Analysis of the Condition-Specific Regulation of Puf3p Activity and Puf3p-Mediated Translational Repression of mRNA in *Saccharomyces cerevisiae*

Melanie A. Miller
*University of Missouri-St. Louis*

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Analysis of the Condition-Specific Regulation of Puf3p Activity and Puf3p-Mediated Regulation of mRNA Translation in *Saccharomyces cerevisiae*

Melanie A. Miller  
M.S., Biology, University of Missouri – St. Louis, 2007  
B.S., Biology, Southeast Missouri State University, 2004  

Submitted to the Graduate School at the University of Missouri - St. Louis  
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Analysis of the Condition-Specific Regulation of Puf3p Activity and Puf3p-Mediated Translational Repression of mRNA in *Saccharomyces cerevisiae*

**ABSTRACT**

The Puf family of proteins regulates diverse cellular processes in eukaryotes such as gametogenesis, embryonic development, and memory formation by promoting translational repression and/or degradation of targeted mRNAs. One member, yeast Puf3p, regulates mitochondria biogenesis and function by modulating the stabilities of nuclear-transcribed mitochondrial mRNAs in response to different carbon sources. In the presence of dextrose, Puf3p promotes rapid deadenylation and degradation of its mRNA targets in the cytoplasm via decay complex recruitment. Alternatively, in the presence of ethanol, galactose, or raffinose, mRNA targets are stabilized, as Puf3p-mediated decay activity is severely inhibited or abolished. In this work, I have established that carbon source-induced inhibition of Puf3p activity is not due to decreased levels of transcription or translation, but post-translational mechanisms such as phosphorylation likely regulate the status of Puf3p activity. In addition to a role in mRNA decay, Puf3p reduces the translational efficiency of the mRNA target COX17 in dextrose conditions when mRNA decay is blocked. However, binding interactions between the Puf3 repeat domain (Puf3RD) and the deadenylation factors Ccr4p and Pop2p are disrupted in carbon sources that inhibit Puf3p activity, while interactions with COX17 mRNA are maintained. Analysis of Puf3p localization demonstrated that Puf3p aggregates in multiple cytoplasmic foci in all carbon source conditions, but these foci increase in size in conditions that inactivate Puf3p. Furthermore, Puf3p aggregate size is increased in all P-body inducing conditions, with concomitant co-localization of Puf3p with P-bodies.
However, the co-localization of Puf3p aggregates with mitochondria is only observed in Puf3p inactivating conditions.

These observations present multiple schemes to regulate Puf protein activity, such that post-translational phosphorylation may serve as the molecular switch that regulates Puf3p activity and allows rapid changes in the repression of mRNA targets. Inhibition of Puf3p activity at the molecular level may be explained by the inability of Puf3p to recruit deadenylation and decay factors to a target mRNA. In Puf3p activating conditions, Puf3p is predominately expressed ubiquitously in the cell cytoplasm such that Puf3p can bind target mRNAs and recruit deadenylases, presumably to repress translation. Subsequently, Puf3p-bound transcripts targeted for decay localize within multiple P-bodies where they are decapped and degraded. Alternatively, when Puf3p is inactive, altered Puf3p localization to P-bodies in Puf3p inactivating conditions might serve as a regulatory mechanism to temporarily store inactive Puf3p pools. Finally, this work suggests that Puf3p may shuttle its mRNA targets to mitochondria for translation and subsequent import of nascent proteins into mitochondria in the absence of its decay activity. Together, this work provides a greater understanding of the role of Puf3p in mRNA decay regulation, and provides insight into the conditional control of Puf3p activity and how Puf3p accomplishes fine tuning of mitochondrial protein production.
DEDICATION

I dedicate this Ph.D. dissertation to my mother Marnette who has selflessly supported me throughout my graduate studies.
ACKNOWLEDGEMENTS

Completion of this project would not have been possible without the continuous support from all of my friends and family. I extend immense gratitude for three special individuals who gave me the strength, love and support necessary to complete this project: my mother Marnette Miller and friends Rafeiza Khan and Florencia Lopez Leban. Thank you all for your encouraging words and prayers, and I am grateful to be surrounded by my supportive mother and lifelong friends that are truly intelligent, hard-working, humble, altruistic, genuine women.

I will never forget the numerous times that my mother has selflessly sacrificed her time to cater to my needs during my graduate studies, and ultimately ensure completion of this dissertation. Thank you for the numerous times that you insisted upon coming with me to the lab in the middle of the night/early morning to ensure my safety, despite having to attend your job sleep deprived as a consequence. When I was badly injured in a car accident, you somehow managed to adjust your schedule to shuttle me to and from the lab on several occasions. I cannot forget the time you proofread my comprehensive exam grant proposal for me and gave me your feedback. Thank you again for everything. I cannot forget the enormous amount of support and prayers that my family has given me. Thank you everyone.

I would like to thank my advisor Dr. Wendy Olivas for affording me the opportunity to conduct research in her laboratory and for providing scientific expertise, scholarly guidance, and support. I am grateful for her patience, compassion, and willingness to work with me during the struggles I encountered after a car accident. Her kindness and thoughtfulness will never be forgotten.
Additionally, I would like to thank my dissertation committee members, Dr. Marc Spingola, Dr. Lisa Schechter, and Dr. Bethany Zolman, for their constructive criticism and support regarding this work.

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I must also express thanks to the following individuals for providing expression constructs and yeast strains that were essential for the completion of my work: members of the Roy Parker lab for providing pRP1186, pRP1162, ywo185 and ywo186; Yuan Su at UMSL for providing pBin35SRed1; and previous lab member S. Sean Houshmandi for creating the Myc-tagged yeast strains used in the co-immunoprecipitation experiments.
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CHAPTER I: INTRODUCTION
The majority of the information presented in this introduction to Puf protein function and regulation is published in my Puf protein review article [1], and excerpts from this publication are presented below in quotation. Material relating to recent scientific advances in the area of Puf proteins or additional information relating to mRNA decay is presented without quotation.

“Proper gene expression requires that protein production occur at the right time, in the correct amount, and in the proper location in the cell. Such tight regulation involves not only transcriptional regulation, but also multiple levels of post-transcriptional control. In particular, the ability to modulate mRNA stability and/or translation allows rapid alteration of protein production in response to cellular changes [2]. Therefore, this type of regulation is common in somatic cells and plays a critical role during early development. The sequence elements that influence mRNA translation and decay rates are often located within the 3’ UTR. Such sequences direct the dynamic assembly of proteins and microRNAs on a particular mRNA, leading to unique ribonucleoprotein (mRNP) complexes [3, 4]. An important field of study is understanding how the binding of factors to these sequences, or the “mRNP code”, leads to functional changes in the mRNAs.

Puf proteins represent a conserved family of RNA-binding factors that are key regulators of mRNA translation and stability across the eukaryotic kingdoms. The first Puf proteins identified, Pumilio in Drosophila melanogaster and FBF in Caenorhabditis elegans, provided the Puf family name. Puf proteins play important roles in stem cell maintenance, cell development, and differentiation by binding conserved elements within target mRNAs, resulting in mRNA degradation and/or translational repression [5-7].
CHARACTERISTIC FEATURES OF PUF PROTEINS

The primary characteristic of Puf proteins is a highly conserved Pumilio homology domain (Pum-HD), often referred to as the Puf repeat domain (PufRD). The canonical PufRD is located near the protein’s C-terminus and consists of eight tandem Puf repeats of ~36 amino acids each, plus flanking half-repeats [8-13, 14, 15, 16, 17, 18, 19]. The PufRD physically binds the 3’UTR of mRNAs [10, 13, 20-25], and for many Puf proteins, the PufRD alone is sufficient to regulate mRNA translation and decay [20, 23, 26]. Regions outside the PufRD are not well conserved, although many Pufs contain glutamine-rich motifs, which may promote aggregation to regulate Puf protein activity [12, 14, 27-29].

Hundreds of Puf proteins have been identified based on the characteristic Puf repeat domain in diverse eukaryotic organisms, and they appear to be evolutionarily conserved from fungi, molds and parasites, to flowering plants, moss, and metazoans. Within metazoans, Pufs are present in insects, nematodes, amphibians, birds, fish and mammals. Some organisms such as Drosophila express only one Puf protein, while others such as C. elegans, S. cerevisiae, T. brucei, and Arabidopsis thaliana express six or more Puf proteins. With continuing advances in sequencing, it is likely that the number of Puf proteins is underestimated, even within an organism. For example, it has been thought that mice and humans each express only two Puf proteins. However, recent analysis of novel exons in humans, along with comparative genomics studies with mice and zebrafish, has revealed two novel classes of Puf proteins, Puf-A and C14orf21 [30]. Thus, mice and humans may each actually express four Puf-related proteins: the
canonical Pum1 and Pum2 proteins, as well as Puf-A and C14orf21. The zebrafish, human, and murine Puf-A homologs are structurally distinct from the canonical 8-repeat Puf repeat domain based on computer modeling of the human Puf-A repeat domain [30]. Specifically, Puf-A is modeled to contain six Puf repeats, three on each side of two repeat-like structures. Moreover, Puf6p in budding yeast is predicted to be a Puf-A homolog [30], as its non-conventional repeat domain contains only seven repeats [22].

The primary role of Puf proteins is to negatively regulate target mRNA expression by stimulation of mRNA decay and/or inhibition of translation. In accordance with this role, Puf proteins are predominately localized within the cytoplasm of cells [9, 11, 31-40]. Two exceptions are that *Saccharomyces cerevisiae* Puf6p is present in both the cytoplasm and nucleus [22], and *Trypanosoma brucei* PUF7 is localized in the nucleolus [41]. Consistent with their role in translational repression, mouse PUM2, *T. brucei* PUF1, and *Trypanosoma cruzi* PUF6 do not associate with polysomes [29, 42, 43]. In contrast to the customary role of Puf proteins as translational repressors, recent studies have shown that in some cases, Puf proteins in *C. elegans*, *T. brucei*, and *Xenopus laevis* display a role in stabilizing transcripts and positively regulating mRNA target expression [31, 44-46].

**BIOLOGICAL ROLES OF PUF PROTEINS**

Puf proteins regulate several aspects of eukaryotic development. In higher eukaryotes, Puf proteins are implicated in regulating several aspects of gametogenesis/gamete maturation, embryogenesis, and neural development and function (Table 1). However, in lower eukaryotes such as *Dictyostelium*, yeast and trypanosomes, Puf proteins inhibit cell differentiation and regulate organelle biogenesis and maintenance
(Table 1). While Puf proteins appear to have diverse functions among different organisms, it is hypothesized that the underlying function of Puf proteins is to support stem cell maintenance and self-renewal [5].

**Puf Mutant Phenotypes in Drosophila**

Extensive analysis of *pumilio* mutants and their phenotypes have elucidated several physiological roles of Puf proteins as summarized in Table 1, with a few key examples described herein. In *Drosophila*, aberrant Hunchback protein distribution and abdominal segmentation defects observed in *pumilio* mutant embryos contributed to the discovery that Pumilio regulates posterior development. Normally, Hunchback protein expression is restricted to the anterior region during early development [47]. However, in *pumilio* mutant embryos, the Hunchback protein gradient is extended to the posterior region of the embryo [8, 47], suggesting that hunchback mRNA is no longer repressed in this region. This finding was supported by the observation that the poly(A) tail of hunchback mRNA isolated from the posterior of *pumilio* mutant embryos is not deadenylated as compared to wild-type embryos, and thus the mRNA is not translationally repressed [48]. As a consequence, *pumilio* mutant embryos exhibit defects in abdominal segmentation [8, 49].

Analysis of *pumilio* mutant embryos also revealed a defective germline cell phenotype. In female embryos and third instar larvae, mutation of *pumilio* impedes the migration of germline progenitors into the ovaries [50], as well as asymmetric division [51] or proliferation [50, 52]. In adults, loss of germline stem cells in the ovaries is noted [53], resulting in sterility. To regulate germline stem cell maintenance as well as
Table 1.1. Diverse Functions of Puf Proteins (Reprinted from [1])

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<tr>
<td>Germine development and differentiation</td>
<td>Drosophila</td>
<td>Pumilio</td>
<td>Nanos</td>
<td>cyclin B</td>
<td>[26, 50-53, 64], [11, 66-71]</td>
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<td>Spermatogenesis/oogenesis switch</td>
<td>C. elegans</td>
<td>FBF-1, FBF-2</td>
<td>NANO1 to NANO3</td>
<td>fem-3</td>
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<td>Oocye maturation/differentiation</td>
<td>Xenopus</td>
<td>Pum1</td>
<td>CPEB, Xcat-2</td>
<td>fog-2(^7)</td>
<td>[72]</td>
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<td></td>
<td>Xenopus</td>
<td>Pum2</td>
<td>DAZL, ePAB</td>
<td>cyclin B1</td>
<td>[17, 44, 73]</td>
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<td></td>
<td>C. elegans</td>
<td>PUF-5 to</td>
<td>?</td>
<td>glp-1</td>
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<td>C. elegans</td>
<td>PUF-8</td>
<td>?</td>
<td>?</td>
<td>[76]</td>
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<td>PUM2</td>
<td>BOL</td>
<td>SDAD1(^8)</td>
<td>[77]</td>
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<td>PUM2</td>
<td>?</td>
<td>CEP3(^5)</td>
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<td>Germline stem cell maintenance/mitotic</td>
<td>C. elegans</td>
<td>FBF-1, FBF-2</td>
<td>gld1(^4)</td>
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<td>A. thaliana</td>
<td>PUM1-PUM6</td>
<td>?</td>
<td>gld-1(^4)</td>
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<td>C. elegans</td>
<td>FBF-1, FBF-2, FBF-8</td>
<td>?</td>
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<td>Repression of filamentous-form cell</td>
<td>S. cerevisiae</td>
<td>Puf5p</td>
<td></td>
<td>?</td>
<td>[81]</td>
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<td>Puf5p and Puf6p</td>
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<td>Organellre biogenesis, maintenance,</td>
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<td>function</td>
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<td>Puf5p</td>
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<td>LRG1</td>
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<td>TOM-20</td>
<td>COX17, PET123</td>
<td>[23, 34, 35, 90-92]</td>
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<td>Peroxisome protein localization</td>
<td>S. cerevisiae</td>
<td>Puf5p</td>
<td>?</td>
<td>PEX14(^5)</td>
<td>[93]</td>
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<td>Organelle copy number regulation, cell-</td>
<td>T. brucei</td>
<td>PUF9</td>
<td>?</td>
<td>PNT1(^4), PNT2(^4), LIGKA(^4)</td>
<td>[31]</td>
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<td>cycle dependent replicative processes</td>
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\(^1\) mRNA contains canonical Puf recognition element in the open reading frame [55, 56].

\(^2\) Pumilio and Nanos have opposing functions in regulating GlurIIA mRNA. Pumilio represses GlurIIA, while Nanos induces its expression.
Function of Puf protein has not been elucidated; putative role in spermatogenesis is deduced based on Puf expression in testis [77, 78]; putative role in neural function is deduced based on Puf expression in ventral nerve cord [57].

In rare instances, Puf proteins stabilize and/or positively regulate mRNA expression [31, 45]. FBF-1 and FBF-2 can both negatively and positively regulate gld-1 expression [33, 46].
mRNAs have only been shown to bind Puf proteins [18, 58, 77, 78, 93].

Altered frequency of mating-type switching is observed in PUF5 deletion cells [82]. Pu4p and Pu5p work in combination to repress HO mRNA [83-85].

Germline development and differentiation, Pumilio is required to promote deadenylation and translational repression of cyclin B [26].

*Drosophila* embryos expressing either a *bicoid* mRNA transgene mutated in its Pumilio binding site or *pumilio* embryos exhibit mouth hook defects and other defects in head formation, suggesting that Pumilio also regulates anterior patterning of the embryo. Furthermore, analysis of *bicoid* mRNA polyadenylation in *pumilio* mutant embryos or embryos expressing the mutant *bicoid* transgene revealed that *bicoid* transcripts are stabilized, and deadenylation proceeds more slowly as compared to wild-type embryos [65].

Another area of Pumilio regulation is in neuron development and function. In *pumilio* mutant *Drosophila* larvae, a hyperexcitability defect is observed in motoneurons at the neuromuscular junction [54] This hyperexcitability phenotype is attributed to an increase in the persistent component of the voltage-gated Paralytic sodium current that corresponds with an increase of *paralytic* mRNA levels in *pumilio* mutant flies [55]. The motoneuron hyperexcitability defect is suppressed by overexpression of Pumilio and thereby repression of *paralytic* mRNA [56].

Pumilio specifically localizes to central nervous system neurons and the post-synaptic side of the neuromuscular junction in muscle fiber. In *pumilio* loss of function larvae, an abnormal bouton phenotype is observed in muscle. The size of terminal
boutons is increased, along with reduction of bouton number and decreased synaptic span. Additionally, an increase in the number of eIF-4E aggregates was observed in the muscle fiber of *pumilio* mutants as well as increased expression of the GluRIIA glutamate receptor, suggesting that Pumilio may function to repress translation of this translation initiation factor and neurotransmitter receptor, respectively [38]. This is supported by electrophoretic mobility shift assays in which Pumilio specifically binds to the 3’ UTRs of *eIF-4E* and *GluRIIA* mRNAs [25, 38].

Pumilio also appears to regulate dendrite morphogenesis, as expression of *pumilio* loss of function mutants in dendritic arborization neurons causes class-specific elongation of dendrite spikes and class-specific reduction of higher-order dendrite branches that result in insufficient coverage of the epidermis [59]. In rat neurons, Pum2 co-localizes with mRNAs and with stress granules in dendrites [29]. Knockdown of Pum2 using shRNA results in increased arborization of dendrites, suggesting that Puf proteins in mammals also regulate dendrite morphogenesis. As with *Drosophila* Pumilio, mammalian Pum2 also represses translation of eIF-4E [29].

**Puf Mutant Phenotypes in *C. elegans***

In *C. elegans*, the Puf proteins FBF-1 and FBF-2 were originally identified by their ability to bind a regulatory region in the 3’ UTR of *fem-3* mRNA [11]. The repression of *fem-3* is required to regulate the transition from spermatogenesis to oogenesis [66-68], and such repression was thought to occur post-transcriptionally via the regulatory region in the *fem-3* 3’ UTR [68, 69]. RNAi-mediated knockdown of *fbf* (*fbf-1* and *fbf-2* genes) resulted in increased sperm production with concomitant inhibition of oogenesis and production of abnormal oocytes, suggesting that FBF regulates the
spermatogenesis/oogenesis switch by repressing *fem-3* [11]. The spermatogenesis/oogenesis switch is also regulated by PUF-8. While many *puf-8* mutant hermaphrodites are phenotypically normal, some exhibit increased sperm production and oogenesis is inhibited. However, all *fhf-1 puf-8* double mutants display a masculinized phenotype accompanied by an increase in FOG-2 protein expression, suggesting that FBF-1 and PUF-8 may regulate *fog-2* expression, a gene that acts upstream in germ-line sex determination [72].

Germline cell development and differentiation in *C. elegans* occurs spatially along a distal-proximal axis. In the distal region of the gonad, stem cells proliferate and undergo mitosis. As cells proceed proximally, they enter meiosis at the transition zone and differentiate into gametes. Mutation of *puf-8* or *fhf-1* disrupts the spatial regulation of the mitosis/meiosis decision in hermaphrodites, as the span of the distal mitotic region is shortened concomitant with a reduction of germline stem cells [72, 79] and inhibition of mitosis [32]. In *fhf-1 fhf-2* double mutant hermaphrodites, defects are exacerbated, such that all germline stem cells in the mitotic region inappropriately enter meiosis and begin spermatogenesis [33]. FBF promotes germline stem cell self-renewal by repressing *gld-1*, which in turn, ensures mitosis at the distal end of the gonad [33]. FBF may also function to promote meiosis and differentiation proximally, as *fhf-1 fhf-2* mutant hermaphrodites and males exhibit an abnormal reduction in GLD-1 protein levels in the transition zone, suggesting that FBF also functions to promote *gld-1* expression in the proximal region of the gonad [46].

Multiple Puf proteins act to regulate *C. elegans* oogenesis and embryogenesis. For example, *fhf-1 fhf-2 fem-3* mutant females have abnormally large oocytes, suggesting
that FBF functions to restrict oocyte growth [75]. RNAi knockdown of puf-5 or the nearly identical puf-6 and puf-7 produced small oocytes that have abnormal spatial orientation within the gonad, defects in yolk uptake into oocytes, as well as reduced size and abnormal spatial localization of the nuclei within oocytes [37]. Therefore PUF-5, PUF-6 and PUF-7 regulate several aspects of oogenesis. These mutants also produced embryos that do not hatch, lack an eggshell, failed to undergo cytokinesis, and were incompletely cellularized, suggesting that Puf proteins are required for several aspects of embryogenesis as well [37].

_C. elegans_ FBF-1 also plays a role in neural function. Specifically, FBF-1 localizes within AWC olfactory sensory neurons, which regulate odor sensation and adaptation. Chemotactic behavior towards an odor is normally reduced after long exposures to the odor. However, in _fbf-1_ null mutants, chemotactic adaptation to persistent odors is compromised, with _fbf-1_ mutants retaining chemotactic behavior towards the odor. FBF-1 mediated odor adaptation is achieved by promoting the translation of _egl-4_ mRNA [45].

**Puf Mutant Phenotypes in yeast**

In the single-celled eukaryote _S. cerevisiae_, several physiological functions for Puf5p (previously referred to as Htr1, Uth4, and Mpt5) have been identified. For example, _puf5_ mutants display increased stress tolerance and an increased replicative life span (the number of mitoses a cell accomplishes before death) [86, 87], while causing a decreased chronological life span (duration of cell viability in saturated cultures grown in synthetic media). This defect can be rescued by complementation with _PKC1_, a component of the cell wall integrity pathway [89]. _puf5_ mutants also exhibit detergent
sensitivity and sensitivity to the cell wall stain calcofluor white, consistent with a defect in the cell wall stability/structure [88]. Puf5p may regulate cell wall integrity and aging by repressing the \( LRG1 \) transcript, whose product acts as an upstream inhibitor of Pkc1p [89].

Alterations of \( PUF3 \) in \( S. \) \textit{cerevisiae} revealed several mitochondria-related defects. First, Puf3p appears to be involved in mitochondrial motility and inheritance, as \( puf3 \Delta \) caused an increased occurrence of aggregated and fragmented mitochondria and a decrease in mitochondrial movement to the daughter bud tip [34]. Additionally, over-expression of \( PUF3 \) in cells grown in glycerol, a non-fermentable carbon source, results in growth defects at elevated temperatures. This phenotype is consistent with defects in respiration [34]. Affinity purified Puf3p is enriched for nuclear-transcribed mRNAs that encode mitochondrial proteins [35]. Deletion of \( PUF3 \) results in increased levels of such mRNAs and proteins, including \( COX17 \) [91] and Pet123p [34]. Moreover, \( puf3 \Delta \) disrupts asymmetric localization of Puf3p-associating mRNAs to the mitochondria [92].

**Coordinate Regulation of Functionally Related mRNAs**

Puf protein-mediated regulation of mRNAs involves a network of complex interactions with several regulatory pathways. For example, regulation of individual mRNAs can have a broad impact when the mRNAs targeted by Puf proteins are involved in cell signaling pathways. \( C. \) \textit{elegans} FBF and human PUM2 regulate the MAP kinase pathway by repressing the translation of mRNAs that encode MAPK/ERK proteins. Furthermore, FBF and the MAPK phosphatase LIP-1 work in combination to negatively regulate expression and activity of MPK-1, albeit at different levels of gene regulation [94]. \( S. \) \textit{cerevisiae} Puf5p also negatively regulates the yeast MAPK pathway [81]. \( C. \)
C. elegans PUF-5, PUF-6, and PUF-7 negatively regulate the Notch signaling pathway by repressing glp-1 mRNA, which encodes a Notch receptor (Table 1; [37]). Puf proteins also work in conjunction with other mRNA regulatory pathways, such as microRNAs (miRNAs). mRNA targets that interact with human PUM1 and PUM2 are enriched in high-confidence miRNA binding sites located in close proximity to the Puf binding site (Figure 1.1A; [95, 96]). In addition, C. elegans PUF-9 regulates hbl-1 mRNA, which also contains putative binding sites for members of the let-7 miRNA family (Figure 1.1A; [57]).

In a given organism, a single Puf protein can regulate several mRNAs. C. elegans FBF binds and represses the expression of fem-3 and gld-1 mRNAs [11, 33, 46, 71]. Alternatively, several Puf proteins can act together to target a single mRNA (Figure 1.1B). In S. cerevisiae, Puf4p and Puf5p repress HO mRNA [82-85, 97], Puf1p and Puf5p stimulate decay of TIF1 mRNA, while Puf1p, Puf4p, and Puf5p all function to promote HXK1 mRNA turnover [24]. Such combinatorial control of a single mRNA allows fine-tuned regulation of the transcript by Puf proteins of potentially different activity levels under varying conditions and using potentially different mechanisms of action on the mRNA.

Analysis of mRNAs that co-purify with Puf proteins has revealed that Pufs regulate specific classes of mRNAs that are often functionally related (Figure 1.1C). This finding supports the RNA regulon theory, which states that functionally related mRNAs are co-regulated through the binding of trans-factors to these mRNAs to coordinate post-transcriptional events [3, 98, 99]. Within a single organism, the mRNA classes bound by Pufs are typically involved in distinct functions within a particular
Figure 1.1. Modes of Puf-mediated regulation of transcripts. (Reprinted from [1]). Puf-mediated regulation of mRNAs often involves interactions with other regulatory pathways, other Puf proteins, and recognition of specific classes of transcripts. A) In some cases, one Puf protein is sufficient to regulate a single transcript [8, 25, 26, 54, 56, 64, 65, 91]. In other cases, the Puf protein may repress the transcript by working in conjunction with another regulatory pathway, such as the miRNA regulatory system [57, 96]. The Puf protein (green arc) binds conserved UGUR elements within the 3’UTR of the mRNA target, while the miRNA (red nucleotides) binds to a complementary sequence in the 3’UTR. The combinatorial functions of the Puf and the miRNA can result in both deadenylation and decay of the mRNA target, as well as translational repression. B) Multiple Puf proteins can work together to promote repression and/or turnover of a single mRNA target [24, 37, 72, 83-85]. C) A single Puf protein can regulate several mRNAs that belong to a specific class of transcripts [35]. Different Puf proteins recognize and interact with distinct classes of mRNAs (represented as different colored lines).
subcellular compartment/organelle and/or regulatory pathway. For example, affinity tagged Puf1p-Puf5p in *S. cerevisiae* interact with mRNAs that function within a particular subcellular compartment or organelle. Puf1p and Puf2p predominantly bind mRNAs that encode membrane-associated proteins, Puf3p physically associates with nuclear-encoded mRNAs that encode mitochondrial proteins, and Puf4p and Puf5p associate with mRNAs that encode nucleolar or nuclear proteins, respectively [35]. In another single-celled eukaryote, *T. brucei*, purified TAP-tagged PUF9 was enriched for mRNAs involved in DNA replication, and these transcripts were coordinately regulated by the mitotic cell cycle [31].

In multicellular eukaryotes such as *Drosophila*, the gene ontology annotations of the mRNAs that associate with Pumilio are divided into two major functional groups: mRNAs encoding nuclear proteins involved in nucleotide metabolism and transcriptional regulation, and mRNAs that encode proteins that localize to the membrane of organelles [100]. Specifically, a subset of these latter transcripts encodes several subunits of the vacuolar V-type ATPase. Human Pum1 associates with mRNAs encoding both positive and negative regulators of the G2/M transition of the cell cycle, transcripts that encode other transcriptional, posttranscriptional and posttranslational regulatory proteins [40], and several components of the Ras and Wnt signaling pathways [96]. Based on these analyses, Pum1 may indirectly affect the translation of a large number of downstream mRNA targets by directly modulating other regulators that are upstream of these transcripts in their associated pathways [40]. In contrast, *C. elegans* FBF associated with various transcripts involved with meiosis, the Ras/MAPK pathway, apoptosis, and in germline stem cell maintenance and development [101].
Many of the mRNAs that associate with Puf proteins from humans (Pum1 and Pum2) [96], *Drosophila* [100], and yeast Puf3p [35] contain a very similar Puf binding motif (Figure 1.2), which may be attributed to the high degree of sequence conservation within the repeat domain [10, 14]. However, as described above, the functions of the proteins encoded by the mRNAs containing this motif are not always conserved between different organisms, even if homologs or orthologs of a Puf-bound transcript from one organism is present in another organism [40, 96, 100]. For example, more than half of the Pum-associated transcripts in adult flies contain a Puf binding motif that is also conserved in mRNAs that co-purify with *S. cerevisiae* Puf3p (Figure 1.2; [100]). In yeast, the motif is present within 73% of nuclear-transcribed mRNA targets that encode mitochondrial proteins and physically associate with Puf3p [35], while this motif is only present in 8% of *Drosophila* nuclear genes that encode mitochondrial proteins [100].

Conservation of some mRNA targets and their biological functions have been observed between *C. elegans* FBF and human Pum proteins [101], and between *Drosophila* Pumilio and human Pum1 [40]. However, while *Drosophila* Pumilio and human Pum1 may associate with transcripts that have similar functions, the mRNAs bound by these Pufs are not always homologous mRNAs [96]. Moreover, both *C. elegans* FBF [94, 101] and human Pum2 regulate the MAPK pathway [94]. Yet FBF represses *mpk-1* mRNA, while human Pum2 represses translation of Erk2 and p38a mRNAs [94]. Therefore, Puf-mediated regulation of some biological processes appears to be conserved across species, even if the actual mRNAs targeted are not conserved.
PUF PROTEIN STRUCTURE AND RNA BINDING MECHANISMS

The means by which Puf proteins recognize, bind and regulate transcripts of a particular class is dependent on the specificity of the Puf protein for its binding sequence within the target transcript. Puf proteins specifically bind conserved UGUR elements, usually located in the 3’UTRs of mRNA targets [10, 12, 21-23, 25, 31, 33, 56, 61, 65, 71, 74, 79]. Mutation of these elements abolishes binding [10, 11, 17, 20, 22, 23, 25, 26, 56, 61, 71, 79, 82] and Puf-mediated regulation [20, 22-24, 26, 60-62, 73, 82]. Sequences that flank the core UGUR element determine the specificity of Puf protein-mRNA target interactions [20, 23, 24, 61, 71, 102]. The minimal Puf binding element is much larger than the canonical UGUR tetranucleotide. For example, Drosophila Pumilio, human PUM1 and PUM2, and S. cerevisiae Puf3p share an identical UGUANAUA binding consensus sequence based on bioinformatic predictions and analysis of confirmed mRNA target sequences [12, 23, 35, 40, 56, 96, 100, 103]. As shown in Figure 1.2, Puf binding sites contain similar yet distinct AU-rich sequences downstream of the core UGUR element, while some Puf sites such as for yeast Puf3p have conserved upstream sequences as well. Though the minimal RNA binding sequence is typically 8-10 bases, high affinity Puf binding often requires a larger sequence. For instance, the C. elegans FBF proteins require an expanded 22 nucleotide target sequence for maximum binding [71]. In a novel finding, paralytic mRNA, which is targeted by Drosophila Pumilio, contains a putative conserved Puf binding element in the open reading frame versus the 3’UTR [56].

Although Puf proteins typically recognize distinct recognition elements, Puf protein specificity can be relaxed, whereby multiple Puf proteins within an organism can
bind the same element within an mRNA target. For example, both yeast Puf1p and Puf5p can bind and regulate through the same binding site in the 3’ UTR of TIF1 mRNA [24]. Considering that a single UGUR element will only be bound by one Puf protein at a time, it is hypothesized that relaxed Puf specificity may ensure the availability of Puf proteins to regulate a transcript, should the expression or activity of one Puf protein be altered [24].

**Binding Specificity Revealed from Puf Protein Crystal Structures**

The original crystal structures of Puf proteins were determined in 2001. Since then, the structures of several Puf proteins bound to target mRNAs have been elucidated, which provide key insight into how Puf proteins elicit specificity of binding to mRNA target sites. The structures of the Puf repeat domains alone from *Drosophila* Pumilio [104] and human PUM1 [105] revealed an arc-shaped molecule. Each of the eight, 36 amino acid repeats folds into three alpha helices, with the helices of successive repeats stacking together to produce layers of alpha helices on the inner concave and outer convex surfaces. The sequences within the helices on the concave surface are the most conserved between different Puf proteins, and this concave region also has a high percentage of positive charge. From these crystal structures, it was hypothesized that the inner concave surface contacts RNA, while protein-protein interactions involve the outer convex surface [104, 105]. The curved Puf structure is similar to other helical repeat proteins composed of HEAT repeats or Arm repeats, though the Puf repeat domain is the first such structure to bind RNA versus other proteins [104, 105].
Figure 1.2. Puf protein binding element consensus sequences. (Reprinted from [96]). Consensus sequence weight matrices were developed based on sequences in the 3’UTRs of mRNAs associated with human PUM1 and PUM2, *Drosophila* Pumilio, and *S. cerevisiae* Puf3p, Puf4p and Puf5p. The height of the nucleotide represents the probability that it will occur at that position. Positions that are conserved across organisms and proteins are highlighted in yellow.
The first structure of a Puf protein bound to an RNA target utilized human PUM1 (HsPUM1), and it confirmed the hypothesis that RNA binds to the inner concave surface of the protein (Figure 1.3A; [106]). This structure suggested that Puf protein binding was modular, whereby each successive repeat domain utilizes conserved amino acid positions to bind a consecutive base in the target RNA [106]. Specifically, the 8 bases of the target RNA, 1-8, are contacted by protein repeats 8-1, with the critical UGU sequence recognized by repeats 8, 7 and 6, respectively. Within each repeat, amino acid position 13 forms stacking interactions between successive bases, while positions 12 and 16 make base-specific hydrogen bond or van der Waals contacts (Figure 1.3A, E). As expected from the similar RNA target sequences bound by Pufs, these amino acid positions are highly conserved between different Puf proteins, and mutations at these positions in PUM1 predictably altered binding specificity [106]. In fact, a detailed study to engineer PUM1 to bind altered RNA target sequences revealed that mutation of only the base-contacting amino acids (and not the stacking amino acids) could transform binding specificity [107]. However, the mutants could not always bind their targets with as strong an affinity as wild-type PUM1, implying that RNA binding involves more complicated interactions than just those two positions [107]. Similar complexity was revealed by a study comparing the binding specificities of yeast Puf3p and Puf5p [13]. Mutation of Puf3p RNA-binding amino acids to those of Puf5p could enhance binding to a Puf5p target sequence, yet these mutations did not disrupt binding interactions with a Puf3p target sequence. Thus, while RNA recognition by Puf proteins is clearly based on
a modular set of binding coordinates, binding specificity is often more complex than the simplistic two amino acid to one base contacting scheme.

Puf proteins have high conservation of amino acids at the key RNA-binding positions, yet different Puf proteins attain distinct RNA binding specificities, especially in those organisms that contain multiple Puf proteins, each with a unique RNA target set. Insight into the mechanism of such specificity has been achieved through mutational and crystal structure analysis of additional Puf family members. In C. elegans, the nine expressed Puf proteins can be divided into four clusters of related proteins based on the similarity of their RNA binding domains: FBF-1/2; PUF-3/11; PUF-5/6/7; and PUF-8/9 [108]. Each cluster of proteins appears to contain structural features that promote distinct target specificities. For example, both FBF and PUF-8 bind an RNA target site containing UGU at the 5’ end and AUA at the 3’ end. However, PUF-8 binds an 8 base target using a consecutive one base to one repeat unit arrangement, while FBF binds a 9 base target [109]. Through RNA target screening and mutational analysis, it was hypothesized that the non-conserved 5th base of the FBF target sequence is flipped out from the protein binding surface in order to accommodate a structural distortion between repeats five and six of the protein [109]. Crystal structure analysis of FBF confirmed this flipped base hypothesis and demonstrated that FBF is less curved than HsPUM1 due to a flattened surface in the central repeat region (Figure 1.3B, E; [110]). This flattened surface requires flipping of the central bases in the mRNA target in order for the 5’ UGU and 3’ AU elements to bind FBF properly (Figure 1.3E). Thus, one mechanism of promoting specificity of binding is the formation of unique surface curvatures in Puf proteins that may induce flipping of bases out from the RNA target sequence, even when the amino
Figure 1.3. Puf protein-mRNA binding schemes. (Reprinted from [1]). Puf proteins bind recognition elements in either a one base to one repeat modular-manner or by inclusion of spacer/flipped bases. A) Co-crystal structure of the canonical human PUM1-RD bound to a Nanos Response Element (NRE) from *Drosophila hunchback* mRNA (Reprinted from [106]) B) Co-crystal structure of *C. elegans* FBF-RD bound to a Puf recognition element (PRE) in *gld-1*. Arrow denotes flipped RNA base that does not interact with FBF amino acids. (Reprinted from [110]) C) Co-crystal structure of yeast Puf4RDp bound to the HO PRE. Arrow denotes flipped RNA base that does not interact with Puf4p amino acids. (Reprinted from [111]) D) Co-crystal structure of yeast Puf3RDp bound to a PRE in *COX17*. Arrow denotes novel interaction between an upstream cytosine base and amino acids of Puf repeat 8’. (Reprinted from [102]) E) Binding interactions between RNA bases and amino acids of PufRDs. Conserved interactions are represented in black. Amino acid-RNA base interactions and spacer/flipped bases that are unique to each Puf protein are indicated by color: *C. elegans* FBF (red), human PUM1 (gold), yeast Puf4p (green), and yeast Puf3p (orange). (Reprinted from [110])
acids that contact RNA are nearly identical between Pufs. Moreover, it is hypothesized that flipped bases may be points of interaction with Puf protein cofactors, and the nature of the flipped base could promote unique protein complexes to form on different mRNA targets [110].

In addition to the FBF and PUF-8 clusters of *C. elegans* Puf proteins, the other clusters of Pufs appear to utilize further variations of the flipped base mechanism of RNA target recognition. The PUF-5/6/7 proteins bind a unique 10 base RNA binding site that contains UGU sequences at each end [108]. It is proposed that one or more bases between the UGU sequences are flipped out and act as spacers between the specific RNA contact points [108]. PUF-11 takes RNA recognition variability to the extreme, and is able to bind three types of RNA binding sites, each with the core UGU element located different distances from a downstream AU element [112]. In one RNA site class, PUF-11 acts like PUF-8 and binds consecutive nucleotides with each of its repeat domain units. In the other two classes, an extra non-conserved base is located between the UGU and AU elements in the binding sites, either at +4 or +5 relative to the UGU, and is proposed to flip out from the Puf binding surface [112]. To accommodate these distinct binding sites, it is hypothesized that PUF-11 can change its curvature. The alternative Puf structures on different Puf target sites may allow differential recruitment of cofactors, resulting in multiple regulatory mechanisms for even a single type of Puf protein [112]. Additional support for a single Puf protein able to bind different types of target sites comes from crystal structures of HsPUM1 bound to 9 base, noncognate RNA sequences [113]. While PUM1 typically uses a one base to one repeat unit recognition scheme, PUM1 was able to bind 9 base target sites by flipping out the extra base. Noncanonical
bases within the binding site were also accommodated by alternative hydrogen bonds with PUM1. Together, these mechanisms of promiscuity may explain the large number of RNAs with variable sequences that appear to be targets of Puf protein regulation [113].

The ability to attain unique RNA binding specificities has also been elucidated for two of the six Puf proteins in the yeast *S. cerevisiae*. Consensus binding sequences for yeast Pufs 3, 4 and 5, which were compiled from sets of RNAs that physically associate with these Pufs, contain 8, 9, or 10 bases, respectively (Figure 1.2; [35]). Crystal structures of Puf4p revealed that like FBF, its flattened surface accommodates an extra base between the conserved 5’ UGUA and 3’ UA sequences in the Puf4p target sequence. Specifically, an extra base at position +7 is flipped out, while another non-conserved base at position +5 lacks hydrogen bond contacts with repeat 4 (Figure 1.3C, E; [111]). From these results, it is thought that any non-conserved position within an RNA target sequence is likely flipped out from the binding surface, or at least not making base-specific contacts with the Puf protein. Thus for Puf5p, it is predicted that one or more bases are flipped out from the 10 base recognition sequence [111]. In contrast to Puf4p, the crystal structure of Puf3p demonstrates the simple one base to one repeat unit binding scheme, with an overall curvature similar to PUM1 (Figure 1.3A, D; [102]). However, the unique feature of the Puf3p structure is a binding pocket located between repeats 8 and 8’ that accommodates a cytosine at the -2 position from the core UGU element (Figure 1.3D, E). This cytosine is not only well conserved in Puf3p RNA target sequences [35], but is required for high affinity binding of RNA to Puf3p in vitro, and regulation of RNA stability by Puf3p in vivo [102]. Crystal structures of PUM1 and Puf4p indicate that the positioning and identity of amino acids in these proteins would
prevent a binding pocket from forming, and therefore the cytosine binding pocket in Puf3p is a key determinant of RNA target specificity [102].

An auxiliary mechanism of differential RNA regulation by Puf proteins was suggested by structural studies of PUM1 binding to the Drosophila mRNA binding sequence termed the Nanos Response Element (NRE) [114]. The NRE contain two consecutive UUGU Puf binding sites separated by only 6 bases. Modeling of its molecular structure based on analytical ultracentrifugation suggests a circular structure of two Puf proteins bound to the consecutive sites [114]. Other mRNAs targeted by Puf proteins contain either single Puf binding sites or multiple binding sites at increased distances from each other. It is therefore hypothesized that Puf proteins form different topographies on their mRNA targets depending on the spacing between sites, and these alternative structures might allow differential recruitment of cofactors to the mRNAs [114].

**MECHANISMS OF RNA REPRESSION THROUGH PROTEIN INTERACTIONS**

There are several possibilities for how Puf proteins repress an mRNA target. Pufs could simply be scaffolds for binding other proteins that actually perform the function of repressing the RNA, or Puf proteins themselves could either block translation factor binding or recruit mRNA decay machinery. Pufs could also alter mRNP structure in such a way to increase access to mRNA decay factors or prevent translational complexes from forming. Evaluation of the mechanisms of Puf protein action across eukaryotes has revealed that all of the above possibilities are valid, even within a single organism.
**Puf Interactions with Protein Partners**

Puf proteins in higher eukaryotes typically mediate repression of their mRNA targets via interactions with additional cofactors such as Brat and the RNA-binding proteins Nanos, CPEB, DAZ, DAZL, and BOL. The requirement for these protein partners appears to be organism and transcript-specific, as homologs of such protein partners are not present in *S. cerevisiae*. Nanos homologs have been identified in *Drosophila*, humans, *C. elegans*, and *Xenopus*, and are required for the repression of several mRNA targets (Figure 1.4A-D). The formation of Nanos/Puf protein complexes may or may not require interactions with the target transcript, as Nanos/Puf complex formation in *Xenopus*, *C. elegans*, and humans is RNA-independent [17, 36, 70], while complex formation in *Drosophila* requires the *hunchback* NRE [63]. Moreover, Nanos proteins interact specifically [26] or non-specifically [17, 36, 62, 63, 70] with the mRNA target while directly interacting with the Puf repeat domain region to maintain stable complexes [63, 104]. In *Drosophila*, translational repression of some mRNAs, such as *hunchback*, are dependent on the formation of quaternary Brat/Nanos/Pumilio/mRNA complexes [48, 63, 64], while other Puf complexes, such as on *cyclin B*, exclude Brat (Figure 1.4A, B; [26, 64]). In *Drosophila* neurons, Brat, which functions in neuronal differentiation, is included or excluded in Puf repression complexes depending on the type of neuron [56]. Alternatively, in *Xenopus*, Puf repression complexes on *cyclin B1* mRNA include the Nanos homolog Xcat-2, Pum1, and cytoplasmic polyadenylation element-binding (CPEB) proteins (Figure 1.4D; [17, 44, 73, 115]). Thus, the translation of *Xenopus cyclin B1* versus *Drosophila cyclin B* is repressed via different mechanisms.
(Figure 1.4B, D), resulting from the formation of mRNP complexes containing different Puf protein partners.

Human PUM2 interacts with several Puf protein partners, including the Deleted in Azoosperma (DAZ), Deleted in Azoosperma-Like (DAZL), and the BOULE (BOL) RNA-binding protein family, which regulates germ cell maintenance, development, and differentiation in eukaryotes. Human DAZL interacts with mRNAs involved in growth regulation and spermatogenesis [116], and PUM2 and DAZL can form a complex with the NRE sequence from *Drosophila* Pumilio targets as detected by a yeast three-hybrid assay [39]. However, while PUM2 and DAZL specifically bind cis-elements in the human *SDAD1* 3’UTR as detected by *in vitro* gel shift assays [116], PUM2 and DAZL cannot form a stable complex on the *SDAD1* 3’UTR as detected by a yeast three-hybrid assay. Alternatively, BOL and PUM2 can form stable complexes with both the *Drosophila* NRE and *SDAD1* 3’UTR sequences via yeast three-hybrid detection [77]. Although such complexes have not been shown to regulate these mRNAs, the observations imply that regulation of human mRNA targets may require different protein partners and complex formations than determined for *Drosophila* Pumilio. The recruitment of PUM2 protein partners and stable complex formation on a target may be regulated by the nature of the PUM2-protein partner interactions, as DAZL association with PUM2 requires repeats 2, 4 and 8, while BOL interaction may involve repeat 1 or a region outside of the repeat domain [77]. DAZ also interacts with the PUM2RD, and this association minimally requires PUM2 repeat 8 as determined by analysis of PUM2 truncation mutants. However, the exact regions of PUM2 required for this interaction have not been fully elucidated [39].
Clearly, Puf protein partners such as Nanos, DAZ, DAZL, and BOL make important structural interactions with Puf repeat domains to elicit regulatory responses. However, the interactions occurring between the protein partner and the mRNA target tend to vary among organisms and targeted transcripts. The specificity of these interactions on a particular mRNA may largely be due to the ability of the protein partner to bind the RNA itself and/or to recognize specific Puf conformations such as protein curvature or flipped bases that are dependent on the sequence of the transcript. Stabilization of these translationally repressive mRNP complexes is thus due to the overall topography of the mRNPs. Moreover, the formation of different Puf protein complexes can promote different mechanisms of translational repression and RNA decay.

**Transcript and mRNP-Specific Mechanisms of RNA Repression**

As described above, the mechanisms involved in translational repression and decay of *hunchback* and *cyclin B* mRNAs in *Drosophila* are induced by the formation of different mRNP complexes. As shown in Figure 1.4A, Pumilio binds specifically to the NRE of *hunchback* via the repeat domain [10, 20, 21, 60, 61] and recruits Nanos to this complex via interactions with an outer loop structure between repeats 7 and 8 [63, 104]. Interactions between Nanos and *hunchback* are mediated by the C-terminal CCHC motif of Nanos and the mRNA [62, 63]. Brat is recruited to the Nanos/Pumilio/*hunchback* tertiary complex by interactions between the NHL domain of Brat and the outer loop structure of Pumilio [64, 104, 117]. The Brat/Nanos/Pumilio complex can facilitate both translational repression and deadenylation of *hunchback* mRNA via two distinct mechanisms. Brat may repress *hunchback* mRNA by binding the eIF4E-like translational inhibitor d4EHP [118], which competes with eIF4E for interaction with the 5’ cap.
Alternatively, Pumilio and Nanos can promote deadenylation of *hunchback* [48], by recruiting decay machinery to the transcript.

In contrast, Pumilio-mediated repression of *cyclin B* mRNA requires interactions with Nanos, while Brat is dispensable (Figure 1.4B; [26, 64]). In this case, the sequence of the target mRNA may dictate the conformation of the Nanos/Pumilio/RNA complex, which inhibits Brat binding [64]. Once Pumilio recruits Nanos to the transcript, Nanos subsequently recruits the NOT4 subunit of the CCR4-POP2-NOT deadenylase complex to the transcript [26]. Pumilio also associates with POP2 [26], so it is hypothesized that the entire CCR4-POP2-NOT complex is recruited to the mRNA. Deadenylation of the mRNA by this complex could inhibit translation and/or promote further degradation.

Distinct from the mechanisms involved in the translational repression of *Drosophila* Pumilio targets, translational repression of *C. elegans* *fem-3* mRNA may employ the action of multiple NANOS proteins. *C. elegans* FBF binds to the *fem-3* 3'UTR [11, 71] and recruits NANOS-3 to the transcript. The central portion of NANOS-3 interacts with the FBF-1 repeat domain, while NANOS-3 binds *fem-3* mRNA non-specifically (Figure 1.4C; [70]). The formation of this tertiary NANOS-3/FBF-1/*fem-3* complex promotes repression of *fem-3*. NANOS-1 and NANOS-2 also contribute to the spermatogenesis to oogenesis switch, but through an unknown mechanism not involving *fem-3* or FBF-1 binding [70]. The mechanism of repression by FBF likely involves deadenylation of the target mRNA, as FBF interacts with CCF-1, the *C. elegans* homolog of Pop2p, and complexes of FBF with yeast Pop2p stimulate deadenylation in vitro [46]. It is proposed that FBF also represses *gld-1* mRNA through CCF-1 recruitment and
Figure 1.4. Mechanisms of Puf protein-mediated mRNA repression and decay. (Reprinted from [1]).
Puf-mediated repression of mRNA targets requires Nanos, Brat, CPEB and/or other protein partners on a transcript-specific basis. A) Model of *Drosophila hunchback* translational repression. For simplicity, only one NRE is shown. Repression of *hunchback* mRNA requires the formation of a quaternary complex including *hunchback* mRNA, Pumilio (green arc), Nanos (gold rectangle) and Brat (blue triangle) proteins [64]. Brat interacts with the cap binding protein d4EHP, which in turn may disrupt eIF4E-cap interactions and prevent translation initiation [118]. B) Model of *Drosophila cyclin B* translational repression. Repression of *cyclin B* mRNA requires the formation of a ternary complex including *cyclin B* mRNA, Pumilio (green arc), and Nanos (gold rectangle) proteins [64]. Nanos interacts with NOT4 [26], while Pumilio associates with POP2, and these interactions likely recruit the entire CCR4-POP2-NOT deadenylase complex to the mRNA [26]. C) Model of *C. elegans fem-3* translational repression. Repression of *fem-3* mRNA requires the formation of a ternary complex including *fem-3* mRNA, FBF (green arc) and NANOS-3 (gold rectangle) proteins [11, 70]. FBF interacts with the POP2 homolog CCF-1 *in vitro* to stimulate deadenylation of *gld-1* mRNA [46], and this deadenylation mechanism is likely to occur with *fem-3* mRNA as well. D) Model of *Xenopus cyclin B1* translational repression. For simplicity, only one cytoplasmic polyadenylation element (CPE) is represented. Repression of *cyclin B1* mRNA requires the formation of a large complex including *hunchback* mRNA, Pumilio1 (green arc), Xcat-2 (gold rectangle), and CPEB (purple) proteins [17, 44, 73]. Small light blue circles represent poly(A)-binding proteins to which eIF4G binds. Pumilio1 binds UGUR elements and interacts with CPEB to stabilize its interactions with the transcript via CPE sequences. CPEB interacts with Maskin (tan ellipse), which prevents eIF4E-eIF4G interactions, thus inhibiting translation initiation [119]. Arrowheads in models denote stimulation of deadenylation.
deadenylation [46]. In contrast, FBF may act to upregulate gld-1 mRNA in certain cells by interaction with the GLD-2 poly(A) polymerase [46].

Translational repression of the maternal cyclin B1 mRNA in Xenopus oocytes involves interactions with CPEB, the Nanos homolog Xcat-2, and Pumilio1 (XPum1) (Figure 1.4D; [17, 44, 73, 115]). The XPum1RD binds cyclin B1 UGUR elements [17] and physically interacts with unphosphorylated CPEB proteins [73], which are implicated as the major regulators of cyclin B1 translational repression and activation. In immature oocytes, cyclin B1 is stored in a translationally repressed form, harboring a short poly(A) tail. This repression is mediated, in part, by unphosphorylated CPEB proteins that bind cytoplasmic polyadenylation elements (CPE) in the 3’UTR [17, 44, 73]. CPEB proteins interact with maskin protein, which in turn binds eIF4E to prevent its association with eIF4G. Such inhibition prevents the recruitment of the 40s subunit, resulting in translational repression [119]. XPum1 enhances CPEB-mediated repression [44, 73]. However, multiple Puf binding sites are necessary to mediate repression enhancement [44]. Although XPum1’s role in repression has not been elucidated, it is hypothesized that XPum1 may either temporally regulate cyclin B1 translational activation, function to promote a short poly(A) tail length [73], or enhance repression by stabilizing CPEB proteins on cyclin B1 [1, 44].” The latter hypothesis is more plausible, as phosphorylation of Xenopus Pum1 is coupled with the dissociation of CPEB-Pum1 binding interactions, resulting in translational activation of cyclin B1 mRNA [115]. “Consistent with a hypothesized role in deadenylation, XPum1 interacts with the Nanos homolog Xcat-2 [17]. Based on the roles of the Drosophila Pumilio-Nanos complexes, Xcat-2 may recruit the deadenylation machinery to cyclin B1, as overexpression of
XPum1 results in *cyclin B1* transcripts with short poly(A) tails similar to that observed in immature oocytes [73].

In contrast, *Xenopus* Pum2 represses the translation of the RINGO/Spy mRNA by interacting with two UGUR elements within the 3’UTR. Repression by XPum2 may require DAZL proteins, since DAZL co-immunoprecipitates with Pum2, although DAZL’s potential role has not been determined [74]. Alternatively, XPum2 is thought to prevent translational activation of the RINGO/Spy mRNA by competing with eIF4E for interaction with the mRNA cap (Figure 1.5A). Interactions between Pum2 and a cap analog require a critical tryptophan residue in the Pum2 N-terminus [120], which defines a unique function of the Puf protein N-terminal region [1].”

Human Puf proteins can also indirectly promote translational repression of mRNA targets in somatic cells in conjunction with miRNAs. As shown in Figure 1.5A, the 3’UTR of p27 mRNA contains a miR-221/222 recognition element (miRNA RE) downstream of the PUM1 recognition element. When p27 is translationally active, the PUM1 and miR-221/222 elements may form a stem loop that renders the miR-221/222 RE inaccessible to the microRNAs. Notably, PUM1 is not phosphorylated and has a reduced affinity for its binding site. When PUM1 is phosphorylated, its RNA-binding activity is turned on, and subsequent interaction with its binding site stimulates a conformational change in the p27 3’UTR that now renders the miRNA RE accessible for miR-221/222 binding. Together, these interactions allow translational repression or decay of p27 by miRNAs (Figure 1.5B; [121]).
“General Mechanisms of mRNA Repression

Disruption of mRNA interactions with translation initiation machinery appears to be a conserved mechanism of Puf-mediated repression across organisms. Yeast Puf6p represses the translation of \textit{ASH1} mRNA either by interacting with the nonessential translation factor Fun12p/eIF5B or by competing for interactions with other translation initiation factors. Furthermore, \textit{in vitro} translation assays reveal that Puf6p prevents the formation of the 80S ribosome complex during translation (Figure 1.6B; [122]).

Recruitment of mRNA decay machinery is another conserved mechanism of Puf-mediated repression of mRNA targets. Similar to the recruitment of the CCR4-POP2-NOT deadenylase complex by the \textit{Drosophila} Pumilio-Nanos complex [26], \textit{S. cerevisiae} Puf proteins recruit and directly bind Pop2p, which bridges interactions between the Puf and Ccr4p, and presumably the NOT complex (Figure 1.6C; [83-85, 123]). Recruitment of Ccr4p, the catalytic subunit of the deadenylase complex, by Puf3p, Puf4p or Puf5p results in deadenylation of the mRNA target [83-85, 123]. Binding of Puf proteins to Pop2p is also conserved in \textit{C. elegans} and humans [26, 46, 83], implicating the importance of this mechanism of mRNA stability control by Pufs.

Mechanisms of RNA repression that do not require Ccr4p and/or Pop2p have also been identified in yeast. For Puf3p, deadenylation can not only be stimulated through the recruitment of Ccr4p, but also through a Ccr4p-independent mechanism that may involve alterations in the poly(A)-binding protein (PAB1)-mRNP structure [123]. Furthermore, while the Puf4p-Pop2p complex requires recruitment of Ccr4p for repression of the \textit{HO} mRNA target, Puf5p is able to repress \textit{HO} mRNA even in the absence of Ccr4p [83-85]. An explanation for this activity may at least partly derive from the binding of Puf5p-
Pop2p to the decapping factor Dcp1p and the Dhh1p helicase [83-85]. Similar decay factor recruitment is seen in *T. cruzi*, where PUF6 immunoprecipitates with the Dhh1 helicase [43]. From these interactions, the subsequent removal of the 5’ cap and probable remodeling of the mRNP structural confirmation likely aids in the repression and degradation of the transcript, though interactions with translation initiation factors may also play a role in repression. In fact, Puf5p-mediated translational repression of *HO* mRNA has been replicated *in vitro*, and the observed decrease in *HO* mRNA levels was independent of a mRNA decay mechanism [124]. Puf5p may even act through a Pop2p-independent mechanism, as appears to be the case for its role in regulating DNA replication stress, and such a mechanism may involve mRNA localization for co-regulated translation [125]. Thus, a single Puf protein may utilize multiple different mechanisms to achieve fine-tuned regulatory control over a target mRNA, though some mechanisms may be target-specific [1].”

**RNA Repression, Processing Bodies and Stress Granules**

The processes of translational repression and mRNA decay are spatially controlled within the cells of various eukaryotes such as mammals, yeast, and trypanosomes, as non-translating mRNAs and associated decay factors are sequestered into cytoplasmic foci termed processing bodies (P-bodies) [126]. These aggregates have been studied extensively in yeast, and are dynamic structures that can serve as temporary storage sites for pools of non-translating mRNAs such that mRNP aggregates can disassemble and associate with polysomes for reentry into translation (Figure 1.7; ([127]; reviewed in [126, 128]). Alternatively, P-bodies are also the terminal destination for mRNAs that are targeted for decay. While P-bodies are constantly present within cells
Figure 1.5. Roles of PUM1 in microRNA-mediated regulation of mRNAs [121]. Human PUM1-p27 mRNA binding allows post-transcriptional regulation of p27 by miRNAs. The PUM1 recognition element is represented as UGUR, and the miR-221/222 recognition element is represented as miRNA RE. A) When PUM1 is inactive and p27 is translationally active, the PUM1 and miR-221/222 elements may form a stem loop that renders the miR-221/222 RE inaccessible to microRNAs. miRNAs sequence is hypothetically represented by GACUGCAAC sequence and does not represent the actual miRNA sequence that regulates p27 mRNA. B) When PUM1 is phosphorylated, its RNA-binding activity is turned on, resulting in disruption of the stem loop to allow post-transcriptional regulation of p27 by a miRNA.
Figure 1.6. Translational repression and decay mechanisms through direct interactions with Puf proteins. (Reprinted from [1]). A) XPum2 prevents translational activation of the RINGO/Spy mRNA by competing with eIF4E for interaction with the mRNA cap [120]. B) Yeast Puf6p represses the translation of ASHI mRNA by either interacting with the translation factor Fun12p/eIF5B or by competing with it for interactions with other translation initiation factors. Puf6p also prevents the formation of the 80s ribosome complex [122]. C) Yeast Pufs directly recruit the Pop2p subunit of the Ccr4p-Pop2p-Notp deadenylase complex, which in turn binds Ccr4p and presumably the rest of the Not complex. The Puf protein also recruits Dcp1p, which cleaves the 5’ cap, and Dhh1, a regulator of mRNA degradation [83-85]. After deadenylation and decapping of the transcript, the 5’→3’ exonuclease Xrn1p rapidly degrades the mRNA [132].
[129], they are often difficult to observe under the microscope, as they are small in size. Optimal microscopic visualization of P-bodies requires subjecting yeast cells to stressful cellular conditions that block translation or decay such as glucose deprivation, osmotic stress, high cell density and diauxic shift, ultraviolet light, mutations in translation initiation machinery [129], deletion of decay factors [130] or microtubule disruption [131]. For example, stresses such as glucose deprivation have been shown to greatly increase P-body size [129], thus enhancing their detection using confocal microscopy.

P-bodies are characterized by the presence of mRNPs containing the decapping enzyme subunits Dcp1p/Dcp2p and the Xrn1p exonuclease [130]. Notably, ribosomal proteins [129], translation factors [129], murine and human Puf proteins [29, 40], and the deadenylase subunit Ccr4p [130], which catalyzes the first step of the major decay pathway in yeast, are excluded from P-bodies [130]. Therefore, the association of Puf3p and the deadenylation complex to a transcript targeted for decay may precede P-body formation. mRNA degradation in P-bodies likely occurs during the steps that follow deadenylation, such as decapping and rapid exonucleolytic digestion.

While yeast Puf3p was initially reported to be excluded from P-bodies in glucose deprivation conditions [130], Puf3p has recently been shown to colocalize with P-bodies after glucose depletion [133]. It is possible that some Puf proteins could be components of P-bodies. Several decay factors that compose P-bodies contain glutamine and asparagine-rich domains that enhance P-body accumulation [134]. Similarly, Puf proteins also contain glutamine-rich domains that may allow the formation of protein aggregates [12, 14, 27-29]. Alternatively, mouse PUM2 and human PUM1 are excluded
from P-bodies [29, 40]. Instead, mammalian PUM proteins [29, 40] along with microRNAs [135], colocalize with stress granules.

Stress granules are storage sites for non-translating mRNPs that are distinct from P-bodies. In contrast to P-bodies, stress granule formation requires a cellular stress that compromises translation initiation. Furthermore, stress granules contain mRNAs bound by translation initiation and termination factors, unlike P-bodies (Figure 1.7 stress granule mRNPs; [135, 136]). In yeast, stress granules form from existing mRNP aggregates found within P-bodies [136]. In contrast, mammalian stress granules can form independently from P-bodies [135].

As shown in Figure 1.7, mRNAs can cycle between polysomes, P-bodies, and stress granules or be terminally sequestered to P-bodies for mRNA decay. Specifically, a pool of translationally repressed mRNAs and associated decay factors, possibly including Puf proteins, form P-bodies where the transcripts can be degraded. Alternatively, the non-translating mRNAs within the P-body can undergo mRNP remodeling to form a stress granule. During the remodeling process, the transcript-associated decay factors dissociate and the mRNAs acquire translation initiation factors to prime for reentry into translation [136]. This transition from P-body to stress granule formation is likely initiated or controlled by an unknown mRNA binding protein. When cellular conditions are favorable, the stress granules can disassemble, and the translationally silent mRNAs associate with the complete translation initiation complex and polysomes to execute several rounds of translation. In mammals, translationally repressed mRNPs including PUM proteins can aggregate into stress granules independently of P-body formation.
Figure 1.7 Model of mRNA movement through states of translation, P-bodies and stress granules in the cytoplasm. (Reprinted and modified from [136]). In yeast, translationally repressed mRNAs associate with decay factors and aggregate to form P-bodies, which may or may not include Puf proteins. The mRNPs can be degraded in the P-body or undergo mRNP remodeling where the mRNAs exchange decay factors for translation initiation factors and form stress granules. In order to reenter translation, stress granules disassemble and the mRNPs associate with additional translation factors to form the preinitiation complex. In mammals, mRNPs can directly form stress granules.
“REGULATION OF PUF PROTEIN ACTIVITY

With most Puf proteins regulating groups of functionally related mRNAs, it seems logical that such mRNAs should not be repressed at all times in the cell. Instead, such mRNAs are likely expressed in response to certain cellular or environmental signals, then are repressed under other cellular conditions. A simple model to attain differential repression of these mRNAs is to alter the activity of the Puf proteins under the different conditions. In fact, eukaryotes have developed a variety of mechanisms to modulate Puf protein expression and the ability of Puf proteins to interact with and regulate mRNA targets. Puf expression and activity are controlled at every level of gene expression: transcription, post-transcription, translation, and post-translation, suggesting that Puf protein activity is tightly regulated.

It is hypothesized that *C. elegans* has developed an autoregulatory mechanism for post-transcriptionally regulating *fhl-1* and *fhl-2* mRNA levels via binding of the FBF proteins to their 3’ UTRs. In *fhl-1* mutants, an increase in FBF-2 levels is observed. Similarly, FBF-1 levels are increased in *fhl-2* mutants. This hypothesis is further supported by the presence of FBF binding elements in the 3’UTRs of *fhl* mRNAs. These findings suggest that FBF-1 and FBF-2 proteins may negatively regulate the expression of their own transcripts, as well as each other’s (Figure 1.8A; [79]). Pum1 and Pum2 mRNAs associate with affinity purified human Pum1 protein [40], suggesting that the mechanism of Puf autoregulation is also conserved in humans.

As documented for rat PUM2, Puf expression can also be inhibited post-transcriptionally by microRNA interactions (Figure 1.8B; [137]). miR-134 promotes dendritic outgrowth of rat hippocampal neurons by repressing translation of PUM2
mRNA. The 3’UTRs of PUM2 homologs in rat, mouse, and human contain conserved miR-134 binding sites, suggesting that microRNA-mediated repression of Puf proteins may be a conserved mechanism in vertebrates [137].

In addition to altering levels of Puf proteins, Puf activity can be altered by post-translational modifications [1].” Phosphorylation of *Xenopus* Pum1 is coupled with the dissociation of CPEB-Pum1 binding interactions, resulting in translational activation of *cyclin B1* mRNA [115]. “In budding yeast, Puf6p represses translation of *ASH1* mRNA and assists in its asymmetrical localization within the daughter cell bud tip [22]. N-terminal phosphorylation of Puf6p by protein kinase CK2 relieves translational repression of *ASH1* mRNA, indicating that in this case, phosphorylation turns off Puf activity (Figure 1.4C; [122]) [1].” In contrast, phosphorylation of human PUM1 enhances RNA-binding activity, suggesting that phosphorylation can also turn on Puf activity [121]. “In further support of post-translational modification, *Xenopus* Pum1 is phosphorylated during oocyte maturation, suggesting that post-translational modifications may also control the stability of mRNP complexes [73].

Another common mechanism of inhibiting Puf activity is inhibition of Puf-mRNA interactions. The trans-acting factors GLD3, Bam, and Bgcn proteins in *C. elegans* and *Drosophila* bind Puf proteins and counteract their repressive activity (Figure 1.8D; [138, 139]). *C. elegans* GLD-3 binds specifically to the FBF repeat domain *in vitro* via an RNA-independent manner. Yeast three-hybrid analysis demonstrated that GLD3 impedes binding interactions between FBF and the *fem-3* mRNA 3’UTR [138]. In *Drosophila*, Bam and Bgcn form a ternary complex with the N-terminal domain of Pumilio (not containing the repeat region). Bam directly interacts with the N-terminal
Figure 1.8 Regulation of Puf protein activity. (Reprinted from [1]). A) PUF mRNAs can autoregulate their protein levels. In this model of fhl mRNA post-translational regulation, FBF-1 or FBF-2 (green arc) may bind to the FBF recognition element (UGUN) in the 3’UTR of fhl-1 or fhl-2 mRNA to promote deadenylation and inhibit translation [79]. B) PUF mRNAs can be regulated post-transcriptionally by the miRNA regulatory system. A miRNA (red RNA bases) may bind to the 3’UTR of PUF mRNA (green RNA bases), resulting in translational repression or degradation [137]. C) Puf protein activity can be negatively regulated post-translationally. Phosphorylation of yeast Puf6p can turn off its activity [122]. D) Puf-mediated translational repression can be inhibited by disrupting interactions with the mRNA target [138, 139]. In this model, Bam or GLD3 (yellow circle labeled X) interacts with the Puf protein (green arc) and prevents it from binding the Puf recognition element in the 3’UTR of the targeted transcript. E) Puf protein-mRNA interactions and activity are regulated by environmental stimuli. In Xenopus oocytes, progesterone disrupts Pum2 interaction with its mRNA target [74]. Yeast Puf3p-mediated mRNA decay occurs in the presence of dextrose, but its activity is inhibited in the presence of ethanol [23, 91, 140]. F) Puf proteins can form aggregates mediated by the Q-rich domain (modeled as green barbells with Q-rich and repeat domains) [27], which potentially prevent or promote Puf-mediated regulation of mRNA targets [56, 74]. These aggregates could be sequestered from the mRNA target, preventing binding interactions and alleviating RNA repression.
region of Pumilio, and this binding inhibits the Puf repeat domain-dependent repressive activity [139]. It is possible that the interactions between Pufs and inhibitory factors simply block binding of the Puf to mRNA or other cofactors. Alternatively, binding may result in structural changes to the Puf that abolish mRNA or protein binding. Similarly, mutations to the outer surface of the Puf3RD inhibit binding to its mRNA target, likely through structural changes [13].

Environmental factors can also regulate Puf protein levels or function. *Dictyostelium* PufA represses PKA-C mRNA and inhibits cell differentiation. However, during starvation the YakA kinase represses PufA mRNA levels, thus alleviating the repression of PKA-C mRNA [141]. Additionally, Puf protein levels can be regulated by cell signaling pathways, as FBF-2 protein levels in *C. elegans* are altered by GLP-1/Notch signaling [79]. Hormones can inhibit Puf protein-mRNA interactions, as *Xenopus* Pum2 dissociates from its target RINGO/Spy mRNA in oocytes following progesterone treatment, which stimulates oocyte maturation (Figure 1.8E; [74]). This destabilization of Pum2-RINGO/Spy mRNA complexes allows translation of the transcript [74]. In yeast, the presence of different carbon sources can positively or negatively regulate Puf activity (Figure 1.8E). Yeast Puf3p, which regulates mitochondrial biogenesis and function [34, 35], promotes rapid degradation of mRNAs involved in mitochondrial function, such as *COX17*, in the fermentable sugar dextrose [23, 91, 140]. However, in ethanol, a non-fermentable carbon source that stimulates mitochondrial biogenesis [34], Puf3p is inactivated. This allows Puf3p target mRNAs such as *COX17* to be stabilized for increased protein production and mitochondrial function [140].
Control of Puf protein activity may involve the formation of protein aggregates. Analysis of glutamine/asparagine (Q/N)-rich domains in *Drosophila* Pumilio suggests that these domains may autoregulate Pumilio activity. Expression of the Pumilio Q/N-rich domain facilitates the formation of prion-like protein aggregates in yeast and disturbs endogenous postsynaptic Pumilio activity in *Drosophila* neuromuscular junctions (Figure 1.8F; [27]). It is hypothesized that the Q/N-rich domain of *Drosophila* Pumilio may have a dominant-negative effect by disrupting Q/N-dependent Pumilio-protein interactions that are required for translational repression of mRNA targets [27]. Thus far, *Drosophila* Pumilio has been shown to bind the 3'UTR of eIF4E mRNA and repress eIF4E accumulation in the neuromuscular junction [38]. Mammalian PUM2 has also been shown to repress the translation of eIF4E and *scn1a* mRNAs in hippocampal neurons [58]. However, the requirement of Q/N rich domains for the repression of these neuronal transcripts is not clear. Injection of *Xenopus* oocytes with a truncated Pum2 protein containing the N-terminal region results in the translational activation of a RINGO/Spy mRNA reporter, implying that the Pum2 N-terminus has a dominant negative effect on the activity of endogenous Pum2 [74]. However, this model conflicts with the observation that Q-rich domains in mammalian Pum2 induce the formation of stress granules in neurons [29], suggesting that these Q-rich domains in mammals do not inhibit Puf activity, considering that stress granules contain translationally repressed mRNAs. In *Drosophila* motoneurons, Pumilio is required to reduce *paralytic* expression. The PumilioRD is not sufficient to downregulate *paralytic* mRNA expression as determined by real-time RT-PCR, indicating that sequences outside of the PumilioRD are required for translational repression [56]. It is possible that the Q/N motifs in Pumilio are
important for this activity. Overall, Q/N motifs can regulate Puf activity, positively or negatively; however, the proper conditions or stimuli that disrupt or enhance potential Pumilio Q/N interactions with other factors must be examined [1].”

**PREVIOUS STUDIES OF YEAST Puf3p**

Prior to my M.S. thesis research, Puf3p was identified as a post-transcriptional regulator of a single mRNA, *COX17* [91], and the putative function of Puf3p was ascertained primarily from computational analyses and Puf3p tandem affinity purification assays [35, 140]. Global analysis of Puf3p-mRNA interactions revealed that Puf3p physically associated with 162 predicted Puf3p mRNA targets that were nuclear-transcribed and encode mitochondrial proteins [35], including *COX17* [142, 143]. In an effort to experimentally validate these mRNAs as bona fide targets of Puf3p-mediated decay during my thesis work, I monitored the stability of 15 putative Puf3p target transcripts in wild-type (WT) and *puf3Δ* strains, thus identifying 10 new mRNA targets of Puf3p. *COX17* mRNA was used as a positive control in these experiments [144]. These results demonstrated that Puf3p plays a global role in mitochondrial function through its regulation of this class of functionally related transcripts. My results also suggested that both microarray and computational analyses may overestimate the number of Puf3p targets, possibly due to the identification of false positives or mRNAs that associate with Puf3p for a purpose other than regulation of decay.

Computational analysis of publicly available microarray datasets revealed that the steady-state levels of mitochondrial mRNAs containing the Puf3p binding element are coordinately expressed in response to ~750 environmental stresses, including heat shock, starvation, and carbon source [145]. Specifically, the analyses predicted that steady-state
expression of putative Puf3p targets is downregulated in repressing carbon source conditions (i.e. dextrose), and upregulated in non-repressing conditions (i.e. galactose, raffinose and ethanol) [140].

To address this prediction, I selected two of the transcripts that I newly identified as targets of Puf3p-mediated decay, CYT2 and TUF1, as well as COX17 to analyze changes in stabilities under different carbon source conditions. I monitored the decay of these mRNAs in wild-type and puf3Δ strains grown in dextrose, ethanol, galactose, and raffinose conditions (Figure 1.9A, B and C; [144]). Growth in ethanol caused all three mRNAs to decay with extended half-lives that were similar between wild-type (WT) and puf3Δ strains, demonstrating complete inactivation of Puf3p activity in this carbon source (Figure 1.9A). Galactose was the only one of the four tested that did not induce complete inhibition of Puf3p activity on all three mRNA reporters (Figure 1.9B). Thus, galactose severely inhibits Puf3p activity, but target mRNAs are not affected equally by the residual Puf3p activity in this carbon source. Like ethanol, raffinose appears to completely inactivate Puf3p for all three mRNAs tested (Figure 1.9C). In the presence of ethanol or raffinose, the mRNA targets are stabilized, with their extended half-lives indistinguishable between wild-type and puf3Δ strains, except for CYT2 in raffinose. The stabilization of CYT2 mRNA in puf3Δ-raffinose conditions is likely due to the presence of multiple AU-rich elements within the 3’UTR that are known to stabilize mRNAs only in certain carbon sources due to differential protein binding [151]. Specifically, I hypothesized that in the absence of Puf3p in raffinose conditions, an unidentified trans-acting factor could bind the AU-rich element within the CYT2 3’UTR to exert its stabilizing effect. To ensure that the stabilization of CYT2, TUF1, and COX17 mRNAs in
ethanol, galactose and raffinose conditions is due to altered Puf3p activity and not indirect effects of the carbon sources, we also monitored the decay of STE3 mRNA, a highly unstable transcript that is not regulated by Puf3p (Figure 1.9D; [144]). I found no stabilization due to galactose or raffinose, and only a minor stabilizing effect by ethanol, which was expected based on publicly available microarray data [145]. The stability of a different mRNA that is not regulated by Puf3p, MFA2 mRNA, is not altered in ethanol conditions [140]. Therefore, the observed stabilization of Puf3p targets in non-fermentable carbon sources is not due to a global effect, but reflects changes in Puf3p activity [140]. Together, the decay analysis of CYT2, TUF1, and COX17 mRNAs in different environmental conditions suggest that Puf3p-mediated decay activity is condition-specific wherein Puf3p activity is severely inhibited or abolished in ethanol, galactose, and raffinose conditions.

Next, I examined the kinetics of Puf3p activation and inactivation by changing the carbon source. In these experiments, TUF1 and CYT2 mRNAs were used as reporters to analyze Puf3p activity [144]. To assess how quickly Puf3p decay activity could be activated, wild-type yeast were grown in galactose or raffinose (inhibitors of Puf3p activity) to log phase then switched to dextrose 10 or 2 minutes before transcriptional repression. Experiments were also performed to determine how quickly Puf3p could be inactivated, in which wild-type yeast were grown in dextrose (activator of Puf3p activity) to log phase then switched to galactose or raffinose 10 or 2 minutes before transcriptional repression. Transcripts exhibiting short mRNA half-lives similar to that observed in continuous dextrose growth conditions would indicate that Puf3p activity is stimulated. Alternatively, transcripts that exhibit long mRNA half-lives similar to that observed in
Figure 1.9. Puf3p-mediated decay of CYT2, TUF1, and COX17 mRNAs is conditionally regulated. Shown are graphical representations of the average half-lives of CYT2, TUF1, and COX17 mRNAs from a wild-type (WT) strain or a puf3Δ strain grown in media supplemented with dextrose (dex), A) ethanol (eth), B) galactose (gal), or C) raffinose (raff). The average half-lives of STE3 mRNA were determined from a wild-type strain. Minutes after transcriptional repression are noted below each set of experiments, and the mRNA half-lives with SEM are determined from a minimum of 3 experiments.
continuous galactose or raffinose growth conditions would indicate that Puf3p activity is inhibited.

For both mRNAs and all carbon sources tested, Puf3p could be activated (Figure 1.10) or inactivated (Figure 1.11) in 2 minutes, with full activation or inhibition decay regulation occurring within 10 minutes [144]. Together, these experiments demonstrated that the status of Puf3p activity is rapidly altered simply by changing the available carbon source.

Together, the data obtained from my previous studies demonstrates that Puf3p regulates a specific class of nuclear-transcribed mitochondrial mRNAs. These transcripts are stabilized in certain environmental conditions, including the presence of ethanol, galactose and raffinose. Moreover, these changes in mRNA stability reflect alterations to Puf3p-mediated decay activity as well, such that Puf3p activity is turned on in dextrose conditions, but is severely inhibited or inactivated in galactose, raffinose and ethanol conditions. Finally, Puf3p activity can be rapidly turned on or off by changing the available carbon source. These results are consistent with the phenomenon of dextrose repression in yeast. In this fermentable carbon source, glycolysis is performed and mitochondrial respiration is not required. Thus, dextrose triggers the repression of genes involved in several aspects of mitochondrial adenosine triphosphate (ATP) production, such as mitochondrial electron export and oxidative phosphorylation [146-149]. My work implicates Puf3p as an additional level of mitochondrial regulation in response to the available carbon source. Specifically Puf3p is active in dextrose to mediate rapid degradation of its target mRNAs, thereby downregulating mitochondrial protein production. Alternatively, when yeast cells require additional processing of non-
Figure 1.10. Puf3p can be rapidly activated within 2 minutes. (Reprinted from [144]). A, B, and C) Wild-type (WT) or puf3Δ strains were grown continuously in galactose or raffinose followed by a 2 minute incubation in dextrose (Puf3p Activating Conditions) prior to transcriptional repression. A minimum of 3 northern blot analyses of CYT2 and TUF1 mRNAs were performed, and the average mRNA half-lives with SEM were determined.
Figure 1.11. Puf3p can be rapidly inactivated within 2 minutes. (Reprinted from [144]). A, B, and C) Wild-type (WT) or puf3Δ strains were grown continuously in dextrose followed by a 2 minute incubation in galactose or raffinose (Puf3p Inactivating Conditions) prior to transcriptional repression. A minimum of 3 northern blot analyses of CYT2 and TUF1 mRNAs were performed, and the average mRNA half-lives with SEM were determined.
repressing carbon sources for metabolic functions and therefore increased production of mitochondria, Puf3p is inactivated so that target mRNAs are stabilized and the translation of nuclear-encoded mitochondrial mRNAs is upregulated.

**DISSERTATION OVERVIEW**

The research presented in this dissertation was performed to understand the molecular pathways that regulate mRNA-specific rates of decay. This work is a continuation of my M.S. thesis where I identified 10 new mRNA targets of yeast Puf3p-mediated decay and identified environmental factors that affect the activity of Puf proteins. In this dissertation, I present work that focuses on identifying the molecular switch that regulates Puf3p activity, and determining the aspect of Puf3p function that is altered when Puf3p activity is inhibited. In chapter III, I focused on elucidating the molecular basis of yeast Puf3p inactivation by identifying altered Puf3p-decay factor and Puf3p-mRNA target interactions in conditions that inactivate Puf3p. I also present Puf3p localization studies, in which I examined the possibilities that Puf3p inactivation might result from the formation of Puf3p aggregates or altered localization of Puf3p to P-bodies or mitochondria. In Chapter IV, I present research that examines novel functions of Puf3p relating to translational efficiency of one of its mRNA targets, *COX17*. Chapter V presents a summary of the research conducted in this dissertation, including models for Puf3p regulation and function, and recommendations for future studies.
REFERENCES


CHAPTER II: GENERAL METHODOLOGY
Chapter II presents a general overview of the experimental methods performed to address the dissertation research questions that are presented in Chapter III and IV. Additionally, all of the yeast strains, plasmids, and oligonucleotides used in this dissertation are presented in Tables 2.1, 2.2, and 2.3, respectively. Moreover, this chapter provides detailed explanations about specific methods, including the rationale for performing the experiment, a synopsis of how the experiment is performed, and interpretations of hypothetical results. A more detailed account of each experimental protocol, including concentrations of buffers and reagents, is explained in the Material and Methods section of its respective chapter (Chapter III or IV).
Table 2.1. Yeast strains used in this dissertation.

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<th>Description</th>
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Table 2.2. Plasmids used in this dissertation.

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owo653  PGK1 SYBR green reverse TTGTCAGGAACCTTGGAGCACCAC
STEADY-STATE TRANSCRIPTIONAL DECAY ANALYSIS

To determine if Puf3p affected the stability of mRNAs, steady-state transcriptional decay analysis was performed in vivo to monitor the decay rate of mRNAs in both wild-type and puf3Δ background strains. mRNAs that are regulated by Puf3p-mediated decay should exhibit differential decay rates in wild-type and puf3Δ strains. Therefore, targeted RNAs are expected to decay rapidly in the presence of Puf3p, and should be stabilized in a puf3Δ strain.

Analysis of endogenous transcripts using temperature-sensitive yeast strains

This experimental procedure was performed as previously described [3, 13]. To analyze the stabilities of endogenous yeast mRNAs, I utilized yeast strains that harbor an rpb1-1 temperature sensitive mutation in RNA polymerase II [13]. These mutants transcribe mRNAs at the permissive temperature of 24°C, and transcription can be shut off by shifting the cultures to the restrictive temperature for the mutation at 37°C (Figure 2.1A). Wild-type and puf3Δ steady-state cultures were grown to log phase at 24°C in rich yeast extract-peptone (YEP) media supplemented with dextrose. Next, the cultures were harvested, pelleted, and resuspended in 37°C YEP-dextrose media to repress transcription. At this time (0 minutes after transcriptional repression), a sample of the cultures were taken, pelleted, aspirated, and stored in dry ice. Additional samples were collected at designated time intervals (Figure 2.1A). The cells collected in each sample were lysed, and total RNA was prepared from each sample by phenol-chloroform extraction and was separated on an agarose gel. The total RNA was transferred and covalently cross-linked to nylon membrane for probing with a radiolabeled oligo. A ~25nt DNA oligo that was complementary to a region near or within the mRNA 3’ UTR
was designed and end labeled using $\gamma^{32}$P ATP to detect the endogenous mRNA. The Northern blots were normalized for total RNA loading using the RNA polymerase III transcript 7S, whose expression is not repressed at 37°.

**Analysis of fusion transcripts expressed from GAL inducible/repressible plasmids**

Alternatively, mRNA decay analysis of transcripts expressed from plasmids was performed using a repressible promoter to control transcription. Specifically, I utilized plasmid pwo25 (Table 2.2) expressing hybrid transcripts in which the 3’UTR of COX17 mRNA was fused to the open reading frame (ORF) of another mRNA that is not affected by Puf3p (Figure 2.1B). It was previously shown that Puf3p interacted with elements located within the COX17 3’UTR to stimulate the decay of this fusion mRNA [8]. Additionally, the chimeric transcript was expressed from the plasmid under the control of the inducible/repressible GAL upstream activating sequence (UAS). Transcription of this mRNA could be induced by the addition of galactose to the media and subsequently repressed by the addition of dextrose (Figure 2.1B). Wild-type and puf3Δ strains transformed with the GAL plasmid were grown to log phase at 30°C in selective synthetic media supplemented with galactose. Next, the cultures were harvested, pelleted, and resuspended in selective synthetic media supplemented with dextrose media to repress transcription. At this time (0 minutes after transcriptional repression), a sample of the cultures were taken, pelleted, aspirated, and stored in dry ice. Additional samples were collected at designated time intervals (Figure 2.1B). The cells collected in each sample were lysed, and total RNA was prepared from each sample by phenol-chloroform extraction and was separated on an agarose gel. The total RNA was transferred and covalently cross-linked to nylon membrane for probing with radiolabeled oligos. A
~25nt DNA oligo that was complementary to the junction of the ORF and the COX17 3’UTR (owo303; Table 2.3) was designed and end labeled using $\gamma^{32}$P ATP to exclusively detect the fusion transcript. The Northern blots were normalized for total RNA loading using the RNA polymerase III transcript 7S. COX17 and 7S RNAs were detected using a phosphorimager.

In Figure 2.1C, the decay analysis of Puf3p target MRPL6 is presented as an example. Time 0 represents the steady-state pool of MRPL6 transcripts that were present at the time of transcriptional repression. At this time, the expression of additional mRNAs was blocked. As the time post-repression increases, the intensity and size of MRPL6 in the wild-type strain (WT) rapidly diminishes, while the intensity and size of MRPL6 in the puf3Δ strain diminishes more slowly. The time at which 50% of the initial pool of MRPL6 at time zero is degraded is noted as the mRNA half-life (T½). Therefore, MRPL6 is rapidly degraded in the presence of Puf3p, given a half live of 2.9 minutes. When Puf3p is deleted, MRPL6 is stabilized >2-fold to a half life of 6.7 minutes, suggesting that Puf3p promotes rapid decay of MRPL6.

STEADY-STATE GENE EXPRESSION ANALYSIS

To determine if PUF3 expression is downregulated in conditions that inhibit its activity (ethanol, galactose and raffinose), wild-type yeast strains were grown in different carbon source conditions to log phase, then steady-state PUF3 mRNA and Puf3p protein levels were assayed. If condition-specific inactivation of Puf3p is the result of decreased transcription or translation, then I would expect that PUF3 mRNA and protein levels would be reduced in ethanol, galactose and raffinose conditions when compared to levels in dextrose.
Figure 2.1. Illustration of steady-state transcriptional shut off analysis. A) Transcriptional shut off experiments utilizing temperature sensitive yeast strains requires shifting the cultures from 24°C to 37°C to repress transcription. B) Transcriptional shut off experiments utilizing a fusion transcript expressed from a GAL inducible/repressible requires shifting the culture from galactose to dextrose to repress transcription. In these experiments, the fusion transcript must be detected by a radiolabeled oligo that is complementary to the ORF-3'UTR junction. C) Shown is an example Northern Blot analysis of Puf3p target MRPL6 mRNA in wild-type (WT) and puf3Δ strains that would result from the transcriptional shut off analysis. Puf3p promotes rapid decay of MRPL6, and the half-lives (T½) of the mRNA in both strains are represented in minutes.
Analysis of mRNA levels

Wild-type yeast strains were grown in YEP media supplemented with dextrose, ethanol, galactose, or raffinose to log phase, as defined by an optical density of 0.4 at a wavelength of 600nm. Cultures were harvested and pelleted, and cells were lysed by mechanical shearing using acid washed glass beads. Total RNA was prepared from each sample by phenol-chloroform extraction and was separated on an agarose gel. The gel was stained with ethidium bromide to detect the 28S and 18S ribosomal RNAs and normalize for total RNA loading. The total RNA was transferred and covalently cross-linked to nylon membrane for probing with a $^{\gamma}$32P end-labeled oligo that was complementary to the coding region of PUF3 mRNA. PUF3 mRNA was detected using a phosphorimager. 7S mRNA was not used as a loading control, as its expression was varied among the different growth conditions.

Analysis of protein levels

Wild-type yeast strains were grown in YEP media supplemented with dextrose, ethanol, galactose, or raffinose to log phase, as defined by an optical density of 0.4 at a wavelength of 600nm. Cultures were harvested, pelleted, and resuspended in a sample buffer containing $\beta$-mercaptoethanol to mimic the reducing conditions in which Puf3p is expressed. Cells were lysed in 2ml tubes by mechanical shearing using acid washed glass beads, and were heated at 100°C to inactivate cellular proteases. A hole was made in the bottom of the sample tubes using a syringe, and the sample tubes were centrifuged in 15ml conical tubes to collect the cell extract. The clear supernatant was collected, and the cell pellet was resuspended in sample buffer in a separate tube. The supernatant and pellet fractions were separated on a denaturing SDS polyacrylamide gel and were
transferred to a nitrocellulose membrane by electroblotting overnight. Protein transfer and total protein loading was detected by Ponceau S staining. The Western Blot was hybridized with anti-Puf3p antibodies produced in rabbit, followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies. To control for loading, the Western Blot was stripped and reprobed with anti-Tfp1p antibodies that were produced in mouse. Cross-reacting proteins were visualized by a secondary reaction with anti-mouse IgG antibodies. Puf3p and Tfp1p were detected by incubating the blot with a chemiluminescent horseradish peroxidase substrate and exposing it to X-ray film.

**CO-IMMUNOPRECIPITATION ASSAYS**

**Analysis of Puf3p and decay factor interactions in vivo**

The condition-specific inactivation of Puf3p in galactose, ethanol and raffinose conditions might be due to an inability to interact with decay factors to promote rapid mRNA decay of its targets. To address this hypothesis, I expressed a FLAG-PUF3RD expression construct (pwo16; Table 2.2) in individually Myc-tagged decay factor strains (Myc-CCR4 (ywo187), Myc-POP2 (ywo191), or Myc-DCP1 (ywo188); Table 2.1) that have a deletion of *PUF3* and performed co-immunoprecipitation assays with FLAG-Puf3RDp from yeast grown in dextrose (Puf activating) versus galactose (Puf inactivating) conditions to ascertain if Puf3p-decay factor binding interactions are disrupted in inactivating conditions. If these interactions were disrupted, I would be unable to detect the Myc-tagged factor with anti-Myc antibodies.

In these experiments, I utilized three *puf3Δ* strains in which the endogenous *CCR4, POP2* and *DCP1* genes were individually Myc-tagged by homologous recombination as previously described (Table 2.1; [18]). Each of the three strains were
transformed with a plasmid that constitutively expressed FLAG-Puf3p or an empty vector that constitutively expressed the FLAG peptide (Figure 2.2 B). Each of the strains expressing FLAG-Puf3p or empty vector were grown in selective synthetic media supplemented with dextrose or galactose to log phase. Cultures were subsequently pelleted and resuspended in immunoprecipitation buffer containing β-mercaptoethanol to mimic the reducing conditions in which the proteins are expressed, Igepal-630 to improve the stringency of the co-immunoprecipitation, and a protease inhibitor cocktail. Cells were lysed using glass beads, and were incubated on ice to prevent protein degradation. Total protein in the cell extracts were quantified by colorimetric assay using Bradford reagent. Equal mg of total protein extract resuspended in equal volumes of IP buffer were incubated with anti-FLAG affinity resin, with rocking to allow FLAG-Puf3p (and any associated Myc-tagged factor) to bind the anti-FLAG antibodies that are conjugated to the resin. (Figure 2.2C) The reaction was washed with a buffer containing detergent to remove any proteins that non-specifically interacted with the resin. FLAG-Puf3p and associated proteins were eluted from the anti-FLAG affinity resin by competition with an excess of the 3X FLAG peptide (Figure 2.2C). The eluates were separated on a denaturing SDS polyacrylamide gel and were transferred to a nitrocellulose membrane by electroblotting overnight. The Western blot was hybridized with anti-Myc antibodies produced in mouse to detect the Myc-tagged decay factor that co-immunoprecipitated with Puf3p, followed by incubation with horseradish peroxidase-conjugated anti-mouse antibodies and chemiluminescent detection. Loading was normalized by loading equal µg of the total protein extracts on denaturing SDS polyacrylamide gels, electroblotting,
**Figure 2.2. Illustration of co-immunoprecipitation assay using Puf3RDp.**

A) Western blot analysis of co-immunoprecipitation assays performed with FLAG-Puf3RDp. Decay factors were detected using anti-Myc antibodies (Sean Houshmandi, unpublished data).

B) In a puf3Δ strain, endogenously Myc-tagged Pop2p is co-expressed with FLAG-Puf3p (from plasmid). Other cellular proteins that do not interact with Puf3p are represented as gray geometric shapes. Yeast cells are lysed to prepare cell extracts containing total protein.

C) Cell extracts are incubated with a FLAG affinity resin that is composed of beads that are covered with anti-FLAG antibodies, which bind the FLAG portion of the FLAG-Puf3p protein. Proteins that do not express the FLAG epitope do not bind the anti-FLAG antibodies, but may non-specifically interact with the bead to which the antibodies are attached. The resin is washed with detergent to remove any proteins that do not interact with Puf3p or non-specifically interact with the resin bead. An excess of a 3X FLAG epitope is added to the resin to compete for binding with the anti-FLAG antibodies attached to the bead, resulting in elution of FLAG-Puf3p and any associating proteins, including Myc-Pop2p.
and detecting Puf3p using anti-Myc primary antibodies made in mouse followed by anti-mouse secondary antibodies.

**Analysis of Puf3p and COX17 mRNA interactions in vivo**

The condition-specific inactivation of Puf3p in galactose, ethanol and raffinose conditions might be due to an inability to bind its RNA targets. To address this hypothesis, I performed in vivo co-immunoprecipitation assays with cells grown in dextrose and galactose conditions that expressed FLAG-Puf3p to determine if COX17 mRNA co-purifies with Puf3p from cell extracts. I isolated RNA from the co-immunoprecipitation eluate, and used this to perform both semi-quantitative and quantitative RT-PCR to amplify COX17 cDNA. If Puf3p RNA-binding activity is reduced in galactose conditions, then I would expect reduced levels of COX17 cDNA binding, when compared to COX17 levels in dextrose conditions. Alternatively, if COX17 cDNA levels in galactose are similar to or greater than COX17 levels in dextrose, then this suggests that Puf3p can still maintain physical interactions with its mRNA targets in inactivating conditions.

**Semi-quantitative RT-PCR analysis**

A puf3Δ strain expressing FLAG-Puf3p or empty vector expressing the FLAG epitope with a Myc-tagged decay factor were grown in selective synthetic media supplemented with dextrose or galactose to log phase (Figure 2.3A). Cultures were subsequently pelleted and resuspended in IP buffer containing β-mercaptoethanol to mimic the reducing conditions in which the proteins are expressed, Igepal-630 to improve the stringency of the co-immunoprecipitation, a protease inhibitor cocktail, and an RNase inhibitor to prevent RNA degradation. Cells were lysed using glass beads, and were
briefly incubated on ice to prevent protein degradation. Total protein in the cell extracts were quantified by colorimetric assay using Bradford reagent. Equal mg of total protein extract resuspended in equal volumes of IP buffer were incubated with anti-FLAG affinity resin, with rocking to allow FLAG-Puf3p (and any associated mRNAs) to bind the anti-FLAG antibodies that are conjugated to the resin. (Figure 2.3B) The reaction was washed with a buffer containing detergent to remove any proteins or RNAs that non-specifically interacted with the resin (Figure 2.3C). FLAG-Puf3p and associated proteins were eluted from the anti-FLAG affinity resin in acidic conditions using glycine-HCl, pH 3.5 (Figure 2.3D). Total RNA was prepared from the co-immunoprecipitations and 10% volume of cell extracts used in the co-IPs by a hot phenol-chloroform extraction, and was precipitated with ethanol and the carrier glycogen. Total cDNA transcription was performed using an poly d(T) primer on DNase-treated total RNA by the addition of a reverse-transcriptase (Figure 2.3E). COX17 cDNA along with the housekeeping gene ACT1 or PGK1, were amplified with gene-specific primers (Figure 2.3F), and gene expression was verified by semi-quantitative PCR analysis visualized on an agarose gel.

**Quantitative Real-time PCR analysis**

A puf3Δ strain expressing FLAG-Puf3p or empty vector expressing the FLAG epitope with a Myc-tagged decay factor were grown in selective synthetic media supplemented with dextrose or galactose to log phase, and were subsequently incubated with formaldehyde to cross-link mRNPs (Figure 2.4A). The cross-linking reaction was quenched with the addition of glycine, and the cultures were pelleted and resuspended in a high stringency RIPA buffer containing β-mercaptoethanol to mimic the reducing
Figure 2.3. Illustration of co-immunoprecipitation assay to analyze Puf3p interactions with COX17 mRNA using semi-quantitative RT-PCR. A) FLAG-Puf3p is expressed from a plasmid in a puf3Δ strain. Other cellular proteins and RNAs that do not interact with Puf3p are represented as gray geometric shapes and lines, respectively. A) Yeast cells are lysed to prepare cell extracts containing total protein. B) Cell extracts are incubated with FLAG affinity resin beads. C) The resin is washed with detergent to remove any proteins or RNAs that do not interact with Puf3p or non-specifically interact with the resin bead. D) An excess of glycine-HCl, pH 3.5 is added to the resin to elute FLAG-Puf3p-target mRNA complexes. E) Total RNA is prepared from the eluate and cDNAs are reverse-transcribed using the mRNAs that co-purified with Puf3p as templates and poly d(T) primer that binds the poly(A) tails of the mRNAs. F) COX17 cDNA, along with an RNA that does not contain any Puf3p binding sites, is PCR amplified with gene specific primers and Taq polymerase. PCR products are visualized on an agarose gel to determine levels of COX17 cDNAs.
Figure 2.4. Illustration of co-immunoprecipitation assay to analyze Puf3p interactions with COX17 mRNA using real-time RT-PCR. A) FLAG-Puf3p is expressed from a plasmid in a puf3Δ strain. Other cellular proteins and RNAs that do not interact with Puf3p are represented as gray geometric shapes and lines, respectively. A) Yeast cells are lysed to prepare cell extracts containing total protein. B) Cell extracts are incubated with FLAG affinity resin beads. C) The resin is washed with detergent to remove any proteins or RNAs that do not interact with Puf3p or non-specifically interact with the resin bead. D) An excess of a 3X FLAG epitope is added to the resin to elute FLAG-Puf3p-target mRNA complexes. E) The eluate is incubated with NaCl to reverse cross-linking, and proteinase K is added to digest FLAG-Puf3p. Digested protein is removed by phenol-chloroform extraction. F) cDNAs are reverse-transcribed using the mRNAs that co-purified with Puf3p as templates and poly d(T) primer and random hexamer primers that bind several regions and the poly(A) tails of the mRNAs. G) COX17 cDNA is PCR amplified with gene specific primers and Taq polymerase included in SYBR green PCR mix.
conditions in which the proteins are expressed, a cocktail of detergents to improve the stringency of the co-immunoprecipitation, a protease inhibitor cocktail, and RNase inhibitor. The co-immunoprecipitation was performed as described for the analysis of Puf3p-decay factor interactions, except that washes were performed with RIPA buffer (Figure 2.4B, C and D). After elution of the mRNPs, the cross-links were reversed by incubating with sodium chloride at 65°C, and FLAG-Puf3p was degraded by incubation with proteinase K (Figure 2.4E).

Total RNA was prepared from the co-immunoprecipitations and 10% volume of cell extracts used in the co-IPs by a hot phenol-chloroform extraction, and further purified using a column to remove contaminating genomic DNA and proteins. Total cDNA transcription was performed using an poly d(T) primer along with random hexamer primers on DNase-treated total RNA by the addition of a reverse-transcriptase (Figure 2.4F). COX17 cDNA along with the housekeeping gene TDH1 (GAPDH), were amplified with gene-specific primers (Figure 2.4G) using SYBR green supermix, which contains SYBR green dye, dNTPs and Taq DNA polymerase. During quantitative real-time PCR analysis, COX17 is synthesized by Taq polymerase using COX17-specific primers while SYBR green intercalates within the dsDNA, and the DNA amplification process can be monitored during each cycle in real-time by excitation of the fluorescent dye. Considering that the thermocycler measures the fluorescence early in the amplification cycle at the point when the fluorescence exceeds background levels, this method prevents plateauing of the COX17 PCR product.
MICROSCOPY OF FLUORESCENTLY LABELED Puf3p

Changes in Puf3p activity may be due to differential localization or aggregation of Puf3p within the cell (Figure 2.5A). Alternatively, Puf3p might asymmetrically localize to the cytoplasmic face of mitochondria as previously described [14] in conditions in which Puf3p is inactive to perform an alternative mRNA shuttling function.

To address the matter of potential Puf3p colocalization with P-bodies or stress granules, I obtained endogenously C-terminal green fluorescent protein (GFP)-tagged PUF3 and GFP-tagged DCP2 strains [4], and utilized Dcp2p-GFP as a marker for the detection of P-bodies. Furthermore, I created a PUF3-RFP expression construct to transform the Dcp2p-GFP strain, which would allow for monitoring of possible Puf3p-Dcp2p colocalization within P-bodies (Figure 2.5B).

Yeast strains transformed with the appropriate plasmid were grown in selective minimal media supplemented with dextrose, galactose or ethanol to log phase. P-body and stress granule formation was induced by depleting the media of the carbon source and resuspending the cells in fresh media lacking the carbon source [15]. Alternatively, P-body formation was induced by osmotic stress by supplementing the media containing the appropriate carbon source with potassium chloride to a final concentration of 1M [15]. Cell suspensions were applied to an agarose pad made with synthetic minimal media supplemented with amino acids and ammonium sulfate. The microscope slide was sealed with a coverslip, and the cells were immediately visualized by confocal microscopy.

To analyze potential Puf3p association with the mitochondria, I obtained a red fluorescent mitochondrial stain that could be used with the PUF3-GFP yeast strain. Yeast
strains were grown in complete minimal media supplemented with dextrose, galactose or ethanol to log phase. Mitotracker Deep Red (Invitrogen) mitochondrial stain was added to the cultures and incubated with shaking to allow cell uptake of the stain (Figure 2.5C). The cultures were washed to remove excess stain in the media, resuspended in fresh complete media supplemented with the appropriate carbon source, and visualized using confocal microscopy.

Under the microscope, yeast mitochondria appear as long tubules that may lie along the periphery of the cell or stretch centrally throughout the cell (Figure 2.5C). Using confocal microscopy techniques, the individual fluorescent proteins and dye are excited at different wavelengths of light by lasers. Therefore, the excitation/emission spectra for GFP would not overlap with that of RFP. In turn, they emit fluorescence at a different wavelength that is detected by the microscope. The visualization of the different proteins or mitochondria are enhanced by false coloring of GFP and RFP as green or red, respectively (Figure 2.5A, B, and C). Therefore, Puf3p colocalization with processing bodies, stress granules, or mitochondria would be visualized as yellow or orange foci that are the products of the overlap of both false colored red and green fluorescence (Figure 2.5B, and C).

**POLYSOME PROFILE ANALYSIS OF COX17 mRNA**

Puf3p may interact with actively translating mRNAs in dextrose conditions to reduce their translational efficiency in preparation for translational repression and its subsequent role to promote decay (Figure 2.6A). Furthermore, Puf3p may enhance the translational efficiency of its mRNA targets in galactose conditions, when Puf3p decay activity is turned off. To address these hypotheses, I performed poly-ribosome
Figure 2.5. Illustration of confocal microscope imaging. Fluorescently-tagged Puf3p is used to analyze Puf3p interactions with P-bodies, stress granules or mitochondria. For simplicity, hypothetical images of P-bodies and mitochondria are represented. Stress granule formation would appear similar to that of P-bodies using the microscope. A) Green fluorescent protein (GFP)-tagged Puf3p is represented by false green coloring, and was previously reported to be diffusely expressed throughout the yeast cytoplasm in dextrose conditions [4]. If Puf3p is forming protein aggregates, as hypothesized in galactose (Gal), ethanol (EtOH), or raffinose (Raf) conditions, it will appear as foci in the cytoplasm. B) Yeast cells grown in media supplemented with dextrose, galactose or ethanol co-express Puf3p-RFP and Dcp2p-GFP, which are false colored red and green, respectively. Dcp2p-GFP is a component of yeast P-bodies, and is used as a marker for P-body formation under osmotic or nutrient deprivation conditions. If Puf3p co-localizes with P-bodies, then the foci will appear yellow colored. Alternatively, if Puf3p does not co-localize with P-bodies, then the Puf3p-RFP and Dcp2p-GFP foci will remain their respective colors. C) Yeast cells expressing Puf3p-GFP and grown in media supplemented with dextrose, galactose or ethanol are incubated with the mitochondrial stain Mitotracker Deep Red. If Puf3p co-localizes with mitochondria, then the foci will appear yellow colored. Alternatively, if Puf3p does not co-localize with mitochondria, then the Puf3p-GFP foci and the mitochondria will remain their respective colors.
(polysome) profiling experiments in \textit{ccr4\textDelta} strains, which inhibits Puf3p-mediated deadenylation-dependent mRNA decay, to observe the translational efficiency of \textit{COX17} mRNA. If Puf3p reduces the translational efficiency of \textit{COX17}, then I would expect that \textit{COX17} mRNAs would be enriched with the ribosomal subunits or single ribosome (monosome) fractions. Alternatively, if Puf3p enhances the translational efficiency of \textit{COX17}, then I would expect that \textit{COX17} mRNAs would be enriched with the polysome fractions.

I performed polysome profiling of cell extracts isolated from dextrose and galactose conditions by sucrose gradient ultracentrifugation and fractionation. To ensure that I analyzed the role of Puf3p in repressing or activating the translation of its mRNA targets independent of its role in decay, I utilized strains that are deleted of \textit{CCR4}, a deadenylation factor (ywo13, Table 2.1; [2]). In a \textit{ccr4\textDelta} strain, all of the mRNAs should be polyadenylated, which will essentially block the major (deadenylation-dependent) mRNA decay pathway in yeast. As a control, I will also perform these experiments using a \textit{ccr4\textDelta puf3\textDelta} strain, in which all Puf3p mRNA targets should be efficiently translated. If \textit{COX17} mRNA is translationally activated by Puf3p in galactose conditions and is translated efficiently, then a pool of these transcripts will associate with polysomes. Conversely, if \textit{COX17} mRNA is translationally repressed or if translational efficiency is reduced by Puf3p in dextrose conditions, then a pool of these transcripts will only associate with the individual 40S and 60 ribosomal subunits or 80S monosome (Figure 2.6C). This data would suggest that Puf3p represses the translation of its mRNAs in dextrose independent of its decay stimulating activities. Moreover, if translational
efficiency is increased in galactose conditions, Puf3p may also enhance the translation of COX17 when its decay-stimulating function is turned off. If the mRNA targets of Puf3p show similar translational efficiencies in the dextrose and galactose conditions, as well as in the ccr4Δpuf3Δ strain, then this suggests that Puf3p does not act directly to repress the translation of its targets. These experiments would also determine if Puf3p-mediated translational repression or enhanced translation is conditionally regulated as well.

puf3Δ or puf3Δccr3Δ strains were grown in rich YEP media supplemented with dextrose or galactose to log phase. The translation elongation inhibitor cycloheximide was not added to the cultures, as it disrupts Puf3p mRNA target-mitochondria interactions [16]. Cultures were pelleted and mechanically lysed using chilled, acid washed glass beads. Cell extracts were incubated with potassium chloride and puromycin to stimulate the release of Puf3p mRNA targets from the mitochondria [16], which associate with the insoluble pellet fraction of the cell extract. Therefore, this treatment allows Puf3p mRNA targets to move to the soluble supernatant fraction of the cell extract. The cell extracts were loaded onto 15% to 50% sucrose gradients and subjected to ultracentrifugation, which separates smaller complexes that include mRNAs bound to 40S or 60S ribosomal subunits, or a single 80S ribosome, from large complexes that include mRNAs bound by multiple ribosomes (polysomes). The smallest complex, mRNA-40S ribosomal subunit, sediments more slowly than the largest complex, an mRNA bound by several polysomes, which sediments the fastest (Figure 2.6B). Fourteen fractions were collected from the sucrose gradient, from the top to the bottom, and each fraction was precipitated with guanidine HCl and ethanol. The fractions were resuspended in Tris buffer, and equal amounts of each fraction were separated on an
agarose gel. The gel was stained with ethidium bromide to detect the abundance of the

Figure 2.6. Illustration of polysome profiling experiment. A) Rationale and experimental design. Experiments are performed in ccr4Δ background strains to monitor Puf3p’s putative function as a regulator of target mRNA translational efficiency independent of its mRNA decay function. In dextrose conditions, when Puf3p decay activity is turned on, Puf3p may also reduce the translational efficiency of its targets prior to promoting decay. Alternatively, in galactose conditions, when Puf3p decay activity is severely inhibited, it is proposed that Puf3p might enhance the translational efficiency of its targets. B) Cell extracts containing mRNAs associate with ribosomal subunits or polysomes are separated on a sucrose gradient. mRNAs that are associated with ribosomal subunits, and are therefore relatively small and light complexes, sediment slowly in the gradient and remain at the top of the gradient. mRNAs that are associated with polysomes, and are therefore relatively heavy and large complexes, sediment quickly in the gradient and migrate toward the bottom of the gradient, depending on the number of associated ribosomes. C) Examples of polysome profile data. In top panel, example of ethidium bromide staining of total complexes without cycloheximide treatment; notice enrichment of mRNAs associated with ribosomal subunits and monosome. In second panel from top, example of ethidium bromide staining of total complexes with cycloheximide treatment; notice accumulation of polysomes in fractions 9-14 (Reprinted from [17]). In third and fourth panels from top, examples of mRNA translational profiles. In third panel from top, mRNA translation is inefficient, as the mRNA is enriched in fractions associated with ribosomal subunits and monosomes (fractions 1-8). In fourth panel from top, mRNA translation is efficient, as mRNA is enriched in fractions associated with ribosomal subunits and monosomes (fractions 9-14).
28S and 18S ribosomal RNAs. Ethidium bromide staining was performed to distinguish the translationally inefficient monosome fractions from the translationally efficient polysome fractions as described below (Figure 2.6C).

The 28S RNA is a component of the large 60S ribosomal subunit, while 18S RNA is a component of the small 40S ribosomal subunit. Therefore, on the ethidium bromide stained gel, the 18S RNA of the 40S subunit would appear first in the fractions that correspond to the top of the gradient (Figure 2.6C). In the next fraction, the 28S RNA of the 60S subunit would appear on the gel. On the gel, the 80S subunit would be represented by an equal abundance of 28S and 18S RNAs. Considering that cycloheximide was not used to trap polysomes on the mRNAs during translation elongation, the polysomes will quickly exit the mRNA as rounds of protein synthesis are completed (Figure 2.6C, top EtBr panel). Furthermore, the polysomes will not be highly visible on the ethidium bromide stained gel. Alternatively, if cycloheximide had been used in these experiments, the polysome fractions would have been represented by an increased abundance of both the 28S and 18S mRNAs (Figure 2.6C, bottom EtBr panel).

To analyze the translational efficiency of COX17 mRNA, the total RNA was transferred and covalently cross-linked to nylon membrane for probing with a $\gamma^{32}$P end-labeled oligo that was complementary to the 3’UTR of COX17 mRNA (owo2; Table 2.3). COX17 mRNA was detected using a phosphorimager. Alternatively, total cDNA transcription of each fraction was performed on DNase-treated total RNA by the addition of a reverse-transcriptase. COX17 cDNA was amplified with gene-specific primers and verified by semi-quantitative PCR analysis visualized on an agarose gel (Figure 2.6C). In either case the Northern blot or agarose gel images could be compared with the ethidium
bromide staining image to assess translational efficiency.
REFERENCES


CHAPTER III:

CONDITION-SPECIFIC Puf3p ACTIVITY IS REGULATED BY Puf3p-DECAY FACTOR INTERACTIONS AND SUBCELLULAR LOCALIZATION
Experimental analyses revealed that Puf3p activity is altered or reduced in the presence of different carbons source conditions [1]. In this chapter, I analyzed the molecular nature of Puf3p inactivation by determining if $PUF3$ expression is reduced in inactivating conditions, or if Puf3p activity is regulated post-translationally. Furthermore, I tested the RNA-binding activity of Puf3p as well as its ability to interact with decay factors under different carbon source conditions. I also analyzed if changes in localization or aggregation affect Puf3p activity. Together, these experiments should help identify the molecular switch that regulates Puf3p activity, and help determine the aspect of Puf3p function that is altered in inactivating conditions.

**ANALYSIS OF CONDITIONAL $PUF3$ EXPRESSION AND PHOSPHORYLATION**

**Changes in Puf3p Condition-Specific Activity Are Not Regulated at the Transcriptional or Translational Levels**

In my M.S. thesis work, I demonstrated that the status of Puf3p activity may be quickly altered by changing the available carbon source. I hypothesized that the observed changes in Puf3p activity upon exposure to different environmental conditions could be due to changes in $PUF3$, either at the transcriptional or translational level. Therefore, the observed inhibition of Puf3p activity upon incubation with galactose, raffinose, and ethanol might be due to downregulation of $PUF3$ expression in these conditions.

To examine this question, I examined steady-state levels of $PUF3$ mRNA and Puf3 protein from wild-type yeast strains grown in rich media supplemented with 2% dextrose, ethanol, galactose or raffinose. An excerpt of these results from my submitted manuscript [1] is presented in the following text, in which F. Lopez Leban and I contributed equally to the experiments. “As shown in Figure 3.1A, steady-state $PUF3$ mRNA levels in ethanol, galactose and raffinose conditions were slightly elevated as
compared to *PUF3* levels in dextrose conditions, suggesting that inhibition of Puf3p activity by ethanol, galactose, and raffinose is not a consequence of reduced transcription.” Recently, these observations were supported by real-time RT-PCR studies performed by an independent lab, in which *PUF3* cDNA levels were increased in ethanol conditions, when compared to dextrose [2].

“Similarly, Puf3 protein levels in ethanol, galactose and raffinose conditions were not decreased as predicted, but were increased with the detection of additional bands corresponding to Puf3p degradation products, especially in the insoluble pellet fractions, when compared to Puf3p levels in dextrose (Figure 3.1B, top panel). To control for loading, we analyzed levels of Tfp1p, a subunit of the vacuolar ATPase V1 domain, which is minimally affected in dextrose, ethanol, galactose, and raffinose conditions (<1.3-fold variation) according to microarray datasets provided at http://genome-www.stanford.edu/yeast_stress/explorer.shtml [3]. Furthermore, the 3’UTR of *TFP1* mRNA does not contain any putative Puf3p binding sites. Western analysis of Tfp1p showed reduced levels in ethanol, galactose and raffinose conditions when compared to dextrose conditions, which is opposite to the pattern of Puf3p levels. (Figure 3.1B, middle panel). Ponceau S total protein staining further controlled for loading, showing a similar pattern to the Tfp1p levels (Figure 3.1B, bottom panel). It is not apparent why Puf3p levels increase under inactivating conditions. However, these results clearly demonstrate that inactivation of Puf3p by ethanol, galactose and raffinose is not accomplished by reduction of transcription or translation in these conditions, but may result from other regulatory mechanisms such as post-translational modification or altered localization [1].”
Figure 3.1. *PUF3* expression is not downregulated at the mRNA or protein level. A) Representative Northern blot analysis of *PUF3* mRNA levels from wild-type cells grown in YEP media supplemented with 2% dextrose, ethanol, galactose, or raffinose is shown with normalized fold changes in expression levels relative to dextrose indicated. Loading of *PUF3* mRNA was normalized to levels of 28S rRNA detected by ethidium bromide staining. B) Puf3p from wild-type cells grown in YEP media supplemented with 2% dextrose, ethanol, galactose, and raffinose was visualized in the top panel by Western blot analysis using antibodies against Puf3p. Western blots were stripped and Tfp1p was visualized in the second panel using antibodies against Tfp1p. Equal OD600 units of cells prior to preparation of protein extracts were calculated for loading onto SDS polyacrylamide gels. Total protein loading was visualized in the bottom panel by Ponceau S staining. In panels A) and B), *PUF3* expression was determined from a minimum of 6 experiments.
The Puf3 Repeat Domain is Phosphorylated in Activating and Inactivating Conditions

Puf protein phosphorylation has been observed in human fibroblasts [7], *Xenopus* oocytes [4], and yeast [5]. These phosphorylation modifications have been shown to either negatively [5, 6] or positively [7] affect Puf protein activity. Computational prediction analysis of yeast Puf3RDp has identified putative sites of phosphorylation as well. Specifically, NetPhos 2.0 was first used to predict the locations of phosphorylated residues on the Puf3RD. Additionally, an alignment of the repeat domains of Puf3p, Puf4p, and Puf5p was created, since Puf4p and Puf5p are well-conserved with Puf3p and have been shown to also be conditionally regulated. From these analyses, seven to nine putative phosphorylation sites were predicted for each Puf, and four of these sites were conserved between at least two of the Puf proteins, suggesting that these residues may have a functional role (Lopez Leban, F. and Olivas, W., personal communication), and may be responsible for regulating Puf3p activity. Based on these observations and computational predictions, I wanted to determine if Puf3p is differentially phosphorylated in activating versus inactivating carbon sources.

To accomplish this, I affinity purified FLAG-tagged Puf3RDp from yeast grown in media supplemented with dextrose or ethanol, and visualized the purified protein by SDS- polyacrylamide gel electrophoresis (SDS-PAGE) and coomassie staining (Figure 3.2A, bottom panel). Additionally, I detected the presence of the ~37kDa FLAG-Puf3RDp using the sensitive total protein stain SYPRO ruby (Figure 3.2A, red boxes in middle panel). After verifying that the FLAG-Puf3RDp immunoprecipitation was successful, I stained an additional Western gel with Pro-Q Diamond phosphoprotein stain, which adheres to and detects phosphate groups that are attached to serine, threonine or tyrosine
residues. Furthermore, Pro-Q Diamond is sensitive, as it can detect ng amounts of phosphorylated protein in the gel bands, and the intensity of the stained bands is directly correlated with the number of phosphate groups attached to the protein. Additionally, the phosphoprotein marker Peppermint Stick was used as a control for the detection of phosphorylated proteins with Pro-Q Diamond, as this marker includes phosphorylated ovalbumin and β-casein, with molecular weights of 45kDa and 23.6 kDa, respectively (Figure 3.2A, top panel). Interestingly, Puf3RDp, which theoretically has a molecular weight around 37kDa, is phosphorylated in both dextrose (activating) and ethanol (inactivating) conditions (Figure 3.2A, top panel, arrow). Unexpectedly, the stain also detected a second larger band in both the dextrose and ethanol conditions. This larger band is likely due to co-immunoprecipitation of proteins that non-specifically bound the anti-FLAG resin or Puf3RDp, as total protein staining with the highly sensitive SYPRO Ruby gel stain revealed that many proteins co-purified with FLAG-Puf3RDp (Figure 3.2A, middle panel). However, FLAG-Puf3RDp could be distinguished from the protein contaminants when compared to the Peppermint Stick molecular weight standards, in which loading of Puf3RDp purified from ethanol conditions may have been greater than that of Puf3RDp-dextrose, as determined by the presence of an additional band that likely corresponded to degraded Puf3RDp (Figure 3.2A, middle panel, red boxes).

Although I could not quantitatively assess the number of phosphorylation groups attached to Puf3RDp-dextrose and Puf3RDp-ethanol, the results of this experiment verified that the Puf3RDp is phosphorylated in both activating and inactivating conditions. I hypothesize that phosphorylation is altered at specific sites in dextrose
Figure 3.2. The Puf3 repeat domain is phosphorylated in activating and inactivating conditions. A) Shown are representative SDS-PAGE gels of affinity purified Puf3p from yeast grown in activating dextrose or inactivating ethanol conditions. Gels are stained for the detection of phosphate groups (Pro-Q Diamond) and total protein (SYPRO Ruby). In the bottom panel, elution of affinity purified Puf3p was detected with coomassie staining of a separate gel. Puf3RDp phosphorylation was determined from 3 experimental replicates. B) Puf3RDp is predicted to be differentially phosphorylated in activating (dextrose) vs. inactivating (ethanol) conditions, where one or a few phosphorylated resides may modulate Puf3RDp activity.
versus ethanol conditions, such that one or a few critical and unique phosphorylation sites are responsible for controlling Puf3p activity (Figure 3.2B).

**ANALYSIS OF Puf3p INTERACTIONS WITH PROTEINS AND mRNA IN INACTIVATING CONDITIONS**

Puf3RD Interactions with Some Decay Factors Appear to Be Altered in Inactivating Conditions

I hypothesized that changes in Puf3p activity might be due to the reduction of Puf3RDp’s binding affinity for the mRNA decay factors in inactivating conditions. For example, translational repression of *hunchback* mRNA is dependent on PumilioRD-mediated recruitment and binding with the Nanos and Brat proteins. Specifically, PumilioRD-protein partner binding interactions are mediated by an outer loop that lies on the convex surface of the RD between repeats 7 and 8, as mutation of this loop disrupts interactions between Pumilio, Nanos and Brat [11-16]. Similarly, yeast Puf3p contains an outer loop between repeats 7 and 8 (Figure 1.3D, [17]; Figure 3.3) that is required for turnover of *COX17* mRNA [18]. Specifically, mutation of this outer loop results in extreme stabilization of *COX17* mRNA, with a half-life identical to that observed in a *puf3Δ* strain, and these mutations do not alter Puf3p’s binding affinity for *COX17* [18].

In previous studies, FLAG-Puf3RDp was shown to interact with the Myc-tagged deadenylation factors Ccr4p and Pop2p, the Dcp1p subunit of the Dcp1p/Dcp2p decapping holoenzyme, and the accessory decay factors Lsm1p and Dhh1p that stimulate decapping [31] in dextrose conditions (Figure 2.2A; Houshmandi, S. and Olivas, W., unpublished data) in an RNA-independent manner (Lopez Leban, F. and Olivas, W. personal communication). As shown in Figure 3.3, Puf3p-mediated decay of *COX17* mRNA involves the assembly of a Puf3p-decay factor complex in which Pop2p directly interacts with the Puf3RDp outer loop between repeats 7 and 8 and bridges interactions
with Dhh1 (Lopez Leban, F. and Olivas, W., personal communication), as determined by co-immunoprecipitation using FLAG-Puf3RDp in a pop2Δ strain or a FLAG-Puf3RD construct harboring mutations in the outer loop region, respectively. Puf3RDp directly interacts with Dcp1p, which interacts with and cleaves the 5’cap, as well as Ccr4p and Lsm1p (Lopez Leban, F and Olivas, W., personal communication). Interestingly, Pop2p did not bridge interactions with Ccr4p. In contrast, translational repression of HO mRNA by yeast Puf5p involves the formation of a complex, in which Puf5p recruits and directly binds Pop2p, which stabilizes with Ccr4p and possibly the Not complex. Other decay factors, such as Dhh1p and Dcp1p are recruited to the mRNA as well, although direct interaction with Puf5p has not been established ([10]; Figure 3.3).

Based on my findings that Puf3p is phosphorylated, I predicted that a differential Puf3p phosphorylation pattern in inactivating conditions could alter the hydrophobicity or energy landscape of Puf3p, thus altering interactions between hydrophobic and hydrophilic amino acid residues and causing a conformational change in Puf3p structure. This change in Puf3p structure could bury the outer loop within the structure and hide decay factor binding sites on the Puf3RD. This hypothesis is supported by computational and crystal structure studies of the phosphorylated activation loop of CDK2 protein, which suggest that phosphorylation alters the structure of the loop and alters amino acid side chain hydrogen bonding interactions in regions surrounding the loop [8]. Moreover, if interactions with Pop2p, Ccr4p, or Dcp1p are disrupted, then I can determine if Puf3p inactivation prevents mRNA decay at the step of deadenylation or decapping, respectively.

Western analysis of affinity purified FLAG-Puf3RDp from cells co-expressing
Figure 3.3. Model for yeast Puf5p and yeast Puf3p translational repression and decay complexes. (Reprinted and modified from [10]). To repress HO mRNA, Puf5p binds to the Puf5p element in the HO 3’UTR and directly binds the Pop2p deadenylase. Pop2p bridges interactions with other decay factors including the deadenylase Ccr4p, Notp complex, and Dhh1p. Dcp1p is also recruited to the transcript, and may mediate interactions with Dhh1p and the Notp complex [10]. To stimulate COX17 mRNA turnover, the Puf3RD binds to two Puf3p elements in the COX17 3’UTR. For simplicity, only one Puf3 protein and binding element are represented. Similarly as for the Puf5p-decay factor complex, Pop2p directly binds the outer loop between repeats 7 and 8 of the Puf3RD. In contrast, Pop2p was only shown to bridge interactions with Dhh1p, while Dcp1p, Lsm1p and Ccr4p directly interact with the Puf3RD (Lopez Leban and Olivas, personal communication).
Myc-Ccr4p, Myc-Pop2p, or Myc-Dcp2p in dextrose and galactose conditions revealed that decay factor interactions are disrupted, albeit to different extents, when Puf3p activity is inhibited. To ensure that the observed changes in FLAG-Puf3RDp-decay factor interactions reflected specific interactions, the co-immunoprecipitation experiments were also conducted with an empty vector (EV) expressing the FLAG peptide alone. Any non-specific interactions would be detected in the empty vector lanes (Figure 3.4A, B, and C, EV lanes, top panels) after detection with anti-Myc antibodies. As shown in Figure 3.4A, B, and C, non-specific interactions were virtually undetectable in the empty vector lanes. Additionally, 25μg of the total protein extracts, which represented 1/80 of the extract amounts used in the actual co-immunoprecipitation experiments, were probed with anti-Myc antibodies to detect the amount of each Myc-tagged protein that was used in the co-immunoprecipitation experiments (Figure 3.4A, B, and C, bottom panels). Levels of each Myc-tagged factor that co-purified with FLAG-Puf3RDp were normalized to extract input loading, and the normalized changes in decay factor abundance were calculated relative to dextrose conditions, where a value of 1.0 represents 100% protein (Figure 3.4A, B, and C, top panels).

The deadenylase factor Ccr4p was detected using anti-Myc antibodies in the eluates of co-immunoprecipitation extracts derived from yeast grown in both dextrose and galactose conditions. Specifically, Ccr4p protein abundance in galactose conditions was 0.7 or 70% of the protein levels detected in dextrose conditions, suggesting that Puf3RDp-Ccr4p interactions may be somewhat compromised when Puf3p is inhibited (Figure 3.4A). In contrast, analysis of Puf3RDp interactions with the deadenylase factor Pop2p revealed that binding was severely inhibited in the Puf3p inactivating galactose
Figure 3.4. Co-immunoprecipitation of decay factors with Puf3RDp. Myc-Ccr4p, Myc-Pop2p, and Myc-Dcp2p were co-immunoprecipitated with Puf3RDp or an empty vector (EV) from yeast protein extracts that were subjected to Dextrose (Puf3p activating) or Galactose (Puf3p inactivating) conditions, in which 1 biological replicate was of each coimmunoprecipitation experiment was performed. Myc-tagged decay factors that co-purified with FLAG-Puf3RDp were detected using anti-Myc antibodies. Loading of Myc-tagged decay factors was determined by using 1/80 of total protein that was loaded onto the anti-FLAG affinity resin for the co-immunoprecipitation experiments, and detecting each decay factor using anti-Myc antibodies (Myc-decay factor input). Levels of each Myc-tagged factor that co-purified with FLAG-Puf3RDp were normalized to extract input loading, and the normalized changes in decay factor abundance were calculated relative to dextrose conditions, where a value of 1.0 represents 100% protein (Figure 3.4A, B, and C, top panels). A) Shown is the antibody detection of Myc-Ccr4p resulting from co-elution with Puf3RDp (top panel), and FLAG-Puf3RDp loading (bottom panel). B) Shown is the antibody detection of Myc-Pop2p resulting from co-elution with Puf3RDp (top panel), and FLAG-Puf3RDp loading (bottom panel). C) Shown is the antibody detection of Myc-Dcp2p resulting from co-elution with Puf3RDp (top panel), and FLAG-Puf3RDp loading (bottom panel).
conditions, as Myc-Pop2p was virtually undetectable (Figure 3.4B). Unexpectedly, I
detected the presence of a band in all of the immunoprecipitation experiments that
migrated just below Myc-Pop2p, which should theoretically have an approximate
molecular weight of 58kDa. This band located below Pop2p most likely corresponded to
the heavy chain of the anti-FLAG antibody attached to the immunoprecipitation resin,
which I previously detected in immunoprecipitation experiments where I used harsh
elution methods such as boiling the resin and using acidic glycine. As shown in Figure
3.4C, interactions between the Puf3RDp and Dcp1p, a subunit of the decapping
holoenzyme that is encoded by DCP1 and DCP2 in yeast, did not appear to be affected
by Puf3p inactivation in galactose conditions.

Together, these experiments demonstrate that interactions between the Puf3 repeat
domain and the deadenylation factors Pop2p and Ccr4p are disrupted in galactose
conditions, albeit to different extents. Interestingly, Puf3RDp-Ccr4p interactions were
only partially disrupted in Puf3p inactivating conditions by co-immunoprecipitation,
while Puf3RDp-Pop2p interactions were virtually eliminated. Considering that Ccr4p
can function as the catalytic domain of a larger deadenylase complex including the
subunit Pop2p [19], it is unlikely that Puf3p would still be able to simulate deadenylation
of its mRNA targets with an incomplete deadenylase complex. Alternatively, Puf3RDp
interactions with the Dcp1p decapping enzyme subunit were maintained when Puf3p is
inactive. Decapping, the second step in mRNA decay performed by the Dcp1p/Dcp2p
complex, is dependent on prior deadenylation of the transcript targeted for decay [1].
Even though Puf3RDp-decapping factor interactions are maintained in the absence of
Puf3p decay activity, mRNA decay would be blocked since deadenylation, the first step
in mRNA decay, would not occur. In other words, although Puf3RDp may still bind Ccr4p and Dcp1p in Puf3p inactivating conditions, their interactions may not be functional to promote RNA decay. However, it is also important to consider that these immunoprecipitation assays were performed in galactose conditions, in which Puf3p harbors some residual activity to slightly destabilize some of its mRNA targets [1]. Biological replicates of these experiments must be performed not only in galactose, but also in ethanol and raffinose conditions to determine if these results are reproducible, and to definitively establish that some Puf3RD-decay interactions are compromised when Puf3p is inactive.

It is likely that phosphorylation of Puf3p might be regulating Puf3p activity, and possibly binding interactions. Specifically, only one-third of proteins in eukaryotic cells are phosphorylated at a given time, and most phosphorylated proteins are heterogeneously phosphorylated [20]. Therefore, the population of FLAG-Puf3RDp purified from cells subjected to galactose conditions (Puf3p inactivating conditions) may include a small fraction of protein that is differentially phosphorylated and in its active form. Therefore, this may account for the maintenance of some Puf3RDp-decay factor interactions in galactose. Alternatively, these binding interactions may be maintained if an unknown protein partner required for Puf3p-mediated stimulation of decay factors is conditionally regulated. Therefore, it is possible that this unknown regulator may still be functional in galactose conditions, thus mediating interactions between Puf3p and the decay factors.
Changes in Puf3p Activity-Dependent Binding to COX17 mRNA Cannot Be Determined by Semi-Quantitative RT-PCR Analysis

The condition-specific regulation of Puf3p may also be altering its ability to bind its RNA targets. In human fibroblasts, PUM1 phosphorylation stimulates its activity and enhances mRNA target binding [7], which might suggest that Puf3p’s affinity for its targets is reduced when its activity is inhibited. Based on these observations with human PUM1, I hypothesized that Puf3RD interactions with one of its mRNA targets, COX17, would be disrupted when Puf3p decay mediating activity is inhibited.

To address this question, I performed co-immunoprecipitation experiments using cell extracts prepared from yeast cells grown in media supplemented with 2% dextrose (Puf3p activator) or 2% galactose (Puf3p inhibitor). For each carbon source condition, FLAG-Puf3RDp or an empty vector (EV) expressing only the FLAG epitope were used as bait in the experiments. Total RNA was extracted from the eluates and used to perform semi-quantitative RT-PCR, in which total cDNA was synthesized using the extracted RNA and reverse transcriptase. COX17 cDNA was subsequently amplified along with a housekeeping gene using Taq polymerase and gene-specific primers. Specifically, I selected several potential housekeeping genes that did not contain any Puf3p binding sites within the 3’UTRs such as PGK1 and ACT1, as well as other genes that were previously identified as yeast RT-PCR controls TPS1, TPS2, TFP1, TUB1, ORC5 [47]. These genes were examined to identify a proper control for loading in the cell extracts and would allow detection of non-specific interactions with an empty vector in the immunoprecipitation experiments. The abundance of COX17 cDNA and the control cDNA that co-purified with FLAG-Puf3RDp were then visualized on an agarose gel.
While many of the cDNAs could not be detected on agarose gels or were amplified along with several non-specific PCR products, two cDNAs, \textit{ACT1} and \textit{PGK1}, were successfully amplified and detected in these experiments. I selected the common housekeeping gene actin (\textit{ACT1}), as it is a component of the cell cytoskeleton and is not regulated by Puf3p. Additionally, I selected phosphoglycerate kinase (\textit{PGK1}) as a housekeeping control, as \textit{PGK1} mRNA stability is not affected by Puf3p. To account for loading errors in the cell extracts and determine the specificity of Puf3p-mRNA interactions in the immunoprecipitation experiments, I amplified \textit{COX17} cDNA along with \textit{ACT1} or \textit{PGK1} cDNAs. In theory, the \textit{ACT1} or \textit{PGK1} controls should only be amplified from total cDNAs synthesized from the cell extracts. Furthermore, if technical errors were not made during these experiments, the size and intensity of \textit{ACT1} or \textit{PGK1} cDNAs should be similar in both carbon source conditions, regardless if Puf3RDp or the EV was expressed. Alternatively, in the co-immunoprecipitation (IP) experiments, \textit{ACT1} or \textit{PGK1} should not be amplified.

As shown in Figure 3.5A, \textit{COX17} cDNA levels were reduced in galactose IP conditions, suggesting that some Puf3RDp-\textit{COX17} mRNA binding interactions are reduced when Puf3p activity is inhibited. Unexpectedly, the control \textit{ACT1} was shown to specifically co-elute with \textit{COX17} mRNA in both dextrose and galactose conditions when Puf3RDp was used as bait (Puf Dex IP and Puf Gal IP). However, Puf3p has been shown to interact with actin-related proteins in conjunction with a role in mitochondrial motility [21]. Therefore, it is possible that actin mRNA could be specifically interacting with Puf3RDp as well for a purpose other than decay regulation. Similarly as for \textit{COX17} cDNA, the abundance of \textit{ACT1} was reduced in galactose conditions, which rendered the
Figure 3.5. Changes in Puf3p Activity-Dependent Binding to COX17 mRNA Cannot Be Determined by Semi-Quantitative RT-PCR Analysis. In panels A) and B), shown are semi-quantitative agarose gels of COX17, ACT1, and PGK1 cDNAs resulting from Puf3RDp or empty vector (EV) co-immunoprecipitations (IP) performed in dextrose (Dex) and galactose (Gal) conditions. To control for loading of mRNAs used in the IP experiments, COX17 and control cDNAs were amplified from cell extracts. A minimum of 3 biological replicates were performed. A) COX17 cDNA and the control ACT1 were amplified from co-IP eluates and cell extracts expressing FLAG-Puf3RDp or an empty vector (EV) expressing FLAG only. DNA-free RNA from the FLAG-Puf3RDp Dex IP experiment was used to amplify COX17 and ACT1 using Taq polymerase (-RT). B) PGK1 was amplified from co-IP eluates and cell extracts expressing FLAG-Puf3RDp or an empty vector (EV) expressing FLAG only. DNA-free RNA from the FLAG-Puf3RDp Dex IP experiment was used to amplify PGK1 using Taq polymerase (-RT). C) Diagram of IP interactions. During IP, ACT1 mRNA specifically interacted with Puf3RDp in Dex and Gal experiments, while PGK1 non-specifically interacted with the anti-FLAG resin. IP experiments were eluted in harsh acidic glycine conditions, which stripped the bead of PGK1 mRNA and other non-specific interacting transcripts.
results of these co-immunoprecipitation experiments inconclusive. Therefore, I was not able to determine if Puf3RDp interactions with COX17 mRNA were disrupted when Puf3p is inactive. To eliminate the possibility that the observed COX17 and ACT1 bands were due to amplification of contaminating genomic DNA, COX17 and ACT1 were amplified from total RNA with Taq DNA polymerase (Figure 3.5A, -RT lane). Neither COX17 nor ACT1 was detected in the minus reverse transcriptase (-RT) lane, demonstrating that only cDNAs were amplified.

To identify a more suitable control cDNA, I next performed the experiments and amplified PGK1, which is not regulated by Puf3p. As shown in Figure 3.5B, PGK1 cDNA amplified in both the IP and extract experiments with equal intensity and abundance. Replicates of experiments performed using PGK1 revealed that PGK1 was detected in the EV Dex IP lane with an intensity equal to that demonstrated in the other experimental and control lanes. However, I could not locate the images that demonstrated this result. These non-specific interactions were likely due to PGK1 interacting non-specifically with the resin (Figure 3.5C). To improve the yield of mRNAs that co-eluted with Puf3RDp and subsequent detection of the cDNAs in these experiments, the Puf3RDp IP complexes were eluted in harsh conditions using glycine-HCl, pH 3.5. I previously performed co-IP experiments with FLAG-Puf3RDp and Myc-tagged decay factors using this elution method, which revealed that the anti-FLAG antibodies were stripped from the IP resin in the presence of acidic glycine, as the heavy and light antibody chains were detected along with the Myc-tagged decay factors. Therefore, I expected that these harsh elution conditions likely eluted every transcript that non-specifically interacted with the resin.
Puf3p-COX17 mRNA Interactions Appear to Be Maintained in Puf3p Inactivating Conditions Using Quantitative Analysis

To eliminate these non-specific interactions and quantitatively assess Puf3RDp-COX17 mRNA interactions in dextrose and galactose conditions, I performed the experiments by crosslinking mRNPs in vivo while the yeast were growing in the respective carbon sources. Therefore, Puf3RDp-COX17 mRNA interactions would be maintained during cell lysis and the IP experiments. To eliminate non-specific RNA interactions with Puf3RDp or the anti-FLAG bead resin, the IP experiments were performed with extremely stringent RIPA buffer containing the detergents SDS and sodium deoxycholate. The mRNPs were eluted gently by competition with an excess of 3X FLAG peptide, and crosslinking was reversed to allow for phenol-chloroform based RNA extraction. To control for loading, I assessed the expression of PGK1 and the yeast homolog of GAPDH (TDH1) in different carbon sources using the publically available yeast microarray data set (http://genome-www.stanford.edu/yeast_stress/explorer.shtml); [3]). The TDH1 mRNA 3’UTR does not contain any Puf3p regulatory elements or any of the minimal UGUA Puf protein binding elements.

As shown in Figure 3.6, expression of ACT1 and PGK1 is repressed about 4-fold in continuous galactose growth conditions when compared to continuous dextrose growth conditions (ACT1 and PGK1, yellow boxes). Furthermore, the extent of ACT1 repression was further increased in continuous ethanol conditions, indicating that it would be difficult to conclude results from IP experiments performed in ethanol conditions. However, TDH1 expression was minimally altered in galactose versus dextrose conditions (Figure 3.6, TDH1 yellow box and arrow), and therefore, was used as the
Figure 3.6. Image of yeast microarray data for continuous carbon source conditions. Modified from ([3]; companion website). Global gene expression microarray for yeast cells subjected to continuous growth in yeast extract peptone (YP) media supplemented with ethanol, galactose, raffinose, sucrose and fructose is shown, and expression levels are compared to continuous YP dextrose conditions. If gene expression levels in the different carbon sources are similar to that of dextrose conditions, then they are represented by black color. However, if gene expression levels in the different carbon sources are increased (induced) relative to dextrose conditions, then they are represented by varying shades of red color. Alternatively, if gene expression levels in the different carbon sources are decreased (repressed) relative to dextrose conditions, then they are represented by varying shades of green color. The fold difference of gene induction or repression in comparison to dextrose conditions are noted in the legend. To perform quantitative analysis of \(COX17\) eluted from Puf3RDp IPs in dextrose versus galactose conditions, the microarray was used to identify an appropriate housekeeping gene that did not display altered expression in galactose conditions. \(ACT1, PGK1,\) and \(TDH1\) (GAPDH) genes are highlighted by blue rectangles, and \(TDH1\) is highlighted in yellow with bold font. In the microarray data, the corresponding YP Galactose vs. YP Dextrose experiments are boxed in yellow, and \(TDH1\) is identified by a yellow arrow. Both \(ACT1\) and \(PGK1\) are represented by green coloring for this experiment, and comparison to the legend reveals that these genes are repressed about 4-fold in comparison to dextrose growth conditions. However, \(TDH1\) is represented by mostly black coloring, indicated that its expression levels are minimally altered by galactose conditions.
housekeeping gene to normalize COX17 expression. To eliminate the possibility of technical errors that are associated with semi-quantitative RT-PCR analysis, I used quantitative real-time PCR to assess Puf3RDp-COX17 mRNA interactions with three technical replicates. Data was normalized, quantitated and generated using Bio-Rad CFX Manager software.

As shown in Figure 3.7A, COX17 expression was statistically similar in dextrose and galactose conditions, suggesting that Puf3RDp-COX17 mRNA interactions are not disrupted when Puf3p is inhibited. These IP results mirrored the pattern of COX17 expression in cell extracts expressing FLAG-Puf3RDp in dextrose and galactose conditions (Figure 3.7B). While this experiment reveals that Puf3p interactions are not altered by inactivating conditions, biological replicates must be performed to conclusively support this finding.

ANALYSIS OF Puf3p SUBCELLULAR LOCALIZATION IN INACTIVATING CONDITIONS

In my condition-specific studies of Puf3p activity and mRNA target stability regulation, I found that the decay rates of COX17 and TUF1 mRNAs in ethanol conditions are identical to those observed in dextrose conditions with a PUF3 deletion strain ([1]; Chapter I, Figure 1.9). It is possible that under ethanol and galactose conditions, Puf3p may be differentially localized or form aggregates so that it may no longer bind to some decay factors or other protein partners in vivo. Alteration of Puf3p localization would not necessarily be exclusive of the possibility that post-translational modification of Puf3p in yeast may inhibit Puf3p decay factor-binding activity in ethanol.
Figure 3.7. Puf3RDp and COX17 mRNA interactions are not altered when Puf3p activity is partially inhibited using quantitative analysis. In panels A) and B), data reflects 3 technical real-time qPCR replicates resulting from a single co-immunoprecipitation experiment. A) Shown are graphical representations of COX17 cDNA levels resulting from Puf3RDp or empty vector (EV) co-immunoprecipitations (IP) performed in dextrose (Dex) and galactose (Gal) conditions. COX17 cDNA and the control TDH1 were amplified from co-IP eluates expressing FLAG-Puf3RDp or an empty vector (EV) expressing FLAG only. COX17 cDNA expression was normalized to TDH1 expression levels in all experiments. B) Shown are graphical representations of COX17 cDNA levels from cell extracts that were used in the IP experiments. COX17 cDNA expression was normalized to TDH1 expression levels in all experiments.
To address this matter, I obtained a GFP-tagged Puf3p strain from Roy Parker, who originally reported that GFP-tagged Puf3p is localized throughout the cytoplasm in dextrose conditions [31]. I visualized the subcellular localization of Puf3p-GFP under dextrose, ethanol, and galactose conditions using epi-fluorescence microscopy to determine if Puf3p subcellular localization and/or aggregation is altered when Puf3p is inactivated in galactose and ethanol conditions. The Dcp2p subunit of the decapping holoenzyme, which is encoded by \( DCP1 \) and \( DCP2 \), has been shown to accumulate in cytoplasmic foci in dextrose conditions, and Dcp2p-GFP serves as a marker for the presence of P-bodies [31]. P-bodies, which function as storage sites for some mRNA decay factors such as Dcp1p/Dcp2p and sites of mRNA decay, are dynamic structures that constantly form and disassemble in the cell cytoplasm. While P-bodies are always present within cells, they are difficult to detect by fluorescence microscopy methods due to their small size. P-body size can be dramatically increased by subjecting cells to different stresses, such as osmotic stress, which improves visualization of P-bodies under the microscope [31].

I increased P-body size and formation as well as possible Puf3p aggregate formation in the respective Dcp2p-GFP and Puf3p-GFP strains by growing the strains in media supplemented with galactose, raffinose, and ethanol, followed by washing with water, and an additional 5 minute incubation in water (Miller, M. and Lopez Leban, F., unpublished data, galactose and raffinose not shown). The strongest Puf3p aggregation phenotype was demonstrated in ethanol conditions, as shown in Figure 3.8. In dextrose and ethanol conditions, about 1 to 2 large Dcp2p foci were detected within the cells, indicative of the formation of P-bodies. Similarly, the subcellular localization of GFP-
Figure 3.8. Epi-fluorescence microscopy of Puf3p-GFP in P-body inducing conditions. Shown are representative epi-fluorescence microscope images of DCP2-GFP (middle panel) and PUF3-GFP (right panel) yeast strains that result from a minimum of 4 biological replicates. Cells expressing Dcp2p-GFP or Puf3p-GFP were grown in dextrose or ethanol to log phase, then briefly incubated in water to induce the formation of processing bodies as previously performed [31]. As a control to establish background levels of auto-fluorescence, a yeast strain lacking genomically integrated PUF3-GFP was also imaged (left panel). The cytoplasmic foci apparent in the Dcp2p-GFP strain are processing bodies. Cells were imaged using a YFP fluorescence filter and a 100X objective.
Puf3p was most severely altered in ethanol conditions, with the appearance of a single cytoplasmic Puf3p focus in many of the cells that contrasted the diffuse cytoplasmic subcellular localization of Puf3p in dextrose conditions (Figure 3.8, red arrows). Unfortunately, the use of water to stimulate P-body formation created enough osmotic stress to kill some of the yeast cells, which auto-fluoresced and created a strong background signal that was not due to GFP excitation in the Puf3p-GFP experiments and was a major limitation of epi-fluorescence microscopy. Furthermore, it was not clear whether Puf3p is localizing to P-bodies in ethanol conditions, or if Puf3p is simply forming protein aggregates.

**Puf3p Aggregate Formation is Stimulated by Puf3p Inactivating Conditions, But Only Occurs in a Small Number of Cells**

Based on preliminary Puf3p-GFP studies, I hypothesized that the observed changes in Puf3p activity may be due to aggregation or differential localization of Puf3p within the cell. Epi-fluorescence imaging of Puf3p localization, after incubating the cells with water, demonstrated that the formation of Puf3p foci that may have been the result of osmotic stress. On the contrary, several lines of evidence support my hypothesis that Puf3p may be forming aggregates in inactivating conditions (galactose, raffinose and ethanol). First, several Puf proteins contain glutamine and asparagine-rich motifs [22-26], which are commonly found in prions, and facilitate the formation of amyloid aggregates in prion diseases. Specifically, Puf3p contains two polyglutamine tracts found at amino acid positions 398-409 and 412-418 just upstream of the first Puf3p repeat at residue 549 (Figure 3.9A). Moreover, the formation of Puf aggregates may be important for controlling Puf activity both negatively [26, 27] and positively [28].
To address the possibility that Puf3p was forming aggregates when it is inactive, I utilized a PUF3-GFP yeast strain for confocal microscopy. Furthermore, I wanted to verify that the addition of the GFP tag to the C-terminus of Puf3p did not alter Puf3p functions. I therefore examined the ability of Puf3p-GFP to stimulate decay of the COX17 3’UTR by performing steady-state transcriptional shut off experiments. I utilized a hybrid mRNA construct in which the 3’UTR of COX17 RNA was fused to the coding region of MFA2, which is not regulated by Puf3p. It has been previously demonstrated that the COX17 3’UTR alone is sufficient to confer Puf3p regulation upon MFA2 [29]. Expression of the MFA2-COX17 3’UTR fusion transcript was regulated by the inducible/repressible GAL upstream activating sequence, in which transcription could be induced by the addition of galactose to the media and subsequently repressed by the addition of glucose. Decay analysis revealed that Puf3p-GFP promoted rapid decay of MFA2-COX17 3’UTR, given a half-life of 5.7 ± 0.6 minutes (Figure 3.9A). This half-life was ~2-fold slower than the previously established 2.5 minute half-life for the transcript with wild-type Puf3p, yet significantly shorter than the 10.5 minute half-life in a puf3Δ strain [30]. While the MFA2-COX17 3’UTR decay analysis using wild-type Puf3p and a puf3Δ strain were performed by another individual (Houshmandi, S., published data [30]), mRNA half-lives typically only differ by 1 minute when experiments are performed by different people (Houshmandi, S., Lopez Leban, F., and Olivas, W., personal communication). Therefore, the presence of the GFP tag only had a slight effect on the functionality of Puf3p.

After determining that Puf3p-GFP was still capable of stimulating decay, I utilized a confocal microscope to image the cells, as it has several advantages over using
an epi-fluorescence microscope. First, the confocal microscope utilizes a pinhole to eliminate out of focus light, thus eliminating false auto-fluorescence signal and allowing me to visualize light emitted by GFP excitation. Second, the confocal microscope greatly improves the optical resolution of the yeast images, along with greater magnification capability. Finally, the confocal microscope has the capability of imaging the yeast cells along multiple focal planes (Z-stacking) and can create a three dimensional reconstruction of the series of images to determine the subcellular localization of Puf3p and P-bodies throughout the cells.

I performed confocal microscopy of a PUF3-GFP yeast strain grown continuously in media supplemented with 2% dextrose, galactose, or ethanol, and observed changes in Puf3p aggregation by taking images of 6-10 focal planes in the yeast cells. In dextrose conditions when Puf3p decay activity is turned on, Puf3p was previously shown to be expressed ubiquitously in the cytoplasm [31]. My analysis of Puf3p in dextrose conditions revealed similar results, in which Puf3p was localized throughout the cytoplasm, with a granular, punctate appearance (Figure 3.9B). About 5% of yeast cells in dextrose conditions contained 1 or more Puf3p foci (Table 3.1). Unexpectedly, I found that the subcellular distribution of Puf3p was not greatly altered in galactose or ethanol conditions that severely inhibit Puf3p activity, as Puf3p was ubiquitously expressed in the vast majority of cells. In galactose conditions, 28% of cells displayed ubiquitous Puf3p expression concomitant with the formation of 1-2 foci Puf3p (Figure 3.9B, white arrows; Table 3.1). Similarly, in ethanol conditions, the majority of yeast cells displayed diffuse cytoplasmic expression of Puf3p. However, 20% of yeast cells also displayed a single aggregate that was larger than the aggregates observed in a
Figure 3.9. Puf3p aggregate size is increased in galactose and ethanol conditions, but only in a small subset of cells.  
A) Shown are the decay analyses of MFA2-COX17 3'UTR mRNA (GAL promoter) from wild-type PUF3, PUF3-GFP, and puf3Δ strain grown in selective synthetic media supplemented with galactose, and then briefly incubated with dextrose. The average half-lives of the transcript in the PUF3-GFP yeast strain and standard error of the mean were determined from two experiments, and are represented graphically. The average half-lives of the transcript using a wild-type PUF3 strain and puf3Δ were determined without standard error of the mean by S. Houshmandi [30] and used to compare to the data from the PUF3-GFP strain.  

B) Shown are representative images of a PUF3-GFP strain grown in synthetic media supplemented with dextrose (left panels), galactose (middle panels) or ethanol (right panels) media. GFP fluorescence was performed using the same laser power in all conditions, with master gain units (image brightness) varying by 31 units at most. Master gain units were increased in dextrose conditions, relative to galactose and ethanol. Scale bars are indicated in the fluorescence images. Three biological replicates were performed for each experiment. For each biological replicate, a minimum of 4 fields was observed under the microscope. The formation of larger, more intense
Puf3p aggregates are denoted by white arrows in galactose and ethanol conditions. In the bottom panel, shown are differential interference contrast (DIC) images of the yeast cells. Large vacuoles are designated by yellow arrows in galactose and ethanol conditions. Images shown were taken from a single focal point or the reconstruction of 6-10 Z-slices using a confocal microscope. C) Diagram of Puf3p protein and location of domains. The polyglutamine repeat domain may be required for Puf3p aggregate formation. Zinc finger is represented as (Zn), while central poly-glutamine repeats are represented as (Q). The repeat domain (RD) is represented as 8 black boxes.

<table>
<thead>
<tr>
<th># of Cells Analyzed</th>
<th># of Biological Replicates</th>
<th>Carbon Source</th>
<th>Puf3p-GFP Aggregates (# green foci)</th>
<th>% Cells Containing 1 or more Puf3p-GFP foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>107</td>
<td>3</td>
<td>Dextrose</td>
<td>5</td>
<td>5%</td>
</tr>
<tr>
<td>101</td>
<td>3</td>
<td>Galactose</td>
<td>28</td>
<td>28%</td>
</tr>
<tr>
<td>103</td>
<td>3</td>
<td>Ethanol</td>
<td>21</td>
<td>20%</td>
</tr>
</tbody>
</table>

The images in Figure 3.9 are representative of the quantitated results in this table.

1 Puf3p-GFP foci were counted in images taken from a 3 biological replicates for the indicated carbon source. The total number of Puf3p foci are indicated, in which a minimum of 101 cells were analyzed.

2 The percentage of cells containing 1 or more Puf3p foci were determined by dividing the total number of cells analyzed by the total number of Puf3p aggregates identified.
small population of cells grown in dextrose and galactose conditions (Figure 3.9B, white arrows). Based on these observations, the subcellular localization of Puf3p is only partially altered in Puf3p activating (dextrose) vs. Puf3p activating conditions (ethanol and galactose), as Puf3p is expressed uniformly in the majority of yeast cells. However, 1-2 punctate Puf3p foci were detected in 5% of the cells subjected to dextrose conditions, whereas ≥ 20% of the cells in galactose and ethanol conditions contained Puf3p aggregates (Table 3.1). The most noticeable differences between the carbon sources were the size of the Puf3p aggregates, in which aggregate size was noticeably increased in ethanol conditions when compared to dextrose and galactose (Figure 3.9B).

I hypothesized that the occasional occurrence of large Puf3p aggregates in ethanol conditions could be a response to cellular stresses stimulated by the presence of ethanol or galactose. Consistent with this hypothesis, ethanol has been shown to alter vacuole morphology, with the formation of a single, large vacuole within a single yeast cell [32]. Similarly, vacuolar morphology was altered in galactose and ethanol conditions, with the presence of large vacuoles as determined by differential interference contrast (DIC) imaging (Figure 3.9B, DIC yellow arrows). In contrast, several very small vacuoles were detected in some yeast subjected to dextrose conditions, and in many of these cells I did not observe any vacuoles (Figure 3.9B, DIC dextrose).

Since Puf3p displays a granular, punctate appearance when it is active in dextrose conditions, this phenotype may be attributed to the polyglutamine repeat within the protein (Figure 3.9C). Together these experiments demonstrate that Puf3p aggregate formation is minimal in yeast cells in all conditions, although the size of aggregate formation is increased in ethanol conditions when Puf3p is inactive. Therefore, it does
not appear that Puf3p aggregation serves as the most prominent mechanism to sequester Puf3p from its mRNA targets or protein partners when Puf3p is inactive.

**Puf3p Colocalizes with P-Bodies in All Conditions, But More Puf3p Aggregates Associate with P-bodies in Inactivating Conditions**

Puf3p may be localizing to P-bodies or stress granules in inactivating conditions. While yeast Puf3p was initially reported to be excluded from P-bodies in glucose deprivation conditions [31], Puf3p has recently been shown to colocalize with P-bodies [33]. The latter observation may be a more accurate account of Puf3p subcellular localization, as several decay factors that compose P-bodies also contain glutamine and asparagine-rich domains that enhance P-body accumulation [34]. Similarly, mammalian stress granule formation also requires the glutamine repeats of Pum2 [25]. In yeast, P-bodies serve as precursors for the formation of stress granules [35]. Thus, it is likely that Puf3p may accumulate in stress granules as well.

The observation that Puf3p-P-body interactions occur in Puf3p activating conditions [33] actually supports the roles of Puf3p in mRNA decay. In dextrose conditions, Puf3p promotes the rapid decay of nuclear-transcribed mitochondrial mRNAs that are involved in several aspects of translation and ATP production inside the mitochondria [1]. For example, these mRNAs are important for upregulation of mitochondrial DNA genes and the formation of mitochondrial enzyme complexes, as they encode subunits of mitochondrial ribosomes, mitochondrial translation elongation factors, cytochrome c oxidase subunits, and subunits of the F1F0 ATPase [1]. Puf3p stimulates the first step of mRNA decay, deadenylation [1, 29], which occurs in the cell cytoplasm, as the catalytic deadenylase subunit Ccr4p is excluded from P-bodies [31]. Puf3p also stimulates the second step of mRNA decay, decapping [1, 29], which is
performed by Dcp1p/Dcp2p in P-bodies. Therefore, I hypothesized that Puf3p is primarily localized in the cell cytoplasm, with a small portion of the protein localized within P-bodies when it is active in dextrose conditions. Alternatively, I hypothesized that when Puf3p is inactive in ethanol and galactose conditions, the majority of Puf3p would sequester itself inside P-bodies for temporary storage to allow translation of its mRNA targets.

To analyze these hypotheses, I grew a PUF3-GFP yeast strain that was transformed with a DCP2-RFP construct in synthetic minimal media supplemented with 2% dextrose, galactose, or ethanol, and induced large P-body formation by washing and incubating the cells with media lacking the respective carbon sources to prevent osmotic cell death as described in [36]. In these confocal microscopy experiments, analysis of a minimum of 123 cells from each carbon source condition revealed that the number of P-bodies were increased in dextrose conditions, when compared to ethanol or galactose or conditions (Table 3.2). Presumably, the increased number of P-bodies in dextrose conditions reflected multiple sites where nuclear-transcribed mitochondrial mRNAs and other non-mitochondrial transcripts that are involved in various cell processes are degraded. In dextrose P-body inducing conditions, Puf3p is expressed ubiquitously in the majority of cells, with the formation of very small Puf3p aggregates within the majority of cells. In P-body inducing dextrose conditions, visually detectable Puf3p foci were prevalent with 55% (69 foci per 125 cells) of cells containing one or more Puf3p foci (Table 3.2; Figure 3.10) In contrast, 5% of yeast that were subjected to continuous dextrose conditions contained 1 or more Puf3p aggregates (Table 3.1; Figure 3.9). Additionally, the cellular stress resulting from large P-body induction greatly increases,
Figure 3.10. Puf3p aggregates asymmetrically localize to P-bodies in both Puf3p activating and inactivating conditions. Shown are representative images of a *PUF3*-GFP strain expressing the P-body marker Dcp2p-RFP grown in synthetic media supplemented with dextrose, ethanol, and galactose. Scale bars are indicated in each image. Large P-body induction was performed by depleting the carbon sources from the media. GFP fluorescence was performed using the same laser power in all conditions. A minimum of 3 biological replicates were performed for each experiment. For each biological replicate, a minimum of 4 fields were observed under the microscope. Puf3p foci and P-bodies that are shown to co-localize are denoted by white arrows. In dextrose conditions, Puf3p foci that are found adjacent to P-bodies are denoted by yellow arrows, and are shown enlarged in the white boxes. Images shown were taken from a single focal point or the reconstruction of 6-10 Z-slices using a confocal microscope.
Table 3.2. Puf aggregate localization to P-bodies.

<table>
<thead>
<tr>
<th># of Cells Analyzed</th>
<th># of Biological Replicates</th>
<th>Carbon Source</th>
<th>Puf3p-GFP Aggregates (# green foci)(^1)</th>
<th>P-bodies (# red foci)(^2)</th>
<th># Colocalized Foci by Merge (# yellow/orange foci)(^3)</th>
<th>% Puf3p Foci Colocalized with P-bodies(^4)</th>
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<tbody>
<tr>
<td>125</td>
<td>3</td>
<td>Dextrose</td>
<td>69</td>
<td>102</td>
<td>43</td>
<td>62%</td>
</tr>
<tr>
<td>126</td>
<td>3</td>
<td>Galactose</td>
<td>49</td>
<td>72</td>
<td>38</td>
<td>77%</td>
</tr>
<tr>
<td>123</td>
<td>4</td>
<td>Ethanol</td>
<td>46</td>
<td>65</td>
<td>37</td>
<td>80%</td>
</tr>
</tbody>
</table>

The images in Figure 3.10 are representative of the quantitated results in this table.

1 Puf3p-GFP foci were counted in images taken from a minimum of 3 biological replicates for the indicated carbon source. The total number of Puf3p foci are indicated, in which a minimum of 123 cells were analyzed.

2 P-body foci (Dcp2p-RFP) were counted in images taken from a minimum of 3 biological replicates for the indicated carbon source. The total number of Dcp2p foci are indicated, in which a minimum of 123 cells were analyzed.

3 Colocalization of Puf3p-GFP and P-bodies (Dcp2p-RFP) were determined by counting yellow/orange foci in the Merge panel (Figure 3.10).

4 Percentage of Puf3p foci that colocalized with P-bodies was determined by dividing the number of “Colocalized Foci by Merge” by the total number of counted “Puf3p-GFP Aggregates” and multiplying by 100.
with the formation of large vacuoles that are represented as black areas in the center of the cells (compare Puf3p-GFP dextrose, Figures 3.9 and 3.10). 3D imaging of Dcp2p revealed the presence of 1 to 2 P-bodies within many of the cells. Imaging of 6-10 focal planes within the cells (Z-stacking) did not reveal the presence of additional P-bodies within the cells. A small pool of Puf3p colocalized with P-bodies in many cells, (Figure 3.10A, dextrose merge, white arrow) suggesting that Puf3p is transported along with target mRNPs to P-bodies, where the mitochondrial transcripts are degraded. Presumably, Puf3p binds the target mRNA and recruits Ccr4p as part of the deadenylase complex, which shortens the poly(A) tail and then dissociates from the mRNP prior to its localization within P-bodies for subsequent decapping and exonucleolytic digestion. Interestingly, Puf3p was found adjacent to P-bodies in some cells (Figure 3.10A, dextrose conditions, yellow arrows and enlarged foci in white box), perhaps demonstrating the transport of Puf3p-bound mitochondrial mRNAs to P-bodies, where the transcripts are decapped and degraded.

In galactose P-body inducing conditions, the number of Puf3p foci were reduced (49 Puf3p foci per 126 cells) when compared to dextrose (69 Puf3p foci per 125 cells) (Table 3.2). However, Puf3p foci in galactose P-body inducing conditions were consistently larger and more pronounced when compared to dextrose conditions (Figure 3.10). The number of P-bodies within cells were also reduced in these conditions as determined by P-body quantitation in over 100 yeast cells (Table 3.2). In contrast to dextrose conditions, a larger percentage of the large Puf3p aggregates colocalized with P-bodies in galactose conditions (77%), given the appearance of several large yellow/orange foci in the galactose merge panel (Figure 3.10, white arrows; Table 3.2).
In ethanol P-body inducing conditions, yeast cells expressed similar numbers of large Puf3p aggregates in comparison to galactose conditions (Table 3.2; Figure 3.10). Additionally, in these conditions, 80% of Puf3p foci colocalized with P-bodies. (Figure 3.10A, ethanol merge, white arrows; Table 3.1).

In Puf3p inactivating/large P-body inducing conditions, Puf3p noticeably formed a single large focus in nearly 40% of cells, which is in contrast to the numerous smaller aggregates shown in dextrose P-body inducing conditions (Figure 3.10, compare Puf3p-GFP dextrose versus galactose and ethanol). In Puf3p inactivating/large P-body inducing conditions, it is possible that the cellular stress resulting from carbon source depletion (large P-body induction) causes the large insoluble Puf3p aggregates to be targeted for decay by autophagy in the vacuole. This hypothesis is supported by the observation that a pool of Puf3p was degraded in insoluble cell fractions of yeast subjected to Puf3p inactivating conditions by Western analysis (Figure 3.1). Autophagy, which is triggered by cell stresses including nutrient starvation and oxidative stress, is a tightly regulated degradative mechanism that is involved in the removal of cytosolic proteins, as well as damaged or obsolete organelles. Furthermore, autophagy mediates cellular homeostasis by modulating the physiological levels of proteins and organelles in response to the metabolic needs of the organism (reviewed in [37]). Analysis of Puf3p in ethanol P-body inducing conditions shows that the Puf3p aggregates are excluded from the vacuoles, eliminating the possibility that Puf3p was being targeted to the vacuole by autophagy of cytoplasmic contents, as determined by merging of Puf3p-GFP and DIC images (Figure 3.11). Alternatively, I hypothesize that the increase of Puf3p degradation in inactivating conditions (Figure 3.1) is mediated by the proteasome. Together these
Figure 3.11 Cellular stresses that result from P-body induction do not target Puf3p aggregates for autophagy in cells depleted of ethanol. Shown are representative Puf3p-GFP fluorescence and corresponding DIC images of yeast cells grown in ethanol conditions that were depleted of the carbon source prior to microscopy. Scale bars are indicated in each panel. Three biological replicates were performed for each experiment. For each biological replicate, a minimum of 4 fields were observed under the microscope. Some Puf3p aggregates are marked with a white arrow in the Puf3p-GFP panel. In the DIC panel, the vacuoles are visualized as large circles within yeast cells. Two vacuoles are denoted by a (v) in the center of the vacuole and a white arrow. Images shown were taken from a single focal point or the reconstruction of 6-10 Z-slices using a confocal microscope.
experiments demonstrate that Puf3p aggregate localization to P-bodies is independent of
the status of Puf3p decay activity, although the size of aggregates is increased when
Puf3p is inactive.

Condition-specific regulation of Puf3p localization may function to regulate
Puf3p accessibility to its mRNA targets or protein partners. Yet Puf3p inactivating
conditions alone do not appear to be the major molecular switch that triggers Puf3p
aggregate formation or altered localization, as Puf3p aggregate foci formation was only
observed in < 30% cells in both activating and inactivating conditions. While Puf3p is
ubiquitously expressed in the cell cytoplasm in dextrose conditions, Puf3p still maintains
a rather granulated appearance, in which tiny aggregates may be forming in Puf3p
activating conditions. It is likely that this Puf3p aggregate phenotype is exacerbated in
conditions that inhibit decay activity. In the inactivating conditions galactose and
ethanol, the formation of larger insoluble Puf3p aggregates may be triggered by the
presence of the polyglutamine tract located in the center of Puf3p.

Additionally, the cellular stress applied to the cells to induce P-body formation
exacerbates the Puf3p foci phenotype, with an increase in both the size and number of
Puf3p aggregates in all conditions. The increased number of Puf3p aggregates in
dextrose P-body inducing conditions may reflect Puf3p-mediated decay of mRNAs
targets that occurs in P-bodies. Alternatively, it is possible that the number of Puf3p
aggregates in dextrose P-body inducing conditions are not actually increased, but that
large P-body inducing conditions may also increase the size of the tiny Puf3p aggregates,
which makes them easier to visualize in the cell cytoplasm. Interestingly, conversion of
the yeast Sup35 protein to its prion form is enhanced by Lsb2p, which forms aggregates
in response to heat shock stress and interacts with the actin cytoskeleton. Furthermore, disruption of Lsb2p-actin interactions prevents the formation of Lsb2p aggregates and abolishes its ability to stimulate prion formation [38]. Similarly, Puf3p, which forms large aggregates in response to stress, also displays interactions with mitochondria-associated actin related protein complexes [21], and actin mRNA in co-immunoprecipitation studies (Figure 3.5A).

In Puf3p inactivating conditions, Puf3p aggregate accumulation in P-bodies may serve as a mechanism to temporarily store pools of inactive Puf3p proteins, thus allowing translation of mitochondrial transcripts. Furthermore, I hypothesize that Puf3p can re-enter the cell cytoplasm in dextrose conditions where decay stimulating activities are required for proper regulation of mitochondrial mRNAs. This mechanism would be similar to the regulation of non-translating mRNAs that are temporarily stored in P-bodies until translation initiation resumes in the cytoplasm [50]. Alternatively, in Puf3p activating conditions, I hypothesize that part of the Puf3p population is transported to P-bodies with targeted mRNAs to facilitate mRNA decapping by Dcp1p/Dcp2p and subsequent degradation.

Recently, researchers have demonstrated that 1M potassium chloride can be added to cells growing in glucose, resulting in large P-body induction without needing to perform carbon source depletion [36]. Based on this knowledge I performed similar experiments in dextrose and ethanol conditions using the genomically integrated PUF3-GFP strain expressing Dcp2p-RFP from a plasmid. Unfortunately, P-body formation of plasmid expressed Dcp2p could not be induced using the potassium chloride method, although a genomically integrated DCP2-GFP strain did produce P-bodies in response to
1M potassium chloride, dextrose conditions. I therefore created and expressed a PUF3-RFP plasmid in the genomically integrated DCP2-GFP strain, but this construct failed to produce stably-expressed Puf3p.

Some Puf3p aggregates Co-localize with Mitochondria in Puf3p Inactivating Conditions

Recently, Puf3p has been implicated as a regulator of mitochondrial biogenesis and function, in which Puf3p interacts with the mitochondrial outer membrane and displays physical and genetic interactions with several mitochondria-associated complexes [21] including protein import [39]. Additionally, Puf3p was shown to facilitate the asymmetric association of nuclear-translated mitochondrial transcripts to the mitochondria in galactose conditions [40]. Based on these observations, an alternative function of Puf3p was hypothesized, in which Puf3p shuttles mitochondrial mRNAs to mitochondria in conditions that turn off Puf3p’s decay stimulating activity (galactose, raffinose and ethanol) thereby upregulating genes involved in mitochondrial respiration and ATP production [41-45], as well as genes involved in translation occurring within the mitochondria [1]. Specifically, Puf3p-mediated localization of mitochondrial mRNAs would place the transcripts in close proximity to the mitochondria, where the mRNAs are translated, and nascent proteins could be imported into mitochondria during biogenesis.

To address this hypothesis, I wanted to determine if Puf3p asymmetrically interacted with mitochondria in ethanol or galactose conditions, when Puf3p-mediated decay activity is inhibited. The PUF3-GFP yeast strain was grown in synthetic complete media supplemented with 2% dextrose or ethanol to log phase, followed by the addition of Mitotracker Deep Red FM mitochondrial stain to the cultures. As shown in Figure
3.12, Puf3p expression was observed diffusely throughout the yeast cytoplasm in dextrose conditions, while mitochondria were observed as tube-like structures that were mainly localized around the periphery of the cell membrane (dextrose panel). Overlapping, or merging, of the two images revealed that Puf3p did not asymmetrically co-localize with mitochondria in dextrose conditions, consistent with Puf3p’s role in cytoplasmic mRNA decay. Unexpectedly, numerous Puf3p foci were identified in ethanol conditions (Figure 3.12; Table 3.3) that exceeded the number of Puf3p foci that were detected in ethanol P-body inducing conditions (compare Tables 3.3 and 3.2). Mitochondrial biogenesis was greatly upregulated in ethanol conditions to the extent that it was difficult to distinguish a single mitochondrion from another. This prevented accurate quantitation of Puf3p aggregate localization with mitochondria. Unexpectedly, merging of a visually optimized image revealed that the vast majority of Puf3p did not appear to asymmetrically co-localize with the numerous mitochondria (Figure 3.12, ethanol panel, white arrow), whereas a small pool of Puf3p aggregates was shown to co-localize with mitochondria in one yeast cell (Figure 3.12, ethanol, yellow arrows).

I also analyzed the cells in galactose conditions, in which yeast cells utilize fermentation, but also require mitochondrial activity to efficiently metabolize this sugar (Johnston, M., personal communication). Therefore, less mitochondrial activity would be required in galactose conditions in comparison to ethanol. Accordingly, I expected that the number of mitochondria would be reduced in galactose conditions, thus allowing more accurate visualization of Puf3p-GFP and mitochondrial localization. Indeed, mitochondrial biogenesis was not upregulated to the extent as previously seen in ethanol.
Figure 3.12. Some Puf3p aggregates co-localize with mitochondria in Puf3p inactivating conditions. Shown are representative images of a PUF3-GFP strain grown in media supplemented with dextrose, ethanol, and galactose media and subjected to mitochondria staining (Mitotracker Deep Red FM). Scale bars are indicated in each panel. Three biological replicates were performed for each experiment. For each biological replicate, a minimum of 4 fields were observed under the microscope. GFP fluorescence was performed using the same laser power in all conditions. Master gain (image brightness) units were decreased in ethanol conditions, relative to dextrose and galactose. Puf3p foci and mitochondria that are shown to co-localize are denoted by yellow arrows, whereas Puf3p foci that do not colocalize with mitochondria are denoted by white arrows. Images shown were taken from a single focal point or the reconstruction of 6-10 Z-slices using a confocal microscope.
<table>
<thead>
<tr>
<th># of Cells Analyzed</th>
<th># of Biological Replicates</th>
<th>Carbon Source</th>
<th>Puf3p-GFP Aggregates (# green foci)</th>
<th># Colocalized Foci by Merge (# yellow/orange foci)</th>
<th>% Puf3p Foci Colocalized with Mitochondria</th>
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<tr>
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<td>Could Not Be Determined</td>
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</table>

The images in Figure 3.12 are representative of the quantitated results in this table.

1 Puf3p-GFP foci were counted in images taken from 3 biological replicates for the indicated carbon source. The total number of Puf3p foci are indicated, in which a minimum of 100 cells were analyzed.

2 Colocalization of Puf3p-GFP and mitochondria in dextrose and galactose conditions were determined by counting yellow/orange foci in the Merge panel (Figure 3.12). Colocalization of Puf3p with mitochondria could not be determined in ethanol conditions due to strong mitochondrial fluorescence.

3 Percentage of Puf3p foci that colocalized with mitochondria was determined by dividing the number of colocalized foci by merge by the total number of counted Puf3p-GFP foci and multiplying by 100.
conditions, as I could identify distinct mitochondria within the cells. Some of the cells expressing Puf3p throughout the cytoplasm also contained 1 to 3 Puf3p foci, while many of the cells only expressed Puf3p ubiquitously. In the cells expressing Puf3p foci, a small pool of Puf3p specifically colocalized with mitochondria (Figure 3.12, galactose panel, small yellow arrows), while the rest of the Puf protein did not (Figure 3.11, galactose panel large white arrows).

Together, the results of these mitochondria localization experiments suggest that some Puf3p foci colocalize with mitochondria when Puf3p is inactive. Specifically, Puf3p-mitochondria colocalization was only observed in 30% of cells subjected to galactose growth conditions, which contradicts the results of previous Puf3p and mitochondria microscopy studies in dextrose conditions [21], in which the entire population of Puf3p-GFP colocalized with mitochondria. The observations from this previous study are counterintuitive, as mRNA decay [1] and microscopy [31] analyses reveal that Puf3p stimulates rapid decay of its mitochondria mRNA targets in the cytoplasm in the presence of dextrose. However, the disparity between my results and the Garcia-Rodriguez et al. (2007) studies may be a consequence of the manner in which the experiments were performed. In the Garcia-Rodriguez et al. studies, PUF3-GFP cells co-expressed PreF0ATPase-[subunit9]-DsRed protein, thus eliminating the need to wash the cells [21]. On the contrary, in my experiments, cells were washed with 1X PBS dissolved in synthetic media to remove excess Mitotracker Deep Red FM stain and prevent high background fluorescence during imaging. Subsequently, cells were imaged on a synthetic media agarose pad supplemented with 2% of the respective carbon source. The use of 1X PBS likely caused osmotic stress to the cells, as evidenced by a dramatic
increase in the number of Puf3p foci visualized in galactose and ethanol conditions (compare Table 3.1 and Table 3.3). This increase in Puf3p foci was similar to the Puf3p aggregation phenotype observed in galactose and ethanol large P-body inducing conditions. However, unlike the P-body inducing experiments, the yeast cells in the mitochondria microscopy conditions were not depleted of the respective carbon sources. Although the number of Puf3p aggregates was exaggerated in these experiments, observed co-localization of Puf3p and mitochondria in galactose conditions may actually be reflective of the carbon source, as Puf3p foci detected in Puf3p activating dextrose conditions failed to colocalize with mitochondria.

Together, these microscopy studies demonstrated that the majority of inactive Puf3p aggregates co-localize with P-bodies (≥ 77%; Table 3.2), whereas a fraction of inactive Puf3p aggregates co-localize with mitochondria (30%; Table 3.3). The mechanism of altered Puf3p localization is not an exclusive event in which Puf3p asymmetrically localizes to a single subcellular compartment. In the absence of decay activity in ethanol and galactose conditions, I propose that a small pool of Puf3p aggregates assists the localization of mRNA targets to the mitochondria where they are translated and imported, and that the excess pools Puf3p aggregates move into P-bodies for temporary storage. When mitochondrial function is no longer needed in dextrose conditions, Puf3p aggregates can exit the P-bodies and re-enter the cytoplasm, and also dissociate from mitochondria to stimulate decay of the mitochondrial transcript targets.

**COMPLETE MODEL FOR CONDITIONAL REGULATION OF Puf3p**

Together, the work presented in this chapter has provided insight into the detailed mechanisms of Puf protein function and regulation. First, this work demonstrated that
the environmental signals (carbon sources) that activate or inactivate yeast Puf3p mRNA decay stimulating activity likely trigger a signaling cascade that results in post-translational phosphorylation of the Puf3RD. Next, this work examined the implications of inhibiting Puf3p activity in galactose and ethanol conditions, and demonstrated that multiple aspects of Puf3p function are altered at the molecular level, such as interactions with the decay machinery and subcellular localization. Overall, this work demonstrates how yeast Puf3p is modulated to accomplish tightly controlled regulation of mitochondrial biogenesis and function. A model explaining the significance of this work is detailed below.

In the presence of dextrose (Puf3p Activating Conditions), the yeast cell does not require mitochondrial activity for efficient cell growth, and utilizes glycolysis and fermentation metabolism pathways to obtain cellular energy. As a consequence, Puf3p is phosphorylated in its active state, and localizes throughout the cell cytoplasm to bind and promote rapid deadenylation and decay of nuclear-transcribed mitochondrial mRNAs, likely through interactions with the Pop2p/Ccr4p deadenylase complex in the cell cytoplasm (Figure 3.13). Shortening of the mRNA poly(A) tail represses translation of the mRNAs, and Puf3p-bound transcripts subsequently associate with Dcp2p decapping complexes and exonucleases in P-bodies, where they are degraded.

Alternatively, in the presence of non-fermentable ethanol, or galactose and raffinose (Puf3p Inactivating Conditions; Figure 3.13), the yeast cell requires mitochondrial function to utilize these carbon sources efficiently for cell energy and growth. Puf3p mRNA targets encode proteins required for the upregulation of mitochondrial DNA genes (mitochondrial ribosome subunits and mitochondrial
translation elongation factors) as well mitochondrial function (cytochrome c oxidase subunits, and subunits of the F₁F₀ ATPase) [1]. Therefore, nuclear-transcribed mitochondrial transcripts would need to be stabilized, translated in the cell cytoplasm, and imported into mitochondria to activate mitochondrial function. Accordingly, Puf3p decay-stimulating activity is inhibited in these conditions, in part by differential phosphorylation of Puf3p and its inability to recruit Pop2p to mRNA targets in the cytoplasm. While some binding interactions between Puf3p and Ccr4p are maintained, Ccr4p deadenylase activity is likely inhibited in the absence of the Pop2p subunit (of the Ccr4p/Pop2p deadenylase complex). As a result, nuclear-transcribed mitochondrial transcripts are stabilized. In these conditions, inactive pools of Puf3p form large aggregates, in which a subpopulation of these aggregates is proposed to direct the association of mRNA targets to the mitochondrial surface, thus facilitating import of nascent proteins into the mitochondria to render this organelle functionally active. Puf3p aggregates that are in excess of the number of transcripts that require shuttling to the mitochondrial surface are temporarily housed in P-bodies until Puf3p activity is turned on again.
Figure 3.13. Current Model for Regulation of Puf3p Activity. Puf proteins are represented as green rainbow shapes with phosphorylation modifications. Decay factors Pop2p, Ccr4p and Dcp2p are represented by orange and yellow shapes. P-bodies are represented by semi-transparent blue ellipses. A single mitochondrion is depicted as a red ellipse. In dextrose conditions (Puf3p Activating Conditions, left diagram), cellular import of dextrose inhibits mitochondrial function by repressing transcription of mitochondrial mRNAs [51-54]. Additionally, dextrose likely triggers phosphorylation and subsequent activation of Puf3p to post-transcriptionally downregulate expression of mitochondrial mRNAs that persist in the cytoplasm. As a result, Puf3p is ubiquitously expressed throughout the cell cytoplasm to maximize its ability to locate and bind nuclear-transcribed mitochondrial mRNAs followed by recruitment and binding of decay factors that repress translation and subsequently degrade the mRNA in cytoplasmic P-bodies. In galactose, raffinose and ethanol conditions (Puf3p Activating Conditions, right diagram), the cell likely alters the phosphorylation sites on Puf3p to turn off Puf3p activity. While the majority of Puf3p remains in the cytoplasm, another subpopulation of Puf3p forms a large aggregate, presumably to promote a conformational change that inhibits binding interactions with some components of the decay machinery but permits binding to mRNA targets. Subsequently, Puf3p-bound mRNAs aggregate, and Puf3p shuttles the mRNA targets to the outer surface of mitochondria, where they are translated and imported independently of any Puf3p decay function. While a subpopulation of Puf3p aggregates is sufficient to support transport of mRNAs to the mitochondria, the remaining Puf3p aggregates localize within P-bodies to sequester the protein for temporary storage.
MATERIALS AND METHODS

Steady-State PUF3 mRNA analysis

Temperature sensitive yeast strains ywo7 (wildtype) and ywo43 (puf3Δ), which express the rpb1-1ts allele for RNA polymerase II, were grown in yeast extract/peptone (YEP) media supplemented with 2% dextrose, galactose, raffinose, or ethanol at 24°C to an OD$_{600}$ of 0.4. Total RNA was separated on 1.25% agarose gels containing formaldehyde and transferred to nylon membrane for probing with the radiolabeled oligo owo124, which is complementary to PUF3 mRNA. The transcript was visualized using a Storm phosphorimager (Molecular Dynamics). Northern blots were normalized for loading by ethidium bromide staining of the 28S and 18S rRNAs.

Steady-State Puf3p Western Analysis

The temperature sensitive yeast strain ywo7 (wild-type) was grown in YEP media supplemented with 2% glucose, galactose, raffinose, or ethanol at 24°C to an OD$_{600}$ of 0.4. Harvested cells were resuspended in sample buffer and were mechanically lysed with glass beads. The cell extract was collected by poking a hole in the bottom of the microfuge tube with a 23G1 syringe, placing the tube into a 15ml centrifuge tube, and centrifuging at 4000rpm. The supernatant and pellet fractions were collected in separate tubes, and equal OD$_{600}$ units of total protein (Biorad assay, Biorad) were loaded onto a 10% denaturing Tris-glycine polyacrylamide gel (Lonza). Gels were electroblotted to nitrocellulose membrane and probed with anti-Puf3p antibodies that were produced in rabbit. Cross-reacting proteins were visualized by a secondary reaction with anti-rabbit IgG antibodies. Blots were stripped and reprobed with anti-Tfp1p antibodies that were produced in mouse. Cross-reacting proteins were visualized by a secondary reaction with
anti-mouse IgG antibodies. Loading of total protein was normalized by staining blot with Ponceau S.

**Puf3RDp Immunoprecipitation and Phosphorylation Analysis**

Yeast strain ywo192 (puf3Δ) expressing FLAG-Puf3RDp grown in synthetic minimal media supplemented with 2% dextrose or ethanol at 30°C to an OD\textsubscript{600} of 0.4. Harvested cells were resuspended in IP buffer treated with a complete mini protease inhibitor cocktail tablet (Roche). Cells were lysed with glass beads, and the cell extract was collected in a 15ml centrifuge tube by poking a hole in the bottom of the 2ml microfuge tube with a 23G1 syringe, and centrifuging at 4000rpm. Total protein quantitation of cell extracts was performed by BioRad assay (BioRad), and equal mg of total protein was nutated with anti-FLAG M2 affinity gel (Sigma) slurry at 4°C. The anti-FLAG affinity resin fraction was washed with IP wash buffer, and FLAG-Puf3RDp was eluted by the addition of 2X SDS gel loading dye to the resin, followed by boiling at 100°C. Equal volumes of the loading dye containing FLAG-Puf3RDp were loaded onto loaded onto two 13% denaturing SDS polyacrylamide gels. Immunoprecipitation of FLAG-Puf3RDp was verified by coomassie staining.

The second polyacrylamide gel was stained with Pro-Q Diamond phosphoprotein stain (Invitrogen) per manufacturer’s directions, and was imaged on a Typhoon 840 scanner (Amersham Biosciences/Molecular Dynamics). Total protein levels were determined by subsequent SYPRO Ruby (Invitrogen) staining and detection using a UV transilluminator.
Analysis of Puf3p and decay factor interactions in vivo

Yeast strains ywo187 (puf3Δ, CCR4-myc), ywo188 (puf3Δ, DCP2-myc), and ywo191 (puf3Δ, POP2-myc) were transformed with empty vector plasmid pAV72 (pwo15) expressing the FLAG epitope by LiOAC transformation method. Yeast strains ywo187, ywo188, and ywo191 were also transformed with the pAV72 vector expressing FLAG-Puf3RDp (pwo16).

Strains were grown in synthetic minimal media supplemented with 2% dextrose or galactose at 30°C to an OD₆₀₀ of 0.4. Harvested cells were resuspended in IP buffer treated with a complete mini protease inhibitor cocktail tablet (Roche). Cells were lysed with glass beads, and the cell extract was collected in a 15ml centrifuge tube by poking a hole in the bottom of the 2ml microfuge tube with a 23G1 syringe, and centrifuging at 4000rpm. Total protein quantitation of cell extracts was performed by BioRad assay (BioRad), and equal mg of total protein was nutated with anti-FLAG M2 affinity gel (Sigma) slurry at 4°C. The anti-FLAG affinity resin fraction was washed with IP wash buffer treated with a complete mini protease inhibitor cocktail tablet (Roche), and FLAG-Puf3RDp was eluted by nutating with 3X FLAG peptide at 4°C. An equal volume of 2X SDS was added to the eluate, and the samples were separated on a SDS polyacrylamide gel.

Gels were electroblotted to nitrocellulose membrane and probed with anti-Myc antibodies that were produced in mouse. Cross-reacting proteins were visualized by a secondary reaction with anti-mouse IgG antibodies. Puf3p input loading was determined by SDS PAGE of equal µg of total protein extract. The Western gel was transferred to nitrocellulose membrane that was probed with anti-FLAG antibodies produced in mouse.
Cross-reacting proteins were visualized by a secondary reaction with anti-mouse IgG antibodies.

**Analysis of Puf3p and** $COX17$ **mRNA interactions** in vivo

**Semi-quantitative RT-PCR**

Yeast strain ywo192 ($puf3\Delta$, $CCR4$-myc) was transformed with plasmid pwo16 expressing FLAG-Puf3RDp or pwo15 (empty vector) expressing the FLAG epitope by LiOAC transformation method. Cultures were grown in synthetic minimal media supplemented with 2% dextrose or galactose at 30°C to an $OD_{600}$ of 0.4. Cells were lysed, total protein quantitation and the co-immunoprecipitation was performed as described in “**Analysis of Puf3p and decay factor interactions** in vivo” except that the Puf3RDp complexes were eluted by nutating the resin with 0.1M glycine-HCl, pH 3.5. Total RNA was prepared by hot phenol-chloroform extraction as previously described in [46]. Equal ng of total RNA were treated with Turbo DNase (Ambion), and the DNase-free RNA was used for cDNA synthesis using the Verso cDNA synthesis kit for semi-quantitative $COX17$ cDNA PCR analysis (Thermo-Scientific). $COX17$ was amplified using 2X Biomix (Bioline) and primers owo7 and 459. Several yeast housekeeping genes for RT-PCR [47] including, $ACT1$ (owo456 and owo457), $TPS1$ (owo555 and 556), $TPS2$ (owo557 and 558), $TFP1$ (owo572 and 573), $TUB1$ (owo574 and 575), $ORC5$ (owo576 and 577), and $PGK1$ (owo553 and 554), were also amplified using 2X Biomix and separated on a 2% agarose gel.

**Quantitative real-time qPCR**

Yeast strain ywo188 ($puf3\Delta$, $DCP2$-myc) was transformed with plasmid pwo16 expressing FLAG-Puf3RDp or pwo15 (empty vector) expressing the FLAG epitope by
LiOAC transformation method. Cultures were grown in synthetic minimal media supplemented with 2% dextrose or galactose at 30°C to an OD$_{600}$ of 0.4. mRNPs were cross-linked with formaldehyde, and the cross-linking reaction was quenched by addition of glycine. Harvested cells were washed and resuspended in RIPA buffer containing sodium deoxycholate, a complete mini protease inhibitor cocktail tablet (Roche) and RNaseOUT (Invitrogen) RNase inhibitor. Cells were lysed, total protein quantitation and the co-immunoprecipitation was performed as described in “Analysis of Puf3p and decay factor interactions in vivo”, except that M2 anti-FLAG affinity gel resin washes were performed with RIPA buffer. Elution from the resin was performed with 3X FLAG peptide. Cross-linking was reversed by incubating the eluate with sodium chloride at 65°C. Protein digestion was performed by incubating with proteinase K.

Eluates from co-immunoprecipitation (IP) experiments and 1/10 of the IP volume of cell extract used in co-immunoprecipitation reactions were resuspended in Complete Buffer A, and a hot phenol extraction was performed as previously described in [46]. The aqueous phase containing total RNA was purified using Direct-Zol RNA MiniPrep columns (ZYMO research) with Turbo DNase digestion (Ambion) performed in the purification columns. To quantitatively assess $COX17$ levels using real-time PCR, total cDNAs were synthesized using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). $COX17$ and the housekeeping control $TDH1$ cDNAs were amplified using iQ SYBR green supermix (Bio-Rad) using gene specific primer pairs that were designed using QuantPrime online software (owo631 and 653) for $PGK1$ (owo630 and owo651) for $COX17$ and (owo632 and owo652) for $TDH1$. Three technical replicates for $COX17$
and *TDHI* cDNAs were performed for each carbon source condition using total cDNAs isolated from immunoprecipitation eluates and cell extracts.

**Steady-State Transcriptional Decay Analysis**

Steady-state transcriptional shut off experiments were also performed using strains ywo185 (Puf3p-GFP) and ywo186 (Dcp2p-GFP), each transformed with a plasmid expressing *MFA2-COX17* 3’UTR mRNA (pwo25). pwo25 was created as described [29, 48], with the *MFA2-COX17* 3’UTR RNA expressed under the control of the GAL1 UAS, which is induces transcription with the addition of galactose, and is repressed by the addition of dextrose. Additionally, ywo186 was co-transformed with a plasmid expressing Puf3p-DsRed (pwo164), under the control of a constitutive GPD promoter.

The DsRed protein sequence was fused to the C-terminus of Puf3p to produce pwo164 as follows: the DsRed gene was amplified from pBin35SRed1 using oligos owo636 and owo637, and the 768bp PCR product was gel purified. owo636 contains the last 39 bases of *PUF3*, excluding the translational stop codon, directly followed by the DsRed sequence. owo637 contains the last 27 bases, including the translational stop codon, of DsRed, which is flanked by pwo13 sequence containing a *SalI* restriction site. pwo13, which expresses Puf3p, was digested with *SalI*, incubated with calf intestinal phosphatase, and gel-purified. Wild-type (ywo5) yeast cells were co-transformed with SalI digested pwo13 and the DsRed PCR product, to allow recombination and insertion of DsRed into the plasmid, thus creating pwo164. This construct was verified by yeast colony PCR and sequencing.
Yeast strains transformed with pwo25 and/or pwo164 were grown at 30°C in YEP media supplemented with 2% galactose to an OD\textsubscript{600} of 0.4. Transcription was rapidly repressed by shifting the culture to YEP media containing 4% dextrose. Total RNA was separated on 1.25% agarose gels containing formaldehyde and transferred to nylon membrane for probing with radiolabeled owo303, which is complementary to the \textit{MFA2-COX17} 3’UTR junction. Northern blots were normalized for loading using 7s RNA [49]. Transcripts were detected using a Storm phosphorimager (Molecular Dynamics) and quantitated using ImageQuant software (Molecular Dynamics).

**Microscopy of Fluorescently Labeled Puf3p**

Yeast strain ywo185 was grown in complete minimal media supplemented with 2% dextrose, galactose, or ethanol at 30°C to an OD\textsubscript{600} of 0.4. Cells were harvested and resuspended in the media supplemented with the appropriate carbon source, pipetted onto agarose pads supplemented with amino acids and 2% of the appropriate carbon source. Microscope slides were covered with a #1.5 coverslip and sealed. Observations were made using a Zeiss confocal microscope with a 63X objective and Zen 2009 software. Some images are a Z-series compilation of 6-10 images in a stack.

**Puf3p and P-body detection using epi-fluorescence microscope**

Yeast strains ywo185 (Puf3p-GFP) and ywo186 (Dcp2p-GFP) were grown at 30°C in complete synthetic minimal media supplemented with 2% dextrose, ethanol, galactose, or raffinose to an OD\textsubscript{600} of 0.4. Cultures were harvested and washed with an excess of water, resuspended in water and incubated for 5 minutes at room temperature. Cells were pipetted onto glass slides coated with poly-L-lysine concanavalin A to immobilize them for imaging. Slides were covered with a glass coverslip and sealed.
Epi-fluorescence imaging was performed using a Nikon microscope with a 100X objective and YFP filter.

**Puf3p and P-body detection using confocal microscope**

Yeast strains ywo185 (Puf3p-GFP) and ywo186 (Dcp2p-GFP) were transformed with plasmids pRP1186 (Dcp2p-RFP) and pwo164 (Puf3p-DsRed), respectively, by LiOAC transformation. Transformed yeast strains were grown at 30°C in synthetic minimal media supplemented with 2% dextrose to an OD$_{600}$ of 0.3-0.35. Cultures were harvested and washed with an excess of synthetic minimal media, resuspended in synthetic minimal media supplemented with 2% dextrose, galactose, or ethanol and were incubated 30°C with shaking to allow for carbon source acclimation. Subsequently, P-bodies were induced in strain ywo185 as previously described in [36]. Harvested cells were immediately observed on agarose pads supplemented with amino acids, covered with a #1.5 glass cover slip, and sealed. After acclimating to the appropriate carbon source, transformed ywo186 was harvested and resuspended in synthetic minimal media containing the appropriate carbon source and potassium chloride to induce P-bodies as previously described in [36]. Cells were immediately observed on complete minimal media agarose pads 1X amino acids and 2% of the appropriate carbon source, covered with a #1.5 glass cover slip, and sealed. Observations were made using a Zeiss confocal microscope with a 63X objective and Zen 2009 software. Some images are a Z-series compilation of 6-10 images in a stack.

**Puf3p and mitochondria detection using confocal microscope**

Yeast strain ywo185 was grown at 30°C in complete minimal media supplemented with 2% dextrose to an OD$_{600}$ of 0.3-0.35. Cultures were harvested and
washed with an excess of complete minimal media, resuspended in minimal media supplemented with 2% dextrose, galactose, or ethanol, and Mitotracker Deep Red FM stain (Invitrogen). Cells were incubated at 30°C with shaking for an additional 30 minutes, and were harvested and washed with 1X PBS dissolved in the appropriate media. Cells were harvested and immediately observed on complete minimal media agarose pads supplemented with amino acids and 2% of the appropriate carbon source. Observations were made using a Zeiss confocal microscope with a 63X objective and Zen 2009 software. Some images are a Z-series compilation of 6-10 images in a stack.
REFERENCES


CHAPTER IV:

CONDITION-SPECIFIC REGULATION OF Puf3p-MEDIATED COX17 mRNA TRANSLATION
Protein expression is regulated by rates of mRNA translation versus mRNA decay. The molecular switch that modulates the transition between these processes is the formation of different mRNA-protein complexes (mRNPs). An actively translating mRNA will associate with different protein factors than an mRNA that is targeted for decay. Therefore, an actively translating mRNA may adopt a different structural conformation than a transcript destined for turnover. Puf proteins have been implicated in both translational activation and repression, suggesting that Puf proteins may be a part of the molecular switch that bridges these processes. Based on this knowledge, I hypothesized that Puf3p may function to regulate the translational efficiency of its mRNA targets, independent of its role in mRNA turnover. In this chapter, I addressed this hypothesis by performing polysome profiling experiments, followed by Northern analyses and/or RT-PCR to monitor the translational efficiency of COX17 transcripts using yeast strains that are defective in mRNA decay. These experiments help elucidate the full mechanism by which Puf3p regulates its mRNA targets.

**Puf3p Reduces COX17 mRNA Translational Efficiency in the Absence of Decay Activity in Dextrose Conditions**

Prior to mRNA turnover, mRNPs must undergo a conformational change that renders the mRNPs in an inactive, non-translating state. Recently, the *C. elegans* Puf protein FBF was shown to be both a negative and positive regulator of gld-1 mRNA expression. FBF activates gld-1 translation by interacting with the cytoplasmic polyadenylase GLD-2/GLD-3 [1]. In other cellular contexts, FBF likely promotes translational repression by dissociation of GLD-2/GLD-3 and subsequent recruitment of deadenylases, which in turn, disrupts interactions between poly (A) binding proteins,
translation initiation factors and the 5’ mRNA cap [1]. Therefore, the cellular events that trigger the formation of different Puf3p complexes may function as a molecular switch that allows Puf3p to regulate both translational activation and repression [2]. Previous studies that monitored the translation of \textit{COX17} mRNA revealed that Puf3p did not affect \textit{COX17} translational efficiency in dextrose conditions (Figure 4.1A; Olivas, W., published data [3]). However, since Puf3p decay activity is turned on in dextrose, the rapid decay of \textit{COX17} may have masked any changes to \textit{COX17} translational efficiency (Figure 4.1A).

Based on these observations, I hypothesized that Puf3p might have a novel role in conditionally regulating the translation of its mRNA targets independent of its mRNA decay function (Figure 4.1B). Specifically, I proposed that in dextrose conditions, when Puf3p stimulates rapid decay of its targets, Puf3p might also function to disrupt translation of its mRNA targets, possibly by disrupting interactions with translation initiation complexes that are bound to the mRNA. Therefore, Puf3p may repress translation of its mRNA targets, prior to its role in mRNA decay. In galactose conditions, when Puf3p-mediated decay activity is inhibited and its targets are stabilized for subsequent translation, I proposed that Puf3p would not disrupt translation initiation factor complex interactions, thus allowing translation of its mRNA targets to proceed. Alternatively, I hypothesized that Puf3p would enhance the translation of its mRNA targets in galactose conditions. To ensure that I analyzed the role of Puf3p in repressing the translation of its mRNA targets independent of its role in decay, I utilized strains that are deleted of \textit{CCR4}, a deadenylation factor. In a \textit{ccr4Δ} strain, all of the mRNAs should be polyadenylated, which essentially blocks the major (deadenylation-dependent) mRNA
Figure 4.1. Polysome profiling analysis of COX17 translation. A) In wild-type (WT) dextrose conditions, when Puf3p decay activity is turned on, COX17 mRNA is translated efficiently, as determined by its association with polysomes. In puf3Δ conditions, COX17 mRNA translation is not altered. If Puf3p were responsible for reducing COX17 mRNA translation, then it would be expected that COX17 mRNA would shift to the right and associate with more polysomes in the absence of Puf3p when compared to the WT polysome profile (Olivas, W., published data; reprinted and modified from [3]). B) Schematic diagram explaining rationale and hypotheses for COX17 translational analysis independent of Puf3p-mediated decay activity.
decay pathway in yeast.

To address these hypotheses, I analyzed COX17 mRNA association with ribosomes along polysome gradients in ccr4Δ strains grown in dextrose or galactose conditions, as well as a ccr4Δpuf3Δ strain grown in dextrose conditions. COX17 transcripts that are efficiently translated would be expected to associate with multiple ribosomes (polysomes), while COX17 transcripts that are inefficiently translated would be expected to associate with individual ribosome subunits or a single ribosome (monosome). In dextrose conditions in a ccr4Δ strain, COX17 mRNA was associated with polysomes, with an enrichment in fractions 8 and 9 as assessed by Northern blotting (Figure 4.2, red bar). COX17 cDNA as assessed by RT-PCR, also associate with polysomes in a ccr4Δpuf3Δ strain in dextrose conditions. In both of these strains, COX17 mRNA is mostly detected within the polysome fractions, suggesting that the absence of Puf3p does not reduce the translation of COX17 in dextrose conditions. In fact, COX17 is shifted deeper into the polysome region, suggesting that COX17 is more efficiently translated in the absence of Puf3p. Unexpectedly, in ccr4Δ galactose conditions, the efficiency of COX17 translation is reduced when compared to the ccr4Δ dextrose profile, as COX17 mRNA is slightly shifted towards the ribosome subunit and monosome gradient, with an enrichment of the 60S ribosomal subunit in fractions 5 and 6 (Figure 4.2, red bar). Based upon these observations, it does not appear that Puf3p enhances COX17 mRNA translation in ccr4Δ galactose conditions, relative to ccr4Δ dextrose conditions. Future experiments must be conducted with a control mRNA that is not regulated by Puf3p, and the polysome profiles must be conducted using the ccr4Δpuf3Δ strain in galactose conditions to formulate any conclusions about the role of Puf3p in
Figure 4.2. Puf3p reduces COX17 translational efficiency in the absence of its mRNA decay activity in dextrose conditions. Shown are representative polysome profile analyses of COX17 mRNA or cDNA in ccr4Δ-dextrose, ccr4Δ-galactose, and ccr4Δpuf3Δ-dextrose conditions. Northern blots of COX17 mRNA from ccr4Δ-dextrose and ccr4Δ-galactose are shown, while an image of COX17 cDNA from ccr4Δpuf3Δ-dextrose conditions is shown. Four biological replicates of polysome profiles were performed in ccr4Δ-dextrose conditions, while two replicates were conducted in ccr4Δ-galactose, and ccr4Δpuf3Δ-dextrose conditions. Fractions in which COX17 mRNA is most enriched are underlined in red. A representative ethidium bromide staining of the gradients, in the absence of cycloheximide treatment is shown above. The location of monosome and polysome fractions were determined by the intensity of ethidium bromide stained 28S and 18S ribosomal RNA bands, which are components of the large and small ribosomal subunit, respectively, and comparison to an identical gradient with cycloheximide treatment.
affecting translation in galactose conditions.

Together, these polysome profiling studies suggest that Puf3p may modulate COX17 translation independent of its mRNA decay stimulating function in dextrose conditions. The efficient translation of COX17 as detected deep into the polysome fractions in the ccr4Δpuf3Δ dextrose conditions versus detection in the early polysome fractions in the ccr4Δ strain in dextrose indicates that Puf3p does play an inhibitory role in COX17 translation. The slight reduction of COX17 mRNA translational efficiency in galactose conditions was unanticipated, considering that the Ccr4p deadenylase was deleted and that COX17 mRNA is stabilized in these conditions [4]. However, it is possible that global rates of translation are reduced in galactose conditions. Testing of this possibility would require repetition of this experiment with co-amplification of COX17 and a control mRNA that is not regulated by Puf3p. In support of this hypothesis, I demonstrated that total protein levels are decreased in continuous ethanol, galactose and raffinose conditions when compared to continuous dextrose conditions as detected by Ponceau S staining on Western blots (Chapter III, Figure 3.1, Ponceau S). Furthermore, Kuhn et al. performed a global analysis of protein synthesis and polysome profiling in yeast that were quickly transferred from dextrose to nonfermentable glycerol conditions [5]. Global protein synthesis was reduced 5 minutes after the shift to glycerol, and protein synthesis was never fully returned to the level observed in dextrose [5]. In an independent study, ribosomal protein gene expression was upregulated in dextrose, but was downregulated in nonfermentable ethanol conditions [6]. Interestingly, ribosomes were globally shifted from polysomes to monosomes and ribosomal subunits after
switching the carbon source to glycerol, including $ACT1$ and several ribosomal protein mRNAs [5].

To assess the impact of Puf3p inactivation on mRNA translational efficiency without worrying about the global effects of carbon source, it will be important to identify the critical phosphorylation sites on Puf3p that are differentially phosphorylated in dextrose vs. galactose or ethanol conditions. Mutation of critical phosphorylation sites that differ between the Puf3p activating and inactivating conditions would allow analysis of inactive Puf3p without changing the carbon source. The constitutively active or inactive Puf3 proteins could be used in polysome profiling experiments in dextrose conditions to determine if Puf3p inactivation enhances $COX17$ translation.
MATERIALS AND METHODS

Polysome Profile Analysis of \textit{COX17} mRNA

Yeast strain ywo267 (\textit{ccr4Δpuf3Δ}) was created by swapping the endogenous \textit{PUF3} gene with \textit{URA3} as follows: the \textit{URA3} was amplified from yeast genomic DNA using oligos owo549 and owo550, and the 926bp product was gel purified. owo549 contains \textit{PUF3} 5’UTR sequence, directly followed by the first 22 bases of \textit{URA3} sequence. owo550 contains the last 25 bases of \textit{URA3}, including the translational stop codon, which is flanked by \textit{PUF3} 3’UTR sequence. pwo15 was digested with BamHI and SalI, incubated with calf intestinal phosphatase, and gel-purified. The \textit{ccr4Δ} strain (ywo13) was transformed with the \textit{URA3} PCR product, to allow recombination and insertion of \textit{URA3} in the place of \textit{PUF3} in the yeast genome, thus creating ywo267. Deletion of \textit{PUF3} was verified by yeast colony PCR.

Yeast strains ywo13 (\textit{ccr4Δ}) and ywo267 (\textit{ccr4Δpuf3Δ}) were grown in YEP media supplemented with 2% dextrose or galactose at 30°C to an OD$_{600}$ of 0.5-0.6, and polyribosome extracts were prepared without cycloheximide as previously described [7] and illustrated in Figure 4.2. Fourteen fractions were collected from the top of the sucrose gradients. Fractions were prepared for Northern or RT-PCR analysis as described in [8]. Equal volumes of fractions were separated on a 1.25% agarose gel containing MOPS buffer and formaldehyde. 28S and 18S rRNA levels in each fraction were detected by ethidium bromide staining, and the gel was transferred to a nylon membrane for probing with the radiolabeled oligo owo2, which is complementary to \textit{COX17} mRNA. The transcript was visualized using a Storm phosphorimager (Molecular Dynamics). Alternatively, total cDNA of each fraction was prepared using DNase-free
RNA and the Verso cDNA synthesis kit (Thermo-Scientific). COX17 was amplified using 2X Biomix (Bioline) and primers owo7 and 459.
REFERENCES


CHAPTER V:

SUMMARY AND FUTURE DIRECTIONS
CONDITIONAL REGULATION OF Puf3p FUNCTION IS DIRECTLY LINKED TO CHANGES IN CELL METABOLIC PATHWAYS

Yeast Puf3p has been implicated as a regulator of mitochondria biogenesis and function by genomic computational studies [1, 2], genetic and physical interactions with mitochondria [3, 4], and decay analysis of the experimentally validated Puf3p target COX17 mRNA [5, 6]. In my M.S. thesis work, I experimentally validated ten new nuclear-transcribed mitochondrial mRNAs that were regulated by Puf3p, demonstrating that a single yeast Puf protein can regulate a class of functionally related transcripts [7]. This is in stark contrast to the observation that most Puf proteins work in a combinatorial manner to promote repression and/or turnover of mRNAs [8-13]. Furthermore, Puf3p-mediated decay stimulating activity is conditionally regulated by environmental conditions, such that Puf3p promotes rapid decay of target mRNAs in the presence of dextrose. Alternatively, in the presence of galactose, ethanol, or raffinose, the half-lives of these transcripts are stabilized demonstrating that Puf3p activity is inhibited in these conditions [7].

A reasonable model for the dynamics of Puf3p-mediated decay activity is that in dextrose conditions, yeast cells do not require mitochondrial respiration to utilize dextrose for efficient growth. Therefore, to conserve energy, cells repress translation of nuclear-transcribed mitochondrial mRNAs such as COX17, TUF1, and CYT2 that must be imported into the mitochondria for maturation and function. To accomplish this repression, Puf3p stimulates rapid turnover of these transcripts. Alternatively, in the presence of non-fermentable carbon sources, which require mitochondrial maturation and ATP production, Puf3p decay stimulating activity is abolished to allow translation and
import of these mitochondrial transcripts. In support of this theory, dextrose has been shown to trigger repression of genes involved in the metabolism of alternative carbon sources such as ethanol and galactose [14] and several aspects of mitochondrial ATP production[15-17] and cytochrome complex subunit COX6 [18]. Puf3p provides another level of regulation beyond transcriptional repression by acting at the level of mRNA stability to control mitochondrial protein production.

**FUNCTIONAL LINK BETWEEN ENVIRONMENTAL STIMULI, Puf PROTEIN PHOSPHORYLATION, AND CHANGES IN Puf PROTEIN DECAY ACTIVITY**

In this dissertation work, I sought to understand the complex molecular mechanisms involved in the regulation of yeast Puf3 protein activity. Specifically, the goal of this work was to elucidate the mechanism of Puf3p decay activity and determine the aspects of Puf3p function that are altered when Puf3p activity is compromised. At the molecular level, environmental signaling and subsequent signal transduction appears to be a critical and central component of the molecular mechanism that regulates Puf protein activity. For example, environmental stimuli, such as hormones, can be recognized by extracellular or intracellular receptors, and in turn trigger signaling cascades that may elicit cellular responses such as protein phosphorylation, to alter levels of gene expression. In chapter III of this dissertation, I demonstrated that PUF3 expression was not downregulated at the levels of transcription or translation, suggesting that inhibition of Puf3p activity was not due to reduced expression in inhibitory conditions. Furthermore, I found that the repeat domain of Puf3p is post-translationally modified by phosphorylation in both Puf3p activating and inactivating conditions. While this data shows that there is not an all or nothing difference in phosphorylation, it is still likely that the repeat domain may be differentially phosphorylated in response to carbon source.
Therefore, I propose that conditional phosphorylation of yeast Puf3p may serve as the molecular switch that modulates Puf3p decay stimulating activity. Puf protein phosphorylation in response to an environmental trigger appears to be a conserved regulatory mechanism in eukaryotes that is critical for controlling Puf protein activity as evidenced in multiple organisms. For example, nutrient starvation in the slime mold *Dictyostelium* triggers phosphorylation activity of the YakA kinase, which prevents PufA from repressing its mRNA target PKA-C [19]. Additionally, the hormone progesterone inhibits Puf protein activity in *Xenopus* oocytes [20]. Finally, in yeast, kinase CK2 phosphorylates an N-terminal region of Puf6p, thus turning off its transcript repressive activity [21]. Together, these observations highlight the role of phosphorylation in regulating Puf decay stimulating and/or translational repression activity.

**IMPLICATIONS OF Puf PROTEIN PHOSPHORYLATION AT THE MOLECULAR LEVEL**

**Altered Puf protein-protein interactions**

Post-translational modifications such as phosphorylation can modify tertiary protein structure by inducing conformational changes and/or alter the charge landscape to inhibit protein-protein interactions. For example, phosphorylation of an activation loop in cyclin-dependent kinase 2 alters the structure of the loop and alters amino acid side chain hydrogen bonding interactions in regions surrounding the loop [22]. Additionally, phosphorylation of Src tyrosine kinase induces a conformational change such that the kinase domain is buried, thus inactivating kinase activity [23]. In *Xenopus*, phosphorylation of Pum1 is coupled with the dissociation of CPEB-Pum1 binding interactions, resulting in translational activation of *cyclin B1* mRNA [24]. Based on these observations, in Chapter III of this dissertation work, I hypothesized that phosphorylation
of the Puf3RD in inactivating conditions causes a conformation or charge landscape change such that the outer loop between repeats 7 and 8 is no longer able to mediate interactions with the decay machinery. This hypothesis was based on the observation that the Puf3p outer loop has been shown to directly bind Pop2p in Puf3p activating conditions (Lopez Leban, personal communication). To address this question, I performed co-immunoprecipitation studies with yeast extracts expressing FLAG-Puf3RDp and Myc-tagged decay factors derived from cells grown in Puf3p activating or inactivation conditions. These experiments demonstrated that interactions between the Puf3 repeat domain and the decay factor Pop2p is disrupted in conditions that inhibit Puf3p-mediated decay activity, while interactions with Ccr4p appear to be partially disrupted. These compromised interactions suggest that Puf3p is defective in stimulating deadenylation and decapping when its function in mRNA turnover is inactivated. Together, these experiments demonstrate that conditional inactivation of Puf3p decay stimulating activity is at least partially the result of reduced interactions with deadenylation and decapping factors.

Despite these observations regarding Puf3RD phosphorylation and altered interactions with the decay machinery, several unanswered questions remain regarding the conditional regulation of Puf3p activity. In the future, it will be important to identify potential phosphorylation site(s) that regulates Puf3p activity using mass-spectroscopy. It is probable that one or a few unique sites are responsible for activating or inactivating Puf3p activity. Furthermore, it is important to analyze full length Puf3p in an effort to identify possible phosphorylation sites outside of the repeat domain, as yeast Puf6p activity is turned off by phosphorylation of a residue in the N-terminus [21]. This
analysis would be critical for creating constitutively phosphorylated or non-phosphorylated Puf3RDp mutants that could be used to determine if phosphorylation activates or inactivates Puf3p, although I hypothesize that phosphorylation would turn off Puf3p activity similarly as for Puf6p. Moreover, these Puf3RDp mutants could be used in deadenylation assays to validate that Puf3p is actually unable to stimulate deadenylation in the absence of its decay activity. Additionally, it will be important to determine the aspects of Puf3p that are structurally altered at the level of amino-acid side chain interactions and bonding forces, which can be determined by co-crystal structure modeling of Puf3p mutant complexes with protein partners. Thus far, a kinase responsible for Puf3RD phosphorylation has yet to be identified, although Puf3p was predicted to be a downstream target in the rapamycin signaling pathway [2]. Together, these important experiments will help create a more detailed portrait about the complexity of Puf protein regulation.

**Altered Puf protein-mRNA interactions**

Protein phosphorylation of mammalian Puf proteins has a direct impact on Puf protein-mRNA target interactions, as unphosphorylated PUM1 has a reduced affinity for its binding site [25]. Additionally, environmental signals such as progesterone, have been shown to trigger *Xenopus* Pum2 dissociation from its target RINGO/Spy mRNA in oocytes [20]. In chapter III of this work, I used both semi-quantitative and quantitative real-time PCR to analyze levels of *COX17* mRNA that co-immunoprecipitated with Puf3RDp in activating and inactivating conditions. The results of the semi-quantitative analysis were inconclusive, but real-time qPCR demonstrated no significant difference between activating and inactivating conditions. Therefore, for yeast Puf3p, altered
mRNA binding interactions do not appear to be an underlying molecular mechanism that contributes to impaired decay stimulating activity in response to carbon source.

**Altered Puf protein subcellular localization**

There are several examples in which phosphorylation has been shown to stimulate differential protein subcellular localization including β-catenin and Snail proteins. For example, unphosphorylated β-catenin is mainly localized to the plasma membrane, with a small fraction of the protein localizing to the cytoplasm and nucleus. Alternatively, phosphorylation of β-catenin by protein kinase D1 increases its localization to the nucleus [26]. Both the activity and subcellular localization of Snail repressor protein are controlled by phosphorylation, such that phosphorylation triggers the movement of the transcription factor Snail from the nucleus to the cytoplasm so that E-cadherin can no longer be repressed in the nucleus [27].

In other cases, phosphorylation is implicated in the conversion of normal prion proteins into the aggregated, disease form [28, 29]. Interestingly, prion conversion is also linked to cellular stress responses, as yeast Sup35 prion conversion is enhanced by a regulator that forms aggregates in response to heat shock [30]. Notably, the N-terminus of Puf proteins also contains a prion-like aggregation domain via glutamine repeats that may regulate Puf3p activity. Expression of the of the *Drosophila* Pumilio glutamine rich domain was previously shown to assist formation of protein aggregates in yeast and disrupt endogenous Pumilio activity in *Drosophila* [31]. Moreover, in this dissertation work, I have shown that full length GFP-tagged Puf3p forms protein aggregates both in activating and inactivating conditions. However, in Puf3p inactivating conditions, Puf3p
aggregation is exacerbated, with an increase in the size of the protein aggregates and a pool of Puf3 protein localizing with mitochondria. These observations starkly contrast that of dextrose conditions, where the majority of Puf3 proteins are ubiquitously expressed throughout the cytoplasm, with a few cells expressing very small Puf3p aggregates. Additionally, cellular stresses such as carbon source depletion can stimulate the formation of large P-bodies after dextrose, ethanol, and galactose have been depleted from the media. While Puf3p localized to P-bodies in all P-body inducing conditions, the percentage of Puf3p aggregates colocalizing with P-bodies was increased in galactose and ethanol conditions.

Recently, researchers have demonstrated that 1M potassium chloride can be added to cells growing in glucose, resulting in P-body induction as an alternative to carbon source depletion [29]. I attempted to repeat these experiments in dextrose and ethanol conditions using the DCP2-GFP strain with plasmid expressed Puf3p-RFP or the PUF3-GFP strain with plasmid expressed Dcp2p-RFP, which failed to correctly express Puf3p or induce P-body localization of plasmid expressed Dcp2p, respectively. In the future, these potassium chloride experiments should be conducted with a yeast strain expressing genomically integrated DCP2-GFP and PUF3-RFP to validate that the results are dependent on carbon source. Despite this setback, these observations demonstrate that Puf3p inactivating conditions can trigger altered Puf3p localization to mitochondria, while Puf3p localization to P-bodies is likely independent of Puf3p functional state.

Based on this work, I propose that Puf3p aggregate formation is facilitated by the presence of glutamine repeats located outside of the repeat domain. Further analysis of the putative Puf3p aggregation domain (glutamine repeats) will be critical for
understanding the function of the regions outside of the repeat domain. It will be interesting to examine aggregation using GFP-tagged Puf3p that has deletions of the glutamine repeats. In addition, analysis of the glutamine mutants in the different Puf3p activating and inactivating conditions will be important in determining whether Puf3p activity is mediated by the glutamine repeats. It will also be important to determine if Puf3p can also localize to stress granules, particularly in galactose and ethanol conditions using the potassium chloride method. I expect that this is a conserved mechanism in most Puf expressing eukaryotes, as mammalian PUM proteins [32, 33] colocalize with stress granules.

Based on the current understanding of Puf protein subcellular localization and the work presented in this thesis, a model relating conditional Puf3p regulation and subcellular localization can be explained as follows: When is activated in dextrose conditions, the majority of Puf3p is diffusely expressed throughout the cell cytoplasm to increase the efficiency of finding, binding, and stimulating deadenylation of its mitochondrial mRNA targets. Puf3p may then direct these transcripts to P-bodies where they are decapped and degraded. In contrast, when Puf3p decay activity is inhibited by ethanol, galactose or raffinose, a pool of Puf3p aggregates may retain the ability to associate with mRNA targets and shuttle them to the mitochondrial surface where the mRNAs are translated and nascent proteins are imported into mitochondria. Excess pools of Puf3p aggregates that are no longer required for shuttling mRNAs to the mitochondria may localize to P-bodies for temporary storage.
Puf PROTEINS AND TRANSLATION

In multicellular eukaryotes, Puf3p proteins have been shown to mediate both translational activation and repression. In *C. elegans* and *Xenopus*, translational activation is mediated by Puf protein interaction with cytoplasmic polyadenylases or cytoplasmic polyadenylation element-binding proteins, respectively [34, 35]. However, cytoplasmic poly (A) polymerases are not conserved in yeast, as *S. cerevisiae* only expresses two poly(A) polymerases that target aberrant transcripts for decay [36]. Therefore, it is probable that these mechanisms of Puf-mediated translational activation or Puf-mediated modulation of translation are not conserved functions in yeast, but are a newer function of Puf proteins that emerged in more complex eukaryotic cells.

In yeast, the 3’UTR of yeast *MFA2* mRNA has been shown to regulate translation by recruiting trans-acting 3’UTR binding factors and differentially recruiting poly(A) tail binding proteins (Pab1p) in response to carbon source [37]. The highly unstable yeast *MFA2* mRNA is post-transcriptionally regulated by RNA-binding proteins that interact with 3’UTR AU-rich elements (ARE) independently of the available carbon source [2, 38]. However, *MFA2* mRNA translation is regulated by carbon source, and is dependent on the presence of the Hog1p kinase. In dextrose conditions, *MFA2* translation is presumably downregulated by altered recruitment of Pab1p and ARE-RNA binding protein related Pub1p to ARE in a Hog1p-dependent manner, which prevents 5’cap-poly(A) tail interactions that are required for translation initiation. In glycerol conditions, it is predicted that Hog1p may indirectly promote Pab1p localization to the poly(A) tail through interactions with an unknown ARE-binding factor, thus stimulating *MFA2* translation [37].
In this work, I wanted to determine if Puf3p could conditionally alter the translational efficiency of COX17 mRNA in the absence of its decay activity. Specifically, I hypothesized that Puf3p would increase the translational efficiency in galactose conditions, when Cox17 protein production would be upregulated versus dextrose conditions. Surprisingly, COX17 mRNA translation was less efficient in galactose conditions when compared to dextrose. However, the decreased translational efficiency of COX17 mRNA is most likely the result of global downregulation of protein synthesis, as previous studies have shown that translation of ribosomal protein encoding transcripts are coordinately downregulated in ethanol conditions, when compared to dextrose [39]. Additionally, shifting yeast cultures from dextrose to nonfermentable glycerol was shown to downregulate global protein synthesis, with a concomitant decrease in the translational efficiency of ribosomal protein encoding mRNAs, and the transcript encoding actin, ACT1 [40].

On the contrary, these studies demonstrated that Puf3p reduced the translational efficiency of COX17 mRNA, when translational repression mediated by Ccr4p deadenylation and subsequent mRNA decay was blocked. This result is particularly important, as it highlights the possibility that Puf3p can also function to disrupt translation of its mRNA targets. One explanation for this result is that Puf3p might disrupt interactions between the translation machinery, and it will be important to analyze the role of Puf3p in regulating mRNA translation in the future. The role of Puf proteins in translational repression has been frequently documented in eukaryotes, such as Drosophila, C. elegans, and yeast. In yeast, genetic assays implicate Puf4p and Puf5p in
repressing the translation of *HO* mRNA in a Pop2p-dependent manner [10-12, 41], while Puf6p represses translation of *ASH1* mRNA [42].

Another important research question regarding Puf proteins concerns identifying the function(s) within the N-terminal regions outside of the PufRD, which may be necessary for Puf proteins to finely tune protein production both spatially and temporally within cells. Prior to my dissertation work, the repeat domain was shown to be sufficient to regulate mRNA binding and stimulate translational repression and decay in yeast and *Drosophila* [6, 43], albeit suboptimal in comparison to the full length Puf proteins. While the region outside of the repeat domain comprises 1/2 to 2/3 of Puf proteins, its functional role has not been clearly determined. This dissertation work highlights one possibility that the conserved glutamine repeats outside of the yeast Puf3p repeat domain may contribute to Puf protein aggregation as a means to modulate Puf protein activity and therefore appropriately regulate protein production. Recently, two highly conserved motifs termed Pumilio Conserved Motifs or PCMα and PCMβ, were identified in the N-termini of Puf proteins ranging from insects to vertebrates, although their roles in Puf protein function remain poorly understood [44]. However, the N-terminal 2/3 of *Drosophila* Pumilio and human PUM1 and PUM2 were shown to stimulate translational repression of a reporter transcript similarly to that of full-length *Drosophila* Pumilio, suggesting that *Drosophila* and human Puf protein repression activity was controlled by an N-terminal region outside of the repeat domain. Moreover, the *Drosophila* Pumilio N-terminus was found to inhibit translational repression to a greater extent than promoting mRNA turnover [44]. Further analyses of these N-terminal domains are critical for painting a complete picture about Puf protein activity regulation and function.
REGULATION OF Puf3p ACTIVITY AT THE MOLECULAR LEVEL

This work has provided insight into the detailed mechanisms of Puf protein function and regulation. First, this work demonstrated that the environmental signals that activate or inactivate yeast Puf3p mRNA decay stimulating activity likely trigger a signaling cascade that results in post-translational phosphorylation of the Puf3RD. Next, this work examined the implications of inhibiting Puf3p activity in galactose and ethanol conditions, and demonstrated that multiple aspects of Puf3p function are altered at the molecular level, such as interactions with the decay machinery, protein aggregation, and subcellular localization. Finally, this work highlighted a previously identified function of Puf3p activity, such that Puf3p may reduce the translational efficiency of its mRNA targets prior to stimulating their decay. Overall, this work demonstrates how several aspects of yeast Puf3p function are modulated to accomplish tightly controlled regulation of mitochondrial biogenesis and function.
REFERENCES


