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Post-transcriptional regulation of gene expression by the DExD/H-box protein Dhh1

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Post-transcriptional regulation of gene expression by the DExD/H-box protein Dhh1

By

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Abstract

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By repressing translation and promoting mRNA decay, cells are able to modulate gene expression and respond swiftly to changing environmental signals and developmental cues. Although translation, storage and degradation of mRNAs are key steps in the post-transcriptional control of gene expression, how mRNAs transit between these processes remains poorly understood.

During my thesis I functionally characterized the DExD/H box ATPase Dhh1, a critical regulator of the cytoplasmic fate of mRNAs. Using mRNA tethering experiments in yeast, I showed that Dhh1 is sufficient to move an mRNA from an active state to translational repression. In actively dividing cells, translational repression is followed by mRNA decay, however, deleting components of the 5' to 3' decay pathway uncoupled these processes. Interestingly, Dhh1's ability to inactivate an mRNA coincided with its ability to move mRNAs into cytoplasmic processing bodies (P bodies).

I also examined the role of ATP hydrolysis in Dhh1's ability to repress translation and activate mRNA decay. While Dhh1's ATPase activity is not essential for translational inhibition and mRNA decay in *dhh1*Δ cells, I found that ATP hydrolysis regulates P body dynamics and the release of Dhh1 from these RNA-protein granules. Surprisingly, I found that the presence of a wild-type copy of Dhh1 rescues the abnormal P-body localization of a Dhh1 ATPase-mutant. Additionally, the Dhh1 ATPase mutant no longer reduces mRNA and protein levels when tethered to an mRNA in the presence of a wild-type copy of Dhh1. My results place Dhh1 at the interface of translation and decay controlling whether an mRNA is translated, stored or decayed.

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Abbreviations

mRNP: messenger ribonucleoprotein complex
P-body: processing body
m^7G cap: 5' 7-methylguanosine cap
miRNA: micro RNA
PP7CP: PP7 coat protein
FRAP: fluorescent recovery after photobleaching.

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♥ Johanna

Chapter 1

General Introduction

Central to the proper regulation of gene expression is the post-transcriptional control of mRNA translation, storage and decay. By repressing translation and promoting mRNA decay, cells are able to rapidly alter the transcripts that are available for protein production and to modulate gene expression accordingly. Given that translation, storage and degradation of mRNAs are key steps in the post-transcriptional control of gene expression, it is critical to understand how mRNAs transit between these processes.

Eukaryotic mRNA decay

The bulk of eukaryotic mRNA turnover initiates with deadenylation, which causes an mRNA to exit translation (Figure 1.1a) (Coller and Parker, 2004). Shortening of the poly(A) tail is the only reversible step in mRNA turnover; transcripts can be readenylated and return to polyosomes to be actively translated (Coller and Parker, 2004; Curtis et al., 1995). However, if an mRNA is destined for decay, deadenylation is followed by mRNA degradation. Degradation occurs through one of two pathways that are conserved across eukaryotes; either the unprotected 3' end is degraded by the exosome, a complex of 3' to 5' exonucleases, or alternatively, and more commonly in yeast, the Dcp1/Dcp2 decapping enzyme cleaves the 5' cap structure, exposing the mRNA to the 5' to 3' exonuclease Xrn1 (Coller and Parker, 2004; Garneau et al., 2007).

Although the majority of mRNAs are degraded in a deadenylation-dependent maner, a number of mRNAs have been shown to circumvent this route by undergoing endonucleolytic cleavage (Figure 1.1b). The process of internal cleavage is essential in RNA-mediated gene silencing (RNAi). During RNAi, an exogenous double-stranded RNA (dsRNA) substrate is recognized by the endonuclease *Dicer* (Liu et al., 2004; Song et al., 2003). *Dicer* cleaves the dsRNA into short 21-23 nucleotide fragments which can then integrated into the RNA-induced silencing complex (RISC). The RNA component of the RISC complex then base pairs with a complementary strand of mRNA inducing cleavage by Argonaut, the catalytic component of the RISC complex. This cleavage results in two fragments that are susceptible to digestion by exonucleases. Endonucleic cleavage has also been shown to control decay of specific transcripts in a non-miRNA dependent manner (Binder et al., 1994; Bremer et al., 2003; Cunningham et al., 2000; Hanson and Schoenberg, 2001; Stoeckle and Hanafusa, 1989; Tomecki and Dziembowski, 2010; van Dijk et al., 2001).

Additionally, two specific mRNA transcripts, *RPS28B* and *EDC1* have been shown to bypass deadenylation, but as opposed to undergoing endonucleolytic cleavage, they are directly decapped (Figure 1.1c) (Badis et al., 2004; Muhlrad and Parker, 2005). Interestingly, these two transcripts appear to activate deadenylation-independent decapping via different mechanisms. *EDC1* mRNA is protected from deadenylation by an association between the poly(A) tail and a poly(U) stretch in its 3'UTR (Muhlrad and Parker, 2005). On the other hand, the Rps28b protein binds directly to the 3'UTR of its own mRNA and recruits proteins which enhance the activity of decapping enzyme (Badis et al., 2004).

mRNA deadenylation

As stated above, the majority of mRNA decay begins with shortening of the poly(A) tail. Poly(A) tail length of mature cytoplasmic mRNAs are fairly uniform; sizes

range from 200 to 250 residues in mammalian cells and from 70 to 80 residues in yeast (Chen, 2011). The major deadenylase complexes Ccr4-Pop2 and Pan2-Pan3 are highly conserved throughout evolution (Dupressoir et al., 2001; Zuo and Deutscher, 2001). The Ccr4-Pop2 complex is the predominant cytoplasmic deadenylase in yeast and contains two core nucleases, Ccr4 and Pop2 as well as a number of accessory proteins, Not1-Not5, Caf4, Caf 16 Caf40 and Caf130. Deleting Ccr4 in yeast or mutating the catalytic domain of the protein abolishes deadenylation activity both *in vivo* and *in vitro* (Chen et al., 2002; Tucker et al., 2002; Tucker et al., 2001). The Pan2-Pan3 complex on the other hand predominates in the nucleus and is responsible for trimming the poly(A) tail in yeast to 70-80 nucleotides during mRNA maturation (Brown and Sachs, 1998). Knocking out Pan2-Pan3 function results in the production of mature mRNAs with abnormally long poly(A) tails (Sachs and Deardorff, 1992).

In addition to the two major deadenylase complexes mentioned above, six additional deadenylases have been identified in metazoa, suggesting that there is great diversity in the biological functions of deadenylases (Goldstrohm and Wickens, 2008). The advantage of having multiple deadeylases remains to be determined; however one intriguing possibility is that different deadenylases target specific mRNA substrates thus regulating the degradation of these mRNAs. Indeed, specific deadenylases have been shown to be required for development in *Xenopus laevis*, *D. melanogaster* and *C. elegans*, while others are required for fertility in mice, *D. melanogaster* and *C. elegans* (Berthet et al., 2004; Korner et al., 1998; Molin and Puisieux, 2005; Morris et al., 2005; Nakamura et al., 2004). What directs the specificity of deadenylases is unclear; however, they are likely controlled via their association with other accessory proteins. For example, the Pan2-Pan3 complex binds to the poly(A) binding protein (PABP) which in turn regulates and stimulates the activity of the deadenylase complex by recruiting it to poly(A) tails (Sachs and Deardorff, 1992). The Ccr4-Pop2 complex on the other hand is inhibited by the presence of PABP (Tucker et al., 2002; Viswanathan et al., 2003). These observations suggest that different deadenylases have preferred mRNA substrates, and therefore distinct populations of mRNAs may differ in their susceptibility to deadenylases thereby influencing their translation and decay.

mRNA translation and decay are regulated by the 5' 7-methylguanosine (m^7G) cap

Decapping is a key step in mRNA decay as the presence of the cap is critical for translation of many transcripts and its removal irreversibly activates decay. In eukaryotes, mRNA is stabilized by both the 5' m^7G cap and the 3' poly(A) tail. The cap-binding protein eIF4E, a component of the eIF4F complex, and PABP interact with the mature transcript preventing its degradation and promoting its association with translation initiation factors (Coller and Parker, 2004; Garneau et al., 2007). For decapping to occur the interaction between eIF4F and the mRNA cap must be antagonized. When this occurs, translation is halted and decapping and degradation can ensue (Figure 1.2).

The decapping proteins Dcp1 and Dcp2 function together as the decapping holoenzyme, with Dcp2 acting as the catalytic subunit (Cohen et al., 2005; Steiger et al., 2003; van Dijk et al., 2002; Wang et al., 2002b). The Dcp1/2 holoenzyme cleaves the m^7GpppX cap of mRNAs to yield m^7GDP and a 5'-monophosphate mRNA. Dcp1/2 associate preferentially with deadenylated mRNAs, and co-immunoprecipitation experiments have demonstrated that Dcp1/2's association with an mRNA occurs

concomitantly with a loss of translation initiation factors from the mRNP (Tharun and Parker, 2001). These data suggests that the decapping machinery and the translation initiation machinery compete to determine the fate of an mRNA. Additionally data supporting this model has shown that the cap binding protein eIF4E, which is critical for initiating translation, inhibits decapping *in vitro* (Schwartz and Parker, 2000; Wilusz et al., 2001), and deletion of eIF4E *in vivo* stimulates decapping (Schwartz and Parker, 2000). These data suggests initial steps triggering RNA decay involve shortening the poly(A) tail and removing translation factors from an mRNP.

Regulators of mRNA decapping

A number of accessory proteins have been identified as activators of mRNA decapping. A complex of Sm-like proteins (LSM1-7) together with the protein Pat1, associates preferentially with the 3' end of deadenylated mRNAs and promotes decapping (Bouveret et al., 2000; Chowdhury et al., 2007; Haas et al., 2010; Tharun et al., 2000; Tharun and Parker, 2001). Pat1 is proposed to act as a link between deadenylation and decapping as it triggers both processes when artificially tethered to an mRNA reporter (Haas et al., 2010). Pat1 has also been implicated as a translational repressor as its overexpression in yeast causes general repression of mRNA translation (Coller and Parker, 2005). Recent studies suggest that Pat1 represses mRNA translation by binding to an mRNA, limiting the formation of the 48S preinitiation complex and subsequently recruiting components of the decapping complex to activate mRNA decay (Nissan et al., 2010).

Another protein implicated in both translational repression and mRNA decay is the DExD/H box protein Dhh1. Functionally, Dhh1 is thought to act as an enhancer of decapping since deletion of Dhh1 in yeast results in a significant stabilization of mRNA transcripts and a severe inhibition of decapping (Coller et al., 2001; Fischer and Weis, 2002). In addition to its involvement in mRNA decay, Dhh1 and its orthologs have also been implicated in both general and miRNA mediated translational repression (Chu and Rana, 2006; Coller and Parker, 2005; Eulalio et al., 2007c; Minshall et al., 2009; Minshall and Standart, 2004; Nakamura et al., 2001; Navarro et al., 2001). Like Pat1, overexpression of Dhh1 causes general translational repression, and co-deletion of Dhh1 and Pat1 inhibits the general repression of mRNAs normally observed upon glucose starvation (Coller and Parker, 2005). Additionally, Dhh1 has been shown to represses translation of a reporter mRNA *in vitro* as well as in *Xenopus* oocytes and Drosophilla S2 cells (Coller and Parker, 2005; Minshall et al., 2009; Minshall and Standart, 2004; Tritschler et al., 2009). How Dhh1 represses translation remains unclear; however, one possibility is that it, together with Pat1 and/or other protein factors, could inhibit formation of the 48S preinitiation complex. Additionally, Dhh1 has been proposed to use its ATPase activity to release the eIF4F complex from an mRNA thus concurrently repressing translation and activating decapping (Franks and Lykke-Andersen, 2008).

Additional proteins which influence mRNA decapping include the enhancers of decapping, Edc1, Edc2 and Edc3. These three proteins are not structurally related, but were instead identified by genetic screens to identify proteins which affect interact with the decapping enzyme (Dunckley et al., 2001; Kshirsagar and Parker, 2004). Edc1 and Edc2 are specific to yeast, whereas Edc3 is conserved across eukaryotes (Cougot et al., 2004; Dunckley et al., 2001; Kshirsagar and Parker, 2004; Schwartz et al., 2003). Edc1

and Edc2 have both been shown to stimulate the decapping activity of Dcp2 *in vitro*, and depletion of Edc3 prevents the decapping of a subset of mRNAs (Badis et al., 2004; Kshirsagar and Parker, 2004; Schwartz et al., 2003; Steiger et al., 2003). Additionally, all the Edc proteins have been shown to interact with decapping factors (Borja et al., 2011; Decker et al., 2007; Dunckley et al., 2001; Tritschler et al., 2009). Interestingly, the Edc proteins seem to act with some specificity; Edc3 has been shown to target specific transcripts for degradation (Badis et al., 2004; Dong et al., 2007), and Edc1 has been implicated in cells' adaptive response to carbon source shifts (Schwartz et al., 2003).

In addition to decapping activators, proteins involved in inhibiting the activity of the decapping enzyme also exist. When purified Dcp2 is combined with human cell extracts, the protein is rendered inactive suggesting the presence of a general inhibitor of decapping (Jiao et al., 2006). The testis-specific protein VCX-A has been identified as one such inhibitor which binds to capped mRNAs and prevents the decapping activity of Dcp2 (Jiao et al., 2006). Because activation of mRNA decapping inevitably leads to decay, regulators of decapping can have a profound influence on gene expression. Therefore, further research is important to determine biological significance and the mode action of enhancers and inhibitors of decapping.

Processing Bodies (P-Bodies): Sites of mRNA storage and decay

mRNAs that are associated with the decay machinery localize to cytoplasmic structures termed processing bodies (P-bodies) (Eulalio et al., 2007a; Parker and Sheth, 2007). All the proteins involved in the 5' to 3' decay pathway localize to P-bodies in addition to factors involved in nonsense mediated decay, ARE mRNA-decay and miRNA mediated repression (Figure 1.3) (Buchan and Parker, 2009; Franks and Lykke-Andersen, 2008). The presence of the mRNA machinery in P-bodies suggests that they could serve as the sites of mRNA decay. Several lines of evidence support this hypothesis. First, mRNA decay intermediates accumulate in P-bodies (Cougot et al., 2004; Sheth and Parker, 2003). Second, blocking deadenylation, an early step in the mRNA decay pathway, causes P-bodies to disappear (Andrei et al., 2005; Sheth and Parker, 2003). Conversely, inhibiting 5' to 3' digestion, the last step in decay, results in an increase in P-body number and size (Andrei et al., 2005; Cougot et al., 2004; Sheth and Parker, 2003; Teixeira et al., 2005). Finally, the integrity of a P-body is dependent on mRNA, as exposure to ribonuclease A *in vitro* and in permeabilized cells causes P-body disassembly (Eulalio et al., 2007c; Teixeira et al., 2005).

It should be noted however, that the presence of an mRNA in a P-body doesn't necessarily result in its decay. For example, it has been shown that some yeast mRNAs, which enter P-bodies upon glucose starvation, can re-enter active translation once glucose is restored (Brengues et al., 2005; Coller and Parker, 2005). Additionally, a human mRNA targeted to P-bodies via a miRNA can re-enter translation upon association with the protein HuR (Bhattacharyya et al., 2006). If, as discussed above, P-bodies are the sites of mRNA decay, this evidence raises important questions as to how some mRNAs escape degradation and reenter the translational pool. The dynamics of P-body formation

P-bodies are highly dynamic, evolutionarily conserved structures found in both lower and higher order eukaryotes which change rapidly according to the cellular environment (Eulalio et al., 2007a). In mammalian cells microscopically visible P-bodies

(ranging from 100-300nm) are constitutively present, but increase in number when cells are exposed to stress (Kedersha et al., 2005; Yang et al., 2004). P-bodies in *S. cerevisiae* cells on the other hand have only been visualized either by deleting or overexpressing various P-body components, or by subjecting cells to stresses like glucose deprivation or hyperosmotic shock (Brengues et al., 2005; Teixeira et al., 2005; Ujwal Sheth, 2006).

Interestingly, subjecting cells to the stresses which increase P-body formation corresponds to a dramatic change in the translational status of the majority of mRNA in the cell. For example, in actively growing yeast cells where P-bodies are not visible, the majority of mRNAs are found in polysomes, which is indicative of mRNAs undergoing active translation (Teixeira et al., 2005). Stressing cells by depriving them of glucose however results in rapid loss of polysomes and an accumulation of P-bodies. This correlation suggests that translationally repressed mRNPs are targeted to P-bodies where mRNA storage and/or degradation can occur. In support of this model, it has been shown that releasing mRNA's from polysomes by treating cells with puromycin results in enhanced P-bodies (Eulalio et al., 2007b). Treating cells with cycloheximide, a drug which blocks translational elongation and therefore traps RNA in the polysome, causes P-bodies to disappear (Cougot et al., 2004; Eulalio et al., 2007b; Teixeira et al., 2005). These results suggest that the dynamics of P-body formation is dependent on the concentration of translationally repressed mRNAs.

Interestingly loss of visual P-bodies does not effect any known mRNA decay or translational repression pathways, suggesting that they are not the only sites of mRNA decay (Decker et al., 2007; Eulalio et al., 2007b). Along these lines, it has been shown that P-body components are found diffusely throughout the cytoplasm as well as within P-bodies (Andrei et al., 2005; Bashkirov et al., 1997; Brengues et al., 2005; Chu and Rana, 2006; Coller and Parker, 2005; Cougot et al., 2004; Eystathioy et al., 2003; Ingelfinger et al., 2002; Sheth and Parker, 2003; van Dijk et al., 2002). And, proteins found in P-bodies, with the exception of Dcp2, dynamically exchange with the cytoplasmic pool (Aizer et al., 2008; Kedersha et al., 2005). Together these results suggest that repression of mRNA translation and activation of decay are not confined to P-bodies. The exact role of these evolutionarily conserved structures in translational repression and mRNA decay is a major unresolved question in the field however.

Factors involved in P-body assembly

The presence of prion-like Glutamine/Asparagine (Q/N)-rich domains in several P-body components suggests that mRNP aggregation is important for P-body formation (Decker et al., 2007; Reijns et al., 2008). Indeed, deletion of the Q/N regions of Ccr4, Pop2 and Dhh1 all resulted in reduced accumulation of these proteins in P-bodies after osmotic shock (Reijns et al., 2008). And even more strikingly, deletion of the Q/N domain of the yeast protein Lsm4 together with a dimerization domain in Edc3, Yjef-N, led to a complete ablation of P-bodies (Decker et al., 2007; Reijns et al., 2008). Interestingly, both Lsm4 and Edc3 interact with the translational repressors Pat1 and Dhh1 respectively, suggesting a model whereby the binding of Dhh1 and/or Pat1 to an mRNA triggers translational repression and promotes assembly of the mRNA into P-bodies via their association with Lsm4 and Edc3 (Franks and Lykke-Andersen, 2008).

The role of the Edc3 appears to be conserved in other eukaryotes as the Yjef-N dimerization domain is highly conserved, and deletion of Edc3 in *Drosophila* S2 cells

results in decreased P-bodies (Tritschler et al., 2009). The Q/N domain of Lsm4 on the other hand is only present in budding yeast suggesting that this protein may act through a different mechanism in higher eukaryotes. However, the conservation of Q/N-rich regions in the metazoan P-body proteins Dcp2, Ge-1/Hedls and GW182 suggests that prion-like domains may play a role in P-body assembly in higher eukaryotes as well (Decker et al., 2007). Indeed, deletion of Ge-1/Hedls or GW182 in human and *Drosophila* cells results in a depletion of P-bodies (Eulalio et al., 2007b; Jakymiw et al., 2005; Liu et al., 2005; Yu et al., 2005).

Once assembled, P-bodies are often highly mobile within the cell, and this motility is dependent on microtubules (Aizer et al., 2008). Although the role motility plays in P-body function remains uncertain, P-bodies have been shown to interact with other mRNP granules such as stress granules (discussed below) suggesting that P-body movement could allow the association of P-bodies with stress granules and therefore could mediate the movement of mRNPs between the two. It should be noted that although the mobility of an entire P-body dependent on microtubules, the dynamic movement of individual proteins in and out of P-bodies is not (Aizer et al., 2008; Sweet et al., 2007).

Other mRNP granules

A number of other mRNP granules are found in eukaryotes during stress, in oocytes and in neuronal cells, and they are all generally thought to be involved in mRNA translational repression (Anderson and Kedersha, 2006). Like P-bodies, stress granules form in response to a variety of stresses which inhibit the majority of mRNA translation including UV irradiation, heat shock and oxidative stress (Anderson and Kedersha, 2006; Kedersha et al., 2005; Kedersha et al., 1999; Kimball et al., 2003). Many of the proteins found in P-bodies are also found in stress granules, although there does appear to be some difference in the composition of P-bodies and stress granules (Figure 1.3) (Buchan and Parker, 2009). Stress granules are distinct from P-bodies in that they typically contain 40S ribosomal subunits as well as translation factors such as eIF4G, eIF4A, eIF3 and PABP (Kedersha and Anderson, 2002; Kedersha et al., 1999). Certain decay proteins such as Dcp1, Dcp2, Lsm1-7 and GW182 seem to exclusively localize to P-bodies on the other hand (Eystathioy et al., 2003; Ingelfinger et al., 2002; van Dijk et al., 2002). Stress granules and P-bodies can co-exist separately in the cytoplasm, but can come in close association and fuse over time suggesting that stress granules and P-bodies could exchange mRNPs between them (Anderson and Kedersha, 2006; Kedersha et al., 2005).

In both oocytes and neurons, specific mRNAs are translationally repressed and transported to particular locations in the cell where they can be stored in mRNP granules. During oogenesis in several organisms many maternal mRNAs are transported to the posterial pole where they associate with germinal mRNP granules (Amiri et al., 2001; Nakamura et al., 2001; Wilhelm et al., 2000). These include P granules in *C. elegans*, *D. melanogaster* and *X. laevis* (also referred to as polar granules, germ cell granules or dense bodies) (Eulalio et al., 2007a). The repression and subsequent activation of these mRNAs are key germ line determinants (Ephrussi et al., 1991; Gavis and Lehmann, 1992). In neurons mRNAs are also translationally repressed and transported to specific locations, like axons and dendrites, in the cell which is critical for localized protein production (St Johnston, 2005). Many of the protein components of P-bodies, P-

granules, stress and neuronal granules overlap, which is not surprising given that these mRNP granules all play a role in silencing mRNA transcripts (Eulalio et al., 2007a).

Specialized decay pathways for cytoplasmic mRNA quality control

Thus far I have focused mostly on the pathways involved in the translational repression and decay of properly transcribed transcripts; however, it should be noted that there are also specialized decay pathways important for mRNA quality control. These pathways all use the same decay enzymes responsible for degrading normal transcripts; however, they differ in their modes of recognizing and targeting defective mRNAs for decay. Aberrant transcripts can arise from errors in transcription and RNA processing, and if they are translated into protein, can have extremely detrimental effects on the cell. mRNA quality control pathways are therefore essential for detecting and degrading these abnormal transcripts.

Nonsense mediated decay (NMD) is responsible for recognizing and degrading mRNAs with premature termination codons (PTC). 3-10% of cellular mRNAs are thought to contain PTCs; therefore, NMD is essential for preventing the accumulation of aberrant truncated proteins (Isken and Maquat, 2007). NMD is initiated by the recruitment of the RNA helicase Upf1 and its cofactors Upf2 and Upf3 to the terminating ribosome. This NMD mRNP then recruits mRNA decay enzymes to initiate degradation by either decapping, deadenylation and/or endonucleic cleavage (Muhlemann and Lykke-Andersen, 2010).

If an mRNA lacks a termination codon, it is targeted for non-stop decay. Without a stop codon, the ribosome will continue translation along the poly(A) tail until it stalls at the 3' end of the RNA. Ski7, a component of the cytoplasmic exosome recognizes and interacts with the stalled ribosome, triggering the mRNA degradation in a 3' to 5' direction via recruitment of remaining exosome components (Frischmeyer et al., 2002; van Hoof et al., 2002). Additionally, translation can terminate in the 3' UTR when the normal stop codon is not recognized (Kong and Liebhaber, 2007). These mRNAs are degraded via the canonical 5' to 3' decay pathway; however the mechanism by which they are recognized as abnormal has yet to be determined.

mRNAs that are improperly transcribed or processed may contain strong secondary structures or other obstacles which slow or halt the translation machinery. No-go decay is a relatively recently discovered mechanism by which the stalled ribosomes are recognized and the mRNA subsequently degraded (Doma and Parker, 2006). Two evolutionarily conserved proteins, Dom34 and Hbs1, which are similar to the translation termination factors eRF1 and eRF3, recognize the stalled ribosome, and activate mRNA decay via endonucleolytic cleavage(Doma and Parker, 2006).

Signals involved in mRNA decay specificity

The various steps of post-transcriptional processing leading to mRNA degradation are highly regulated. Individual transcript half-lives can vary from a few minutes to a number of hours (Jacobson and Peltz, 1996), and decay rates are similar between transcripts that encode for proteins found in common macromolecular complexes (Wang et al., 2002a). The process of mRNA decay is therefore not a default mechanism resulting in random transcript degradation, but instead is precisely coordinated and tightly

controlled. The mechanisms that drive specific sets of mRNAs for targeted decay remains an area of intense investigation in the mRNA decay field.

The decay of many mRNAs are regulated by *cis*-encoded elements in the mRNA which recruit specific *trans*-factors. For example, AU-rich elements (ARE) destabilizing sequences are found in the 3' UTR of many mRNAs that encode growth factors, cytokines, proto-oncogenes and transcription factors (Khabar, 2005). They are comprised of repeats of the AUUUA pentamer or UUAUUUAUU nonamer which mediate mRNA decay via recruitment of ARE-binding proteins. These proteins are thought to activate mRNA decay via recruitment of the cellular mRNA decay machinery as biochemical studies have shown that ARE-binding proteins interact with the 3' to 5' exosome as well as proteins involved in 5' to 3' decay including decapping proteins and deadenylases (Chen et al., 2001; Gherzi et al., 2004; Lai et al., 2003; Lykke-Andersen and Wagner, 2005).

Puf proteins are another family of mRNA binding proteins which regulate mRNA half-life by binding to UG-rich elements and recruiting the Ccr4-Not deadenylase complex (Goldstrohm et al., 2006). These proteins are found in *S. cerevisiae*, but are related the *D. melanogaster* translational regulator Pumilio (Gerber et al., 2006). Interestingly, each of the Puf proteins binds and regulates a set of functionally related mRNA targets (Gerber et al., 2004). For example Puf5 interacts with transcripts encoding for chromatin modifiers and Puf3 binds to mRNAs that encode mitochondrial proteins. This binding specificity suggests that each Puf protein regulates a specific cellular process and raises the question as to whether there are similar proteins which confer degradation specificity in higher order eukaryotes. These data demonstrate that the stability of specific mRNAs can be controlled by *trans*-factors in the cell. In this way these the cell is able to regulate the precise expression of many genes, allowing it to respond and adapt to internal and external stimuli.

Purpose of this study

By repressing translation and promoting mRNA decay, cells are able to modulate gene expression and respond swiftly to changing environmental signals and developmental cues. Although translation, storage and degradation of mRNAs are key steps in the post-transcriptional control of gene expression, how mRNAs transit between these processes remains poorly understood. The question as to what is required to move an mRNA from active translation to storage and/or decay has been central to my thesis. My primary focus has been on the role of the enhancer of decapping, Dhh1 in this process. Dhh1 is DExD/H-box ATPase which has previously been shown to be a key regulator in directing mRNA from an active translation pool into translational repression and ultimately targeting the mRNA for degradation; however, the molecular mechanism of how Dhh1 influences gene expression remains unclear. Central to my research is the hypothesis that Dhh1 regulates the fates of specific mRNAs by changing the composition of messenger ribonucleoprotein (mRNP) complexes and localizing them to P-bodies. To investigate this hypothesis, I developed a tethering assay with which to examine the role of Dhh1 on the translation, decay and localization of a specific mRNA in S. cerevisae. By deleting different components of the mRNA decay machinery, I was able to uncover the core proteins required for Dhh1 to activate mRNA decay. By mutating the ATPase domain of Dhh1, I discovered that Dhh1's ability to hydrolyze ATP plays an important

role in P-body localization. Additionally, I investigated the role of other decay proteins, the enhancers of decapping (Edc1, 2, 3), Pat1 and Xrn1, and found that they exhibit differential effects on mRNA decay and P-body localization. Together these data addresses fundamental questions in the control of gene expression by providing novel insight into the regulation of mRNA turnover and translational repression.

Figure 1.1
The pathways of mRNA decay. (A) Deadenylation-dependent mRNA decay. The majority of mRNAs undergo decay in a deadenylation-dependent manner. The poly(A) tail is first removed by a deadenylase shown here as Ccr4-Not or PARN. Following deadenylation, decay can occur through one of two pathways. Either the mRNA is decapped by the Dcp1/Dcp2 complex and digested in a 5' to 3' direction by the exonuclease Xrn1, or the mRNA is degraded in a 3' to 5' direction by the exosome. **(B)** Endonuclease-mediated mRNA decay. Endonuclease-mediated mRNA decay initiates by internal cleavage of an mRNA generating two RNA fragments which are digested by either the exosome or Xrn1. **(C)** Deadenylation-independent decapping. A few select mRNAs have been shown to undergo decapping prior to deadenylation. Shown here Rps28 binds directly to the 3'UTR of its own mRNA and recruits proteins which enhance the activity of decapping enzyme.

This figure is adapted from Garneau et al. 2007 and Munchel, 2009.

Figure 1.1

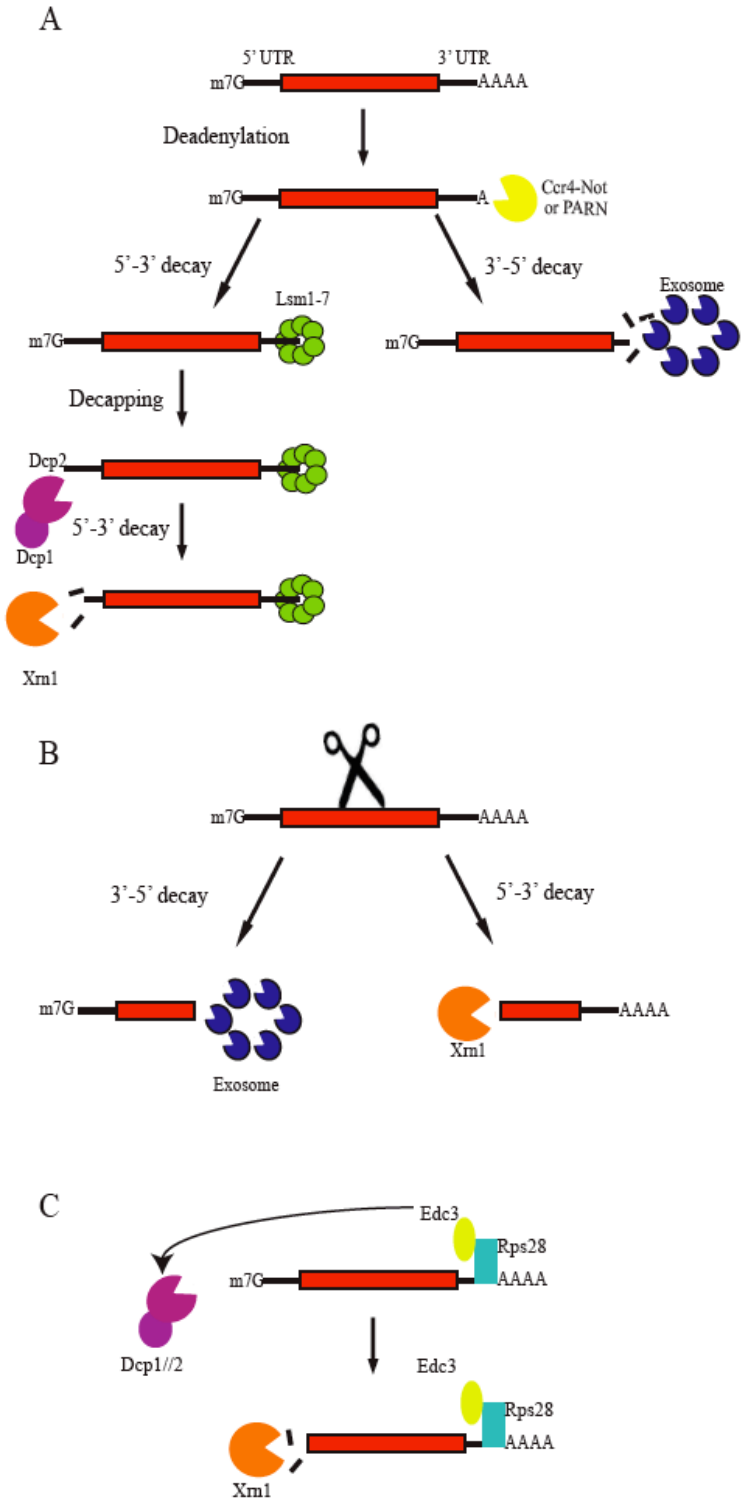

A
5' UTR
3' UTR
m7G
AAAA
Deadenylation
A
Ccr4-Not
or PARN
5'-3' decay
3'-5' decay
Lsm1-7
Exosome
Decapping
Dcp2
Dcp1
Xrn1
B
3'-5' decay
5'-3' decay
C
Edc3
Rps28
Dcp1//2

Figure 1.2
The transition from active translation to decay requires an mRNP composition change. For an mRNA to transition from active translation to decay it is believed that the mRNA must first exit translation via removal of the cap-binding complex and other translation factors. miRNAs and the protein factors Dhh1 and Pat1 are thought to be involved in this process. Once an mRNA has become translationally repressed, additional decay factors are recruited to the mRNP.

Figure 1.2

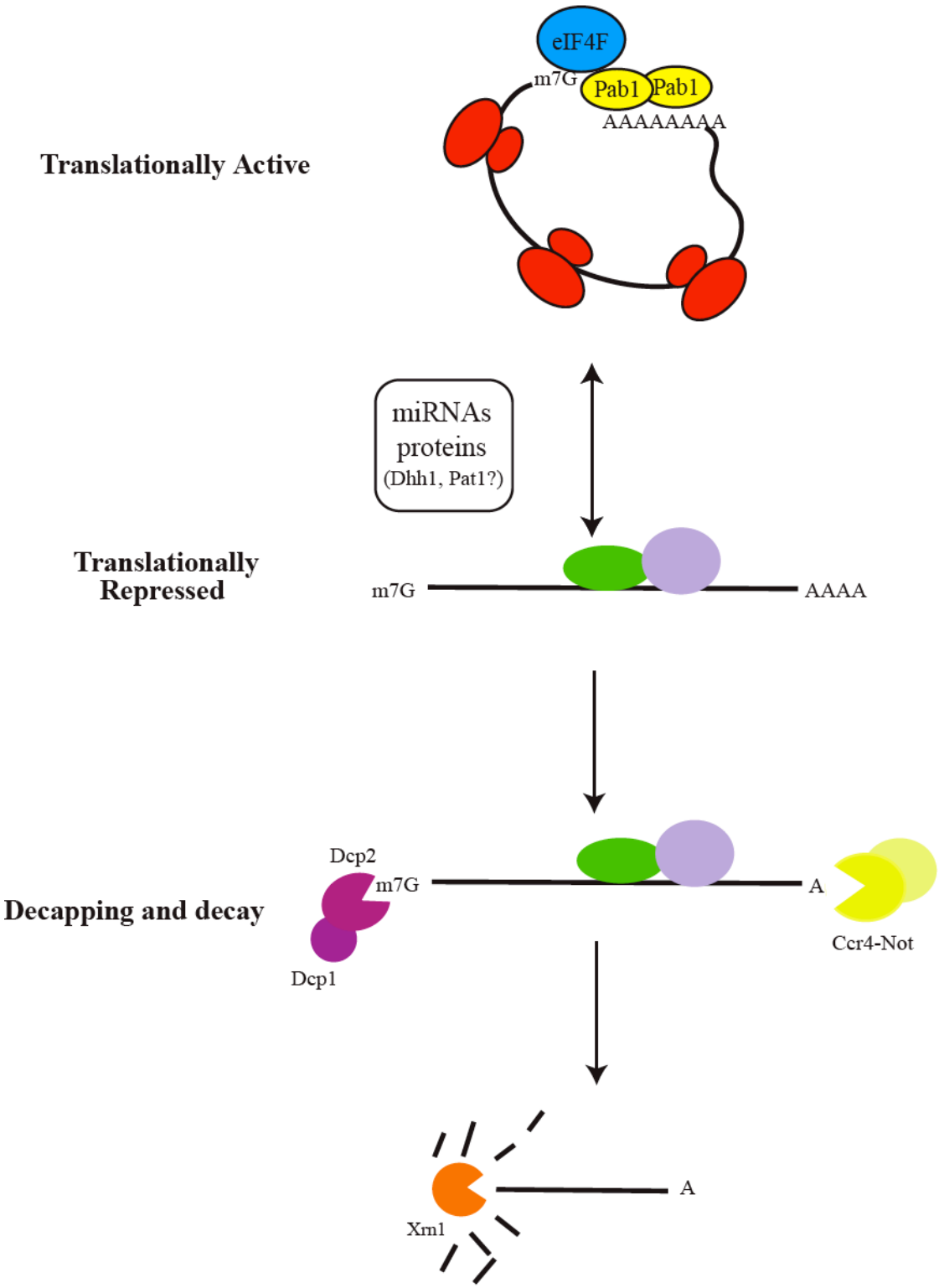
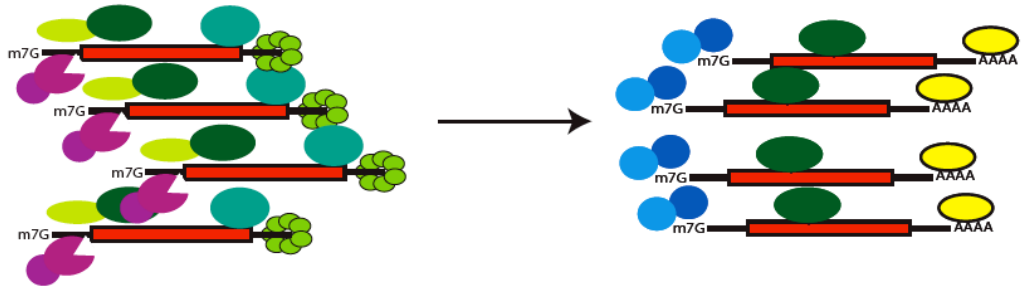

eIF4F
m7G
Pab1
Pab1
AAAAAAAA
Translationally Active
miRNAs
proteins
(Dhh1, Pat1?)
Translationally
Repressed
m7G
AAAA
Dcp2
m7G
A
Ccr4-Not
Decapping and decay
Dcp1
A
Xrn1

Figure 1.3
P-body and stress granule components. P-bodies and stress granules are believed to be distinct structures based on composition. Stress granules typically contain 40S ribosomal subunits as well as translation factors, whereas certain decay proteins exclusively localize to P-bodies (Buchan and Parker, 2010). Proteins solely observed in P-bodies are shown in green, those only in stress granules are in blue and components seen in both foci are shown in cyan.

Figure 1.3



P-Body Aggregate

Dcp2
Dcp1
Lsm1-7
Hedls
GW182
Ccr4
Pop2
Pan2
eIF4E-T
Mex3A
Pat1
Edc1-3

Components of both P-bodies and SGs

Xrn1
Dhh1
Ago
Upf1
eIF4E
TTP
BRF1
Lin28
APOBEC
Mex3b
FAST
Rap55
PCBP2

Stress Granule Aggregate

Pabp
40S subunits
TIA-1/R
HuR
G3-BP
eIF-4G
eIF-4A
Staufen
FMRP
FXR1/2
ZBP1
RSK1
RACK1
TRAF1
Mex67

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Chapter 2

The Function of the DExD/H-box protein Dhh1 in translational repression, mRNA decay and P body dynamics

Background

Central to the proper regulation of gene expression is the post-transcriptional control of mRNA translation, storage and decay. By repressing translation and promoting mRNA decay, cells are able to rapidly alter the transcripts that are available for protein production and to modulate gene expression accordingly.

In eukaryotes, mRNA is stabilized by a 5' methylguanosine cap and a 3' poly[A] tail. The cap-binding protein eIF4E and poly[A]-binding protein (PABP) interact with the mature transcript preventing its degradation and promoting its association with translation initiation factors (Coller and Parker, 2004; Garneau et al., 2007). The bulk of eukaryotic mRNA turnover initiates with deadenylation, which causes an mRNA to exit translation (Coller and Parker, 2004). Shortening of the poly[A] tail is the only reversible step in mRNA turnover; transcripts can be readenylated and return to polyosomes to be actively translated (Coller and Parker, 2004; Curtis et al., 1995). However, if an RNA is destined for decay, deadenylation is followed by mRNA degradation. Degradation occurs through one of two pathways that are conserved across eukaryotes; either the unprotected 3' end is degraded by the exosome, a complex of 3' to 5' exonucleases, or alternatively, and more commonly in yeast, the Dcp1/Dcp2 decapping enzyme cleaves the 5' cap structure, exposing the mRNA to the 5' to 3' exonuclease Xrn1 (Coller and Parker, 2004; Garneau et al., 2007).

Decapping is a key step in mRNA decay as the presence of the cap is critical for translation of many transcripts and its removal irreversibly activates decay. Co-immunoprecipitation experiments have demonstrated that decapping factors, Dcp1/2, Lsm1-7 and Pat1 associate with deadenylated mRNA concomitant with a loss of translation initiation factors from this mRNP (Tharun and Parker, 2001). Additionally, the cap binding protein eIF4E, which is critical for initiating translation, inhibits decapping *in vitro* (Schwartz and Parker, 2000; Wilusz et al., 2001), and deletion of eIF4E *in vivo* stimulates decapping (Schwartz and Parker, 2000). These data suggests that the decapping machinery and the translation initiation machinery compete to determine the fate of an mRNA, and that initial steps triggering RNA decay involve shortening the poly[A] tail and removing translation factors from an mRNP (Franks and Lykke-Andersen, 2008). Therefore a change in the composition of messenger ribonucleoprotein complexes (mRNPs) is likely to be critical for the transition from active translation to decay.

Interestingly, non-translating mRNPs were shown to localize in distinct mRNP granules in the cytoplasm (Anderson and Kedersha, 2006; Parker and Sheth, 2007). One class of these mRNP granules termed processing bodies (P-bodies) contain non-translating mRNAs and various proteins involved in decapping, exonucleolytic decay, nonsense mediated decay and miRNA mediated repression (Eulalio et al., 2007a; Parker and Sheth, 2007). P-bodies are evolutionarily conserved structures found in both lower and higher order eukaryotes (Eulalio et al., 2007a). The formation of P-bodies in cells correlates to the proportion of non-translating mRNPs; the larger the pool of non-translating mRNA, the greater the number of P-bodies (Teixeira et al., 2005). As such, a release of mRNA's from polysomes results in enhanced P-bodies (Brengues et al., 2005; Cougot et al., 2004; Eulalio et al., 2007b; Teixeira and Parker, 2007), while trapping mRNA in polysomes causes P-bodies to disappear (Cougot et al., 2004; Eulalio et al., 2007b; Teixeira and Parker, 2007). The mechanisms involved in the movement of

mRNA from polysomes into P-bodies remains unclear; however, it is assumed that changes in the protein composition of the mRNP are critical for this relocalization to occur.

Likely candidates for remodelling protein-RNA complexes are members of the DExD/H-box family of ATPases. Members of this family are known to be involved in all aspects of post-transcriptional mRNA processing including pre-mRNA splicing, mRNA export, translation and RNA turnover (Cordin et al., 2006; Rocak and Linder, 2004). These proteins all have RNA-dependent ATPase activity, and have been shown to have a wide array of activities including the ability to melt duplex RNA, dissociate proteins bound to RNA (RNPase activity), and function as RNA-binding scaffolds onto which cofactors can bind (Cordin et al., 2006; Rocak and Linder, 2004).

The *Saccharomyces cerevisiae* protein Dhh1 is a DExD/H box protein involved in both translational repression and mRNA decay making it a good candidate for mediating mRNP remodeling steps required to move an mRNA from active translation to a translationally inactive state. Dhh1 is part of a highly conserved subfamily of DExD/H-box proteins which include orthologs in *Schizosaccharomyces pombe* (Ste13), *Spisula solidissima* (clam p47), *Caenorhabditis elegans* (cgh-1), *Xenopus* (Xp54), *Drosophila* (Me31b), and mammals (RCK/p54). Interestingly, over-expression of RCK/p54, Xp54 or Me31b can rescue the loss of Dhh1 in yeast (Maekawa et al., 1994; Tseng-Rogenski et al., 2003; Westmoreland et al., 2003) suggesting that the function of this DExD/H-box protein is conserved across all eukaryotes. Dhh1 and its orthologs interact with proteins essential for decapping, deadenylation and translational repression (Coller et al., 2001; Fischer and Weis, 2002; Maillet and Collart, 2002; Weston and Sommerville, 2006), and localize to P-bodies under conditions of cellular stress (Coller and Parker, 2005; Sheth and Parker, 2003; Teixeira et al., 2005). Functionally, Dhh1 is thought to act as an enhancer of decapping since deletion of Dhh1 in yeast results in a significant stabilization of mRNA transcripts and a severe inhibition of decapping (Coller et al., 2001; Fischer and Weis, 2002). In addition to its involvement in mRNA decay, Dhh1 and its orthologs have also been implicated in both general and miRNA mediated translational repression (Chu and Rana, 2006; Coller and Parker, 2005; Minshall et al., 2009; Minshall and Standart, 2004; Nakamura et al., 2001; Navarro et al., 2001). For example, recombinant Dhh1 can represses translation of a reporter mRNA *in vitro* (Coller and Parker, 2005), and both Xp54 and Me31b can repress translation of a luciferase reporter mRNA in *Xenopus* oocytes and Drosophilla S2 cells respectively (Minshall et al., 2009; Minshall and Standart, 2004; Tritschler et al., 2009). Additionally, Xp54, Me31b and Cgh-1 are all components of stored maternal RNPs and are involved in amassing these RNAs in a translationally repressed state (Coller and Parker, 2005; Minshall and Standart, 2004; Minshall et al., 2001; Nakamura et al., 2001; Navarro et al., 2001).

Together, these observations suggest that Dhh1 plays an important role in regulating the translation status of mRNA; however, the mechanism by which Dhh1 directs the fate of an mRNA remains unclear. To investigate the role of Dhh1 in translational repression and decay *in vivo*, I tethered Dhh1 to endogenous mRNAs in *Saccharomyces cerevisiae*. I found that tethering Dhh1 reduced both steady-state mRNA and protein levels in actively growing yeast cells. The reduction in mRNA, but not protein, depended on components of the 5' to 3' decay machinery. Protein and mRNA localization experiments further revealed that tethering of Dhh1 is sufficient to localize

an mRNA to Dcp2-containing P-bodies. Interestingly, ATP hydrolysis is not required for the ability of Dhh1 to reduce mRNA and protein levels but instead is important for the transition of Dhh1 in and out of P-bodies. My data demonstrate that Dhh1 acts as a translational repressor and decay activator *in vivo* and supports a model where Dhh1 functions to regulate the transition of an mRNA between states of active translation, translational repression and decay.

Results

Steady state RNA and protein levels decrease upon tethering Dhh1 to endogenous yeast mRNAs

Previous studies in multiple organisms have suggested that the DEAD box ATPase Dhh1 can function in both mRNA decay and translational repression (Coller and Parker, 2005; Fischer and Weis, 2002; Minshall et al., 2009; Minshall and Standart, 2004; Nakamura et al., 2001; Tritschler et al., 2009). To dissect the role of Dhh1 in these processes, I examined the effect of directly tethering Dhh1 to endogenous mRNAs *in vivo*. Tethering was accomplished by expressing Dhh1 fused to the bacteriophage PP7 coat protein (PP7CP) in a yeast strain containing an mRNA with a stem-loop binding site for the PP7CP in its 3' UTR (Figure 2.1A). Expression of Dhh1-PP7CP rescued the growth defect of a *dhh1*Δ strain demonstrating that the fusion protein is functional (unpublished data).

I first tethered Dhh1 to an mRNA coding for fructose1, 6-bisphosphate aldolase (*FBA1*), which functions in glycolysis and gluconeogenesis. I chose to tether Dhh1 to *FBA1* mRNA because it is essential, and it is amongst the most highly expressed mRNAs in yeast (Holstege et al., 1998). Insertion of the PP7-loop into the *FBA1* 3'UTR did not cause any apparent growth defects suggesting that the loop itself did not significantly interfere with the expression of *FBA1*. To monitor the effects of Dhh1 tethering on *FBA1*, I first analyzed steady state RNA levels by Northern blotting when Dhh1-PP7CP, GFP-PP7CP or non-tethered Dhh1-GFP was present (Figure 2.1B, left panel). I found that tethering Dhh1 to *FBA1* mRNA resulted in a four-fold reduction in steady-state mRNA levels (Figure 2.1B, left panel). In contrast, tethering GFP had no effect on the *FBA1* mRNA levels compared to the no tether control with both Dhh1-PP7CP and GFP-PP7CP expressed at similar levels (Figure 2.1B and Supplementary Figure 2.1A). Additionally, the reduction in *FBA1* mRNA by Dhh1-PP7CP was dependent on the presence of a PP7 binding loop in the 3' UTR of *FBA1* (Supplementary Figure 2.2A). Thus, tethering of Dhh1 leads to a reduction of steady-state *FBA1* mRNA levels.

Next I compared Fba1 protein levels in cells expressing Dhh1-GFP, GFP-PP7CP or Dhh1-PP7CP (Figure 2.1B, right panel). I found that tethering Dhh1 to *FBA1* mRNA resulted in approximately a four-fold reduction in Fba1 protein levels when normalized to the endogenous control, Xpo1 (Figure 2.1B, right panel). This reduction was again specific to the presence to Dhh1-PP7CP and was dependent on the presence of a PP7 binding loop in the 3' UTR of *FBA1* (Figure 2.1B and Supplementary Figure 2.2B). Together these results show that the association of Dhh1 with *FBA1* mRNA causes a similar fold-reduction in both mRNA and protein levels of *FBA1*.

To test whether Dhh1 tethering also reduced steady state mRNA and protein levels of other mRNAs, we tethered Dhh1 to *RPL25*. *RPL25* is an essential gene, which codes for an rRNA-binding protein in the large ribosomal subunit. While insertion of the

PP7 stem loop into the *RPL25* mRNA by itself had no effect on cell growth, haploid yeast were inviable upon co-expression of Dhh1-PP7CP (unpublished data). I reasoned this was because tethering of Dhh1 to *RPL25* reduces *RPL25* mRNA to levels which are too low to support growth and therefore performed the tethering assay in a diploid strain, which contained one wild-type and one PP7-tagged copy of *RPL25* mRNA. As seen in the *FBA1* experiments, tethering Dhh1 to *RPL25* mRNA results in a four-fold reduction in both protein and mRNA levels when compared to GFP-PP7CP and the non-tethered Dhh1-GFP control (Figure 2.1C). These data demonstrate that binding of Dhh1 to yeast mRNAs is sufficient to cause a significant reduction in both steady state mRNA and protein levels.

The ability of Dhh1 to reduce mRNA levels depends on components of the 5' to 3' decay pathway

Given that Dhh1 can function *in vivo* to reduce steady state mRNA and protein levels, I next wanted to determine (a) if Dhh1 requires the 5' to 3' decay machinery to lower mRNA levels, and (b) whether the reduction in protein levels is merely a consequence of the lower mRNA levels or whether Dhh1 directly represses translation. mRNA decay begins with removal of the polyA tail, followed by cleavage of the 5' cap structure and completed by 5' to 3' exonucleolytic digestion (Coller and Parker, 2004; Garneau et al., 2007). To test whether components of this pathway are required for the Dhh1 induced reduction in RNA and protein levels, I carried out the tethering assay in various yeast mutants deficient in 5' to 3' decay.

I first deleted *CCR4*, a component of the deadenlyation complex needed for mRNA deadenylation and turnover (Tucker et al., 2001). *FBA1* mRNA in *ccr4*Δ yeast was still decreased approximately 3-fold upon Dhh1 tethering (Figure 2.2A, left panel) demonstrating that deadenylation is not required for Dhh1's ability to reduce RNA levels. This suggests that recruitment of Dhh1 bypasses the need for deadenylation, and is consistent with prior results showing that Dhh1 functions downstream of deadenylation (Coller et al., 2001; Fischer and Weis, 2002).

Next, I deleted a component of the decapping complex, *DCP1*, to prevent decapping. Tethering of Dhh1 did not lower *FBA1* mRNA levels in *dcp1*Δ cells compared to the control (Figure 2.2A, middle panel). Similarly, Dhh1 no longer caused a decrease in *FBA1* mRNA when the 5' to 3' exonuclease Xrn1 was deleted (Figure 2.2A, right panel). Thus the ability of Dhh1 to reduce mRNA levels depends on Dcp1 and Xrn1 demonstrating that Dhh1 functions upstream of decapping and exonucleolytic digestion. Together, these results show that tethering of Dhh1 is sufficient to induce to the specific reduction of mRNA levels by activation and/or recruitment of the 5'-3' decay machinery.

Dhh1 can reduce protein levels in the absence of RNA decay

The ability to uncouple the tethering of Dhh1 from the induction of mRNA degradation in *dcp1*Δ and *xrn1*Δ cells allowed me to assess the effects of Dhh1 on protein levels independently of its role in mRNA decay. This was important because Dhh1 has previously been reported to function as a translational repressor (Coller and Parker, 2005; Minshall et al., 2009; Minshall and Standart, 2004). I compared Fba1 protein levels in *ccr4*Δ, *dcp1*Δ and *xrn1*Δ deletion strains when Dhh1 was tethered to *FBA1* mRNA. As

expected, tethering of Dhh1 to *FBA1* mRNA in the *ccr4*Δ strain, resulted in a decrease in Fba1 protein levels corresponding to the decrease in mRNA levels (Figure 2.2A and B, left panel). Intriguingly, I found that tethering Dhh1 to *FBA1* mRNA in both the *dcp1*Δ and *xrn1*Δ strains still resulted in a decrease in Fba1 protein levels (Figure 2.2B, center and right panels) despite the fact that there was no change in *FBA1* mRNA levels in these strains (Figure 2.2A). These results strongly suggest that Dhh1 can act to repress mRNA translation *in vivo*, independent of its ability to activate mRNA decay.

Tethering Dhh1 to *FBA1* mRNA results in an increase in Dcp2-positive P-bodies

In yeast, the mRNA decay machinery localizes to cytoplasmic foci called P-bodies during certain conditions of cell stress (Parker and Sheth, 2007). Current models suggest that changes in the translational status of mRNAs from active translation to decay induce P-body formation and that P-body size therefore correlates with the amount of non-translating mRNAs in cells (Franks and Lykke-Andersen, 2008). Since tethering Dhh1 to *FBA1* mRNA results in translational repression and mRNA decay, I wanted to test whether tethering Dhh1 to the very abundant *FBA1* mRNA effects P-body formation. To visualize P-bodies, I first tagged a known P-body component, the decapping protein Dcp2, with GFP. Dcp2-GFP was co-expressed in *dhh1*Δ cells with either Dhh1-PP7CP or PP7CP in the presence or absence of *FBA1* containing the PP7 binding loop (Figure 2.3A-B). Few Dcp2 positive P-bodies could be detected in cells expressing the PP7CP in either the presence or absence of the PP7 binding loop in the *FBA1* mRNA. Expression of Dhh1-PP7CP alone caused an increase in Dcp2-positive P-body intensity compared to PP7CP. Importantly however, I found that tethering Dhh1 to *FBA1* mRNA results in a twofold increase in the amount of Dcp2-GFP found in P-bodies compared to the non-tethered control (Figure 2.3B). Together these data show that tethering Dhh1 to an abundant mRNA results in increased P-body formation *in vivo*.

Tethering Dhh1 is sufficient to localize *FBA1* mRNA to P-Bodies.

Since tethering Dhh1 to an mRNA promoted P-body formation, I hypothesized that Dhh1 targets translationally inactive mRNAs to P-bodies for degradation. To test this hypothesis I quantified the co-localization of *FBA1* mRNA tethered to Dhh1 with P-bodies marked by Dcp2-GFP. I was unable to detect any enrichment of *FBA1* mRNA in P-bodies in a wild-type strain background however (unpublished data). Since Dhh1 tethering leads to a drastic reduction of steady-state *FBA1* mRNA levels in this background (Figure 1), I reasoned that *FBA1* mRNA turnover might be too rapid to see P-body accumulation or, alternatively, that *FBA1* mRNA levels are potentially too low to be visualized by *in situ* hybridization. I therefore also monitored the localization of *FBA1* mRNA in *xrn1*Δ cells. In this strain background *FBA1* mRNA levels are restored, but tethering Dhh1 to *FBA1* still results in translational repression (Figure 2.2). While deleting *XRN1* causes the constitutive formation of Dcp2 containing P-bodies ((Teixeira and Parker, 2007) and Figure 4A), I found that *FBA1* mRNA tethered to the PP7CP was enriched in only ~5% of the Dcp2 labeled P-bodies (Figure 2.4A-B). By contrast, tethering Dhh1 to *FBA1* mRNA, resulted in a 5-fold increase in the number of P-bodies in which a signal for *FBA1* mRNA could be detected (Figure 2.4A-B). Importantly, targeting of *FBA1* mRNA to P-bodies was specific to tethering Dhh1, as there was no change in localization of *FBA1* mRNA that lacked the PP7 binding loop (Figure 2.4B).

This demonstrates that tethering Dhh1 to *FBA1* mRNA under conditions where it causes translational repression, results in an increased localization of *FBA1* mRNA to Dcp2 positive P-bodies. Thus my results argue that recruitment of Dhh1 to a specific mRNA is sufficient to target this mRNP to a P-body.

Reduction in RNA and protein levels does not require ATP hydrolysis by Dhh1.

My results suggest that targeting of Dhh1 to an mRNA leads to translational inactivation, degradation and localization to P-bodies. Given that Dhh1 is a member of the large family of DExD/H-box ATPases, we wanted to investigate the role of ATP hydrolysis in these processes. To eliminate the ATPase activity, a single point mutation from glutamic acid (E) to glutamine (Q) can be introduced in the DEAD domain of DExD/H-box ATPases (Pause and Sonenberg, 1992). This glutamate residue is essential for the catalytic function of DExD/H ATPases as it positions the nucleophilic water molecule attack the ATP γ-phosphate bond (Ling et al., 2009). We generated a Dhh1^{DQAD} variant which is predicted to still bind to, but no longer hydrolyze ATP, thus locking the protein in an ATP-bound state.

To examine the effects of the Dhh1^{DQAD} variant on mRNA and protein levels a PP7-tagged copy of the DQAD mutant protein (Dhh1^{DQAD}-PP7CP) was expressed in a *dhh1*Δ strain in which *FBA1* was tagged with a PP7 binding loop. Dhh1^{DQAD}-PP7CP and Dhh1-PP7CP were expressed to comparable levels in these cells (Supplementary Figure 2.1). Surprisingly, I found that Dhh1^{DQAD}-PP7CP reduced both mRNA and protein levels to a similar extent as the wild-type protein when we compared it to GFP-PP7CP or the non- tethered Dhh1^{DQAD}-GFP control (Figure 2.5A). These results demonstrate that ATP hydrolysis by Dhh1 is not required to reduce RNA and protein levels when directly targeted to an mRNA.

I next wanted to test whether Dhh1^{DQAD} is able to function in the decay of non-tethered RNAs. To test this, I looked at the decay kinetics of three mRNAs, *RPL25*, *CGH1*, and *ACT1* either in cells in which *DHH1* is deleted, or in cells expressing Dhh1^{wt} or Dhh1^{DQAD} (Supplementary Figure 2.3). In agreement with previous research showing that some mRNAs are stabilized upon deletion of Dhh1 (Coller and Parker, 2001; Fischer and Weis, 2002), I found that all three mRNAs were stabilized in *dhh1*Δ cells, and the presence of Dhh1^{wt} accelerates decay (Supplementary Figure 2.3). The presence of Dhh1^{DQAD} had differential effects on decay depending on the mRNA tested however. Although Dhh1^{DQAD} fully rescued the decay defect of *ACT1*, it only partially rescued the decay defect of *CRH1* and it was not able to rescue *RPL25* decay (Supplementary Figure 2.3). Interestingly, the ability of Dhh1^{DQAD} to rescue the mRNA decay defect corresponds to the overall stability of the mRNA. Dhh1^{DQAD} fully rescues *ACT1*, a stable mRNA, but has no effect on the unstable mRNA *RPL25*. These results suggest Dhh1^{DQAD} is able to activate the decay of some non-tethered mRNAs; however, the ATPase activity of Dhh1 is rate limiting with short-lived mRNAs.

ATP hydrolysis is necessary for the dynamic localization of Dhh1 in P-Bodies.

Although the Dhh1^{DQAD} mutant reduced mRNA and protein levels of tethered mRNAs and partially rescued the decay defects of non-tethered mRNAs in the absence of wild-type Dhh1 (Supplementary Figure 2.3), expression of Dhh1^{DQAD} was unable to rescue the temperature sensitivity growth defect of the *dhh1*Δ strain (Figure 2.5B).

Therefore, it was important to further investigate the function of ATP hydrolysis in the cellular role of Dhh1. To begin to address this question I first tested whether the DQAD mutation affected the interaction with known binding partners of Dhh1. However, no significant difference was seen between the amount of Xrn1-myc or Pat1-myc that co-purified with either Dhh1 or Dhh1^{DQAD} in pull-down assays examined by Western blot (Supplementary Figure 2.4). Similarly, no significant interaction differences between Dhh1 or Dhh1^{DQAD} and Dcp1, Dcp2, Lsm1, Edc3 or Ccr4 were detected when co-purified proteins were analyzed after affinity purifications by MudPIT mass spectrometry (C. Mugler, pers. communication).

I next monitored the localization of Dhh1-GFP or Dhh1^{DQAD}-GFP in *dhh1*Δ cells. Both proteins were expressed at similar levels (Supplementary Figure 2.1). Consistent with previous reports (Teixeira et al., 2005), Dhh1-GFP assumed a diffuse localization throughout the cytoplasm in logarithmically growing yeast, and localized to P-bodies only upon stress (Figure 2.6A). By contrast, we found that the Dhh1^{DQAD} mutant localized to distinct cytoplasmic foci even in logarithmically growing cells (Figure 2.6B). This localization remained unaltered in stress conditions (Figure 2.6B). As shown in Figure 6B, Dhh1^{DQAD} -GFP and Dcp2-RFP foci completely overlapped suggesting that the Dhh1^{DQAD} foci represent *bona fide* P-bodies.

Because abolishing the ATPase activity of Dhh1 leads to the formation of constitutive P-bodies, I hypothesized that the ATPase function of Dhh1 is critical for the recycling of Dhh1 out of P bodies. To test whether P-body dynamics are altered in Dhh1 wild-type versus Dhh1^{DQAD} expressing cells, I performed fluorescent recovery after photobleaching (FRAP) experiments (Figure 2.6C). P-bodies that were induced upon glycerol stress in the presence of wild-type Dhh1 were dynamic in that the Dhh1-GFP P-body signal rapidly recovered (Figure 2.6C, left panel). This suggests that wildtype Dhh1 continuously cycles in and out of P-bodies. In contrast Dhh1^{DQAD}-GFP failed to recover after bleaching P-bodies in either logarithmically growing or glycerol stressed cells (Figure 2.6C, middle and right panels). No significant recovery could be detected even one minute after the bleach. Together these data demonstrate that ATP hydrolysis by Dhh1 is important for normal P body dynamics. My results further suggest that the DQAD mutant protein is trapped in P-bodies, and that ATP hydrolysis is important for the recycling of Dhh1 into and out of P-bodies.

Discussion

The DExD/H-box ATPase Dhh1 has been implicated to function at the interface of translation and decay, but the role of Dhh1 in regulating these processes *in vivo* has remained poorly understood. By tethering Dhh1 to endogenous yeast mRNAs, I have shown here that Dhh1 causes a change in steady-state mRNA and protein levels, and that this coincides with the ability of Dhh1 to localize mRNAs to P-bodies. These findings support a model in which Dhh1 acts as a key regulator of the cytoplasmic fate of mRNAs by regulating the transition of an mRNA from the cellular pool undergoing active translation to an inactive storage and decay pool (Figure 2.7). In this model, binding of Dhh1 to an mRNA first leads to the inhibition of translation. Under normal physiological conditions recruitment of Dhh1 could be triggered through shortening of the poly(A)-tail, through interactions with distinct sequence-specific RNA binding proteins, or in other eukaryotes, through miRNA-mediated recruitment of a RISC complex (Chu and Rana,

2006; Eulalio et al., 2007c; Goldstrohm et al., 2006; Pedro-Segura et al., 2008). The inactivated mRNA-protein complex would then be targeted to mRNA-protein granules in the cytoplasm (either P-bodies or stress granules) where the mRNA could be subjected to one of two fates. Either it could remain translationally repressed and stored until yet unknown cellular signals direct it to reenter the translational pool, or alternatively, the additional recruitment of degradation factors could target the mRNA for decay. ATP hydrolysis releases Dhh1 from the cytoplasmic RNP granules allowing for another cycle of RNA binding and inactivation.

My data suggest that in actively growing yeast cells the translationally repressed complex formed by tethering Dhh1 is immediately targeted for degradation. However, it is likely that in different cellular environments or growth conditions Dhh1's association with an mRNA could result in storage rather than decay. This could be achieved either through the recruitment of additional co-factors, or through a general inhibition of the mRNA decay machinery. In this context it is interesting that two Dhh1 interacting proteins in yeast have recently been shown to modulate mRNA storage and decay specifically during stress (Talarek et al., 2010). Furthermore, tethering the Dhh1 ortholog Xp54 to a reporter mRNA in *Xenopus* oocytes, cells known to have very low decapping activity (Gillian-Daniel et al., 1998), resulted exclusively in translational repression (Minshall et al., 2009; Minshall and Standart, 2004). Consistent with this idea, the ortholog of Dhh1 in *Caenorhabditis elegans,* CGH-1, was found in distinct complexes depending on the developmental status of the organism (Boag et al., 2008). In somatic tissues, Dhh1 associates in a Pat1-dependent manner with P-bodies proposed to function in decapping; whereas, during oogenesis CGH-1/Dhh1 forms complexes with translational regulators and promotes storage of maternal mRNAs.

My model proposes that translational inhibition via Dhh1 functions upstream of mRNA decay. I show here that the reduction of mRNA levels we observe upon tethering of Dhh1 does not require deadenylation, but is strictly dependent on the 5' to 3' decay machinery (Figure 2.2). By contrast, Dhh1 does not require the 5' to 3' RNA decay machinery for its ability to lower protein levels. If the two pathways of mRNA decay and translational inhibition were functioning in parallel and were competing with each other, I would expect that the restoration of mRNA levels should at least partially restore steady state protein levels. However, this is not the case as the Dhh1-mediated decrease in protein levels is identical in wild-type cells and mutants in which the 5'-3' RNA decay pathway is blocked (compare Figure 2.1 and Figure 2.2). This suggests that Dhh1-mediated mRNA decay is epistatic to translational repression.

My model also predicts that concurrent with assembly of a translationally repressed mRNP the inactivated complex is targeted to mRNA-protein granules in the cytoplasm (either P-bodies or stress granules/EGP-bodies). We found here that tethering Dhh1 to an mRNA results in an increase in Dcp2-positive mRNP granules. Furthermore, Dhh1 is sufficient to relocalize an mRNA into P-bodies, presumably sequestering the mRNA away from the translation machinery.

How P-body assembly is mediated remains unclear, however Dhh1 is a member of the DExD/H-box family of ATPases and members of this protein family have previously been shown to use the energy of ATP hydrolysis to remodel RNA-protein complexes (Jankowsky and Bowers, 2006). It is therefore possible that Dhh1 acts as an RNPase facilitating the dissociation of translation factors and/or the binding of

translational repressors and decay factors, which could induce mRNA re-localization into P-bodies. Alternatively, Dhh1 could be functioning more passively as a RNA-dependent scaffold to which decay factors and translational repressors bind. Such a mode of action would be analogous to the function of the DExD/H box protein eIF4AIII, which was shown to act as an ATP-dependent RNA scaffold recruiting cofactors to the exon junction complex upon pre-mRNA splicing (Andersen et al., 2006; Ballut et al., 2005; Bono et al., 2006). As proposed for eIF4AIII, the ATPase function of Dhh1 could be primarily required for complex disassembly, RNA release and recycling. A role for ATP hydrolysis in recycling of Dhh1 would be consistent with localization data showing that a hydrolysis-deficient Dhh1 variant, $Dhh1^{DQAD}$, constitutively localizes to P bodies ((Kramer et al., 2010) and Figure 2.6). Furthermore, whereas the Dhh1 wild-type protein displays a highly dynamic P body localization, $Dhh1^{DQAD}$ is severely impaired in its ability to cycle in and out of P bodies (Figure 2.6)

Although I found here that ATP hydrolysis is not required for Dhh1 to reduce mRNA and protein levels when directly tethered to an mRNA, it should be noted that this result differs from previously published data in *Xenopus* oocytes (Minshall et al., 2009; Minshall and Standart, 2004). When tethering a *Xenopus* p54/Dhh1 ATPase mutant, Minshall *et al*. found that the mutant protein caused an increase in translation of a tethered, microinjected RNA lacking a poly(A) tail. Additionally, these authors showed that mutations in the ATPase function of human Dhh1 led to a decrease in the number of P-bodies in tissue culture cells (Minshall et al., 2009). This result differs from our finding that in yeast P-bodies accumulate upon inhibition of Dhh1's ATPase activity correlating with a defect in P-body recycling (Figure 2.6). At present the reason for the discrepancies between these results remain unclear but they may be explained by differences in the experimental systems since Dhh1 has been proposed to have different functions in various cell types and at different stages of development (see discussion above and (Boag et al., 2008)). Interestingly, an ATPase hydrolysis mutant of a Dhh1 homolog in trypanosomes also led to an increase in P body formation (Kramer et al., 2010), and a similar ATPase mutation in the SF1 RNA helicase Upf1, involved in nonsense mediated decay, induces constitutive P-bodies (Franks et al., 2010; Ujwal Sheth, 2006). During the revision of this manuscript, it was furthermore shown that the ATPase activity of Upf1 is required for mRNP disassembly and completion of nonsense-mediated mRNA decay (Franks et al., 2010). While I have no evidence for the accumulation of a non-degraded mRNA fragment upon tethering of $Dhh1^{DQAD}$, taken together these results suggest that ATP hydrolysis by these RNA helicases plays an important role in enzyme recycling, which in turn is important for the regulation of P body assembly and dynamics.

In summary, I have shown that Dhh1 is a critical regulator of the post-transcriptional fate of mRNAs in yeast. Dhh1 is sufficient to move mRNAs out of the active translation pool into a translational repressed state where mRNAs can be targeted for degradation. At the same time, Dhh1 is sufficient to sequester mRNAs into cytoplasmic RNP granules involved in mRNA storage and decay. The overall function of Dhh1 appears to be highly conserved throughout eukaryotes supported by the high degree of sequence conservation between species and by the finding that the *Xenopus, Drosophila* and mammalian orthologs can rescue the loss of Dhh1 in yeast (Maekawa et al., 1994; Tseng-Rogenski et al., 2003; Westmoreland et al., 2003). Therefore my studies

in budding yeast should provide insight into the function of Dhh1 and the regulation of gene expression in higher eukaryotes. Intriguingly, Dhh1 has also been implicated in miRNA-dependent inactivation of mRNAs (Chu and Rana, 2006; Eulalio et al., 2007c). While the relationship between of miRNA mediated translational repression and decay remains ambiguous (Eulalio et al., 2008; Wu and Belasco, 2008), my work here suggests an epistatic relationship between mRNA decay and translational inhibition. It is therefore tempting to speculate that, as proposed for Dhh1 (Figure 7), miRNAs might also first trigger translational repression, and that inhibition of translation functions directly upstream of mRNA decay. The final outcome of whether mRNAs are stored or degraded in response to miRNA recognition might be highly dependent on cell and tissue type, or environmental conditions, which could partially explain conflicting results present in the current literature. Clearly, future work will be required to elucidate the mechanisms behind the role of Dhh1 and miRNAs in regulating gene expression by translational repression and mRNA decay.

Methods

Construction of yeast strains and plasmids

Construction of plasmids for this study (Table S2.1) was performed using standard molecular cloning techniques. Yeast strains (Table S2.2) were constructed using PCR-based transformation approach with specific primers and integration plasmids (Longtine et al., 1998). Additionally, yeast mutants were constructed by transformation with genomic regions PCR amplified from the corresponding yeast mutant strains or by mating and subsequent dissection of the tetrads.

To generate the plasmid used to tag genes with the FLAG tag, the FLAG peptide was cloned into a yeast integration cassette. Plasmids containing the PP7 loop and PP7CP sequences were gifts from B. Hogg and K. Collins (Hogg and Collins, 2007). To generate the plasmid used for PP7 loop tagging, the PP7 loop was cloned into a yeast integration plasmid downstream of a FLAG tag. FLAG-PP7 tagged genes were obtained by performing PCR based plasmid integration. To generate plasmids for expressing proteins tagged with PP7CP, GFP and CBP-TEV-ZZ, each tag cloned into a plasmid with the Dhh1 promoter and the Adh1 3'UTR. Protein sequences were then subcloned into these plasmids to generate C-terminal tagged proteins.

Dhh1 mutagenesis: The Dhh1^{DQAD} (E196Q) mutation was introduced using a Strategene Quickchange site directed mutagenesis kit using UC 1949 and UC 1950 (Table S3) and confirmed by sequencing. This caused a GAA-CAA mutation at base pair 568, which results in an amino acid change from glutamic acid to glutamine.

PP7 Tethering Assay

Yeast cultures were grown to mid log phase ($O.D._{600}$ 0.4-0.6) at 30°C in synthetic media containing 2% dextrose. Cells were collected by centrifugation and lysed in 1X PBST with protease inhibitors. Lysis was performed with 2x30 second pulses using a mini-beadbeater (Biospec Products). Extract was clarified by centrifugation and extract protein concentration was normalized by Bradford Assay (Biorad). Protein samples were prepared by resuspending extract in SDS sample buffer. RNA was isolated using the Qiagen RNeasy RNA isolation kit (Qiagen).

Nothern and Western Blot analysis

Total RNA levels were measured using a NanoDrop spectrophotometer and equal amounts of total RNA were separated by agarose gel electrophoresis. RNA was transferred to a nitrocellulose membrane and incubated with gene-specific antisense oligonuleotides (Table S3) end labeled with ATP-γ32P using T4 polynucleotide kinase (New England Biolabs). Hybridization was carried out at 46°C in Church buffer; wash steps were performed at 46°C in saline-sodium citrate (SSC) buffer with 0.1% SDS. RNA was visualized using a Typhoon TRIO imager (Amersham) and RNA levels were quantified using ImageQuant software (Molecular Dynamics). All tethered mRNAs were monitored by probing with UC587, an oligonucleotide against the FLAG-tag. Tethered mRNA levels were normalized against an endogenous untethered mRNA.

Protein samples from yeast extract were separated by SDS-PAGE and used for Western blot analysis with the Odyssey infrared imaging system (LI-COR Biosciences). Western blotting was performed with one of the following primary antibodies: anti-FLAG, anti-His, anti-myc, anti-Xpo1 and anti-Pab1. Goat anti-mouse AlexaFluor680 (Invitrogen) or goat anti-rabbit IRDye800 (Rockland Immunochemicals) were used as secondary antibodies. Protein levels were quantified using the Odyssey imaging software. Protein levels of tethered mRNAs were monitored by probing an antibody against the FLAG-tag. Protein levels were normalized against endogenous Xpo1 or Pab1 protein.

mRNA decay measurements

Cell cultures were grown to log phase. Time points were collected following addition of 3ug/ml thiolutin (Enzo Life Sciences). RNA isolation was performed as described above. Following DNase treatment (Ambion) of the RNA, cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen). Quantitative PCR was performed in a StepOnePlus Real-Time PCR system (Applied Biosystems) using gene specific primers and Absolute QPCR SYBR Green ROX Mix (Thermo Scientific). cDNA levels were normalized against levels of the RNA subunit of the signal recognition particle, SCR1.

Myc Purifications

Purifications were carried out as described by (Oeffinger et al., 2007). Briefly, cells were grown to mid log phase and lysed mechanically using a Mixer Mill type 307 (Retsch). Grindate was resuspended in TBT buffer and extract was incubated with IgG coupled magnetic dynabeads (Invitrogen). Beads were washed twice in TBT buffer and resuspended in SDS sample buffer.

Microscopy

Live cell fluorescent microscopy: Cells were grown to mid log phase ($O.D_{600}$ 0.4 – 0.6) at 30°C in synthetic media containing 2% dextrose. Cells were observed using a Nikon Eclipse E600 fluorescence microscope using a 100X oil-immersion objective. Images were captured using an Orca II CCD (C4742-98-24R, Hamamatsu Photonics) controlled by Metamorph 4.6R6 (Universal Imaging Corporation).

For tethering experiments measuring the accumulation of Dcp2-GFP in P-Bodies, the fluorescent intensity of Dcp2 in P-bodies was compared to fluorescent intensity of the

entire cell for approximately 100 cells per indicated condition. The amount of Dcp2 in P-bodies was then normalized to the untethered strain expressing Dhh1-PP7CP for each independent replicate. Quantification was done using ImageJ, a public domain Java image-processing program (http://rsb.info.nih.gov/ij/).

For carbon source shift experiments, cells were washed twice in fresh synthetic media containing either 2% dextrose or 3% glycerol as a carbon source. Cells were resuspended in the same medium used for washing and observed after 15 minutes. Image processing was done using Photoshop (Adobe).

In situ hybridizations: Four oligonucleotides were designed against regions of *FBA1* mRNA (Table S3) and used to create fluorescently labeled RNA probes. The protocol for in vitro transcription and fluorescent labeling was based on manufacturers instructions and published protocols from Robert Singer's lab (http://www.singerlab.org/protocols). RNA probes were synthesized by in vitro transcription using reagents from the MEGAshortscript kit (Applied Biosystems). RNA probes were synthesized by a standard in vitro transcription reaction, except that UTP was replaced by a 1/1 mixture of UTP/amino-allyl UTP. Transcription reactions were then treated with DNase, phenol extracted, ethanol precipitated and resuspended in 1X SSC. Unincorporated nucleotides were removed by gel filtration through NucAway spin columns (Ambion). The probes were then ethanol precipitated and resuspended in 0.1M NaHCO3 buffer, pH 8.8. The four *FBA1* probes were combined into one reaction (1.5 ug of each probe) and labeled using 1 vial of Cy3 monoreactive labeling kit (Amersham). Unincorporated nucleotides were removed by two rounds of ethanol precipitation and resuspended in water.

Cultures were grown to mid log phase ($O.D_{600}$ 0.4 – 0.6) at 30°C in synthetic media with 2% dextrose. 1ml of cell culture was fixed for 15 minutes with 134µl of 37% Formaldehyde (Flukka) and washed twice in buffer A (.01M Potassium Phosphate buffer pH6.5, 0.5mM MgCl2) followed by resuspension in buffer B (.01M Potassium Phosphate buffer pH6.5, 0.5mM MgCl2, 1.2M Sorbitol). Cells were spheroplasted with zymolase T100, plated on a Poly-L-lysine coated slides and incubated in 70% ethanol for 2 hours. Samples were then blocked for 30 minutes at 37° in hybridization buffer and incubated overnight with Cy3 labeled RNA probes against *FBA1* mRNA. Slides were washed with decreasing concentrations of SSC and mounted in vectashield (Vectorlabs). *FBA1* mRNA was colocalized with Dcp2-GFP and the number of overlapping FBA1 and Dcp2 P bodies were counted in approximately 150 cells per condition.

Fluorescent recovery after photobleaching: Photobleaching was performed using a Deltavision microscope (Deltavision Spectris; Applied Precision, LLC) equipped with a 488 nm Deltavision Quantifiable Laser Moduleand and a Cool Snap HQ camera (Photometrics). One Z-stack image (with optical sections 0.5 µm apart) was collected prior to photobleaching. P-bodies to be photobleached were subjected to a 1-2 second pulse from a UV laser. Images were collected at 5 second intervals for one minute post-bleach. Images were deconvolved using SoftWoRx (Applied Precision, LLC) and a maximum projection from each Z-stack was obtained using Softworx image processing software (Applied Precision, LLC). Data analysis was performed using ImageJ.

Figure 2.1:
Tethering Dhh1 to endogenous mRNA's results in a decrease in mRNA and protein levels. (A) Schematic of tethering strategy. Dhh1-GFP, GFP-PP7CP, Dhh1-PP7CP fusion proteins were expressed in strains engineered with one PP7 binding loop in the 3'UTR of an endogenous yeast mRNA. **(B)** Left panel: Northern analysis of *FBA1-PP7* mRNA levels in cells expressing the non-tethered control (Dhh1-GFP), GFP-PP7CP or Dhh1-PP7CP fusion proteins. *FBA1* mRNA levels were quantified with ImageQuant analysis software and normalized to an endogenous mRNA, *RPL37*. Right panel: Western analysis of Fba1 protein levels in these strains. *FBA1* protein levels were measured using the Odyssey imaging system and compared to Xpo1, a non-tethered control. **(C)** Left panel: Northern analysis of *RPL25-PP7* mRNA in cells expressing Dhh1-GFP, GFP-PP7CP or Dhh1-PP7CP fusion proteins. *RPL25* mRNA levels were normalized to *ADH1* loading control. Right panel: Western analysis of Rpl25 protein levels normalized to Xpo1 loading control. In (B) and (C), mean values +/- standard deviations from three independent experiments are shown.

Figure 2.1

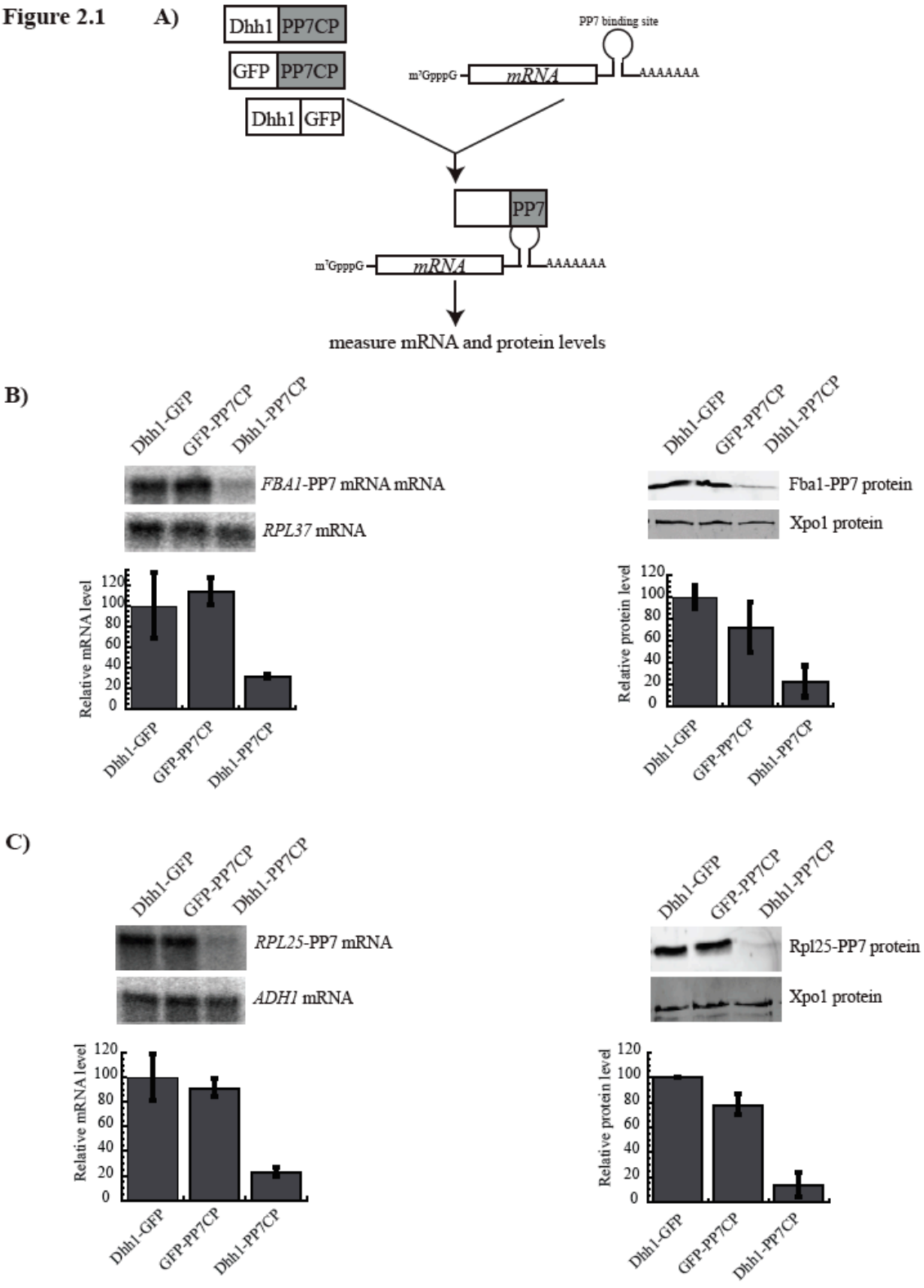

A)
Dhh1 PP7CP
GFP PP7CP
Dhh1 GFP
PP7 binding site
m7GpppG mRNA AAAAAAA
PP7
m7GpppG mRNA AAAAAAA
measure mRNA and protein levels
B)
Dhh1-GFP
GFP-PP7CP
Dhh1-PP7CP
FBA1-PP7 mRNA mRNA
RPL37 mRNA
Relative mRNA level
Fba1-PP7 protein
Xpo1 protein
Relative protein level
C)
RPL25-PP7 mRNA
ADH1 mRNA
Rpl25-PP7 protein
Xpo1 protein

Figure 2.2:
Dhh1's ability to reduce mRNA, but not protein, levels depends on a functional 5' to 3' decay pathway. (A) *FBA1* mRNA levels when GFP-PP7CP or Dhh1-PP7CP is tethered in *ccr4*Δ (left column), *dcp1*Δ (middle column) or *xrn1*Δ (right column) strains. Tethering assay was performed and mRNA levels analyzed as described in Figure 1. **(B)** Effects of tethered Dhh1-PP7CP on Fba1 protein levels in *ccr4*Δ, *dcp1*Δ and *xrn1*Δ strains. Assay was performed and protein levels were analyzed as described in Figure1.

Figure 2.2

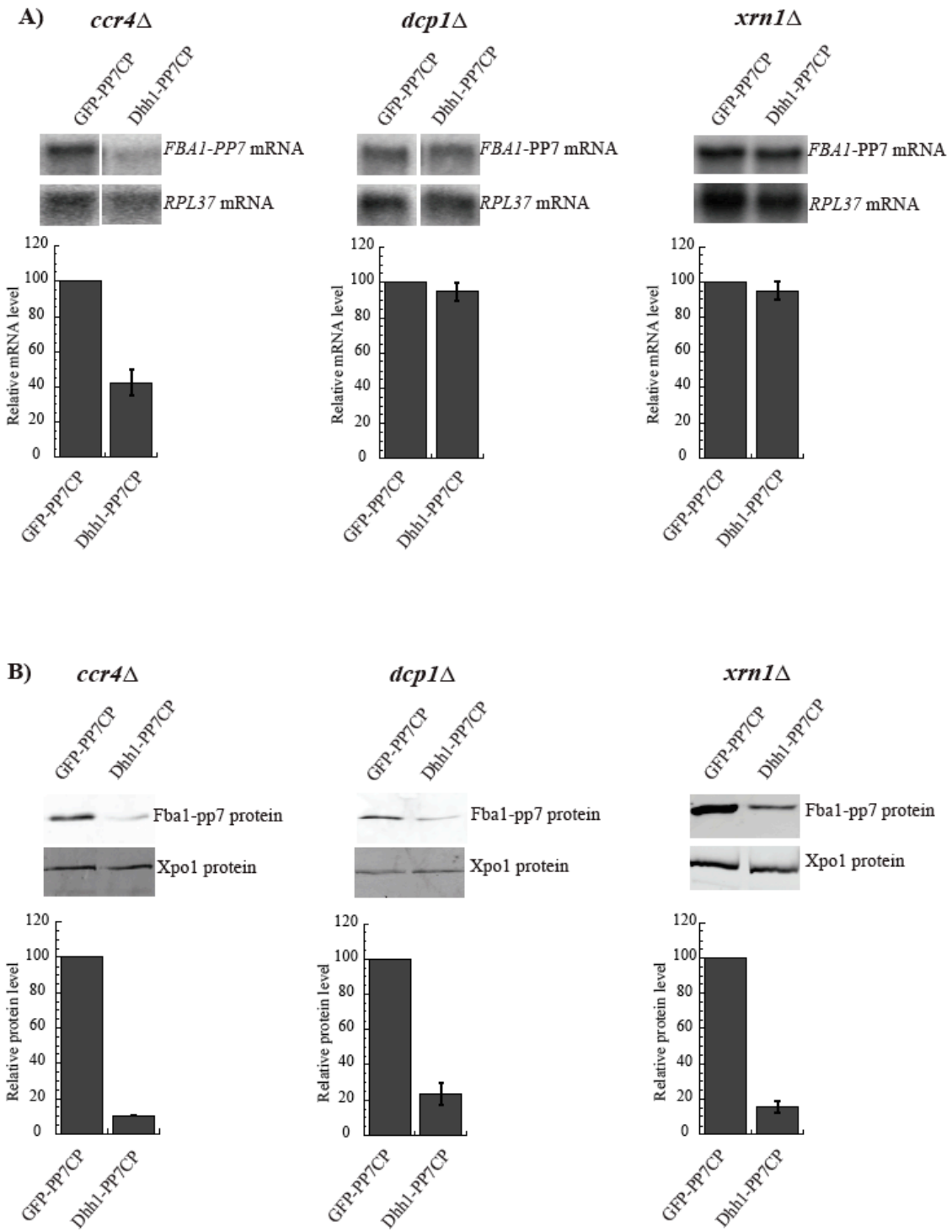

A)
ccr4Δ
dcp1Δ
xrn1Δ
GFP-PP7CP
Dhh1-PP7CP
FBA1-PP7 mRNA
FBA1-PP7 mRNA
RPL37 mRNA
RPL37 mRNA
Relative mRNA level
120
100
80
60
40
20
0
B)
ccr4Δ
dcp1Δ
xrn1Δ
Fba1-pp7 protein
Xpo1 protein
Relative protein level

Figure 2.3:
Tethering Dhh1 to *FBA1* mRNA is sufficient to induce the accumulation of Dcp2-positive P-bodies. (A) PP7CP (left panel) or Dhh1-PP7CP (right panel) proteins were expressed in cells with *FBA1* +/- PP7 binding loop. Cells were grown to mid log phase and P-bodies were visualized with Dcp2-GFP by fluorescence microscopy. **(B)** The fluorescent intensity of Dcp2 P-bodies was measured using ImageJ and compared to total cell fluorescence for approximately 100 cells. The amount of Dcp2 in P-bodies was then normalized to the untethered strain (*FBA1*-no loop) expressing Dhh1-PP7CP for each independent replicate. Values generated represent mean values +/- standard deviations from three independent experiments.

Figure 2.3

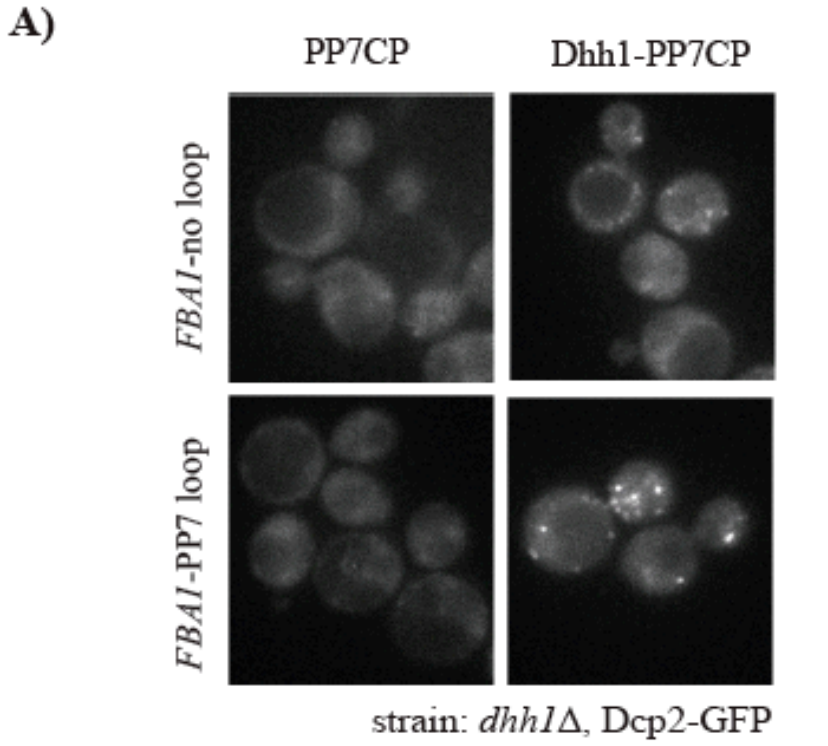
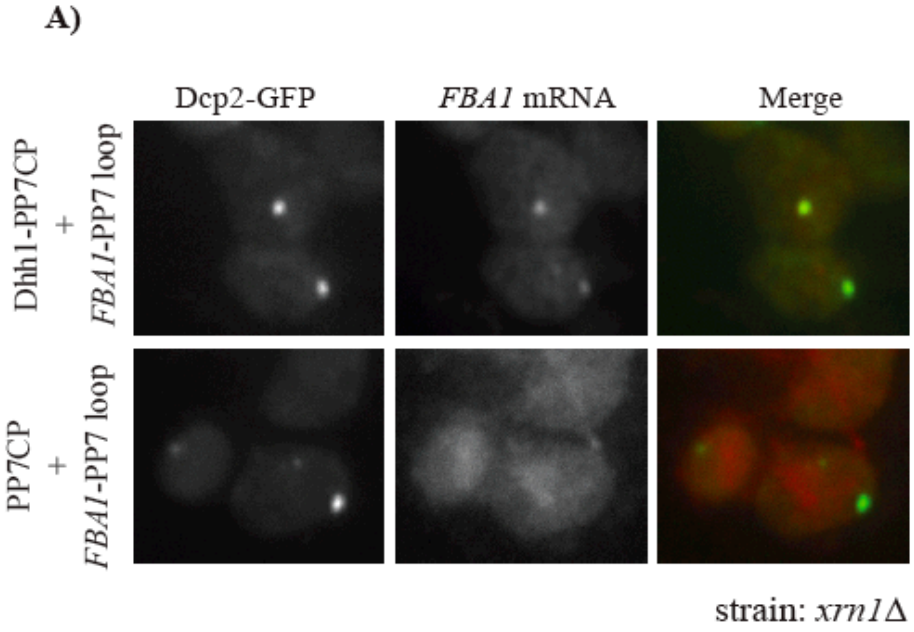

A)
PP7CP
Dhh1-PP7CP
FBA1-no loop
FBA1-PP7 loop
strain: dhh1Δ, Dcp2-GFP
B)
Relative fluorescent intensity of Dcp2 positive P-bodies
2
1.5
1
0.5
0
FBA1-no loop
FBA1-PP7
FBA1-no loop
FBA1-PP7
PP7CP only
Dhh1-PP7CP

Figure 2.4:
***FBA1* tethered to Dhh1 concentrates in Dcp2-positive P-bodies.** PP7CP and Dhh1-PP7CP proteins were expressed in *xrn1*Δ cells with either *FBA1* tagged with a PP7 binding loop or *FBA1* with no loop. **(A)** P-bodies were visualized with Dcp2-GFP (left panel) and *FBA1* mRNA was localized via *in situ* hybridization with fluorescently labeled probes against *FBA1* mRNA (middle panel). **(B)** *FBA1* mRNA was colocalized with Dcp2-GFP and the number of overlapping *FBA1* and Dcp2 P bodies were counted in approximately 150 cells per condition. Graph shows the ratio of *FBA1* P-bodies to the total number of Dcp2-GFP P-bodies. Mean values +/- standard deviations from two independent experiments are shown.

Figure 2.4


A)
Dcp2-GFP
FBA1 mRNA
Merge
Dhh1-PP7CP + *FBA1*-PP7 loop
PP7CP + *FBA1*-PP7 loop
strain: *xrn1*Δ

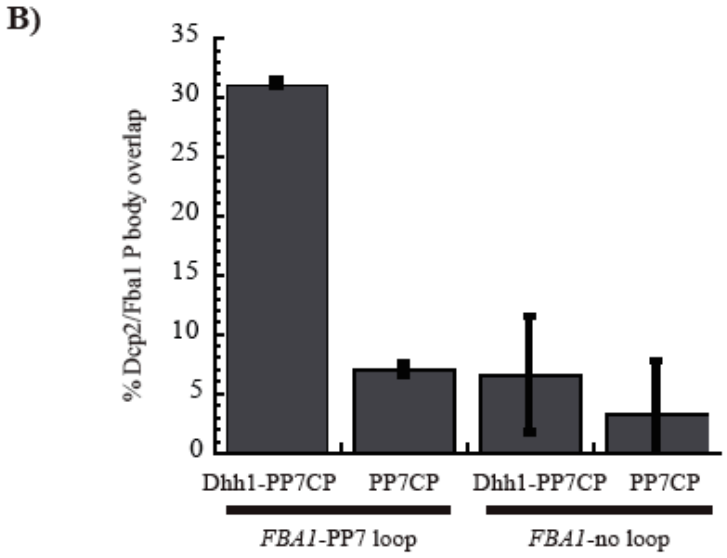

B)
% Dcp2/Fba1 P body overlap
35
30
25
20
15
10
5
0
Dhh1-PP7CP
PP7CP
Dhh1-PP7CP
PP7CP
FBA1-PP7 loop
FBA1-no loop

Figure 2.5:
The ATPase function of Dhh1 is not required for its ability to reduce mRNA and protein levels. (A) Left panel: Northern analysis of *FBA1* mRNA. Dhh1^{DQAD}-GFP, GFP-PP7CP or Dhh1^{DQAD}-PP7CP fusion proteins were expressed in *dhh1*Δ strains engineered with one PP7 binding loop in the 3' UTR of *FBA1*. *FBA1* mRNA levels were normalized against *SCR1* mRNA. Right panel: Western analysis of *FBA1* protein levels. *FBA1* protein levels were compared to Xpo1 protein. Mean values +/- standard deviations from three independent experiments are shown. **(B)** Wild-type Dhh1 or Dhh1^{DQAD} was expressed in a *dhh1*Δ strain background. Growth at 30° and 37° was monitored over a period of 3 days.

Figure 2.5

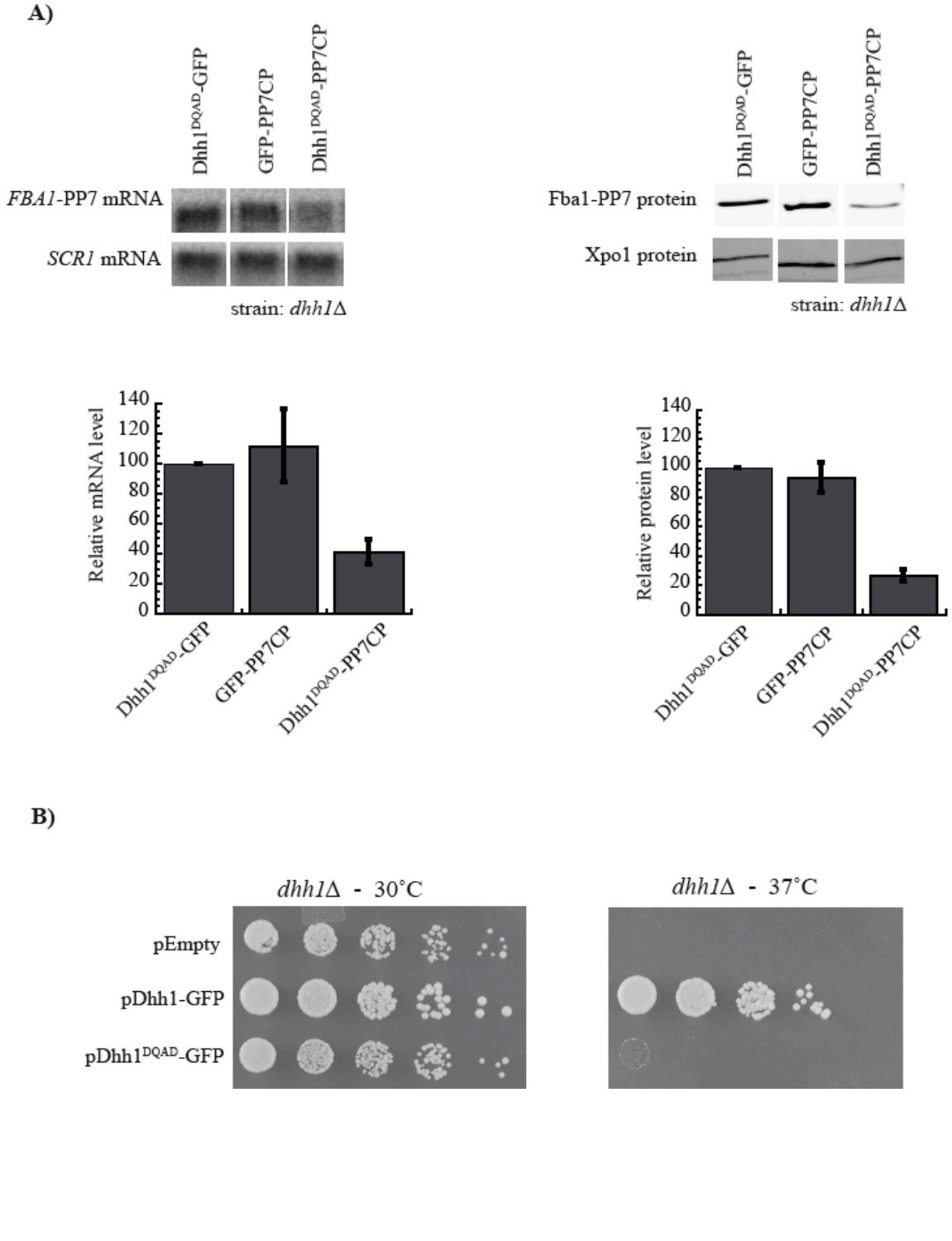
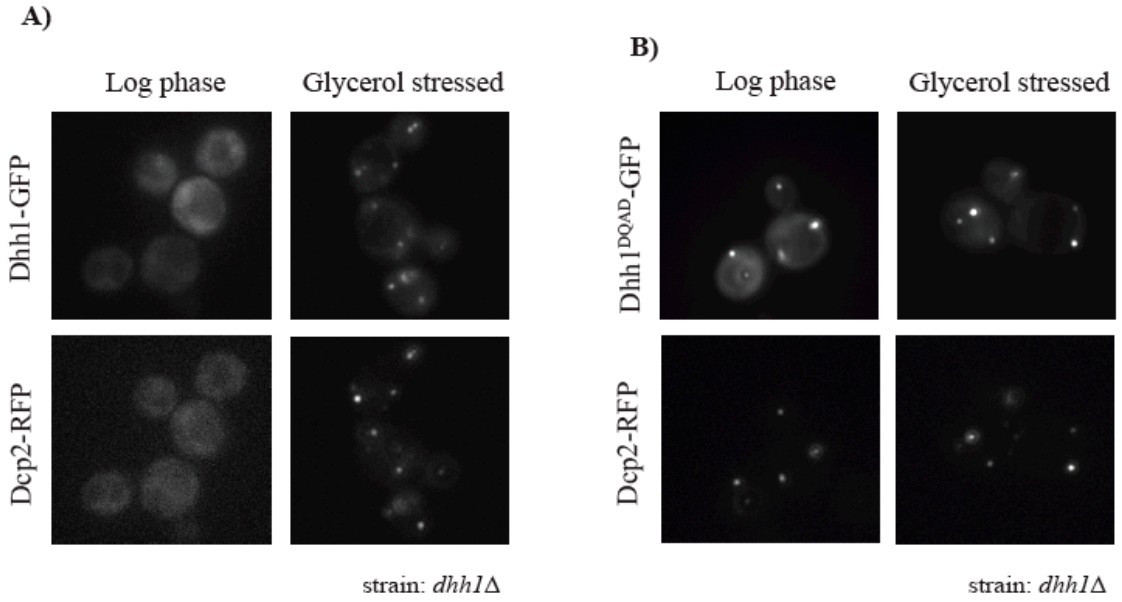

A)
Dhh1DQAD-GFP
GFP-PP7CP
Dhh1DQAD-PP7CP
FBA1-PP7 mRNA
SCR1 mRNA
strain: dhh1Δ
Fba1-PP7 protein
Xpo1 protein
strain: dhh1Δ
Relative mRNA level
Relative protein level
0
20
40
60
80
100
120
140
B)
dhh1Δ - 30˚C
dhh1Δ - 37˚C
pEmpty
pDhh1-GFP
pDhh1DQAD-GFP

Figure 2.6:
The ATPase function of Dhh1 is required for recycling out of P-bodies. Dhh1-GFP **(A)** or Dhh1^{DQAD}-GFP **(B)** fusion proteins were co-expressed with Dcp2-RFP in a *dhh1*Δ strain. Proteins were localized by fluorescent microscopy in yeast actively growing with glucose (A and B, left panel) or after a carbon source shift from glucose to glycerol (A and B, right panel). **(C)** Fluorescent recovery after photobleaching experiments were performed on Dhh1-GFP and Dhh1^{DQAD}-GFP P-bodies in mid-log phase yeast growing with glucose, or after a glucose to glycerol carbon source shift. Individual P bodies were partially bleached and representative recovery curves are shown.

Figure 2.6


A)
Log phase
Glycerol stressed
Dhh1-GFP
Dcp2-RFP
strain: dhh1Δ
B)
Log phase
Glycerol stressed
Dhh1DQAD-GFP
Dcp2-RFP
strain: dhh1Δ

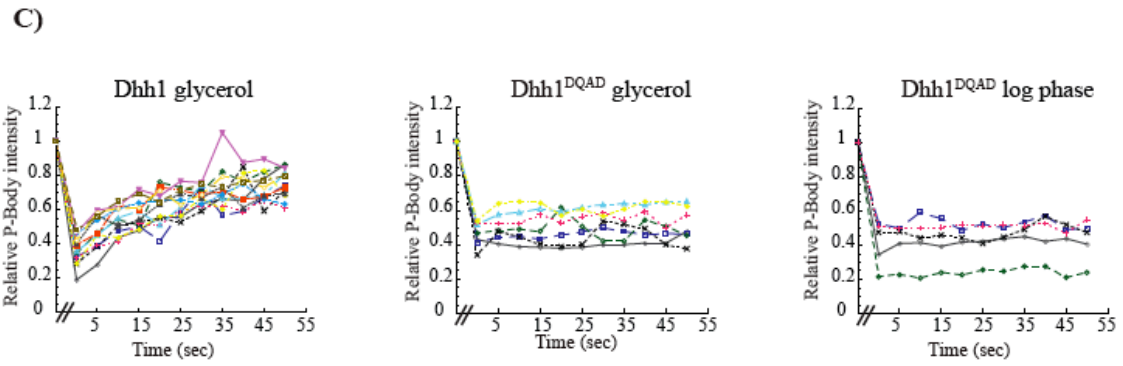
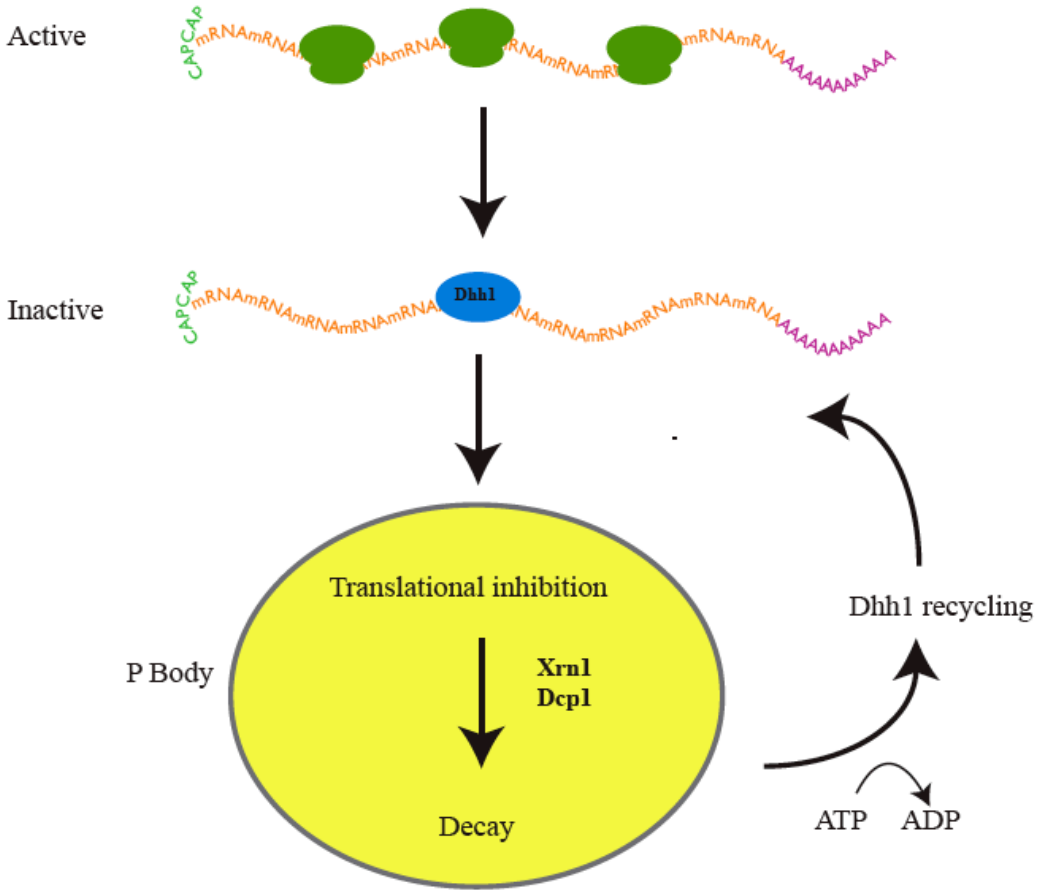

C)
Dhh1 glycerol
Dhh1DQAD glycerol
Dhh1DQAD log phase
Relative P-Body intensity
Time (sec)
0
0.2
0.4
0.6
0.8
1
1.2
5
15
25
35
45
55

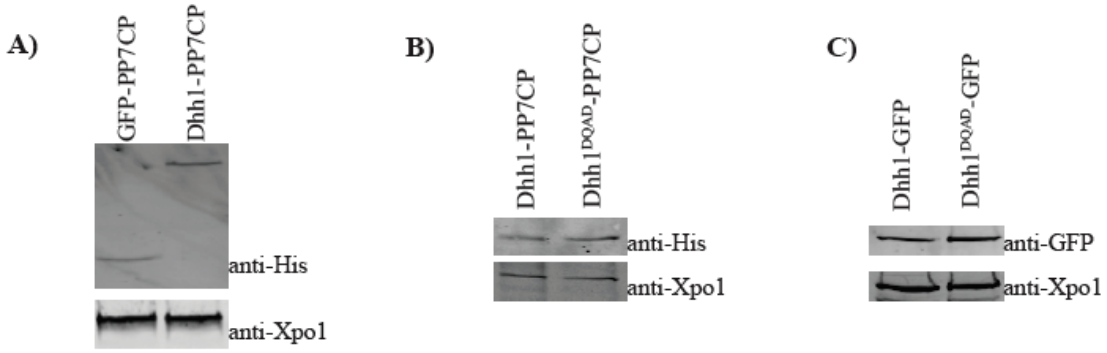
Figure 2.7:
Model for Dhh1 function. Recruitment of Dhh1 to an mRNA leads to the inhibition of translation and facilitates the movement of the mRNA from an active, cytoplasmic pool into an inactive pool located in P-bodies where the mRNA can be stored or undergo decay. In actively growing yeast, the default result of Dhh1 binding is mRNA decay; however, when the decay machinery is inactive, translational repression can occur independent of decay. ATP hydrolysis is necessary for Dhh1 recycling out of the P-body. For details refer to text.

Figure 2.7


Active
Inactive
Dhh1
Translational inhibition
P Body
Xrn1
Dcp1
Decay
Dhh1 recycling
ATP
ADP

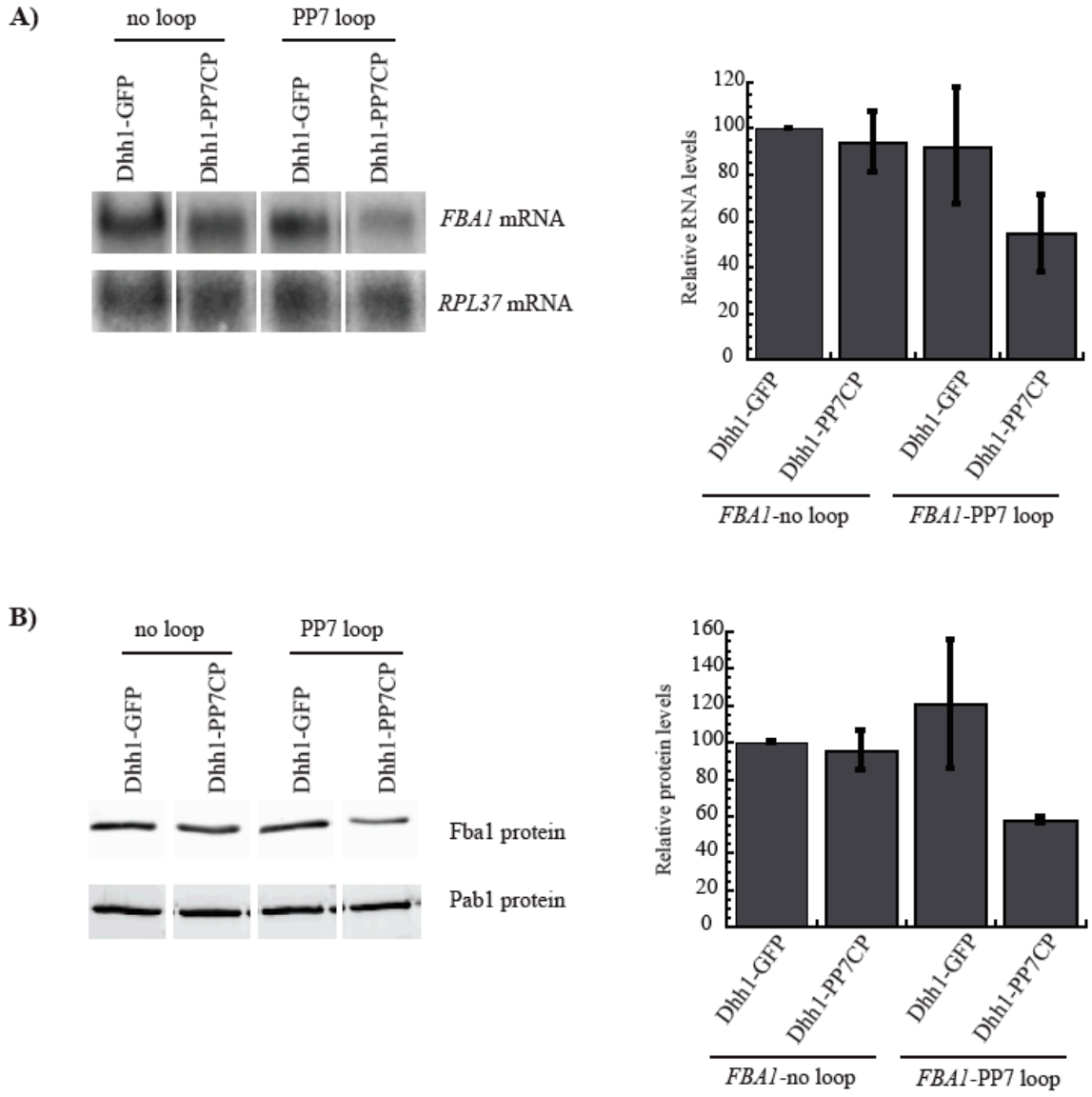
Supplementary figures and tables

Suplementary Figure 2.1



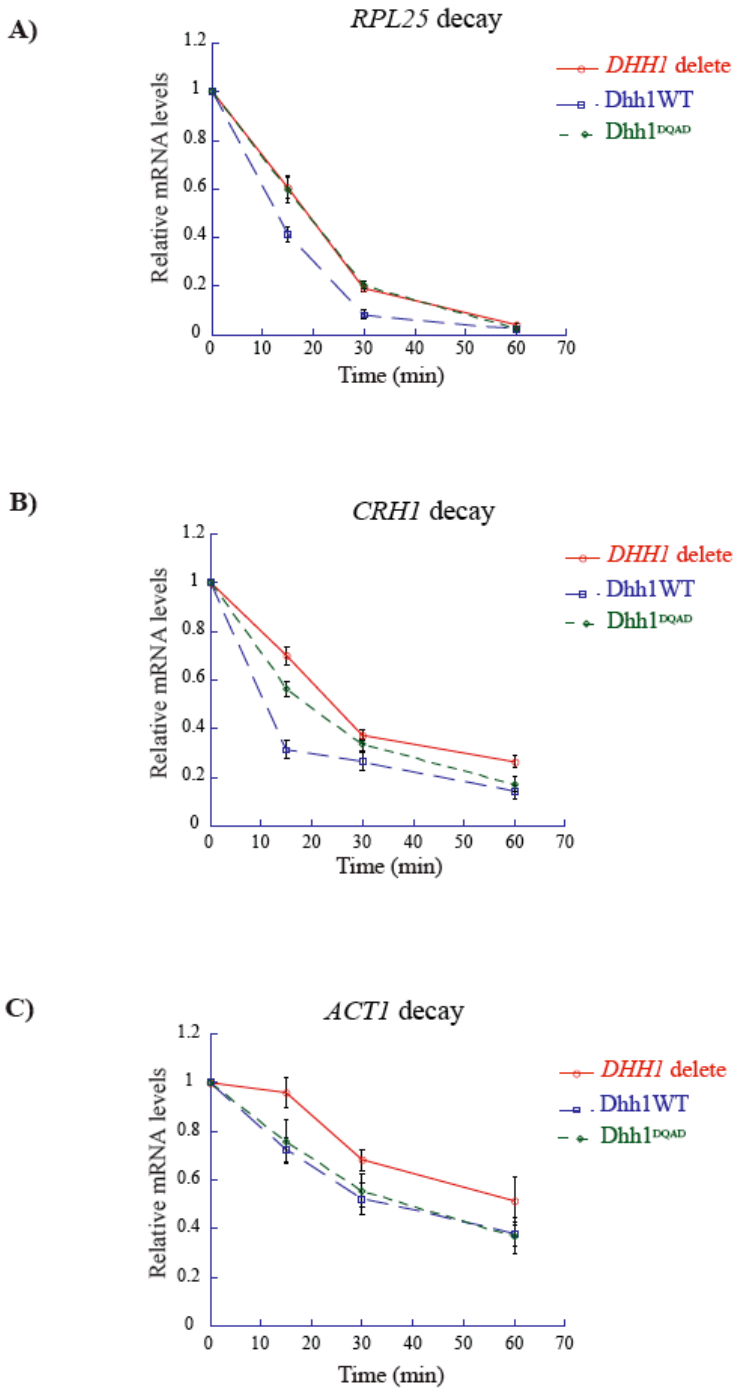
Supplementary Figure 2.1:
Dhh1, Dhh1^{DQAD} and GFP fusion proteins are expressed to similar levels. **(A)** GFP-His-PP7CP and Dhh1-His-PP7CP fusion protein expression in the wildtype strain used for the tethering assay (KWY1571). **(B)** Dhh1-His-PP7CP and Dhh1^{DQAD}-His-PP7CP fusion proteins in *dhh1*Δ yeast used for tethering assay (KWY2532). **(C)** Dhh1-GFP and Dhh1 ^{DQAD}-GFP fusion proteins in *dhh1*Δ yeast used for microscopy. For each Western blot, protein extracts were prepared from mid-log phase yeast and blots were probed with an anti-his antibody.

Supplementary Figure 2.2



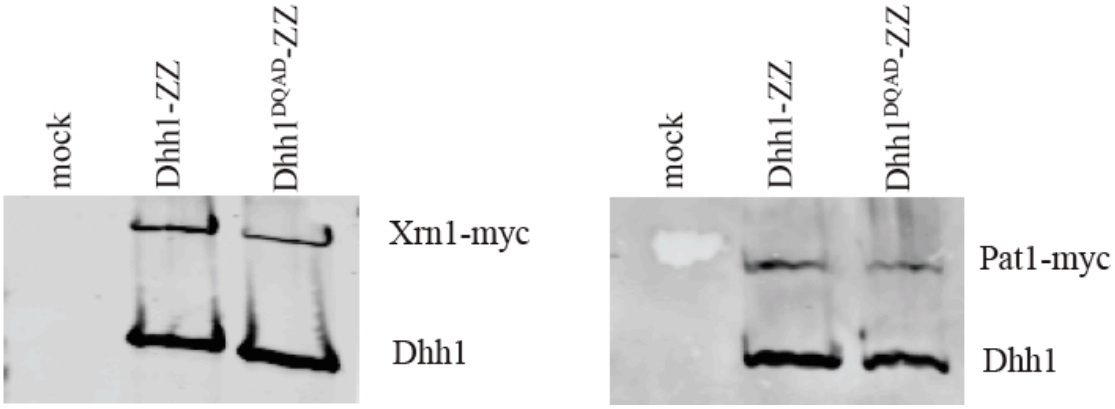
Supplementary Figure 2.2
: Dhh1-PP7CP induced reduction of *FBA1* mRNA and protein levels is specific to tethered Dhh1. (A) Dhh1-GFP or Dhh1-PP7CP proteins were expressed in strains with either *FBA1* tagged with a PP7 binding loop (right two lanes) or *FBA1* with no loop (left two lanes). *FBA1* RNA levels were analyzed by Northern blot and normalized to *RPL37* mRNA. (B) Western analysis of Fba1 protein levels in tethered and non-tethered strains compared to the loading control Pab1. Graphs depict mean protein levels +/- standard deviations from four independent experiments

Supplementary Figure 2.3



Supplementary Figure 2.3:
Dhh1^{DQAD} has differential affects on non-tethered mRNAs. *RPL25* (A), *CRH1* (B), and *ACT1* mRNA levels were measured after transcriptional shutoff upon thiolutin addition. mRNA decay was observed in *DHH1* delete cells, and *DHH1* delete cells expressing wild-type Dhh1 or Dhh1 ^{DQAD}. Graphs depict depict mean mRNA levels +/- standard error from three independent experiments

Supplementary Figure 2.4



Supplementary Figure 2.4:
Equal amounts of mRNA decay factors copurify with wild-type and ATPase mutant Dhh1. Xrn1 (left panel) or Pat1 (right panel) proteins were tagged with a 13x-myc tag in *dhh1*Δ cells. Empty, Dhh1-zz tag or Dhh1^{DQAD}-zz tag proteins were expressed cells and purified from cell extract.

Table S2.1:
Plasmids used in this study

Plasmids	Description	Source
pKW 2304	pRS316 PDhh1-PP7CP	This study
pKW 2306	pRS316 PDhh1-GFP	This study
pKW 2312	pRS316 PDhh1-Dhh1-GFP	This study
pKW 2313	pRS316 PDhh1-Dhh1^{DQAD}-GFP	This study
pKW 2321	pRS316 PDhh1-Dhh1-6xhis-PP7CP	This study
pKW 2322	pRS316 PDhh1-Dhh1^{DQAD}-6xHis-PP7CP	This study
pKW 2420	pRS316 PDhh1-GFP-6xHis-PP7CP	This study
pKW 2421	pRS316 PDhh1-Dhh1-CBP-TEV-ZZ	This study
pKW 2422	pRS316 PDhh1-Dhh1^{DQAD}-CBP-TEV-ZZ	This study
pKW 1008	pFA6a-Flag-KAN	This study
pKW 1606	pFA6a-Flag-PP7-KAN	This study
pKW 2090	pFA6a-yEGFP-HIS5MX	(Sheff and Thorn, 2004)
pKW 1809	pFA6a-mCherry-KAN	(Westfall et al., 2008)
pKW 1061	pFA6a-13myc-KAN	(Bahler et al., 1998)

Table S2.2:
Yeast strains used in this study

Yeast Stains	Genotype	Source
W303	*MAT*a/*a ade2-1 ura3-1 his3-11,15 trp1-1 leu203, 112 can1-100*	
KWY 1570	*W303*a *Fba1-flag::KANMX*	This study
KWY 1571	*W303*a *Fba1-flag-PP7::KANMX*	This study
KWY 2580	*W303*a/*a Rlp25-flag-PP7::KANMX*	This study
KWY 2548	*W303*a *ccr4::KANMX, Fba1-flag-PP7::NAT*	This study
KWY 2257	*W303*a *xrn1::KANMX, Fba1-flag-PP7::NAT*	This study
KWY 2269	*W303*a *dcp1::KANMX, Fba1-flag-PP7::NAT*	This study
KWY 2526	*W303a dhh1::NAT, Fba1-flag-PP7::KANMX, Dcp2-GFP::HIS*	This study
KWY 2530	*W303a dhh1::NAT, Fba1-flag::KANMX, Dcp2-GFP::HIS*	This study
KWY 2459	*W303*a *xrn1::NAT, Fba1-flag::KANMX, Dcp2-GFP::HIS*	This study
KWY 2461	*W303*a *xrn1::NAT, Fba1-flag-PP7::KANMX, Dcp2-GFP::HIS*	This study
KWY 293	*W303*a *dhh1::KANMX*	(Fischer and Weis, 2002)
KWY 2532	*W303a dhh1::NAT, Fba1-flag-PP7::KANMX*	This study
KWY 2193	*W303a dhh1::KANMX, Dcp2-RFP::NAT*	This study
KWY 2907	*W303a dhh1::NAT, Xrn1-13myc::KAN*	This study
KWY 2908	*W303a dhh1::NAT, Pat1-13myc::KAN*	This study

Table S2.3:
Oligonucleotides used in this study

Oligo	Sequence	Description
UC 1949	5'-TGTTCATTATTCATCATGGACCAAGCCG ATAAAATGTTATCTCGT-3'	Quickchange for Dhh1^{DQAD}
UC 1950	5'-ACGAGATAACATTTTATCGGCTTGGTCC ATGATGAATAATGAACA-3'	Quickchange for Dhh1^{DQAD}
UC 587	5'-CGCGCCCTATTTATCGTCATCGTCTT TATAGTCGTTAAT-3'	Antisense to Flag tag for northern probe
UC 1347	5'-TGTAAGATCTGGTCTTAGCAGCTGGAT AACCACAGGAGGAACAGGTCTTCTTTT GAACATGGAAAGAACG-3'	Antisense to Rpl37A for northern probe
UC 3501	5'-TACCTTGAGGAATGTGAGCGGCTTGAA CAGCGTCAGCGGT-3'	Antisense to Adh1 for northern probe
UC 3187	5'-CCAGACAGAGAGACGGATTCCTCACGC CTCCTGCCAACG-3'	Antisense to Scr1 for northern probe
UC 3919	TATGCTTCCAAGGCTGTTCC	Sense to RPL25 for qPCR
UC 3920	GCGGTTTCAGAAGTGATTGG	Antisense to RPL25 for qPCR
UC 3761	TTGCTTCCTCATCAGTCACC	Sense to CRH1 for qPCR
UC 3762	GGAAGCCACTGTTTTCTTGG	Antisense to CRH1 for qPCR
UC 3788	CCTTTGGGCAAGGGATAGTT	Sense to SCR1 for qPCR
UC 3789	CTGCCCAGGACAAATTTACG	Antisense to SCR1 for qPCR
UC 3315	5'-AGACCGGTGTCATCGTTGGTGAAGA TGTCCACAACTTATTCACTTACGCTCC CTATAGTGAGTCGTATTAAATT-3'	Sense to Fba1 with T7 promoter for in vitro transcription reaction.
UC 3316	5'-TCCAGCTTACGGTATCCCAGTTGTC TTACACTCTGACCACTGTGCCAAGAC CCTATAGTGAGTCGTATTAAATT-3'	Sense to Fba1 with T7 promoter for in vitro transcription reaction.
UC 3317	5'-GTCTTCCACGGTGGTTCCGGTTCTA CTGTCCAAGAATTCCACACTGGTATC CCTATAGTGAGTCGTATTAAATT-3'	Sense to Fba1 with T7 promoter for in vitro transcription reaction.
UC 3318	5'-TCCCCGGGTTAATTAACGACTATAA AGACGATGACGATAAATAGGGCGCG CCCTATAGTGAGTCGTATTAAATT-3'	Sense to flag tag with T7 promoter for in vitro transcription reaction.

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Chapter 3

The function and regulation of multiple mRNA decapping activators in the post-transcriptional regulation of gene expression

Background

Regulating the translational status of an mRNA, either though mRNA stability or storage, is a critical way for the cell to control gene expression. For example, it has been estimated that up to fifty percent of the changes in gene expression seen in response to cellular signals occur on the level of mRNA stability (Cheadle et al., 2005; Fan et al., 2002). By stimulating mRNA decay, the cell is able to rapidly change its transcriptome and subsequently to quickly respond to both intra and extracellular signals. There are many potential points of regulation in the mRNA decay pathway, but one key step is removal of the 5' cap structure as the presence of the cap is essential for translation and its removal activates decay (Coller and Parker, 2004). The decapping machinery and the translation initiation machinery are believed to compete to determine the fate of an mRNA(Franks and Lykke-Andersen, 2008). RNA decay is therefore triggered by a change in mRNP composition whereby translation factors dissociate from an mRNP and decay factors bind.

Decapping is carried out by the Dcp1/Dcp2 holoenzyme, but the activity of this complex is enhanced by a number of other factors including Dhh1, Pat1, the Lsm1-7 complex and the Enhancers of Decapping 1-3 (Edc1-3) proteins (Bonnerot et al., 2000; Coller et al., 2001; Dunckley et al., 2001; Fischer and Weis, 2002; Kshirsagar and Parker, 2004; Schwartz et al., 2003; Tharun et al., 2000; Tharun and Parker, 2001). In the Chapter 2, we characterized Dhh1 as a critical regulator of mRNA translation and decay. Like Dhh1, the protein Pat1 has been implicated in post-transcriptional processes, which influence gene expression. Pat1 is an evolutionarily conserved protein which can act both an mRNA decay factor and a translational repressor suggesting that it's involved in the mRNP rearrangement required to move an mRNA from translation to decay (Coller and Parker, 2005; Haas et al., 2010; Marnef and Standart, 2010). Deleting *PAT1* slows the decay of mRNA reporters (Bonnerot et al., 2000; Bouveret et al., 2000). *PAT1* deletion strains accumulate deadenylated, capped mRNAs suggesting that Pat1 works at the level of mRNA decapping (Bouveret et al., 2000). Biochemical evidence also supports a role of Pat1 at the step of decapping. For example, Pat1 interacts with Edc3, Dcp1 and the Lsm1-7 complex (Bonnerot et al., 2000; Bouveret et al., 2000; Pilkington and Parker, 2008; Tharun et al., 2000). Pat1 also associates preferentially with deadenylated mRNAs and interacts with the Dcp1/2 complex in an RNA-dependent manner (Chowdhury et al., 2007; Tharun and Parker, 2001).

Interestingly, co-deletion of Dhh1 and Pat1 cause a more severe block in mRNA decapping than either of the single deletions suggesting that these proteins could function independently to promote mRNA decay (Coller and Parker, 2005). Co-deletion of Dhh1 and Pat1 also causes mRNAs to become trapped in polysomes upon glucose deprivation suggesting that the two proteins are required for translational repression (Coller and Parker, 2005). In support of this hypothesis, overexpression of either Pat1 or Dhh1 leads to global translational repression (Coller and Parker, 2005). Together these data suggest that Dhh1 and Pat1 play critical roles in post-transcriptional gene expression by promoting translational repression and activating mRNA decapping.

In addition to Dhh1 and Pat1, a number of other factors, which accelerate decapping have been identified. These include the enhancers of decapping, Edc1, Edc2, Edc3, and Hedls/Ge-1/Edc4 (Cougot et al., 2004; Dunckley et al., 2001; Fenger-Gron et al., 2005; Kshirsagar and Parker, 2004; Schwartz et al., 2003). These proteins are not

structurally related, but were instead identified by genetic screens to identify proteins which affect the decapping enzyme (Dunckley et al., 2001; Kshirsagar and Parker, 2004). Edc3 is conserved across eukaryotes, whereas Edc1 and Edc2 are specific to yeast and Hedls/Ge-1/Edc4 is found only in metazoans. Edc1, Edc2 and Hedls have all been shown to stimulate decapping by Dcp2 *in vitro* suggesting that they specifically stimulate the cap-binding and hydrolysis step (Fenger-Gron et al., 2005; Schwartz et al., 2003; Steiger et al., 2003). Although Edc3 has not been shown to stimulate the activity of the decapping enzyme *in vitro*, deletion of Edc3 inhibits mRNA decapping *in vivo* suggesting that it also plays a role in the decapping step of mRNA decay (Badis et al., 2004; Kshirsagar and Parker, 2004). Interestingly, Edc3 has been shown to specifically targeting a small number of mRNAs for decapping-mediated decay. For example, the mRNA coding for the ribosomal protein Rps28b and the nuclear export factor Yra1 are both specifically targeted for decay by Edc3 (Badis et al., 2004; Dong et al., 2007). Edc1 has also been implicated in regulating the expression of genes involved in glycolosis and gluconeogenesis suggesting that this protein also acts with some specificity (Schwartz et al., 2003). These data suggest that the enhancers of decapping could provide some specificity to the decay process by binding to target mRNAs and recruiting the decay machinery.

To further investigate the interplay of Dhh1, Pat1 and the enhancers of decapping proteins in the post-transcriptional regulation of gene expression, I took advantage of the tethering assay developed and described in chapter 2. I found that tethering Pat1 and Dhh1, but not the exonuclease Xrn1, to an endogenous yeast mRNA resulted in a significant reduction in steady state mRNA and protein levels. When tethered to an mRNA, Dhh1's ability to reduce mRNA and protein levels is not dependent on either Pat1, Edc1 or Edc3. Upon further investigation into the physiological roles of the enhancer of decapping proteins, we found that the Edc proteins all localize to P-bodies in stationary phase cells. Interestingly, Dhh1 and Edc1 seem to play a critical role in stationary phase, as *dhh1*Δ *edc1*Δ cells do not survive prolonged growth in stationary phase. We also continued to investigate the role of ATP-hydrolysis on Dhh1's function in the cell. Surprisingly, we found that when both a wild-type and ATPase-mutant copy of Dhh1 are present in the cell, the ATPase-mutant no longer localizes to constitutive P-bodies and it cannot reduce mRNA and protein levels when tethered to an mRNA. Together these data shed new light on the control of Dhh1, Pat1 and the enhancers of decapping in regulating gene expression.

Results

Tethering Pat1, but not Xrn1, to an mRNA results in a decrease in both mRNA and protein levels.

In addition to Dhh1, Pat1 has also been implicated in repressing mRNA translation (Coller and Parker, 2005). However, like for Dhh1, at the onset of this study it was unclear how Pat1 is carrying out this activity. Therefore, to dissect the role of Pat1 in mRNA decay and translational repression, we examined the effect of directly tethering Pat1 to an endogenous mRNA *in vivo* using the same tethering assay described in chapter 2. Tethering Pat1-PP7CP to *FBA1* mRNA results in a 60% decrease in steady state *FBA1* mRNA levels compared to the control in which no protein is tethered to the mRNA

(Figure 3.1a compare lanes 2 and 6). This decrease was similar, albeit slightly less, than the 70% decrease in *FBA1* mRNA levels which occurs upon tethering Dhh1-PP7CP (Figure 3.1a compare lanes 2, 4, 6). The reduction in *FBA1* mRNA by both Pat1-PP7CP and Dhh1-PP7CP was dependent on the presence of a PP7 binding loop in the 3' UTR of *FBA1* (Figure 3.1a lanes 1-6). Thus, tethering of both Dhh1 and Pat1 leads to a reduction in steady state mRNA levels.

We also compared Fba1 protein levels in cells expressing Pat1-PP7CP and Dhh1-PP7CP to a non-tethered control (Figure 3.1b). We found that tethering Pat1 to *FBA1* mRNA resulted in approximately a 40% reduction in Fba1 protein levels (Figure 3.1b compare lanes 1 and 5). Tethering Dhh1 also resulted in a decrease (approximately 60%) in steady state protein levels (Figure 3.1b, compare lanes 1 and 3). This reduction was again dependent on the presence of a PP7 binding loop in the 3' UTR of *FBA1* (Figure 3.1 lanes 1-5). Together these results show that Pat1, like Dhh1, lowers steady state *FBA1* mRNA and protein levels when tethered to the mRNA. However, compared to Dhh1 Pat1 displayed a slightly less robust effect on tethered mRNA and protein levels.

Previously, we showed that Dhh1 requires an active 5' to 3' decay pathway in order to stimulate mRNA decay (Figure 2.2). I therefore wanted to address whether tethering the exonuclease Xrn1, the most downstream component of this pathway, was sufficient to activate mRNA decay. Unlike Dhh1 or Pat1, Xrn1 had no effect on steady state mRNA or protein levels (Figure 3.1 a and b). Thus, the mere association of a decay protein with an mRNA is not sufficient to repress translation and activate decay. The reduction in steady state mRNA and protein levels I see upon tethering Pat1 and Dhh1 to an mRNA is therefore specific to these proteins.

Pat1 is not essential for Dhh1's ability to reduce mRNA and protein levels.

Given that tethering both Dhh1 and Pat1 results in a decrease in mRNA and protein levels, I next wanted to see if Dhh1's ability to repress translation and promote decay was dependent on Pat1. To test if Pat1 is required for the Dhh1 induced reduction in RNA and protein levels, I carried out the tethering assay *pat1*Δ cells. *FBA1* mRNA and protein levels were decreased in *pat1*Δ yeast approximately 40% upon Dhh1 tethering (Figure 3.2 a and b). Interestingly, this reduction in mRNA and protein levels is less than the 75% reduction seen when Dhh1 is tethered to *FBA1* in the presence of Pat1 (Figure 2.1). These data indicate that Pat1 is not required for Dhh1's ability to reduce RNA and protein levels, although it could enhance the activity of Dhh1.

The enhancer of decapping proteins Edc1 and Edc3, are not required for Dhh1's ability to activate mRNA decay in *S. cerevisiae*.

Pat1 and Dhh1 have both been shown to influence mRNA decapping (Tharun et al., 2000), and we have previously shown that Dhh1's ability to activate mRNA decay is dependent on the activity of Dcp1 (Figure 2.2). In yeast three additional enhancers of decapping have been identified; Edc1, Edc2, and Edc3, which all physically interact with Dhh1 (cite). To determine if any of these proteins are required for Dhh1's ability to activate mRNA decay, we tethered Dhh1 to *FBA1* mRNA in *edc1*Δ and *edc3*Δ cells. Dhh1 was still able to reduce steady state mRNA levels by 75-80% in the absence of Edc1 and Edc3 (Figure 3.3 a and b). These data demonstrate that Dhh1's ability to

activate mRNA decay is not dependent on Edc1 or Edc3 when directly tethered to an mRNA suggesting that Edc1 and Edc3 function upstream of Dhh1 in the decay pathway.

The enhancer of decapping proteins form P-bodies in stationary phase yeast.

Although we found that Edc1 and Edc3 are not required for Dhh1's ability to activate mRNA decay when tethered to an mRNA in actively growing yeast cells, it is possible their activity is specialized for specific mRNAs or cellular conditions. In fact, it has been shown that Edc3 targets specific transcripts for degradation (Badis et al., 2004; Dong et al., 2007), and Edc1 has been implicated in a cell's adaptive response to carbon source shifts (Schwartz et al., 2003). To begin to address whether the Edc proteins act in specific growth conditions, we first monitored the localization of Edc1, Edc2 and Edc3-GFP proteins in yeast cells subjected to various cellular stresses and compared their localization to that of the P-body marker Dcp2-RFP. Interestingly, although Dcp2 localized to P-bodies in response to both osmotic stress and 5mM DTT, the Edc proteins remained diffusely localized in the cytoplasm upon treatment with these stresses (data not shown). However, all three Edc proteins were found localized to P-bodies in stationary phase yeast, and these foci colocalized with Dcp2-RFP (Figure 3.4a). These data suggest that the enhancer of decapping proteins may play a role in regulating mRNA decay in stationary phase yeast.

Dhh1 and Edc1 are required for stationary phase survival.

Because I found that all three Edc proteins, as well as Dhh1, localize to P-bodies in stationary phase yeast, I next wanted to test whether any of these proteins are required for the cell's ability to survive in stationary phase. To test this I deleted *edc1, edc2, edc3* and/or *dhh1* and tested the ability of these strains to survive prolonged incubation liquid media (Figure 3.4b). In liquid culture, *S. cerevisiae* initially undergo a logarithmic phase of growth in which they generate energy by fermentation (Herman, 2002). Typically, after less than one day, glucose becomes limiting, growth slows significantly, and yeast undergo a diauxic shift and begin to generate energy by respiration. Cells which have undergone the diauxic shift are often referred to as being in stationary phase; however, "true" stationary phase does not occur until four to seven days post diauxic shift when cells run out of all nutrients and stop growing completely.

To test the ability of Edc and Dhh1 mutants to survive in stationary phase, I first diluted cells into rich media containing 2% dextrose and incubated these cultures at 30°C. After 2, 7 and 18 days, I removed a sample from the liquid culture, plated the cells YP-dextrose plates, and observed cell growth on the plates. As previously reported, *dhh1*Δ cells grew somewhat slower than wild-type (Figure 2.5b and Figure 3.4b). However, neither the single *edc1*Δ, *edc2*Δ, *edc3*Δ, nor the double *edc1*Δ*/edc2*Δ, *edc2*Δ*/edc3*Δ strains exhibited a growth defect after 48 hours of growth (Figure 3.4b). The *edc1*Δ*/dhh1*Δ yeast on the other hand grew significantly slower than either *edc1*Δ or *dhh1*Δ single deletion suggesting that these proteins could work in parallel pathways (Figure 3.4b). Interestingly the *edc1*Δ*/dhh1*Δ yeast were dead after prolonged growth at 30° whereas all the other deletion strains exhibited similar survival to the wild-type yeast (Figure 3.4b). These data suggest that Dhh1 and Edc1 are required for stationary phase survival.

A wild-type copy of Dhh1 rescues the abnormal localization of a Dhh1 ATPase mutant.

Previously, I showed that abolishing the ATPase activity of Dhh1 results in constitutive P-body formation (Figure 2.6). Furthermore, the ATPase activity of Dhh1 was found to be required for recycling of the protein into and out of P-bodies suggesting that the ATP hydrolysis activity of Dhh1 is important for normal P-body dynamics. To further investigate the role of ATP hydrolysis by Dhh1 in P-body dynamics, I looked at the localization of a GFP-tagged Dhh1 ATPase mutant (Dhh1$^{\mathbf{DQAD}}$-GFP) in the presence of a wild-type copy of Dhh1 (Figure 3.5). Interestingly, I found that both wild-type Dhh1-GFP and Dhh1$^{\mathbf{DQAD}}$-GFP assumed a diffuse cytoplasmic localization in actively growing cells when a wild-type copy of Dhh1 was present (Figure 3.5, left panel). This was in marked contrast to the mutant protein's localization in *dhh1*Δ cells (Figure 2.6). When cells were stressed by switching their carbon source from glucose to glycerol, both Dhh1-GFP and Dhh1$^{\mathbf{DQAD}}$-GFP relocalized to P-bodies (Figure 3.5, right panel). Together these results argue that the presence of wild-type Dhh1, which is able to hydrolyze ATP, is sufficient to rescue the abnormal P-body localization of Dhh1 $^{\mathbf{DQAD}}$.

Tethering Dhh1$^{\mathbf{DQAD}}$ in the presence wild-type Dhh1 results in an impaired ability to reduce mRNA and protein levels.

I next wanted to test the affect the presence wild-type Dhh1 has on the ability of Dhh1^{DQAD} to reduce mRNA and protein levels when tethered to an mRNA. I had previously shown that in *dhh1*Δ cells, ATP hydrolysis is not required for Dhh1's ability to reduce mRNA and protein levels of a tethered RNA (Figure 2.5). To examine the effects of the Dhh1^{DQAD} variant on mRNA and protein levels a PP7-tagged copy of the DQAD mutant protein (Dhh1^{DQAD}-PP7CP) was expressed in a wild-type strain in which *FBA1* was tagged with a PP7 binding loop. Surprisingly, I found that in the presence of a wild-type copy of Dhh1, Dhh1^{DQAD}-PP7CP was no longer able to reduce *FBA1* mRNA and protein levels to the same extent as Dhh1-PP7CP (Figure 3.6 compare lanes 3 and 4). This result differs significantly from our previous finding in *dhh1*Δ cells and suggests that presence of a wild-type copy of Dhh1 inhibits the ability of Dhh1^{DQAD} to reduce mRNA and protein levels of a tethered RNA.

Tethering Dhh1$^{\mathbf{DQAD}}$ to *FBA1* mRNA inhibits mRNA decay

Because tethering Dhh1 to an mRNA leads to a decrease in steady state mRNA levels, whereas tethering Dhh1^{DQAD} in the presence of wild-type Dhh1 has little effect on steady state mRNA levels, we next tested if there is a difference in the half-life of these tethered mRNAs. To address this question, I examined the turnover rates in wild-type cells of both *FBA1* and *RPL25* mRNA tethered to Dhh1-PP7CP or Dhh1^{DQAD}-PP7CP after transcriptional inhibition with 1,10 phenantroline (Figure 3.7). Because tethering Dhh1 to an mRNA significantly reduces steady state mRNA levels, I was unable to accurately determine the half-life of an mRNA tethered to Dhh1. Interestingly however, I found that tethering Dhh1^{DQAD}-PP7CP to either *FBA1* or *RPL25* resulted in the stabilization of these mRNAs when compared to the non-tethered control (Dhh1-GFP) and GFP-PP7CP (Figure 3.7a and b). In fact, this pool of non-degraded mRNA can, in the case of *FBA1*, persist in the cell five hours after transcriptional shut-off. Expression

of Dhh1^{DQAD}-PP7CP did not increase the half-life of a non-tethered RNA, *RPL37* (Figure 3.7c), suggesting that the stabilizing effect of Dhh1^{DQAD} is due to specifically tethering the protein to an mRNA, not to global changes in RNA decay.

Discussion

Regulating the interface between translation and decay is critical for cells to accurately control gene expression. In chapter 2, I characterized Dhh1 as a critical regulator of this transition. Here, I have shown that Pat1 also functions at the boundary of translation and decay. I demonstrated that tethering Pat1 to *FBA1* mRNA is sufficient to reduce steady state mRNA and protein levels (Figure 3.1). It should be noted that during the course of this study, Haas *et al.* also found that tethering Pat1 to a luciferase reporter mRNA in *D. melanogaster* S2 cells results mRNA decay (Haas et al., 2010). Interestingly, these researchers found that deleting the portion of Pat1 that interacts with Dhh1 reduced Pat1's activity in the tethering assay. This result suggests that Dhh1 may enhance the activity of Pat1. We found that the converse experiment had a similar outcome; deleting Pat1 reduced Dhh1's ability to lower steady state mRNA levels (Figure 3.2). It has previously been suggested that Dhh1 and Pat1 function independently, as co-deletion of Pat1 and Dhh1 in yeast cells causes a more severe block in mRNA decay than either single deletion (Coller and Parker, 2005). However, our results, as well as those of Hass *et al.*, suggest that these proteins may function in concert. This is also consistent with the biochemical interaction that can be observed between Pat1 and Dhh1 (Coller et al., 2001; Fischer and Weis, 2002).

We found that Dhh1 was still able to reduce mRNA and protein levels in the absence of either Edc3 or Edc1 (Figure 3.3). Interestingly, Tritschler *et al.*, showed that mutating the domain of Dhh1 responsible for the interaction of Dhh1, Edc3 and the protein Tra1, abolished Dhh1's ability to repress translation of a luciferase reporter mRNA in *D. melanogaster* S2 cells (Tritschler et al., 2009). We found that deleting the *S. cerevisiae* homolog of Tra1, Scd6, had no effect on Dhh1's ability to reduce mRNA and protein levels (C. Mugler, pers. communication). It will be therefore interesting to test the ability of Dhh1 to reduce mRNA and protein levels in an *edc3*Δ/*scd6*Δ background to see if the interaction between these three proteins is critical for Dhh1's ability to activate decay of a tethered RNA. By determining if Dhh1 requires Edc3 and Scd6 in both yeast and drosophila, we will be able to determine whether Dhh1 has retained the same co-factor requirements throughout evolution.

Although we found that neither Edc1 nor Edc3 alone is required for Dhh1's ability to activate mRNA decay when tethered to an mRNA in actively growing yeast cells, one intriguing possibility is that the activity of the different enhancer of decapping proteins are specialized for specific environmental conditions. Interestingly, I found that Edc1, Edc2 and Edc3 localize to P-bodies in quiescent yeast (Figure 3.4). To our knowledge, this is the first report of Edc1 and Edc2 localizing to P-bodies in response to environmental conditions. Although Edc3 has previously been shown to localize to P-bodies following glucose deprivation (Kshirsagar and Parker, 2004), its localization in stationary phase yeast has not yet been described.

The importance of mRNA turnover in stationary phase quiescence is not well understood; however, it has been shown that nutrient limitation causes changes in mRNA abundance (Albig and Decker, 2001; Hardwick et al., 1999). Additionally, inhibition of

the TOR pathway, a signaling pathway which senses nutrient availability and is turned off when nutrients are limited, causes accelerated decay of specific mRNAs (Albig and Decker, 2001). We found that *edc1*Δ/*dhh1*Δ cells had an extremely slow growth rate, and that these cells were not able to survive stationary phase (Figure 3.4). Deletion of Dhh1 is known to slow mRNA decay (Supplementary figure 2.4 and (Fischer and Weis, 2002), therefore it is possible that mRNA stabilization in *edc1*Δ/*dhh1*Δ cells prevents entry into stationary phase. Future experiments to examine mRNA decay in these cells will help to address this question. In this context, it is interesting that two Dhh1 interacting proteins have recently been shown to play an essential for ensuring the proper initiation of the quiescence by binding Dhh1 and preventing the degradation of newly expressed mRNAs (Talarek et al., 2010). Additional evidence for a role of Dhh1 in nutrient sensing and stationary phase comes from *S. pombe* where it was shown that deletion of the Dhh1 ortholog, Ste13, causes fission yeast to rapidly lose viability after nutrient deprivation (Maekawa et al., 1994). Edc1 has also been implicated in nutrient sensing, as *edc1*Δ cells cannot grow on glycerol and several mRNAs display abnormal expression patterns following carbon source shifts (Schwartz et al., 2003). Additionally, Edc1 was identified as a high copy suppressor of a PAS kinase mutant (Rutter et al., 2002). The PAS kinase is a global regulator of protein synthesis and sugar flux in yeast (Rutter et al., 2002). Although I found that *edc1*Δ/*dhh1*Δ cells cannot survive prolonged incubation, further experiments are needed to show that this is due specifically to a defect in stationary phase survival. Testing the cells' ability to survive treatment with rapamycin, an inhibitor of the TOR pathway, could be one interesting way to further explore the role of these proteins in stationary phase.

While the exact role of ATP hydrolysis in Dhh1's function in the cell remains elusive, I have shown here that it plays an important role in P-body localization and dynamics. I previously showed that the Dhh1^{DQAD} ATPase mutant aberrantly localizes to P-bodies in *dhh1*Δ cells (Figure 2.6). However, this abnormal localization is reversed when a wild-type copy of Dhh1 is present (Figure 3.4). The reason for this change in localization is unclear, however it is possible that the wild-type copy of Dhh1 is able to release the Dhh1^{DQAD} mutant from its locked state in P-bodies. FRAP studies looking at the dynamics of Dhh1^{DQAD} in P-bodies in the presence of a wild-type copy of Dhh1 will provide important insight to this question.

The ability of wild-type Dhh1 to rescue Dhh1^{DQAD} P-bodies could also be due to changing the flux of global mRNA decay. This idea is not unprecedented; it is known that large P-bodies form when components of the mRNA decay machinery are compromised (Teixeira and Parker, 2007). If this is the case, having some wild-type Dhh1 around may be sufficient to shuttle all the mRNA into the decay pathway and therefore prevent the accumulation in P-bodies. My finding that the Dhh1^{DQAD} mutant only partially rescues the mRNA decay defect seen with some mRNAs in *dhh1*Δ cells supports the idea that Dhh1^{DQAD} may not function as efficiently in mRNA decay as wild-type Dhh1 (Supplementary Figure 2.4). Additionally, I found that Dhh1^{DQAD} has an impaired ability to reduce mRNA and protein levels when tethered to an mRNA in the presence wild-type Dhh1, and that these tethered RNAs exhibit slower mRNA decay (Figures 3.5 and 3.6). Surprisingly, I found that in *dhh1*Δ cells both wild-type Dhh1 and Dhh1^{DQAD} are able to reduce mRNA levels to the same extent when tethered to an mRNA (Figure 2.5). This result is perhaps due to the fact that tethering increases the local

concentration of the decay proteins to the RNA and in a sensitized *dhh1*Δ background, this is sufficient to activate mRNA decay. Global analysis of mRNA decay in *dhh1*Δ cells expressing Dhh1^{DQAD} will be important to determine if the ATPase activity of Dhh1 is indeed important for the decay of all non-tethered RNAs and to further dissect the puzzling effects of the wild-type Dhh1 protein on the Dhh1^{DQAD} variant.

In summary, I have shown that Dhh1 and Pat1 are critical regulators of the post-transcriptional fate of mRNAs in yeast. I have found that in the context of the tethering assay, Dhh1 can activate mRNA decay independent of Pat1, Edc1 and Edc3. Interestingly, I found that many of the decay proteins localize to P-bodies in quiescent yeast, and that *dhh1*Δ/*edc1*Δ cells have trouble surviving stationary phase suggesting that these proteins could play a role in regulating mRNA storage or decay in these cells. Together these data, which characterize the role of Dhh1 in regulating mRNA turnover and translational repression in both actively growing and quiescent yeast, provide important insights into the post-transcriptional control of gene expression in various environmental conditions.

Methods

Construction of yeast strains and plasmids

Construction of plasmids for this study (Table 3.1) was performed using standard molecular cloning techniques. To generate the plasmid used to tag genes with PP7CP, the PP7CP was cloned into a yeast integration plasmid downstream of a 6xhis tag. Yeast strains (Table 3.2) were constructed using PCR-based transformation approach with specific primers and integration plasmids (Longtine et al., 1998). Additionally, some yeast mutants were constructed by transformation with genomic regions PCR amplified from the corresponding yeast mutant strains or by mating and subsequent dissection of the tetrads.

PP7 Tethering Assay

Assay was performed as described in Chapter 2. mRNA and protein analysis was performed by northern and western blot as described in Chapter 2 with one exception. *FBA1* mRNA levels in *edc1*Δ cells were analyzed by quantitative PCR instead of northern blot. Quantitative PCR was performed as described in Chapter 2.

mRNA decay measurements

Cell cultures were grown to log phase. Time points were collected following addition of 0.1 mg/ml 1, 10 phenantroline (Sigma). RNA isolation was performed as previously described (Chapter 2). mRNA levels were assessed via northern blot. *FBA1* mRNA levels were normalized against levels of the RNA subunit of the signal recognition particle, *SCR1*.

Growth tests and fluorescent microscopy of stationary phase yeast

Cell cultures were diluted to OD_{600} 0.1 and grown at 30°C in rich media containing 2% dextrose. For growth experiments after 2, 7 and 18 days, a sample was removed from the liquid culture and plated on YP-dextrose plates. Plates were observed for colony growth after 3-4 days. For P-body localization experiments, cells were observed after 48 hours using a Nikon Eclipse E600 fluorescence microscope using a

100X oil-immersion objective. Images were captured using an Orca II CCD (C4742-98-24R, Hamamatsu Photonics) controlled by Metamorph 4.6R6 (Universal Imaging Corporation).

Figure 3.1
Tethering Dhh1 and Pat1, but not Xrn1 to an endogenous mRNA results in a decrease in mRNA and protein levels. (A) Northern analysis of *FBA1* mRNA with or without a PP7-binding loop. mRNA levels are shown in cells expressing no PP7 coat protein (no tether control), Dhh1-PP7CP, Pat1-PP7CP or Xrn1-PP7CP fusion proteins. *FBA1*-PP7 mRNA levels were quantified with ImageQuant analysis software and normalized to an endogenous mRNA, *RPL37*. **(B)** Western analysis of Fba1 protein levels in these strains. *FBA1* protein levels were measured using the Odyssey imaging system and compared to Pab1, a non-tethered control. In (A) and (B), mean values +/- standard deviations from three independent experiments are shown.

Figure 3.1

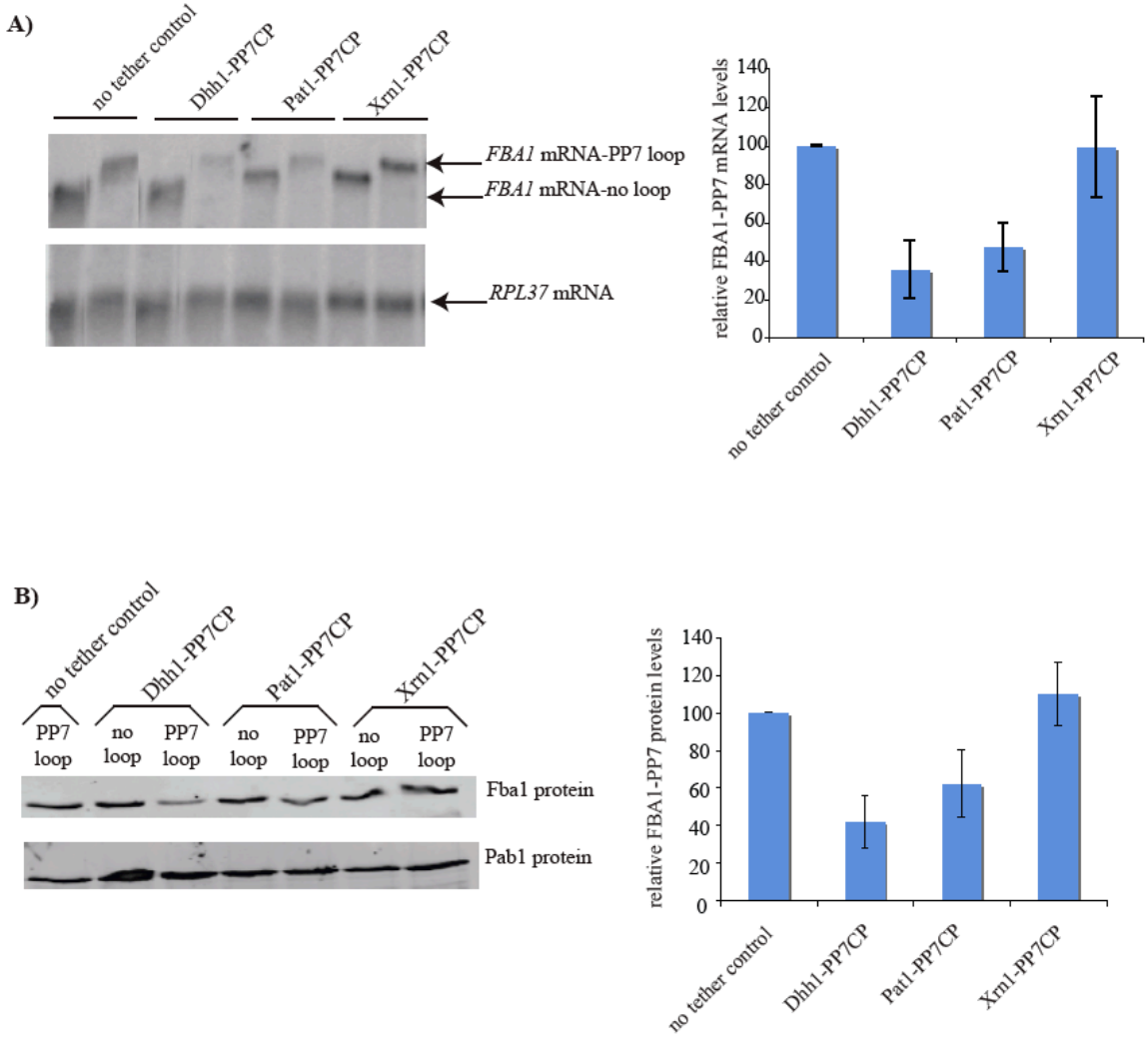

A)
no tether control
Dhh1-PP7CP
Pat1-PP7CP
Xrn1-PP7CP
FBA1 mRNA-PP7 loop
FBA1 mRNA-no loop
RPL37 mRNA
relative FBA1-PP7 mRNA levels
140
120
100
80
60
40
20
0
no tether control
Dhh1-PP7CP
Pat1-PP7CP
Xrn1-PP7CP
B)
no tether control
Dhh1-PP7CP
Pat1-PP7CP
Xrn1-PP7CP
PP7 loop
no loop
PP7 loop
no loop
PP7 loop
no loop
PP7 loop
Fba1 protein
Pab1 protein
relative FBA1-PP7 protein levels
140
120
100
80
60
40
20
0
no tether control
Dhh1-PP7CP
Pat1-PP7CP
Xrn1-PP7CP

Figure 3.2
Dhh1 can reduce mRNA and protein levels in the absence of Pat1. (A) *FBA1* mRNA levels when GFP-PP7CP or Dhh1-PP7CP is tethered in *pat1*Δ strains. Tethering assay was performed and mRNA levels analyzed as described in Figure 3.1 **(B)** Effects of tethered Dhh1-PP7CP on Fba1 protein levels in *pat1*Δ strains. Assay was performed and protein levels were analyzed as described in Figure 3.1.

Figure 3.2

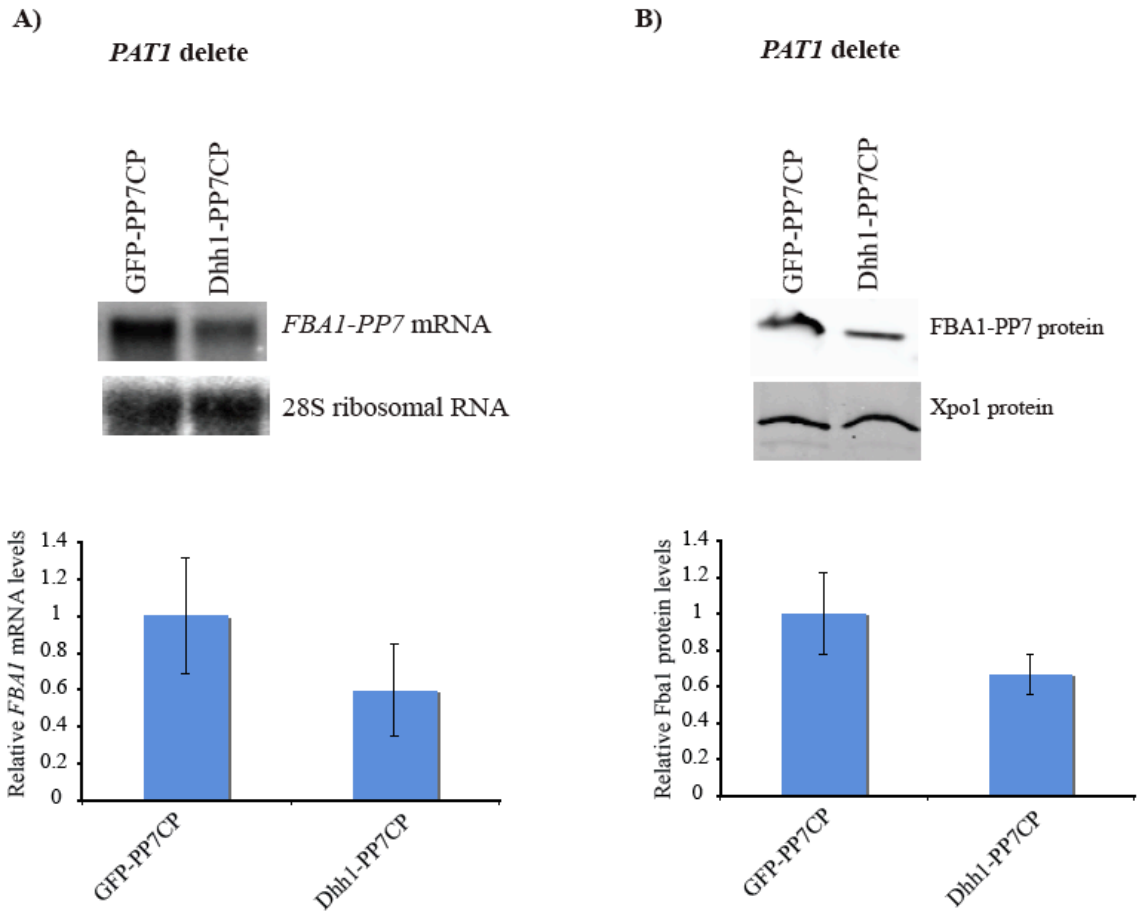
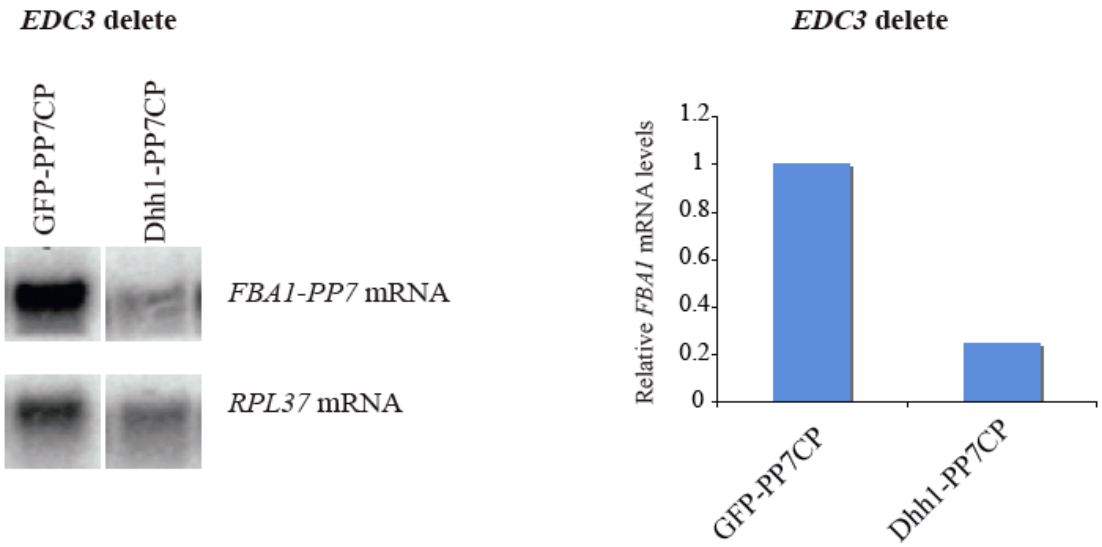

A)
PAT1 delete
GFP-PP7CP
Dhh1-PP7CP
FBA1-PP7 mRNA
28S ribosomal RNA
Relative FBA1 mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GFP-PP7CP
Dhh1-PP7CP
B)
PAT1 delete
GFP-PP7CP
Dhh1-PP7CP
FBA1-PP7 protein
Xpo1 protein
Relative Fba1 protein levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GFP-PP7CP
Dhh1-PP7CP

Figure 3.3
Dhh1's ability to reduce steady state mRNA levels is not dependent on Edc1 or Edc3. (A) *FBA1* mRNA levels when GFP-PP7CP or Dhh1-PP7CP is tethered in *edc3*Δ strains. Tethering assay was performed and mRNA levels analyzed as described in Figure 3.1 **(B)** Effects of tethered GFP-PP7CP or Dhh1-PP7CP on *FBA1* mRNA levels in *edc1*Δ strains. mRNA levels were determined by quantitative PCR.

Figure 3.3

A)


EDC3 delete
GFP-PP7CP
Dhh1-PP7CP
FBA1-PP7 mRNA
RPL37 mRNA
EDC3 delete
Relative FBA1 mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
GFP-PP7CP
Dhh1-PP7CP

B)

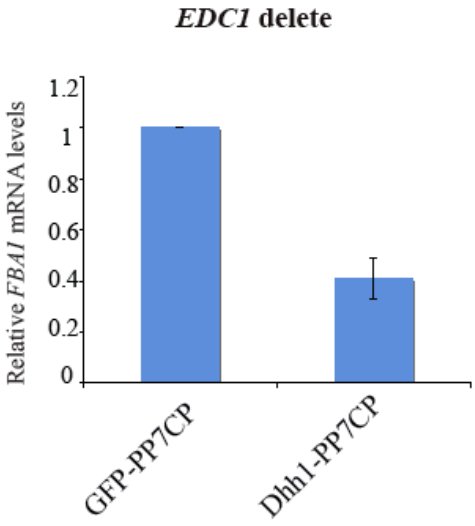
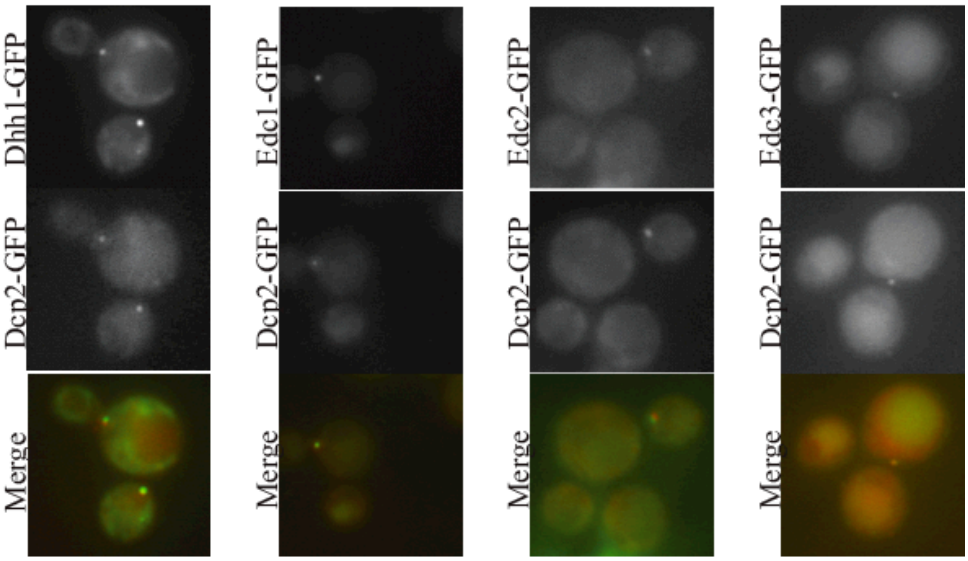

EDC1 delete
Relative FBA1 mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
GFP-PP7CP
Dhh1-PP7CP

Figure 3.4
Dhh1 and the enhancer of decapping proteins localize to P-bodies in stationary phase yeast. (A) Dhh1, Edc1, Edc2 or Edc3 proteins were tagged with GFP (top panel) and colocalized with Dcp2-RFP (lower two panels). Cells were grown to stationary phase (48 hours) and P-bodies were visualized by fluorescence microscopy. **(B)** *edc1, edc2, edc3* and/or *dhh1*Δ strains were growth in YP-dextrose media at 30°. After 2, 7 or 18 days of growth, cells were diluted to OD_{600} 0.1 and plated on YP-dextrose plates. Plates were visualized after 3-4 days.

Figure 3.4

A)


Dhh1-GFP
Dcp2-GFP
Merge
Edc1-GFP
Dcp2-GFP
Merge
Edc2-GFP
Dcp2-GFP
Merge
Edc3-GFP
Dcp2-GFP
Merge

B)

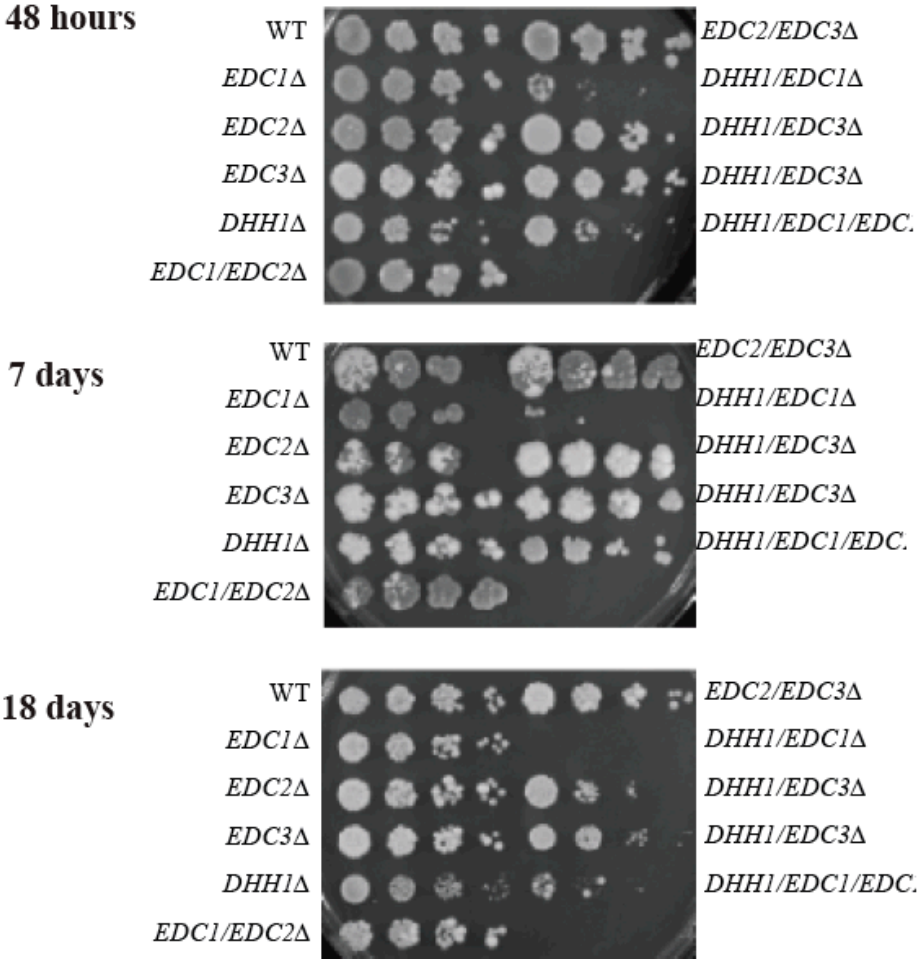

48 hours
WT
EDC1Δ
EDC2Δ
EDC3Δ
DHH1Δ
EDC1/EDC2Δ
EDC2/EDC3Δ
DHH1/EDC1Δ
DHH1/EDC3Δ
DHH1/EDC3Δ
DHH1/EDC1/EDC.
7 days
WT
EDC1Δ
EDC2Δ
EDC3Δ
DHH1Δ
EDC1/EDC2Δ
EDC2/EDC3Δ
DHH1/EDC1Δ
DHH1/EDC3Δ
DHH1/EDC3Δ
DHH1/EDC1/EDC.
18 days
WT
EDC1Δ
EDC2Δ
EDC3Δ
DHH1Δ
EDC1/EDC2Δ
EDC2/EDC3Δ
DHH1/EDC1Δ
DHH1/EDC3Δ
DHH1/EDC3Δ
DHH1/EDC1/EDC.

Figure 3.5

The ATPase function of Dhh1 is not required for P-body localization when a wild-type copy of Dhh1 is present. Dhh1-GFP (top panel) or Dhh1DQADGFP (bottom panel) fusion proteins were co-expressed with Dcp2-RFP in a wild-type strain. Proteins were localized by fluorescent microscopy in yeast actively growing with glucose (left panel) or after a carbon source shift from glucose to glycerol (right panel).

*Photos courtesy of Sarah Munchel

Figure 3.5

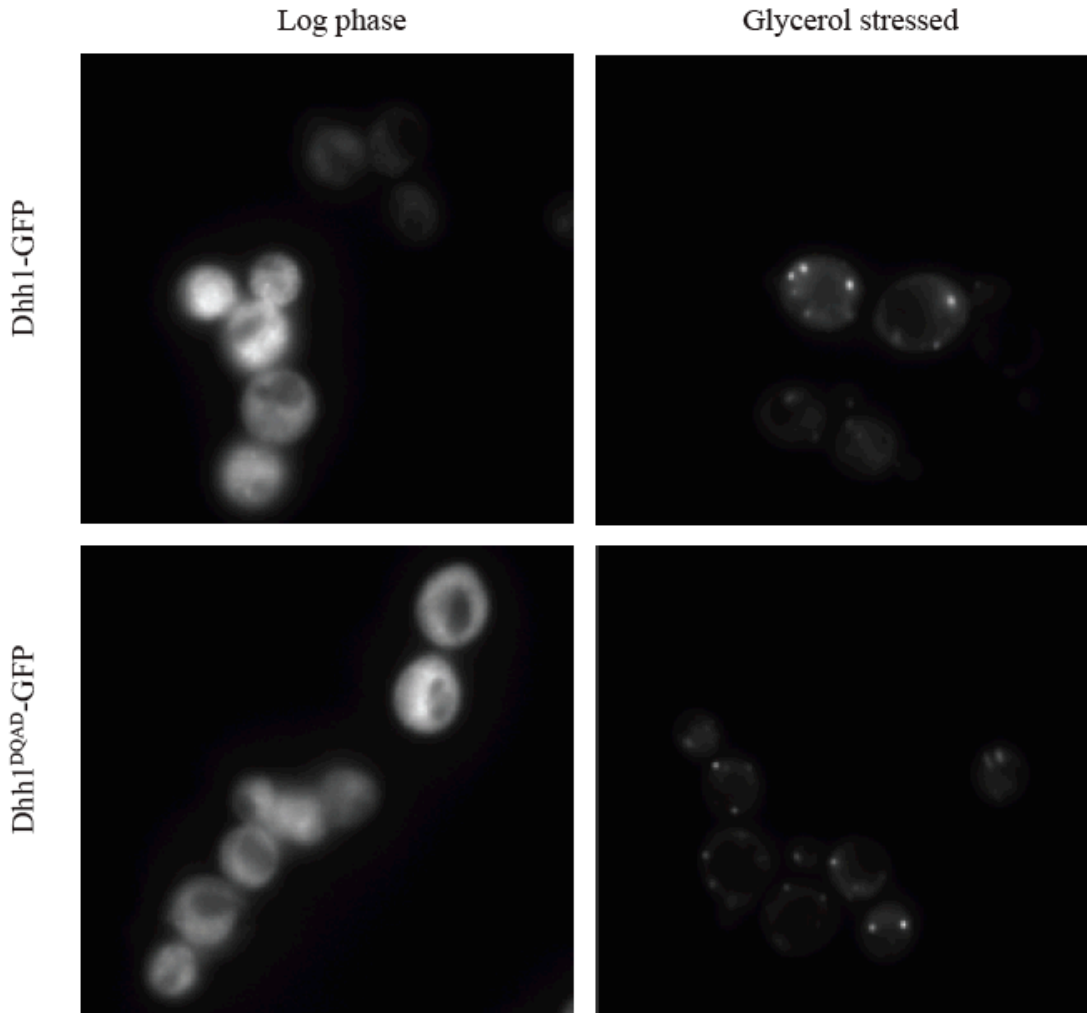


Figure 3.6
Tethering an ATPase-dead Dhh1 mutant results in an impaired ability to reduce mRNA and protein levels when a wild-type copy of Dhh1 is present. (A) Northern analysis of *FBA1* mRNA. Dhh1^{DQAD}-GFP, GFP-PP7CP, Dhh1-PP7CP or Dhh1^{DQAD}-PP7CP fusion proteins were expressed in wild-type strains engineered with one PP7 binding loop in the 3' UTR of *FBA1*. *FBA1* mRNA levels were normalized against *RPL37* mRNA. **(B)** Western analysis of *FBA1* protein levels. *FBA1* protein levels were compared to Xpo1 protein. Mean values +/- standard deviations from three independent experiments are shown.

Figure 3.6

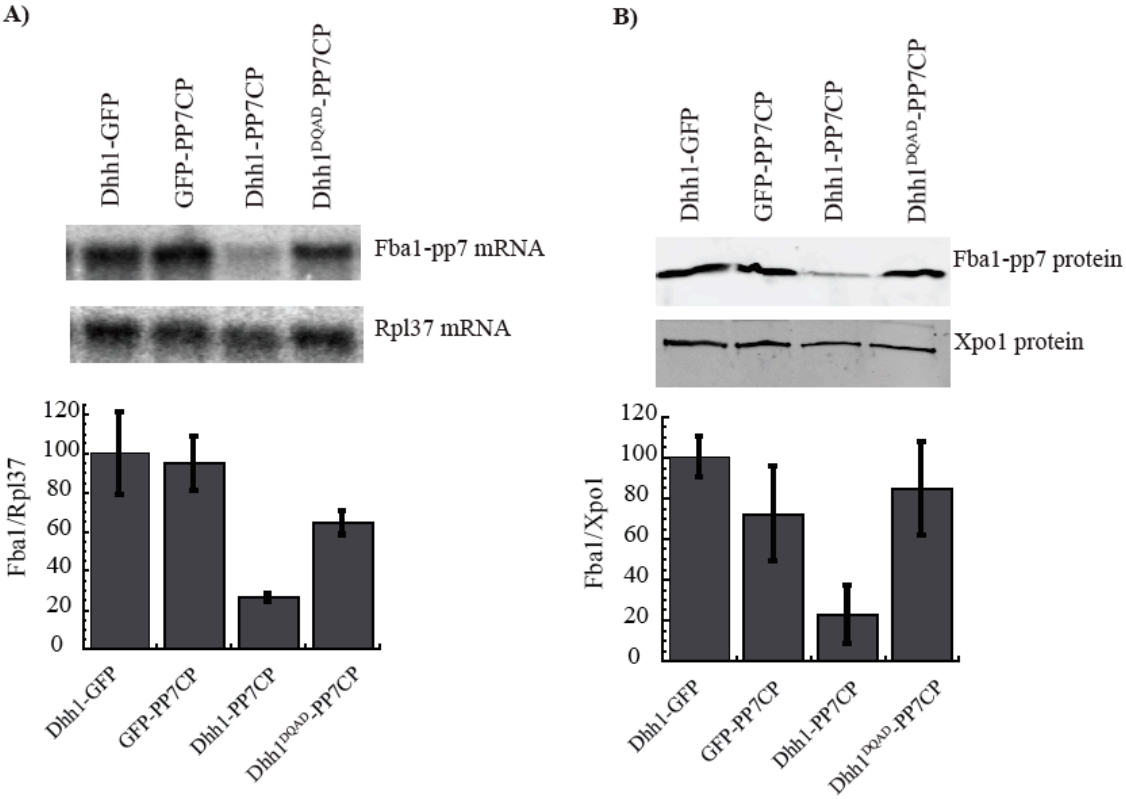
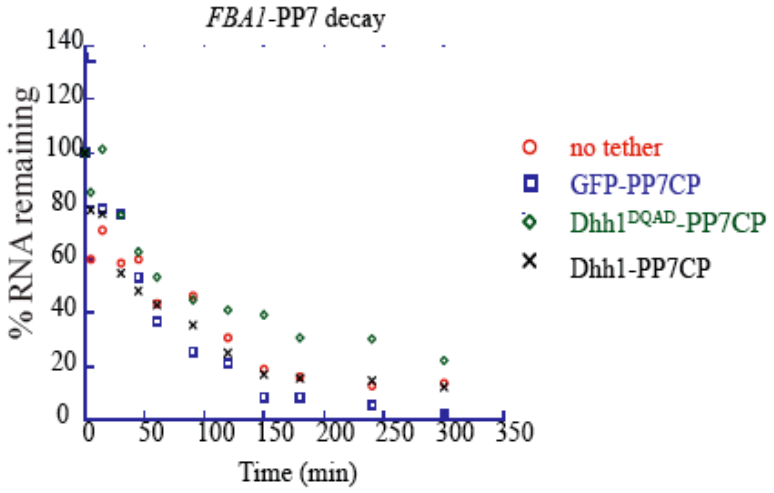

A)
Dhh1-GFP
GFP-PP7CP
Dhh1-PP7CP
Dhh1DQAD-PP7CP
Fba1-pp7 mRNA
Rpl37 mRNA
Fba1/Rpl37
120
100
80
60
40
20
0
Dhh1-GFP
GFP-PP7CP
Dhh1-PP7CP
Dhh1DQAD-PP7CP
B)
Dhh1-GFP
GFP-PP7CP
Dhh1-PP7CP
Dhh1DQAD-PP7CP
Fba1-pp7 protein
Xpo1 protein
Fba1/Xpo1
120
100
80
60
40
20
0
Dhh1-GFP
GFP-PP7CP
Dhh1-PP7CP
Dhh1DQAD-PP7CP

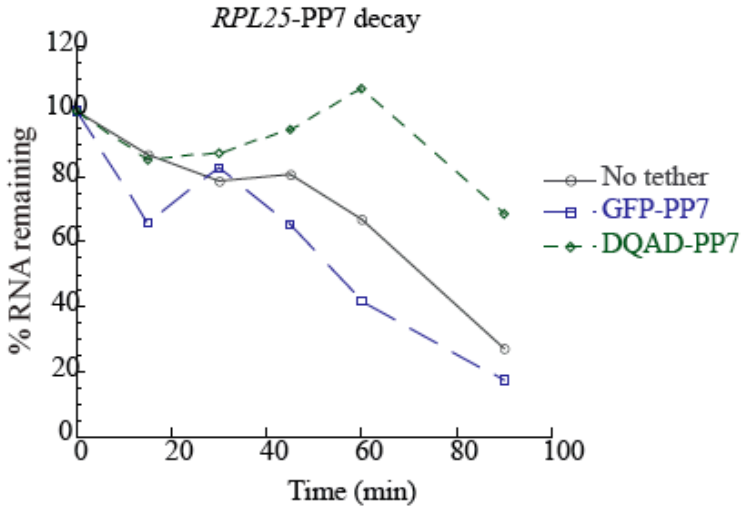
Figure 3.7
Tethering Dhh1^{DQAD} in wild-type cells stabilizes both FBA1 and RPL25 mRNA.
Dhh1-GFP (no tether), GFP-PP7CP, Dhh1-PP7CP or Dhh1^{DQAD}-PP7CP fusion proteins were expressed in wild-type strains engineered with one PP7 binding loop in the 3' UTR of *FBA1* or *RPL25*. *FBA1*-PP7 **(A)**, *RPL25*-PP7 **(B)**, or *RPL37* **(C)** mRNA levels were measured after transcriptional shutoff upon 1,10 phenantroline addition.

Figure 3.7

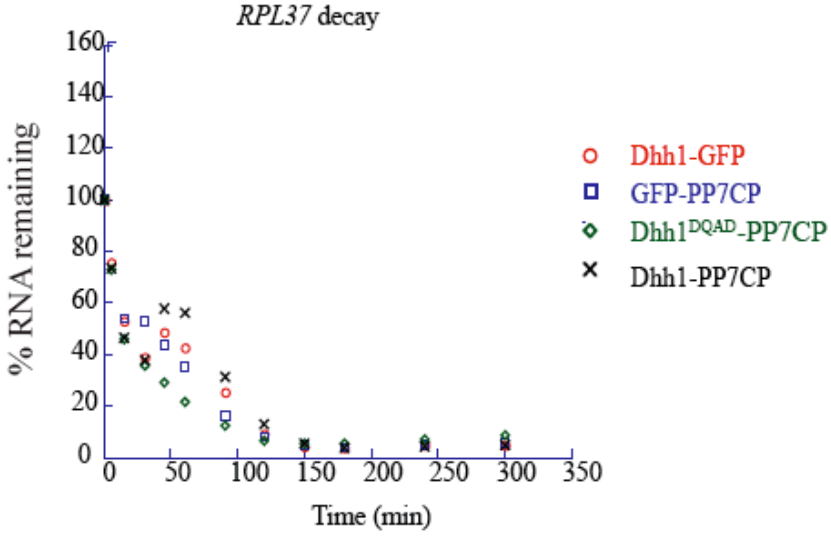
A)



B)



C)



Supplementary Tables

Table S3.1:
Yeast strains used in this study

Yeast Stains	Genotype	Source
W303	*MAT*a/*a ade2-1 ura3-1 his3-11,15 trp1-1 leu203, 112 can1-100*	
KWY 1570	*W303*a *Fba1-flag::KANMX*	(Chapter 2)
KWY 1571	*W303*a *Fba1-flag-PP7::KANMX*	(Chapter 2)
KWY 1956	*W303*a/*a Fba1-flag::KANMX, Dhh1-PP7CP-his::HIS*	This study
KWY 1958	*W303*a/*a Fba1-flag-PP7::KANMX, Dhh1-PP7CP-his::HIS*	This study
KWY 2092	*W303*a/*a Fba1-flag::KANMX, Pat1-PP7CP-his::HIS*	This study
KWY 2093	*W303*a/*a Fba1-flag-PP7::KANMX, Pat1-PP7CP-his::HIS*	This study
KWY 2096	*W303*a/*a Fba1-flag::KANMX, Xrn1-PP7CP-his::HIS*	This study
KWY 2097	*W303*a/*a Fba1-flag-PP7::KANMX, Xrn1-PP7CP-his::HIS*	This study
KWY 2261	*W303*a *pat1::NAT, Fba1-flag-PP7::KANMX*	This study
KWY 2546	*W303a edc3::LEU, Fba1-flag-PP7::KANMX*	This study
KWY 1574	*W303*a *Edc1-GFP::HIS, Dcp2-mCherry::KANMX*	This study
KWY 1556	*W303*a *Edc2-GFP::HIS, Dcp2-mCherry::KANMX*	This study
KWY 1557	*W303*a *Edc3-GFP::HIS, Dcp2-mCherry::KANMX*	This study
KWY 1576	*W303*a *Dhh1-GFP::HIS, Dcp2-mCherry::KANMX*	This study
KWY 1358	*W303*a *edc1::HIS*	This study
KWY 1359	*W303*a *edc1::HIS, dhh1::KAN*	This study
KWY 1431	*W303a edc2::LEU*	This study
KWY 1432	*W303a edc1::HIS, edc2::LEU*	This study
KWY 1433	*W303a edc2::LEU, dhh1::KAN*	This study
KWY 1455	*W303a edc3::LEU*	This study
KWY 1457	*W303a edc1::HIS, edc2::LEU, dhh1::KAN*	This study
KWY 1456	*W303a edc1::HIS, edc2::LEU*	This study
KWY 1481	*W303a edc2::LEU, edc3::KAN*	This study

Table S3.2:
Plasmids used in this study

Plasmids	Description	Source
pKW 2304	pRS316 PDhh1-PP7CP	Chapter 2
pKW 2312	pRS316 PDhh1-Dhh1-GFP	Chapter 2
pKW 2313	pRS316 PDhh1-Dhh1^{DQAD}-GFP	Chapter 2
pKW 2321	pRS316 PDhh1-Dhh1-6xhis-PP7CP	Chapter 2
pKW 2322	pRS316 PDhh1-Dhh1^{DQAD}-6xHis-PP7CP	Chapter 2
pKW 2420	pRS316 PDhh1-GFP-6xHis-PP7CP	Chapter 2
pKW 1008	pFA6a-Flag-KANMX	Chapter 2
pKW 1606	pFA6a-Flag-PP7-KANMX	Chapter 2
pKW 2167	pFA6a-PP7CP-6xhis-HIS	This study
pKW 1809	pFA6a-mCherry-KANMX	(Westfall et al., 2008)

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