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The Role of Puf3p and Puf4p in the Regulation of mRNA Decay in Yeast Saccharomyces cerevisiae

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THE ROLE OF Puf3p AND Puf4p IN THE REGULATION

OF mRNA DECAY IN YEAST *Saccharomyces cerevisiae*

by

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ABSTRACT

Proper regulation of gene expression at a cellular level is required in all organisms for their successful adaptation and survival to physiological or environmental changes. In eukaryotes, a convenient way of regulating gene expression is achieved by post-transcriptionally adjusting the decay rates of different mRNAs. The Puf family of proteins in yeast belong to a widespread group of eukaryotic RNA-binding proteins that regulate the lifespans of target mRNAs by sequence specifically binding to 3' untranslated regions (UTRs) and modulating their decay rates. For example, the yeast Puf3 protein binds the *COX17* 3'UTR, stimulating its deadenylation and subsequent decay. However, the specific mechanism by which Puf3p regulates these decay processes was not known.

In this research, insight was gained on Puf3 protein interactions and its mechanism of action for *COX17* mRNA regulation. Through biochemical and genetic approaches, several decay factors involved in decapping and deadenylation events were identified to bind Puf3p via protein-protein interactions. Specifically, a four amino acid loop structure on the outer surface of Puf3p (R7A loop) was found to be the interaction point to which Pop2p directly (and Dhh1p indirectly through Pop2p) binds the Puf3RD. Other decay factors were found to bind Puf3RD independent of the R7A loop and Pop2p. Puf3p activity was also analyzed under different environmental conditions. While Puf3p was inactivated by ethanol, galactose and raffinose, Puf3 protein levels were not decreased. Instead, the different carbon sources are likely triggering a post-translational

modification, such as a change in the phosphorylation state of the protein that would account for its change in activity. Finally, four new Puf4p mRNA targets (Rrs1, YJL122W, Ebp2 and Pus7) were experimentally determined. All of these Puf4p target RNAs were also regulated by Puf5p, suggesting that combinatorial regulation of RNAs by Puf4p and Puf5p is a common mechanism.

In conclusion, the results from this research provide insight into the mechanism of Puf protein action and contribute to the understanding of Puf3p interactions that function to regulate mRNA decay in yeast. In addition, this research provides evidence that physiological conditions play a key role in post-translational regulation of Puf3p activity and therefore mRNA decay. Given the structural and functional similarities between Puf proteins, these results will significantly increase our understanding of the role of Puf proteins in yeast and other eukaryotes.

DEDICATION

This work is dedicated to Elaine and Frank Moss for

their love and unconditional support.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter I

Introduction

Regulation of gene expression

In prokaryotes as well as in unicellular eukaryotes as yeast, gene regulation allows cells to respond economically and appropriately to sudden changes of environmental conditions and efficiently adapt to the unique features of each environment (Gasch et al, 2000). For example, in natural conditions yeast typically shift from fermentation (anaerobic way of processing fermentable sugars with ethanol production) to respiration (aerobic metabolism using respiratory chain in mitochondria) when the media is depleted of glucose and ethanol becomes the main carbon source (DeRisi et al., 1997). This change in metabolism is known as the diauxic shift and requires an adequate and coordinate regulation of the expression of the yeast genome. With the help of high throughput assays such as DNA microarrays, gene expression can be explored on a genomic scale. A wide variety of signature patterns can be identified under different conditions tested in which diverse sets of genes are being up or down-regulated coordinately. In the particular case of the diauxic shift, genes involved in mitochondrial biogenesis such as cytochrome c-related genes and those required for the TCA/glyoxylate cycle and carbohydrate storage are all induced under glucose depletion. This up-regulation of mRNAs and their proteins involved in mitochondrial functionality metabolically makes sense because their activity should be increased under circumstances in which the cell needs to use its mitochondria to obtain energy. On the other hand, genes involved in protein synthesis, ribosomal and tRNA biogenesis, and translation elongation and initiation factors, all show a decrease in their expression patterns under these conditions (DeRisi et al., 1997). The down-regulation of all these genes involved in anabolic processes when switching from fermentation to respiration also makes much

sense metabolically speaking, since environmental growth conditions are unfavorable and thus the cell responds in an energy conserving way. In this metabolic reprogramming that occurs after the diauxic shift, the coordinate response of large groups of functionally related genes shows the way yeast successfully adapts to environmental changes to achieve survival.

While the main concern in unicellular organisms is their adaptation to growth conditions, in higher eukaryotes multicellularity comes associated with cell differentiation and specialization and the requirement to regulate gene expression in each type of cell. Cells differentiate in tissues by activating different subsets of genes. Due to the existence of numerous different tissues in multicellular organisms, and the need to adapt to the environment for survival in the unicellular organisms, regulation of gene expression becomes very complex and to achieve the goal, this regulation occurs at different levels.

Gene expression covers the whole process from transcription through protein synthesis and is ultimately measured by the amount of protein present in the cell able to perform the function specified by that particular gene. The modulation of gene expression includes not only the rate of transcription of the gene and translation of the mRNA, but also the rates of RNA processing and export from the nucleus to the cytoplasm. In addition, the rates of protein and mRNA decay contribute to the amount of protein present in the cell. All of these processes are highly regulated. In fact, it has been recently accepted that the customized modification of the messenger ribonucleoprotein particle (mRNP) composition contributes significantly to the different gene expression profiles that are achieved by regulation at a post-transcriptional level. mRNPs are composed of

mRNAs associated with RNA-binding proteins and/or small non-coding RNAs such as microRNAs and siRNAs (Moore, 2005). The "RNA operon theory"describes an efficient and flexible way of post-transcriptionally regulating gene expression through coordinated regulation of multiple RNAs at the level of mRNA stability and translation. This regulation occurs through the combinatorial interaction of trans-acting factors targeting multiple mRNA regulatory elements known as USERs (untranslated sequence elements for regulation). In this way, mRNAs that encode functionally related proteins are coordinately regulated during cell growth and differentiation as post-transcriptional RNA operons, orchestrated by the RNP composition (Keene, 2007).

Problems in the proper regulation of gene expression may lead to the development of certain types of cancers in mammals. This can be due to the pathological stabilization of mRNAs that encode for growth factors, cyclins and proto-oncogenes. In normal resting mammalian cells, the turnover of these mRNAs is achieved by the presence of A-U-rich elements (AREs) in their 3' untranslated regions (UTRs) that allow a rapid recruitment of the decay machinery through RNA-binding zinc finger proteins such as tristetraprolin (TTP). It has been experimentally shown that the presence of AREs as antioncogenic targets can affect mRNA turnover, and tumor suppression can be achieved (Stoecklin et al., 2003).

Several other 3' UTR elements exist that regulate mRNA turnover – Puf binding elements are one of them. Taking advantage of the conservation across eukaryotes of several of these elements and proteins, allow us to use yeast as a simple eukaryotic model system to understand their mechanism of action.

mRNA life cycle in eukaryotes

In eukaryotic cells, pre-mRNAs are transcribed in the nucleus where they are capped at the 5' end, polyadenylated at the 3' end, spliced if necessary, and exported to the cytoplasm. Here the mRNAs are translated into proteins in the ribosomes; the longer these mRNAs persist in the cytoplasm, the more they will be translated and thus the larger the amount of protein produced (Fig.1). These mRNAs will finally be degraded, with the length of their lifespans being determined by their translational efficiency and by specific sequences present in their 3' untranslated regions (UTRs). These 3'UTR control elements regulate mRNA translation and decay of a wide variety of transcripts, with lifespans varying by more than two orders of magnitude (Cabrera et al., 1984). Eukaryotic mRNA turnover is physiologically important for the cell not only for regulating gene expression, but also for providing quality control of mRNA biogenesis and antiviral defenses.

Decay pathways for mRNAs in eukaryotes

In eukaryotic cells there are multiple pathways by which polyadenylated mRNAs can be degraded. In yeast, the main degradation pathway begins with the deadenylation of the poly(A) tail at the 3' end, followed by decapping and then 5' to 3' digestion of the transcript (Beelman et al, 1996; Decker & Parker, 1993; Muhlrad et al, 1994) (Fig.2).

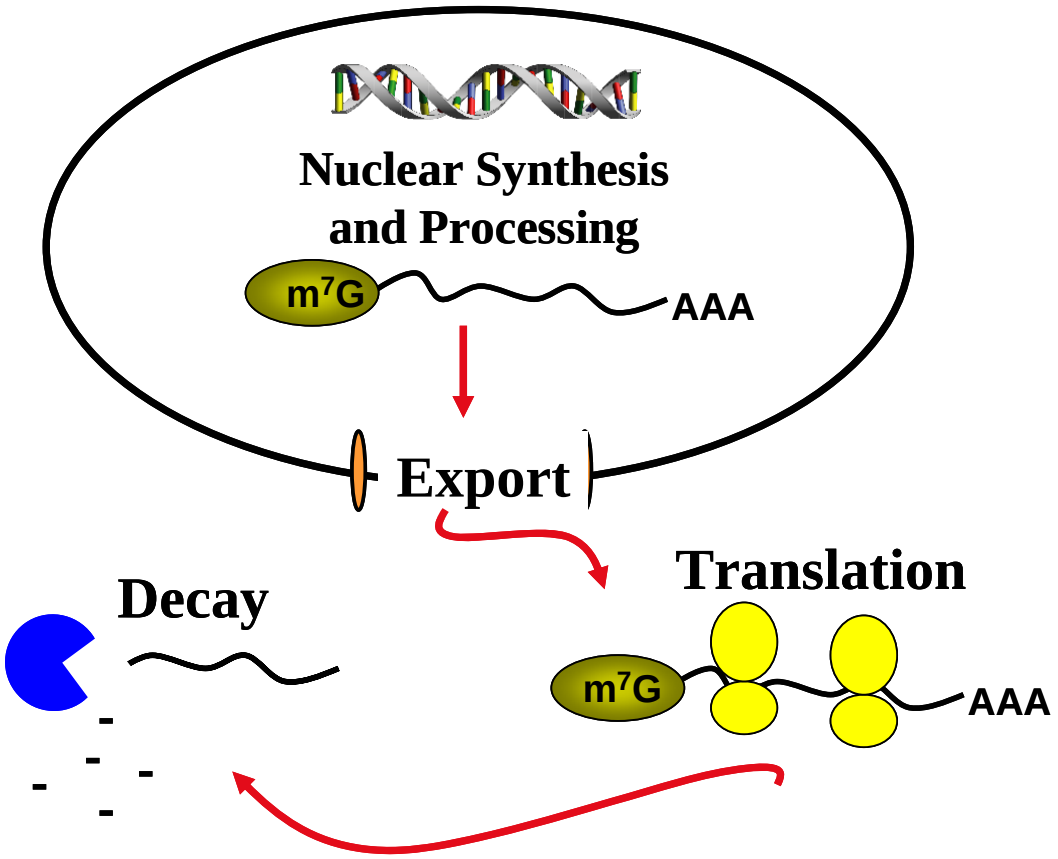


Fig.1 Schematic of a eukaryotic mRNA life cycle. mRNAs are synthesized in the nucleus where they are processed, undergoing splicing, the addition of a 5' cap structure and a 3' polyA tail. These now mature mRNAs are next exported to the cytoplasm where they go through several rounds of translation into proteins in the ribosomes and are finally degraded by the decay machinery of the cell (Figure adapted from Pat Hilleren).

Deadenylation is performed by two mRNA deadenylase complexes: PAN (composed of PAN2p and PAN3p) is involved in the initial shortening of the poly(A) tail, while the Ccr4-Pop2-Not complex deadenylates the remainder of the poly(A) tail. This latter multi-protein complex is built around a core of seven proteins: Ccr4p and Pop2p (both containing a nuclease domain and therefore potentially responsible as deadenylators) and five Not proteins (Not 1 – 5). Published data suggests that Ccr4 provides most of the nuclease activity in yeast (Chen et al, 2002; Tucker et al, 2002). The loss of the poly(A) tail and its associated poly(A) binding (PAB) proteins disrupts the interaction of Pabp with eIF-4F at the 5' end of the mRNA, thus enabling the linearization of the transcript and exposure of the cap to the Dcp1/Dcp2 decapping holoenzyme, with Dcp2p as the catalytic subunit. The MutT motif located within of Dcp2 is found in a class of phosphatases and is necessary and sufficient for Dcp2 to perform its decapping function (Dunkley & Parker, 1999). For efficient decapping in vivo, other proteins that interact with Dcp1/Dcp2 are also required. The heteroheptameric ring composed of Lsm1-7p is thought to rearrange, facilitating the mRNP assembly and activation of the decapping machinery (He & Parker, 2000). Pat1p is required for efficiency of both decapping and formation of processing bodies (P bodies) *in vivo*. P bodies are subcytoplasmic compartments that contain deadenylated non-translating mRNAs and are sites of mRNA degradation (Sheth and Parker, 2003). Another protein, Dhh1p, which is a member of the ATP-dependent DEAD/H box helicase family, is also required for efficient decapping in vivo, as well as interactions with the deadenylation complex ((Hata et al., 2001; Fischer and Weis, 2002). Dhh1p physically interacts in an RNA independent manner with Pop2p, Dcp1p, Lsm1p and Pat1p (Coller et al, 2001). In addition, a protein group known as

enhancer of decapping proteins formed by Edc1p and Edc2p assist decapping (Dunckley et al., 2001). Finally, once the cap structure is removed, the transcript is degraded by Xrn1p exonuclease in a 5' to 3' manner (Coller and Parker, 2004) (Fig.2). There are still many other proteins suggested to be involved in mRNA turnover, but their functions remain unclear.

An alternative decay pathway is 3' to 5' degradation after deadenylation (Fig. 3). This degradation is catalyzed by the cytoplasmic exosome, a large complex of 3' to 5' exonucleases. While this pathway appears to be slower than the decapping and 5' to 3' decay steps for most yeast mRNAs, 3' to 5' decay occurs on all mRNAs when decapping is blocked, and may be the primary pathway for some mRNAs. In mammalian cells, the 3' to 5' decay pathway may be more predominant, and the residual cap structure resulting from such decay is then hydrolyzed by the scavenger decapping enzyme DcpS **(**Decker and Parker, 2002).

In eukaryotic cells, the same degradation machinery is used to process aberrant mRNA transcripts (Fig. 3). Through the nonsense-mediated decay (NMD**)** pathway, transcripts containing premature translational stop codons are detected and rapidly degraded either in a deadenylation-independent decapping fashion performed by Dcp2 (Dunkley & Parker, 1999) or by an accelerated deadenylation and 3' to 5' exonucleolytic digestion. mRNAs that do not possess a stop codon are degraded in a process known as nonstop decay (NSD**)** through a rapid 3' to 5' degradation by the cytoplasmic exosome (Parker and Song, 2004).

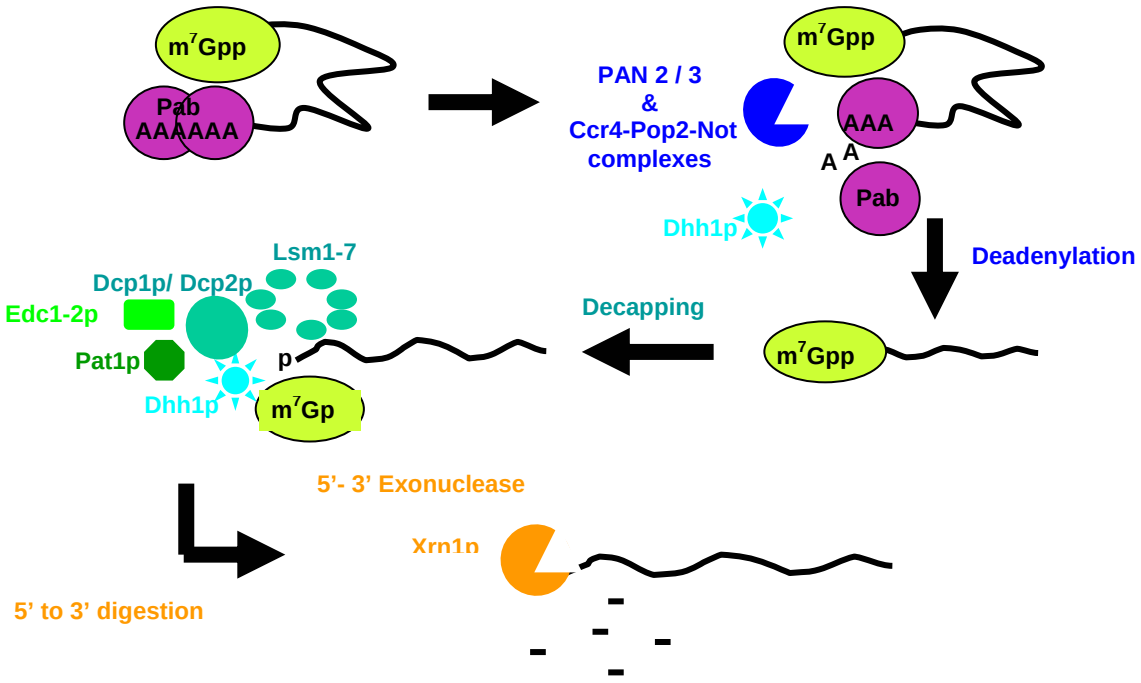


Fig.2 Main mRNA degradation pathway in yeast. The first step is deadenylation, which is performed by two deadenylase complexes: PAN and Ccr4-Pop2-Not. With the loss of the poly(A) tail, the poly A binding proteins (Pab) fall off the RNA, losing cap and tail interaction and thus allowing linearization of the transcript. The second event, decapping, now takes place mainly performed by Dcp1/Dcp2, but aided by several other proteins: Lsm1-7 (hetero-heptameric ring), Dhh1, Pat1 and enhancers of decapping Edc1-2p. The last step involves the exonucleolytic degradation in a 5' to 3' direction by Xrn1p.

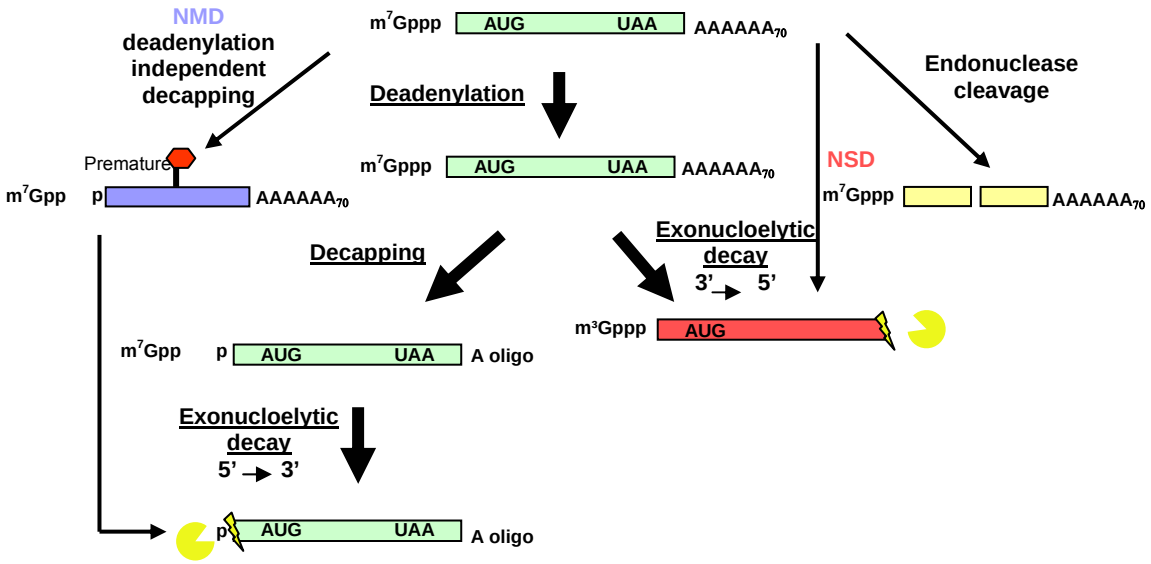


Fig.3 Pathways of mRNA decay (adapted from Parker and Song, 2004). The main degradation pathway in yeast begins with deadenylation, followed by decapping and finishes with 5' to 3' exonucleolysis. An alternative pathway that is more prominent in mammalians starts with deadenylation and is followed by 3' to 5' exonucleolysis by the exosome; DcpS (scavenger decapping enzyme) degrades the residual cap structure. These two first pathways are deadenylation-dependent, while the three remaining are independent of the deadenylation step. The lack of a stop codon triggers fast exosome degradation through non-stop decay (NSD). The detection of a premature stop codon in a transcript signals to the nonsense-mediated decay (NMD) pathway, responding with the rapid decapping of the aberrant mRNA species that has not been deadenylated. Another alternative begins with an endonucleolytic cleavage.

RNA-binding proteins: the Puf family of proteins

General properties and characteristics

There are several different types of RNA-binding proteins that specifically interact with sequences in 3'UTRs to regulate the decay of the transcripts to which they bind. The Puf family of RNA-binding proteins is one such family that is widely conserved throughout the eukaryotic kingdom. The Puf family is characterized by eight imperfect repeats present in the RNA binding region known as the Pumilio homology domain (PUM-HD). Each repeat consists of 36 amino acids arranged in three alpha-helices, with a "core consensus" of aromatic and basic residues (Zhang et al, 1997; Zamore et al, 1999). Wang et al. have solved the crystal structure of the PUM-HD from human Pumilio1 bound to an mRNA target (Fig.4). They show a crescent shape of this domain in which the concave surface binds to the RNA while the outer convex surface interacts with other proteins.

Puf proteins have been studied in different organisms such as human, mouse, frog, fly, worm and yeast. Pumilio in Drosophila (Dm-PUM) and FBF in C. elegans were the founding members of the family. All Puf proteins studied to date bind regulatory elements in the 3'UTRs of their target mRNAs. By doing so, they post-transcriptionally control expression by stimulating decay and repressing translation. In Drosophila and C. elegans, Pufs target mRNAs that encode key regulators of development. In both cases, Dm-PUM and FBF require interactions with other proteins to regulate the expression of their target mRNAs. Pumilio together with Nanos and Brat generate a gradient of hunchback mRNA expression necessary for normal development of posterior embryonic patterning in flies (Wharton & Struhl, 1991; Murata & Wharton, 1995). Similarly, FBF in

C. elegans physically interacts with NOS-3 and participates in the sperm-oocyte switch by forming a regulatory complex that controls fem-3 mRNA (Kraemer, et. al. 1999). Not only are Drosophila and C. elegans Pufs involved in cell development and differentiation, but human and Xenopus Pufs also regulate germ cell development and oocyte maturation (Moore, 2003; Nakahata, 2003). By repressing translation of cyclin B mRNA in Drosophila and gld-1 mRNA in C. elegans, Pufs achieve regulation of germline stem cell development and maintenance (Forbes and Lehmann, 1998; Crittenden, 2002). In another area Dm-Pum affects neurons by regulating proper neuronal excitability, dendrite morphogenesis, long-term memory formation, and synaptic growth and plasticity (Schweers, 2002; Mee, 2004; Ye, 2004; Dubnau, 2003; Menon, 2004). Human Pum-2 is also present in neurons and has been shown to participate in dendritic stress granule formation (Vessey, 2006). More recent results show that while both human Pufs (PUM1 and PUM2), have a 91% amino acid identity in their homology domain and share association with functionally related groups of mRNAs, they also uniquely target particular set of transcripts. For example, PUM1 targets angiogenesis-related transcripts, while PUM2 targets transcripts linked to Parkinson's disease (Galgano et al, 2008). Predicted miRNA binding sites seem to be significantly enriched in 3'UTRs of PUM1 and PUM2 experimentally-determined targets. These findings suggest possible functional interactions between human Pufs and the miRNA regulatory system as a way of combinatorial mRNA regulation. This hints towards a higher, more precise way of regulation that is achieved through network crosstalk between different post-transcriptional regulatory systems.

In *Saccharomyces cerevisiae*, six Pufs have been identified (Fig.5). Puf proteins 1-5 appear to primarily regulate the decay of target mRNAs, while the known mRNA target of Puf6p is regulated at the level of translation. Puf3p controls mitochondrial function and metabolism (Glerum et al, 1996; Olivas and Parker, 2001), Pufs 4, 5 and 6 regulate different aspects of mating type switching (Sil and Herskowitz, 1996; Tadauchi et al, 2001; Gu et al, 2004; Goldstrohm et al, 2006), while 1, 4 and 5 regulate mRNA targets involved in translation efficiency (*TIF1)* and sugar metabolism (*HXK1)* (Ulbricht and Olivas, 2008)

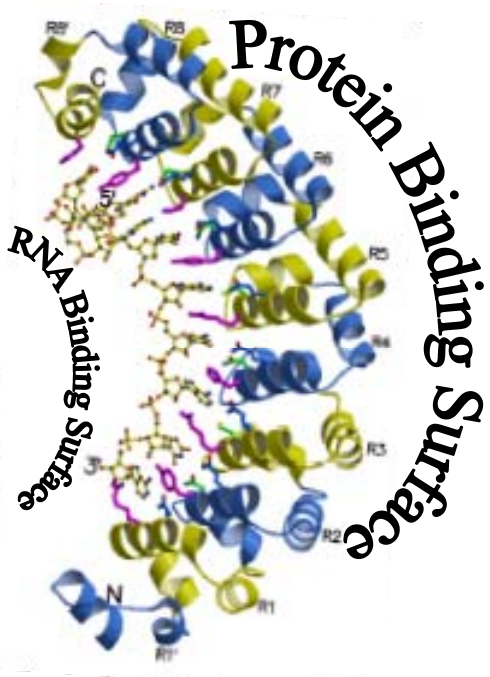


Fig.4 Human PUM-HD crystal structure bound to RNA. The concave inner surface of the protein binds the RNA, while the convex outer surface interacts with other proteins (adapted from Wang et.al. Cell, 2002).

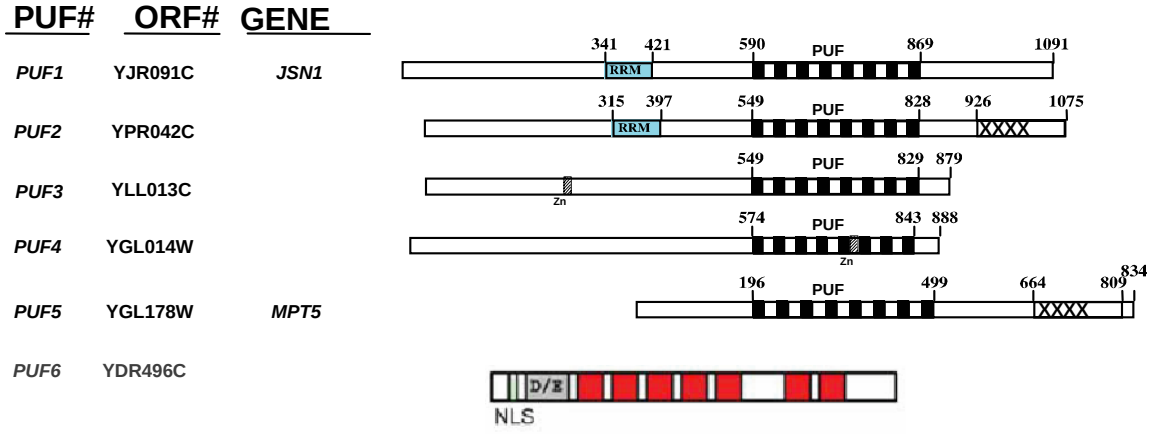


Fig.5 Yeast Puf proteins. Alignment and sequence elements of the five yeast Puf proteins. Each Puf repeat region is denoted as eight black (or red) vertical rectangles. Puf1p and Puf2p also contain putative RRM RNA-binding domains (blue boxes), while Puf3p and Puf4p contain putative zinc finger domains (box labeled Zn). A C-terminal sequence region related in Puf2p and Puf5p is denoted by boxes labeled XXXX (adapated from Olivas et al., 2000). Puf6p consists of 656 amino acids and contains seven Puf repeats between amino acids 171 and 419. The D/E region is rich in aspartic and glutamic acids. A nuclear localization signal (NLS) is also present (adapted from Gu et al., 2004)

Puf-mediated decay in yeast

COX17 mRNA encodes a protein that transports copper into the mitochondria for cytochrome oxidase assembly, and this mRNA was experimentally shown to be a target of Puf3p-mediated decay. Puf3p specifically promotes *COX17* mRNA decay by binding two conserved UGUANAUA sequences present in the 3'UTR of *COX17* mRNA, thereby promoting the mRNA's rapid deadenylation and decay (Olivas and Parker 2000; Jackson et al, 2004). Transcriptional shut off experiments were carried out with WT and *puf3Δ* strains, which have a temperature sensitive RNA polymerase II, in order to compare *COX17* mRNA half-lives in both strains. In these strains, transcription can be arrested by switching from a permissive (24°C) to a non-permissive (37°C) temperature. Several samples of the culture are collected at different experimental time points after transcription is stopped, and the RNA from each sample is extracted and visualized by Northern blot analysis to monitor how quickly an mRNA decays over time. The results showed more than a 5-fold difference in the half-life of *COX17* mRNA from the WT strain (3 minutes) versus the *puf3Δ* strain (17 minutes), indicating that Puf3p promotes *COX17* mRNA decay.

Recently in our lab several new Puf3p mRNA targets have been experimentally confirmed, including *TUF1* and *CYT2* (Melanie Miller, personal communication). These targets had previously been identified as possible Puf3p candidates through co-immunoprecipitation experiments and microarray analyses. These types of high throughput experiments identified several hundreds of functionally related mRNAs that were pulled down with each one of the five TAP-tagged Pufs. Results from these experiments showed that Puf3p preferentially bound mRNAs encoding mitochondrial proteins, while Puf4p associated mainly with mRNAs encoding nucleolar

ribosomal RNA-processing factors. Puf1p and Puf2p were mainly pulled down together with mRNAs that encoded membrane associated proteins and Puf5p preferentially bound mRNAs encoding chromatin modifiers (Gerber et al, 2004). It has also been recently shown that Puf3p co-immunoprecipitates with the mitochore (required for cytoskeletal interactions) and the Arp2/3 complex (required for mitochondrial movement towards the bud using actin cables) (Garcia-Rodrigez, L. J., 2007).

Candidate Puf target RNAs were also identified by an alternative computational method. The Bussemaker lab developed a high throughput algorithm termed Matrix REDUCE (for Regulatory Element Detection Using Correlation with Expression), which was applied to a set of ~ 700 microarray experiments. This algorithm identifies mRNAs whose levels are coordinately regulated and contain similar 3'UTR sequence elements. As a result, several candidate mRNA 3'UTR cis-regulatory elements were identified, including predicted binding sites for Puf3p and Puf4p (Foat et al, 2005).

Though *HO* mRNA had originally been identified as a Puf5p target (Tadauchi et al., 2001)), it has been recently shown that both Puf4p and Puf5p stimulate its decay by enhancing its deadenylation. *HO* mRNA is greatly stabilized in a *puf4-puf5* double deletion strain compared to WT or *puf5*Δ (Goldstrohm et al., 2006). In our lab two other mRNAs, *TIF1* and *HXK1*, have been established as Puf-mediated decay targets with multiple Puf proteins involved in their regulation. *TIF1* mRNA is regulated both by Puf1p and Puf5p while *HXK1* mRNA requires Puf1p, Puf4p and Puf5p for its full decay stimulation. The absence of any of the Pufs is sufficient for obtaining partial decay phenotypes. This suggests a coordinated regulation rather than a redundant performance (Ulbricht and Olivas, 2008).

Pufs and their interaction with the decay machinery

We have shown that the repeat domain of Puf3p is sufficient both for binding and regulating *COX17* mRNA decay (Jackson, et. al., 2004). We therefore speculated that Puf3p may interact directly or indirectly with the decay machinery (such as deadenylation and decapping enzymes) through its repeat domain. Co-immunoprecipitation experiments were performed by Sean Houshmandi in the Olivas lab to determine whether proteins that play a role as decay factors were interacting with Puf3p, specifically with its repeat domain. He tested the deadenylation factors Ccr4p and Pop2p, and the decapping factors Dcp1p, Lsm1p and Dhh1p. For these experiments he endogenously myc-tagged each one of these factors in a *puf3*Δ strain, then each individually myc-tagged strain was transformed with a plasmid expressing FLAG-tagged Puf3RD. The assay involved the immunoprecipitation of FLAG-Puf3RD onto an anti-FLAG resin, along with any other proteins that might be interacting with Puf3RD. After several washes, the eluates from the resin were electrophoresed on an SDS-PAGE gel and blotted. The western blot obtained was hybridized with anti-myc antibodies to visualize any myc-tagged decay factors that had co-immunoprecipitated with Puf3RD. The results showed a positive interaction between Puf3RD and all five proteins tested, suggesting that the repeat domain may regulate *COX17* mRNA decay by binding to the decay machinery (Fig.6) (Sean Houshmandi, unpublished data).

Studies were done to further understand the key amino acids within the repeat domain that are involved in the specific *COX17* mRNA binding and decay regulation. In vitro binding and in vivo functionality studies of several Puf3RD mutant proteins were performed. A Puf3RDp structure was created using Swiss-Model and aligned with Hs-

Pum and Dm-Pum of known structure (Houshmandi and Olivas, 2005). It is known that NANOS and BRAT in Drosophila bind the outer surface loops of PumRD between repeats 6, 7 and 8 (Edwards et al., 2001). Similar loop structures on the outer convex surface of Puf3RDp were identified. Therefore, amino acids located in these loops were predicted to be involved in protein interactions, so four different loop deletion mutants were created and tested (R6A, R6B, R7A and R7B) (Fig.7, Houshmandi and Olivas, 2005). The R6B and R7B mutants were shown to inhibit proper RNA binding and decay, and were thought to alter overall Puf3RD structure. The R6A mutation showed no effect on *COX17* binding or regulation. However, the R7A mutation did not inhibit binding, but eliminated proper regulation. Specially, the Puf3RDp-R7A mutant in a puf3Δ strain was unable to rescue rapid decay of the COX17 mRNA as did the WT Puf3RDp. This suggests that the R7A loop region may be critical for interactions with proteins involved in RNA decay. In my work, I analyze the effect of the R7A mutant on binding of Puf3p to the myc-tagged decay factors. In addition, I use two other alternative methods, the yeast-two hybrid assay and modified yeast three-hybrid approach, to further study Puf3RD protein interactions.

Similar to the findings of Puf3RD interacting with the decay machinery, TAP-tagged Puf5p was recently shown to co-immunoprecipitate with T7 epitope-tagged deadenylation and decapping factors (Ccr4, Pop2, Dcp1 and Dhh1). In vitro experiments showed that Puf5RD bound directly to Pop2p in an RNA independent manner (Goldstrohm et al., 2006). Using our FLAG-tagged Puf3RD, my work has analyzed the RNA dependence of its interactions, as well as the directness of its protein interactions.

Puf5p in yeast stimulates *HO* mRNA deadenylation. For in vivo and in vitro regulation both Pop2p and Ccr4 are required, even when the enzymatic activity is performed by Ccr4. It is hypothesized that Pop2p plays a role in bridging Ccr4 as the catalytic deadenylator to Puf5p and the *HO* mRNA in this event. Ccr4 by itself in a *Pop2*Δ strain has a non-specific deadenylation activity, and the presence of Puf protein is unable to enhance this activity. Thus, the presence of Pop2p is required to promote *HO* mRNA deadenylation by Ccr4 in a Puf-mediated manner (Goldstrohm et al., 2006).

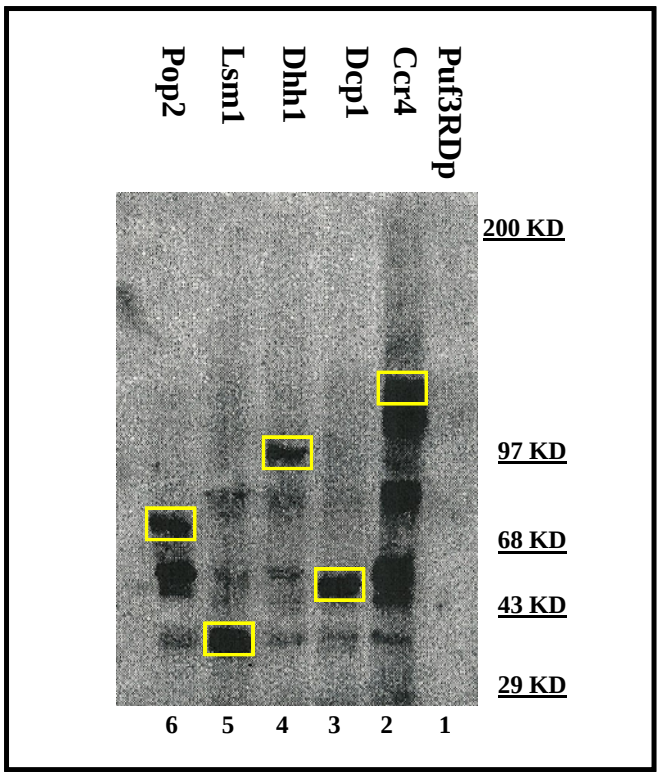
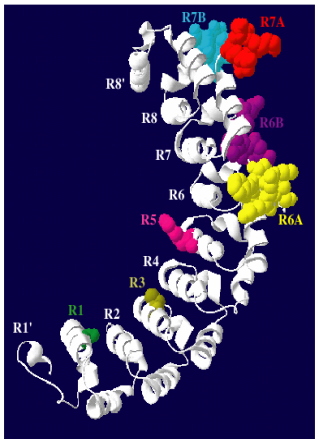


Fig.6 Puf3p interaction with decay factors as analyzed by co-immunoprecipitation experiments. Western blot showing the myc-tagged decay factors (Ccr4, Dcp1, Dhh1, Lsm1 and Pop2) that have been pulled down by interaction with FLAG-Puf3RD. The decay factors were detected using anti-myc antibodies. FLAG tagged Puf3RD was immunoprecipitated using anti-FLAG resin. (Sean Houshmandi, unpublished data).

Fig.7 Predicted 3-D structure of Puf3-RD showing the regions where deletions were made. R6A and R6B are colored in yellow and purple respectively. R7A and R7B are shown in red and blue. These four mutations are localized on the outer surface of Puf3-RD. The R7A loop mutation was the only one that did not inhibit binding of the protein to RNA yet did affect its decay rate (Houshmandi et. al., 2005).



The effect of environmental conditions on Puf3p activity

Not only can proteins that interact with Puf3p affect its ability to regulate *COX17* mRNA, but environmental conditions can alter Puf3p activity as well. It was experimentally verified that the ability of Puf3p to destabilize its target mRNA is dependent on a fermentable carbon source as predicted by computational analyses of microarray data. Through transcriptional shut-off experiments it was shown that a hybrid transcript (*MFA2/COX17*) that contains the *COX17* 3'UTR, and is thus under Puf3p regulation, has a half-life four-fold longer in ethanol compared to glucose conditions. In contrast, the *MFA2* transcript that is not Puf–regulated does not show any significant difference in its decay between these conditions (Foat et al, 2005) (Fig.8). This indicates that Puf3p is not functioning to enhance decay in ethanol conditions. In my work, I further investigate the mechanisms by which Puf3p activity is altered by different environmental conditions.

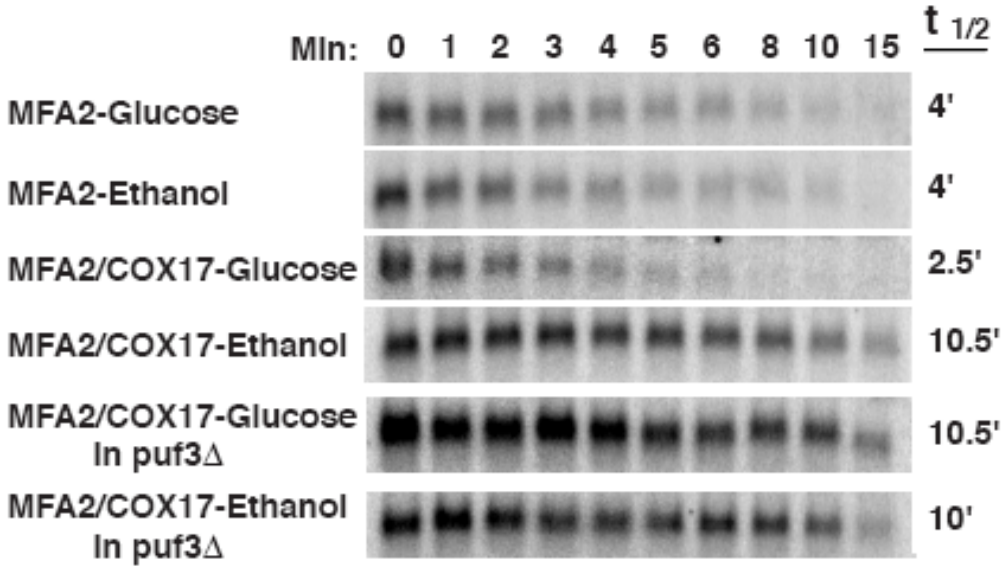


Fig.8 Puf3p activity is altered in different carbon sources. Northern blot analyses of the decay of *MFA2* mRNA and the hybrid *MFA2/COX17* in both 2% glucose and 2% ethanol media are shown in WT and *puf3*Δ strains. t½ indicates the half-lives of the mRNAs after transcription is shut off at the 0 minute time point (Foat et. al, 2005).

Dissertation overview

In Chapter II, the general experimental methodology used for this research is presented. Chapter III focuses on the mechanism of action of Puf3p to mediate rapid *COX17* mRNA decay. Protein-protein interactions between Puf3RD and the decay machinery are shown both through co-immunoprecipitation experiments, directed yeast two-hybrid and modified yeast three-hybrid assays. These interactions with the deadenylation and decapping factors are RNA independent and the R7A loop of the Puf3RD is found to be required for binding of Pop2p and Dhh1p, but not the other decay factors. *COX17* mRNA decay rate is shown to be dependent on all the tested decay factors including Dcp1p, Dhh1p and Lsm1p. Strains deleted of these decay factors show a half-life similar to that of a *puf3*Δ. It is also shown that *COX17* mRNA decay, is independent of the NMD (nonsense mediated decay) pathway.

In Chapter IV, Puf3p activity is analyzed by comparing the decay rate of *COX17* mRNA from a WT strain grown under different environmental conditions. Amounts of Puf3 protein and mRNA are compared under glucose, ethanol, galactose and raffinose conditions. Both Puf3 protein and mRNA levels seem to be equal or even higher under the latter three conditions in which the *COX17* mRNA decay phenotype corresponds to that of an inactive protein. This suggests that Puf3p activity is controlled post-translationally. This hypothesis is addressed by investigating whether post-translational modification of the protein through phosphorylation might be involved. Results indicated Puf3RD can be phosphorylated. Using a comparative mutational analysis of particular serines or tyrosines of the Puf3RD, different decay phenotypes are found. Using a C-terminal GFP-tagged Puf3p fusion protein, experiments are conducted to visualize any

difference in localization or aggregation of the protein under different environmental conditions.

Chapter V discusses the identification of Puf4p mRNA targets and the investigation of its activity under different conditions. Together, all of this information will help us better understand the mechanism of action and the role of Puf proteins as important regulators of mRNA metabolism.

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Chapter II

General experimental methodology

Comparing mRNA decay rates

Transcriptional shut-off analysis

The transcriptional shut-off experiment is one of the main tools used to analyze and compare mRNA decay rates between different yeast strains. The strains used for these experiments carry a mutation in RNA polymerase II (rpb1-1) which makes the polymerase functional at 24˚C, but inactive at 37˚C. Taking advantage of this mutation, transcription of mRNAs can be stopped by switching the yeast culture from a permissive temperature of 24˚C to the non-permissive 37˚C. Specifically, yeast cultures are grown to mid-log phase (OD 0.4) at 24˚C, then the cells are harvested by centrifugation and resuspended in 37˚C media. An aliquot of the culture is taken at this time (the 0 minute time point), representing the steady-state RNA pool at the time of transcriptional arrest. Culture aliquots are then collected at increasing time points after transcription is arrested (Fig.1). RNA is extracted from cells and visualized by northern blot analysis (Olivas and Parker, 2000). Equal amounts of total RNA are loaded on 1% agarose gels, electrophoresed and transferred to a nylon membrane to which the RNA is cross-linked by UV-light exposure. *7S* is used as a loading control and both the RNA of interest and the *7S* are detected using radio-labeled probes with P32. An example of this technique is shown in Figure 2, where the *COX17* mRNA is analyzed in a WT strain versus a *puf3*Δ strain. The half-life of an mRNA is determined as the time it takes for half of the steady–state pool of mRNA at time zero to decay away.

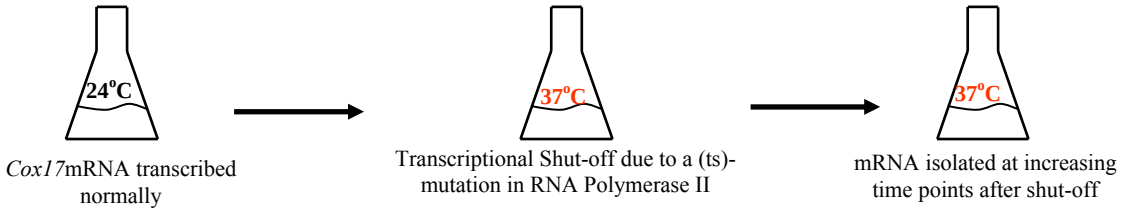

Fig.1 Diagram showing the different steps for performing a transcriptional shut-off experiment. The strain that is used has an RNA Polymerase II that is temperature sensitive, meaning that it is only functional at 24˚C. When the yeast cells are shifted to 37˚C, transcription is arrested (pol II is non-functional) and from that point onwards no more mRNA will be produced. Decay rates of mRNAs can be compared in different strains by gathering samples of the culture at increasing time points and visualizing RNA on a northern.

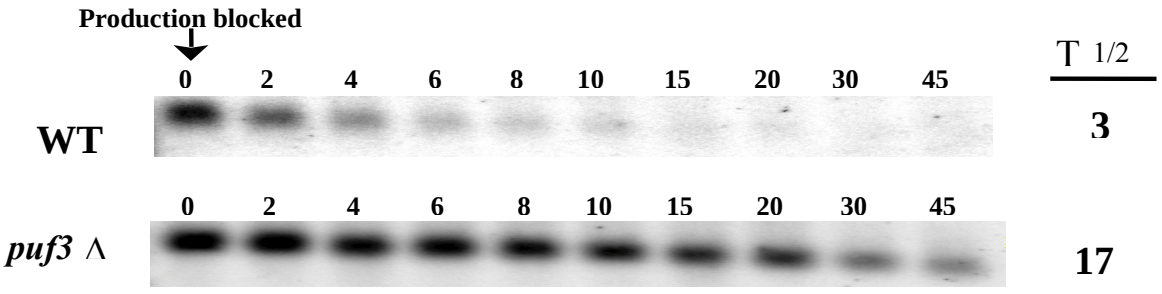

Fig.2 Northern blot analysis of transcriptional shut-off experiments in WT and *puf3*Δ strains. Puf3p promotes rapid decay of *COX17* mRNA. T ½ indicates the half-life of the mRNA in each strain (Olivas and Parker, 2000).

Analysis of protein levels

For measuring the amount of Puf3 protein present in yeast cultures growing in different carbon sources, proteins were extracted using the protein boil prep method or the YPER method. The main difference between the two methods is that while the YPER method only extracts the soluble portion of the proteins, the protein boil prep accounts for the extraction of both soluble and insoluble proteins. In the latter method, pelleted cells from a 10 ml yeast culture are resuspended in 100 µl of sample buffer in a 1.5 ml tube. Glass beads are added, and cells are disrupted by three consecutive cycles of vortexing and boiling at one minute each. A needle hole is made in the bottom of the tube, the sample is centrifuged at 4000 rpm for 2 minutes and the clear supernatant (protein extract) is collected.

In the YPER method, the cell pellet obtained form the culture is resuspended in a buffer that consists of the YPER solution (Pierce) with the addition of 10% DTT and a mini-complete protease inhibitor cocktail tablet for every 10 ml of solution. Then the sample is rocked at room temperature for 1 hour to disrupt the cells. The samples are centrifuged and the clear supernatant (protein extract) is collected.

Identifying specific protein-protein interactions

Directed Yeast two-hybrid assay

This is one of the assays used to determine protein-protein interactions with Puf3p. “The two-hybrid system utilizes two plasmid-borne gene fusions that are co-transformed into a host yeast strain containing inducible reporter genes” (James et al.,

1996). A positive physical interaction between both fusion proteins would bring the binding and activation domains of each fusion protein in close proximity, resulting in the transcription of a reporter gene. To analyze interactions with Puf3p, different regions of Puf3p were cloned as gene fusions to the DNA binding domain of *GAL4* and were expressed from the high copy plasmid pGBDU-C2 containing a *URA3* marker. These fusions were used as baits. The different proteins to be tested for interactions with Puf3p were cloned as gene fusions to the DNA activation domain of *GAL4* and were expressed from the high copy plasmid pGAD-C2 containing a *LEU2* marker. These fusions were used as the prey proteins. The bait plasmids were transformed into PJ694α, mating type α, while the prey plasmids were transformed into PJ694a, mating type a. The mating of the transformed parental strains containing bait and prey plasmids resulted in a diploid that is co-transformed with both plasmids and selected by growth in –leu,-ura media. Positive protein interaction is screened by growth on selective –leu,-ura,-his plates, where the histidine gene *HIS3* is the reporter gene (Fig.3).

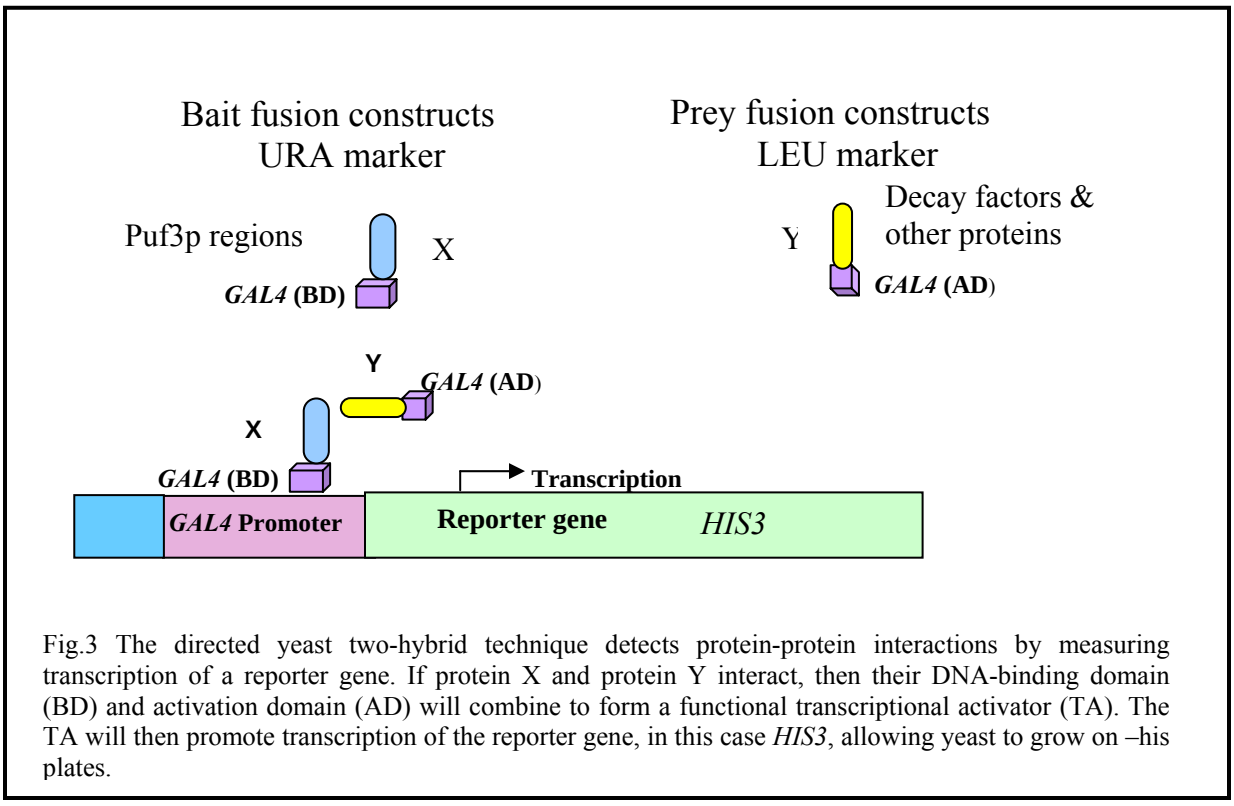

Fig.3 The directed yeast two-hybrid technique detects protein-protein interactions by measuring transcription of a reporter gene. If protein X and protein Y interact, then their DNA-binding domain (BD) and activation domain (AD) will combine to form a functional transcriptional activator (TA). The TA will then promote transcription of the reporter gene, in this case *HIS3*, allowing yeast to grow on –his plates.

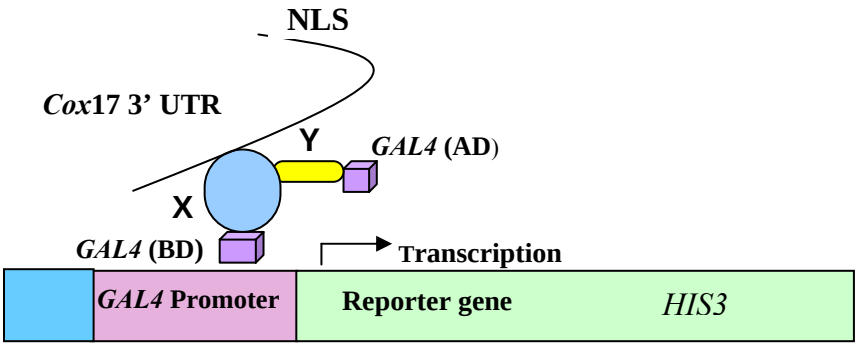

Fig.4 The modified yeast three hybrid assay requires the presence of the target RNA in the nucleus where transcription occurs. This will allow any conformational change induced by RNA binding to occur in the bait protein in order for it to interact with the prey. A positive interaction is detected by the expression of the reporter gene.

Modified yeast- three hybrid assay

Some RNA-binding proteins must bind to their RNA target before they are able to stably interact with other proteins. To analyze this kind of protein-protein interaction, a modified yeast three-hybrid assay is used. The assay utilizes the same concept as the directed yeast-two hybrid, but with the addition of the expression of a target RNA containing a NLS (nuclear localization signal) that will allow it to enter and interact with the bait/prey proteins in the nucleus. In this assay, the pIIIMS2-2 plasmid (*TRP1* marker) expressing *COX17* 3'UTR was co-transformed into strains containing the Puf3-Repeat Domain fusion bait construct. The diploids obtained from the mating of this strain with strains containing the prey plasmids were selected on –trp,-ura,-leu plates. To test for protein-protein interactions in the presence of the *COX17* 3'UTR, yeast colonies were selected on –trp,-ura,-leu,-his plates, with *HIS3* as the reporter gene (Fig.4).

Co-immunoprecipitation assays

An alternative procedure used to analyze protein-protein interactions was the co-immunoprecipitation assay. All strains used for this assay were *puf3*Δ strains transformed with a plasmid expressing FLAG-tagged Puf3p. Five different yeast strains were used in which the genes coding for decay factors *CCR4, DCP1, DHH1,* LSM1 or POP2 were individually myc-tagged by homologous recombination (Sean Houshmandi, unpublished data). The strains used in these co-immunoprecipitation experiments are shown on table 1 p.55 Ch. III. Yeast cells pelleted from 400 ml cultures were resuspended in 800 µl of IP buffer with protease inhibitor and 10% glycerol. Glass beads were added to these pellets and five consecutive cycles of vortex – ice of 2 minutes each allowed cell lysis. Protein

extracts were quantitated using the colorimetric assay to measure equal amounts of crude protein extracts at the beginning of the assay. The assay was performed by incubating the different protein extracts obtained from each one of the strains for 45 minutes with the anti-FLAG resin and immunoprecipitating FLAG-Puf3RD onto the resin, along with any other proteins that might be interacting with Puf3RD. After several washes the eluates from the resin were electrophoresed on an SDS-PAGE gel and blotted. The western blot obtained was hybridized with specific anti-myc antibodies then HRP-tagged anti-mouse secondary antibodies to visualize any myc-tagged decay factors that had co-immunoprecipitated with Puf3p (Fig. 5).

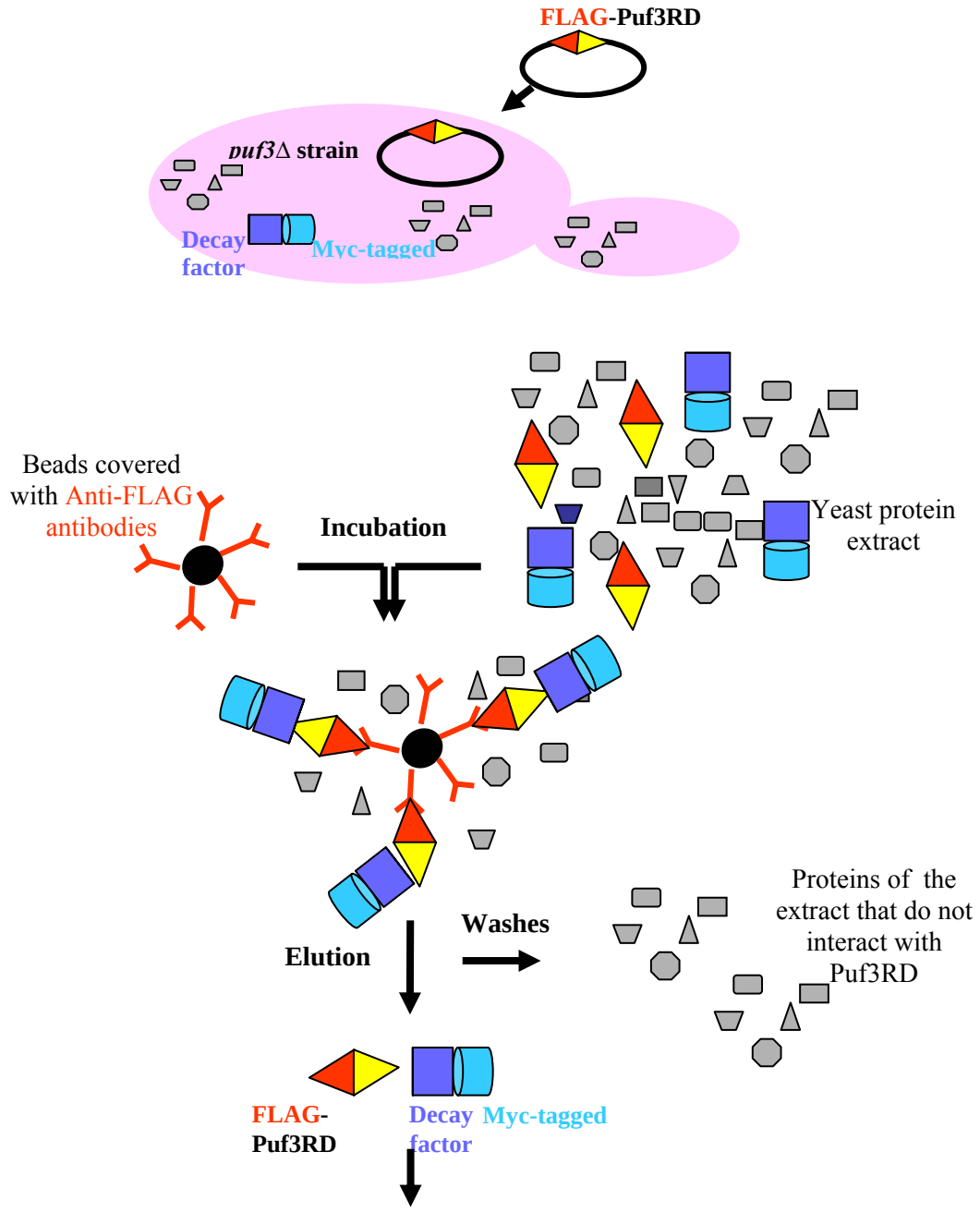

Fig.5 Diagram showing the main steps of the co-immunoprrecipitation experiment. Protein extracts obtained from yeast cultures expressing both FLAG-Puf3RD and a myc-tagged decay factor are incubated with anti-FLAG resin. The anti-FLAG antibodies will pull down FLAG-tagged Puf3RD and any interacting myc-tagged decay factor. After three consecutive washes of the beads, the only remaining proteins should be the ones interacting with Puf3RD. The eluted samples are run on an SDS-PAGE and electroblotted on a nitrocellulose membrane.

Epi-fluorescence microscopy

Fluorescence is based on the property fluorophores have in absorbing short wavelength light and producing a longer wavelength light which is emitted and detected. Epi-fluorescence is a type of excitation-emission configuration, in which both the illumination and emission light travel through the objective and reaches the specimen from above. In our experiments we used Green Fluorescent Protein (GFP) as the fluorophore molecule that was fused to the protein of interest (Puf3p) to be able to detect differences in appearance or localization of Puf3p-GFP when grown in minimal media using different carbon sources.

A mercury or xenon lamp produces excitation light with several wavelengths. An excitation filter removes the unwanted frequencies from the light emitted except those that will be used to cause the sample to fluoresce. A dichroic beam splitter (mirror) reflects blue light (~ 395nm for GFP) down through the objective lens and onto the specimen. GFP molecules in the specimen (yeast cells) emit fluorescence (~509nm), which passes up, through the objective lens, through the dichroic mirror to be detected by eye or a camera. The barrier filter just above the dichroic mirror selects a particular wavelength range so that background noise is eliminated. Figure 6 is an example for visualizing GFP expression of Dcp2-GFP tagged protein that is known to localize to P-bodies versus a negative control strain.

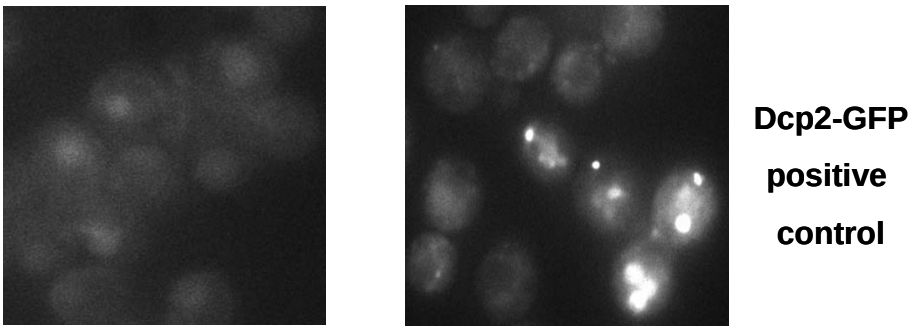

Fig.6 GFP tagging. The image on the left corresponds to a yeast strain used as a negative control that lacks a GFP protein. The image on the right shows a Dcp2-GFP tagged strain; Dcp2 localizes to discrete cytoplasmic foci known as p-bodies.

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Chapter III

How does Puf3p regulate decay?

Introduction

One way of regulating gene expression in the cell is by affecting mRNA turnover. In yeast, the main mRNA decay pathway involves deadenylation-dependent decapping followed by 5' to 3' exonucleolytic degradation by Xrn1p. The deadenylation of an mRNA not only includes the poly(A) tail removal by Ccr4-Pop2, the main deadenylator in yeast, but also implies a reorganization of the mRNP ribonucleoprotein, which will mean a transition from a translating mRNA to a transcript targeted for degradation (Schwartz and Parker, 1999; Tharun et al, 2000). This transition involves the loss of Pab1p associated with the poly(A) tail and the loss of translation initiation factors eIF4E and eIF4G associated with the cap structure. These changes allow the mRNA to associate with Dhh1p and the Lsm1-7p complex, which enhance the interaction of the mRNA with the Dcp1/Dcp2 decapping enzyme complex (Tharun and Parker, 2001). Physiologically, mRNAs can thus be described as belonging to one of two sets of functionally different groups in the cell, one group associated with polysomes involved in translation and the other as a non-translating pool sequestered in discrete cytoplasmic foci where the mRNAs are substrates for decapping and subsequent decay. Sheth and Parker were the first to identify these foci in yeast, which they called processing bodies or p-bodies (Sheth and Parker, 2003). In these sites, decapping and 5' to 3' exonucleolysis occur, being therefore sites of congregation of Dhh1p, Dcp1/2p, Xrn1p, Lsm1p and Pat1p with a pool of deadenylated, non-translating mRNAs that are no longer bound to translation initiation factors eIF4E and eIF4G.

Dhh1p, which is required for efficient decapping of mRNAs after deadenylation, belongs to a highly conserved subfamily of DEAD box helicases. Dhh1p has been shown

to physically interact in an RNA- independent manner with Pop2p, Dcp1p, Lsm1p, Pat1p and Xrn1p (Coller et al., 2001)**.** Though Dhh1p is not essential for yeast viability, it has been described as a regulator of mRNA decapping (Fischer et al., 2002) and is present in p-bodies**.** Yeast strains lacking Dhh1p tend to accumulate transcripts with an intact cap structure, but that have lost the poly(A) tail. The Dhh1p homolog in Xenopus, Xp54, is also a DEAD-box helicase described as an integral component of mRNP particles of Xenopus oocytes (Weston et al, 2006). Since Dhh1p co-localizes to p-bodies, there could be mechanistic similarities between mRNA turnover in the deadenylation-dependent decay pathway in yeast and maternal mRNA storage in higher eukaryotes. These helicases are considered key regulators of post-transcriptional gene expression since they modulate mRNA metabolism by remodeling mRNPs for entry into translation, storage or decay pathways (Weston et al., 2006)**.**

In this chapter, I dissect the molecular mechanism of Puf3p-mediated *COX17* mRNA decay. Utilizing genetic and biochemical approaches, I propose that Puf3p plays a central role in the recruitment of both deadenylators and decapping factors to promote mRNA degradation by directing protein-protein interactions between the Puf3RD and the decay machinery.

Goldstrohm et al have recently shown that Puf4p and Puf5p regulate *HO* mRNA decay by enhancing its deadenylation rate (Goldstrohm et al., 2006). This mRNA encodes a DNA endonuclease required for mating type switching in yeast. The authors showed that TAP-tagged Puf5p co-immunoprecipitated with T7 epitope tagged decay factors: Ccr4, Pop2, Dcp1 and Dhh1. The Puf5p-decay factor interactions were all RNA independent. They also demonstrated that only Pop2 can bind Puf5p in vitro, and found

the interactions to be evolutionarily conserved by testing Pufs and Pop2p from different species. They proposed that Pop2p is the bridging molecule between Puf5p and all the other decay factors. The presence of Pop2p is required to promote *HO* mRNA deadenylation by Ccr4 in a Puf-dependent mechanism. *HO* mRNA deadenylation occurs at a slow basal rate in a puf4-5Δ strain since the Ccr4-Pop2-Not complex is not efficiently recruited to its 3' UTR (Hook et al., 2006). In addition, the absence of Ccr4p, Puf5p, but not Puf4p, was still able to repress mRNA expression into protein by an unknown mechanism independent of deadenylation but dependent on Pop2p.

In our lab, the Puf3p repeat domain alone has been shown to be sufficient and essential for both in vitro binding to the *COX17* 3'UTR and in vivo stimulation of *COX17* mRNA decay (Jackson et al, 2004). The Puf3RDp-R7A mutant was created by deletion of the R7A outer surface loop of the Puf3RDp. This mutant was shown to be incapable of mediating rapid *COX17* mRNA decay, but was still capable of mRNA binding. This region was thus predicted to be involved in protein-protein interactions important for signaling to the decay machinery (Houshmandi and Olivas, 2005). The focus of this chapter is to study the role Puf3RDp plays in recruiting the decay machinery to promote *COX17* mRNA decay. Through in vivo co-immunoprecipitation experiments (co-IPs), Puf3RDp was found to interact in an RNA-independent manner with Ccr4p, Dcp1p, Dhh1p, Lsm1p and Pop2p. All of these proteins were found to be actively involved in the deadenylation and decapping of *COX17* mRNA. The R7A loop was also identified as the region of the repeat domain involved in the binding of Pop2p and Dhh1p. Furthermore, co-IPs were performed in *pop2Δ* strains, demonstrating that Pop2

bridges Dhh1 binding to the Puf3RD through the R7A loop, but Pop2p is not required for Dcp1p binding to Puf3RD.

Puf3-mediated *COX17* mRNA decay involves rapid deadenylation and decapping rates (Olivas and Parker, 2000). In the yeast cell, the decay machinery is shared by different decay pathways, including the Nonsense Mediated Decay (NMD) pathway, which is in charge of the degradation of aberrant mRNAs as well as many normal mRNAs with short lifespans. To determine whether the NMD pathway was involved in the rapid *COX17* mRNA decay, transcriptional shut-off assays were used to analyze *COX17* decay in a *upf1Δ* strain, which is NMD deficient. However, *COX17* mRNA decay was found to be NMD independent. Thus, the data of this chapter suggests that Puf3p mediates rapid mRNA decay by recruiting the standard mRNA decay factors to the Puf3p-bound mRNA.

Together this research implies that Puf3RD regulates *COX17* mRNA decay by binding its 3'UTR and recruiting Ccr4 and Pop2 (deadenylators), as well as Dhh1, Lsm1 and Dcp1 (decapping factors). All these interactions between Puf3RD and the decay machinery are RNA independent, being therefore direct protein-protein interactions. In contrast with Puf5p regulation of *HO* mRNA decay, in which Pop2 is thought to bridge the interactions between Puf5p and all the remaining decay factors, this does not seem to be the case for Puf3p. Instead, our data suggests a different model for Puf3p regulation, whereby the R7A loop region is required for Pop2 binding and indirectly for Dhh1 binding, while Dcp1 and Ccr4 appear to bind Puf3p independently of the R7A loop and Pop2p. While all Puf proteins appear to be engaged in the decay of their mRNA targets,

each one has its particular way of interacting with the decay machinery and achieving this regulation.

Experimental Procedures

Directed yeast two-hybrid assay

Full length Puf3p, the C terminal half of Puf3p (containing the repeat domain) and different portions of the N-terminal half of Puf3p were used as baits in the yeast two-hybrid assay (see Fig.3 for a schematic of the baits used). These different Puf3p fragments were each expressed as a gene fusion to the DNA binding domain of GAL4 from the high copy plasmid pGBDU-C2 containing a URA3 marker. The different prey proteins were each expressed as a gene fusion to the DNA activation domain of GAL4 from the high copy plasmid pGAD-C2 containing a *LEU2* marker (see table 1 for the genes cloned for prey protein expression). The mating of the parental strains transformed with either the bait or the prey plasmid resulted in a diploid that contained both plasmids and was selected by growth in –leu,-ura media. The parental strains were PJ694α for the baits and PJ694a for the preys. Positive protein interaction was screened by growth on selective –leu,-ura,-his plates, where the histidine gene *HIS3* was the reporter gene under the control of the *GAL* promoter. *HIS3* is a leaky gene, so 0.5mM 3-AT (3-amino-1,2,4-triazole) was added to the selective plates to prevent growth of false positives. 3-AT competitively inhibits imidazole glycerol phosphate dehydratase, a His biosynthetic enzyme (Hilton et al., 1965), and therefore can limit histidine biosynthesis and growth. The HIS3 gene encodes the enzyme activity inhibited by 3-AT. In the

directed yeast two-hybrid assays that use a HIS3 as a reporter gene, the high expression that arises from a successful two-hybrid interaction can overcome the growth-inhibitory effect of 3-AT in the medium. When 3-AT is added to yeast media (0.5-10 mM), it will limit histidine biosynthesis and is used in two-hybrid screens to "fine tune" leaky expression of the HIS3 reporter gene. Therefore, the use of 3-AT and the HIS3 reporter enables positive selection for successful two-hybrid interactions.

Modified yeast three-hybrid assay

In the modified yeast three-hybrid assay, interactions between the Puf3 protein and a prey protein that occur in the presence of the Puf3p target mRNA were tested by expressing the *COX17* 3' UTR fused to the MS2 RNA for nuclear localization, with the bait and prey proteins. The Puf3-RD fusion bait expressed from pGBDU-C2 (*URA3* marker) was co-transformed into the PJ694α strain together with the pIIIMS2-2 (*TRP*1 marker) plasmid expressing *COX17* 3'UTR RNA tagged with the MS2 sequence containing a nuclear localization signal (NLS). This bait strain was mated with PJ694a strains individually transformed with plasmids expressing 53 different candidate prey proteins. Each prey protein was expressed as a gene fusion to the DNA activation domain of *GAL4* from pGAD-C2 (*LEU2* marker). The diploids obtained from the mating of the transformed PJ694α and PJ694a strains were selected on –trp,-ura,-leu plates. To test for protein-protein interactions in the presence of *COX17* 3'UTR RNA, yeast colony growth was screened on –trp,-ura,-leu,-his plates with the addition of 0.5mM ATZ, where *HIS3* is the reporter gene under the control of the *GAL* promoter.

Co-immunoprecipitation experiments

The myc-tagged strains lacking endogenous Puf3p were transformed with the FLAG- Puf3RD expressing construct and 400 ml cultures were grown to an OD of 0.4. Protein extracts were prepared from each strain by applying consecutive cycles of vortex ice, to break open the cells and obtain the soluble cytoplasmic extract. These samples are incubated with an anti-FLAG resin that will trap the FLAG-tagged Puf3RD proteins along with any other protein that might be interacting with it. After a series of washes, the eluates from the resin are electrophoresed on an SDS-PAGE gel and blotted. The western blot obtained is hybridized with specific anti-myc antibodies to bind any co-immunoprecipitated decay factors. Since the primary anti-myc antibody was developed in mouse, a secondary anti-mouse antibody developed in goat that is HRP tagged allowed the detection of these proteins on the western.

Transcriptional shut-off analysis

Transcriptional shut off experiments were carried out to analyze *COX17* mRNA half lives in a WT strain versus *puf3*Δ*, dcp1Δ, dhh1Δ* and *lsm1Δ* strains. All strains were transformed with pST30/COX17pG in which *COX17* is expressed under the *GAL* UAS. Cultures were grown at 30°C in 2% galactose overnight to activate the control of the expression and then switched to 4% glucose to shut transcription off. Culture samples were gathered at specific time points once transcription is arrested. Then RNA is extracted from these cells, electrophoresed on 1% agarose gels and visualized and compared by northern blot analysis.

TABLE 1. Strains used in this study.

Deletion	Strain	Genotype	Source
*puf3*Δ	yWO 186	MAT a, *trp1, ura3-52, leu2-3,112, his4-539,cup1:LEU2/PM, puf3::NEO*	Olivas &Parker(2000)
*puf3*Δ	yWO 187	MAT a, *ura3-52, leu2-3,112, his4-539,cup1:LEU2/PM, puf3::NEO,Ccr4-myc::TRP1*	This study
*puf3*Δ	yWO 188	MAT a, *ura3-52, leu2-3,112, his4-539,cup1:LEU2/PM, puf3::NEO,Dcp1-myc::TRP1*	This study
*puf3*Δ	yWO 189	MAT a, *ura3-52, leu2-3,112, his4-539,cup1:LEU2/PM, puf3::NEO,Dhh1-myc::TRP1*	This study
*puf3*Δ	yWO 190	MAT a, *ura3-52, leu2-3,112, his4-539,cup1:LEU2/PM, puf3::NEO,Lsm1-myc::TRP1*	This study
*puf3*Δ	yWO 191	MAT a, *ura3-52, leu2-3,112, his4-539,cup1:LEU2/PM, puf3::NEO,Pop2-myc::TRP1*	This study
W T		MAT a, *ura3-52, his3-11,15, trp1-1, leu2-3,112*	Invitrogen
*puf3*Δ		MAT a, *ura352, his3-11,15, trp1-1, leu2-3,112,puf3::KanMX*	Invitrogen
*dhh1*Δ	yWO 207	MAT a, *ura352, his3-11,15, trp1-1, leu2-3,112, dhh1::KanMX*	Invitrogen
*dcp*Δ		MAT a, *ura352, his3-11,15, trp1-1, leu2-3,11, dcp1::KanMX*	Invitrogen
*lsm1*Δ		MAT a, *ura352, his3-11,15, trp1-1, leu2-3,112, lsm1::KanMX*	Invitrogen
W T	yWO 225	MAT a, *ura3-52, his3-11,15, trp1-1, leu2-3,112*	Audrey Atkin
*upf1*Δ	yWO 226	MAT a, *his3-11,15, trp1-1, leu2-3,112, upf1::URA3*	Audrey Atkin
*pop2*Δ	yWO 223	MAT a, *ura3-52, leu2-3,112, his4-539,cup1:LEU2/PM, puf3::NEO, Dcp1-myc::TRP1, pop2::NAT*	This study
*pop2*Δ	yWO 224	MAT a, *ura3-52, leu2-3,112, his4-539,cup1:LEU2/PM, puf3::NEO, Dhh1-myc::TRP1, pop2::NAT*	This study

TABLE 2. Genes cloned in the pOAD for prey protein expression for yeast two and three- hybrid assays.

AIR1	CDC39	LSM2	NOT3	PUF4	TUB3
AIR2	CDC50	LSM5	NRG2	PUF5	YHR035W
ARP2	CRZ1	LSM7	PAB1	REX1	YPR013C
CAF4	DCP1	MIG1	PAT1	REX2	YER130C
CAF17	DHH1	MOT2	PBP1	RPA14	YKL1
CAF40	EDC1	MSN2	POP2	RPB4	ALPHA-1
CAF130	EDC3	MSN4	PRP4	SIG1	YHR121W
CCR4	ELA1	NAM7	PSU1	SMB1	
CDC28	HRR25	NHD2	PUF1	SPB8	
CDC36	LSM1	NIL2	PUF3	SPP381	

Results

Rapid *COX17* mRNA decay is dependent on Dhh1p, Dcp1p and Lsm1p decay factors.

COX17 mRNA has been used as a model to study Puf3p-mediated decay in yeast. It was previously shown through pulse chase experiments that the rapid deadenylation of *COX17* mRNA, which is stimulated by Puf3RD, requires the Pop2p and Ccr4p deadenylation factors (Tucker et al., 2002). It was likely that *COX17* decay was also dependent on the standard decapping complex, Dcp1p/Dcp2p, as well as the enhancers of decapping, Dhh1p and the Lsm1-7p complex. However, it was possible that Puf3p-mediated decay utilized some other mechanism of decay stimulation that bypassed the need for one or all of these factors. We therefore investigated whether *COX17* mRNA decay was slowed in strains deleted of *DCP1, DHH1* or *LSM1*. To measure mRNA decay, transcriptional shut-off assays were performed. Five different strains (WT, *puf3*Δ*, dcp1*Δ*, dhh1*Δ and *lsm1*Δ) were transformed with pWO 153 that has *COX17*pG under a *GAL* promoter, so that *COX17* with a 3' UTR poly(G) tag is expressed in galactose and its transcription can be shut-off when switched to glucose. The results in Fig.1 demonstrate that all three of these decay factors are required for rapid *COX17* mRNA decay. The individual deletion of either *DHH1* or *LSM1* results in a two fold increase in the *COX17* mRNA half-life, similar to the *puf3*Δ strain. The *DCP1* deletion results in a more drastic effect, causing an 8 fold increase in the half-life of *COX17* mRNA. Clearly, Puf3p cannot bypass the need for these factors in promoting rapid decay of *COX17* mRNA. Instead, Puf3p likely recruits or stimulates the activity of these factors on the mRNA.

***COX17* mRNA decay is NMD-independent.**

In cells there exists a mechanism of mRNA surveillance known as non-sense mediated decay (NMD). In *Saccharomyces cerevisiae,* NMD is responsible for detecting mRNAs with premature stop codons as well as some wild-type mRNAs and targeting them for accelerated decay (Lelivelt and Coulbertson, 1999). NMD uses the standard degradation machinery of the cell but with the addition of Upf1p, Upf2p and Upf3p. The deletion of any of these three *UPF* genes results in a similar phenotype of knocking out the NMD pathway (Atkin et al., 1997). Thus, in any of these NMD mutants, mRNAs that are usually degraded by the NMD pathway will accumulate to higher levels compared to a WT strain.

In order to determine whether *COX17* mRNA decay depended on NMD or followed the typical steps of deadenylation, decapping and 5' to 3' degradation, transcriptional shut-off experiments were performed. In this case, transcription of *COX17* was repressed by switching temperature from 24°C to 37°C in WT and *upf1*Δ strains since both of them have a temperature sensitive mutation in RNA polymerase II (*rpb1*-1). The results in Fig.2 show that the half-life of *COX17* mRNA is similar in both strains, implying that the NMD pathway is not involved in the rapid *COX17* mRNA decay. Rather, Puf3p is stimulating *COX17* mRNA decay through the classical deadenylation, decapping and 5' to 3' decay as occurs in most yeast transcripts (Fig.2).

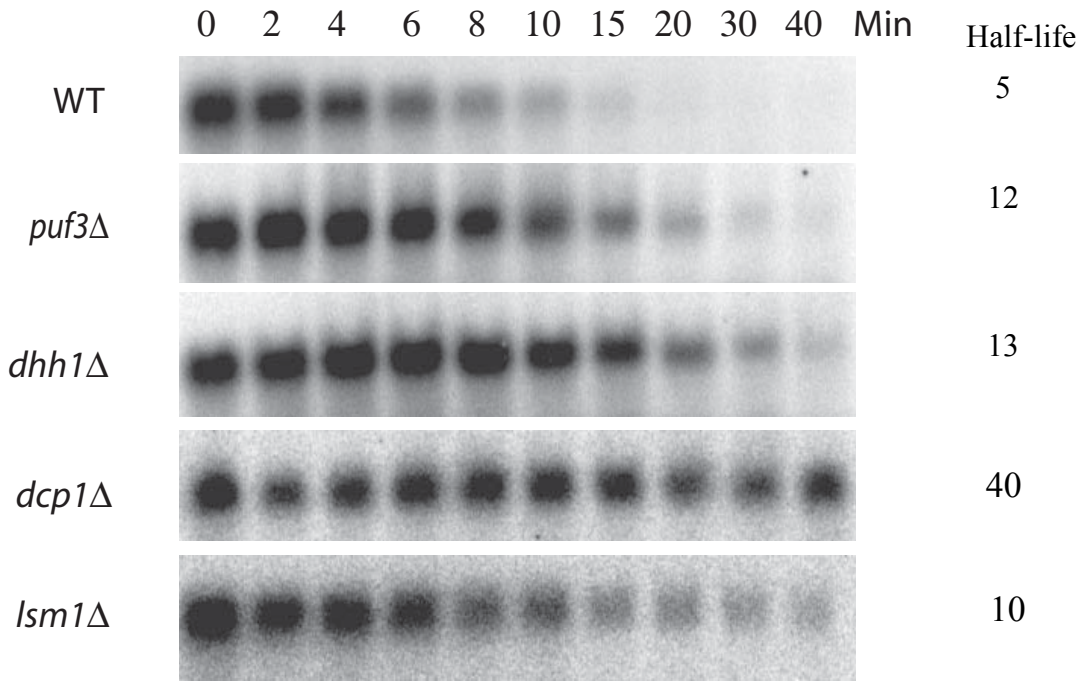


Fig.1 Puf3p-mediated rapid decay of *COX17* mRNA requires Dhh1p, Dcp1p and Lsm1p. At the top, time in minutes is shown after transcription was shut off and samples of the culture were taken for the analysis. On the right, half-life in minutes of *COX17* mRNA decay is shown in different deletion strains compared to WT and *puf3*Δ strains.

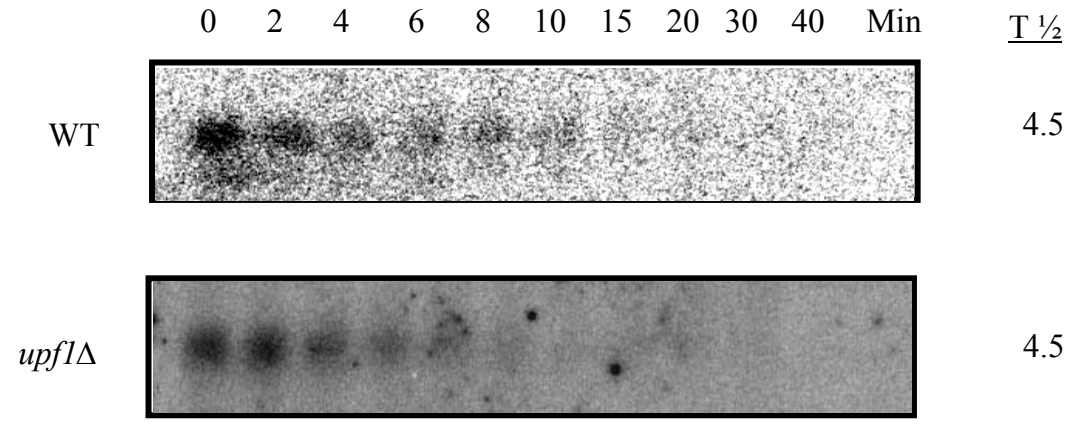


Fig.2 *COX!7* mRNA decay is NMD independent. Nortrhern blots of transcriptional shut-off experiments for WT and *upf1*Δ strains. At the top, time points are shown in minutes after transcription was repressed and samples taken. On the right, the half-life of *COX17* mRNA decay is shown in minutes for each of the strains.

Puf3p interacts with decay factors involved both in deadenylation and decapping. Genetic approach for detecting protein-protein interactions using directed yeast two-hybrid and modified yeast three-hybrid assays.

Puf proteins from *Drosophila* and *C. elegans* must interact with other regulatory proteins, such as Nanos and Brat, to mediate repression of their target mRNAs. Though there are no Nanos or Brat homologs in yeast, it is possible that Puf3p might also be interacting with other regulatory proteins to mediate decay of *COX17* mRNA. Alternatively, Puf3p may directly interact with mRNA decay factors, such as those involved in deadenylation (Ccr4, Pop2) and/or decapping (Dcp1/Dcp2, Dhh1, Lsm1-7 and Pat1). To identify proteins that interact with Puf3p, a yeast two-hybrid library screen was first performed by Andrew Van Brunt in the Olivas lab (See Materials and Methods for overview of the yeast two-hybrid assay). In this assay, the Puf3RDp was used as bait against a genomic library of prey proteins, however no significant interactions were detected (unpublished data). I then tested through a directed yeast two-hybrid assay the interaction of Puf3p with various known mRNA decay factors and several other proteins that through systematic analyses had been identified as potential Puf interacting proteins. The results from the directed yeast two-hybrid assay were not very revealing (Fig.3). The Puf3RDp did not show detectable interactions with any of the prey proteins, while the full-length Puf3p interacted with all the proteins, including the negative controls. I determined that the first 65 amino acids of the protein were responsible for this non-specific binding, since this 65 amino acid region interacted with all proteins, while a construct lacking this region could not bind to any of the tested prey proteins.

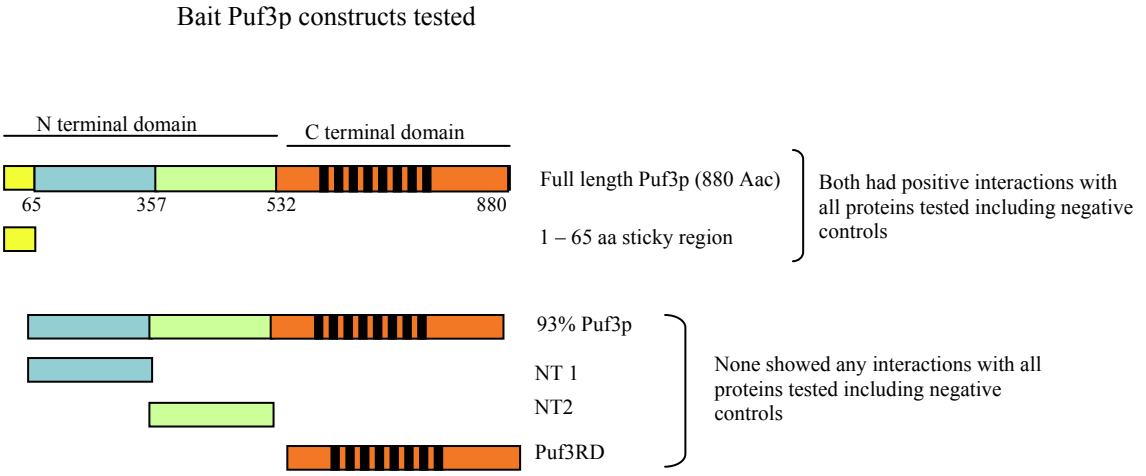


Fig.3 Schematics of Puf3p baits. Full-length Puf3p and different regions of the protein were used as baits in the directed-yeast two hybrid assay.

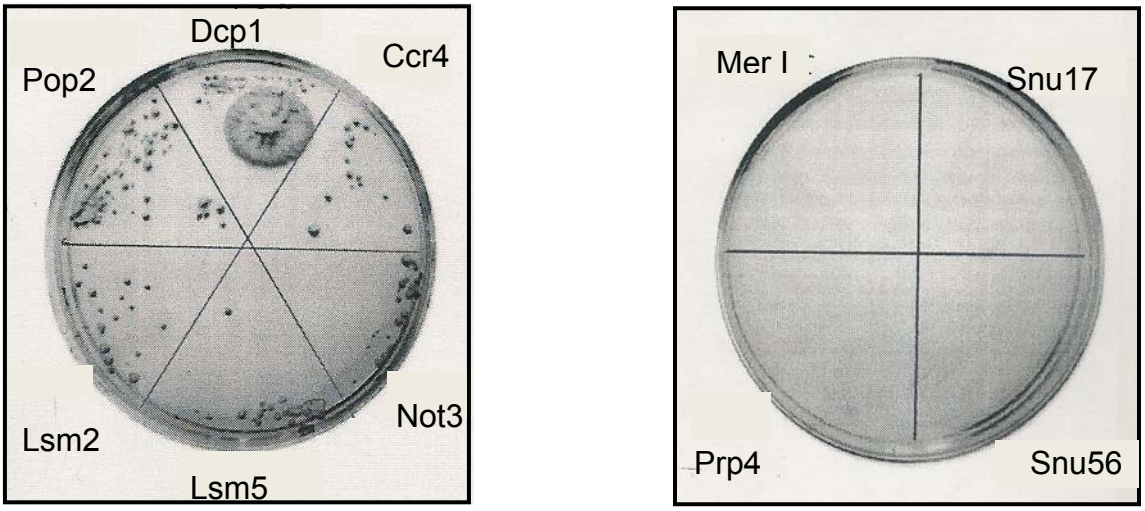


Fig.4 Modified yeast three-hybrid assay. Left plate shows four positive interactions between Puf3RD and Lsm2, Pop2, Dcp1 and Ccr4. Lsm5 and Not3 show weak interactions. On the right plate, negative controls do not grow, showing no interaction.

It is important to note that protein interaction in the yeast-two hybrid assay must occur in the cell's nucleus, which is not where Puf proteins normally function to bind and regulate mRNA targets. Therefore, it was possible that the absence of an mRNA target in the nuclear two-hybrid assay was detrimental to the ability of the proteins to interact. In fact, studies have shown that the *Drosophila* Puf protein, Pumilio, requires binding to its mRNA target prior to binding to the Nanos and Brat proteins (Wharton et. al., 1998). Therefore, to test whether Puf3RDp requires binding to its mRNA target in order to be competent for protein interaction, a modified yeast three-hybrid assay expressing a nuclear localized *COX17* 3' UTR RNA was performed as described in Materials and Methods. The results obtained showed interactions (albeit weak) between Puf3RDp and several of the prey proteins tested. Since protein interaction is assayed by the ability to activate expression of the *HIS3* reporter gene, positive interactions were observed as growth on selective plates lacking histidine as compared to no growth of the negative controls. Addition of 0.5 mM amino tetrazolium was used to inhibit leaky expression of the *HIS3* reporter gene. Positive interactions with Puf3RDp included proteins involved in mRNA degradation: Pop2, Dcp1, Ccr4, Lsm2, Lsm7 and Caf4. These proteins belong to the deadenylation and decapping machinery in yeast. Other proteins also identified through the directed yeast three-hybrid assay that are not related to mRNA decay were: Tub3, YHR035W, HRR25, α1, Smb1, Rpb4, YHR121W, Ela1 and Mig1. The rest of the 39 proteins tested did not show any interaction with Puf3RDp (Fig. 4).

Co-immunoprecipitation experiments

Since the yeast-three hybrid assay showed interactions with deadenylating and decapping factors, but also with other proteins not related to the decay machinery, we wanted to use another experimental approach to test these interactions between the Puf3RD and the decay factors. Therefore, co-immunoprecipitation experiments were developed in which Puf3RD was FLAG-tagged and expressed from the pG-1 vector, while the decay factors were endogenously myc-tagged at their C-terminal ends by homologous recombination. The overall scheme is described in a diagram shown in Fig.5 of Chapter II- General Experimental Methodology. In brief, protein extracts are applied to anti-FLAG resin that will trap the FLAG-tagged Puf3RD proteins along with any other protein that might be interacting with the Puf3RD. After a series of washes, the eluates from the resin are electrophoresed on an SDS-PAGE gel and blotted. The western blot obtained is hybridized with specific anti-myc antibodies to visualize any co-immunoprecipitated decay factors. Sean Houshmandi in the lab originally demonstrated that the five myc-tagged decay factors (Ccr4p, Dcp1p, Dhh1p, Lsm1p and Pop2p) all co-immunoprecipitated with Puf3RD (Fig.5). Similar results were obtained by another research group in which the Ccr4p, Dcp1p, Dhh1p and Pop2p decay factors epitope-tagged with T7 were pulled down by TAP-tagged Puf5 (Goldstrohm et al, 2006). Together, these results suggest a conserved mechanism of yeast Puf proteins binding directly with mRNA decay machinery, and these interactions likely act to recruit the machinery to the target mRNA for promotion of decay.

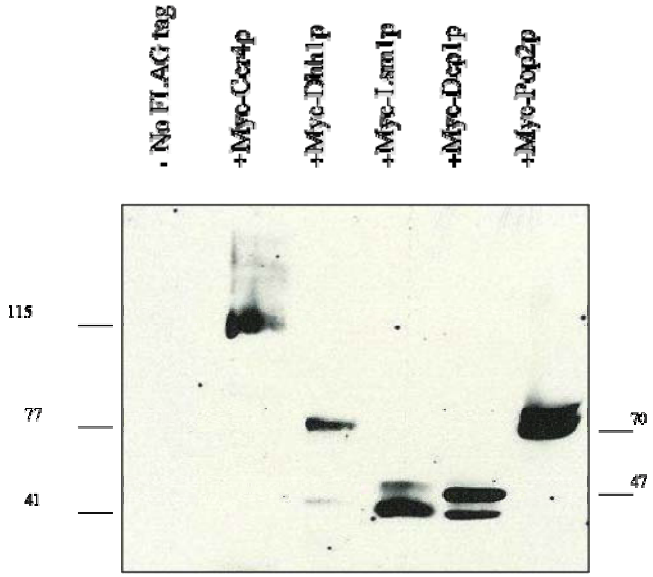


Fig.5 Puf3RD co-immunoprecipitates with decay factors. These decay factors (Ccr4, Dcp1, Dhh1, Lsm1 & Pop2) that are endogenously myc-tagged co-immunoprecipitate with FLAG-Puf3RD and are detected with anti-myc antibodies (Houshmandi, S. unpublished data).

Puf3p interactions with decay factors are RNA independent and Puf3p binds Dhh1p and Pop2p through the 7A loop region of the repeat domain (RD).

After having detected the interactions of Puf3RD with the various decay factors through co-immunoprecipitation assays, we wanted to further evaluate the nature of the interactions. First, we wanted to determine whether the Puf3p/decay factor interactions were really due to protein-protein interactions, or whether the co-immunoprecipitation results were simply due to proteins binding to different points along a single mRNA molecule. To differentiate between these possibilities, protein extracts were incubated with RNase A before performing the co-immunoprecipitation. The results show no difference in the binding of Puf3RD to any of the decay factors in the presence or absence of RNase A (Fig.6A). This data suggests that interactions between Puf3p and the decay factors are mediated through protein interactions.

Next, previous work in the lab indicated that a deletion of four amino acids on the outer surface of Puf3RD, the R7A mutant, failed to promote rapid decay of *COX17*, while another deletion mutant of an outer surface loop of Puf3RD, the R6A mutant, had no effect on *COX17* decay (Houshmandi and Olivas, 2005). We wanted to determine whether the failure of the R7A mutant to promote rapid decay was due to an inability to interact with one or more decay factors. Therefore the myc-tagged strains lacking endogenous Puf3p were transformed with each of the FLAG-tagged Puf3p constructs: WT Puf3RD, Puf3R6A mutant and Puf3R7A mutant.

Co-immunoprecipitation experiments with the R6A mutant demonstrated that it is able to bind Ccr4p, Dcp1p, Dhh1p, Lsm1p and Pop2p, thus behaving in a similar way as WT Puf3RD (results not shown). In contrast, the R7A mutant disrupted

interactions with two of the decay factors (Pop2p and Dhh1p), while interactions with Dcp1p, Lsm1p and Ccr4p were maintained (Fig.6B). This indicates that the R7A loop is the specific region of Puf3RD to which Pop2p and Dhh1p bind. It is possible that each one of these proteins binds directly to the R7A loop. However, other work with Puf5p indicates that Pop2p acts as a bridge between Puf5p and all other decay factors (Goldstrohm et al., 2007). This suggests that Pop2p may be acting as a bridge between the Puf3RD-R7A loop and Dhh1p. To test this hypothesis, *POP2* was deleted from the myc-tagged *DHH1* and *DCP1* strains, then the strains were transformed with vector alone or with the Puf3RD expressing construct. The results of Co-Ip experiments with these *pop2*Δ strains clearly show that Dhh1p binding to Puf3RD is disrupted in a *pop2*Δ strain, while Dcp1p is still able to bind (Fig.7). These experiments provide evidence that Dhh1p, but not other decay machinery is recruited via Pop2p to Puf3p. It might be that different Pufs interact with decay factors in a customized mechanism for achieving unique modes of decay of their target mRNAs.

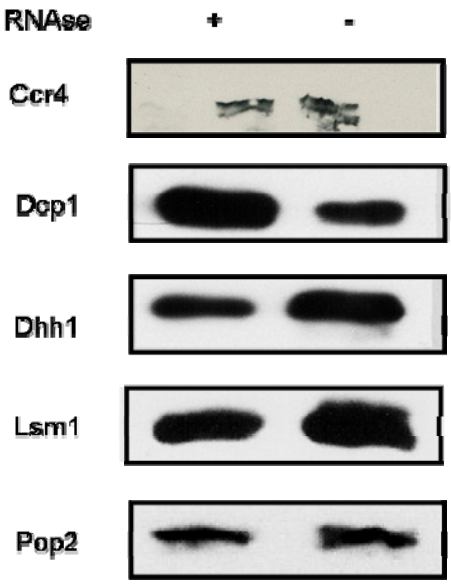


Fig.6A Puf3RD interactions with decay factors are RNA independent. Western blot of Co-Ip experiments with and without RNAse treatment. Myc-tagged decay factors (Ccr4, Dcp1, Dhh1, Lsm1 and Pop2) were detected with anti-myc antibodies.

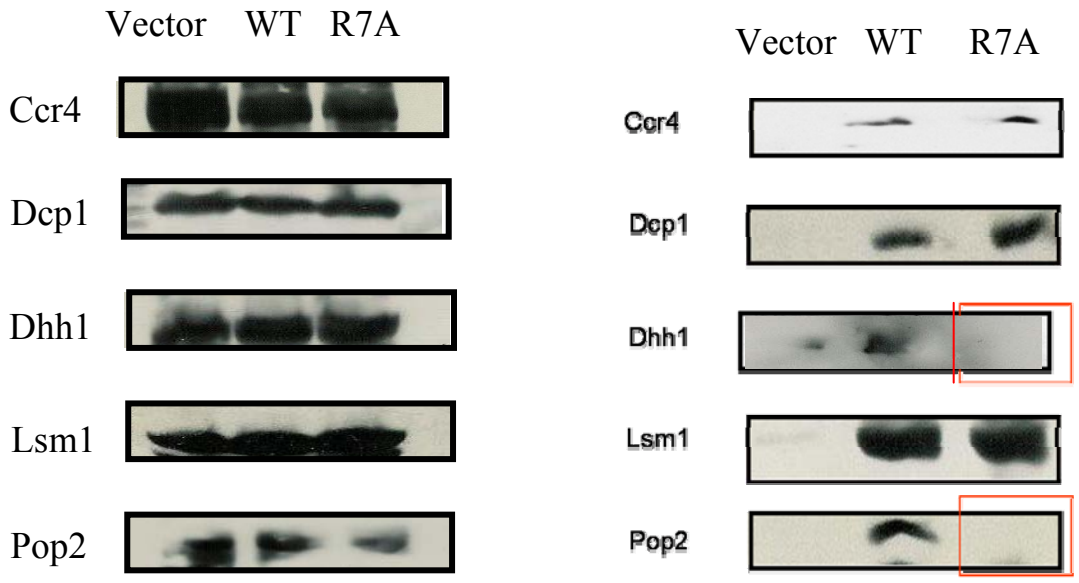


Fig.6B Extracts and Co-Ip for Puf3RD and Puf3R7A. On the left, western blots show protein extracts that were loaded onto the resin. On the right, the results after performing the Co-Ip are shown comparing the pull-down with WTRD and that by the mutant RD that contains the 7A loop deletion. Myc-tagged decay factors (Ccr4, Dcp1, Dhh1, Lsm1 and Pop2) were detected with anti-myc antibodies. Red boxes denote interactions inhibited by the 7A mutation.

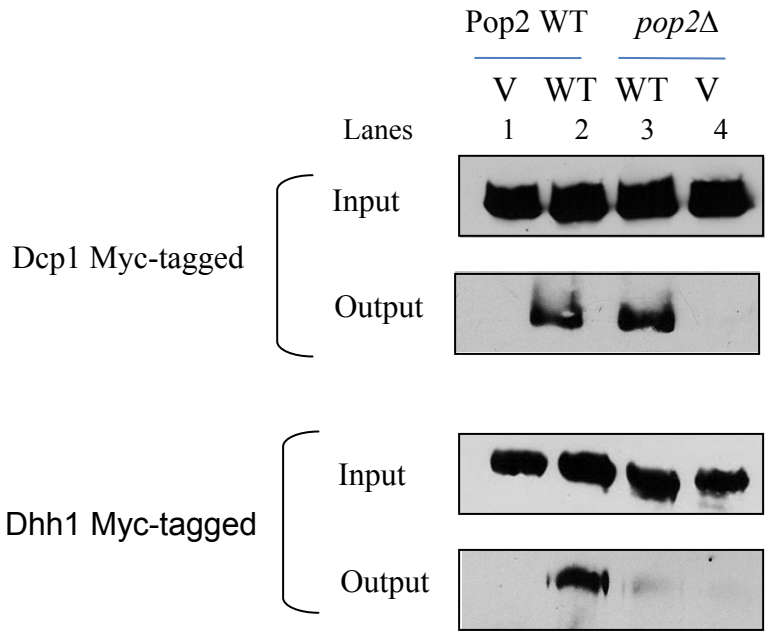


Fig.7 Pop2p is required for Dhh1p binding. Western blots of Co-Ip experiments comparing a WT strain versus a *pop2*Δ.strain with Dcp1 or Dhh1 myc-tagged decay factors. Anti-myc antibodies were used for detecting the decay factors. Input denotes extracts that were loaded onto the resin, and output denotes the elutions from the co-immunoprecipitations. Lanes 1 and 4 correspond to the results obtained with the strains transformed with vector alone, while 2 and 3 show the results with the strains expressing Puf3WTRD.

Discussion

Puf 3p mechanism of action

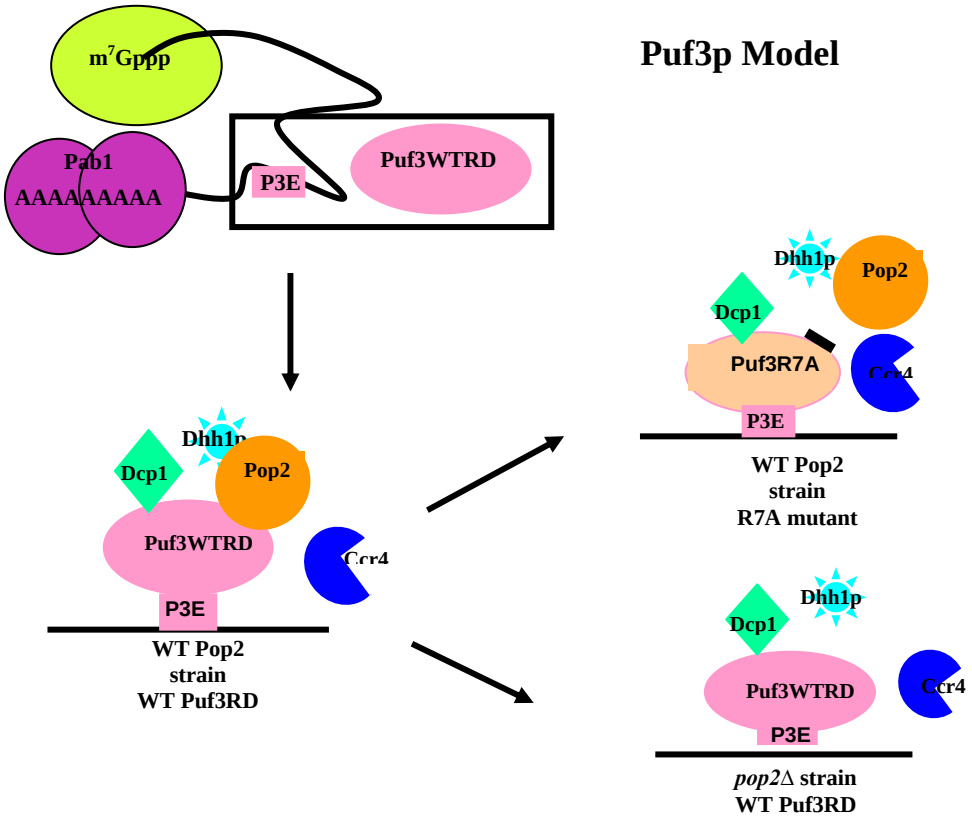
COX17 mRNA decay shows a prolonged half-life in a *puf3Δ* strain compared to a WT strain. It has been shown that the deadenylation rate of *COX17* in the *puf3Δ* strain is slower and at least partially accounts for the mRNA stabilization (Olivas and Parker 2000). A similar phenotype is observed in strains lacking either *CCR4* or *POP2*, which are the main yeast deadenylators (Tucker et al, 2002). The decapping step of *COX17* mRNA also appears to be slowed by Puf3p (Olivas and Parker, 2000). In this research we show that deletions of *DCP1, DHH1* and *LSM1*, which encode proteins involved in decapping, all promote *COX17* mRNA stabilization. Deletion of *DCP1*, which encodes part of the holoenzyme responsible for decapping, showed the most drastic effect, prolonging the *COX17* mRNA half-life 8-fold. Thus, the promotion of rapid *COX17* mRNA decay by Puf3p requires the action of each of these deadenylation and decapping factors. In addition, we show that this decay is NMD independent since the impairment of the NMD pathway in a *upf1*Δ strain does not affect rapid *COX17* mRNA decay.

Through co-immunoprecipitation experiments, we demonstrated that Puf3p directly interacts with the Ccr4p, Pop2p, Dcp1p, Dhh1p and Lsm1p deadenylation and decapping factors. Previous work had shown that the four amino acid deletion of the Puf3RD-R7A mutant disrupted Puf3p's ability to promote mRNA deadenylation (Houshmandi and Olivas, 2005). Here we demonstrate that this mutant prevents the binding of Pop2p and Dhh1p to the Puf3RD, yet maintains binding to Dcp1p, Lsm1p and Ccr4p. To analyze the arrangement of Pop2p and Dhh1p binding to the R7A outer surface loop, Co-Ip experiments were performed in the absence of Pop2p. These results

revealed that Pop2p is required to bridge Dhh1p binding to Puf3p, while Pop2p likely binds directly to the R7A loop region. In contrast, Dcp1p was shown to independently bind Puf3RD without the presence of Pop2p. This result confirms that Dcp1p binds Puf3RD in a different region other than the R7A loop, and the interaction is not bridged by Pop2p (Fig.8). Previous work has shown that Pop2p is required for rapid deadenylation of *COX17* (Tucker et al., 2002). So it makes sense that the R7A mutant that cannot bind Pop2p would also be defective in rapid *COX17* deadenylation. Thus, even if Ccr4p can independently bind to the Puf3RD-R7A mutant, this interaction apparently cannot override the need for Pop2p to stimulate Ccr4p catalytic activity for deadenylation. However, it is unclear whether the R7A mutant can still promote mRNA decapping through Dcp1p binding, or if the absence of Dhh1p binding to this mutant prevents such mRNA stimulation as well. Together, our results of Puf3RD interactions with the decay machinery provide a similar yet distinct model as compared to the model of Puf5p interactions (Fig.8) in which Pop2p is the only factor to directly bind Puf5p and thus bridges interactions with all the rest of the decay machinery (Ccr4, Dhh1 and Dcp1) (Goldstrohm et al., 2007).

Future work will be necessary to determine the unique arrangement of interactions for other Puf proteins and any conserved bridging role of Pop2p. The Puf3p protein interaction results from the yeast two-hybrid and three hybrid assays suggest that the presence of the target RNA is required in order for the Puf3RD to interact with the decay factors. Yet the Co-Ip results show that RNAse treatment does not affect Puf3RD's protein interactions. This treatment assures that both proteins being pulled down are physically interacting and not just being tethered to different parts of a single RNA

molecule. It might still be that the Puf3RD is required to first bind the RNA target in order to recruit the decay machinery, as has previously been shown for the *Drosophila* Pum to first bind hunchback mRNA before recruiting Nanos (Sonada et al., 1999; Sonada et al., 2001). Once the Puf protein binds the RNA target, there might be conformational changes in the Puf that will then favor the interactions of its outer surface with other proteins. So in the case of Puf3p, even after RNAse treatment, the small portion of RNA to which the Puf3RD may be attached would be protected from RNAse degradation and could still be allowing a conformational change in the Puf3RD to enable it to directly interact with decay factors. It would be very interesting in future work to test binding-incompetent mutants such as the Puf3RD-R1 mutant (Houshmandi and Olivas, 2005) in Co-Ip experiments to determine if such a mutant can still interact with the decay machinery, or if protein binding indeed requires prior mRNA interaction.



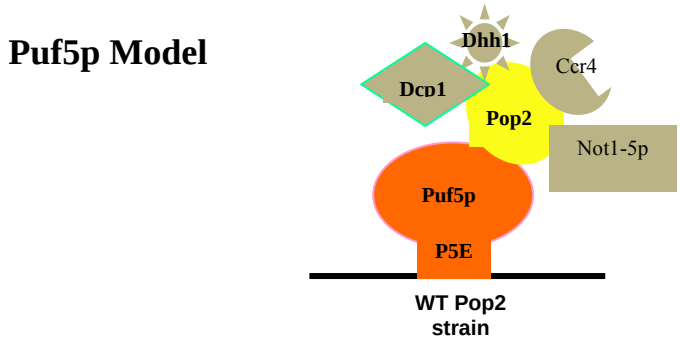


Fig.8 Models comparing Puf3p vs. Puf5p interactions with decay factors. Pop2p binds the Puf3RD at the R7A outer surface loop. Either by the R7A loop deletion or the deletion of Pop2p, Dhh1p binding is prevented. However, Dcp1p binding remains unaffected, being independent of both the R7A loop and Pop2p. Puf5p binds all the shown decay factors through Pop2p.

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Chapter IV

Puf3p activity is affected by the type of carbon source present in the media

Introduction

The regulation of mRNA stability by cis-regulatory elements (CREs) is a dynamic process and is affected by several environmental stimuli. DNA microarrays in *Saccharomyces cerevisiae* have allowed the analysis of high throughput genomic data in which different expression profiles can be observed and compared under different conditions. It is known that in the adaptation of the cell to environments that have poor conditions for growth and survival, the environmental stress response program (ESR) is triggered (Gasch et al., 2000). This is a survival skill of the cell in which a broad spectrum of genes are turned on to respond in a positive way, adapting the cell's metabolism to the new physiological requirements. Mechanisms of DNA repair, protein folding, defense against reactive oxygen species, energy generation, and storage are increased functions while cell growth and protein synthesis are arrested (Gasch et al., 2000). Although ESR is a stereotypical response in which the cell will automatically turn on and off particular sets of genes, other environmental stimuli will specifically target other genes, rendering a unique gene expression pattern customized for each specific condition (DeRisi et al., 1997; Brown et al., 2000). These signature patterns for individual environmental conditions give the possibility of grouping genes according to whether they are up- or down-regulated under a specific condition, and the patterns display at a genomic network level the response to environmental changes through coordinated changes in gene expression profiles. These data can be useful for acquiring information about which genes respond coordinately with a group of genes, versus those that belong to a different group that responds in an opposite manner. Gaining knowledge

on the coordinated regulation of these gene networks will allow us to better manipulate gene expression to trigger the desired response at a cellular level.

In an attempt to study and classify gene expression in gene clusters and to better analyze the information obtained by high throughput data, the Matrix REDUCE algorithm (Regulatory Element Detection Using Correlation with Expression) was created by Foat et al. This algorithm was applied to 200 bp sequences downstream of every ORF of the yeast genome and to the microarray expression profiles of these genes tested under 700 different conditions. As a result, groups of different genes containing the same CRE were detected as coordinately regulated in a similar fashion under different environmental conditions (Foat et al., 2005). Predictions were made for the functional role of the P3E (Puf3 element) present in most of the mitochondrial-related transcripts. According to the results obtained through the algorithm, these transcripts would be down-regulated under growth conditions where a fermentable carbon source is present and thus mitochondria would not be necessary, while the transcripts would be up-regulated under a non-fermentable carbon source when mitochondria are required for respiration. These predictions were experimentally confirmed in the Olivas lab. The *COX17* mRNA half-life was four fold higher in ethanol, in which mitochondria need to be functional to obtain energy through the respiratory chain, compared to glucose where fermentation takes place (Foat et al., 2005)).

In this work I was seeking to understand the mechanisms involved in the regulation of Puf3p activity by different environmental conditions. For this purpose, I measured the amount of Puf3 mRNA and protein present in the different conditions. Finding that protein and mRNA quantities in both ethanol and glucose were similar (or

even higher in ethanol versus glucose), led to further investigations searching for post-translational modifications in Puf3p. In fact, it has been shown in *Dictyostelium* that Puf activity is regulated through a change in its phosphorylation state (Mendes Souza et al., 1999). Puf6p in yeast, which is involved in *ASH1* mRNA translational repression, was also recently shown to be regulated by the kinase CK2, which releases Puf6p repressive activity by phosphorylation (Deng et al., 2008). I discovered that Puf3p is phosphorylated when recovered from yeast cultures grown under different carbon sources by using phospho-specific antibodies. Specific conserved amino acids of the Puf3RD that were predicted to be phosphorylated were mutated to alanines, and these nutants provided evidence that phosphorylation might be affecting Puf3p activity. Future work to understand the role of post-translational modifications could include: mass-spectrometry study of tryptic digests of protein purified from different conditions; analysis of Puf3p activity in different kinase mutants; and testing the rescue of the phosphorylation mutants by substituting the alanines with aspartic acid to test whether this mimic of the phosphorylated state restores Puf3p activity.

Other possible post-translational mechanisms of regulating Puf3p activity by the available carbon source included differences in localization or differences in solubility. We observed Puf3-GFP localization under different carbon sources and found that non-functional Puf3p (in ethanol, galactose and raffinose) appears with a more punctate pattern in the cytoplasm rather than a homogeneouos one. We were also able to show that much of the non-functional Puf3p was in an insoluble state by comparing protein extracts obtained using different protein extraction protocols.

Experimental Procedures

Transcriptional shut-off analysis

Transcriptional shut-off experiments were used to analyze and compare mRNA decay rates between different yeast strains. The strains used for these experiments carry a mutation in RNA polymerase II (*rpb1*-1), which makes the polymerase functional at 24˚C, but inactive at 37˚C. This allows mRNA transcription to be arrested by switching from permissive to non-permissive temperature. The entire procedure is explained under general methodology. In these experiments, two *rpb1*-1 strains were used. yWO 7 is a WT Puf3p strain in which *COX17* mRNA's half-life was measured from cultures grown in different carbon sources. yWO 51 is a *puf3Δ* strain used as a control .

Analysis of the amount of Puf3p in different conditions

For measuring the amount of Puf3 protein present in yeast cultures growing in different carbon sources, proteins were extracted using the protein boil prep method or the YPER method. The main difference between the two methods is that while the YPER method only extracts the soluble portion of the proteins, the protein boil prep accounts for the extraction of both soluble and insoluble proteins. In the latter method, pelleted cells from a 10 ml yeast culture are resuspended in 100 µl of sample buffer in a 1.5 ml tube. Glass beads are added, and cells are disrupted by three consecutive cycles of vortexing and boiling at one minute each. A needle hole is made in the bottom of the tube, the sample is centrifuged at 4000 rpm for 2 minutes and the clear supernatant (protein extract) is collected.

In the YPER method, the cell pellet obtained form the culture is resuspended in a buffer that consists of the YPER solution (Pierce) with the addition of 10% DTT and a mini-complete protease inhibitor cocktail tablet for every 10 ml of solution. Then the sample is rocked at room temperature for 1 hour to disrupt the cells. The samples are centrifuged and the clear supernatant (protein extract) is collected.

Phosphorylation experiments

Eluates obtained from the Co-Ip experiments that contained a higher concentration of Puf3RD were used as substrates for detecting the phosphorylation state of the protein. Lambda phosphatase was used to treat these elution samples obtained from cultures grown in different carbon sources. Samples were incubated with or without phosphatase for 20 minutes at 30°C, then were separated via SDS-PAGE. Westerns were developed using anti-Serine or anti-Tyrosine phospho-specific antibodies (Qiagen).

Site-directed mutagenesis

In vitro site-directed mutagenesis was performed to mutate individual amino acids present in the Puf3RD in pWO 14 using the QuickChange XL site-directed mutagenesis kit (Stratagene). Two Serine residues located in repeat 1 (R-1A and R-1B) were mutated to Ala using two set of primers oWOs 351 and 352 for R-1A and oWOs 353 and 354 for R-1B. A Tyrosine residue in repeat 3 (R-3) was mutated to Ala using primers oWOs 355 and 356, and another Ser in repeat 8 (R-8) was changed using oWOs 357 and 358. Different combinations of mutagenesis were performed to obtain double,

triple and quadruple mutants. All resulting mutants were confirmed by sequencing (Table 2).

GFP tagged proteins

Green fluorescent protein (GFP) fusion proteins of full-length yeast Puf3p and Dcp2p created in the Parker Lab were used to study Puf3p-GFP localization and appearance under different carbon sources. These fusion proteins are labeled at the C-terminal and are functional. Puf3-GFP was shown to be uniformly distributed throughout the cytoplasm while Dcp2-GFP was concentrated in discrete cytoplasmic foci when visualized from cultures grown in glucose (Sheth and Parker, 2003). In our work, yWO 5, which is not GFP-tagged, was used as a negative control strain. Puf3-GFP was tested under different carbon sources and Dcp2-GFP was used as a positive control.

50 ml yeast cultures were grown in minimal media with the addition of a unique carbon source (glucose, ethanol, galactose or raffinose) at 30°C and harvested at an OD of 0.4. The total volume was split into two 50 ml falcon tubes and centrifuged. One pellet was resuspended in 3 ml minimal media while the other was resuspended in water, and both were incubated at room temperature for 5 minutes. Aliquots from each sample (2 ul) were applied to clean slides previously covered with concanavaline A + lysine (fixer), then squeezed below the cover-slip to obtain a thin layer of yeast cells for microscopic observation.

Table 1. Strains used in these studies.

Deletion	Strain	Genotype	Source
WT	yWO 7	MAT x, *ura3, leu2, rpb1-1*	Caponigro et al. 1993
*Puf3*Δ	yWO 51	MAT a, *trp1-1, ura3(-1 or-52), leu2-3,112, his4-539,rpb1-1, COX17::TRP, PUF3::NEO*	Olivas and Parker 2000
Puf3-GFP	yWO 185	MAT a, *trp1-1, ura3-52, leu2-3,112, his4-539, cup1::LEU2/PGK1pG/MFA2pG, PUF3-GFP (NEO)*	Sheth and Parker 2003
Dcp2-GFP	yWO 186	MAT a, *trp1-1, ura3-52, leu2-3,112, his4-539, cup1::LEU2/PGK1pG/MFA2pG, DCP2-GFP (NEO)*	Sheth and Parker 2003
WT	yWO 5	MAT a, *ura, his4, trp, leu, CUP1::LEU2/PM*	Olivas and Parker 2000

Table 2. Puf3RDp phosphorylation site mutants

Protein	Mutation	Yeast plasmid
Puf3RDp	Wild type	pWO 14
Puf3RDp/ R1A	S553A	pWO 144
Puf3RDp/R1B	S563A	pWO 145
Puf3RDp/R3	Y634A	pWO 146
Puf3RDp/R8	S835A	pWO 147
Puf3RDp/R1B-3	S563A and Y634A	pWO 148
Puf3RDp/R3-8	Y634A and S835A	pWO 149
Puf3RDp/R1A-3-8	S553A, Y634A and S835A	pWO 150
Puf3RDp/R1B-3-8	S563A, Y634A and S835A	pWO 151
Puf3RDp/R1A-1B-3-8	S553A, S563A, Y634A and S835A	pWO 152

Results

Puf3p activity depends on the carbon source available in the media

It had already been experimentally shown in our lab that Puf3p is inactive when yeast is grown in ethanol as a carbon source. This experimental result matched the computational model prediction made by the matrix REDUCE algorithm (Foat et. al., 2005). This algorithm also made other predictions for Puf3p activity under several more conditions which had not been experimentally tested. Theoretical modeling provides a fast mechanism for obtaining global genome expression results, in this case predicting the response of an organism to changes in the environment through coordinated regulation of its gene expression. However, these predictions, though useful, can sometimes be mistaken since not all the variables that exist in a living organism can be taken into account when developing an algorithm.

In this research I have further experimentally tested the computational prediction results obtained through the matrix REDUCE algorithm. Using transcriptional shut-off experiments performed under different environmental conditions, I show that Puf3p activity can be altered by several conditions. Confirming the model's predictions, Puf3p is similarly active under glucose, fructose or sucrose conditions (fermentable sugars), thus mediating rapid decay of the target *COX17* mRNA, which is seen as a half-life of 3-6 minutes (Fig. 1). In contrast, *COX17* mRNA half-life is extended to 10-13 minutes in ethanol, galactose, raffinose, acetate and glycerol, indicative of inactive Puf3p (Fig.1). These results show that the regulation of gene expression by Puf3p is greatly affected by the carbon source present in the environment. It is to be expected that unicellular microorganisms such as yeast need this type of regulation in response to environmental

changes, since the adaptation to the medium will assure their survival. Results from mRNA decay experiments in which yeast were switched from glucose to galactose, or from galactose to glucose for two minutes, showed that rapid inactivation or activation of Puf3p was achieved (Melanie Miller personal communication), implying that Puf3p activity is altered post-translationally.

The amount of Puf3p and *PUF3* mRNA present in ethanol, galactose or raffinose conditions is similar to that in glucose.

The proper regulation of gene expression is necessary at a molecular level in the cell to achieve the adequate response to adapt to the environment and survive. In multicellular organisms this regulation allows not only for cell development and differentiation into a variety of tissues, but ultimately allows the expression of life itself. Understanding the basic mechanisms of gene regulation is of key importance for future development of stem cell research, gene therapy and cancer treatment, which has already brought a new perspective for more effective and targeted treatment for a wide variety of human diseases.

To better understand one aspect of gene regulation in yeast, we wished to find the cause of the inactivation of Puf3p depending on which carbon source is available. Based on the hypothesis that Puf3p may be non-functional in ethanol conditions due to less protein being expressed under such conditions, I first wanted to measure and compare the amount of *PUF3* mRNA and of Puf3 protein between the different conditions.

Steady state experiments were performed in which 100 ml yeast cultures in YEP media with different carbon sources (glucose, ethanol, galactose and raffinose) were

grown to an OD of 0.4 and harvested. 70 ml of each culture was used for mRNA extraction, and the remaining 30 ml was used for making protein extracts. The analysis of *PUF3* mRNA by northern blots and Puf3 protein by western blots indicated that both the amount of *PUF3* mRNA and Puf3p measured in ethanol, galactose or raffinose is equal or greater than that measured in glucose (Fig.2). These findings suggest that the extended *COX17* mRNA half-life in these carbon sources compared to glucose is likely due to a post-translational alteration of Puf3p activity.

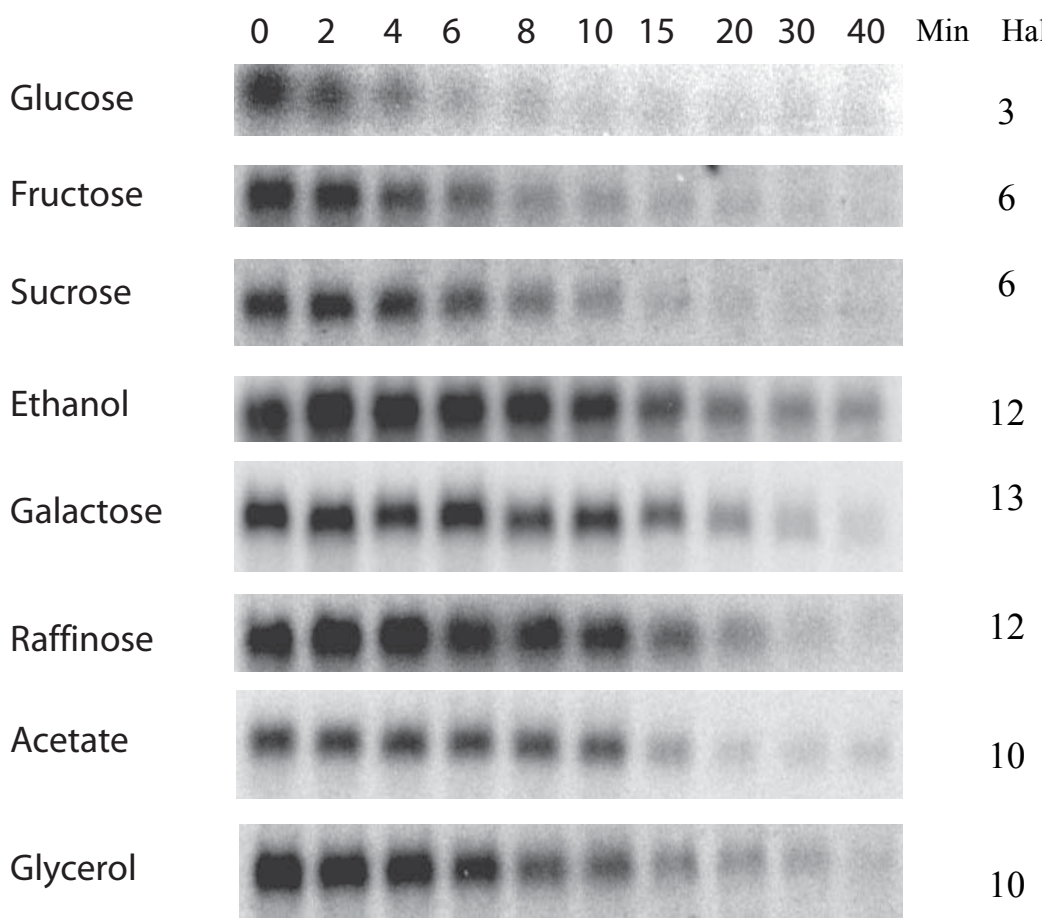

Fig.1 Analysis of Puf3p activity under different conditions as measured by *COX17* mRNA decay. The destabilizing activity of Puf3RD is dependent on the available carbon source. At the top, time in minutes is shown after transcription was shut off and samples of the culture were taken for the analysis. On the right, half-life in minutes of *COX17* mRNA decay is shown in different carbon sources in the WT strain.

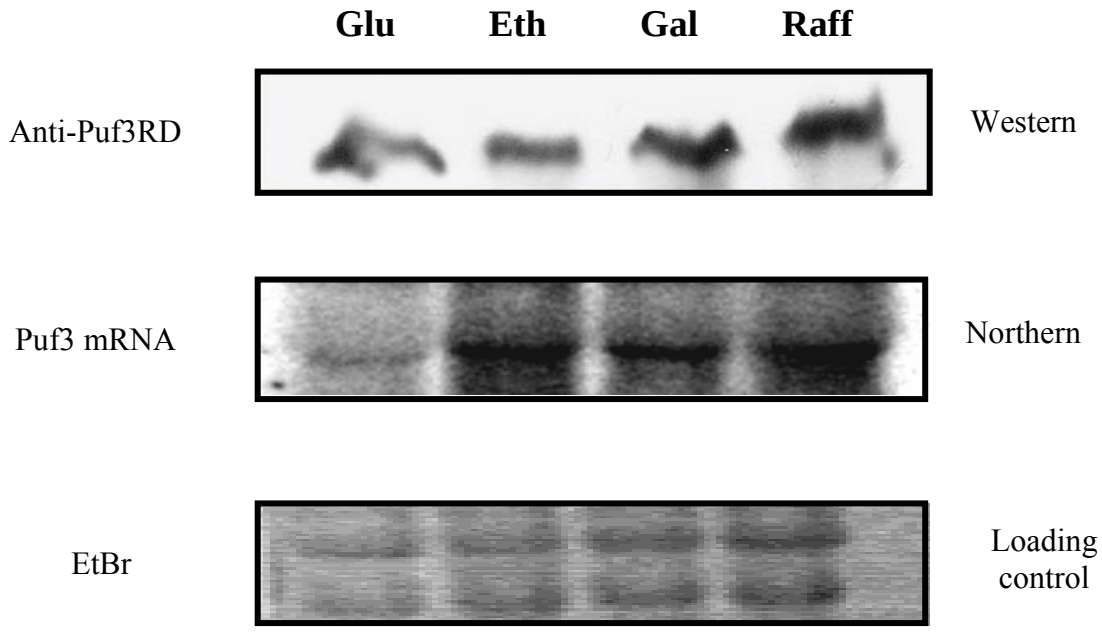

Fig.2 Puf3p and *PUF3* mRNA levels in different conditions. In ethanol (Eth), galactose (Gal) and raffinose (Raff) conditions, the levels of both Puf3p and *PUF3* mRNA are similar or even higher than in glucose (Glu) conditions. Puf3p was detected with anti-Puf3RD antibodies. *PUF3* mRNA was detected with radiolabeled Puf3 oligo probe. Ethidium bromide (EtBr) staining of ribosomal RNA shows amount of RNA loaded onto Northern.

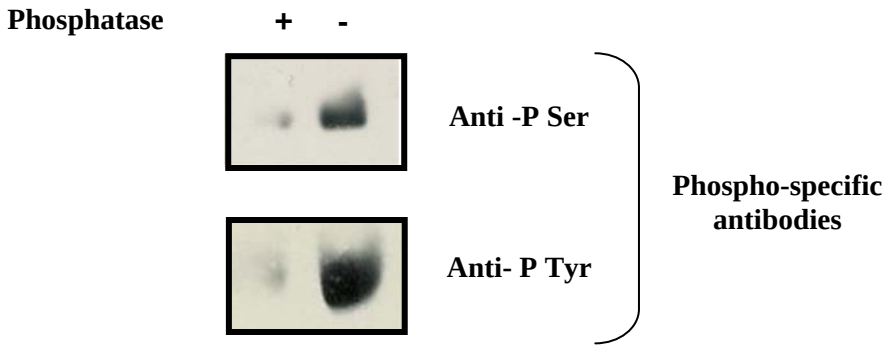

Fig.3 Puf3p is phosphorylated. Western blots of FLAG-tag purified Puf3RDp treated with and without phosphatase. Phosphorylated amino acids were detected using anti-phospho serine or anti-phospho tyrosine as primary antibodies. Shown is protein purified from glucose cultures.

Puf3p is phosphorylated.

In Dictyostelium, Puf A binds the 3' end of *PKA-C* mRNA, thereby regulating *PKA-C* translation. Under starvation conditons, Dictyostelium responds by repressing Puf A via phosphorylation by kinase Yak A. This relieves the negative control Puf A exerts on *PKA-C*, which is required for initiation of development (Mendes Souza et al., 1999). It might be that in a similar way as the Dictyostelium Puf, yeast Puf3p activity is regulated by its phosphorylation state. We first wanted to determine whether phosphorylation was one of the post-translational modifications occurring on Puf3p when purified from yeast cultures grown under different carbon sources. We wanted to see if there was a correlation between Puf3p activity and a possible difference in its phosphorylation state. We obtained protein extracts from yeast cultures, one grown in glucose and another in galactose as carbon sources. To enrich the extracts for Puf3RD, equal amounts of extract were used to perform immunoprecipitations in which FLAG-tagged Puf3RD was captured and eluted, then treated with or without phosphatase. The westerns of these treated samples were developed using phospho-specific antibodies (anti-P-Ser and anti-P-Tyr). Both Ser and Tyr residues were found to be phosphorylated in Puf3p recovered from cultures grown with either glucose or galactose as carbon sources (Fig.3 and data not shown). This data establishes that Puf3p is phosphorylated. However, because multiple sites on the protein may be phosphorylated, this result does not distinguish whether there is a particular site that is differentially phosphorylated under the different conditions. Future mass spectrometry work analyzing tryptic digests of Puf3p purified from different carbon sources might shed light on any differential phosphorylation event.

Different phosphorylation mutants of Puf3RD show different *COX17* mRNA decay phenotypes.

Using the phospho-specific antibodies, I was unable to detect a particular difference in phosphorylation state. Thus, I took an alternative approach to try and identify any critical amino acid residues in the RD that might alter Puf3RD activity when phosphorylated. We took advantage of a bioinformatic program (NetPhos 2.0) that utilizes the protein's primary structure to predict Ser, Thr or Tyr residues most likely to be phosphorylated according to their surrounding sequence. Three Ser and one Tyr residue within the Puf RD were selected for subsequent mutation into Ala through site-directed mutagenesis. Two of the Ser were located in repeat 1 and the mutants were identified as R1A and R1B. The third mutation was made in Tyr of the third repeat, and the last one corresponded to a Ser of repeat 8; each was named as R3 and R8, respectively. Different combinations of these single mutations were made, resulting in double, triple and quadruple mutants. Each of the plasmids expressing the Puf3RD with one or more mutations was transformed into a *puf3*Δ strain, and transcriptional shut off experiments were performed with each of the resulting strains (Fig.4).

Results show that by mutating different conserved amino acid residues present in the Puf3RD that could be phosphorylated, several *COX17* mRNA decay phenotypes can be detected. With the experiments done so far, the most significant differences were seen in the R1A, R3 and R8 individual mutations, as well as the respective triple mutant in affecting Puf3RD activity. It is possible that the substitution of Ser or Tyr for Ala is not altering the phosphorylation of the Puf3p protein, but may simply be causing conformational changes in the protein that affect its ability to bind to the RNA or the

decay factors. Future work should analyze these amino acids by mutating them to Asp in order to mimic phosphorylation. If this mutation restores Puf3p activity versus the Ala mutant, then this would indicate that it is the phosphorylation state of the amino acid that is altering activity and not just a structural defect.

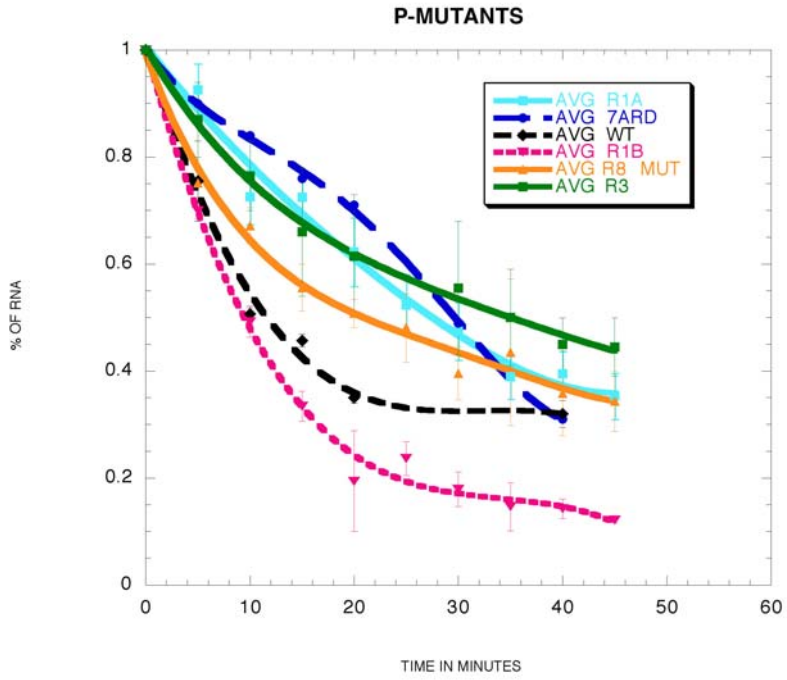

Fig.4 Graph comparing decay rates of the Puf3RD phosphorylation site mutants. Time after transcription shut-off is represented in minutes on the x axis. % of RNA remaining is represented on the y axis. Half-life for WT is approximately 14 minutes, similar to R1B. Mutants R1A and R3 have half-lives twice as long as the WT. The R8 mutant displays an intermediate half-life phenotype.

Puf3-GFP images under different environmental conditions may show a difference in protein aggregation.

P-bodies have been defined as sites of mRNA storage, decapping and decay. In order to visualize these decay factors in the cell, several GFP fusion constructs of different yeast mRNA decay factors were observed in vivo. Puf3-GFP was shown to be evenly distributed throughout the cytoplasm, while Dcp2-GFP was found in P-bodies. All fusion constructs have been shown to be functional and were made with full-length proteins GFP labeled at the C terminal (Sheth and Parker, 2003).

Another hypothesis to explain the change in the activity of Puf3p under different conditions may be that alteration of protein localization prevents it from performing its function. I used the same GFP tagged strains as described above to evaluate any difference in localization of Puf3-GFP in yeast grown under different carbon sources. Dcp2-GFP was used as a positive control, and yWO5, a WT yeast strain that is not GFP tagged, as a negative control. Though the imaging of Puf3-GFP does not show a radical difference in protein localization when comparing ethanol, galactose, and raffinose conditions versus glucose, the images do show a more clumpy phenotype under the first three conditions. This punctate pattern may be reflecting aggregated protein versus homogeneously distributed protein. However, this punctuate pattern is clearly not the same type as the P-body loci seen with Dcp2-GFP (Fig.5). These results are supported by experiments in which the amount of Puf protein isolated was measured under different conditions. Using the protein boil prep method, in which both soluble and insoluble protein is recovered, the westerns show a greater amount of Puf3 protein present in conditions other than glucose. On the other hand, when using the YPER method, in

which most of the protein recovered is in a soluble state, the greater amount of Puf3 protein is recovered in glucose compared with the other conditions (Fig.6). This means that even though there might be more Puf3 protein in ethanol, galactose and raffinose compared to glucose, most of this protein may be in an insoluble, non-functional state. To conclude, non-functional protein can be visualized and compared to the functional version obtained in glucose media by using a hybrid Puf3p with a GFP tag, which allows the observation of a clumpy versus a homogeneous cytoplasmic distribution correlated to media and protein activity.

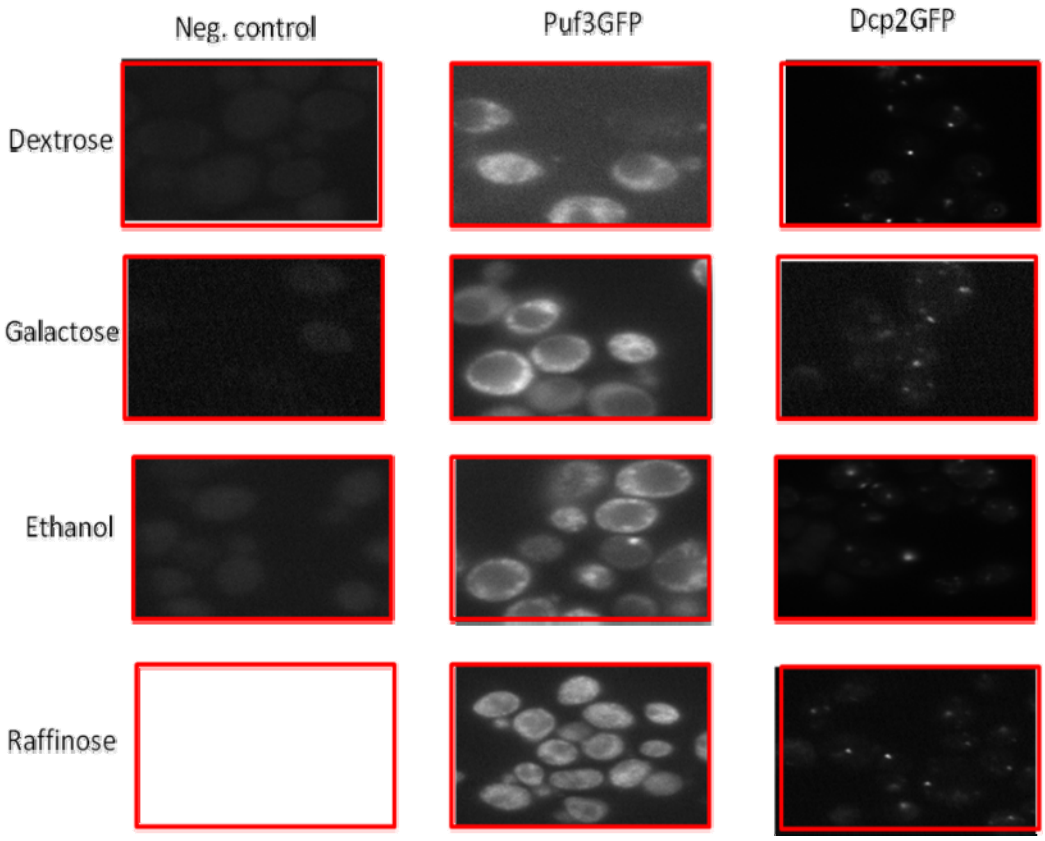

Fig.5 Images of Puf3-GFP in different carbon sources (central column). Images on the left column correspond to a negative control strain tested in glucose, galactose, ethanol and raffinose N/D. Images on the right column correspond to Dcp2-GFP, which was used as a positive control and localizes in very distinctive spots known as P-bodies. The central column shows the distribution and appearance of Puf3-GFP under the conditions tested.

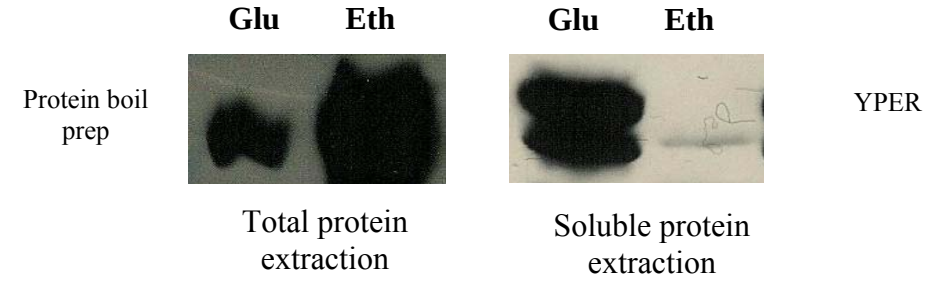

Fig.6 Puf3p recovered using different extraction protocols. Western blots developed with anti Puf3RD antibodies of protein extracts recovered from cultures grown in glucose and in ethanol. Extractions were performed using protein boil prep protocol on the left and YPER protocol on the right.

Discussion

Previous research in our lab has shown Puf3RD's activity being dependent on the type of carbon source available. It was shown to be inactive in ethanol and active in glucose, with activity measured by comparing *COX17* mRNA half-life (a Puf3-mediated decay target) in the different conditions. This half-life was shown to be 2.5 minutes in glucose and 10 minutes in ethanol (Foat, et al, 2005). So far we have detected similar levels of both Puf3 protein and mRNA under ethanol, galactose and raffinose conditions in which the protein was in an inactive state compared to glucose. This information, together with the observation of very rapid Puf3p activity changes in response to different carbon sources in the media, led us to consider one of the most common post-translational modifications, phosphorylation, as a way of quickly responding to these environmental changes. In fact, I demonstrated that Puf3RD is phosphorylated by performing a phosphatase treatment of purified Puf3p and using phospho-specific antibodies to detect phosphorylated serines or tyrosines. Further site–directed mutagenesis analysis, in which four of the most conserved potential phosphorylation residues within the Puf3RD were mutated to alanines, allowed us to evaluate their functional role in Puf3p activity through their ability to rescue a *puf3Δ* strain. Several decay phenotypes were detected, but mutations in R1A, R3 and R8 seem to be very important in promoting Puf3RD activity. These point mutations in the serines or tyrosine might alternately be responsible for a general conformational change affecting RNA binding or the recruitment of the decay factors. Further studies should be performed with the R1A-R3-R8 triple P-mutant since preliminary data suggests that these three mutations together might be decreasing Puf3RD activity to a greater extent. Substitution of these

specific alanines to aspartic acid to mimic a phosphorylated state should show a re-gain in function if it is indeed phosphorylation that controls activity. To further confirm any possible role of phosphorylation on Puf3p activity, a set of kinases involved in nutrient response will be tested for their role in Puf3p activity through transcriptional shut-off experiments using different kinase knockout strains under different carbon sources and detecting any differences in the *COX17* mRNA decay rate.

In this work we also addressed the possibility of associating an inactive state of Puf3p with it becoming inaccessibly localized or aggregated. Visualizing Puf3-GFP protein from yeast cultures grown in glucose demonstrated a cytoplasmic and homogeneous distribution in the yeast cells, while a difference in distribution was detected under galactose, raffinose and ethanol conditions that coincided with inactive Puf3p, which all demonstrated a more punctate appearance. These differences were shown to correlate with differences in solubility. Two protein extraction protocols showed that even when the amount of Puf3p present in ethanol, galactose and raffinose is greater than in glucose, most of it is in an insoluble, non-functional state. On the contrary, most of the protein recovered from yeast cultures grown in glucose was soluble and functional.

As it has been previously shown in *Dictyostelium* in which activation of PufA was achieved by a different phosphorylation state of the protein to allow developmental changes (Mendes Souza et al., 1999). This research further confirms that this type of post-translational regulation may also occur in Puf3p in yeast. By better understanding the mechanisms by which yeast and other lower eukaryotes are able to regulate gene expression in their response to changing environmental conditions, a stronger basis will

be established that might be applicable to the regulation of Puf activity in higher eukaryotes.

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Chapter V

Experimental identification of bona fide Puf4p mRNA targets in yeast and analysis of Puf4p role in mRNA decay

Introduction

Genome-wide RNA-protein interaction analyses have shown the presence of conserved sequence elements in the 3'UTRs of distinct groups of mRNAs that help provide a coordinated control of gene expression in response to different physiological and environmental conditions. One such post-transcriptional regulation through RNA-binding proteins in yeast is the Puf family of proteins. There might be a general mechanism for controlling in a coordinated way localization, translation and decay of a particular set of mRNAs as an integral part of the global gene expression program. Through DNA microarrays, conserved sequence elements in the 3'UTRs of mRNAs that were pulled down were identified that are needed for Puf3p, Puf4p and Puf5p binding (Gerber et al, 2004). This UGUANAUA sequence was also experimentally confirmed to be required for Puf3p binding in our lab; in fact, *COX17* 3' UTR contains two distinct binding sites for recruiting Puf3p and stimulating its decay (Jackson et al. 2004). Results obtained from this genome-wide analysis of the mRNAs pull-down by individually tagged Pufs showed a preferential association of Puf1p and Puf2p with mRNAs encoding membrane associated proteins, Puf3p with cytoplasmic mRNAs encoding mitochondrial proteins, Puf5p with mRNAs encoding chromatin modifiers and components of the spindle pole body and Puf4p with mRNAs encoding nucleolar ribosomal RNA-processing factors (Gerber et al. 2004). Puf4 protein has also been shown to somehow be involved in localization of Sir proteins to the nucleolus, and thus, in the regulation of aging in yeast (Kennedy et al., 1997), its mode of function is yet unknown.

Puf4p in yeast consists of 888 amino acids, is approximately 97 KDa and as a characteristic feature of the Puf family it contains the Pumilio homology domain,

comprised of eight α-helical repeats each of which recognizes one RNA base. Recently Puf4p crystal structure has been solved showing an altered RNA binding specificity that allows the protein to accommodate an extra nucleotide by allowing the rotation of one of the bases away from the RNA binding surface (Miller et al, 2008). At the beginning of this study, there were no experimentally determined Puf4p mRNA targets known. Taking advantage of previous high throughput studies, I wanted to experimentally test mRNA candidates that were ranked as top Puf4p targets by both co-immunoprecipitation/microarray studies (Gerber et al., 2004) and computational predictions using Matrix REDUCE (Foat et al., 2005). The information obtained from these types of studies is very useful to help narrow down the selection of possible candidates for targeted decay by a particular yeast Puf. However, experiments in our lab have shown that not all the hundreds of mRNAs that are predicted to be targets of a specific Puf turn out to be real Puf-mediated decay targets. It could be that in the complexity of the cell, factors other than Pufs might be influencing the expression of these Puf predicted targets under these conditions, or that these targets are indirectly being affected by Pufs.

In this research I experimentally identified targets of Puf4p-mediated decay and analyzed the mechanism of Puf4p action in yeast. This is especially interesting given that expression profiles of predicted Puf4p targets are so different than for targets of the well-studied Puf3p based on the inferred activities of Puf3p and Puf4p under different environmental conditions from the Matrix REDUCE results (Foat et.al, 2005). I first compared the steady state levels of each mRNA candidate from a wild type strain and a *puf4*Δ strain. Having identified differentially expressed candidates, transcriptional shut

off experiments were performed to see whether there were differences in the decay rate of the transcripts in the presence or absence of Puf4p. These targets were found to be co-regulated by both Puf4p and Puf5p.

Experimental Procedures

Steady state experiments

Seven mRNA candidates were initially chosen based on two lists of possible Puf4p target mRNAs. One list from the Bussemaker lab was computationally selected using microarray data and the 200bp sequence downstream of every yeast ORF as input, while the other list from the Brown lab was obtained from co-immunoprecipitation of mRNAs with Puf proteins. Complementary DNA probes were designed for each one of these mRNAs and were proven to be unique using the PATmatch search from the Saccharomyces Genome Database. The mRNAs tested included, *PWP1, HPT1, ECM1, YJL122W, PUS7, EBP2* and *RRS1*.

To perform these steady state experiments, wild type (yWO 7) and *puf4*Δ (yWO 105) strains were each grown in either rich YEPD (dextrose) or YEPE (ethanol) media at 24˚C to an OD of 0.4, at which time they were harvested. The cells were then processed for RNA extraction, 40µg of each RNA sample was separated on 1% agarose gels, and Northern blots were prepared and probed for each candidate mRNA. Blots were also probed for *7S* RNA to control for loading.

Transcriptional shut-off experiments

In order to determine if the steady-state differences were due to the Puf4p affecting the decay rate of these mRNAs, a set of transcriptional shut-off experiments were carried out to monitor the decay rate of a pool of steady-state mRNA following repression of their transcription. These experiments were originally attempted in strains containing the temperature sensitive RNA polymerase II, where transcription is repressed by a shift of the culture media to 37˚C.

An alternative method of performing transcriptional shut-offs is to clone each gene of interest into a yeast vector under the control of a repressible promoter. For example, a *GAL* promoter can be induced by the addition of 2% galactose and repressed by switching to a media containing 4% glucose. The *TET* promoter can be repressed by the addition of doxycycline (2µg/ml) to the culture media (Belli et al, 1998). Both promoters were tested since there is some evidence from microarrays that Puf4p may be inactivated by galactose, while the *TET* promoter construct is more difficult to clone and ensure expression. In the case of *YJL122W* and *PUS7,* which are both non-essential genes, the endogenous genes were knocked out of the wild type and *puf4*Δ strains so that only the plasmid derived mRNA would be visualized on Northern blots. To make the knock-outs, the *TRP1* gene was PCR amplified from pRS200 as a template using primers that were designed to be complementary to either side of the *TRP1* gene at their 3' ends, but contained ~ 40 nt of secquence complementary to either side of the target gene to be knocked out. oWOs 369 and 370 were complementary to *YJL122W,* and oWOs 371 and 372 were complementary to *PUS7*. The PCR products obtained with the corresponding flanking homologous regions were used to knock out *YJL122W* or *PUS7* in each of the

strains yWO 5 (WT) and yWO 22 *(puf4*Δ). However, for the essential genes *EBP2* and *RRS1*, unique sequence tags have to be inserted into the plasmid genes so that probes complementary to these sequences will allow visualization of only the plasmid derived mRNAs on Northerns. Both *RRS1* genes flanked by BamH I and Hind III one with a *GAL10* promoter and the other with a *TET* promoter were inserted in pGEM3Z and they were cut at the Acc I site for annealing an oWO for tagging purposes.

Table 1 Strains used in these studies

Deletion	Strain	Genotype	Source
W T	yWO 5	MAT a, *trp1, ura3-52, leu2, his4-539,cup1:LEU2/PM*	Olivas &Parker(2000)
W T	yWO 7	MAT x, *ura3-52, leu2, rpb1-1*	Olivas &Parker(2000)
*Puf4*Δ	yWO 22	MAT α, *ura3-52, leu2-3,112, trp1, lys2, cup1:LEU2/PM, YGLO14w::LYS2*	Olivas &Parker(2000)
*Puf4*Δ	yWO 105	MAT α, *ura3,his4,rpb1-1, YGLO14w::LYS2*	Ulbricht & Olivas (2008)

Results

Steady state experiments

An initial analysis of the seven selected mRNA candidates indicated that only four (*YJL122W, PUS7, EBP2 and RRS1*) showed a 2 to 3 fold difference in levels between the WT and *puf4*Δ strain when grown in glucose (Fig.1). There were no significant differences in mRNA levels between both strains grown in ethanol. Since mRNA levels were higher in the WT versus *puf4*Δ grown in glucose, these results suggest a stabilizing activity of Puf4p on its targets. Moreover, the equally low mRNA levels in both WT and *puf4*Δ strains grown in ethanol suggest a loss of stabilizing activity of Puf4p when grown in a non-fermentable carbon source. These results also showed that not every candidate mRNA is a true target of Puf4p.

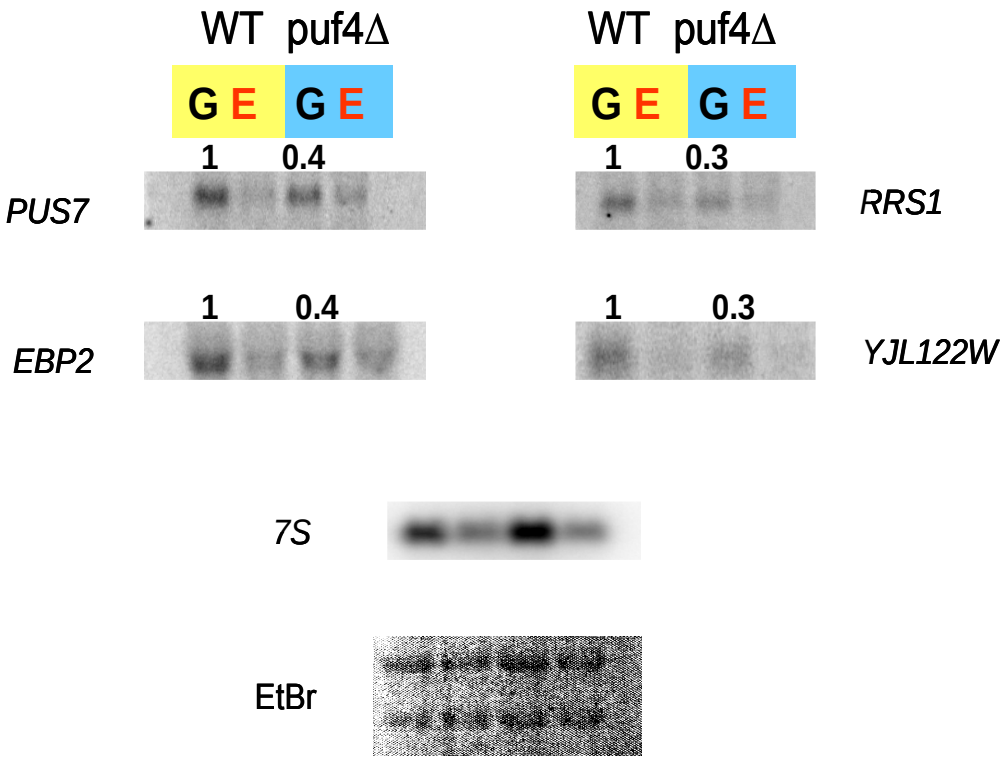

Fig.1 Northern blots of steady state experiments. mRNA levels compared between WT and *puf4*Δ strains of yeast cultures grown in media with glucose (G) or ethanol (E) as carbon sources. *PUS7* and *EBP2* are shown on the left, *RRS1* and *YJL122W* are shown on the right. *7S* and ethidium bromide staining of ribosomal RNA are used as loading controls. The numbers on top of the corresponding lanes show RNA quantification after normalization to *7S*.

Transcriptional shut-off experiments.

The four mRNAs *YJL122W, PUS7, EBP2 and RRS1* that showed a difference in steady-state mRNA levels in WT versus puf4Δ strains all encode proteins involved in ribosomal RNA biogenesis. *EBP2* and *RRS1* are essential genes in the cell, while *YJL122W* and *PUS7* are non-essential. Rrs1p (Regulator of Ribosome Synthesis) is an essential protein that binds ribosomal protein L11 and is required for 25S rRNA maturation and 60S ribosomal subunit assembly and export from the nucleolus to the cytoplasm (Miyoshi, K. 2002; Mizuta, K., 2004). *RRS1* was originally found as the gene responsible for signaling transcriptional repression for rRNA and ribosomal proteins because of a blockage in the secretory pathway (Horigome et al. 2008). It has been shown in mouse model of Huntington's disease (HD) in which the expression of mutant 111-glutamine murine huntingtin protein leads to a dominant phenotype, that is correlated with an elevated *RRS1* mRNA expression that can be used to detect very early onset of HD (Fossale et al. 2002). Ebp2p is also an essential protein that might be Rrs1p target for performing 25 S rRNA maturation and 60 S subunit assembly (Tsuyii et al. 2000). Ebp2p, the yeast homolog of human Epstein-Barr virus nuclear antigen 1- binding protein was shown to interact Rps16p (ribosomal protein S16) and Utp11p (the 40 S ribosomal subunit assembly factor) (Shirai et al. 2004). Ebp2p similarly to Rrs1p has been found to be implicated in the secretory response (Horigome et al. 2008). *PUS7* is a non-essential gene that encodes for a pseudouridine –synthase Pus7p that in yeast acts catalyzing U to ψ conversion both in U2 snRNA and in pre-tRNA (Behm- Ansmant et al. 2003).

In order to determine if the steady-state differences are due to the Puf4p affecting the decay rate of these mRNAs, a set of transcriptional shut-off experiments were carried

out to monitor the decay rate of a pool of steady-state mRNA following repression of their transcription. First a set of experiments was done in strains containing the temperature sensitive RNA polymerase II, where transcription is repressed by increasing the temperature to 37˚C. However, all mRNA targets tested decayed fairly rapidly in both wild-type and *puf4*Δ strains. We believe that this result was due to the Puf4p being inactivated by the heat shock. Microarray analysis showed that candidate Puf4 target mRNA levels are low in cultures grown at high temperature, which suggests an inactivation of Puf4p stabilizing activity (Foat et.al. 2005).

Next, two alternative ways of performing transcriptional shut-offs were done in which each gene of interest was cloned into a yeast vector under either a *GAL* or *TET* repressable promoter. *YJL122W* and *PUS7,* which are both non-essential genes, were knocked out of the wild type and *puf4*Δ strains so that only the plasmid derived mRNA would be expressed. Since *EBP2* and *RRS1* are essential genes, unique sequence tags were inserted in the cloned genes so that specific complementary probes would allow visualization of the plasmid derived mRNAs. The results in figure 2 show the decay rates of the plasmid-derived *YJL122W* under a *GAL* promoter in WT and *puf4*Δ strains as an example. The half-lives of the four different mRNAs tested (*YJL122W, EBP2, RRS1 and PUS7)* when comparing WT to *puf4*Δ were very similar for each mRNA in both strains. Further research performed by two undergraduates under my supervision (Jeanne Pitts and Susana Pulido-Fernandez), showed that these mRNA targets were being co-regulated by both Pu4p and Puf5p. Preliminary results indicate that Puf4p activity might be inhibited by galactose and ethanol, while Puf5p retains its activity for decay stimulation under these conditions.

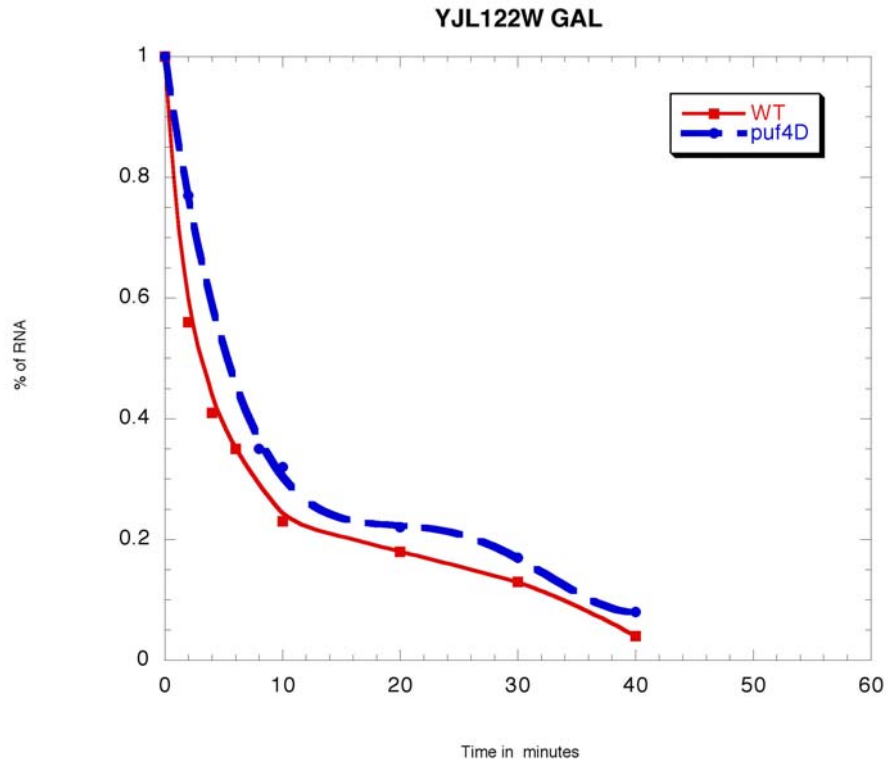

Fig. 2 Decay rates of *YJL122W* under *GAL* promoter. Time in minutes is represented in the x axis and % of RNA is represented on the y axis. WT and *puf4*Δ strains are represented.

Discussion

During the process of this research, evidence of redundant regulation by Puf4p and Puf5p was shown for *HO* mRNA (Goldstrohm et al., 2007). The fact of having found in our lab two new Puf targets, *TIF1* and *HXK1*, which are both regulated by Puf1p and Puf5p with the addition of Puf4p for *HXK1* also showed the possibility of a coordinated regulation, since all these Pufs are needed for full decay stimulation of their target mRNA (Ulbricht and Olivas, 2008).

So far, the steady state analyses using strains grown both at 24ºC and at 30ºC indicate that the identified Puf4p targets are also regulated by Puf5p. Though both Pufs seem to be active and redundantly regulating their targets in glucose, their activities appear to be differently affected by the presence of other carbon sources. These results together with the recent finding of Puf4p's altered RNA-binding specificity that allows it to bind either a eight or nine nucleotide sequence, supports the idea of redundant regulation. On the other hand, the fact of Pufs showing different activity profiles under different carbon sources aligns with the idea of a combinatorial way of regulation. Probably both types of regulation provide the cell with a robust and also flexible mechanism to better respond to changing environmental conditions.

Future work will need to determine whether the combination of Puf4p and Puf5p is really altering target mRNA levels via changes in RNA decay rates. A broader spectrum of strains including WT, *puf4*Δ*, puf5*Δ*, puf4&5*Δ and all *5pufs*Δ, should be used for performing transcriptional shut-off experiments to evaluate under different carbon sources, the extent of each one of the Puf's regulation in a particular environment.

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Chapter VI

Summary and future directions

Organisms have to adapt to changing environmental conditions in order to survive. A precise and efficient regulation of gene expression is required to obtain the optimal response at the lowest cost of energy. The Puf protein family of RNA binding proteins has been identified as an important and widespread group that regulates post-transcriptional steps of the gene expression pathway. Regulation is achieved by altering the decay and translation rates of specific target mRNAs. In *Drosophila*, Pumilio has not only been shown to be critical for embryo patterning by establishing a gradient of hunchback mRNA through translational repression, but has also been shown to regulate neuronal excitability by affecting the expression of a Na+ channel gene (Schweers et al, 2002; Mee, 2004). Together the Puf protein family is involved in mRNA expression regulation for mediating several developmental decisions such as cell differentiation and stem cell maintenance, as well as involved in neuronal plasticity accounting for long-lasting structural and functional modifications in the synapsing neurons that are required for the acquisition of long-term memory. However, the mechanism of Puf protein action in these processes is largely unknown. In yeast, Puf proteins were found associated with hundreds of different mRNA targets, though the functional significance of most of these interactions is unclear (Gerber et al., 2004). Of the six Pufs identified in *Saccharomyces cerevisiae*, Puf3p has been the best characterized to date. Puf3p regulates *COX17* mRNA decay by specifically binding two conserved UGUANAUA sequences present in its 3' UTR and thereby stimulating both deadenylation and decapping. It has also been shown that the Puf3p repeat domain alone is sufficient not only for *in vitro* binding to the 3'UTR, but also for *in vivo* stimulation of *COX17* mRNA decay (Jackson et al, RNA, 2004).

In this research, insight was gained on Puf3p interactions with other proteins that contribute to its mechanism of action for *COX17* mRNA regulation. Similar to the Puf5p mechanism of action for achieving *HO* mRNA regulation, Puf3p binds through protein interactions with several members of the decay machinery including, Pop2p, Ccr4p, Lsm1p, Dcp1p and Dhh1p. I found the R7A outer surface loop to be the precise region to which Pop2p directly and Dhh1p indirectly through Pop2p binds the Puf3RD, while the other decay factors bind to Puf3RD independently of this loop region and Pop2p. This differs from the model of Puf5p interactions in which Pop2p is thought to bridge between Puf5p and all the deadenylating and decapping factors, and no specific binding site has yet been described (Goldstrohm et al, 2007). The interactions between Puf3RD and the decay machinery were verified through both modified yeast three-hybrid assays and co-immunoprecipitation experiments. I also confirmed that at least in the case of Puf3-mediated decay of *COX17* mRNA, the process is independent of the nonsense-mediated decay pathway (NMD).

Puf3p activity was next analyzed under different environmental conditions to better understand how its activity responds to the carbon source available. Research in our lab shows Puf3p inactivity under ethanol, galactose and raffinose conditions versus glucose, yielding extended mRNA half-lifes of Puf3p targets under these inactivating conditions. The high levels of both Puf3 protein and mRNA found under ethanol, galactose and raffinose in which the protein was in an inactive state compared to glucose, led us to consider post-translational modifications of Puf3p as a way of quickly responding to the environmental changes. In fact, other work from the Olivas lab has observed that Puf3p activity can be rapidly turned on and off by simply changing the

carbon source in the culture media (Miller, unpublished data). Puf3 protein was found to be consistently phosphorylated under different carbon sources. This could be one of the most common post-translational modifications to regulate its activity. Both phosphorylated Ser and Tyr residues were detected using phospho-specific antibodies, but our assays were unable to determine whether specific sites were being differentially phosphorylated under different conditions. Further work will involve using mass spectrometry for detecting any specific modification sites on tryptic fragments of the Puf3RD purified from different conditions. It will also be interesting to test interactions between Puf3RD and all the decay factors under different carbon sources to determine whether the same protein interaction network is maintained under conditions when the protein is active versus when it is inactive. Another experiment that could be used for detecting differences in Puf3p protein interactions is a translational repression assay procedure (TRAP). For this assay, a GFP reporter construct is designed such that it has a binding site for the protein being tested at its 5' UTR. If the protein is able to bind, then ribosome recruitment is inhibited and there will be no GFP translated. The difference in binding capability due to the carbon source will be reflected in the amount of GFP measured by flow cytometry.

To further analyze the effect of phosphorylation on Puf3p activity, potential phosphorylation sites were identified through bioinformatic analyses, and four of the most conserved sites within the Puf3RD were mutated to alanines to test their function in Puf3p activity. Each mutant demonstrated a less than 2-fold effect in extending the *COX17* mRNA half-life, suggesting that phosphorylation of these sites is likely not the key trigger for activating Puf3p activity. Instead, mutation of these sites may be causing

structural changes to the protein that cause a slight decrease in protein efficiency. It is possible that phosphorylation acts to inhibit Puf3p activity, so further studies could mutate these sites to aspartic acid to mimic a phosphorylated state and determine if this inhibits activity. To further analyze any possible role of phosphorylation on Puf3p activity, yeast strains deleted of different kinases known to be involved in nutrient response could be screened for any altered *COX17* mRNA decay rate in glucose versus ethanol conditions to test a role in Puf3p activation or inhibition.

This work also addressed the possibility that the localization or aggregation state of Puf3p may be involved in the inactivation of Puf3p. Puf3-GFP localization under different carbon source conditions appears to be cytoplasmic. However, the protein seems to have a more punctate pattern under inactivating conditions (galactose, raffinose and ethanol). This might indicate a more aggregated or insoluble state of the protein in these conditions compared to glucose. In support of this hypothesis, more soluble Puf3 protein is extracted from cells grown in glucose versus ethanol using the YPER extraction protocol that preferentially obtains soluble protein, while more Puf3p protein is actually produced in ethanol versus glucose conditions as obtained from the boil prep extraction procedure that collects both soluble and insoluble protein. This suggests that even when the amount of Puf3p present in ethanol is greater than in glucose, much of it may be in an insoluble, non-functional state.

Another aspect of this research focused on experimentally confirming new Puf4p target mRNAs and evaluating whether Puf4p activity might also be affected by environmental conditions. Computational predictions by the Matrix REDUCE algorithm showed a complete opposite expression pattern for transcripts containing Puf3p and

Puf4p binding elements. Under conditions with fermentable carbon sources when Puf3p regulated transcripts are down-regulated, Puf4p target transcripts are up-regulated. These expression pattern predictions make sense metabolically. Puf3p target transcripts code for proteins involved in mitochondrial function, so these proteins are only needed under non-fermentable carbon sources such as ethanol. Thus, the Puf3p target transcripts should be down-regulated in glucose when mitochondria are not needed. In contrast, many predicted Puf4p target transcripts code for proteins involved in ribosomal biogenesis. In glucose conditions when the growth rate is fast, there is a need for high numbers of ribosomes for protein production. Thus, the Puf4p target transcripts should be up-regulated in such conditions.

Four Puf4p mRNA targets (out of seven) were experimentally validated using analysis of steady-state RNA levels from different conditions. These targets were: YJL122W, RRS1, PUS7 and EBP2. However, transcriptional shut-off experiments to measure mRNA decay rates of these targets revealed no significant differences between wild-type and *puf4*Δ strains. It is possible that these mRNA targets are not regulated by Puf4p at the level of mRNA decay, or that the experimental treatments used to shut off transcription altered Puf4 activity. However, the Olivas lab and others have shown that many Puf target mRNAs are regulated by combinations of Pufs, including *HXK1* and *HO* mRNAs both regulated by Puf4p and Puf5p (Ulbricht and Olivas 2008, Goldstrohm et al. 2006; Hook et al. 2007). I therefore supervised studies performed by two undergraduates (Jeanne Pitts and Susana Pulido Fernandez) that provided evidence from steady-state RNA analyses that all of the identified Puf4p target RNAs are also regulated by Puf5p, and these two Pufs may be acting redundantly under glucose conditions. One or both Puf

protein activities may then be altered under different carbon source conditions to cause changes in RNA levels. Future work will need to determine whether the combination of Puf4p and Puf5p is really altering target mRNA levels via changes in RNA decay rates, and how different carbon sources may affect Puf4p and Puf5p activity.

Up to date we know that Puf proteins belong to a family of RNA-binding proteins that are widely conserved across eukaryotes. We can assign them a central role in regulating mRNA stability of several targets that contain specific control elements in their 3' UTRs. Through this regulation, two main roles are fulfilled: one involved in cell development and differentiation, and the other related to neuronal morphology and physiology. Although we and others have shown the importance and sufficiency of the Puf repeat domain for binding and stimulating mRNA decay, we should also keep in sight that there may be some important yet currently unknown role for the rest of the Puf protein outside the repeat domain.

Puf proteins in humans have been shown to be expressed in the brain and form complexes with different proteins, one of them being CPEB (Cytoplasmic Polyadenylation Element Binding protein), which controls translation of synaptic mRNAs. Human Pum 2 has specifically been shown to be present in dendritic stress granules (SG) in mammalian hippocampal neurons. The induction of these granules depends on both the RNA-binding domain and the glutamine-rich N terminus of Pum 2, which makes it prone to aggregation. Both Pumilio and Staufen, another protein also present in SGs, are involved in the process of acquiring long term memory (Dubnau, 2003). CPEB's presence in hippocampal cells is important for regulating local protein synthesis essential for late phase of long term potentiation. The conversion of CPEB to a

prion-like state in stimulated synapses contributes to the long-term maintenance of a self-sustaining synapse-specific change associated with memory storage. Since this prion-like state is self-perpetuating, it no longer requires continued signaling either by kinases or phosphatases. We have shown in yeast that different environmental conditions are responsible for an active or inactive Puf protein. Similarly, particular local conditions in the mammalian brain that occur to induce a change to the aggregated prion-like state for acquisition of long term memory may also be regulating Puf activity. We have seen how certain carbon sources in yeast induce a punctate, possibly aggregated form of Puf3p. It is possible that the N-terminal portion of the protein that is glutamine-rich might make it prone to aggregation. A speculative hypothesis suggests that aggregation of Puf proteins may in one case cause a loss of function (*COX17* mRNA regulation) and on the other hand may create a gain of function in the neuron for memory storage.

In conclusion, Puf proteins are involved in several important processes in eukaryotic cells. Given their role in stem cell maintenance and cell differentiation, Puf protein activity may be an important factor to address in the growing field of stem cell therapy research. Knowledge of Puf protein activity in neural function may also be important in the development of new therapies for neurodegenerative diseases such as Alzheimers, Huntingtons, Parkinsons and several of the prion-related illneses that cause neuronal damage and loss of memory. The research that we have performed to understand the detailed mechanisms of Puf protein function in yeast cells should provide critical pieces of the puzzle of Puf protein regulation in higher eukaryotes.

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