

10. Mutation of Thr97 of Pop2p Results in Constitutive Decay Phenotypes of mRNAs

As stated earlier, the phosphorylation status of Pop2p is important to cell growth. After depleting glucose and transitioning to other carbon sources, cells bearing a T97A or T97D/E mutation fail to slow down growth as do WT cells (Moriya et al.,
Figure 2.9 Yak1p Association with Puf3p is Stronger in Galactose and Does Not Require Pop2p. (A) Showing FLAG-Puf3RD IP and associated Yak1-myc Co-IP in dextrose and galactose. 3RD lanes, FLAG-Puf3RD vector present; EV lanes, empty 2μ vector alone. (B) Showing FLAG-Puf3RD IP and associated Yak1-myc Co-IP in the absence of Pop2p. Lane labels indicate the same as in A. Co-IPs were performed twice with similar results between the two trials.
To deepen our understanding of the phosphorylation of Thr97 of Pop2p achieved by Yak1p, we decided to investigate the effect of blocking phosphorylation or mimicking constant phosphorylation at this site. To this end, we deleted *pop2* from WT cells with a temperature sensitive mutation to *rpb1-1*. We then expressed either a WT *POP2*, a *pop2* T97A mutant (which blocks phosphorylation to this site), or a *pop2* T97D mutant (which mimics constant phosphorylation because of the negative charge on aspartate) in this strain exogenously from a plasmid under the ADH promoter. The cells were grown in dextrose or galactose and the half-life of *COX17* mRNA measured to assess the effects of the *pop2* mutations. As shown in figure 2.10, the half-life of *COX17* mRNA with the WT *POP2* was longer in galactose (11.84 minutes) than in dextrose (6.37 minutes), as expected. Analysis of the T97A mutant revealed a similar decay rate for *COX17* in dextrose; however, in galactose the half-life was shortened to 7.64 minutes, indicating a destabilization in this growth condition. Conversely, the T97D displayed an increased half life in dextrose of 14.19 minutes; the half life in galactose was also modestly extended compared to WT Pop2p (16.14 minutes versus 11.84 minutes). To determine whether this phenomenon was unique to Puf3p targets, we also tested *CBS1* mRNA decay, which is also a nuclear encoded mitochondrial transcript like *COX17* but does not have a Puf3p binding site and is not under its decay control. Interestingly, we also observed a constitutive lengthening of the mRNA when the T97D mutant form of Pop2p was expressed. This indicates that phosphorylation of Pop2p at this residue influences decay of all mRNAs, and points to a global regulatory mechanism whereby Yak1p phosphorylation of Pop2p results in stabilized transcripts.
**Figure 2.10** Mutation of Pop2p Thr97 Results in Consitutive Decay Activity. Decay analysis of COX17 (upper panel) and CBS1 (lower panel) in cells with a pop2 deletion expressing either WT Pop2p or the Thr97 mutation indicated constitutively from a plasmid. Error bars represent SEM and are the result of ≥3 independent trials. T1/2 = measured half-life of the indicated mRNA.
11. Constitutive Decay Phenotypes of Pop2p Thr97 Mutants Require the Actions of Puf3p for Optimal Destabilization

To further assess the effects of the Pop2p Thr97 mutations on decay, we assayed COX17 decay indirectly using a growth assay. Briefly, his3 mutant strains are transformed with a plasmid bearing the HIS3 ORF with a COX17 3’UTR, then plated on synthetic media lacking histidine. Any differences in growth can be attributed to the expression levels of HIS3, and thus the level of decay stimulation by the Pop2p mutatants. We decided to use endogenous pop2 mutants for Thr97, which were constructed using the delitto perfetto method (Storici and Resnick, 2006), a method that creates targeted mutations in an isogenic background (i.e., the only difference between these three strains genetically is three or less point mutations, see Materials and Methods). As shown in figure 2.11, both the WT and T97D forms of Pop2p were able to grow on plates lacking histidine (upper panel), with no discernible differences between the two. However, the T97A mutant failed to show any growth. This result indicates that the chimeric HIS3-COX17 mRNA is likely being degraded so rapidly that insufficient amounts of HIS3 protein are being made, and the cells are unable to survive. To ascertain whether this decay phenotype required the actions of Puf3p, we mutated the two Puf3p binding sites in the COX17 3’ UTR from UGUA to ACAC, as prior studies from our lab have indicated these sites are required for Puf3p recruitment to the mRNA (Jackson et al., 2004). Upon mutation of these sites, we again observed little discernible difference of growth between the WT and T97D forms of Pop2p (lower panel). We did see growth of the T97A mutant this time, although the growth was not as substantial as
**Figure 2.11 Pop2p Requires Puf3p Mediated Recruitment for Optimum Destabilization of COX17 mRNA.** HIS3 growth studies in cells bearing plasmids with either WT COX17 3’UTR (upper panel) or a COX17 3’UTR in which the two predominant UGUA binding sites for Puf3p had been mutated to ACAC (lower panel). Leftmost panel (-ura, -leu) is shown to confirm all cells were growing equally. ATZ, 3-amino-1,2,4-triazole, added to control leaky HIS3 expression and amplify differences between experimental conditions (see Materials and Methods).
the WT or T97D Pop2p. The results indicate that the actions of Puf3p are needed to mediate rapid decay of Puf3p target mRNAs via recruitment of Pop2p to the mRNA, and recruitment of a Pop2p with unphosphorylated Thr97 enables more rapid decay than phosphorylated Thr97. The T97A mutant strain was likely not able to rescue growth fully when presented with a HIS3-COX17 mutant 3’ UTR because this Pop2p mutant is still constitutively active for decay. There is likely an equilibrium normally in the cell of phosphorylated/non-phosphorylated Pop2p, however with a T97A mutation, every time an mRNA encounters Pop2p the protein is constitutively active for rapid decay. The results further confirm that phosphorylation of Thr97 of Pop2p plays a pivotal role in regulating decay of mRNA.

Discussion

The results of this study give rise to the model depicted in Figure 2.12. When cells are grown in dextrose, Puf3p exhibits a strong association with Pop2p, which is required for binding of decay factors Dcp1p, Ccr4p, and Dhh1p to Puf3p by acting as a bridging molecule. These associations result in rapid decay of the mRNAs targeted by Puf3p. Phosphorylation of Puf3p at Ser563 and Ser566 also likely plays an unknown role. It may be that phosphorylation at these residues leads to decreased association of Puf3p with Yak1p in cells grown in dextrose as compared to galactose. As the vectors used to investigate phosphomimetics of Puf3RDp were made from pWO16, the vector used in the co-IP studies, the vector bearing the S563D and S566D mutations is ideal to use in the same co-IP system to investigate whether these mutations affect binding of Puf3p to Yak1p. When the cells are grown in galactose, Puf3p has a lower association
Figure 2.12 Mechanism of Puf3p Activity Alteration. When cells are grown in dextrose, Yak1p is less associated with Puf3p, and Pop2p is more associated with Puf3p, than in galactose. Pop2p serves as a bridging molecule for the decay factors indicated. In galactose, Pop2p affinity for Puf3p is decreased, which may lead to less-than-optimal recruitment of decay factors. Yak1p may modulate this.
with Pop2p. Interestingly, there does not appear, by Western blot analysis, to be a marked decrease in the association of the other decay factors with Puf3p when cells are grown in galactose. It may be that the reduced amount of Pop2p still allows a fair amount the of decay factors to associate with Puf3p, although optimum association with all decay factors is required for the decay stimulation seen in dextrose. Puf3p also has a higher association with Yak1p in galactose; i.e. every time a transcript comes in contact with Puf3p there is a higher likelihood of it being associated with Yak1p. Yak1p is responsible for phosphorylating Pop2p, therefore in galactose the Pop2p that comes into contact with Puf3p has a higher likelihood of being phosphorylated at Thr97. It is still unclear what effect the phosphorylation status of Thr97 has on the actions of Pop2p. It is unlikely to interfere with the nucleolytic activities of Pop2p, as this residue lies upstream of the catalytic portion of Pop2p (Daugeron et al., 2001). It may be that this residue is important for binding of other factors; reduced Ccr4p binding would certainly explain why the deadenylation step of the chimeric MFA2-COX17 3’ UTR is inhibited in galactose. Two competing theories are that this phosphorylation event may affect binding of Pop2p to Puf3p, or it may affect binding of Pop2p to the other decay factors. Given the fact that an exogenously produced Pop2p T97D mutant showed extended stability of CBS1 mRNA, which is not a target of Puf3p regulation, this points to the latter theory, that general binding of decay factors is being altered. This is also why the endogenous Pop2p T97A mutant was able to grow on the HIS growth assay plates with the HIS3 reporter plasmid containing mutations in the Puf3p binding sites, although not as well as the WT and T97D mutant. If the T97A mutant is allowing decay factors
to always bind to Pop2p, then decay of all mRNAs will be affected, but without the benefit of Puf3p to guide the complex to the mRNA, some expression of HIS3 is allowed. These two competing theories are not necessarily mutually exclusive, as the phosphorylation event may be altering binding to both Puf3p and the decay factors. We have created exogenous N-terminal HA-epitope tagged versions of Pop2p to determine (in a pop2Δ strain) whether these constitutive mutants alter the association of Pop2p with Dcp1p, Dhh1p, or Ccr4p. To investigate the role of Thr97 in the binding of Pop2p to Puf3p, we have made the same endogenous mutations to Pop2p using the delitto perfetto method in the Pop2p-myc strain background (yWO191), to ascertain whether the mutants show reduced or increased binding of Puf3p. We have also made the same mutations in our WT rpb1-1TS strain (yWO7) in order to further validate the decay phenotypes seen with the exogenous Pop2p in an endogenous setting. These experiments are currently underway.

It is still unclear which kinase may be phosphorylating Puf3p. Previous evidence indicates both Sch9p (through mTOR) and PKA (Tpk1p and Tpk2p) pathways may be involved in the phosphorylation of Puf3p, as inhibition of either of these two pathways has been shown to reduce overall Puf3p phosphorylation (Lee and Tu, 2015), although these were identified for the full-length Puf3p. This study mainly used an exogenously expressed repeat domain of Puf3p, which contains only a few of the phosphorylation sites identified in the full-length protein. We have shown that mimicking phosphorylation at Ser563 and Ser566 in this repeat domain results in destabilization of Puf3p target COX17 mRNA in non-fermentative conditions where the transcript would
be normally stable, and nearly destabilized to the extent seen when cells are grown in fermentative conditions (dextrose). This indicates these are the two major sites affecting Puf3p activity, and suggests that although previous studies have established that phosphorylation of residues N-terminal of the repeat domain promote translation of Puf3p targets and stimulate proper mitochondrial biogenesis, phosphorylation of the Ser563 and Ser566 sites in the repeat domain may stimulate Puf3p’s decay-promoting activity. This also suggests that phosphorylation may be playing a dual role in modulating the activity of Puf3p based on where the phosphorylation takes place, and again raises the question of which kinase is acting at which time, depending on particular signaling cascades.

**Materials and Methods**

**Strains, Oligonucleotides, and Plasmids**

Strains, oligonucleotides, and plasmids used in this study can be found on Table 2.1, 2.2, and 2.3, respectively. All transformations in this study were performed using the LiOAc/PEG/ssDNA method as previously described (Gietz and Schiestl, 2007). myc-tagged strains for use in IP were made by transforming yWO18 with PCR amplicons produced from pCH985 (a tagging vector coding for nine consecutive c-myc peptides and the *TRP1* ORF. This epitope tagging construct has since been moved into pBS and is now pWO251) with oWO 190/191, oWO844/845, oWO848/849, oWO842/843, or oWO838/839 to create a Dcp1, Ccr4, Dhh1, Pop2, or Yak1-myc tagged strain, respectively. Successful transformants were selected by plating on media lacking tryptophan, as the tagging amplicon includes the ORF of *TRP1*. Transformants were
further verified via western blot of crude lysate using an anti-c-myc antibody (see Western blots section for details) to assure correct size of tagged protein. pop2Δ and yak1Δ strains were made by amplifying the nourseothricin resistance gene (hereafter NTCR) from pWO241 with oWO834/835 and oWO800/801, respectively, and transformed into cells. Successful transformants were selected by plating cells on plates containing the broad spectrum antibiotic nourseothricin sulfate (GoldBio Catalog#N-500-100) and selecting colonies. Colonies were further verified to contain the deletion by PCR amplification of genomic DNA (extracted using Qiagen’s Puregene Yeast/Bacterial Kit B, Catalog#1042607, following manufacturer’s protocol) with oWO832/833 and oWO830/833, respectively, which only produces an amplicon if the deletion occurred. Amplicon was confirmed for correct size by electrophoresing on a 1% agarose 1X TAE gel stained with EtBr alongside a DNA size marker (New England Biolabs Catalog#N3232S). yWO313 and 314 were made using the delitto perfetto method. Protocol and primer design were performed as described by the authors (Storici and Resnick, 2006). yWO211 was transformed with a PCR amplicon made from pWO250 with oWO905/906, allowed to recover 3 hours in YEPD liquid media, and plated on plates containing 200 µg/ml G418 (Geneticin, GibcoBRL Catalog#11811-031). Colonies were picked and plated on YEPD plates and grown in YEPD media, had genomic DNA extracted as described before, and PCR was performed with oWO482/954 to verify the cassette had been inserted into POP2 at the codon coding for Thr97. After verification of insertion, the resultant strain was transformed with oWO907/908 or oWO909/910 induce the T97A or T97D mutation, respectively. The oligonucleotide
pairs are reverse complements of each other, and both remove the pCORE-UK cassette and introduce the mutation at the same time. The entire transformation was plated on a YEPD plate, allowed to grow overnight, and replica plated the following day on SC complete plates plus 2% dextrose, 60mg/L uracil, and 1mg/ml 5-fluoroorotic acid (5-FOA). 5-FOA is converted into a toxic substance by the URA3 gene, which is also in the pCORE-UK cassette; resultant colonies growing on this media have lost the cassette and incorporated the desired mutation. Colonies were again picked, plated on YEPD, grown in YEPD for genomic DNA extraction, and this DNA was PCR amplified with oWO482/483 to create an amplicon containing the POP2 ORF for sequencing purposes. This amplicon was sequenced with oWO879 by Eurofins and the sequence was analyzed with the free software FinchTV to verify that the codon coding for Thr97 now coded for either alanine or aspartate.

Puf3RD phosphomimetic plasmids were constructed as follows. pWO16 was subjected to PCR amplification using the Stratagene Quikchange XL site-directed mutagenesis kit as per manufacturers instructions. The S543D, S563D/S566D, S563D, Y634D, and S834D mutations were made using oligo pairs oWO490/491, oWO492/493, oWO566/567, oWO494/495, and oWO496/497, respectively. Resultant transformants that grew on LB plates containing ampicillin were picked, grown in LB-Amp liquid, and plasmid DNA was extracted using the Zyppy Miniprep kit as per the manufacturers instructions. The plasmids were subjected to sequencing to verify the indicated mutations. Sequences were analyzed as described above. pop2 T97 mutant Zeo URA3 plasmids were constructed similarly, using oWO877/878 and oWO875/876 to create
pWO228 and 229, respectively. Sequencing of the plasmids and analysis took place as described before to verify the correct mutation of Thr97. To create pWO242 and 243, the COX17 3’UTR was amplified either from genomic DNA or pWO9, respectively, with oWO733/734. pWO118 and the amplicons were cut with the enzymes SpeI and HindIII, and ligated together. Ligations were transformed into E. coli and the transformed cells were plated on LB plates containing ampicillin. Colonies were picked, grown in LB-Amp liquid media, and plasmids were extracted with the Zyppy Miniprep Kit. The resultant plasmids were cut with the enzyme NotI, as was pWO111 to excise the HIS3 ORF. The ORF and the cut plasmids were ligated and transformed into E. coli as described earlier. The transformations were plated on LB-Amp plates, colonies picked and grown in LB-Amp liquid, plasmid DNA extracted, and cut with BamHI and NdeI to determine correct orientation of the HIS3 ORF. Plasmids bearing the correct orientation were subjected to sequencing to verify the COX17 3’UTR sequence was correct, with pWO243 being checked to ensure mutation of the PRE to ACAC.

**Confocal Fluorescent Microscopy**

Endogenously GFP tagged Puf3p (yWO185) cells were grown in YEP media supplemented with 2% dextrose, galactose, or ethanol at 30°C. The cells were allowed to reach an OD$_{600}$ before being incubated with 50 nM MitoTracker Deep Red FM (Invitrogen Catalog #M22426) for 30 minutes. Cells were fixed with 3.7% formaldehyde for 1H, then washed twice with 1X phosphate buffered saline (PBS) and resuspended in a final volume of 8ml 1X PBS. One ml was placed on a circular glass slide, which had been previously coated with 1% polyethyleneimine and allowed to dry. The cells were
allowed to settle for 5 minutes. The solution was aspirated from the edge of the slide, and the slide was dipped twice in 1X PBS to remove non-adherent cells. A glass coverslip was placed on top of the glass slide. Cells were visualized with a Zeiss LSM-700 confocal microscope at 100X oil immersion for eGFP and mRFP signal using excitation/emission wavelengths of 488/507 nm and 584/607 nm, respectively. Ten vertical slices each 6.42 µm were taken through the Z plane of the cells, and flattened using the FIJI Is Just ImageJ (FIJI) software Z project function at maximum intensity. The signal intensity for both eGFP and mRFP channels was normalized for all samples by adjusting the maximum cutoff under Brightness and Contrast options panel for the ethanol samples, and setting the dextrose and galactose samples to the same cutoff. Average eGFP fluorescence was obtained by the measuring the fluorescence signal of a signal divided by the area of the cell and averaging the results. Average fluorescences and for galactose and ethanol samples were normalized to dextrose samples by dividing the average fluorescence of these samples by the average fluorescence of cells grown in dextrose (NAFG and NAFE, respectively). Normalized relative standard error was also calculated. Briefly, relative standard error for dextrose (RSED) was calculated by dividing the standard error of the mean by the average. Relative standard errors were calculated for galactose (RSEG) and ethanol (RSEE) were calculated in the same manner, and normalized (NSEG and NSEE) by the following equations:

\[ \text{NSEG} = \sqrt{(\text{RSED}^2 + \text{RSEG}^2)} \times \text{NAFG} \]

\[ \text{NSEE} = \sqrt{(\text{RSED}^2 + \text{RSEE}^2)} \times \text{NAFE}. \]
RNA-Immunoprecipitations and qPCR Analysis

RNA-IPs were performed as previously described (Gerber et al., 2004) with some alterations. Briefly, endogenously TAP-tagged Puf3p (yWO73) cells were grown in 2 L YEPD at 30°C. Cells were allowed to reach an OD$_{600}$ of 0.4, before being harvested and washed twice with 25ml of ice-cold Buffer A (20mM Tris-HCl [pH 8.0], 140mM KCl, 1.8mM MgCl$_2$, 0.1% Nonidet P-40, 0.02mg/ml heparin) and then frozen at -80°C. The next day, cells were thawed on ice and resuspended with 5 ml of ice-cold Buffer B (Buffer A with 0.5mM dithiothreitol and 1X Complete Mini Protease Inhibitors [Roche, Catalog #11-836-153-001], 40U/ml Recombinant RNasin Ribonuclease Inhibitor [Promega Catalog #N2511]). Cells were vortexed at max speed in the presence of glass beads for 1 minute and placed on ice for 1 minute a total of five times. Lysates were clarified by centrifugation at 7000 x g for 5 minutes. Supernatant was recovered and protein concentration was measured using a Bradford assay with BioRad Protein Assay Dye Reagent Concentrate (Bio-Rad Catalog # 5000006). The lysates were normalized to contain 1.625 mg in a volume of 5 ml, and 1% fraction V bovine serum albumin (BSA) and 50 µg yeast tRNA were added. Lysates were incubated with 400 µl of 50% IgG Sepharose 6 Fast Flow (GE Healthcare Catalog# 17-0969-010) which was previously blocked for 1 hour in 1 ml of Buffer B plus 1% BSA and 50 µg yeast tRNA. Beads were rocked end over end for 2 hours at 4°C, washed once with 5 ml of Buffer B for 15 minutes by rocking and three times with 5 ml of Buffer C (20mM Tris–HCl [pH 8.0], 140mM KCl, 1.8mM MgCl$_2$, 0.01% Nonidet P-40, 0.5mM DTT, 12U/ml Recombinant RNasin Ribonuclease Inhibitor) for 15 minutes by rocking. The sepharose beads were
then resuspended in 400 µl of Buffer C and 80 U of ProTEV Plus (Promega Catalog #V6101) was added and mixed. The beads were incubated for 2 hours at 16°C. The beads were pulse spun down, the supernatant drawn off, and total RNA was isolated via the hot acid phenol extraction method (Wei et al., 2009). 20 µg of glycogen was added to assist precipitation of the RNA. An additional set of IPs was performed in the same manner to recover protein in order to demonstrate equal recovery of the Puf3-TAP protein, as shown by western blot. The entirety of the recovered RNA from the IPs was treated to remove DNA with the Turbo DNA-free Kit (Ambion Catalog #AM1907) according to manufacturer’s protocol. Reverse transcription of RNA was performed with the BioRad iScript 5X Supermix (Bio-Rad Catalog#1708890) according to manufacturer’s specifications to create RT and No-RT cDNA pools for qPCR. qPCR was performed on the BioRad CFX96 Real-Time system using previously optimized cycling parameters with SYBR Green detection chemistry of amplicons using Bio-Rad SSOAdvanced 2X qPCR Mix (Bio-Rad Catalog#1725270). Gene-specific primers for CBS1, COX17, STE3, and housekeeping gene TDH1 are listed in table 2.2. qPCR experiments were conducted in biological and technical triplicates. Analysis for qPCR expression levels from the RNA IP’d was calculated by taking the IP’d levels of STE3 and CBS1 and normalizing them to the relative amount of STE3 and CBS1 mRNA present in total RNA as compared to COX17 (levels were found to differ between dextrose and galactose samples and in comparison to the amount of COX17 transcript present). The 2^-(ΔΔCt) method was used to calculate all fold differences in expression levels. After this normalization, enrichment values were calculated by normalizing COX17 and CBS1 to the amount of
STE3 IP’d to obtain relative amounts of COX17 and CBS1 that were IP’d. Normalized dextrose vs. galactose levels of COX17 mRNA IP’d were calculated by normalizing the difference in IP’d COX17 between dextrose and galactose against the baseline expression differences of COX17 in dextrose and galactose.

**Deadenylation Assays**

Transcriptional pulse chase assays to measure deadenylation rates were performed essentially as previously described (Decker and Parker, 1993). Briefly, a cox17Δ strain (yWO50) was transformed with pwO25, which places expression of this chimeric RNA under the control of the GAL-UAS. The cells were grown in 200ml SC-leucine plus 2% raffinose at 30°C until an OD$_{600}$ 0.4 was reached. Cells were harvested and resuspended in 20 ml SC-leucine media plus 2% galactose to induce transcription, and a -8 time point aliquot of cells were immediately taken. After 8 minutes, the cells were again harvested and resuspended in 20ml SC-leucine plus 2% dextrose to shut off transcription, and cells were incubated with shaking at 30°C. Aliquots of cells were taken at 0, 1, 2, 4, 6, 8, 10, 15, 20, 30, and 40 minutes after resuspension, and RNA was extracted using the hot phenol method described earlier. 40 µg of total RNA was incubated with oWO1 and RNaseH (and oligo-dT to create the oligo-dT sample which indicates fully deadenylated RNA), and RNA was reextracted and separated on a 6% denaturing polyacrylamide gel at 300V for six hours. The RNA was then transferred to a nylon membrane and blocked, probed with oWO303, and washed as previously described (Caponigro et al., 1993). Blots were exposed to Phosphor storage screens and signal intensity was analyzed with the Storm Phosphorimager.
Co-Immunoprecipitations and Western Blots

All Co-IPs took place with the same protocol. The respective strains were first transformed with pWO16 and plated on SC-uracil agar media supplemented with 2% dextrose. Successful transformants were grown in 200ml SC-uracil liquid media supplemented with 2% dextrose, or where indicated, galactose. Cells were harvested by centrifugation at 3,200 x g for 2 minutes, the supernatant was discarded, and the cells were resuspended in the remaining liquid and transferred into a 2 ml conical screw top eppendorf tube filled with 0.5 ml acid-washed glass beads. The cells were centrifuged at 21,000 x g for 30 seconds, the supernatant was completely removed, and the cells were stored at -80°C. Cells were later thawed on ice and resuspended in 500 µl IP buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 2mM MgCl₂, 0.1% Nonidet P-40, 10% glycerol) plus 1 Pierce Protease Inhibitor Mini tablet per 10 ml (ThermoFisher catalog #88665), and vortexed at max speed for one minute and placed on ice for one minute a total of five times. Lysate was clarified by centrifugation at 3,200 x g for 10 minutes. Supernatant was removed and subjected to Bradford total protein analysis as previously described. 1500 mg of protein in 500 µl total volume of IP buffer was prepared from each sample. 40 µl of this was mixed with 60 µl IP buffer and 100 µl 2X Laemmli load buffer (4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, 0.004% Bromophenol Blue, 0.125M Tris-HCl pH 6.8) to be used as input on western blot. The remaining volume was loaded onto 50 µl anti-FLAG M2 Agarose Affinity Gel (Sigma-Aldrich catalog #A2220) which had been equilibrated with 1 ml IP buffer plus 1% BSA for 1 hour with end over end rocking at 4°C before pulse spin and removal of supernatant.
Samples were rocked end over end at 4°C for 1 hour before pulse spin and removal of lysate sample. Samples were then washed a total of five times for five minutes each with Wash Buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 0.5% NP-40) with end over end rocking at 4°C. Samples were eluted by adding 100 µl of Wash Buffer and 4 µl of 5mg/ml 3X FLAG Peptide (Sigma-Aldrich Catalog #F4799) and rocking end over end for 20 minutes at 4°C. Eluate was passed through a 0.45 µm Spin-X filter column (Sigma-Aldrich Catalog#CLS8162) to separate resin from liquid eluate, and the eluate was mixed with an equal volume of 2X Laemmli sample buffer. Proteins were separated on a 7.5% SDS-PAGE handcast gel at 125V for 90 minutes in 1X Tris-Glycine Buffer (25mM Tris, 192mM Glycine, 0.1% SDS). 60 µl of input and elution was used for detection of c-myc, and 20 µl and 10 µl of input and elution respectively was used for FLAG-Puf3RD detection. Gels were transferred to nitrocellulose membrane overnight for 30 minutes in Transblot buffer (25mM Tris, 192mM Glycine, 20% methanol). Blots were blocked blocking buffer (1X PBS with 0.1% Tween-20 and 5% skim milk powder) for 1 hour. Blots were then probed with 1:5000 monoclonal anti-FLAG antibody (Sigma-Aldrich Catalog#F3165) or monoclonal anti-c-myc antibody (Sigma-Aldrich Catalog#M4439) in blocking buffer for 1 hour with gentle orbital shaking. Blots were washed five times in 1X PBS plus 0.1% Tween-20 for three minutes each with gentle orbital shaking. Blots were then probed with anti-mouse secondary antibody (Sigma-Aldrich Catalog #A9917) in blocking buffer for 1 hour with gentle orbital shaking. Blots were washed again as described. Blots were briefly drained and subjected to incubation with SuperSignal West Dura Extended Duration Substrate (ThermoFisher
Catalog#34075) as per manufacturers instructions. Blots were then exposed to autoradiography film (Midwest Scientific Catalog#BX810) to capture chemiluminescence, and developed (Sigma-Aldrich Catalog # P7042) and fixed (Sigma-Aldrich Catalog#P7167) as per manufacturers specifications.

**In Vivo Decay Analysis of mRNAs**

All strains used in mRNA decay analysis bear a temperature sensitive mutation to RNA polymerase subunit B, *rpb1-1*<sup>TS</sup>, which allows cells to transcribe mRNA at room temperature. Upon shifting the culture to 37°C, mRNA transcription stops and no new transcripts are made. Cells were grown in either SC media supplemented with 2% galactose to induce expression of chimeric mRNAs or YEPD when analyzing endogenous transcripts, both at 24°C. Cells were allowed to reach OD<sub>600</sub> 0.4, and were then harvested and resuspended in 10 ml of either SC or YEP media, and combined with the same media supplemented with 4% dextrose at 50°C to achieve a final temperature of 37°C. 2 ml cell aliquots were taken at 0, 2, 4, 6, 8, 10, 15, 20, 30, and 40 minutes into the assay, and RNA was extracted using the hot phenol method. 40µg total RNA was loaded onto a 1.25% agarose 1X MOPS (20mM 3-(N-morpholino) propanesulfonic acid, 5mM NaOAc, 10mM EDTA) gel with 6.66% formaldehyde and separated at 70V for 4 hours in 1X MOPS buffer. RNA was transferred via capillary action to nylon membrane with 10X SSC buffer overnight. Membranes were UV-C crosslinked twice, and blocking, probing, and washing steps were performed as described earlier (Caponigro et al., 1993). Probing took place with both the oligonucleotides to detect the mRNA in question, and after stripping and reblocking with the 7S (scRI) loading control.
Membranes were exposed to Phosphor Storage screens and analyzed by the Storm Scanner as described earlier. Data was analyzed using ImageQuant (Molecular Dynamics) software to quantify signal intensity at each time point. Half lives were calculated as the time when the mRNA was at half the quantity as that at time point 0.

**HIS3 Growth Assays**

Strains were transformed with pWO16 and either pWO242, pWO243, or pWO58 (pWO58 serves as a negative control for the growth studies which has LEU2 and thus the cells can survive on media lacking leucine, but fail to grow on media lacking histidine). The transformations were plated on SC-uracil-leucin agar media supplemented with 2% dextrose. Successful transformants were grown in SC-uracil-leucine liquid media supplemented with 2% dextrose overnight at 30°C. The following day, cultures were diluted into 20 ml of the same media and grown overnight again until an OD$_{600}$ of 0.4-0.6 was reached; timing of cultures was such that all cultures being tested on a particular day reached this density at approximately the same time. Cells were harvested and resuspended in 20ml of SC-uracil-leucine-histidine media. The OD$_{600}$ was measured again, and 16.675 was divided by this reading; it was previously determined that the resulting figure was how many µl of culture needed to be diluted into 1 ml of culture to yield 500,000 cells/ml. Three 10 fold dilutions of cells were made and 20 µl of each dilution and original was plated in series on SC-uracil-leucine agar media supplemented with 2% dextrose (to verify equal growth of all cultures) and SC-uracil-leucine-histidine agar media supplemented with 2% dextrose and either 3 or 5 mM 3-amino-1,2,4-trizaole (ATZ). ATZ is a toxic substance that is broken down by His3p,
and its addition to the media can help differentiate between minor differences in
growth by making them more apparent; it also controls for leaky expression of His3p
from the vector. After plating, cells were allowed to dry into the plate and grown for 2-
3 days at 30°C, scanned, and qualitatively analyzed for growth differences.

Table 2.1: Yeast Strains

<table>
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<th>Yeast Strain</th>
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<td>yWO185</td>
<td>Puf3-GFP</td>
<td>trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), PUF3-GFP (NEO)</td>
<td>(Miller et al., 2014)</td>
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<td>yWO73</td>
<td>Puf3-TAP</td>
<td>trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), PUF3-TAP-TRP1</td>
<td>(Miller et al., 2014)</td>
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<td>yWO50</td>
<td>cox17Δ</td>
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</table>


yWO189  Dhh1-myc  

\( \text{trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), puf3::NEO}^R, \text{DHH1-MYC(9)-TRP1} \)  

This Study

yWO191  Pop2-myc  

\( \text{trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), puf3::NEO}^R, \text{POP2-MYC(9)-TRP1} \)  

This Study

yWO290  pop2\( \Delta \) Dcp1-myc  

\( \text{trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), puf3::NEO}^R, \text{DCP1-MYC(9)-TRP1, pop2::NTC}^R \)  

This Study

yWO285  pop2\( \Delta \) Dhh1-myc  

\( \text{trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), puf3::NEO}^R, \text{DHH1-MYC(9)-TRP1, pop2::NTC}^R \)  

This Study

yWO291  pop2\( \Delta \) Ccr4-myc  

\( \text{trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), puf3::NEO}^R, \text{CCR4-MYC(9)-TRP1, pop2::NTC}^R \)  

This Study

yWO43  puf3\( \Delta \) rpb1\( -1^T_S \)  

\( \text{trp1-1, leu2-3,112, ura3-52, his4-539, rpb1\( -1^T_S \), cup1::LEU2(PM), puf3::NEO}^R \)
yWO7  WT rpb1-1<sup>TS</sup>  leu2-3,112, ura3-52, rpb1-1<sup>TS</sup>, yak1::NTC<sup>R</sup>

yWO292  yak1Δ rpb1-1<sup>TS</sup>  leu2-3,112, ura3-52, rpb1-1<sup>TS</sup>, yak1::NTC<sup>R</sup>  This Study

yWO297  Yak1-myc  trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), puf3::NEO<sup>R</sup>, YAK1-MYC(9)-TRP1  This Study

yWO319  pop2Δ Yak1-myc  trp1-1, leu2-3,112, ura3-52, his4-539, cup1::LEU2(PM), puf3::NEO<sup>R</sup>, YAK1-MYC(9)-TRP1, pop2::NTC<sup>R</sup>  This Study

yWO304  pop2Δ rpb1-1<sup>TS</sup>  leu2-3,112, ura3-52, rpb1-1<sup>TS</sup>, pop2::NTC<sup>R</sup>  This Study

yWO211  WT (BY4741)  his3Δ0, leu2Δ0, lys2Δ0, ura3Δ0  Research Genetics

yWO313  BY4741 pop2 T97A  his3Δ0, leu2Δ0, lys2Δ0, ura3Δ0, pop2 T97A  This Study

yWO314  BY4741 pop2 T97D  his3Δ0, leu2Δ0, lys2Δ0, ura3Δ0, pop2 T97D  This Study

Table 2.2: Oligonucleotides

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<tr>
<td>Location</td>
<td>Gene</td>
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Fischer, Anthony, UMSL, p.98

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Fischer, Anthony, UMSL, p.100

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Puf3RD
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Directed Up
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Directed
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cbs1 mRNA
Probe

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Fischer, Anthony, UMSL, p.101

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**References**


Chapter 3: Multiple Puf Proteins Regulate the Stability of Ribosome Biogenesis Transcripts

The work described in this chapter was performed entirely by me and can be found published in its entirety at https://doi.org/10.1080/15476286.2018.1521211
**Introduction**

Ribosome biogenesis is a highly complex process requiring efforts from multiple regulatory proteins performing excision, processing, and folding events of the ribosomal RNAs, as well as assimilation of ribosomal proteins into the ribosomal subunits to create the final products. Life as a ribosome begins with transcription of ribosomal RNA from a large tract of DNA known as rDNA. RNA Polymerase I transcribes the large ~6.6kb portion of rDNA that later becomes the 25S, 18S, and 5.8S rRNA species, while RNA Polymerase III transcribes the small portion of rDNA in the opposite direction that becomes the 5S rRNA. Within the large rRNA tract transcribed by RNA PolI, sequences are excised from between the three rRNA species and are termed internal transcribed spacers (ITS), while excised sequences that border on the outside of the central tract are referred to as 5’ or 3’ external transcribed spacers (ETS). (Osheim et al., 2004; Woolford and Baserga, 2013). Along the path to ribosome biogenesis, multiple ribosomal proteins attach and release both in the nucleus and after export to the cytoplasm to ensure correct structure and function of the final translational machinery. Many proteins involved in ribosome biogenesis are essential for growth (Steffen et al., 2012), and their expression must be carefully regulated to ensure correct levels of protein at all times. The demands for production are high; in rapidly dividing *Saccharomyces cerevisiae* yeast, a pre-ribosome is released every two to three seconds (Warner, 1999). Posttranscriptional regulatory mechanisms play a key role in such expression control.

Puf proteins are a eukaryotic family of posttranscriptional regulatory proteins that bind conserved sequences in the 3’ untranslated region (3’ UTR) of their target
mRNAs. The canonical sequence bound by Puf proteins normally includes a UGU trinucleotide sequence followed by an A/U rich downstream region, although some Puf proteins bind to less well-conserved sequences, which serves to broaden their repertoire of targets (Murata and Wharton, 1995; Wreden et al., 1997; Zamore et al., 1997; Souza et al., 1999; Zamore et al., 1999; Nakahata et al., 2001; Tadauchi et al., 2001; Wang et al., 2001; Wang et al., 2002; Gerber et al., 2004; Jackson et al., 2004; Qiu et al., 2014; Wilinski et al., 2015). The RNA-binding domain of Puf proteins, also known as the Pumilio homology domain, is typically composed of eight imperfect repeats of approximately 36 amino acid residues. Structural studies of Puf proteins bound to their target mRNAs have revealed that the complex adopts a crescent shape in which conserved residues of the Puf protein contact consecutive bases in the target in a one base per residue stacking manner (Wang et al., 2002; Miller et al., 2008; Wang et al., 2009; Zhu et al., 2009; Koh et al., 2011). Some Puf proteins are able to increase the pool of transcripts they bind by forcing one or more bases away from the binding face of the Puf protein such that targets without perfect retention of the binding sequence may still be bound by Puf proteins (Edwards et al., 2001; Wang et al., 2001; Wang et al., 2002; Miller et al., 2008; Wang et al., 2009; Zhu et al., 2009). Upon binding, Puf proteins typically accelerate decay or inhibit translation of their targets by forming protein-protein interactions with decay machinery to stimulate deadenylation and decapping, or with proteins that inhibit cap binding events of translation initiation (Goldstrohm et al., 2006; Goldstrohm et al., 2007; Lee et al., 2010b; Miller and Olivas, 2011; Blewett and Goldstrohm, 2012). The Pumilio homology domain, or repeat domain (RD) of yeast Pufs
1, 3, 4, and 5 has been shown to be sufficient for both RNA binding and binding to decay factors for regulation of mRNA decay (Jackson et al., 2004; Ulbricht and Olivas, 2008; Russo and Olivas, 2015). Some Puf proteins have additional motifs, such as RRMs and glutamine-rich motifs, but these are not well conserved, and little is known of their contributions, if any, to mRNA decay regulation (Olivas and Parker, 2000; Miller and Olivas, 2011).

Several global-scale studies have identified hundreds of transcripts physically bound by Puf proteins in S. cerevisiae and other eukaryotes (Gerber et al., 2004; Foat et al., 2005; Wilinski et al., 2015; Lapointe et al., 2017). These studies indicate that Puf proteins tend to bind functionally related classes of mRNAs. In S. cerevisiae, Puf3p binds nuclear-encoded transcripts involved in mitochondrial function, Puf4p binds targets involved in ribosome biogenesis, and Puf5p binds targets involved in regulation of gene expression and cell wall maintenance. While these studies highlight the myriad of targets bound by Puf proteins, binding does not always result in regulation (Miller et al., 2014).

Puf and Puf-like proteins have previously been shown to be involved in various aspects of ribosome biogenesis. Nop9 is a Puf-like protein in S. cerevisiae required for 18S rRNA synthesis and is associated with the pre-40S ribosomal subunit (Thomson et al., 2007). It bears 11 pumilio-like repeats and binds both in the pseudoknot region of 18S and close to the Nob1 cleavage site at the 5’ end of ITS1 (between cleavage sites D and A2). Its presence at the D-A2 site prevents premature cleavage of the 20S rRNA. It is unclear what the function of Nop9 is at the pseudoknot region, but it must be released
from the 18S rRNA prior to full maturation so that correct folding may occur (Wang and Ye, 2017). A puf6Δ in S. cerevisiae results in accumulation of 35S, 27S, and 7S precursor rRNA intermediates and causes defects in nuclear and nucleolar trafficking of large and small ribosomal subunits (Li et al., 2009). Mutation of basic residues of Puf6p conserved between Puf6p and human Puf-A results in similar rRNA processing defects and accumulations (Qiu et al., 2014). Caenorhabditis elegans Puf proteins puf-5, puf-8, and puf-9 act coordinately with ncl-1 and nos-2, the homologues to Drosophila melanogaster’s Brat and Nanos, respectively, to downregulate expression of fib-1 and control nucleolus size and the rRNA pool (Yi et al., 2015). Knockdown of PUF7 in Trypanosoma brucei decreases cleavage of the initial 9.2kb rRNA transcript and decreases abundance of the 2.6kb rRNA of the small subunit, indicating a defect in cleavage of the 3.4kb precursor (Droll et al., 2010). Knockdown of PUF7 and PUF10 in T. brucei decreases abundance of the 5.8S rRNA and its immediate 0.6kb precursor (Schumann Burkard et al., 2013). In Arabidopsis thaliana, T-DNA insertion mutants of APUM23 show accumulation of 35S pre-rRNA and unprocessed 18S and 5.8S rRNAs, as well as U3 and U14 snoRNAs, which are involved in maturation of the 18S rRNA (Abbasi et al., 2010). In mice, PUM2 was found to bind PRP-2 mRNA, which has one perfect and two imperfect PREs for PUM2. High PRP-2 expression is linked to decreased expression of several ribosomal protein mRNAs, and it is thought that PUM2 may affect global translation through modulation of PRP-2 mRNA stability (Scott et al., 2005).

In this study, we focused on understanding the role of S. cerevisiae Puf4p in ribosome biogenesis, given that many mRNAs physically bound to Puf4p are involved in
this process. We selected four of the top scoring transcripts binding Puf4p with regard to conservation of their consensus Puf4p binding sequence for further analysis (Gerber et al., 2004). Each of these transcripts is involved in some aspect of ribosome biogenesis. We show that these transcripts are not only regulated by Puf4p, but by a combination of Puf proteins. Pufs 2, 4 and 5 all contribute to destabilization of the mRNAs, while Pufs 1 and 3 act to stabilize the mRNAs. We also show that a single consensus site in the 3’ UTR of these transcripts corresponding to the canonical Puf4p binding sequence is critical not only for Puf-mediated decay regulation, but also decay regulation in the absence of Puf proteins. Unlike other targets of Puf4p regulation, decay of these ribosome biogenesis targets is not inhibited by differing carbon sources. Finally, we demonstrate that overexpression of Puf4p delays ribosomal RNA processing and inhibits ribosomal subunit trafficking. This work may provide a basis for understanding the roles of Puf proteins in ribosome biogenesis in more complex eukaryotes.

**Results**

**Targets involved in ribosome biogenesis that physically bind Puf4p are regulated redundantly by Puf4p and Puf5p**

Of the coordinately regulated transcripts that contain Puf4p binding elements in their 3’ UTRs as identified in (Foat et al., 2005), roughly 20% (148 of the 752) were involved in some aspect of ribosomal RNA processing or ribosome maturation and export. Of the transcripts found physically associated with Puf4p as found in (Gerber et al., 2004), one quarter of the 205 genes were involved in such ribosome biogenesis.
processes, with the rest of the targets falling into other functional categories well below 25% of the total. We therefore selected four of the top ranked targets identified in both studies that were involved in ribosome biogenesis: \textit{ALB1}, \textit{EBP2}, \textit{PUS7} and \textit{RRS1}. Each of these targets has one highly conserved Puf4 binding element in its 3’ UTR. We began investigating Puf4p’s role in regulating these targets by analyzing their steady-state mRNA levels in a wild-type (WT) and a \textit{puf4Δ} strain. Unexpectedly, the mRNA levels of these four targets were similar when comparing the two strains (Fig. 3.1). Some Puf proteins, including Puf4p and Puf5p, are known to bind in a promiscuous nature and can flip out nucleotides from the mRNA binding surface to accommodate a larger pool of binding partners (Opperman \textit{et al.}, 2005; Miller \textit{et al.}, 2008; Koh \textit{et al.}, 2009; Wang \textit{et al.}, 2009; Valley \textit{et al.}, 2012). It is also known that multiple Puf proteins can bind and regulate a single target, although usually more than one cognate Puf binding element exists in the 3’ UTR (Hook \textit{et al.}, 2007; Ulbricht and Olivas, 2008; Russo and Olivas, 2015). Specifically, there is significant overlap between mRNAs that are bound and regulated by both Puf4p and Puf5p (Gerber \textit{et al.}, 2004; Hook \textit{et al.}, 2007; Ulbricht and Olivas, 2008; Russo and Olivas, 2015; Lapointe \textit{et al.}, 2017). We therefore tested whether deletion of \textit{PUF5} influenced the target mRNA levels. While a single deletion of \textit{PUF5} failed to increase the target mRNA pools, a double deletion (\textit{puf4Δpuf5Δ}) noticeably increased the steady-state levels of all four mRNAs (Fig. 3.1), thus indicating that Puf4p and Puf5p are redundant for regulation of these targets. Previous studies have suggested that the binding pattern of one type of Puf site binds primarily to Puf4p,
while another pattern binds both Puf4p and Puf5p (Valley et al., 2012), while more recent studies suggest that Puf5p is more flexible in its binding, dependent on the

![Graphs showing mRNA levels for ALB1, RRS1, EBP2, and PUS7](image)

**Figure 3.1** Puf4p and Puf5p act redundantly to regulate steady-state levels of ribosome biogenesis Factor mRNAs. Steady-state levels of endogenous mRNAs in wild-type (WT), single *puf* deletion, and double *puf4Δ/puf5Δ* deletion strains are shown. Calculated mRNA levels are relative to WT levels for each mRNA.
curvature of its binding domain (Wilinski et al., 2015). Interestingly, the Puf sites in all four targets tested resemble the Puf4p binding pattern rather than the Puf4p+Puf5p pattern. Our results therefore demonstrate that the plasticity of Puf regulation is greater than previously described.

Next, we wished to test whether the increased levels of the four transcripts were a result of an altered decay rate mediated by the Puf proteins. We performed transcriptional shutoff assays in WT, puf1Δ, puf4Δ, puf5Δ, and puf4Δpuf5Δ strains that also contain a temperature-sensitive mutation in the RNA polymerase II Subunit B (rpb1-1), which allows mRNA half-life analysis on steady-state mRNA pools following a shift to the non-restrictive temperature to block further transcription. The results mirrored those seen by steady-state analysis in that half-lives of all four transcripts were similar between the WT and single puf deletion strains, but were lengthened in the puf4Δpuf5Δ strain (Fig. 3.2A). Surprisingly, when we tested decay of the target mRNAs in a strain deleted of PUFs 1, 2, 3, 4, and 5 (puf1-5Δ), the half-lives of all four mRNAs resembled the short half-lives seen in the WT strain (Fig. 3.2A). Previous studies have shown that all other Puf target mRNAs tested are stabilized in the puf1-5Δ strain (Ulbricht and Olivas, 2008; Russo and Olivas, 2015). The current findings indicate that the decay rates of these targets are regulated redundantly by Puf4p and Puf5p, and suggest a yet undiscovered secondary mechanism whereby rapid decay of the transcripts is rescued in the absence of Puf proteins 1-5.
Figure 3.2. mRNAs involved in ribosome biogenesis are regulated redundantly by Puf4p and Puf5p. Decay analyses are shown of endogenous mRNAs (A) or reporter mRNAs constructs PGK1-ALB1 3’ UTR and PGK1-RRS1 3’ UTR (B) in wild-type (WT), single puf deletion, double puf4Δ/puf5Δ deletion, puf1-5Δ deletion, and puf1-6Δ strains. Representative Northern blots are in the left panels, with average half-life (T1/2) listed to the right of each blot, and a graphical representation of the average half-lives in the right panels. Minutes following transcriptional repression at time 0 are indicated above the blots. Error bars represent standard error of the mean (SEM) and are representative of ≥3 trials. Asterisks indicate the only significantly different half-life in the group as determined by one-way ANOVA with Tukey’s post-hoc test (p<0.005).
The 3' UTR of regulated transcripts is sufficient to confer Puf regulation on reporter mRNAs

Unlike the four mRNAs studied here, other mRNAs that are regulated by both Puf4p and Puf5p are at least partially stabilized in single PUF deletion strains (Ulbricht and Olivas, 2008; Russo and Olivas, 2015). One possible explanation for this difference is that the four mRNAs in this study are expressed at lower levels, and thus the amount of either Puf4p or Puf5p in the cell may be sufficient to regulate their decay. To test this hypothesis and also determine if the 3’ UTR sequences of these mRNAs are sufficient to confer Puf-mediated decay regulation onto a reporter transcript, 500 nt of the ALB1 or RRS1 3’ UTR was cloned onto the PGK1-82Δ open reading frame, which alone is not under Puf regulation. The reporter transcripts were expressed from a GAL-UAS promoter on a high copy vector (2µ), and would thus be expressed at higher levels than the endogenous mRNAs. When decay of these reporter transcripts (PGK1/ALB1 and PGK1/RRS1) was analyzed in the WT, puf1Δ, puf2Δ, puf3Δ, puf4Δ, puf5Δ, puf4Δpuf5Δ, and puf1-5Δ strains (Fig. 3.2B), the patterns of half-lives observed resembled that of the endogenous targets seen in Figure 3.2A. Deletion of any single PUF failed to stabilize the transcripts beyond WT levels; however, deletion of both puf4Δ and puf5Δ stabilized both transcripts (3.3-fold half-life increase for PGK1/ALB1 and 2.1-fold increase for PGK1/RRS1). Half-lives of the transcripts in the puf1-5Δ strain were again similar to those seen in WT, further supporting a secondary mechanism responsible for stimulating decay in the absence of these Puf proteins. There are six Puf proteins in S. cerevisiae, and while decay stimulation has not been attributed to Puf6p thus far, a role
in ribosome biogenesis has been documented (Li et al., 2009). To test whether Puf6p was responsible for the secondary mechanism mediating rapid decay in the absence of Pufs 1-5, the reporter transcripts were analyzed in a puf1-6Δ strain. However, the half-lives of the transcripts were still similar to those in WT, eliminating Puf6 as the secondary decay mechanism (Fig. 3.2B). Together, these results show that the 3’ UTRs of these target mRNAs are sufficient to confer Puf regulation, and the redundant regulatory actions of Puf4p and Puf5p are not due to the low level expression of the endogenous mRNA targets.

**Reporter mRNAs are not stabilized in galactose**

When cells are grown in galactose, global transcript turnover is not significantly altered as compared to in cells grown in dextrose, with a few exceptions (Munchel et al., 2011). However, prior work from our lab has demonstrated that transcripts whose decay is regulated by Puf proteins in dextrose are stabilized in galactose, frequently over 2-fold (Miller et al., 2014; Russo and Olivas, 2015). Posttranscriptional modifications of Puf proteins such as phosphorylation are known to inhibit or stimulate activity (Deng et al., 2008; Kedde et al., 2010), and phosphorylation of yeast Puf3p upon glucose depletion has been shown to stimulate translation of its targets (Lee and Tu, 2015). These findings provide an attractive model whereby Puf activity is regulated quickly by the changing carbon source (Miller et al., 2014). We therefore wished to determine if the transcripts in this study were also stabilized in galactose. As shown in Figure 3.3A, the reporter transcripts PGK1/ALB1 and PGK1/RRS1 were not substantially stabilized in galactose. For PGK1/ALB1, the half-life decreased 1.5-fold in galactose versus dextrose,
Figure 3.3. Reporter mRNAs are not stabilized in galactose, while Puf protein overexpression rescues rapid decay. Decay analyses are shown of reporter constructs PGK1/ALB1 3’ UTR and PGK1/RRS1 3’ UTR in (A) the WT strain in the presence of dextrose or galactose, or (B) in the double puf4Δ/puf5Δ deletion strain containing an empty vector (EV) or a vector overexpressing Puf4p or Puf5p. Representative Northern blots are in the left panels, with average half-life (T1/2) listed to the right of each blot, and a graphical representation of the average half-lives in the right panels. Minutes following transcriptional repression at time 0 are indicated above the blots. Error bars represent SEM (n=3). The asterisk in (A) indicates a significant difference as determined by Student’s t-Test (p<0.05). The letters next to the bar graph in (B) indicate significant differences as determined by one-way ANOVA with Tukey’s post-hoc test (PGK1-ALB1, p<0.005; PGK1-RRS1, p<0.05).
while the half-life of $PGK1/RRS1$ increased only 1.4-fold. Since the decay stimulation by Puf4p and Puf5p on other mRNAs is inhibited by galactose (Russo and Olivas, 2015), these results suggest that the secondary mechanism acting to regulate decay of these transcripts in the absence of Puf proteins may also be acting here in a carbon source independent manner.

**Puf4p expression in a puf4Δpuf5Δ strain rescues rapid decay**

We next sought to determine if exogenous expression of either Puf4p or Puf5p individually in the $puf4Δpuf5Δ$ strain could rescue rapid decay of the target mRNAs. As shown in Figure 3.3B, complementation of the $puf4Δpuf5Δ$ strain with Puf4p expressed from a CEN vector dramatically shortened the half-lives of the mRNAs as compared to the $puf4Δpuf5Δ$ strain with an empty vector. The $PGK1/ALB1$ half-life decreased 16.2-fold, while the $PGK1/RRS1$ half-life decreased 6.6-fold. In fact, these half-lives are 3.1-fold and 2.1-fold shorter than those of the respective reporter mRNAs in the WT strain. Since $PUF4$ expression from the CEN vector was 20-fold higher than endogenous $PUF4$ levels as measured by qPCR (Fig. 3.4), these results suggest that endogenous levels of Puf4p are limited in the cell, as higher exogenous levels promoted increased decay stimulation. Exogenous Puf5p expression from a CEN vector displayed partial rescue of decay, decreasing the $PGK1/ALB1$ half-life 1.5-fold and decreasing the $PGK1/RRS1$ half-life 1.4-fold versus the empty vector strain (Fig. 3.3B). $PUF5$ expression from the CEN vector was 15-fold higher than endogenous $PUF5$ levels as measured by qPCR (Fig. 3.4). Thus, given equally increased expression of both exogenously-expressed $PUF4$ and
Figure 3.4. qPCR analysis of PuF4p and PuF5p overexpression. *PUF4* mRNA and *PUF5* mRNA steady-state levels are compared between WT cells versus WT cells overexpressing *PUF4* or *PUF5* from a CEN vector. $C_T$ values were normalized to *TDH1* mRNA values and set relative to the amount in WT. Error bars represent SEM (n=3)
PUF5, exogenous Puf4p appears to be much more active in stimulating decay of the mRNA targets.

Other Puf proteins play contrasting roles in decay regulation

Given our data showing that the target reporter transcripts were stabilized in a *puf4Δpuf5Δ* strain but destabilized in a *puf1-5Δ* strain back to a half-life mirroring WT, it is possible that the loss of Puf1p, Puf2p, Puf3p, and/or Puf6p could be responsible for this destabilization. Specifically, one or more of these proteins may have a stabilizing role, unique to these targets. While the vast majority of Puf proteins stimulate decay upon binding to their targets, some Puf proteins under certain circumstances act to stabilize target transcripts (Archer et al., 2009; Suh et al., 2009). To investigate this possibility, we deleted *PUF1, PUF2, PUF3,* or *PUF6* in the *puf4Δpuf5Δ* background and analyzed the decay of *PGK1/ALB1* and *PGK1/RRS1*. The deletion of *PUF2* in addition to *PUF4* and *PUF5* further stabilized both mRNAs 1.6-fold over the respective half-lives seen in the *puf4Δpuf5Δ* strain (Fig. 3.5A). This result identifies Puf2p as a third Puf protein that acts to promote decay of these targets. Like Puf4p and Puf5p, the individual removal of Puf2p had no effect on target decay (Fig. 3.2B), but all three appear to play redundant roles in stimulating decay, with combinations of their loss additive. In contrast, deletion of *PUF1, PUF3,* or *PUF6* individually in the *puf4Δpuf5Δ* background had little effect on decay of the targets, as the half-lives of the triple mutants were similar to the *puf4Δpuf5Δ* double mutant (Fig. 3.5A).

Although the individual removal of *PUF1* or *PUF3* in the *puf4Δpuf5Δ* background did not alter target decay, it is possible that one of the remaining Pufs in the triple
Figure 3.5. Puf2p acts with Puf4p and Puf5p to stimulate mRNA decay, while Puf1p and Puf3p stabilize mRNA targets. Decay analyses are shown of reporter constructs PGK1–ALB1 3′ UTR and PGK1–RRS1 3′ UTR in (A) triple mutant strains or (B) in the puf1-5Δ strain containing an empty vector (EV) or a vector overexpressing the Puf1p repeat domain (Puf1RD) or full length Puf1p (Puf1FL) or the Puf3p repeat domain (Puf3RD) or full length Puf3p (Puf3FL). Representative Northern blots are in the left panels, with average half-life (T₁/₂) listed to the right of each blot, and a graphical representation of the average half-lives in the right panels. Minutes following transcriptional repression at time 0 are indicated above the blots. Error bars represent SEM (n=3). Asterisks in (A) represent the only significantly different half-life as compared to the double puf4Δpuf5Δ half-life as determined by one-way ANOVA with Tukey’s post-hoc test (p<0.05).
mutant is sufficient to stabilize the mRNAs as compared to the rapid decay in the quintuple PUF mutant. To test whether the presence of one of these Pufs alone can stabilize the target transcripts, we expressed either full-length or the Puf repeat domain of Puf1p or Puf3p from a 2µ vector in the *puf1*-5Δ strain. As shown in Figure 3.5B, expression of either Puf1p or Puf3p was able to stabilize the reporter transcripts. Of the two, Puf1p acted more strongly to stabilize the transcripts, with half-lives increased 4- to 10- fold. The full-length Puf1p was the most potent stabilizer, though the Puf1p repeat domain alone was sufficient for stabilization. The repeat domain of Puf3p stabilized the transcripts 2- to 3- fold, while the full-length Puf3p stabilized only the *PGK1/ALB1* transcript 3-fold. Both Puf1p and Puf3p have previously been shown to stimulate decay of their target mRNAs (Olivas and Parker, 2000; Ulbricht and Olivas, 2008; Miller *et al.*, 2014). Therefore, these results demonstrate a novel role for Puf1p and Puf3p in stabilizing these particular transcripts, and the first evidence of a stabilizing role for Puf proteins in the yeast *S. cerevisiae*. Moreover, the secondary mechanism that may be responsible for stimulating decay in the absence of Puf proteins may be inhibited by the presence of either Puf1p or Puf3p.

**The PREs in the target mRNAs are necessary for decay regulation**

Each of the four endogenous mRNA targets contains a single Puf binding site, also termed a Pumilio Response Element (PRE), shown in Figure 3.6, which matches the canonical Puf4p PRE (Gerber *et al.*, 2004; Valley *et al.*, 2012). To investigate the necessity of these sites for mRNA decay regulation, the conserved UGUA sequences within the PREs of the *PGK1/ALB1* and *PGK1/RRS1* 3’ UTRs were mutated to ACAC, then
Figure 3.6. Cloned 3’ UTRs of ALBI and RRS1. Shown are the 3’UTRs of (A) ALBI and (B) RRS1 that were cloned behind the PGK1 short ORF to create the reporter constructs. The canonical PuPp PRE is underlined, and the conserved UGUA (which is altered in the mutant PRE construct to ACAC) is highlighted in red.
the half-lives of the transcripts examined. As seen in Figure 3.7, the *PGK1/ALB1* mutant PRE transcript was stabilized 4-fold over its respective native PRE transcript, and the *PGK1/RRS1* mutant PRE transcript was stabilized 3-fold over its respective native PRE transcript in the WT strain containing all Puf proteins. In addition, the *PGK1/ALB1* and *PGK1/RRS1* mutant PRE transcripts were stabilized 3-fold and 2-fold, respectively, in the *puf1-5Δ* strain. Thus, the canonical PREs are necessary not only for decay regulation by Puf proteins, as expected, but also for decay regulation by the secondary mechanism that is acting in the absence of Puf proteins.

**Overexpression of Puf4p causes ribosomal RNA processing defects**

The four mRNA targets examined in this study are all involved in various aspects of ribosome biogenesis. Ebp2p is involved in some of the initial rRNA excision events, as its removal causes accumulation of the 35S transcript (Huber et al., 2000). Rrs1p is also involved in rRNA excision events, as its deletion results in kinetic defects not only in 35S processing, but also in processing 27S to 25S and 20S to 18S (Tsuno et al., 2000). Given our data showing that overexpression of Puf4p shortens the half-lives of target transcripts below those seen in WT cells, we hypothesized that such alterations in half-lives would negatively impact the production of protein from those transcripts. Decreased levels of ribosome biogenesis regulatory factors might then result in slower rRNA processing. To examine this possibility, steady-state levels of rRNA processing intermediates were analyzed from WT cells containing either an empty CEN vector or a CEN vector expressing PUF4. As shown in Figure 3.8A and B, overexpression of Puf4p caused a greater than 2-fold accumulation of the initial 35S precursor rRNA transcript.
Figure 3.7. A single Puf Response Element (PRE) in the 3' UTR is critical for decay regulation. Decay analyses are shown of native PRE reporter constructs PGK1-ALB1 3' UTR and PGK1-RRS1 3' UTR and respective mutant PRE reporter constructs (Mut PRE) in the WT and puf1-5Δ strains. In the mutant PRE constructs, the canonical UGUA portion of the PRE was mutated to ACAC. Representative Northern blots are in the left panels, with average half-life (T_1/2) listed to the right of each blot, and a graphical representation of the average half-lives in the right panels. Minutes following transcriptional repression at time 0 are indicated above the blots. Error bars represent SEM (n≥3).
Figure 3.8. Pu4p overexpression causes accumulation of rRNA processing intermediates. (A) Representative gel images are shown of the steady state levels of rRNA processing intermediates/precursors in the WT strain in the presence of a CEN empty vector (CEN EV) or a CEN vector overexpressing Pu4p (Pu4 OE). The scRI loading controls for each set of rRNA species is shown directly underneath the corresponding images. The diagrams to the right of each gel image are visual representations of the sequence regions within the rRNA intermediates/precursors. The three final species of rRNA originating from the Pol1 transcript (18S, 25S and 5.8S) are labeled in white; sites of processing are labeled in black letters A-D. (B) Graphical representation of the rRNA intermediate/precursor band intensities in (A). Error bars represent SEM (n=3). Asterisks indicate significant differences between reported levels for EV and OE as determined by Student’s t-test (p<0.01). (C) Representative gel images of rRNA processing intermediates/precursors following an L-[Methyl-3H]-Methionine pulse-chase labeling assay in a WT strain in the presence of a CEN empty vector (CEN EV) or a CEN vector overexpressing Pu4p (Pu4 OE). Minutes following the addition of unlabeled methionine at time 0 are indicated above the gels (n=3).
In accordance with a slowed processing of 35S, overexpression of Puf4p caused a significant decrease in the levels of 20S, the immediate precursor to the final 18S rRNA, and also a decrease in 7S, the precursor to the final 5.8S rRNA (see Figure 3.9 for the rRNA cleavage and excision pathway). These alterations are very similar to those found in prior work analyzing mutations in ribosome processing factors (de la Cruz et al., 1998; White et al., 2008). Moreover, like our results, these prior studies typically did not detect differences in the levels of the large, stable pools of 18S and 5.8S final rRNA products due to limitations in the assay and the fact that the kinetics of processing are slowed, but not blocked. Overexpression of Puf4p also caused a moderate increase in the levels of the 23S precursor. The 23S variant is an aberrant excision product that occurs when the cell fails to excise the 5’ ETS at sites A₀ and A₁, and uses alternative cleavage at site A₃. Increased 35S and 23S precursor and decreased 20S precursor are consistent with other mutations that result in inhibition of cleavage at site A₂.

Together, these results demonstrate that Puf4p overexpression causes rRNA processing defects, including slowed 5’ ETS excision and A₂ cleavage inhibition, likely due to the destabilization of mRNAs coding for ribosome biogenesis factors.

To further analyze the effect of Puf4p overexpression on the kinetics of rRNA processing, a pulse-chase rRNA labeling assay was performed. Ribosomal RNA is methylated on the 2’-O-ribose group of many of its bases (Decatur and Fournier, 2002). Using L-[³H-Methyl]-Methionine as a tritiated methyl donor, the rRNA was radiolabeled, then samples were collected at increasing times after a chase with unlabeled methionine. As seen in Figure 3.8C, the kinetics of processing 27S into 25S rRNA and
Figure 3.9. rRNA processing in *S. cerevisiae*. A simplified view of the nucleolytic processing steps yielding the precursor and final rRNAs described in Figure 5.
20S into 18S rRNA were similar when comparing strains with the empty vector versus Puf4p overexpression. However, the original 35S transcript is barely detectable even at the beginning of the chase in the empty vector strain due to rapid processing, while in the strain overexpressing Puf4p, the 35S precursor has accumulated and persists two minutes into the chase. This data provides additional support that Puf4p overexpression slows the kinetics of rRNA processing at the earliest cleavage events, such as 5’ ETS excision, as a result of destabilization of the mRNAs coding for factors that are involved in such events.

**Overexpression of Puf4p causes mislocalization of ribosomal subunits**

In addition to factors involved in rRNA cleavage events, some mRNAs targeted for destabilization by Puf4p code for proteins involved in ribosome subunit trafficking. Alb1p is vital to the export of ribosomal subunits, functioning along with Arx1p to recycle the anti-association factor Tif6 to the nucleus (Lebreton et al., 2006). Rrs1p also has a role in the export of ribosomal subunits; rrs1 mutants display 60S export defects (Miyoshi et al., 2004). We therefore investigated whether overexpression of Puf4p disrupts export of ribosomal subunits. To this end, confocal microscopy was used to visualize the localization of the ribosomal subunits in a WT strain +/- an empty CEN vector, or a WT strain overexpressing Puf4p. GFP-tagged versions of the large ribosomal subunit protein RPL11B or small ribosomal subunit protein RPS2 were used as markers for the large and small ribosomal subunits, and SIK1-mRFP was used as a nucleolar marker. As shown in Figure 3.10A, RPL11B (large subunit) was largely diffuse between the nucleus and cytoplasm in WT cells with or without the empty CEN vector. However,
Figure 3.10. PuF4p overexpression results in nuclear accumulation of ribosomal subunits. (A) Representative panels are shown from confocal microscopy of cells from the WT strain, or the WT strain containing an empty vector (EV) or a vector overexpressing PuF4p (4OE). Labels above the panels indicate the stain/protein being observed. Arrows on the upper set of panels indicate the position of nuclear foci as detected by RPL11B-eGFP. Arrows on the lower set panels indicate the position of nuclei in the cells as detected by a decreased signal of RPS2-eGFP. (B) Graphical representation of the nuclear to cytoplasmic ratio of GFP fluorescence intensity in WT cells +/- empty vector or vector overexpressing PuF4p. Letters a and b indicate significant differences using one-way ANOVA (p<0.005) and Tukey's post-hoc test. Error bars represent SEM and are representative of ≥250 cells spread across 3 individual growth trials.
overexpression of Puf4p caused punctate nuclear foci of RPL11B to appear in many cells (white arrows) that were largely absent in the WT strain +/- empty CEN vector. Overexpression of Puf4p also caused more nuclear accumulation of RPS2 (small subunit). In WT cells +/- empty CEN vector, there is a noticeable spatial deficit of RPS2 that coincides with the position of the nucleus (white perpendicular symbols). Upon overexpression of Puf4p, the incidence of these areas decreased, indicating more RPS2 was residing in the nucleus.

To quantitate the nuclear retention of large and small ribosomal subunits upon Puf4p overexpression, GFP signal was analyzed in the microscopy images using CellProfiler software (Kamentsky et al., 2011). Briefly, the software identified the position of the nucleus from the DAPI channel, and the intensity of the GFP signal corresponding to either RPL11B-eGFP or RPS2-eGFP was calculated for the nuclear and cytoplasmic areas of the cell. As shown in Figure 3.10B, there was a statistically significant increase in the mean fluorescent intensity ratio of nuclear to cytoplasmic signal for both RPL11B and RPS2. The results indicate that overexpression of Puf4p causes mislocalization of large and small ribosomal subunits, likely a result of the inhibition of their export from the nucleus due to destabilization of the mRNAs coding for ribosome export factors.

**Discussion**

Cell growth is heavily dependent on ribosome production, and changing growth conditions necessitates strict control of ribosome biogenesis through both transcriptional and post-transcriptional regulation. This work focused on the role of Puf
proteins in the post-transcriptional regulation of ribosome biogenesis factors, and thereby ribosome production in *Saccharomyces cerevisiae*. Previous work found that Puf4p physically bound multiple mRNAs involved in various aspects of ribosome biogenesis, both in the maturation of pre-ribosomal RNA and the export of pre-ribosomes to the cytoplasm. Our analyses of the top-scoring mRNAs bound by Puf4p demonstrate that these transcripts are uniquely destabilized by a combination of Puf proteins 2, 4 and 5. Our results also provide the first evidence of the role of Puf proteins 1 and 3 in stabilizing transcripts in *S. cerevisiae*. Decay regulation of each transcript appears to occur through a single canonical Puf4p binding site in the 3' UTR, indicating competition between Puf proteins for binding access. We also show evidence for a Puf-independent secondary mechanism of decay regulation that uses the same binding site. The importance of Puf-mediated decay regulation of ribosome biogenesis factor transcripts was further established by demonstrating that Puf4p overexpression causes ribosomal RNA processing delays and inhibits nuclear export of pre-ribosomal subunits.

The data presented suggest the following model for Puf-mediated regulation of the ribosome biogenesis transcripts and the resulting cellular implications (Fig. 3.11). In a normal cell with all Puf proteins present, there is likely a competition between different Puf proteins for binding the single consensus Puf Response Element (PRE) in the 3’ UTR, though it is possible that Pufs may be binding non-canonical elements. Binding of Puf2p, Puf4p, or Puf5p stimulates decay, while binding of Puf1p or Puf3p results in stabilization. Since the half-lives of the target transcripts are short in a wild-type cell expressing all Puf proteins, the actions of Puf2p, Puf4p, or Puf5p to stimulate
Figure 3.11. Puf proteins regulate mRNA decay to ensure proper ribosome biogenesis. (A) Puf2p, Puf4p, and Puf5p can each act to stimulate mRNA decay of ribosome biogenesis factors through binding to a single PRE site (UGU). Puf1p and Puf3p act through the same PRE to stabilize these mRNAs, potentially by blocking access of another decay factor to the PRE. In the absence of Puf proteins, a secondary mechanism that also requires the PRE site acts to stimulate decay. (B) At physiological levels of Puf proteins, processing of rRNA transcripts occurs normally. When Puf4p is overexpressed, processing is slowed, resulting in higher levels of the initial 35S and aberrant 23S transcripts, and lower levels of 20S and 7S intermediates. (C) At physiological levels of Puf proteins, ribosomal subunits are shuttled from the nucleus at a normal rate. When Puf4p is overexpressed, trafficking of ribosomal subunits is inhibited, resulting in abnormal nuclear retention of ribosomal subunits.
decay supersede the stabilizing activities of Puf1p or Puf3p. Furthermore, since the presence of Puf1p or Puf3p can stabilize the transcripts in the absence of the other Pufs, the stabilizing activities of Puf1p or Puf3p supersede the secondary Puf-independent mechanism of decay stimulation.

The mechanism of action by which Puf1p and Puf3p stabilize these transcripts is unclear. Some possibilities include stabilization through stimulation of translation, the blocking of access of the PRE to decay stimulatory factors, or the inability to modify the mRNP to stimulate decay. We do not believe that Puf1p and Puf3p are stimulating translation under the glucose replete conditions used in this study, as all previous work has demonstrated the roles of these Puf proteins in decay stimulation under such conditions (Miller et al., 2014; Russo and Olivas, 2015), and only in glucose depleted conditions has Puf3p been shown to stimulate translation (Lee and Tu, 2015). It is possible that Puf1p and Puf3p actively stabilize these transcripts by recruiting stabilizing factors. However, we think it more likely that Puf1p and Puf3p simply occupy the PRE and prevent other non-Puf decay stimulating factors from binding. It is possible that the overexpression of Puf1p and Puf3p in these studies are exaggerating the effect that these Pufs may have at endogenous levels. However, endogenous Puf3p has been shown to physically bind ALB1 (Freeberg et al., 2013), EBP2, and PUS7 (Kershaw et al., 2015) mRNAs, although the PREs do not represent canonical Puf3p PREs. Puf proteins that bind non-canonical PREs may be in a non-functional conformation, preventing them from recruiting decay factors, while still occupying the site. Another possibility is that the local mRNP structure around non-canonical targets prevents these Pufs from
activating decay. For example, Pub1p, another 3' UTR binding protein, promotes decay of some transcripts while stabilizing others, dependent on the sequence of the mRNA. Remodeling of the mRNP structure by Pub1p has been suggested to be at least partly responsible for this alteration in mRNA stability (Duttagupta et al., 2005). Puf3p has also been shown to require the Poly(A)-Binding protein Pab1p to promote proper activities of poly(A)-tail deadenylation by Pan proteins and Ccr4p, suggesting that perturbations to overall mRNP structure could inhibit Puf stimulation of decay (Lee et al., 2010a).

The data also suggest that Puf expression levels are important to maintaining appropriate levels of proteins involved in ribosome biogenesis. Overexpression of Puf4p inhibited both pre-rRNA processing events and trafficking of ribosomal subunits from the nucleus to the cytoplasm (Fig. 7). The decreased lifespan of target mRNAs upon Puf4p overexpression is concomitant with this phenotype, suggesting that decreased protein production caused by increased decay stimulation of these and likely other targets is responsible for these defects.

A recent study to globally analyze yeast Puf binding affinity to mRNAs utilized a novel tagging system in which Puf3p, Puf4p, and Puf5p were fused to the Polyuridine-Polymerase (PUP) gene from C. elegans, and all mRNAs that bore poly-U tails as a result were analyzed (Lapointe et al., 2017). The mRNAs were scored for the length of the poly-U tails, which is an indirect measurement of their association time with the Puf protein. All four targets studied here are canonical Puf4p targets with 9-residue binding elements; ALB1, RRS1, and EBP2 were top scorers bound by Puf4p, while PUS7 scored lower, which may be why in our studies it did not respond as dramatically to the double
puf4Δpuf5Δ deletion. While none of the targets contain the canonical 10-residue Puf5p binding element, most were found bound by Puf5p, albeit in a lower scoring group than found with Puf4p; ALB1 and RRS2 scored above EBP2, while PUS7 was not identified by the Puf5p analysis. These results support our data showing that Puf4p overexpression in a puf4Δpuf5Δ strain is better at rescuing rapid decay of target mRNAs than overexpression of Puf5p.

Puf2p's discovered role in regulation of these transcripts was unexpected. A previous study suggested the consensus Puf2p binding sequence is UAAU-XXX-UAAU, where X is usually an A or U (Yosefzon et al., 2011). This sequence is unlike those of other PUF proteins, which necessarily include the UGU trinucleotide. The ALB1 3’UTR does not contain this sequence, while the RRS1 3’ UTR contains dual UAAU motifs separated by a less conserved 9 nucleotide linker, yet both seem to be negatively regulated by Puf2p. While studies have analyzed Puf2p binding (Yosefzon et al., 2011), no study has yet implicated Puf2p in decay stimulation of a specific mRNA target, and deletion of puf2 does not alter the lifespans of other tested mRNAs (Ulbricht and Olivas, 2008; Russo and Olivas, 2015). Thus, the current findings represent the first bona fide targets of Puf2p regulation of decay.

Puf6p has previously been shown to play a role in ribosome biogenesis, as its deletion causes rRNA processing and ribosomal subunit trafficking defects (Li et al., 2009). In the absence of Pufs 1-5, rapid decay of the target mRNAs was rescued, suggesting that either Puf6p stimulates decay in the absence of other Pufs, or there exists a non-Puf secondary mechanism of decay stimulation. Since in the deletion of
Pufs 1-6, mRNAs were still rapidly decayed, Puf6p is not involved in this secondary mechanism. The current understanding of Puf6p activity is that it inhibits translation of ASH1 mRNA (Gu et al., 2004; Deng et al., 2008). It is also thought to be required, along with Loc1p, for efficient loading of Rpl43p onto pre-60S particles; Puf6p and Loc1p are released upon Rpl43p loading (Yang et al., 2016). Deletion of Puf6p results in 7s precursor rRNA accumulation, although the mechanism by which Puf6p ensures proper 7s to 5.8S processing is still unclear (Qiu et al., 2014). Thus, Puf6p’s mechanism of action in ribosome biogenesis appears to be quite different than the mRNA decay regulatory activities of Pufs 1-5.

The nature of the non-Puf secondary mechanism acting to promote decay of the target mRNAs in the absence of Pufs is unclear. We show that the mechanism acts through the Puf Response Element, as mutation of the conserved UGUA to ACAC results in stabilization of the mRNA in the absence of Pufs. There are other proteins known to bind similar AU-rich elements (AREs) in 3’ UTRs for decay regulation, such as Vts1, Cth1/2p, Whi3p, and Pub1p (Vasudevan and Peltz, 2001; Duttagupta et al., 2005; Aviv et al., 2006; Pedro-Segura et al., 2008; Rendl et al., 2008; Cai and Futcher, 2013; Holmes et al., 2013). There are numerous other uncharacterized RNA-binding proteins that may also be responsible for the non-Puf secondary mechanism of decay of the target mRNAs. The fact that there are multiple mechanisms all acting to carefully control the lifespans of ribosome biogenesis factor transcripts demonstrates the importance of this regulation for cell fitness in varying environments.
Most studies of ribosome biogenesis gene expression focus on the transcriptional control of these genes, including through the actions of the TORC1 complex in response to nutrient availability (Urban et al., 2007; Huber et al., 2011). There is also some evidence for translational control of ribosome-associated transcripts (Hong et al., 2017; Lahr et al., 2017). This work demonstrates a new layer of regulation of ribosome biogenesis factors through mRNA decay control by both Puf-mediated and non-Puf mechanisms. Further studies will elucidate any cooperation between these two mechanisms and dissect the combinatorial activities of Puf proteins on this regulation.

**Materials and Methods**

**Plasmids, Oligonucleotides, and Yeast Strains**

All plasmids, oligonucleotides, and yeast strains used in this study are listed in Supplementary Tables 3.1, 3.2, and 3.3, respectively. Strain yWO260 was created by mating yWO3 and yWO7, and strain yWO289 was created by mating yWO260 and yWO261 as described (Codon et al., 1995). The diploids were sporulated, the resulting tetrads dissected, and the spores genotyped. Strain yWO261 was created by transforming yWO39 with BamHI digested pWO183 to replace the URA3 gene with the KanMX3 G418 resistance cassette and plating the transformants on yeast extract/peptone/dextrose (YEPD) containing 300µg/ml Geneticin (Life Technologies). Transformations were performed via LiOAc-mediated high efficiency transformation as described (Gietz et al., 1995). Strains yWO306, yWO307, and yWO308 were created by PCR amplification of the nourseothricin resistance cassette (NAT1) contained in pWO241 (Goldstein and McCusker, 1999) using primers oWO869/870, oWO918/919,
and oWO911/912, respectively, and the resultant products were transformed into yWO289 as previously described (Caponigro et al., 1993). yWO317 and yWO318 were created in a similar manner by PCR amplification of the nourseothricin resistance cassette with oWO857/858 and transformation into yWO289 and yWO205, respectively. Transformants were allowed to recover overnight in YEPD media, then plated on YEPD containing 100µg/ml nourseothricin (Jena Bioscience) to select for replacement of PUF genes by the nourseothricin resistance cassette. Gene replacement was verified by PCR amplifying a region spanning upstream of the insertion site to inside the resistance cassette using primers oWO901/833, oWO919/833, oWO913/833, and oWO863/833.

Creation of pWO226 was achieved by digesting pWO165 with XhoI/EcoRI, then transforming the resultant fragment and pWO13 into yWO3. Transformants were selected on media lacking uracil. Plasmids were recovered from yeast cells by digestion with Zymolyase (MP Biomedicals) at 37°C for 30 minutes, harvesting the cells, and proceeding with alkaline lysis using the Zyppy plasmid miniprep kit (Zymo Research) per the manufacturer’s instructions. Creation of the PGK1-ALB1 3’ UTR (pWO231) and PGK1-RRS1 3’ UTR (pWO232) reporter constructs was performed by PCR amplifying ~500nt of the 3’ UTRs of the respective genes while introducing a 5’ ClaI site and a 3’ HindIII site for ALB1, and a 5’ EcoRV and 3’ HindIII site for RRS1. The digested PCR products were ligated into the PGK1Δ82 ORF vector (pWO102) and the resulting constructs sequence verified. Creation of the PGK1-ALB1 3’ UTR (pWO233) and PGK1-RRS1 3’ UTR (pWO234) reporter constructs was performed by digesting pWO167 with SmaI and transforming the resultant linear DNA with pWO231 or pWO232 into yWO3.
Cells with recombinant plasmids were selected on media lacking leucine. Plasmids were recovered from yeast cells by digestion and alkaline lysis as described above. The \textit{PGK1-ABL1} and \textit{PGK1-RRS1} mutant PRE reporter constructs were created by site directed mutagenesis using the Quikchange II-XL site directed kit (Agilent Technologies) per manufacturer’s instructions with primers oWO786/oWO787 and oWO788/oWO789, respectively. Creation of pWO238 was performed by digesting pWO166 with \textit{SmaI} and transforming the resultant linear DNA and pWO237 into yWO236. Cells with recombinant plasmids were selected on media lacking histidine, and plasmids recovered as described above. Creation of pWO253 was performed by amplifying \textit{PUF5} from pWO196 using primers oWO936 and oWO938, then transforming the resultant PCR product and pWO116 linearized by \textit{HindIII/XhoI} into yWO3. Cells with recombinant plasmids were selected on media lacking leucine.

\textbf{Steady State Analysis of Endogenous mRNAs}

\textit{WT} (yWO3), \textit{puf4Δ} (yWO22), \textit{puf5Δ} (yWO17), or \textit{puf4Δpuf5Δ} (ywo39) cells were grown in 20 ml YEP media containing 2% dextrose at 30°C until an \textit{OD}_{600} of 0.4 was reached. Cells were then harvested and total RNA was isolated as previously described (Caponigro \textit{et al.}, 1993). 40\(\mu\)g of total RNA was loaded onto a 1.25\% agarose gel (containing formaldehyde and MOPS) and electrophoresed at 70V for 4 hours. RNA was transferred to a membrane (Biobond Plus Nylon Membrane, Sigma-Aldrich), UV crosslinked, and probed with \textsuperscript{32}P \textit{5'}-end labeled oligonucleotides complementary to \textit{ALB1} (oWO318), \textit{PUS7} (oWO319), \textit{EBP2} (oWO320) or \textit{RRS1} (oWO321) mRNA, and with an oligonucleotide complementary to \textit{scRI} RNA (oWO21) as a loading control.
Membranes were exposed to phosphorimager screens, and imaging and quantification of RNA levels were performed using ImageQuant Software (Molecular Dynamics).

**In Vivo Decay Analysis of Endogenous mRNAs**

WT (yWO7), *puf4*Δ (yWO106), *puf5*Δ (yWO49), *puf4*Δ*puf5*Δ (yWO289), or *puf1-5*Δ (yWO268) cells were grown in 200 ml YEP media plus 2% dextrose at 24°C until an OD$_{600}$ of 0.4 was reached. These strains harbor a mutation in RNA polymerase II subunit b (*rpb1-1*), which causes temperature-sensitive inactivation of the polymerase at 37°C. Cells were harvested and resuspended in 10 ml YEP media at 24°C. 10 ml of YEP media plus 8% dextrose at 50°C was added to raise the temperature to 37°C and effectively stop transcription. 2 ml aliquots of cells were collected at time points up to 40 minutes, and total RNA was isolated as previously described (Caponigro *et al.*, 1993). Northern analysis, detection and quantification proceeded as described above.

**In Vivo Decay Analysis of Reporter mRNAs**

Decay analysis of reporter mRNAs from pWO231, 232, 233, 234, 235, and 236 was performed in the same manner as decay analysis of endogenous mRNAs. Briefly, indicated strains were transformed with the reporter construct and any other indicated plasmids and grown in media lacking uracil, leucine, or both uracil and leucine as noted. The temperature of the culture was shifted to 37°C to stop new transcription, cell aliquots at the indicated time points were collected, and total RNA was extracted. Northern analysis, detection and quantification proceeded as described above. *PGK1-ALB1* and *PGK1-RRS1* reporter mRNAs were detected with oWO521 and oWO522,
respectively, which are complementary to the junction between the end of the
PGK1Δ82 ORF and beginning of the ALB1 or RRS1 3’ UTR.

**Steady State Analysis of rRNA**

20 ml cultures of yWO3 containing pWO58 or pWO116 were grown at 30°C to an OD$_{600}$
of 0.4, then the cells harvested and frozen on dry ice. Total RNA was extracted via a hot
phenol method previously described (Wei et al., 2009), and 10μg of RNA was loaded on
a 1.25% agarose gel (containing formaldehyde and MOPS) for large rRNAs and on a 6%
polyacrylamide (19:1 acryl:bis) 8M urea TBE for the 7S and 5.8S rRNAs. The agarose gel
was electrophoresed for 8 hours at 70V and the polyacrylamide for 6 hours at 300V.
The RNA was transferred to nylon membrane as previously described for the agarose gel
and electroblotted for polyacrylamide gel in 0.5X TBE at 10.5V for hours. The
membranes were UV crosslinked, and probed with $^{32}$P radiolabeled oligonucleotides
oWO714-724, and scRI loading control as previously described. Membranes were
exposed to phosphorimager screens, and imaging and quantification of RNA levels were
performed using ImageQuant Software (Molecular Dynamics).

**Pulse Chase Labeling of rRNA**

Pulse chase labeling of rRNA was performed essentially as described (de la Cruz
et al., 1998; White et al., 2008). Briefly, 20 ml cultures of yWO3 containing pWO58 or
pWO116 were grown at 30°C in SC-Met-Leu media containing 2% dextrose to an OD$_{600}$
of 0.4. 10 ml of cells were harvested then resuspended in 3 ml SC-Met-Leu media
containing 2% dextrose. Cells were allowed to grow at 30°C for 25 min. 250μCi of L-
$[^{13}$Methyl]-Methionine (Perkin-Elmer) was added and cells were allowed to grow two
minutes. L-Methionine was then added to a final concentration of 0.6 mM. A 600μl cell aliquot was immediately collected, spun down for 15 sec at 3,000 rpm, supernatant removed, and placed in a dry ice/methanol bath. Time points were collected in the same manner at 1, 2, 4, and 8 minutes after methionine chase. Total RNA was extracted via hot phenol method as previously described. 20,000 cpm from each time point was loaded on a 1.25% agarose gel (containing formaldehyde and MOPS) and electrophoresed for 8 hours at 70V. The RNA was then transferred to a nylon membrane, UV crosslinked, and the membrane allowed to dry. The membrane was then coated four times with ENHANCE (Perkin-Elmer), allowing 15 minutes for drying in between each coat and after the final coat, as per manufacturer’s instructions. Autoradiography film was exposed to the membrane for 96 hr at -80°C prior to development.

Confocal Fluorescent Microscopy Analysis of Subcellular Localization of RPL11Bp and RPS2p

A 10 ml culture of yWO236 containing pWO238 (RPL11B-GFP) or pWO239 (RPS2-GFP) and pWO240 (SIK1-mRFP) and either pWO58, pWO116, or no plasmid were grown in SC-His-Met-Leu or SC-His-Met, respectively, containing 2% dextrose to an OD600 of 0.4. 1 ml of cell suspension was collected and 122 μl of 37% formaldehyde was added to bring the concentration to 4%. Cells were fixed at room temp with end-over-end rocking for 20 minutes. Cells were then washed with 1X PBS three times and resuspended in 30 μl 1X PBS. Cells were mounted onto a cleaned glass slide essentially as described (Atkin, 1999). Briefly, a 9 mm circle was etched into the center of a glass
slide. Glass chips were removed and the slide was cleaned in 95% ethanol and allowed to air dry. 30 μl of 1% polyethyleneimine (Sigma-Aldrich) was pipetted onto the etched circle. The slide was allowed to sit for 5 minutes, at which point the polyethyleneimine was removed by aspiration and the remaining solution was allowed to air dry. The 30 μl of cells were then pipetted onto the polyethyleneimine and allowed to settle for 30 minutes. Nonadherent cells were removed by two washes with 1X PBS by covering the slides with the PBS and shaking on an orbital shaker for five minutes. The cells were allowed to dry briefly before 50 μl of mounting media (1X PBS, 50% glycerol, 100ng/ml DAPI, and 2.73mg/ml p-phenylenediamine) was placed onto a glass coverslip and the coverslip placed over the cells. Excess mounting media was removed by blotting with absorbent paper and the coverslip was sealed with clear acrylic nail polish. Cells were viewed for GFP, mRFP, and DAPI fluorescence within 48 hr using a Zeiss LSM-700 confocal microscope. A single plane view comprising 4 μm was captured for each fluorophore. Overlays of sample cells were created using Photoshop image software.

Subcellular nuclear/cytoplasmic localization ratios for RPL11Bp and RPS2p were calculated using Cellprofiler analysis software (Kamentsky et al., 2011). Briefly, to assist the software in identifying nuclei, DAPI channels were adjusted for signal threshold by creating a custom pipeline using the auto threshold method. A second custom pipeline was then created wherein signal from the DAPI channel was used to identify nuclei, and signal from the GFP channel was used to identify the cytoplasm (extranuclear space). Mean GFP fluorescent intensity (MFI) was calculated for each compartment and was expressed as a ratio of nuclear MFI/cytoplasmic MFI. Averages and SEM were
calculated for each data set and ANOVA with Tukey’s Honestly Significant Differences posthoc test statistics were calculated using the R statistical software package. All Cellprofiler pipelines and the R code for the ANOVA statistics can be found at http://openwetware.org/wiki/OlivasLab.

**PUF Overexpression qPCR**

Relative levels of *PUF4* or *PUF5* mRNA expression were analyzed by qPCR via the following method. A 20 ml culture of yWO3 containing pWO58, pWO116, or pWO253 was grown to an OD$_{600}$ of 0.4 in SC-leucine containing 2% dextrose. RNA was extracted via the hot phenol method described previously. Equivalent masses of RNA were DNase treated with the Turbo DNA-Free Kit (ThermoFisher) per manufacturer’s instructions. Equivalent masses of DNase-free RNA were reverse transcribed into cDNA using iScript Reverse Transcription Supermix for RT-qPCR (BioRad) per manufacturer’s instructions. Equivalent volumes of equivalent dilutions of resultant cDNA were loaded into 15 µl qPCR reactions containing PowerUP SYBR Green Master Mix (Applied Biosystems) with oWO939 and oWO940 for *PUF4* amplification, oWO941 and oWO942 for *PUF5* amplification, and oWO632 and oWO652 for *TDH1* amplification. PCR cycling and fluorescence analysis took place on the CFX96 Real Time System (BioRad) per manufacturer’s instructions. Relative expression levels were calculated with *TDH1* expression as the reference gene using CFX Manager (BioRad).

**Acknowledgements**

We would like to thank Dr. David Stillman for pWO183 within the set of marker swap vectors (Voth *et al.*, 2003), Dr. Pamela Silver for the RPL11B-GFP expression vector, Dr.
Marvin Wickens for the Puf4FL/CEN plasmid, and Drs. Arlen Johnson and Edward Marcotte for the RPS2-GFP and SIK1-mRFP expression vectors. We would also like to thank members of the Olivas Lab for helpful discussions and reviews of the manuscript.

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**Disclosure of Interest**

The authors report no conflict of interest.

**Table S1: Plasmids**

<table>
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<th>Plasmid</th>
<th>Description</th>
<th>Marker(s)</th>
<th>Source</th>
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<tr>
<td>pWO13</td>
<td>FLAG-Puf3 2µ</td>
<td>TRP1, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Jackson <em>et al.</em>, 2004)</td>
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<td>pWO15</td>
<td>Empty 2µ vector</td>
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<td>pWO16</td>
<td>FLAG-PUF3RD 2µ</td>
<td>URA3, TRP1, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Russo and Olivas, 2015)</td>
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<td>pWO58</td>
<td>Empty CEN Vector</td>
<td>LEU2, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Brachmann <em>et al.</em>, 1998)</td>
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<td>pWO102</td>
<td>pGAL-UAS-PGK1Δ82 ORF</td>
<td>URA3, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>pRP227 (Heaton <em>et al.</em>, 1992)</td>
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<tr>
<td>pWO114</td>
<td>FLAG-PUF1 2µ</td>
<td>URA3, TRP1, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Russo and Olivas, 2015)</td>
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<tr>
<td>pWO115</td>
<td>FLAG-PUF1RD 2µ</td>
<td>URA3, TRP1, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Russo and Olivas, 2015)</td>
</tr>
<tr>
<td>pWO116</td>
<td>p415-GPD-PUF4 CEN</td>
<td>LEU2, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Hook <em>et al.</em>, 2007)</td>
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<td>pWO165</td>
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<td>pWO166</td>
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<td>HIS3, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pWO167</td>
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<td>ATCC</td>
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<td>pWO183</td>
<td>URA3::KanMX3 Marker swap</td>
<td>KanMX3, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Voth <em>et al.</em>, 2003)</td>
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<td>pWO194</td>
<td>FLAG-PUF4 2µ</td>
<td>URA3, TRP1, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Russo and Olivas, 2015)</td>
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<td>pWO196</td>
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<td>pWO200</td>
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Table S2. Oligonucleotides

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<td>oWO21 scRI Probe</td>
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<td>(Caponigro et al., 1993)</td>
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<td>oWO318 ALB1 Probe</td>
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<td>oWO319 PUS7 Probe</td>
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<td>oWO320 EBP2 Probe</td>
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<td>WT</td>
<td>MAT a, leu2-3, lys2-201, ura3-52, trp1-1, his4-539</td>
<td>(Olivas et al., 1997)</td>
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<td>yWO7 WT rp1-1</td>
<td>WT rp1-1</td>
<td>MATα, leu2-3, ura3-52, rp1-1</td>
<td>(Caponigro et al., 1993)</td>
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<td>yWO17 puf5Δ</td>
<td>puf5Δ</td>
<td>MATα, trp1, ura3-52, leu2-3, 112, his4-539, cup1::LEU2(PM), puf5::TRP1</td>
<td>(Olivas and Parker, 2000)</td>
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<td>yWO22 puf4Δ</td>
<td>puf4Δ</td>
<td>MATα, trp1, ura3-52, leu2-3, 112, his4-539, cup1::LEU2(PM), puf4::LYS2</td>
<td>(Olivas and Parker, 2000)</td>
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<tr>
<td>yWO39 puf4Δpuf5Δ</td>
<td>puf4Δpuf5Δ</td>
<td>MATα, trp1-1, ura3-52, lys2, cup1::LEU2/PM, puf4::LYS2, puf5::URA3</td>
<td>(Olivas and Parker, 2000)</td>
</tr>
<tr>
<td>yWO43 puf3Δ rp1-1</td>
<td>puf3Δ rp1-1</td>
<td>MATα, ura3-52, trp1-1, his4-539, leu2-3, 112, cup1::LEU2(PM), rp1-1, puf3::NEO</td>
<td>(Ulbricht and Olivas, 2008)</td>
</tr>
<tr>
<td>yWO48 puf2Δ rp1-1</td>
<td>puf2Δ rp1-1</td>
<td>MATα, leu2-3, 112, ura3-52, his4-539, rp1-1, puf2::URA3</td>
<td>(Ulbricht and Olivas, 2008)</td>
</tr>
<tr>
<td>yWO49 puf5Δ rp1-1</td>
<td>puf5Δ rp1-1</td>
<td>MATα, leu2-3, trp1-1, ura3-52, rp1-1, puf5::URA3</td>
<td>(Ulbricht and Olivas, 2008)</td>
</tr>
<tr>
<td>yWO102 puf1Δ rp1-1</td>
<td>puf1Δ rp1-1</td>
<td>MATα, leu2-3, trp1-1, ura3-52, cup1::LEU2/PM, puf1::NEO</td>
<td>(Ulbricht and Olivas, 2008)</td>
</tr>
<tr>
<td>yWO105 puf4Δ rp1-1</td>
<td>puf4Δ rp1-1</td>
<td>MATα, his4-539, lys2-201, ura3-52, rp1-1, puf4::LYS2</td>
<td>(Ulbricht and Olivas, 2008)</td>
</tr>
<tr>
<td>yWO236 WT BY4741</td>
<td>WT BY4741</td>
<td>MATα, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0</td>
<td>Research Genetics</td>
</tr>
<tr>
<td>yWO260 WT rp1-1</td>
<td>WT rp1-1</td>
<td>MATα, ura3-52, lys2-201, his4, leu2, rp1-1</td>
<td>This study</td>
</tr>
<tr>
<td>yWO261 puf4Δpuf5Δ</td>
<td>puf4Δpuf5Δ</td>
<td>MATα, leu2-3, trp1-1, ura3-52, lys2, cup1::LEU2/PM, puf4::LYS2, puf5::URA3, ura3::KanMX3</td>
<td>This study</td>
</tr>
<tr>
<td>yWO268 puf1-5Δ rp1-1</td>
<td>puf1-5Δ rp1-1</td>
<td>MATα, his4-539, leu2-3, lys2-201, trp1-1, ura3-52, rp1-1, puf1::NEO, puf2::TRP1, puf3::NEO, puf4::LYS2, puf5::URA3, ura3::KanMX3</td>
<td>(Russo and Olivas, 2015)</td>
</tr>
<tr>
<td>yWO289 puf4Δpuf5</td>
<td>puf4Δpuf5</td>
<td>MATα, leu2-3, trp1-1, ura3-52, lys2,</td>
<td>This study</td>
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</table>
Δ rpb1-1  his4, trp1-1, puf4::LYS2, puf5::URA3, ura3::KanMX3, rpb1-1

γWO306  puf1Δ puf4Δ puf5Δ rpb1-1
          MATa, leu2-3, trp1-1, ura3-52,lys2, his4, trp1-1, puf4::LYS2, puf5::URA3, ura3::KanMX3, rpb1-1, puf1::NTC^R This study

γWO307  puf2Δ puf4Δ puf5Δ rpb1-1
          MATa, leu2-3, trp1-1, ura3-52,lys2, his4, trp1-1, puf4::LYS2, puf5::URA3, ura3::KanMX3, rpb1-1, puf2::NTC^R This study

γWO308  puf3Δ puf4Δ puf5Δ rpb1-1
          MATa, leu2-3, trp1-1, ura3-52,lys2, his4, trp1-1, puf4::LYS2, puf5::URA3, ura3::KanMX3, rpb1-1, puf3::NTC^R This study

γWO317  puf4Δ puf5Δ puf6Δ rpb1-1
          MATa, leu2-3, trp1-1, ura3-52,lys2, his4, trp1-1, puf4::LYS2, puf5::URA3, ura3::KanMX3, rpb1-1, puf6::NTC^R This study

γWO318  puf1-6Δ rpb1-1
          MATa, his4-539, leu2-3, lys2-201, trp1-1, ura3-52, rpb1-1, puf1::NEO, puf2::TRP1, puf3::NEO, puf4::LYS2, puf5::URA3, puf6::NTC^R This study

References


de la Cruz, J., Kressler, D., Tollervey, D., and Linder, P. (1998). Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3' end formation of 5.8S rRNA in Saccharomyces cerevisiae. The EMBO journal 17, 1128-1140.


Chapter 4: Broader Impacts
Broader Impacts and Contributions

The work presented here is the culmination of several years’ worth of work toward a deeper understanding of the mechanisms underlying Puf protein regulation, the unique ways in which Puf proteins regulate their targets, and the cellular processes that are affected by this regulation. While phosphorylation of Pop2p by Yak1p has been known for several years, and the importance of the phosphorylation state of Pop2p Thr97 demonstrated, it was not previously known how phosphorylation alters Pop2p mRNA decay activity. As stated earlier, it is unlikely, although possible, that this event alters the intrinsic exonucleolytic activities of Pop2p, since Thr97 lies upstream of the catalytic domain of Pop2p. It is more likely that this phosphorylation alters binding of Pop2p to other protein cofactors. The interplay of Pop2p and its binding partners as it relates to phosphorylation state remains a mystery for future work to solve.

The finding that Puf proteins in yeast are able to stabilize their targets is profound. While this activity has been described rarely for other organisms, it has never before been described in yeast. The delicate interplay of the Puf proteins while they jostle for position on the one consensus PRE in the 3’ UTR gives rise to the idea of competing Pufs. Even more interesting is the finding that in the absence of the Puf proteins, which when present influence the decay of ribosome biogenesis transcripts, the half-life of the targets is still short. This was completely unexpected, as targets of Puf regulation usually are stabilized in the absence of Puf proteins. This raises the question of a secondary non-Puf mechanism that still utilizes, at least in part, the same binding site as the Puf proteins. Puf proteins are only some of many 3’ UTR element
binding proteins. It will be interesting to try and discover if ribosome biogenesis genes are regulated post-transcriptionally by other mechanisms. Energy-wise, it is advantageous to the cell to have multiple redundant mechanisms to control ribosome creation; spending cellular resources on ribosome biogenesis under nutrient deprivation is unwise, and studies have shown that upon nutrient deprivation or activation of mTOR and PKC pathways, ribosome biogenesis gene expression is downregulated. This work also makes the link between transcripts that Puf4p binds and the role of Puf4p in their regulation, indicating an active role for Puf4p in proper ribosome biogenesis. There is a role for Puf proteins in ribosome biogenesis in nearly every organism in which Puf protein function has been studied. This also begs the question of the evolution of this relationship; i.e. how Puf regulation of ribosome biogenesis adapted through the ages.

The work presented here has made the above advancements in the knowledge of the regulation and roles of Puf proteins. It has also led to the establishment of several protocols within the Olivas lab that will undoubtedly be of further use for future research. Through the compilation of several primary literature articles and experimentation, I have established protocols for fixing, mounting, and visualizing cells in a confocal microscope, and for the post-imaging analysis using quantitative methods via the CellProfiler suite of software. I have also made detailed notes of how to analyze large data sets for ANOVA statistical significance and post-hoc analysis of individual differences in the R statistical software package. I have brought a new method, the pulse-chasing of rRNA with tritiated methionine as a methyl donor, into the lab. Also, the use of the delitto perfetto method of gene manipulation (Storici and Resnick, 2006)
is a powerful tool to analyze a number of genome alterations, from miniscule changes to alter an amino acid residue to larger C-terminal or N-terminal truncations and even whole gene deletions, without the need for selective markers. This could prove to be an invaluable tool should a yeast strain “run out” of selectable markers. I have also compiled protocols detailing the dissection of sporulated yeast and the general genotyping process, using the most efficient environmental stimuli for sporulation of diploid yeast. During work with Dr. Joseph Russo, I also established a detailed protocol outlining the procedures for RNA immunoprecipitation.

Our lab has made great strides in recent years to working with human cell culture. It will be interesting to investigate how human Pum proteins, which share the most homology with Puf3p, are involved in these mechanisms, and how evolution has altered these mechanisms. Pop2p in more complex (multicellular) eukaryotes has lost the N-terminal portion of the protein that houses the T97 residue studied here, and it is unclear how this deletion affects interaction with human Pums. As described before, at least human Pum-A is tentatively involved in ribosome biogenesis, although the work done has been using homologous studies in yeast. In this study, mutation of conserved residues between Pum-A and Puf6p in yeast Puf6p led to ribosome biogenesis inhibition (Qiu et al., 2014). As stated earlier, in animals (and especially humans), the interaction of Pum proteins and the miRNA machinery has been described. While Pum proteins do not act exclusively through the miRNA machinery to achieve their regulation, they are thought to assist in exposing seed regions upon binding. We are not sure how Pum proteins influence ribosome biogenesis and by what means in humans, nor how
evolution has changed these processes from an organism with six Puf proteins (yeast) to one with two (humans). While Pum proteins are not specifically implicated in human ribosomal diseases, it does bear mentioning that many of these diseases are not of the ribosomal proteins or RNA, but of the biogenesis factors (Freed et al., 2010), and yeast have been used as model organisms in which to gain an initial understanding of multiple ribosomal diseases (Woolford and Baserga, 2013). Pum proteins may have undiscovered roles in the progression of such diseases.

It is also unclear how Puf proteins fit into the nutrient response pathways concerning ribosome biogenesis. Ribosome biogenesis is known to be regulated at least in part by the TOR signaling pathway (Kos-Braun et al., 2017), and some Puf proteins are thought to be rapamycin sensitive (Foat et al., 2005). Further studies will undoubtedly link these two cooperative pathways together.

References