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No Nonsense: The Protection of Wild-Type mRNAs From Nonsense-Mediated mRNA Decay in *Saccharomyces cerevisiae*

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by

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Gene regulation in eukaryotes is tightly controlled at multiple levels to ensure proper expression and cellular homeostasis. Misregulation of gene expression is a common source of genetic disease. One mechanism by which cells are able to control gene expression is through the synthesis and degradation of the mRNA molecules encoding the genes. The transcription and degradation of mRNA molecules controls the pool mRNAs that are available to the translational machinery. One of the well-studied mRNA decay pathways is the Nonsense-Mediated mRNA Decay pathway (NMD). Originally, NMD was discovered as a posttranscriptional mRNA surveillance mechanism responsible for the deadenylation-independent decapping and rapid 5'→3' degradation of mRNAs that harbor premature termination codons (PTCs). Approximately one-third of all inherited genetic disease and cancers are related to NMD. It is now known that NMD plays a much larger role in the stability and expression of wild-type mRNAs as well. Wild-type mRNAs with NMD-targeting signals, which include 1) a translated uORF, 2) a long 3' UTR, 3) leaky scanning leading to out-of-frame initiation of translation, 3) programmed ribosome frameshift sites, and 5) regulated alternative splicing variants, are rapidly destabilized by NMD. It has also been observed that some wild-type mRNAs contain NMD targeting signals but are not degraded by NMD due to protecting mechanism. Here we show that the *SSY5* mRNA in *Saccharomyces cerevisiae* is a wild-

type mRNA with multiple NMD targeting signals but is not degraded by NMD. None of the current models for NMD protection explain the *SSY5* mRNA stability so the mechanism of protection is likely to be novel. Additionally, we show the *SSY5* mRNA is primarily degraded 5'→3'. We also explore two additional mRNAs, *YAP1* and *GCN4*, in *S. cerevisiae* that also contain at least one NMD-targeting signal but are not degraded by NMD. Elucidating the mechanism of protection from NMD of these three mRNAs will provide valuable insight to the underlying molecular mechanisms of NMD, which despite thorough investigation remain unclear. Understanding the molecular intricacies of the NMD pathway will allow for the efficient development of NMD-related disease therapies with minimal risks and side-effects.

Graphical Abstract

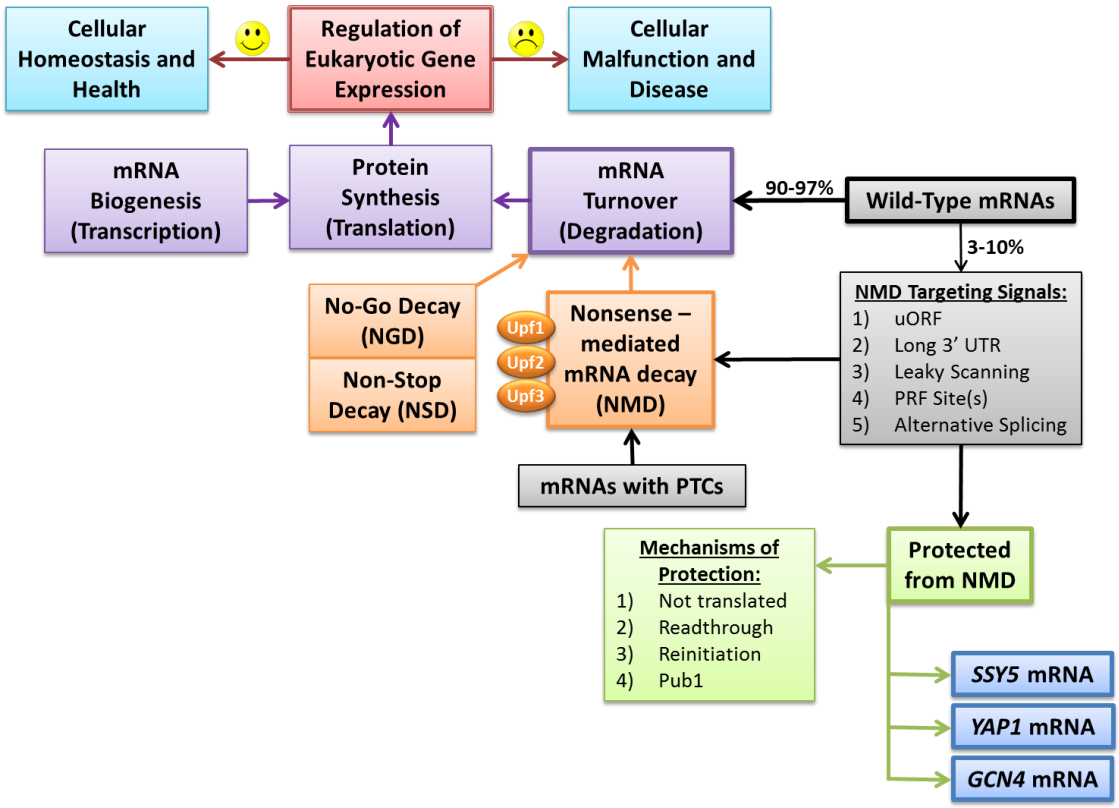

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1:

Introduction

1.0 Overview

Eukaryotic gene expression is an incredibly intricate process and is controlled with high fidelity at many different levels. When something goes awry at any level in the tight control of gene expression the result can be detrimental not only to the cell but to the organism as a whole manifesting in cell death and disease. The most obvious level of control is at the level of protein synthesis from the mRNA transcript (translation). However, a level of control even before that of translation occurs with the biogenesis of the mRNA transcripts (transcription) and also turnover of the mRNA transcripts (degradation). The processes of mRNA transcription and degradation are also tightly controlled. Eukaryotes have evolved several quality control (QC) mechanisms to help maintain the fidelity of gene expression by quickly riding the cell of aberrant mRNAs (Lykke-Andersen and Bennett, 2014). It is now known that these pathways can also be exploited to regulate gene expression of wild-type mRNAs. One of the most extensively studied QC mechanisms is the nonsense-mediated mRNA decay (NMD) pathway. However, despite extensive study and characterization over the past several years the exact molecular mechanisms of NMD are not entirely known.

The NMD pathway was originally characterized for its role in the rapid degradation of mRNAs that contain a premature termination codon (PTC). If these PTC-containing mRNAs were not rapidly removed from the translatable pool they would lead to the build-up of C-terminally truncated proteins, which could result in deleterious consequences (Akimitsu, 2008; Baker and Parker, 2004; Behm-Ansmant et al., 2007; Chang et al., 2007; Frischmeyer and Dietz, 1999; Hentze and Kulozik, 1999; Hilleren and

Parker, 1999; Muhlemann et al., 2008; Schweingruber et al., 2013; Shyu et al., 2008). The NMD pathway plays a vital role in the regulation of gene expression and exists in all eukaryotes that have been examined including yeast, *Drosophila*, *Caenorhabditis elegans*, *Arabidopsis*, and humans (Bedwell et al., 1997; Grimson et al., 2004; Hall and Thein, 1994; He et al., 2003; Hentze and Kulozik, 1999; Kalyna et al., 2012; Kurihara et al., 2009; Maquat and Carmichael, 2001; Maquat and Serin, 2001; Mendell et al., 2004; Pulak and Anderson, 1993; Rayson et al., 2012; Rehwinkel et al., 2005; Sun and Maquat, 2000). Importantly, it is now recognized that the NMD pathway is also responsible for the degradation of a significant portion of wild-type mRNAs (non-PTC-containing) as well. Studies in *Saccharomyces cerevisiae*, *Drosophila*, *C. elegans*, *Arabidopsis*, and humans have revealed that a significant portion of the transcriptome is affected when the NMD pathway is inactivated (Guan et al., 2006; He et al., 2003; Johansson et al., 2007; Kalyna et al., 2012; Lelivelt and Culbertson, 1999; Mendell et al., 2004; Mitrovich and Anderson, 2005; Peccarelli and Kebaara, 2014; Rayson et al., 2012; Rehwinkel et al., 2005).

The NMD pathway requires to coordinated action of the three core *trans*-acting factors, up-frameshift proteins Upf1, Upf2 and Upf3, which have orthologs in all eukaryotes examined (Applequist et al., 1997; Cali et al., 1999; Cui et al., 1995; Denning et al., 2001; Gatfield et al., 2003; He and Jacobson, 1995; Hodgkin et al., 1989; Isken and Maquat, 2008; Jeong et al., 2011; Leeds et al., 1991; Leeds et al., 1992; Lykke-Andersen et al., 2000; Pulak and Anderson, 1993). It has been shown that mutation or deletion in any of the genes coding for these three factors results in the stabilization of NMD-

substrates (Cui et al., 1995; He and Jacobson, 1995; Leeds et al., 1991; He et al., 1997; Lee and Culbertson, 1995; Lelivelt and Culbertson, 1999; Maderazo et al., 2000). Upf1 is the most highly conserved of the three NMD factors and is enriched in binding to NMD substrates (Johansson et al., 2007). This ~109 kDa protein exhibits both RNA-dependent ATPase activity and 5'→3' ATP-dependent RNA helicase activity in addition to RNA binding (Altamura et al., 1992; Bhattacharya et al., 2000; Czaplinski et al., 1995; Weng et al., 1996a, b). Upf1 interacts with the eukaryotic translation release factors eRF1 and eRF3, and upon recognition of an NMD substrate interacts with Upf2 (directly) and Upf3 (indirectly) to form a surveillance complex (Czaplinski et al., 1999; Czaplinski et al., 1998; Ivanov et al., 2008; Kadlec et al., 2006). There is evidence to support the idea that the formation of the surveillance complex occurs through the staged assembly of two subcomplexes—1) Upf1/eRF1/eRF3 and 2) Upf2/Upf3—which come together to form a complete NMD mRNP (Atkin et al., 1997). Additionally, Upf1 has also been shown to interact with mRNA decay factors Dcp1 and Dcp2 as well as Ski7, an exosome-associated protein (Lykke-Andersen, 2002; Takahashi et al., 2003). Upf1 localizes predominantly to the cytoplasm, but can shuttle between the nucleus and the cytoplasm (Lykke-Andersen et al., 2000; Mendell et al., 2002). Upf2 interacts with both Upf1 and Upf3 likely serving as a bridge between the two proteins (He et al., 1997; Lykke-Andersen et al., 2000). The N-terminal domain of Upf2 contains multiple nuclear localization signals but the protein localizes predominantly to the perinuclear region of the cytoplasm (Lykke-Andersen et al., 2000; Mendell et al., 2002; Muhlemann et al., 2008). Upf3 is the smallest and least conserved of the three core NMD factors and

contains multiple nuclear localization and nuclear export signals (Lee and Culbertson, 1995; Lykke-Andersen et al., 2000; Serin et al., 2001; Shirley et al., 1998).

The exact mechanism by which the cellular machinery identifies an NMD-substrate, whether the mRNA contains a PTC or is a wild-type mRNA with an NMD-targeting signal (discussed below), among all mRNAs remains unclear despite thorough investigation. However, it is known that targeting of an mRNA for degradation by NMD begins with the recruitment and assembly of the NMD mRNP, which consists of the three core factors discussed above (Nicholson et al., 2010; Rebbapragada and Lykke-Andersen, 2009). Upf1is the first to interact with the terminating ribosome and release factors eRF1 and eRF3. Upf1 then binds to the Upf2/Upf3 subcomplex, mRNA decay factors are recruited, and mRNA decay is initiated through deadenylation-independent decapping by the Dcp1/Dcp2 decapping complex and 5'→3' mRNA decay by the exonuclease Xrn1 (Coller and Parker, 2004; Decker and Parker, 1993; Hsu and Stevens, 1993; Maderazo et al., 2000; Muhlrad et al., 1994, 1995; Nicholson et al., 2010; Rebbapragada and Lykke-Andersen, 2009). It has been shown that presence of poly(A)-binding protein (Pab1), which is bound to the 3' poly(A) tail of the mRNA, is able to inhibit assembly of the NMD mRNP when in close proximity to the terminating ribosome (Amrani et al., 2004; Behm-Ansmant et al., 2007; Beilharz and Preiss, 2007; Gallie, 1991; Silva et al., 2008; Wilusz et al., 2001). However, the presence of Pab1 is not absolutely required for the recognition of NMD substrates by the cellular machinery (Meaux et al., 2008; Roque et al., 2015). Additionally, some mRNAs with long 3' UTRs—which distances the interaction between the terminating ribosome and Pab1—are not affected by NMD-

mediated mRNA degradation (Kebaara and Atkin, 2009; Nicholson et al., 2010; Obenoskey et al., 2014; Rebbapragada and Lykke-Andersen, 2009, Chapter 2). These observations suggests that other mechanisms exist that are able to antagonize activation of the NMD pathway. Because of the critical role of NMD in the regulation of gene expression and in the development of many human diseases it is critical in moving forward to identify the exact mechanism(s) by which the cellular machinery distinguishes an NMD substrate from the rest of the mRNA pool.

2.0 NMD in Human Health

Due to the vital role of NMD in the fidelity of gene expression it is not surprising that as many as 33% of all genetic disorders and inherited cancers are directly linked to NMD (Culbertson, 1999; Frischmeyer and Dietz, 1999). The majority of these result as a consequence of the cell not being able to produce sufficient levels of full-length protein. A few of these prominent disorders include β-thalassemia, Duchene's and Becker's Muscular Dystrophy, Marfan Syndrome, and Cystic Fibrosis (Bhuvanagiri et al., 2010; Frischmeyer and Dietz, 1999; Khajavi et al., 2006).

β-thalassemia, a disorder of hemoglobin production, is an example that portrays the protective effects of NMD. This disease has multiple genetic and phenotypic variants all related to NMD and PTCs in the *HBB* mRNA which codes for the β-globin protein. A homozygous PTC mutation able to trigger NMD in both β-globin alleles results in a deficiency of tetrameric hemoglobin, which requires both α-globin and β-globin, and leads to severe anemia. However, individuals that are heterozygous for the NMD-

competent PTC in the β-globin gene are usually able to produce sufficient β-globin and remain physically healthy. Both of these cases, in which the PTC elicits NMD, result in a recessive mode of inheritance. In contrast, a dominant negative form of β-thalassemia arises when an individual is heterozygous for an NMD-incompetent PTC (Hall and Thein, 1994; Thein et al., 1990a; Thein et al., 1990b). In this case the PTC does not elicit NMD due to a positional effect of its location in the last exon (exon 3) of the β-globin gene (Nagy and Maquat, 1998; Thermann et al., 1998). One model of NMD in mammalian cells posits that in order to trigger NMD in mammalian cells a PTC must be at least 50-55 nt upstream of the final exon junction that is marked by an exon-junction complex (EJC; Neu-Yilik et al., 2011; Thermann et al., 1998). Because this mutant β-globin gene does not trigger NMD it results in the production of toxic levels C-terminally truncated β-globin which accumulates in precipitation bodies (Neu-Yilik et al., 2011; Peixeiro et al., 2011). These individuals are severely anemic and sometimes require transfusions to survive among other developmental complications (Cao and Galanello, 2010).

Marfan Syndrome is a connective tissue disorder that results from mutations in fibrillin 1 mRNA, and is another example of the protective effects of NMD. Nonsense mutations in the fibrillin 1 mRNA that trigger NMD leading to significantly reduced accumulation of functional protein are associated with a milder disease phenotype. However, when mutant mRNA escapes degradation by NMD and truncated protein is allowed to build up the disease phenotype becomes much more severe (Dietz, 1993; Dietz et al., 1993).

The other side of NMD in human disease is the aggravation of disease severity due to haploinsufficiency because of the degradation of mRNAs that could potentially produce proteins with partial function. Duchenne's muscular dystrophy (DMD) is a severe form of muscular dystrophy that is caused by lack of functional dystrophin protein in skeletal and cardiac muscles. As many as 98% of the mutations in the dystrophin open reading frame result in the introduction of a PTC and subsequent elimination of the mRNA by NMD. However, a less severe form of muscular dystrophy, Becker's muscular dystrophy (BMD), results when mRNAs with PTCs are able to escape NMD and produce partially functionally C-terminally truncated dystrophin protein (Kerr et al., 2001).

NMD also plays a role in a portion of Cystic Fibrosis patients as well. Cystic fibrosis results from mutations in the mRNA that codes for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. Several different mutations in the mRNA can result in the clinical manifestation of Cystic Fibrosis. Different mutations in the mRNA are categorized into classes and affect the CFTR protein at different stages of development. Class I mutations are those that result in a PTC and cause the degradation of the mRNA through the NMD pathway. Thus, the CFTR protein is never able to reach the cell membrane where it is needed. These mutations represent approximately 10% of the mutations that cause Cystic Fibrosis (Bhuvanagiri et al., 2010; Rogan et al., 2011).

2.1 Nonsense Codon Readthrough Therapies

Given the role of NMD in the clinical outcome of a significant portion of detrimental diseases it is of no surprise the NMD pathway is a targeted area of clinical research. As discussed above, NMD-associated disease occurs when 1) mRNAs with PTCs are rapidly removed from the translatable pool of mRNAs so necessary levels of functional protein are never translated, 2) mRNAs with PTCs are able to avoid degradation by NMD but still harbor the PTC resulting in the production of only partially functional protein, or 3) mRNAs containing PTCs escape NMD which leads to the build-up of toxic levels of truncated protein.

Different therapies are being investigated based on two classes of nonsense mutations: 1) those that introduce a PTC as the result of point mutations so the rest of the mRNA can still be translated in the native reading frame, and 2) those that introduce a PTC as the result of a frameshift mutation leaving the remainder of the mRNA to be translated in an alternate reading frame. The end goal, which is subject to overcoming many hurdles, is the synthesis of full-length functional protein from the mutant mRNA without perturbing the translation of any other mRNAs.

Some of the therapies currently being investigated are the use of aminoglycosides, small molecule drugs that promote ribosome readthrough at nonsense codons (e.g. Ataluren), suppressor tRNAs, targeted gene repair, antisense oligonucleotides (AOs), and the upregulation of proteins that could compensate for loss of the functional protein (Kuzmiak and Maquat, 2006; Peltz et al., 2013). The latter two of these approaches are being investigated specifically for the nonsense codons that introduce a PTC as a result of

a frameshift mutation, as these provide the more challenging case for therapy development.

These therapies are promising—some have even made it through late-stage clinical trials—and provide hope for individuals living with NMD-associated debilitating diseases. However, there are still many questions related to the basic underlying mechanism of the NMD pathway that remain unanswered, which makes therapy development both risky and challenging. Elucidating the basic molecular mechanism of NMD and how the cell is able to distinguish NMD substrates from non-NMD substrates will be provide crucial information for the development of nonsense therapies as well as help to understand the potential risks and side effects of new and current therapies.

3.0 Wild-Type mRNA Degradation by NMD

One way by which we can continue to untangle the underlying mechanism of the NMD pathway is by studying the category of wild-type mRNAs that are regulated by NMD. Several wild-type mRNA NMD-targeting signals have already been described. These include: 1) a long 3' UTR (Amrani et al., 2004; Kebaara and Atkin, 2009; Muhlrad and Parker, 1999), 2) translation of an upstream open reading frame (uORF; Amrani et al., 2006; Barbosa et al., 2013; Gaba et al., 2005; Nyiko et al., 2009), 3) a start codon in a suboptimal context which can lead to leaky scanning and out of frame initiation of translation (Welch and Jacobson, 1999), 4) the presence of programmed ribosome frameshift (PRF) sites (Plant et al., 2004) and 5) the presence of pre-mRNA introns and

regulated alternative splicing resulting in PTCs (He et al., 1993; Lewis et al., 2003; McGlincy and Smith, 2008; Ni et al., 2007).

3.1 NMD-targeting through the presence of a translated uORF

Many mRNAs contain one or more short open reading frames in the 5' leader region of the mRNA known as a uORFs (Nagalakshmi et al., 2008). These uORFs can have many functions but they are best known for their role in regulating mRNA translation and mRNA turnover (Arribere and Gilbert, 2013). A genome-wide study conducted in *S. cerevisiae* projected that out of 220 predicted direct NMD substrates 135 (~61%) contain putative uORFs (Guan et al., 2006). The presence of a uORF, when translated, leads to the introduction of a stop codon that is far upstream of the native ORF stop codon and, thus, may be recognized as a PTC targeting the mRNA for degradation by NMD. It has been shown that active translation of the uORF is required to elicit NMD while those mRNAs that contain untranslated uORFs are immune to NMD (Hurt et al., 2013).

At least three mRNAs in *S. cerevisiae* have been verified as wild-type mRNAs that are targeted to NMD by a uORF: the *CPA1*, *FZF1*, and *ALR1* mRNAs (Gaba et al., 2005; Guan et al., 2006; Johansson and Jacobson, 2010). The *FZF1* mRNA, which encodes a transcription factor involved in sulfite metabolism, provides an interesting case in which the uORF begins in the 5' leader and ends in the coding ORF (Breitwieser et al., 1993; Guan et al., 2006). The uORF has two potential start codons at -64 and -58 and the stop codon appears in the ORF at position +29. Interestingly, when each of the two

uORF start codons was mutated (ATG→AGG) individually the *FZF1* mRNA remained a target for NMD. However, when both uORF start codons were mutated simultaneously the *FZF1* mRNA sensitivity to NMD was abolished. This data indicates that the uORF, as long as both uORF start codons are able to be utilized, is an NMD-targeting feature for the *FZF1* mRNA (Guan et al., 2006).

The *CPA1* mRNA encodes the small subunit of carbamoyl phosphate synthetase, which plays a role in the synthesis of citrulline—an arginine precursor (Pierard et al., 1979). The *CPA1* mRNA was originally identified as a wild-type mRNA that is sensitive to NMD (He et al., 2003; Ruiz-Echevarria and Peltz, 2000) and was later shown to be targeted to NMD by presence of the uORF (Gaba et al., 2005). The study by Gaba et al., (2005) showed that mutation of the uORF start codon (AUG→UUG) in a luciferase reporter resulted in a 2-fold increase in steady-state accumulation compared to the reporter with the wild-type uORF AUG codon in NMD+ cells. Additionally, the reporter with the wild-type uORF AUG codon showed a ~7-fold increase in accumulation in an *nmd*- strain compared to the NMD+ strain (Gaba et al., 2005). These results provide evidence that the *CPA1* mRNA is targeted to NMD by presence of the uORF in the 5' leader.

Another important example of the regulation of an mRNA by NMD in response to the presence of a uORF is the case of the *ALR1* mRNA. The *ALR1* mRNA codes for a magnesium (Mg^{2+}) transporter at the plasma membrane and is responsible for magnesium homeostasis in yeast (Graschopf et al., 2001; MacDiarmid and Gardner, 1998). A study by Johansson and Jacobson in 2010 showed that yeast are able to control the fidelity of

translation, in part, by controlling magnesium uptake. The control of magnesium uptake is regulated by the product of the *ALR1* mRNA, which is in turn regulated by NMD. The regulation of the *ALR1* mRNA by NMD was shown to depend on the presence of a uORF in the 5' leader of the mRNA. The *ALR1* mRNA actually contains three uORFs (and one of which has both an A and a B form), but mutational analysis revealed that NMD-sensitivity could be incurred by the 3'-most uORF (uORF3) alone (Johansson and Jacobson, 2010).

The idea of regulation of mRNA susceptibility to NMD by the presence of a uORF is not exclusive to *S. cerevisiae*. Studies in *Saccharomyces pombe, C. elegans*, mammals and plants all show evidence of uORF-containing mRNAs that are upregulated when NMD is inactivated (Calvo et al., 2009; Kochetov et al., 2002; Mendell et al., 2004; Nyiko et al., 2009; Ramani et al., 2009).

3.2 NMD-targeting through the presence of PRF sites

Programmed ribosome frameshift (PRF) sites are *cis*-acting mRNA sequences that cause a ribosome to shift reading frames by one nucleotide either upstream (5' direction) or downstream (3' direction), -1 or +1 PRF site, respectively. A -1 PRF site appears to be the most common phenomenon and is the best characterized of the two. The -1 PRF site consists of a "slippery site," which is a heptameric mRNA sequence of the form X XXY YYZ (in the original reading frame where X is any three identical nucleotides, Y is either a triplet A or triplet U sequence, and Z is an A, U or C), followed downstream by an mRNA secondary structure of two or more stem-loops known as a

pseudoknot (Baranov et al., 2002; Jacks and Varmus, 1985; Namy et al., 2006). The slippery site and the pseudoknot are separated by a region of 5-9 nucleotides known as the "spacer" region (Dinman et al., 1991). The pseudoknot structure causes the ribosome to pause and shift its reading frame on the slippery site (Namy et al., 2006). The binding strength of the nucleotides that compose the pseudoknot is directly correlated with the efficiency at which the structure is able to induce ribosomal frameshifting (Hansen et al., 2007). However, if the strength of the pseudoknot is too strong then it can actually decrease the efficiency of translation downstream (Tholstrup et al., 2012). The +1 PRF site, which is less common, consists of a slippery site and at least one additional stimulatory element such as a pseudoknot (Ivanov et al., 2000).

In recent years it has become increasingly evident that both +1 and -1 PRF sites are being used to control gene expression in a variety of organisms (Dinman, 2012; Namy et al., 2004). It has been well-studied that viruses use -1 PRF sites to synthesize polyproteins from polycistronic mRNAs as a method of genome consolidation (Baril et al., 2003; Brierley and Dos Ramos, 2006; Dulude et al., 2002; Jacks and Varmus, 1985; Leger et al., 2007). Additionally, PRF sites are responsible for the generation of an *Escherichia coli* DNA polymerase III subunit (Blinkowa and Walker, 1990) and release factor 2 (RF2; Baranov et al., 2002), two yeast telomerase-associated proteins (Aigner et al., 2000; Morris and Lundblad, 1997), actin-binding protein Abp140 in yeast (Asakura et al., 1998), and all metazoan ornithine decarboxylase antizymes (Ivanov et al., 2000). A computational study identified the -1 PRF motif at 260 locations in the *S. cerevisiae* genome, which is ~5.2-fold greater frequency than random chance (Hammell et al.,

1999). Further analysis indicated that many of the *S. cerevisiae* -1 PRF sites would result in the ribosome encountering a PTC after resuming translation following the frameshift event (Plant et al., 2004). In a study by Plant et al., in 2004 the yeast *PGK1* mRNA, which is an extensively characterized and very stable wild-type mRNA, was destabilized by the addition of a -1 PRF site. This destabilization was NMD-dependent as the construct regained stability in an *nmd*- strain. Additional confirmation was done to confirm that the destabilization in NMD+ strains was the result of a -1 PRF event (Plant et al., 2004). This same study also showed that there in an inverse correlation between mRNA stability (half-life) and the efficiency of ribosome frameshifting. Further, steady-state mRNA accumulation is drastically reduced when -1 PRF efficiency is high (Plant et al., 2004).

Thus, the frequency of PRF events among all organisms is greater than originally perceived and PRF events could potentially be used as a gene regulatory mechanism (Hammell et al., 1999; Plant et al., 2004). PRF events that cause the ribosome to encounter a PTC can subject the mRNA to rapid degradation by NMD which limits the translational pool of the mRNA.

3.3 NMD-targeting through leaky scanning

The sequence context that surrounds an AUG start codon is largely responsible for the ability of a ribosome to properly initiate translation at a particular AUG. The -6 to +6 sequence surrounding the AUG is important in determining translation initiation efficiency, with the strongest influence at -3 position (Cigan et al., 1988; Yun et al.,

1996). The optimal start codon initiation consensus sequence appears to be (A/U)A(A/C)AA(A/C)**AUG**UC(U/C) (Hamilton et al., 1987). Should the surrounding sequence of the AUG codon be suboptimal the ribosome can bypass the AUG in search of an AUG in a better context. This concept is referred to as "leaky scanning." If the ribosome initiates at a different downstream start codon the initiation may occur in an alternate reading frame, which is referred to as out-of-frame initiation of translation. Leaky scanning resulting in out-of-frame initiation of translation may lead to the introduction of a PTC in the alternate reading frame being translated, which would destabilize the mRNA as a consequence of NMD.

In 1987 Sharp and Li developed a mathematical model, called the codon adaptation index (CAI), which measured the bias of synonymous amino acid codon (different codons that code for the same amino acid) usage which had been shown to have a nonrandom distribution (Sharp and Li, 1987). Later, the CAI equation was adapted to model start codon context resulting in the $A_{UG}CAI$ equation to show that there is a relationship between codon usage bias and translation initiation context (Miyasaka, 1999). Finally, the $A_{UG}CAI$ equation was revised into the $A_{UG}CAI_{(r)}$ equation, which was used to test the leaky scanning model for NMD (Guan et al., 2006). This equation takes into consideration the weighted contribution of each nucleotide at each position (from -6 to +6) and how frequently each nucleotide is used at each position. The result is a value from 0 to 1 that measures the likelihood of translation initiation, and consequently the likelihood of leaky scanning, to occur at a particular AUG with 0 being poor and 1 being efficient (Guan et al., 2006). Further, limited experimental analysis led to the general

conclusion that AUG codons with an $A_{UG}CAI_{(r)}$ score of ≤0.32 might be good candidates for leaky scanning while those with a score ≥0.44 would be poor candidates for leaky scanning (Guan et al., 2006). No examples were shown that have scores in the 0.32-0.44 range, so whether AUG codons with values in that range are subject to leaky scanning remains to be determined.

The *SPT10* mRNA is wild-type mRNA in yeast that exhibits sensitivity to NMD. The *SPT10* mRNA does contain a uORF in the 5' leader region, however, mutational analysis of the uORF start codon (AUG→CCC) revealed that translation of this uORF has little to do with the susceptibility of the mRNA to NMD (Welch and Jacobson, 1999). To determine the *cis*-element responsible for the *SPT10* mRNA susceptibility to NMD chimeric reporters were constructed which contained portions of the *SPT10* mRNA and portions of the well-characterized and stable *PGK1* mRNA. These experiments indicated that the mRNA destabilizing element was located within the 5' UTR and first 94 nucleotides of the ORF of the *SPT10* mRNA. Further analysis revealed that the ORF AUG of the *SPT10* mRNA is in a suboptimal context with an $A_{UG}CAI_{(r)}$ value of 0.32 (Guan et al., 2006). Given this it was confirmed that the ribosome is able to bypass the first AUG and initiate at an AUG in a better context downstream in the +1 reading frame. When the ribosome initiates at the second AUG a PTC is encountered just 15 nucleotides downstream which targets the mRNA for degradation by NMD (Welch and Jacobson, 1999).

3.4 NMD-targeting through the presence of a long 3' UTR

It has also been shown that wild-type mRNAs with long 3' UTRs are substrates for degradation by NMD in *S. cerevisiae*, *C. elegans*, *Drosophila*, plants and mammals. Studies report mRNAs that contain mutations resulting in a long 3' UTR and synthetic mRNAs that terminate at a normal stop codon but contain a long 3' UTR are both substrates for NMD (Amrani et al., 2004; Muhlrad and Parker, 1999).

This has been most extensively studied in *S. cerevisiae* in which the normal 3' UTR length is 50-200 nucleotides with a median length of 121 nucleotides (Graber et al., 1999). A screen of wild-type mRNAs in S. cerevisiae revealed that 56 mRNAs were predicted to have a 3' UTR of longer than 350 nucleotides (an arbitrary cut-off). Of these 56 mRNAs 11 were experimentally tested. Of these 11 mRNAs 10 accumulated to significantly higher levels in a *upf1Δ* strain compared to wild-type. Additionally, half-lives were determined for 5 of the 11 mRNAs and all 5 tested had a significantly longer half-life in the *upf1Δ* strain compared to wild-type (Kebaara and Atkin, 2009). The one anomaly was the *SSY5* mRNA.

The presence of a long 3' UTR has also been identified as a *cis*-acting NMD-targeting element in *Nicotiana benthamiana* and *Arabidopsis* (Kalyna et al., 2012; Kertesz et al., 2006). In plants, increasing the length of the 3' UTR correlates with an increase in NMD efficiency (Kertesz et al., 2006). The same correlation has been observed in murine embryonic stem cells as well (Hurt et al., 2013). Additionally, human mRNAs with long 3' UTRs are also subject to NMD and some of these mRNAs are those that code for crucial NMD factors, which predicts a feedback loop (Longman et al., 2013;

Yepiskoposyan et al., 2011). Further studies show that this regulatory feedback loop is conserved across different organisms (Longman et al., 2013).

The collective data from many of these studies supports a model developed by Amrani *et al.*, in 2004 known as the *faux* 3' UTR model. This model is based on the idea that the cellular machinery recognizes a PTC because the terminating ribosome and associated factors are positioned too far upstream from the poly(A) tail (Amrani et al., 2004; Muhlrad and Parker, 1999). The distance between the termination complex and the poly(A) tail fails to initiate the normal interaction of the ribosome-bound eRF3 with poly(A) tail-bound protein Pab1 (Behm-Ansmant et al., 2007). Because this interaction fails to occur the termination complex is subject to recruitment of the NMD factors and subsequent degradation (Amrani et al., 2004; Brogna and Wen, 2009). Further, artificial tethering of Pab1 in close proximity to a prematurely terminating ribosome is able to abrogate NMD-sensitivity (Amrani et al., 2004). The *faux* 3' UTR model is able to explain the degradation of mRNAs by NMD that contain PTCs and also those mRNAs that are substrates for NMD because of a long 3' UTR.

This regulation of wild-type mRNAs by NMD due to the presence of a long 3' UTR may also be physiologically relevant as revealed by the *PGA1* mRNA. The *PGA1* mRNA codes for an essential component of the GPI-mannosyl-transferase II, which is involved in the synthesis of GPI anchors that are added to proteins for membrane attachment (Sato et al., 2007). The *PGA1* mRNA also has a 3' UTR of ~750nt and is a substrate for NMD (Kebaara and Atkin, 2009). Interestingly, NMD has also been shown to regulate the expression of a set of mRNAs involved the assembly of GPI-anchored cell

wall proteins (Guan et al., 2006). Consistent with this, cells of the *upf1Δ* strains are much more sensitive to Calcofluor White (a fluorescent stain that binds to cellulose and chitin in cells walls and can compromise cell wall integrity at high concentrations; Kebaara and Atkin, 2009).

3.5 NMD-targeting through pre-mRNA introns and alternative splicing

Wild-type pre-mRNAs that escape to the cytoplasm still containing introns as well as regulated alternative splicing are related events that also provide wild-type mRNAs that become NMD substrates. In yeast, the *CYH2, RP51B,* and *MER2* pre-mRNAs are inefficiently spliced and are substrates for the NMD pathway. In wild-type cells a very small accumulation of all three pre-mRNAs is observed while in *upf1Δ* cells the pre-mRNAs accumulate to significantly higher levels and have longer half-lives (He et al., 1993). It was also shown that the three mRNAs associate with ribosomes, which is consistent with 1) these inefficiently spliced mRNAs are escaping to the cytoplasm, and 2) the notion that NMD is a translation-dependent mechanism (He et al., 1993; Peltz et al., 1993). The inefficient splicing of these pre-mRNAs and their subsequent association with ribosomes results in the translating ribosome, which begins at the normal ORF translation initiation site, terminating at a PTC, which triggers NMD.

Similarly, many alternative splicing events result in mRNAs that introduce a PTC in the normal reading frame. Because these alternative splicing events are regulated it is likely that alternative-splicing induced NMD serves an intentional role in gene regulation. Studies of regulated alternative splicing events showed that this is indeed the case in

mammalian cells where NMD is exploited to achieve post-transcriptional regulation by destabilizing certain mRNA isoforms (Cuccurese et al., 2005; Lejeune et al., 2001; Sureau et al., 2001; Weischenfeldt et al., 2012; Wollerton et al., 2004). Notably, the polypyrimidine tract binding protein, PTBP1, and the SC35 protein are regulators of alternative splicing and the proteins themselves are encoded by mRNAs that are subject to alternative-splicing induced NMD in HeLa cells (Sureau et al., 2001; Wollerton et al., 2004). Alternative-splicing induced NMD is also responsible for the regulation ribosomal genes in mammalian cells and in *C. elegans* (Cuccurese et al., 2005; Mitrovich and Anderson, 2000).

Although a very small percentage of *S. cerevisiae* mRNAs contain introns, a recent study by Kawashima and colleagues in 2014 found that of those mRNAs that do contain introns many produce nonproductive alternatively spliced mRNAs that resulted in degradation by NMD (Kawashima et al., 2014). Studies also confirm alternatively spliced mRNA isoforms that are subject to NMD in *Drosophila, Arabidopsis* and zebrafish (Hansen et al., 2009; Jaillon et al., 2008; Kertesz et al., 2006).

4.0 Mechanisms of Protection of Wild-Type mRNAs from NMD

Although it has been widely shown that wild-type mRNAs containing certain NMD-targeting features (and there are likely additional features that remain to be discovered) are substrates for NMD, there are exceptions. Some mRNAs have one or more NMD-targeting element discussed above but show no significant difference in steady-state accumulation or half-life in wild-type versus *upf1Δ* cells (Kebaara and Atkin,

2009; Obenoskey et al., 2014; Ruiz-Echevarria et al., 1998; Vilela et al., 1998) (Chapter 2). A few mechanisms that are able to protect mRNAs with NMD-targeting signals from degradation by NMD have been discovered. Given that these mechanisms are not able to explain the stability of all mRNAs that are protected from NMD (e.g. *SSY5* mRNA) future studies are likely to expand this list. It is important that we identify all of the wild-type mRNA NMD-targeting mechanisms as well as the features that are able to protect an mRNA from degradation by NMD so that we can gain a deeper more comprehensive understanding of the molecular mechanisms of gene regulation by NMD. This information can then be exploited for safer and more efficient therapy development for individuals that are affected by NMD-related diseases.

4.1 Inhibition of translation

Several observations have led to the idea that NMD is a translation-dependent process. First, NMD is perturbed by translation elongation inhibitors such as cycloheximide (Herrick et al., 1990; Zhang et al., 1997; Zuk and Jacobson, 1998). Additionally, NMD is able to resume once the drug is removed (Zhang et al., 1997). Second, mutations that block translation elongation, such as the addition of strong stem-loops (pseudoknots) or mutations in the tRNA nucleotidyltransferase, also inhibit NMD (Peltz et al., 1992; Tholstrup et al., 2012). Third, PTC-containing mRNAs do co-fractionate with polyribosomes in a pattern that reflects the position of the PTC (i.e. early PTCs result in mRNAs in lighter fractions whereas later PTCs result in mRNAs in the heavier fractions; He et al., 1993). Fourth, PTC suppression or stop codon readthrough

promoted by suppressor tRNAs results in the stabilization of nonsense containing mRNAs (Gozalbo and Hohmann, 1990; Keeling et al., 2004). Fifth, eukaryotic release factors eRF1 and eRF3 are essential for NMD and only interact with a terminating ribosome when a stop codon is recognized in the A site during translation (Bertram et al., 2001; Czaplinski et al., 1998; Kisselev et al., 2003; Rospert et al., 2005). Together these observations lead us to the conclusion that if an mRNA is not actively translated then the mRNA is not likely to be degraded by the NMD machinery.

4.2 Stop Codon Readthrough

Translation termination begins when any of the three stop codons (UAA, UGA and UAG) move into the ribosomal A site. When the stop codon appears in the A site a release factor binds since there is no tRNA that corresponds to any of the three stop codons. Binding of the release factor mediates the cleavage of the polypeptide chain from the tRNA in the ribosomal P site. In eukaryotes the two release factors are eRF1 (encoded by the *SUP45* mRNA) and eRF3 (encoded by the *SUP35* mRNA). eRF1, a class I release factor, recognizes all three stop codons and eRF3, a class II release factor, forms a complex with eRF1 to mediate termination through GTP-dependent hydrolysis (Bertram et al., 2001; Kisselev et al., 2003; Rospert et al., 2005).

Just as the start codon of an mRNA has an optimal and suboptimal surrounding sequence context that varies translation initiation efficiency, the efficiency at which the stop codon is recognized is also influenced by the surrounding sequence context. In *S. cerevisiae* the nucleotide with the most prominent influence is the one immediately

following (3') the trinucleotide stop codon. The nucleotide at this position can influence the ability of the ribosome efficiently terminate at the stop codon by at least 100-fold (Bonetti et al., 1995). This has led to the idea that translation termination is actually directed by a tetranucleotide sequence rather than a trinucleotide sequence. Additionally, the six nucleotides upstream and downstream of the stop codon can also influence termination efficiency, but to a smaller degree (Namy et al., 2001). A ribosome can fail to terminate translation at a termination codon by incorporating a near-cognate aminoacyl-tRNA, which complements the sequence of the stop codon at two out of three nucleotide positions, into the ribosomal A site instead of the release factor (Fearon et al., 1994). This occurrence is known as stop codon suppression, also frequently referred to as translational readthrough or leaky termination. The result is a continually growing C-terminally extended polypeptide chain that can have significantly altered function, even if only a few amino acids are added (DePristo et al., 2005; Namy et al., 2002). The ORF stop codon of many yeast mRNAs is followed immediately by more in-frame stop-codons which can serve as a secondary protection if readthrough of the first stop codon were to occur (Williams et al., 2004).

It is interesting, but not surprising, that the ability of the ribosome to readthrough a PTC is able to suppress degradation of the mRNA by NMD. Reading through a PTC may cause the incorporation of one wrong amino acid, but the rest of the polypeptide remains unchanged since the reading frame was not altered (Keeling et al., 2004). Once the ribosome bypasses the PTC by incorporating a near-cognate aminoacyl-tRNA instead of a release factor translation is able to proceed until the normal ORF stop codon is

recognized. This is the basis for the nonsense suppression therapies that target readthrough of PTC so the cells can produce full-length functional protein (Finkel, 2010; Gunn et al., 2014; Peltz et al., 2013). A potential challenge to this approach is the ability to cause readthrough of the PTC without perturbing translation termination at the natural stop codon, especially if the PTC and natural Stop codon are the same triplet codon. The idea of stop codon readthrough can also be applied to those mRNAs that are targeted for degradation by a long 3' UTR. If the *faux* 3' UTR model is correct, which there is much evidence to support this, then translation termination at a normal stop codon followed by a long 3' UTR would place the terminating ribosome in an improper special context for the termination complex to interact with Pab1 (Amrani et al., 2004; Behm-Ansmant et al., 2007; Brogna and Wen, 2009; Muhlrad and Parker, 1999). Thus, if readthrough of the stop codon were to occur—even though the stop codon is not a PTC—and terminate at another in frame stop codon downstream (i.e. in closer proximity to the poly(A) tail), it would result in two important outcomes: 1) a C-terminally extended polypeptide which could have altered function, either harmful or beneficial, and 2) the mRNA, which originally had a long 3' UTR, would no longer be a substrate for NMD.

4.3 Translation Reinitiation

Another mechanism by which mRNAs can avoid degradation by NMD is by reinitiation of translation at a downstream AUG after termination at a PTC. Evidence of reinitiation as an NMD-antagonist comes from a studies of the human β-globin mRNA. In humans, NMD appears to be positional dependent. It has been shown that typically

mRNAs with PTCs at the 3' end of the mRNA fail to trigger NMD which can result in the build-up of C-terminally truncated proteins. This follows an exon-junction complex (EJC) model in which presence of an EJC triggers degradation of an mRNA if termination occurs more than 50-55nt upstream of the final EJC (Chamieh et al., 2008; Le Hir et al., 2001; Lykke-Andersen et al., 2001; Shibuya et al., 2004; Singh et al., 2007). EJC-independent NMD has also been observed, but most of these examples are based on the improper spatial context of the terminating ribosome and the poly(A) tail. This follows the *faux* 3' UTR model in that PTCs located near the 3' end of the mRNA, thus in closer spatial context to the poly(A) tail, may fail to trigger NMD because the termination event would provide normal termination interactions between eRF3 and the poly(A) binding protein (PABP in humans, Pab1 in yeast; Amrani et al., 2004; Behm-Ansmant et al., 2007; Eberle et al., 2008; Ivanov et al., 2008; Singh et al., 2008).

When a PTC is located near the 3' end of the β-globin mRNA, allowing the mRNA to escape NMD, a C-terminally truncated polypeptide is produced that acts in a dominant-negative manner and results in a severe form of heterozygous symptomatic β-thalassemia (Thein et al., 1990, Hall and Thein 1994). In contrast, PTCs that are located more 5' in the β-globin mRNA are able to trigger NMD which limits the build-up of toxic C-terminally truncated polypeptides. The result is an asymptomatic form of heterozygous β-thalassemia (Nagy and Maquat, 1998; Thermann et al., 1998). However, there is one particular set of mutations in the β-globin mRNA that do not fit this pattern. Nonsense mutations in the first exon of the β-globin mRNA, which should activate NMD, do not trigger degradation of the mRNA by NMD and do not result in

symptomatic β-thalassemia (Inacio et al., 2004; Romao et al., 2000). In 2011 Neu-Yilik and colleagues solved this mystery by showing that PTCs in the first exon do not trigger NMD because the ribosome is able to reinitiate translation in exon 2 at Met55. This Met55 is the only in-frame downstream start codon and is located within an optimal initiation context. Importantly, they showed that mutation of the downstream Met55 start codon restores the NMD-sensitivity of the mRNA when a PTC is present upstream. Additionally, PTCs that are located downstream of this Met55 do trigger NMD (Neu-Yilik et al., 2011). Together, these results confirm translation reinitiation as a method of protecting mRNAs from degradation by NMD, but further examples have not yet been demonstrated.

4.4 *Trans*-acting factor Pub1

Two examples of wild-type mRNAs with NMD targeting signals that are not destabilized by NMD are the *YAP1* and *GCN4* mRNAs in *S. cerevisiae*. These two mRNAs contain translated uORFs but show no significant difference in mRNA accumulation or half-life (Michel et al., 2014; Ruiz-Echevarria et al., 1998; Vilela et al., 1998; Chapter 4). A study by Ruiz-Echevarria and Peltz in 2000 identified the *trans*-acting factor poly(U)-binding protein, Pub1, as the mechanism responsible for the protection of these two mRNAs from NMD. They determined that Pub1 bound to stabilizer elements (STEs) located in the 5' leader region of the mRNA. When Pub1 was removed from the cells (*pub1Δ* strains) the mRNAs were significantly destabilized and, importantly, this destabilization was dependent on NMD factor Upf1 (Ruiz-Echevarria

and Peltz, 2000). However, this data was not reproducible in a follow-up study (unpublished data, Chapter 4).

5.0 Perspective and Discussion

Although originally discovered as a surveillance mechanism, further studies of NMD provide evidence that this mechanism of mRNA turnover plays a significant role in regulating the fragile and intricate process of eukaryotic gene regulation. When gene regulation is in check and expression is occurring normally then organisms achieve cellular homeostasis which leads to overall health. However, when gene regulation goes awry it most often results in cellular malfunction and the development of disease. The NMD pathway has been shown to play a role in the development of a significant number of diseases. Interestingly, NMD has been shown to play both a protective and aggravating role in the development of disease. If the C-terminally truncated proteins produced from an mRNA with a PTC would be toxic to the cell then NMD serves a protecting role. However, if the truncated protein would still retain enough activity to be at least partially functional, then continually degrading the mRNA leads to a severe deficiency of the protein. Because inherited PTCs play a role in the development of a significant number of genetically related diseases and cancers researchers are actively pursuing ways of modulating the cellular machinery in order to suppress or readthrough the nonsense codon. Because PTCs can arise through a variety of mechanism (point mutations, insertions or deletions, errors in alternative splicing) the approach to nonsense suppression is not likely to be universal for all PTCs. Accordingly, it is much easier to

approach nonsense suppression of PTCs that have arisen due to a point mutation than it is to approach the suppression of PTCs that have arisen in a way that would cause a shift in the downstream reading frame. The latter of these also poses the complication introducing multiple downstream PTCs due to the shift in reading frame.

It is important to point out that we currently do not have a thorough understanding of the molecular mechanisms of NMD. Notably, it remains elusive as to exactly how a cell is able to distinguish a normal termination event from a premature termination event. Several models have been proposed but so far none of them are able to fully explain all of the observed results of the summation of NMD studies. Considering the diversity of results in studies aiming to elucidate the mechanism behind PTC versus normal termination codon (NTC) differentiation it is reasonable to hypothesize that this mechanism may be cell/tissue-specific. Indeed the *faux* 3' UTR model seems to provide the most comprehensive model and is supported by a significant number of studies and observations.

Additionally, only recently have we begun to appreciate the full breadth of wild-type gene regulation by NMD. Genome-wide studies in various organisms all provide evidence that NMD plays a significant role outside of mRNA surveillance. As the studies of wild-type gene regulation by NMD continue to expand our knowledge we are likely to gain a better understanding of additionally NMD-targeting mechanisms as well as NMD-protection mechanisms. These studies are important as they may provide valuable insight into understanding the underlying mechanisms of NMD.

Understanding the intricate molecular mechanisms of NMD will provide a more solid foundation for the treatment of NMD-related diseases. Therapies can be developed with greater efficiency and, with a better understanding of the overall role of NMD in the cell, we can minimize side-effects and risks associated with disease therapy.

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CHAPTER 2

Saccharomyces cerevisiae SSY5 mRNA is a wild-type mRNA with multiple NMD-targeting signals but is not degraded by NMD

Abstract

Nonsense-mediated mRNA decay is a translation-dependent surveillance mechanism responsible for rapidly degrading mRNAs with premature termination codons (PTCs). However, there is a significant portion of mRNAs that do not contain a PTC but are substrates for the NMD pathway. The underlying mechanisms of how the cellular machinery determines whether or not to degrade an mRNA via the NMD pathway are not well understood. Here we present the case of the *Saccharomyces cerevisiae SSY5* mRNA, which is a wild-type mRNA containing multiple NMD-targeting signals, but is not degraded by the NMD pathway. We demonstrate that known mechanisms for the protection of mRNAs from NMD do not apply to *SSY5* mRNA as the mRNA is translated and we do not find evidence of stop codon readthrough or translation reinitiation. Thus, the protection of the *SSY5* mRNA from NMD is likely due to a novel mechanism that may provide further clues in unraveling the mystery of gene regulation and substrate recognition by NMD.

Introduction

Nonsense-mediated mRNA decay (NMD) was historically identified as a post-transcriptional surveillance mechanism responsible for the rapid degradation of mRNAs containing premature termination codons (PTCs; Gonzalez et al., 2001; Isken and Maquat, 2008; Muhlemann et al., 2008). The identification of a termination event as premature and the subsequent activation of the NMD machinery is critical for cellular homeostasis as this process ensures that truncated proteins, which can otherwise have dominant-negative deleterious effects, are not allowed to build up in the cell (Hentze and Kulozik, 1999; Hilleren and Parker, 1999; Maquat and Serin, 2001). Additionally, a role for NMD is implicated in as many as one third of inherited genetic diseases and cancers (Frischmeyer and Dietz, 1999).

NMD requires the coordinated activity of three core factors: Upf1, Upf2 and Upf3. Mutations or deletions in one or more of the genes encoding these factors stabilizes NMD substrates (Cui et al., 1995; He et al., 1997; He and Jacobson, 1995; Lee and Culbertson, 1995; Maderazo et al., 2000). Importantly, NMD is conserved from yeast to humans and has been observed in all eukaryotes examined (Bedwell et al., 1997; Hall and Thein, 1994; He et al., 2003; Hentze and Kulozik, 1999; Maquat and Carmichael, 2001; Maquat and Serin, 2001; Mendell et al., 2004; Pulak and Anderson, 1993; Rehwinkel et al., 2005). However, the mechanism underlying the identification of a termination event as premature and the subsequent rapid activation of the NMD pathway remains largely obscure. Moreover, it has also been observed that NMD has an impact on the accumulation of 3-10% of yeast mRNAs, many of which do not contain

PTCs (Culbertson, 1999; Guan et al., 2006; Lelivelt and Culbertson, 1999). Thus, we are beginning to appreciate a broader role for NMD in the overall regulation of gene expression. Given this, it has become increasingly important to understand the underlying mechanisms of the NMD pathway and how an mRNA is able to trigger degradation by NMD.

Aside from the presence of a PTC in an aberrant mRNA, there are several known features that can target a wild-type mRNA for degradation by NMD: 1) a long 3' UTR (Amrani et al., 2004; Kebaara and Atkin, 2009; Muhlrad and Parker, 1999), 2) translation of an upstream open reading frame (uORF; Amrani et al., 2006; Barbosa et al., 2013; Nyiko et al., 2009), 3) a start codon in a suboptimal context which can lead to leaky scanning and out of frame initiation of translation (Welch and Jacobson, 1999), 4) the presence of programmed ribosome frameshift (PRF) sites (Plant et al., 2004), and 5) the presence of pre-mRNA introns and regulated alternative splicing resulting in PTCs (He et al., 1993; Lewis et al., 2003; McGlincy and Smith, 2008; Ni et al., 2007). All of these NMD-targeting signals can result in either the ribosome terminating in a context that is different from a normal termination event (i.e. proximity to the poly(A) tail; signals 1-2) or in the introduction of a PTC (signals 2-4). Comparing wild-type mRNAs that have NMD-targeting signals and are degraded by NMD with those that have NMD-targeting signals and are not degraded by NMD may provide important insights as to the underlying mechanisms of NMD.

Currently, there are four known mechanisms that can protect a wild-type mRNA that has an NMD-targeting signal from degradation by NMD, but there are likely more to

be discovered. These NMD-protecting mechanisms include: 1) inhibition of translation, 2) translational readthrough (also known as stop codon suppression), 3) reinitiation of translation, and 4) *trans*-acting factors (Carter et al., 1995; Dang et al., 2009; Keeling et al., 2004; Neu-Yilik et al., 2011; Noensie and Dietz, 2001; Ruiz-Echevarria and Peltz, 2000; Wang et al., 2001). In support of the idea that there are more NMD-protecting mechanisms yet to be discovered, we have identified an mRNA, *SSY5*, in *Saccharomyces cerevisiae* that has multiple NMD-targeting signals but is protected from decay by the NMD pathway through a novel mechanism.

The *SSY5* mRNA codes for an essential component of the SPS amino acid sensor in *S. cerevisiae*. The SPS sensor consists of three subunits: integral membrane protein Ssy1, and peripheral membrane proteins Ssy5 and Ptr3. When extracellular amino acids are present a signaling cascade is initiated by the binding of an amino acid to Ssy1, which then transduces a signal via Ptr3 to Ssy5. Ssy5 is a serine protease that consists of two domains: an inhibitory N-terminal pro domain and a catalytic C-terminal cat domain. During the biogenesis of Ssy5 the two subunits are autolytically cleaved but remain noncovalently associated until an activation signal is received (Andreasson et al., 2006; Conrad et al., 2014; Ljungdahl, 2009). Once activated the Ssy5 pro domain is degraded by the proteasome and the cat domain cleaves the N-terminal domain of transcription factors Stp1 and Stp2, which allows them to enter the nucleus and activate the expression of the amino acid permease (AAP) genes (Ljungdahl, 2009). AAP gene products then facilitate the uptake of extracellular amino acids (Fig 1). Given the importance of Ssy5

and the intricacies of the SPS amino-acid-sensing pathway, it is likely that the regulation of *SSY5* mRNA levels is tightly controlled and has physiological significance.

Here we show the wild-type *SSY5* mRNA, which has multiple NMD-targeting signals, is not degraded by NMD. Based on previous studies, at least one of the targeting signals—the long 3' UTR—is able to target a reporter mRNA for degradation by NMD (Obenoskey et al., 2014). We demonstrate that many of the current mechanisms for protection of an mRNA from NMD do not apply to the *SSY5* mRNA. This provides evidence that the *SSY5* mRNA is actually protected from degradation by NMD through an unknown mechanism. Thus, the *SSY5* mRNA provides a unique and intriguing case to help elucidate the underlying mechanisms of gene regulation by the NMD pathway.

Materials and Methods

Yeast strains

The yeast strains used in this study are listed in Table 2-1. All yeast transformations were done using Lithium Acetate-Mediated transformation as previously described (Gietz and Woods, 2002). AAY590 was constructed by transforming AAY538 with the *upf1Δ2* fragment from pAA70 using primers oAA48 and oAA79.

5'-tagged *SSY5* mRNA constructs: AAY561 was constructed by transforming BY4743 with the fragment amplified from the pFA6a-His3MX6-PGAL1-GFP plasmid using primers F4 and R5 containing sequence to target the product to the *SSY5* mRNA 5' leader (Longtine et al., 1998). AAY568 was constructed by sporulation and random spore analysis of AAY561. AAY625 was constructed by transforming AAY568 with the

upf1Δ2 fragment from pAA70 using primers oAA48 and oAA79. AAY630 was constructed by transforming BY4743 with the fragment amplified from the pFA6a-His3MX6-PGAL1 plasmid using primers F4 and R2 containing sequence to target the product to the *SSY5* mRNA 5' leader to make AAY559 (Longtine et al., 1998). AAY630 was the result of the sporulation and random spore analysis of AAY559. AAY632 was constructed by transforming AAY630 with the *upf1Δ2* fragment from pAA70 using primers oAA48 and oAA79.

3'-tagged *SSY5* mRNA constructs: AAY572 was constructed by transforming BY4741 with the fragment amplified from pFA6a-GFP(S65T)-HIS3MX6 plasmid using primers F2 and R1 containing sequence to target the product in-frame just before the *SSY5* mRNA stop codon (Longtine et al., 1998). AAY623 was constructed by transforming AAY572 with the *upf1Δ2* fragment from pAA70 using primers oAA48 and oAA79. AAY581 was constructed by transforming BY4741 with the fragment amplified from pFA6a-GFP(S65T)-HIS3MX6 plasmid using primers F2 and R1 containing sequence to target the product in-frame just before the *SSY5* mRNA stop codon that would be used if reinitiation were to occur in the 3'UTR (Longtine et al., 1998). AAY585 was constructed by transforming BY4741 with the fragment amplified from pFA6a-GFP(S65T)-HIS3MX6 plasmid using primers F2 and R1 containing sequence to target the product in-frame just before the *SSY5* mRNA stop codon that would be used if readthrough were to occur (Longtine et al., 1998). AAY572, AAY581 and AAY585 were sequenced to verify that the GFP sequence was inserted into the correct reading frame.

SSY5 mRNA terminator constructs: AAY576 was constructed by transforming BY4741 with the fragment amplified from pFA6a-GFP(S65T)-HIS3MX6 plasmid using primers F3 and R1 containing sequence to target the product to the *SSY5* mRNA stop codon. AAY601 was constructed by transforming AAY576 with the *upf1Δ2* fragment from pAA70 using primers oAA48 and oAA79.

Growth conditions

Unless otherwise noted yeast cells were grown using standard techniques with mild agitation equivalent to 225rpm at 30˚C. When cells are stated as being grown in a certain type of media, the same media was used to grow plate cultures from frozen stock and all subsequent liquid cultures. In this study, YAPD media consists of: 1% yeast extract, 2% Bacto-Peptone, 2% dextrose, and 100mg/L Adenine hemisulfate salt; YAPG media consists of: 1% yeast extract, 2% Bacto-Peptone, 2% galactose, and 100mg/L Adenine hemisulfate salt; and minimal media (SD+amino acids) consists of: 0.67% yeast nitrogen base without amino acids, 2% dextrose, 20 mg/L L-Histidine, 30 mg/L L-Leucine, 20 mg/L L-Methionine, 20 mg/L Uracil, and 30 mg/L L-Lysine.

Utilization of the GWIPS-viz Ribo-seq Genome Browser

Ribosome profiles were obtained from the QWIPS-viz Ribo-seq Genome Browser: gwips.ucc.ie (Michel et al., 2014). Search: Group [Yeast], Genome [*S. cerevisiae*], Assembly [Apr. 2011 (SacCer_Apr2011/SacCer3)], Position [chrX: 128,943-128,987]. Select options: Reverse, then PS/PDF [Image configuration: text size-18,

image width-1346 pixels, label area width-14 characters, uncheck option to show light blue vertical lines], download current browser in PDF.

RNA Extractions

Yeast strains were grown in 10mL cultures to an OD_{600} of 0.4-0.6. Cells were harvested by centrifugation, washing in DEPC-ddH_20, and flash-freezing in dry ice/ethanol or liquid nitrogen. Cell pellets were stored at -70˚C until used for RNA extractions. RNA extractions were performed as previously described (Kebaara et al., 2012). RNA samples were diluted to 1µg/µl in DEPC-ddH_20 and stored at -70˚C. RNA quality check gels are performed for every RNA sample (1µl of 1µg/µl Total RNA is run through a 0.8% agarose gel to check for degradation).

Quantitative Northern Analysis

10µg of Total RNA mixed with 3µl Formaldehyde loading dye (Ambion, cat. no. 8552) was separated through a 1.0% agarose gel containing 5.6% Formaldehyde and 1% MOPS (10X MOPS: 0.2 M sodium morpholinopropanesulfonic acid (MOPS), pH 7.0, 0.05 M sodium acetate, 0.01 M EDTA; adjust pH to 7.0 with 10 M NaOH, do not autoclave, store at room temperature in the dark.) RNA was transferred onto a GeneScreen Plus membrane (PerkinElmer) using NorthernMax transfer buffer (Ambion, cat. no. 8672) following the manufacturer's protocol for downward transfer. The lane with the RNA ladder was cut off of the gel before transfer and stained overnight in

0.5µg/ml ethidium bromide. Transfer was allowed to proceed for 2 hours with a 9mm thick RNA gel. Membranes were rinsed in 2X SSC and dried for 15 minutes at 80˚C.

Membranes were hybridized with NorthernMax prehybridization/hybridization buffer (Ambion # 8677). ^{32}P-labelled probes were synthesized using ~25ng of PCR product corresponding to the gene of interest, the RadPrime DNA Labeling system (Invitrogen #18428-011), and ~50 µCi [α-32P]dCTP (3000 Ci/mmol, 10 mCi/ml) (Perkin Elmer) following the manufacturer's protocol. Probes are purified through a Sephadex G-50 column equilibrated with TE pH 8.0. Membranes were hybridized overnight (12-24 hours) and then washed once at room temperature with 2X SSPE and once at 65˚C with 2X SSPE/2% SDS. Membranes were PhosphorImaged™ (GE Healthcare, Typhoon FLA 9500) and quantified using the ImageQuant™ software. All membranes were also autoradiographed using a phosphorescent ruler to determine the size of the bands by comparison to the RNA ladder. Membranes are stripped and stored at -20˚C for re-probing. Detailed protocol for Northern analysis can be found in Kebaara *et al.* (2012).

Westerns

Total protein was extracted as described in Atkin et al., 1995 from 40mls of mid-log culture (OD_{600} of 0.4-0.6). Protein extracts were quantified in triplicate using BSA standards (Pierce #23225). 40µg of total protein was separated through a 7.5% SDS-Polyacrylamide gel and transferred to a .45µM Nitrocellulose membrane. Blocking was done using 5% w/v Carnation® Instant Dry Milk overnight at 4˚C. Primary GFP antibody used was a rabbit polyclonal ChIP-grade antibody (Abcam #ab290) and was

incubated at a concentration of 1:2000 diluted in 5% w/v Carnation® Instant Dry Milk for 1hr at room temperature (per Abcam protocol). Secondary antibody was anti-rabbit IgG, HRP-linked (Cell Signaling #7074) and was used at a concentration 1:6000 diluted in 5% w/v Carnation® Instant Dry Milk for 1hr at room temperature (per Cell Signaling protocol). Pierce ECL Western Blotting Substrate kit (Pierce #32109) was used for detection. Signal was detected using film.

Polyribosome Analysis

Yeast polyribosome analysis was performed as previously described (Atkin et al., 1995). Lysis buffer was composed of 20mM Tris-HCl pH 8.0, 140mM KCl, 1.5 mM $MgCl_2$, 1% Triton X, 0.1 mg/ml Cycloheximide, and 1.0 mg/ml Heparin; Cycloheximide and Heparin were made fresh and added just before use. Gradient buffer (50% and 15% sucrose) was composed of 20mM Tris-HCl pH 8.0, 140mM KCl, 5.0 mM $MgCl_2$, 0.1 mg/ml Cycloheximide, 0.5 mg/ml Heparin, and 0.5mM DTT; Cycloheximide, Heparin, and DTT were made fresh and added just before use. 15%-50% sucrose gradients were made by hand, frozen at -70˚C and thawed at 4˚C overnight just before use. After fractionation RNA was extracted from each fraction using acid phenol/chloroform extraction. Total RNA from each fraction collected was loaded onto an RNA Northern gel and transferred to a GeneScreen Plus membrane as described above. Membranes were hybridized with a ^{32}P-labelled probe as described above.

Results:

***SSY5* mRNA has multiple NMD-targeting signals**

The wild-type *S. cerevisiae SSY5* mRNA was previously identified as an mRNA with a long 3' UTR that is not degraded by the NMD pathway (Kebaara and Atkin, 2009). Upon further inspection, we identified three additional potential NMD-targeting signals present in the *SSY5* mRNA: a translated uORF, a start codon in a poor context, and five predicted ribosome frameshift sites (Fig. 2A-B; Belew et al., 2008; Guan et al., 2006; Michel et al., 2014).

A map of ribosome footprints in the SSY5 uORF was compiled using the GWIPS-viz Ribo-seq Genome Browser (Michel et al., 2014). The compiled ribosome profile (summary profile from all studies) for the *SSY5* mRNA uORF is displayed in Figure 2B. The reading frame for the uORF is identified in the middle row (✱): the ATG start codon begins at position 128, 986 (-34) and the UAA stop codon ends at position 128, 966. The reading frame for the *SSY5* mRNA open reading frame (ORF) is identified in the bottom row (▲) with the ATG start codon beginning at position 128, 952 (+1). The ribosome density peaks (in red) in the *SSY5* mRNA 5' leader region shows ribosomes are present in a periodic pattern that represents translation (Ingolia et al., 2009). When the ribosome terminates translation at the end of the uORF this termination event can be recognized by the cellular machinery as a premature termination event, which would target the mRNA for degradation by NMD (Guan et al., 2006; Hurt et al., 2013).

Targeting to NMD by leaky scanning due to poor translation initiation at the start codon is also a likely possibility for the *SSY5* mRNA. Previously, H. Miyasaka developed a method for quantifying the surrounding context of an AUG codon (Miyasaka, 1999). This value, the A_{UG}CAI (which stands for start Codon Adaptation Index), is built on the concept of the CAI devised by Sharp and Li in 1987 and is used to describe the optimal context of an AUG start codon (Miyasaka, 1999; Sharp and Li, 1987). The A_{UG}CAI takes into account the nucleotide frequency at each position surrounding the AUG from -6 to +6. Guan *et al.*, (2006) revised the A_{UG}CAI to test the leaky scanning model for NMD. They created the A_{UG}CAI(r) (start Codon Adaptation Index revised), which is a score from 0 to 1.0 that takes into account both the frequency at which each nucleotide appears at each position from -6 to +6 and how much each nucleotide contributes to the overall start codon context, in order to determine the efficiency of translation initiation at a particular AUG (Guan et al., 2006). Scores closer to 0 indicate a start codon in a poor context that is more likely to be subject to leaky scanning. Optimal start codon context, defined as (A/U)A(A/C)AA(A/C)**AUG**UC(U/C), is assigned a value of 1.0; however, the frequency of mRNAs with an A_{UG}CAI(r)=1.0 is very low (data not shown; Hamilton et al., 1987). The *SSY5* mRNA has an A_{UG}CAI(r)=0.375. In comparison, the A_{UG}CAI(r) of the *SPT10* mRNA, which is targeted to NMD by leaking scanning, is 0.320 (Guan et al., 2006; Welch and Jacobson, 1999). Based on this observation, Guan *et al.* came to the conclusion that the best candidates for leaky scanning are those with an A_{UG}CAI(r) value $\leq$0.320. While the A_{UG}CAI(r) of the *SSY5* mRNA is slightly above this cutoff, a value of 0.375 is still

relatively low and suggests that the start codon of the *SSY5* mRNA could be subject to leaky scanning.

Programmed ribosome frameshifting (PRF) can also cause an mRNA to become a substrate for NMD. PRF sites are *cis*-elements within an mRNA that cause a ribosome to shift reading frames either in the +1 direction or the -1 direction, although the -1 PRF is much more frequent (Jacobs et al., 2007; Plant et al., 2004). A -1 PRF site is identified by the presence of a heptameric "slippery site" sequence (X XXY YYZ, where X is any three identical nucleotides, Y is either three A nucleotides or three U nucleotides, and Z is either an A, U, or C) followed within eight nucleotides by a sequence that creates an RNA pseudoknot (Dinman et al., 1991; Somogyi et al., 1993; Tu et al., 1992). A previous study found that at least 2.54% of *S. cerevisiae* genes contain a consensus sequence for a -1 PRF site. In contrast to viruses—where the PRF phenomenon was originally discovered—the analysis in this study revealed that most of the -1 PRF signals in yeast would result in the introduction of a PTC into the reading frame (Hammell et al., 1999). We used the Programmed Ribosome Frameshift Database (PRFdb), a database that contains computationally predicted -1 PRF sites in eukaryotic genomes, to analyze the *SSY5* mRNA for the presence of -1 PRF sites (Belew et al., 2008). The PRFdb predicts five potential -1 PRF sites in the *SSY5* mRNA at +18, +132, +324, +333, and +1728. Using the GWIPS-viz Genome Browser, which shows all three reading frames of an mRNA, we noted that any shift in the reading frame of the *SSY5* mRNA results in the introduction of multiple stop codons throughout the ORF (Michel et al., 2014). However, the predicted -1 PRF sites in the *SSY5* mRNA have not yet been experimentally validated.

Finally, long 3' UTRs have been identified in both *S.cerevisiae* and humans as NMD-targeting signals (Amrani et al., 2004; Eberle et al., 2008; Hogg and Goff, 2010; Kebaara and Atkin, 2009; Muhlrad and Parker, 1999; Rebbapragada and Lykke-Andersen, 2009; Yepiskoposyan et al., 2011). One explanation for how PTCs trigger NMD was proposed in the faux 3' UTR model which suggests that early translation termination (e.g. at a PTC) is distinct from normal termination because the terminating ribosome is not in the proper context (e.g. proximity) to interact with 3' UTR-bound poly(A) binding protein, Pab1. The failed interaction between the terminating ribosome and Pab1 leaves the ribosome open for binding of the NMD factors (Upf1, Upf2, and Upf3), which triggers NMD. A long 3' UTR would place the stop codon, even though it is not a PTC, in the same context (i.e. too far from the poly(A) tail for the terminating ribosome to bind Pab1) as if it were a PTC. In *S. cerevisiae*, most 3' UTRs are short ranging in length from 50-200 nt with a median length of 121nt (Graber et al., 1999). The *SSY5* mRNA has a 3' UTR of ~475 nt determined by 3' RACE (Obenoskey et al., 2014). The use of alternative Poly(A) sites is predicted to produce *SSY5* mRNA 3'UTRs that range from 420-500nt (Kebaara and Atkin, 2009).

The long 3' UTR of the *SSY5* mRNA has been validated as an NMD-targeting signal by replacing the native 3' UTR of the *CYC1* mRNA, which is normally NMD-insensitive, with the long 3' UTR of the *SSY5* mRNA. The *CYC1-SSY5* 3'UTR construct is a substrate for NMD indicating that the long 3' UTR of the *SSY5* mRNA is sufficient to target an mRNA for degradation by NMD (Obenoskey et al., 2014). However, the

remaining three potential NMD-targeting signals (Fig. 2A 1-3) have yet to be validated as positive NMD-targeting signals for the *SSY5* mRNA.

Codon optimality of an mRNA was recently identified as a significant determinant of mRNA stability (Presnyak et al., 2015). A higher composition of codons designated as "optimal" results in stable mRNAs whereas a lower composition results in unstable mRNAs and higher turn-over. The optimal codon content of an mRNA also influences the elongation rate of the translating ribosome where a lower content of optimal codons results in a slower elongation rate (Presnyak et al., 2015). The optimal codon content of *SSY5* mRNA is 285 optimal codons out of 698 codons total (subtracting out the AUG start codon and UGA stop codon) yielding a 40.83% optimal codon content. The original study showed that mRNAs with less than 40% optimal codon content are significantly more unstable (median half-life of 5.3 min) than mRNAs with 70% optimal codon content (median half-life of 20.1 min; Presnyak et al., 2015). Thus, *SSY5* mRNA percent optimal codon content falls much unstable mRNA category than to the stable mRNAs. However, it should be noted that codon optimality is subject to exceptions and the contribution of codon optimality specifically to the stability of the *SSY5* mRNA remains to be experimentally determined.

The *SSY5* mRNA has four of the known NMD-targeting features: 1) a translated uORF, 2) a start codon in a poor context based on the AUGCAI(r) value of 0.375, 3) five predicted -1 PRF sites that would all introduce multiple downstream stop codons, and 4) a long 3' UTR that would result in the ribosome terminating a long distance from the poly(A) tail. Additionally, the percent of optimal codons that compose the *SSY5* mRNA

is low, and that alone should destabilize the mRNA. All of this leads to the rational assumption that the *SSY5* mRNA should be a substrate for degradation by NMD. However, below we show this is not the case.

SSY5 mRNA is not degraded by NMD

An initial screen of mRNA half-lives ($T_{1/2}$) in wild-type and *upf1Δ* strains identified the *SSY5* mRNA as being unaffected by the NMD pathway (Kebaara and Atkin, 2009). In order to validate these results we grew *S.cerevisiae* cells in rich growth medium (YAPD) and confirmed by quantitative Northern analysis that steady-state *SSY5* mRNA levels show no significant fold change (FC) between wild-type and *upf1Δ* strains (fold change ratio (FCR) of *upf1Δ*/ wild-type =1.2±0.1) in the BY4741 genetic background (Fig. 3A). Further, the $T_{1/2}$ of the *SSY5* mRNA was not significantly different between wild-type ($T_{1/2}$=33.3min) and *upf1Δ* strains ($T_{1/2}$=38.3min; Fig. 3B). *ENT4* is an mRNA with a long 3' UTR (~600 nt) that was previously identified in *S. cerevisiae* as a substrate for NMD (Kebaara and Atkin, 2009). Here we use the *ENT4* mRNA as well as the *CYH2* pre-mRNA as positive controls for NMD, and *SCR1* serves as the loading control.

It was previously shown that different genetic backgrounds in yeast can influence the relative accumulation of nonsense mRNAs in wild-type versus *upf1Δ* strains to varying degrees (Kebaara et al., 2003). Thus, steady-state *SSY5* mRNA levels were also determined in wild-type and *upf1Δ* strains in the W303 genetic background. Consistent with results in the BY4741 background, there was no significant fold change in steady-

state *SSY5* mRNA levels between wild-type and *upf1Δ* strains (FCR of *upf1Δ*/wild-type =1.1±0.1) in the W303 background (Fig. 3A). Half-lives were not determined for the W303 background in the present study as these were shown in a previous study (Kebaara and Atkin, 2009). The BY4741 and W303 genetic backgrounds are two of the most commonly used genetic backgrounds in NMD work with *S. cerevisiae*. BY4741 strains are derived from S288C and differences between the two are very minor (Brachmann et al., 1998). W303 is a lab strain developed as a model organism and differs from S288C at >8,000 nucleotide positions, but still shares >85% of its genome with S288C (Ralser et al., 2012). Based on these results all subsequent experiments were done using strains with the BY4741 background.

The *SSY5* mRNA does not appear to be a substrate for NMD when cells are grown in rich media (Fig. 3A-B). However, since the *SSY5* mRNA codes for the catalytic subunit of an amino-acid-sensing complex (Fig. 1), we hypothesized that the stability of the *SSY5* mRNA might vary in different growth conditions. To test this hypothesis we grew the cells in minimal media which contained only the amino acids necessary to support the auxotrophies of the strains (Materials and Methods). The steady-state accumulation of the *SSY5* mRNA was not significantly different between wild-type and *upf1Δ* strains (FCR=1.1±0.2; Fig. 3C). Additionally, the $T_{1/2}$ of the *SSY5* mRNA was not significantly different between wild-type ($T_{1/2}$=12.4min) and *upf1Δ* strains ($T_{1/2}$=13.3min; Fig. 3D). Although the $T_{1/2}$ of the *SSY5* mRNA is not significantly different between wild-type and *upf1Δ* strains in both types of media (rich vs. minimal) we did note that the overall $T_{1/2}$ of the *SSY5* mRNA in both strains (wild-type and *upf1Δ*) is much longer in

rich media than in minimal media (Fig. 3B vs. Fig. 3C). This is likely due to the SPS-sensor being in an active state during growth in rich media leading to higher turnover of the Ssy5 component. An increase in demand for Ssy5 could lead to increased mRNA $T_{1/2}$.

Typically, NMD substrates are rapidly degraded in wild-type cells and accumulate to significantly higher levels in *upf1Δ* strains. Additionally, the $T_{1/2}$ of NMD substrates is significantly longer in *upf1Δ* strains compared to wild-type strains (Cao and Parker, 2003; He and Jacobson, 2001; Muhlrad and Parker, 1999). Based on our results for steady-state mRNA accumulation and $T_{1/2}$ analysis of the *SSY5* mRNA in both rich and minimal media we conclude that the *SSY5* mRNA is not degraded by the NMD pathway and this stability is not attributed to differences in environmental amino acid content. However, it should be noted that the minimal media used still contains five amino acids, so true amino acid starvation was not obtained due to auxotrophic requirements of the strains (Materials and Methods).

Replacing the long 3'UTR of *SSY5* mRNA with a short 3'UTR does not influence *SSY5* mRNA susceptibility to NMD

Given that the *SSY5* mRNA has multiple NMD-targeting signals (Fig. 2), and that at least one of these targeting signals (the long 3' UTR) is sufficient to target an mRNA for degradation by NMD (Obenoskey et al., 2014), yet we do not find an increase in steady-state accumulation of *SSY5* mRNA or a difference in $T_{1/2}$ between wild-type and *upf1Δ* strains (Fig. 3A-D) we hypothesize that the *SSY5* mRNA is somehow protected

from degradation by the NMD pathway. As a start to identifying the feature(s) and/or region(s) of the *SSY5* mRNA responsible for protection from NMD we began with the 3' UTR. Since it has already been determined that the long 3' UTR of the *SSY5* mRNA, when placed on a different ORF, is sufficient to target an mRNA to NMD, we began with the reciprocal experiment of replacing the long 3' UTR of the *SSY5* mRNA with a short 3' UTR (Obenoskey et al., 2014). To do this we selected the 180nt 3' UTR of the *ADH1* mRNA (Fig. 4A). The *ADH1* mRNA 3' UTR was selected for two reasons: 1) it has a length that falls within the range of typical *S. cerevisiae* 3' UTRs (50-200nt), and 2) the tools for replacing the 3' UTR of an mRNA with the *ADH1* 3' UTR are readily available and have been validated (Graber et al., 1999; Longtine et al., 1998).

Using quantitative Northern analysis, we found no significant difference in steady-state mRNA accumulation of the *SSY5+ADH1* 3' UTR mRNA between wild-type and *upf1Δ* strains (FCR=1.1±0.2; Fig. 4B, lanes 2 and 3). Likewise, there was no significant increase in $T_{1/2}$ of the *SSY5+ADH1* 3' UTR mRNA in the *upf1Δ* strain ($T_{1/2}$=16.3min) compared to the wild-type strain ($T_{1/2}$=12.3min; Fig. 4C). However, we did observe a higher accumulation of *SSY5+ADH1* 3' UTR mRNA in both wild-type and *upf1Δ* strains compared to the normal *SSY5* mRNA (FCR=1.6±0.2 and FCR=1.7±0.0, respectively; Fig. 4B, compare lanes 2 and 3 with lane 1).

Additionally, because *SSY5* mRNA codes for an essential component of the SPS amino-acid-sensing complex we wanted to see if altering the 3' UTR of the *SSY5* mRNA had any influence on the function of Ssy5. We considered this because sequence elements lead to speculation that readthrough of the annotated *SSY5* mRNA stop codon is

a possibility (discussed below, Fig. 7). If readthrough is occurring, then it is possible that replacing the native 3' UTR of the *SSY5* mRNA could influence Ssy5 structure and function. To determine if Ssy5 enzymatic function is compromised in this strain we chose to look at mRNA accumulation of a downstream target of Ssy5. Ssy5 is responsible for the proteolytic processing of the transcription factors Stp1 and Stp2, which, after being cleaved by Ssy5, enter the nucleus and upregulate the expression of amino acid permease (AAP) genes. The *AGP1* mRNA codes for an amino acid transporter with broad substrate range and is one of the AAP genes upregulated in response to Stp1/2 cleavage by Ssy5 (Fig.1; Ljungdahl, 2009). There is no significant difference in the accumulation of the *AGP1* mRNA between the strain with the native *SSY5* 3' UTR (Fig. 4B, lane 1) and the strains with the *SSY5+ADH1* 3' UTR construct (Fig. 4B, lanes 2 & 3). Thus, using *AGP1* mRNA accumulation as an indicator of Ssy5 activity, there is no evidence of altered Ssy5 function as a result of replacing the *SSY5* mRNA 3' UTR with the shorter *ADH1* mRNA 3' UTR.

Taken together, these results show that replacing the long 3' UTR of the *SSY5* mRNA with a shorter 3' UTR has no impact on *SSY5* mRNA stability. Hence, we conclude that the 3' UTR of the *SSY5* mRNA is not responsible for the protection of *SSY5* mRNA from NMD. This is also significant because a previous study identified a stabilizer element (STE) that is able to protect mRNAs from NMD (Ruiz-Echevarria and Peltz, 2000). This study showed that the STE must be downstream of a stop codon to confer protection from NMD. Here we removed all the *SSY5* 3' UTR sequence downstream of the stop codon, so even if there was an STE in the 3' UTR that is not the

mechanism of protection of the *SSY5* mRNA from NMD. However, the long 3' UTR is not the only NMD-targeting signal present in the *SSY5* mRNA. Investigation of the additional targeting signals may provide insight as to the mechanism of protection of the *SSY5* mRNA from NMD.

Perturbation of the *SSY5* mRNA 5' UTR does not affect *SSY5* mRNA stability

Another potential NMD-targeting feature present in the *SSY5* mRNA is the presence of a translated uORF (Fig. 2B). Since replacing the long 3' UTR of the *SSY5* mRNA with a shorter 3' UTR did not provide evidence of a stabilizing element in the 3' UTR we decided to refocus our attention to the uORF. For this, we hypothesize that if disruption of the 5' UTR influences *SSY5* mRNA stability then there are two likely possibilities: 1) there is another *cis*-acting element present within the 5' UTR responsible for *SSY5* mRNA stability, or 2) there are *trans*-acting factors that bind to specific sequences within the 5' UTR to confer stability. It is also possible that *SSY5* mRNA stability could be the result of a combination of any of these three ideas.

To determine if the *SSY5* mRNA stabilizing element is located in the 5' UTR we utilized a construct that had already been generated in our lab. This construct uses a previously validated method for replacing the 5' UTR of an mRNA with the *S. cerevisiae* galactose (GAL1-10) promoter sequence (pGAL; Longtine et al., 1998). Using this method, 50nt of the *SSY5* 5' UTR are replaced with the pGAL sequence, thus abolishing the *SSY5* uORF and the majority of the *SSY5* 5' UTR sequence.

Using quantitative Northern analysis we measured the steady-state accumulation of the *pGAL-SSY5* mRNA in wild-type and *upf1Δ* strains. No significant difference in accumulation between the two strains (FCR=1.1±0.1) was observed. We also measured the *pGAL-SSY5* mRNA $T_{1/2}$ in wild-type and *upf1Δ* strains. Although there is a slight increase in *pGAL-SSY5* mRNA $T_{1/2}$ in the *upf1Δ* strain ($T_{1/2}$=57.6min) compared to wild-type strain ($T_{1/2}$=41.6min) this difference is too small to be considered significant (Fig 5B). If the *pGAL-SSY5* mRNA does become a substrate for NMD then we would expect to see ≥2-fold increase in $T_{1/2}$ in the *upf1Δ* strain. These results indicate that abolishing the native *SSY5* 5' UTR sequence does not influence *SSY5* mRNA stability, thus the 5' UTR does not contain the stabilizing element.

An important consequence of disruption of the *SSY5* 5' UTR using the above method is that *SSY5* mRNA is now under the control of an inducible promoter. Using this we can also determine the effects of overexpression of the *SSY5* mRNA. When *SSY5* mRNA is placed under the control of the galactose promoter and the cells are grown in rich media containing 20% galactose (YAPG) we can achieve ~65-fold increase in *SSY5* mRNA expression (Fig. 5C). Significantly, this substantial increase in *SSY5* mRNA expression has no influence on *SSY5* mRNA stability (Fig. 5A and B) and also has no consequence on Ssy5 downstream function (Figure 5C, *AGP1* mRNA levels). This indicates that significant overexpression of the *SSY5* mRNA in the cell does not saturate the stabilizing mechanism (e.g. *trans*-acting factors). It also indicates that there is a rate-limiting step before Ssy5 protease activity in the SPS sensor signaling cascade. Also

noted, the cells grow at average rates in liquid media and on plates, so overexpression of the *SSY5* mRNA is not toxic to the cells (data not shown).

SSY5 mRNA is not protected from NMD by inhibition of translation

NMD is a translation-dependent mechanism since targeting of an mRNA to NMD typically involves the interaction of the core NMD factors with the terminating ribosome (Bertram et al., 2001; Czaplinski et al., 1998; Kisselev et al., 2003; Rospert et al., 2005). In line with this, several studies have shown that the inhibition of translation, by blocking translation initiation or elongation, is able to suppress NMD (Carter et al., 1995; Dang et al., 2009; Noensie and Dietz, 2001). We hypothesize that inhibition of translation is not the stabilizing mechanism of the *SSY5* mRNA because *ssy5Δ* mutants are not viable and the Ssy5 protein is an essential component of the SPS sensor, which is required for proper cell homeostasis (Conrad et al., 2014; Ljungdahl, 2009). Nevertheless, it is important to confirm that the *SSY5* mRNA is actively translated in order to rule out this possibility.

Polyribosome analysis was performed using cell extracts from wild-type and *upf1Δ* strains. Density gradient centrifugation of total cell extracts through 15%-50% sucrose gradients was performed followed by gradient fractionation. Total RNA was extracted from each fraction and quantitative Northern analysis was used to determine the mRNA distribution among fractions (Materials and Methods). *SSY5* mRNA is found primarily in the polyribosome fractions (9-15) of both wild-type and *upf1Δ* strains indicating that the mRNA does co-migrate with polyribosomes in both strains, which provides strong evidence in support of *SSY5* mRNA association with polyribosomes (Fig.

6A). The polyribosome analysis of the *CYH2* pre-mRNA and mRNA in wild-type and *upf1Δ* strains is provided for comparison to show how the distribution of an NMD-substrate (*CYH2* pre-mRNA) differs from a non-NMD substrate (*CYH2* mRNA) between wild-type and *upf1Δ* strains (Fig. 6A). In comparison, the *SSY5* mRNA distribution appears to mirror that of the *CYH2* mRNA, which is translated and not an NMD-substrate. However, we cannot ignore the presence of the slower migrating *SSY5* band that is specific to the polyribosome fractions in the *upf1Δ* strain. This band could be a longer or modified form of the *SSY5* mRNA that is actually a substrate for NMD.

To confirm that *SSY5* mRNA co-migration with polyribosomes is in fact indicative of active translation we constructed a strain in which GFP is fused to the 5'end of the *SSY5* mRNA. Because endogenous levels of *SSY5* mRNA and Ssy5 protein are extremely low we again employed the pGAL promoter when constructing these strains. GFP fused to the *SSY5* mRNA under the control of the native promotor was not detectable by fluorescence microscopy or flow cytometry (data not shown). By placing the *GFP-SSY5* mRNA under the control of the *GAL* promoter (*pGAL-GFP-SSY5*) we were able to visualize GFP-Ssy5 using confocal fluorescence microscopy (Fig 6B). GFP can be seen in the strains with the *pGAL-GFP-SSY5* mRNA but not in the strains without the GFP-tagged Ssy5 (Fig. 6B, compare top and bottom rows). Importantly, yeast cells do exhibit green autofluorescence; however we were able to filter out this autofluorescence (Fig. 6B, bottom row) so the green fluorescence seen in the *pGAL-GFP-SSY5* mRNA panels is specific to GFP-Ssy5. Hoechst was used as the nuclear stain. These results confirm *SSY5* mRNA is actively translated.

Furthermore, we confirmed that addition of the *pGAL-GFP* sequence does not alter stability of the *SSY5* mRNA as the *pGAL-GFP-SSY5* mRNA accumulates to similar levels in both wild-type and *upf1Δ* strains (FCR=1.1±0.1; Fig. 6C). This result is consistent with the results observed in Figure 5 for the *pGAL-SSY5* mRNA without the *GFP* sequence.

The *SSY5* mRNA 3' UTR is a good candidate for both stop codon readthrough and reinitiation of translation

Upon closer analysis of the *SSY5* mRNA 3' UTR we find two unique features that are of interest concerning potential mechanisms of escape or protection of the mRNA from NMD. First, the *SSY5* mRNA stop codon is a good candidate for stop codon readthrough. Second, the *SSY5* mRNA 3' UTR has the potential for translation reinitiation. Either of these mechanisms independently would provide an explanation for the stability of the *SSY5* mRNA (Bonetti et al., 1995; Keeling et al., 2004; Neu-Yilik et al., 2011; Wang et al., 2001).

Previously, it was shown that leaky termination at PTCs allows mRNAs to escape degradation by NMD (Keeling et al., 2004). Following this idea, it would make sense that leaky termination at the *SSY5* stop codon could protect the *SSY5* mRNA from NMD as it would reduce the length of the *SSY5* mRNA 3' UTR bringing the terminating ribosome in closer proximity to Pab1. There are three features of the *SSY5* mRNA stop codon and 3' UTR that lead us to hypothesize there is a high probability of stop codon readthrough. First, the *SSY5* mRNA terminates with a UGA stop codon. In yeast, the

UGA stop codon is recognized less efficiently by the terminating ribosome than the UAA or UAG stop codons (Keeling et al., 2004). Second, the *SSY5* mRNA stop codon is in a suboptimal context. Optimal stop codon context occurs when the stop codon is followed immediately by a purine residue (A/G) whereas the *SSY5* mRNA stop codon is followed by a T (Fig. 7; Bonetti et al., 1995). Additionally, it has been shown that very efficient termination occurs when the codon immediately upstream of the stop codon is a UCC (serine) codon and immediately downstream is a GCA (alanine) codon, and the *SSY5* mRNA contains neither of these (Bonetti et al., 1995; Namy et al., 2001). Third, the *SSY5* mRNA UGA stop codon is not immediately followed by another in-frame stop codon or codons—a common feature of efficient termination context (Williams et al., 2004). If the ribosome failed to terminate at the initial UGA stop codon translation would proceed a significant distance (102 codons) before encountering another in-frame stop codon. Moreover, the next downstream in-frame stop codon is a more efficiently recognized stop codon (UAA) and is followed by a purine residue, A (Fig. 7; Keeling et al., 2004).

Previous work on the human β-globin mRNA presented a puzzling group of mutations in the first exon of the mRNA. Typically, nonsense mutations near the 5' end of the mRNA elicit degradation by NMD (Chamieh et al., 2008; Le Hir et al., 2001; Lykke-Andersen et al., 2001; Shibuya et al., 2004; Singh et al., 2007). Interestingly, there are a group of nonsense mutations in the first exon of the human β-globin mRNA that do not trigger degradation by NMD (Inacio et al., 2004; Romao et al., 2000). Further studies showed there is a sharp boundary in the position at which nonsense codons in this

mRNA trigger NMD or do not (Neu-Yilik et al., 2011). It was shown that the reason for this sharp boundary and inability of the first exon nonsense mutations to trigger NMD was due to reinitiation of translation at a downstream AUG (Neu-Yilik et al., 2011). The *SSY5* mRNA 3' UTR has the likely potential for translation reinitiation as there is a start codon not far downstream from the original stop codon but in a different reading frame. This start codon is followed by an 82-codon ORF that terminates with a UAA termination codon in a good context (i.e. followed by a purine residue; Fig. 7). The idea of reinitiation in the mRNA 3' UTR is intriguing as it introduces the possibility of a regulatory downstream open reading frame (dsORF) that may serve similar functions as the uORF.

The *SSY5* mRNA is not protected from NMD by stop codon readthrough

We have identified reasons to speculate there is a good chance for stop codon readthrough of the *SSY5* mRNA UGA codon (discussed above). Thus, we hypothesize that stop codon readthrough may be protecting the *SSY5* mRNA from NMD. To test this idea we designed a reporter system that is similar to the one used previously (Bonetti et al., 1995). Briefly, we placed a *GFP* tag in-frame in the *SSY5* mRNA 3' UTR immediately preceding the downstream UAA stop codon that would likely be used if readthrough of the original UGA stop codon was to occur (Reporter #2; Fig. 8A, orange arrow). As a positive control we also made a reporter construct where the *GFP* tag was inserted in-frame immediately preceding the original *SSY5* mRNA UGA stop codon (Reporter #1; Fig. 8A, green arrow).

Total protein extracts were examined for the presence of GFP in strains containing either reporter using Western analysis. Ssy5 has a molecular weight of 77.5 kDa while GFP has a molecular weight of 26.9 kDa, so the control reporter should appear at 104.4 kDa. Reporter #2 contains the additional sequence of the *SSY5* 3' UTR that would be translated if readthrough were to occur which adds another 0.3 kDa to the total protein size. We were able to detect a band in the positive control at ~104.4 kDa that was not present in the negative control—untagged Ssy5 (Fig. 8B, compare lane one with lane two top asterisk). However, we were not able to detect a band at ~104.7 kDa in the readthrough reporter (Fig. 8B, compare lane two with lane three top asterisks).

Additionally, we looked for GFP signal in strains containing each construct using fluorescence microscopy and flow cytometry. However, in these constructs each reporter is under the control of the native *SSY5* promoter so protein levels are extremely low and we were unable to detect GFP signal even in the positive control using either method (data not shown).

Using total protein extracts from the strains and a ChIP-quality GFP antibody we still observed the presence of numerous non-specific cross-reacting bands that can be seen in Figure 8 B. Conversely, there is one band that is specific to the protein extract from the positive control (Fig. 8B, lane 2 bottom asterisk). In looking for bands at 104.4 kDa and 104.7 kDa we only accounted for the full-length uncleaved Ssy5-GFP. However, during the biogenesis of Ssy5 the protein is autolytically cleaved into the 35.5 kDa catalytic C-terminal cat-domain and the 42.2 kDa inhibitory N-terminal pro-domain (Andreasson et al., 2006). For the GFP reporter constructs used here we inserted the GFP

sequence at the 3' end of the mRNA, which places the GFP tag at the C-terminal end of the cat-domain. So, the band we observe at ~62.2 kDa is specific to Ssy5 and accounts for the GFP-tagged cat-domain of Ssy5 that has been autolytically cleaved from the pro-domain (Fig. 8B, lane 2 bottom asterisk and schematic on the right).

We also analyzed the steady-state mRNA levels in each construct to confirm that addition of the 3' *GFP* sequence in either location does not influence *SSY5* mRNA accumulation. As expected, the stead-state mRNA accumulation is similar between wild type *SSY5* mRNA and both *SSY5-GFP* mRNA constructs (Fig. 8C). Also, addition of the 3' GFP tag does not appear to influence Ssy5 activity as downstream *AGP1* mRNA levels remain unchanged in the Ssy5-GFP constructs compared to the untagged Ssy5 (Fig. 8C).

Here we show that are able to detect Ssy5-GFP by Western analysis through the presence of two specific bands: one at 104.4 kDa and one at 62.2 kDa. However, we are unable to detect the presence of specific Ssy5-GFP bands using the reporter designed to test for readthrough (Fig 8B). This leaves us with two possibilities: 1) readthrough of the *SSY5* mRNA UGA is not occurring or, 2.) *SSY5* mRNA UGA readthrough is not occurring at a high enough frequency for us to be able to detect a readthrough product. Using the positive control reporter we determined that we cannot detect a specific Ssy5-GFP band when ≤10μg of total protein extract is loaded. Based on these results, we can conclude the *SSY5* mRNA is not likely protected from NMD by stop codon readthrough.

The *SSY5* mRNA is not protected from NMD by translation reinitiation

In addition to stop codon readthrough, we have also identified features of the *SSY5* mRNA 3' UTR that are favorable for translation reinitiation (discussed above). Therefore, we hypothesize that translation reinitiation in the *SSY5* mRNA 3' UTR may be protecting the *SSY5* mRNA from NMD. To test this idea we again used our GFP reporter system designed above for the read through analysis. We used the same reporter for the positive control (reporter #1 with *GFP* inserted in frame immediately preceding the *SSY5* mRNA UGA stop codon; Fig. 9A, green arrow). The reporter designed to test for translation reinitiation places *GFP* in-frame with the downstream open reading frame immediately preceding the UAG stop codon that would be used for termination of this 3' UTR ORF (Reporter #3; Fig. 9A, blue arrow).

Total protein extracts were examined for the presence of GFP in strains containing either reporter using Western analysis as results using fluorescence microscopy and flow cytometry proved unsuccessful because of the low level of protein expression under the control of the native *SSY5* mRNA promoter (data not shown). We expected the same bands as before for the positive control at 104.4 kDa and 62.2 kDa. The translation reinitiation product, however, is much smaller and should appear at ~ 36.6 kDa. Since we did not separate the protein extracts using a gradient gel and the reinitiation product is so small, we were only able to distinguish the protein product for the processed GFP-tagged cat-domain in the positive control at 62.2 kDa because the 104.4 kDa was obscured by the group of cross-reacting bands (Fig. 9B). Nevertheless, based on our results in Figure 8B this band still allows us to confirm specificity for an

Ssy5-GFP product. However, we were unable to detect a reinitiation product at 36.6 kDa (Fig. 9B). Again, this means that either reinitiation in the *SSY5* 3' UTR is not occurring or that the reinitiation is occurring at a frequency too low to be detected by our methods. Additionally, it is possible that reinitiation may be occurring in a different reading frame with a different AUG, as there are five additional out-of-frame AUG codons. We tested the most likely position for reinitiation to occur based on start codon context, size of the ORF, and context of the stop codon.

We also analyzed steady-state *SSY5* mRNA levels in all of the constructs to confirm that addition of the 3' UTR *GFP* sequence to test for translation reinitiation did not alter mRNA accumulation (Fig 9C). The small decrease in accumulation in the *SSY5-GFP* reinitiation mRNA construct is not considered significant. The more diffuse banding pattern for the *SSY5* mRNA in all of the 3' *GFP*-tagged constructs in noted, and is likely the result of a more diverse population of mRNAs, but with small variation (Fig, 8C and Fig. 9C). Furthermore, addition of the 3' UTR *GFP* sequence in the reinitiation reading frame does not appear to influence Ssy5 activity as downstream *AGP1* mRNA levels remain unchanged in the Ssy5-GFP constructs compared to the untagged Ssy5 (Fig. 9C).

Thus, we conclude that the *SSY5* mRNA is most likely not being protected from NMD due to translation reinitiation in the *SSY5* 3' UTR at the most favorable potential downstream open reading frame.

SSY5 mRNA is translated equally efficient in both wild-type and _upf1Δ_ cells

The current literature provides evidence that if translation initiation is inhibited then an mRNA is stable and not a substrate for degradation by NMD (Dang et al., 2009; Low et al., 2005; Zuk and Jacobson, 1998). Additionally, translation elongation inhibitors such as cycloheximide and puromycin also block degradation of substrates by NMD (Carter et al., 1995; Herrick et al., 1990; Noensie and Dietz, 2001; Zhang et al., 1997). Finally, if translation proceeds past the ORF stop codon (i.e. stop codon readthrough/nonsense suppression) then NMD is also inhibited and the mRNA is stable (Keeling et al., 2004). Further, mutations in any of the three NMD *trans*-acting factors (Upf1, Upf2, or Upf3) effectively reduce the efficiency of translation termination (Maderazo et al., 2000; Wang et al., 2001). Importantly, it was previously shown that recognition of a nonsense codon in an mRNA leads to an overall decrease in the translational efficiency of the mRNA as a reporter carrying a nonsense mutation yielded significantly higher levels of protein in *upf1Δ* strains compared to wild-type (Muhlrad and Parker, 1999).

Together this information led us to the hypothesis that if the *SSY5* mRNA is being recognized as an NMD substrate, and the mRNA is protected at a point after recognition, then inactivation of the NMD pathway (*upf1Δ* strains) should show significantly higher Ssy5 levels compared to wild-type strains. To test this hypothesis we utilized our SSY5-GFP reporter strains that were constructed above (Fig. 8 and Fig 9). The reporter construct containing the GFP tag in-frame just before the *SSY5* mRNA stop codon (Reporter #1) was transformed into NMD+ (wild-type strains used previously) and *nmd-*

(*upf1Δ*) strains. Total protein extract was analyzed for Ssy5-GFP, which should produce bands at 104.4 kDa and 62.2 kDa (Fig. 10, lanes 2 and 3 asterisks). Nonspecific cross-reacting bands in all samples are shown to verify loading. We were able to detect the specific Ssy5-GFP product in the NMD+ and nmd- strains, however, there does not appear to be a significant difference in the amount of protein present between the two strains (Fig. 10, lanes 2 and 3 asterisks). These results indicate that the NMD pathway is not likely playing a role in the translation of the *SSY5* mRNA. Thus, the *SSY5* mRNA is probably not being recognized as an NMD substrate, which is consistent with the rest of our results.

SSY5 mRNA is not protected from NMD by RNA binding protein Pub1

Finally, we wanted to determine if *SSY5* mRNA is protected from NMD due to the interaction of *trans*-acting factors. The search for potential candidates is a cumbersome task as the pool RNA binding proteins (RBPs) is quite large and there is no collective database that identifies specific binding motifs for the different RBPs. Additionally, it may be an indirect interaction that could be responsible for *SSY5* mRNA protection from NMD in which case identifying RBP binding motifs present in the *SSY5* mRNA would be uninformative. However, a recent study showed that poly(U) binding protein (Pub1) is able to protect two wild-type mRNAs, *GCN4* and *YAP1*, from degradation by NMD (Ruiz-Echevarria and Peltz, 2000). These two mRNAs are targeted for degradation by NMD through the presence of translated uORFs. It was shown that both *GCN4* and *YAP1* mRNAs contain stabilizer elements (STEs) in their 5' UTR which

binds Pub1. The binding of Pub1 protects the mRNA from degradation by NMD (Ruiz-Echevarria and Peltz, 2000).

Using this information we hypothesized that Pub1 could play a role in *SSY5* protection from NMD. To determine if Pub1 is protecting *SSY5* mRNA from degradation by NMD, we analyzed steady-state *SSY5* mRNA accumulation in wild-type, *upf1Δ*, *pub1Δ*, and *upf1Δpub1Δ* strains when cells are grown in rich media (YAPD). Quantitative Northern analysis reveals no significant differences in *SSY5* mRNA accumulation in *pub1Δ* (FCR of *pub1Δ*/wild-type =1.0±0.2) or *upf1Δ pub1Δ* (FCR of *upf1Δpub1Δ*/wild-type =1.2±0.2) cells compared to wild-type cells (Fig. 11A). We also tested the possibility that Pub1 could be protecting *SSY5* mRNA only under nutrient-limiting conditions by analyzing steady-state *SSY5* mRNA accumulation in wild-type, *upf1Δ*, and *pub1Δ* strains when cells are grown in minimal media (amino-acid limiting). We observed the same trend in this condition with no significant difference in *SSY5* mRNA accumulation in *pub1Δ* (FCR of *pub1Δ*/wild-type =1.0±0.2) cells compared to the wild-type cells (Fig. 11B).

Further, we confirmed that there is no change in *SSY5* mRNA stability in wild-type vs *pub1Δ* cells by determining the $T_{1/2}$ of *SSY5* mRNA in each of the strains. The *SSY5* mRNA had a $T_{1/2}$ of 10.2±1.2 min in wild-type strains compared to a $T_{1/2}$ of 14.6±1.7 min in *pub1Δ* (Fig. 11C).

From this we conclude that *SSY5* mRNA is not protected from NMD by the trans-acting factor Pub1. However, a much more thorough analysis of RBPs needs to be conducted to sufficiently analyze the role of *trans*-acting factors in the protection of

SSY5 mRNA from NMD. We are in the early stages of developing a screen using the *S. cerevisiae* RBP mutant collection and our *pGAL-GFP-SSY5* constructs developed for the translation analysis of Ssy5 (Fig. 6B).

Discussion

Here we show the novel case of the wild-type *SSY5* mRNA, which has multiple NMD targeting signals but is not degraded by NMD. Importantly, it was previously shown that the long 3' UTR of the *SSY5* mRNA is able to target a reporter mRNA for degradation by NMD (Obenoskey et al., 2014). Because one of the NMD-targeting signals is able to target an mRNA from degradation by NMD we hypothesized that the *SSY5* mRNA is being protected by some mechanism from degradation by NMD. Elucidating the mechanism that is protecting the *SSY5* mRNA from NMD can add valuable clues to understanding the role of NMD in gene regulation of wild-type mRNAs and the potential physiologic consequences of perturbing the NMD pathway (i.e. nonsense suppression and readthrough therapies).

Here we show that replacing the long 3' UTR of the *SSY5* mRNA with the much shorter 3' UTR of the *ADH1* mRNA does not alter the stability of the mRNA from NMD (Fig. 4). We see the same result upon removal of the translated *SSY5* uORF (Fig. 5). These results confirm that the mechanism of protection of the *SSY5* mRNA is not in the *cis*-elements of the 5' or 3' UTR of the *SSY5* mRNA. This also rules out the role of *tans*-acting factors that would be interfering with NMD by binding to a location within the 5' or 3' UTR of the *SSY5* mRNA.

Previous studies have identified several ways in which an mRNA can be protected from degradation by NMD. One of the ways in which an mRNA is protected from NMD is by inhibition of translation (Carter et al., 1995; Dang et al., 2009; Low et al., 2005; Noensie and Dietz, 2001). The *trans*-acting NMD factors (Upf1, Upf2 and Upf3) are activated by a terminating ribosome, thus, the translation machinery must be active for NMD to occur (Amrani et al., 2004; Bertram et al., 2001; Czaplinski et al., 1998; Kisselev et al., 2003; Rospert et al., 2005). If an mRNA is not actively translated the formation of the NMD mRNP will not be initiated. We did expect the *SSY5* mRNA to be translated due to the vital role for the Ssy5 protease in *S. cerevisiae* in maintaining cellular homeostasis by initiating extracellular amino acid uptake via the activation of AAP genes (Fig. 1). We confirmed by polyribosome analysis and fluorescence microscopy that the *SSY5* mRNA is actively translated (Fig. 6). However, endogenous Ssy5 protein levels are quite low as we are unable to detect GFP signal when *SSY5* mRNA is under the control of its native promoter.

We also tested the ideas that the *SSY5* mRNA could be protected from NMD by translational readthrough or by translation reinitiation in the 3' UTR. The stop codon context of the *SSY5* mRNA and the unique features of the *SSY5* mRNA 3' UTR are all conducive to either possibility (Fig. 7). However, we did not find evidence of translational readthrough or translation reinitiation for the *SSY5* mRNA within our detection limits (Fig. 8 and Fig. 9). Further, it was previously shown that yeast mRNAs are destabilized when there is a very low frequency of translational readthrough events on an mRNA (≤0.5%; Keeling et al., 2004). So, if an mRNA is stabilized by translational

readthrough the readthrough must occur at a frequency ≥0.5%, which should yield a detectable product in our Western analysis. In support of this, the majority of studies providing ribosome footprint data do not indicate detectable ribosome footprints within the *SSY5* mRNA 3' UTR (Michel et al., 2014).

We began our search for the involvement of *trans*-acting factors in the protection of the *SSY5* mRNA from NMD by looking at RBP Pub1. Pub1 was previously shown to protect both *GCN4* and *YAP1* mRNAs from degradation by NMD (Ruiz-Echevarria and Peltz, 2000). We show that Pub1 is not involved in the protection of the *SSY5* mRNA from NMD by looking at both steady-state accumulation and mRNA half-lives in wild-type and *pub1Δ* cells (Fig. 11). However, during these experiments we attempted to use the *GCN4* and *YAP1* mRNAs as positive controls. In doing this we were unable to reproduce the result that Pub1 is solely responsible for the protection of *GCN4* and *YAP1* mRNAs from NMD (Chapter 4). One explanation could be that we were not able to obtain the exact strains used in the original study (Ruiz-Echevarria and Peltz, 2000). We did, however, obtain the parent strains for the ones used in the original study and reconstructed the strains, but were still unable to show that Pub1 definitively protects *GCN4* and *YAP1* mRNAs from NMD (data not shown).

For all of our studies, we were careful to take into consideration the physiologic role of Ssy5 when growing the cells (Fig. 1). Because Ssy5 is a critical component of the SPS amino-acid sensing complex, we are aware that changes in the amino acid composition of the media may cause variations in *SSY5* mRNA levels and possibly in susceptibility to NMD. However, we do not find that growing cells in rich media

(YAPD/YEPD) vs minimal media (amino-acid limiting) has any effect on *SSY5* mRNA levels or susceptibility to NMD (Fig. 3 and Fig. 11). Although it is important to note that true amino-acid starvation, which would result in an inactive SPS-sensor and reduce the need for *SSY5* mRNA translation, was not able to be evaluated as the strains used in this study require supplementation with amino acids to support the auxotrophic requirements (Materials and Methods).

Further, in this study, we characterize a new mRNA that can be used as a control in future NMD experiments. The *ENT4* mRNA was previously identified in a screen as an mRNA with a long 3' UTR that is an NMD substrate. Here we show in repeated experiments that the *ENT4* mRNA is an NMD substrate, as steady-state mRNA accumulation is increased by ≥2.0-fold in *upf1Δ* cells compared to wild-type cells and the $T_{1/2}$ is also ≥2.0 fold longer in *upf1Δ* cells compared to wild-type cells (Figs. 3A-C, Fig. 5A, Fig. 6C, Fig. 8C, and Fig. 9C). Thus, the *ENT4* mRNA can serve as a positive NMD control for wild-type gene regulation by NMD.

Here we have presented a novel case for an mRNA that should be targeted for degradation by NMD, but is not, and the protection of the mRNA cannot be explained by current models. Moving forward, it will be valuable to the NMD field to identify how this mRNA is protected from NMD. We have provided the framework for narrowing down the mechanism and now the studies can proceed in a more focused manner. We have also developed valuable tools that can be used as we move forward. Designing a screen for *trans*-acting factors that stabilize/destabilize the *SSY5* mRNA will be very informative, and we now have the GFP constructs to be able to do so. Once stabilizing

trans-acting factors are identified we can then determine whether they protect the *SSY5* mRNA by direct or indirect binding and if the factors provide interference of the NMD mRNP or remodeling of the NMD mRNP. This information will also help identify at which step in the NMD pathway that protection is incurred - before substrate recognition, or after substrate recognition but before mRNA degradation. Determining if the NMD mRNP ever forms on the *SSY5* mRNA would also provide valuable information. In addition to looking further into *trans*-acting factors, it will be crucial to identify if certain *cis*-elements are playing a role. Specifically, changing the start codon context, manipulating the predicted -1 PRF sites, and changing the termination codon context could all provide valuable results.

It is interesting to note that the human fungal pathogen *Candida albicans* also possesses all of the components of the SPS sensor (Martinez and Ljungdahl, 2004). Studies have shown that when *C. albicans* is unable to uptake extracellular amino acids the fungus is not able to establish infection in a mouse model (Martinez and Ljungdahl, 2004). Further, the ability of *C. albicans* to establish infection requires Stp1, Ssy5 and Ssy1 {Davis, 2011 #130. This raises the possibility that the regulation of *SSY5* mRNA is involved in pathogenicity of *C. albicans*. Going forward, it would be interesting to analyze the *SSY5* mRNA and its susceptibility to NMD in *C. albicans*.

Figure legends

Fig. 2-1. Ssy5p, encoded by the *SSY5* mRNA is a major component of the yeast amino acid SPS sensor. The SPS sensor is the primary complex of the yeast SPS-sensing pathway, which is responsible for the sensing and indirect uptake of extracellular amino acids. The SPS sensor consists of the integral membrane protein Ssy1 and two peripheral membrane proteins Ptr3 and Ssy5. Free extracellular amino acids bind to Ssy1 inducing a conformational change, which activates this sensing protein. When Ssy1 becomes activated a signal is transduced via Ptr3 to the serine protease Ssy5. Ssy5 has a large N-terminal Pro-domain and catalytic C-terminal Cat-domain. These two domains are autolytically cleaved but remain noncovalently associated as an inactive complex. When activated, Ssy5 cleaves the N-terminal domain of the cytosolic transcription factors Stp1 and Stp2, which then enter the nucleus and activate the expression of amino acid permease genes required to generate amino acid transporters {Ljungdahl, 2009 #109}(Conrad et al., 2014).

Fig. 2-2. *SSY5* mRNA has multiple NMD-targeting signals. **A**) Schematic representing the *SSY5* mRNA and the approximate location of the NMD-targeting signals. **B**) Ribosome profile of a region of the *SSY5* mRNA 5' leader region showing the compiled data from all ribosome profiling studies. All three reading frames are shown. Start codon methionines are denoted with "M" (green). (✱) Indicates the reading frame of the *SSY5* mRNA uORF and (▲) indicates the reading frame of the *SSY5* ORF. Image was downloaded from the GWIPS-viz browser (Michel et al., 2014).

Fig. 2-3. *SSY5* mRNA is not degraded by NMD. **A**) Northern blot analysis of the steady-state accumulation of the *SSY5* and *ENT4* (a wild-type NMD substrate) mRNAs in wild-type (AAY277) and *upf1Δ* (AAY363) strains in the BY4741 background and in wild-type (AAY187) and *upf1Δ* (AAY320) strains in the W303 background. Strains were grown in rich media (YAPD). *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials. **B**) Northern blot analysis of the *SSY5* and *ENT4* mRNA half-lives in wild-type (AAY277) and *upf1Δ* (AAY363) strains from the BY4741 background. Strains were grown in rich media (YAPD). 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay. **C**) Northern blot analysis of the steady-state accumulation of the *SSY5* and *ENT4* mRNAs in wild-type (AAY277) and *upf1Δ* (AAY363) strains in the BY4741 background. Strains were grown in minimal media (SD + his, leu, met, ura, lys). **D**) Northern blot analysis of the *SSY5* and *ENT4* mRNA half-lives in wild-type (AAY277) and *upf1Δ* (AAY363) strains from the BY4741 background. Strains were grown in minimal media (SD + his, leu, met, ura, lys). 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay and are the average of three independent trials.

Fig. 2-4. Replacing the long 3' UTR of *SSY5* mRNA with the short 3' UTR of the *ADH1* mRNA does not destabilize the mRNA. **A**) Schematic of constructs. The native 475nt-long 3' UTR of the *SSY5* mRNA was replaced with the 180nt 3' UTR of the *ADH1* mRNA. **B**) Northern blot analysis of the steady-state accumulation of the native *SSY5* mRNA in the wild-type strain (AAY277), and the *SSY5+ADH1 3' UTR* mRNA in wild-type [NMD+] (AAY576) and *upf1Δ* (AAY601) strains in the BY4741 background. The *AGP1* mRNA is a downstream target (AAP gene) of Ssy5 activity and is shown to confirm that manipulation of the *SSY5* mRNA 3' UTR did not influence the enzymatic activity of the protein product. Strains were grown in rich media (YAPD). *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type (lane 1); values are an average of three independent trials. **C**) Northern blot analysis of the *SSY5+ADH1 3' UTR* mRNA half-life in wild-type [NMD+] (AAY576) and *upf1Δ* (AAY601) strains. Strains were grown in rich media (YAPD). 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay.

Fig. 2-5. Disrupting the 5' UTR of the *SSY5* mRNA does not destabilize the mRNA. **A**) Northern blot analysis of the steady-state accumulation of the *pGAL-SSY5* mRNA in wild-type [NMD+] (AAY630) and *upf1Δ* (AAY632) strains in the BY4741 background.

The *ENT4* mRNA is a wild-type mRNA that is an NMD substrate. Strains were grown in rich media with galactose (YAPG). *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type and are an average of three independent trials. **B**) Northern blot analysis of the *pGAL-SSY5* mRNA in wild-type [NMD+] (AAY630) and *upf1Δ* (AAY632) strains in the BY4741 background. Strains were grown in rich media with galactose (YAPG). 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay. **C**) Northern blot analysis of steady-state mRNA accumulation of the native *SSY5* mRNA in the wild-type strain (AAY277) and the *pGAL-SSY5* mRNA in the wild-type [NMD+] background (AAY630) to show the increase in accumulation as a result of placing the *SSY5* mRNA under the control of the galactose promoter. Strains were grown in rich media with galactose (YAPG). Fold change values are normalized and determined as a fold change ratio from wild-type. The native *SSY5* mRNA appears as a band at ~2.6 kb while the *pGAL-SSY5* mRNA is slightly larger and appears as a band at ~3.0 kb.

Fig. 2-6. *SSY5* mRNA is translated. **A**) Polyribosome analysis of *SSY5* mRNA (top) and *CYH2* pre-mRNA and mRNA (bottom) in wild-type (AAY187) and *upf1Δ* (AAY320) strains. Strains were grown in YAPD. Whole cell lysate was centrifuged through a 15-50% sucrose gradient. Total RNA was extracted from each fraction collected and total

RNA from fractions 2-19 was transferred to a membrane for Northern analysis. Fractions corresponding to the 40S ribosomal peak are in lanes 4-5, fractions corresponding to the 60S ribosomal peak are in lanes 6-7, the fraction corresponding to the 80S ribosomal peak is in lane 8 and polyribosome fractions are in lanes 9-15. The graph represents the distribution of the mRNA in each fraction based on the relative corrected volume of the mRNA present in each lane. In lanes where two bands were present only the band corresponding to the size of the expected band for the mRNA was quantified (~2.6 kb for *SSY5* mRNA). Polysome analysis was repeated twice with similar results. **B**) Confocal microscopy of haploid cells containing *pGAL-GFP-SSY5* mRNA (AAY561; top) or untagged *SSY5* mRNA (AAY527; bottom). Strains are of the BY4743 background. Cells were grown to saturation in YAPG, washed, fixed in 3.7% formaldehyde and stained with 1.0 µg/ml Hoechst (33258) in the dark. Cells were immediately imaged. Scale bar=5µM. **C**) Northern analysis of steady-state mRNA accumulation of the *pGAL-GFP-SSY5* mRNA to confirm that there were no significant differences in accumulation of the mRNA between wild-type background [NMD+] (AAY568; haploid of AAY561) and *upf1Δ* (AAY625) strains. Strains were grown in YAPG. Fold change values are normalized and determined as a fold change ratio from wild-type and are an average of three independent trials.

Fig. 2-7. The *SSY5* mRNA 3' UTR is a good candidate for both stop codon readthrough and reinitiation of translation. The 475nt sequence of the *SSY5* mRNA 3' UTR. The ORF stop codon (TGA) is in red with a single underline. Triplet nucleotide codons are

highlighted in alternating gray and black corresponding to the main ORF reading frame. The sequence underlined with a dotted blue line and terminating at the TAA stop codon with a double red underline is the sequence that would be translated if stop codon readthrough of the ORF TGA codon were to occur. The ATG in green is a downstream start codon that could be used for the ribosome to reinitiate translation. Sequence in pink that terminates with the TAG codon in red is the sequence that would be translated if reinitiation were to occur in the *SSY5* mRNA 3' UTR.

Fig. 2-8. There is no evidence of *SSY5* mRNA stop codon readthrough. **A**) Schematic of the reporter constructs that were made along with a sequence snapshot to show exactly which reading frame the GFP sequence was added in (arrows). Reporter #1 was made by placing the GFP sequence in-frame just before the *SSY5* mRNA TGA stop codon. Reporter #2 was made by placing the GFP sequence in-frame just before the stop codon that would be used if translational readthrough were to occur. The dark grey box represents the *SSY5* mRNA ORF while the light gray box represents the additional sequence that would be translated should readthrough occur. **B**) Western blot analysis of the Ssy5-GFP product from each strain; untagged Ssy5, lane 1 (AAY277), Ssy5-GFP before the stop codon (reporter 1), lane 2 (AAY572), Ssy5-GFP to test for readthrough (reporter 2), lane 3 (AAY585). Asterisks indicate where expected bands should be. Schematics show why we see the presence of two bands (at ~104.4 kD and ~62.2 kD) for Ssy5-GFP. The top band is unprocessed Ssy5-GFP while the bottom band is the C-terminal domain of the autolytically cleaved Ssy5-GFP. 40µg of total protein was added

per lane. Non-specific bands serve as an informal loading control; membranes were also stained with Ponceau S (not shown). Western blots were done with three independent trials and results were reproducible. **C**) Northern analysis of steady-state mRNA accumulation of the *SSY5*, *AGP1* and *ENT4* mRNAs in the strains used in part (**B**) to confirm that there were no significant differences in accumulation of the mRNAs between the strains and also to confirm that addition of the GFP tag did not influence the enzymatic activity of Ssy5. *ENT4* mRNA is a wild-type mRNA that is a substrate for NMD and *AGP1* mRNA is a downstream target (AAP gene) of Ssy5. Untagged *SSY5* mRNA appears as a band at ~2.6 kb while the *SSY5-GFP* mRNA is larger and appears as a band at ~2.8 kb. Strains were grown in rich media (YAPD). Fold change values are normalized and determined as a fold change ratio from wild-type (lane 1) and are an average of three independent trials.

Fig. 2-9. There is no evidence of translation reinitiation in the *SSY5* 3' UTR. **A**) Schematic of the reporter constructs that were made along with a sequence snapshot to show exactly which reading frame the GFP sequence was added in (arrows). Reporter #1 is the same as in figure 8 and was made by placing the GFP sequence in-frame just before the *SSY5* mRNA TGA stop codon. Reporter #3 was made by placing the GFP sequence in-frame just before the stop codon that would be used if the ribosome were to reinitiate translation in the small ORF present in the *SSY5* mRNA 3' UTR. The dark grey box represents the *SSY5* mRNA ORF while the light gray box represents the small ORF that would be translated should the ribosome reinitiate translation. **B**) Western blot analysis

of the Ssy5-GFP product from each strain; untagged Ssy5, lane 1 (AAY277), Ssy5-GFP to test for reinitiation (reporter 3), lane 2 (AAY581), Ssy5-GFP before the stop codon (reporter 1), lane 3 (AAY572). Asterisks indicate where expected bands should be. Here we only show the bottom band of the Ssy5-GFP (~62.2 kD), which is the C-terminal domain of the autolytically cleaved Ssy5-GFP, and the bottom portion of the membrane because the reinitiation product is very small with an expected size of ~36.6 kD. 40µg of total protein was added per lane. Non-specific bands serve as an informal loading control; membrane was also stained with Ponceau S (not shown). Western blots were done with three independent trials and results were reproducible. **C**) Northern analysis of steady-state mRNA accumulation of the *SSY5*, *AGP1* and *ENT4* mRNAs in the strains used in part (**B**) to confirm that there were no significant differences in accumulation of the mRNAs between the strains and also to confirm that addition of the GFP tag did not influence the enzymatic activity of Ssy5. *ENT4* mRNA is a wild-type mRNA that is a substrate for NMD and *AGP1* mRNA is a downstream target (AAP gene) of Ssy5. Untagged *SSY5* mRNA appears as a band at ~2.6 kb while the *SSY5-GFP* mRNA is larger and appears as a band at ~2.8 kb. Strains were grown in rich media (YAPD). Fold change values are normalized and determined as a fold change ratio from wild-type (lane 1) and are an average of three independent trials.

Fig. 2-10. *SSY5* mRNA is translated equally efficient in wild-type and *upf1Δ* strains. Western analysis of untagged Ssy5 (AAY277), and Ssy5-GFP in wild-type (AAY572) and *upf1Δ* (AAY623) strains to determine if NMD has an influence on the translation

efficiency of the *SSY5* mRNA. The construct used in this analysis was reporter #1 from figures 8 and 9 so the expected sizes are the same (at ~104.4 kD and ~62.2 kD) and are indicated with red asterisks. 40µg of total protein was added per lane. Non-specific bands serve as an informal loading control; membranes were also stained with Ponceau S (not shown). Western blots were done with three independent trials and results were reproducible.

Fig. 2-11. *SSY5* mRNA is not protected from NMD by *trans*-acting factor Pub1. **A**) Northern blot analysis of the steady-state accumulation of *SSY5* mRNA in wild-type (AAY277), *upf1Δ* (AAY363), *pub1Δ* (AAY538), and *pub1Δupf1Δ* (AAY590) strains in the BY4741 background. Strains were grown in rich media (YAPD). *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials. **B**) Northern blot analysis of the steady-state accumulation of *SSY5* mRNA in wild-type (AAY277), *upf1Δ* (AAY363), and *pub1Δ* (AAY538) strains in the BY4741 background. Strains were grown in minimal media (SD + his, leu, met, ura, lys). **C**) Northern blot analysis of *SSY5* mRNA half-lives in wild-type (AAY277) and *pub1Δ* (AAY538) strains in the BY4741 background. Strains were grown in minimal media (SD + his, leu, met, ura, lys). 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA

remaining at each time point during the exponential decay and are the average of three independent trials.

Figure 2-1

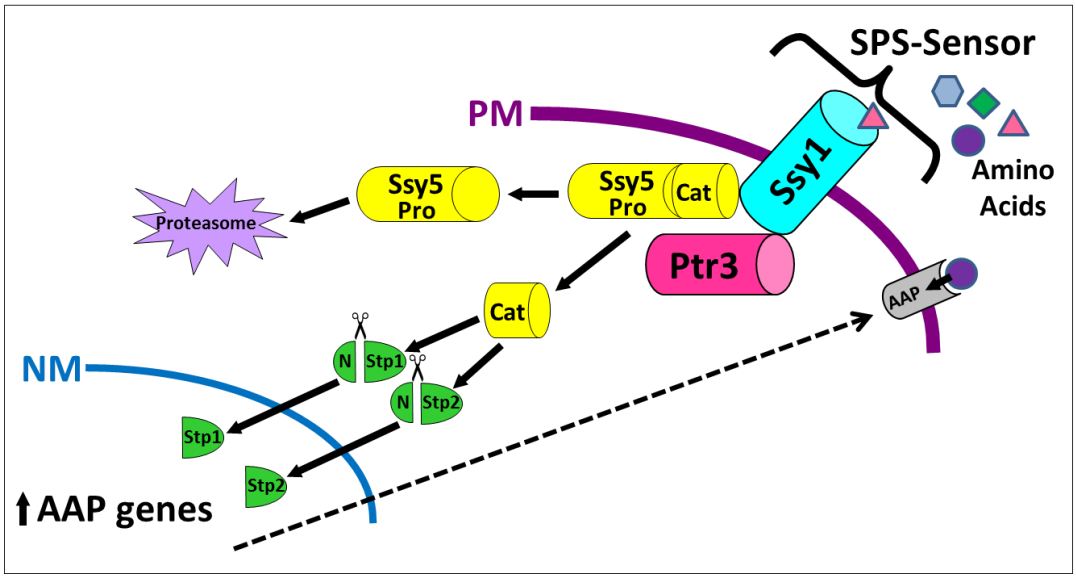
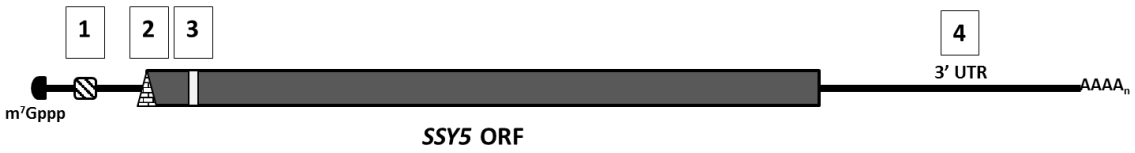


Figure 2-2

A.



NMD-targeting signals in *SSY5* mRNA:	
1	Presence of a translated upstream open reading frame (uORF)
2	Start codon in a poor context ($A_{UG}CAI_{(r)}$ =.375)[1]
3	Five predicted -1 programmed ribosome frameshift (PRF) sites (+18, 132, 324, 333, 1728)
4	Long 3′ untranslated region (UTR): 384-464bp

B.

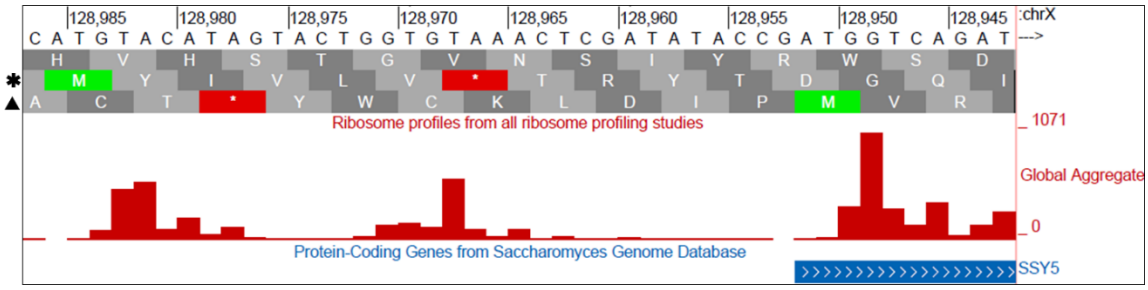


Figure 2-3

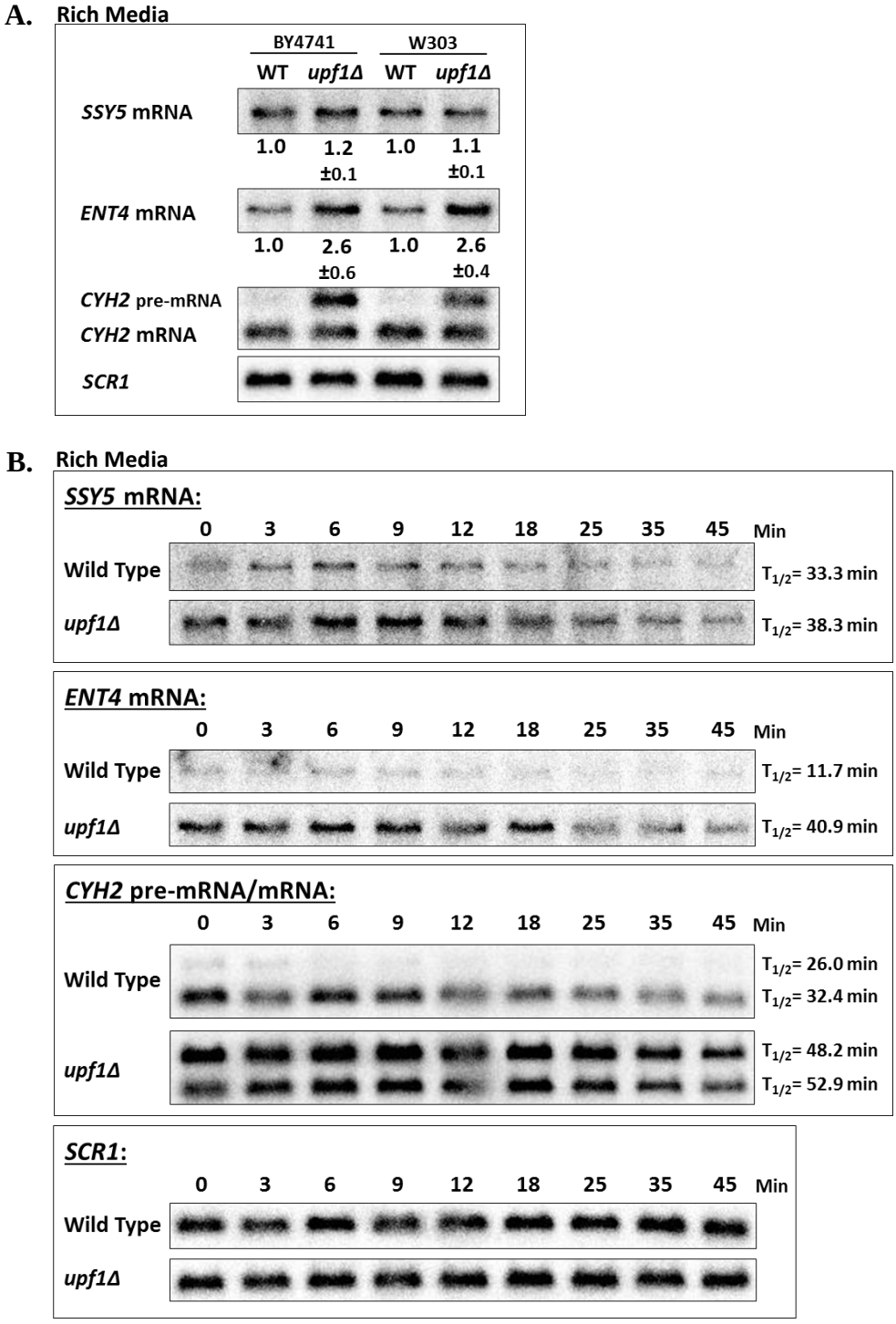
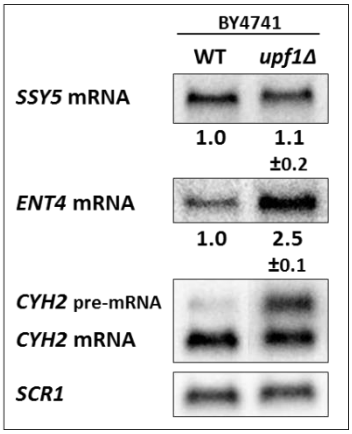
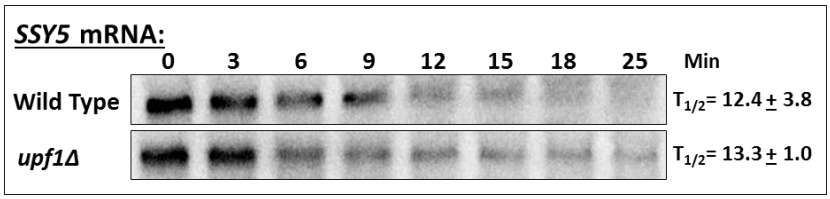


Figure 2-3 (continued)

C. Minimal Media



D. Minimal Media



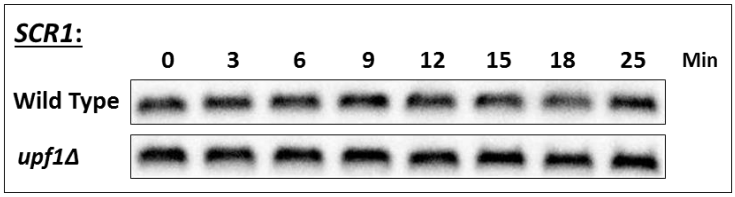


Figure 2-4

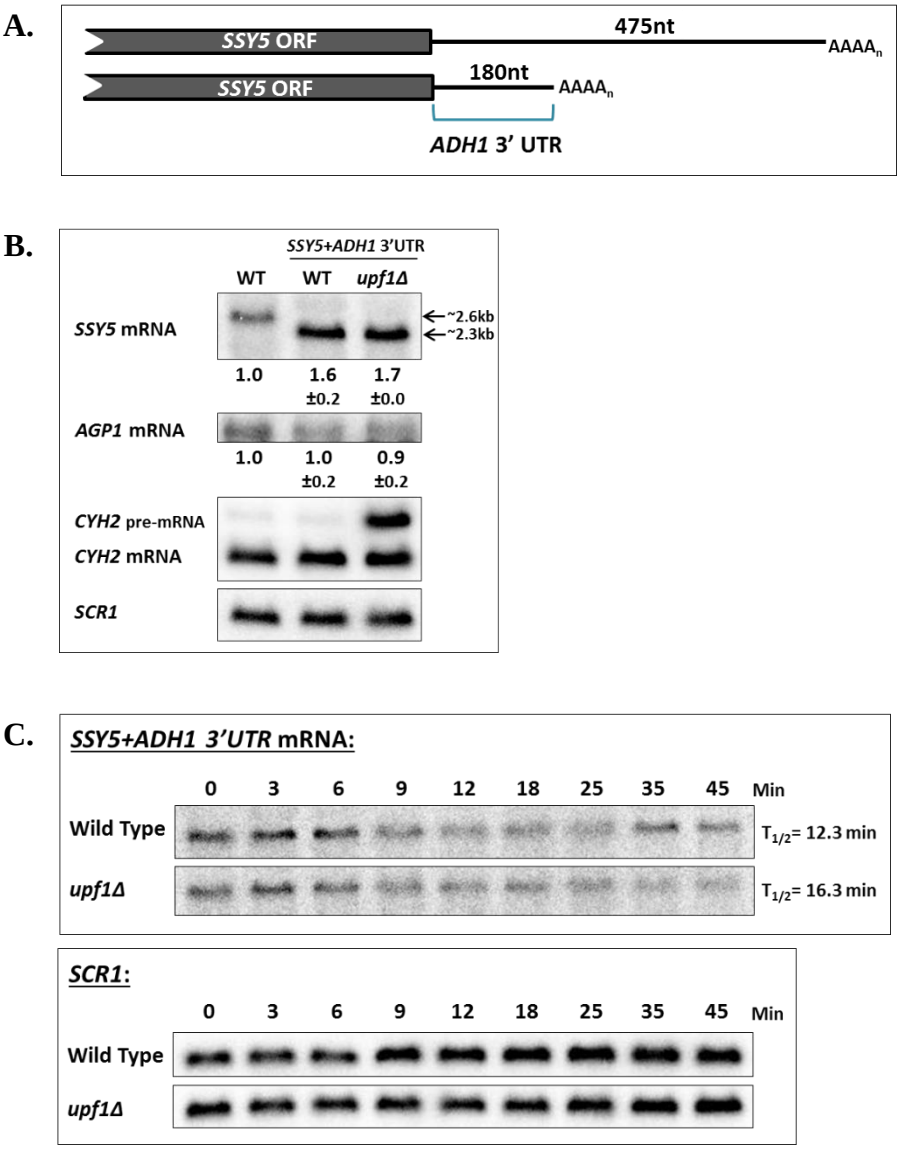


Figure 2-5

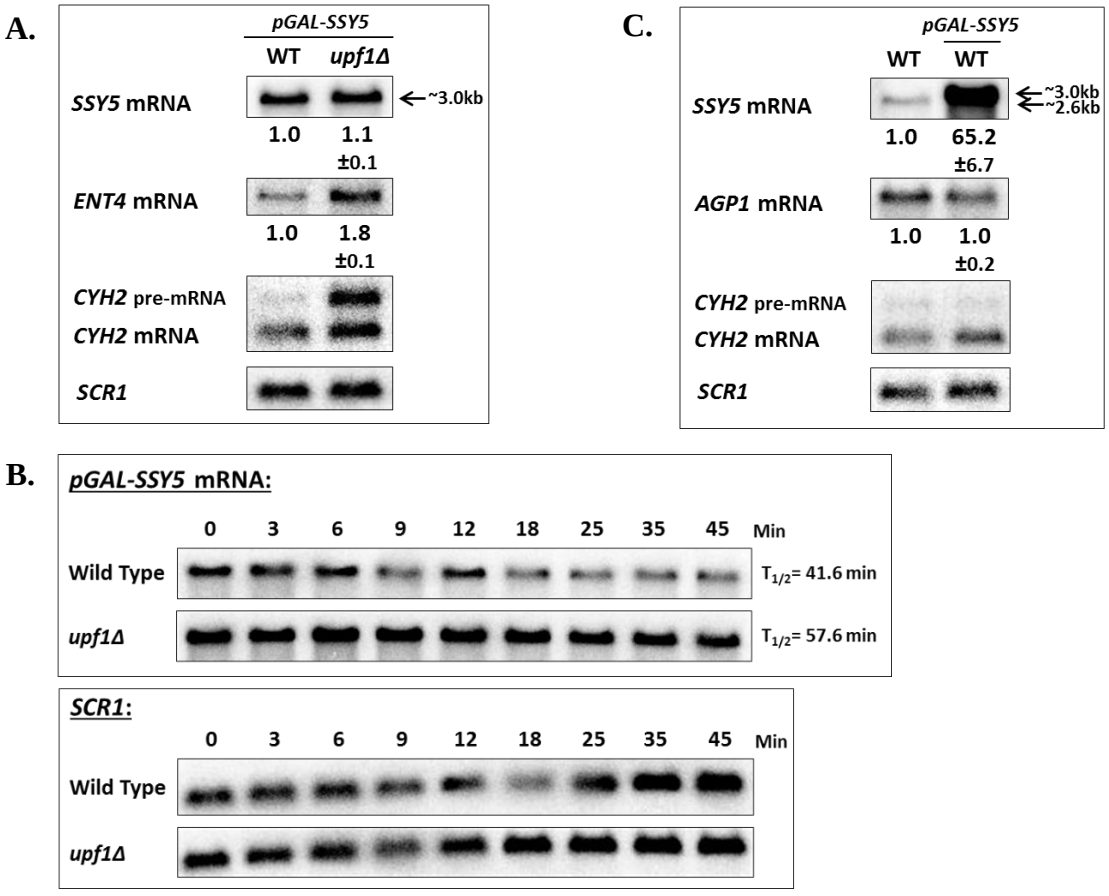
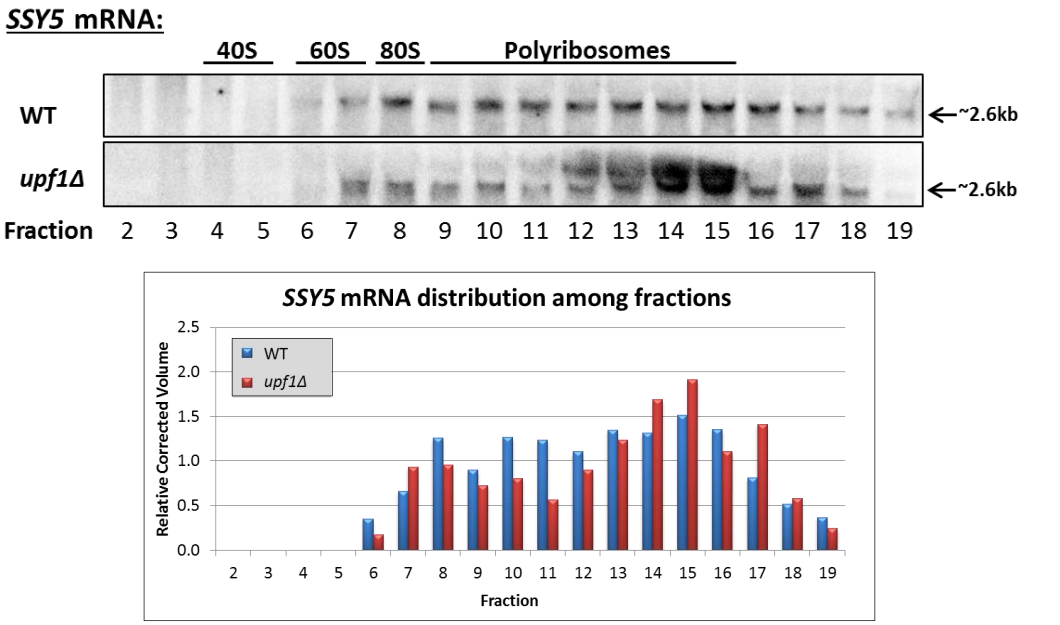


Figure 2-6

A.



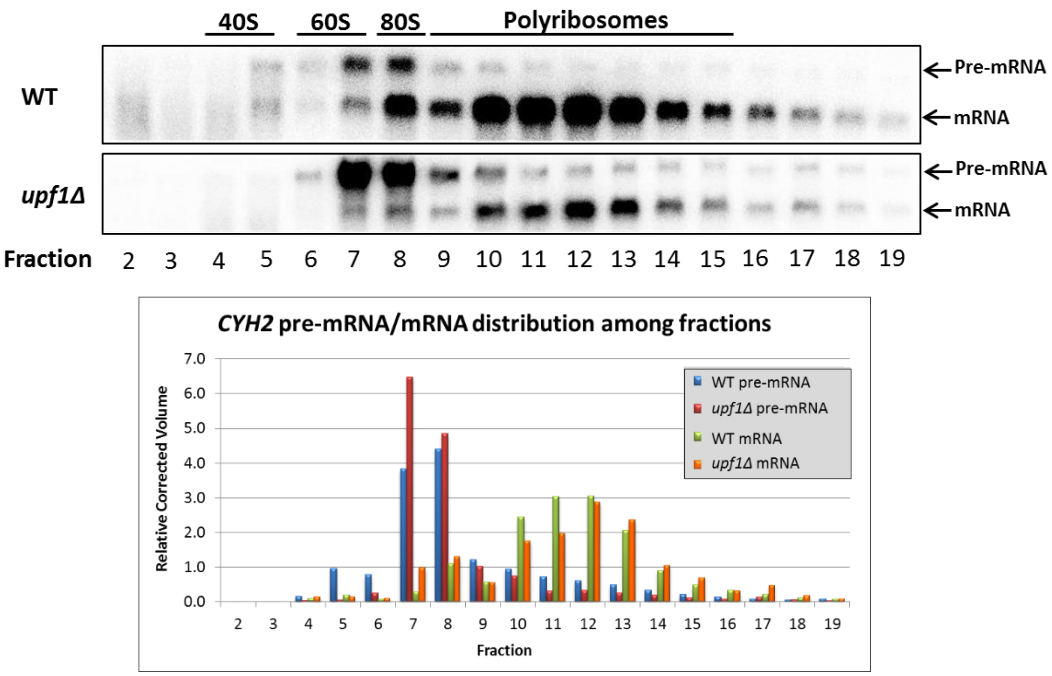
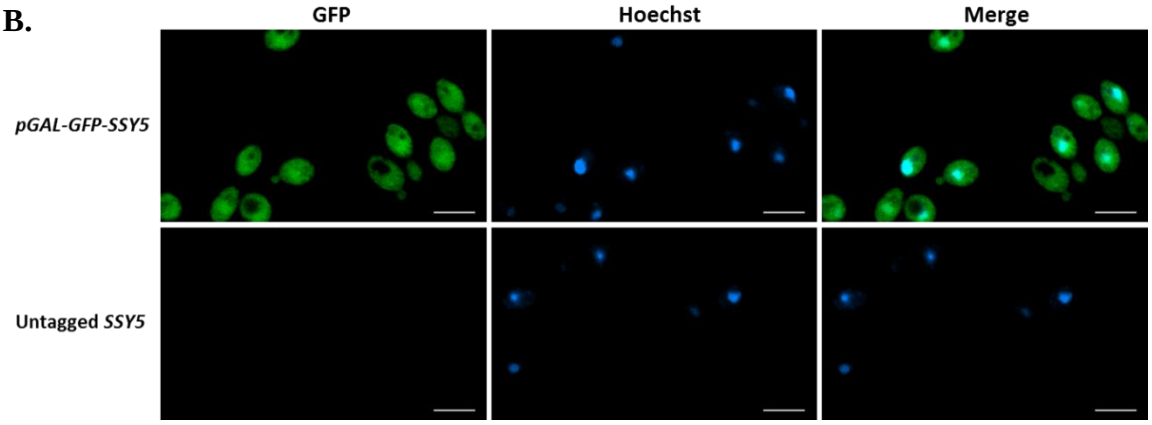


Figure 2-6 (continued)

B.



C.

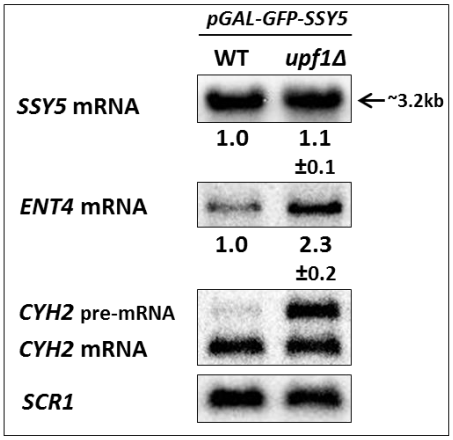


Figure 2-7

Figure 2-8

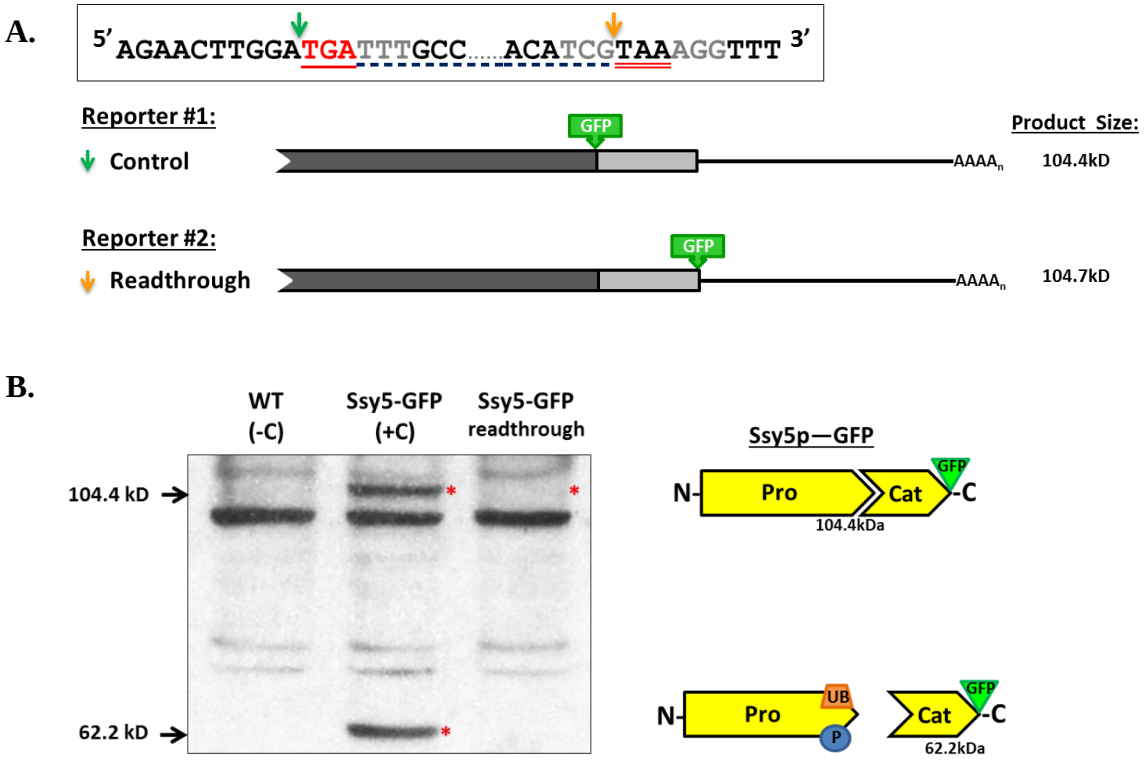
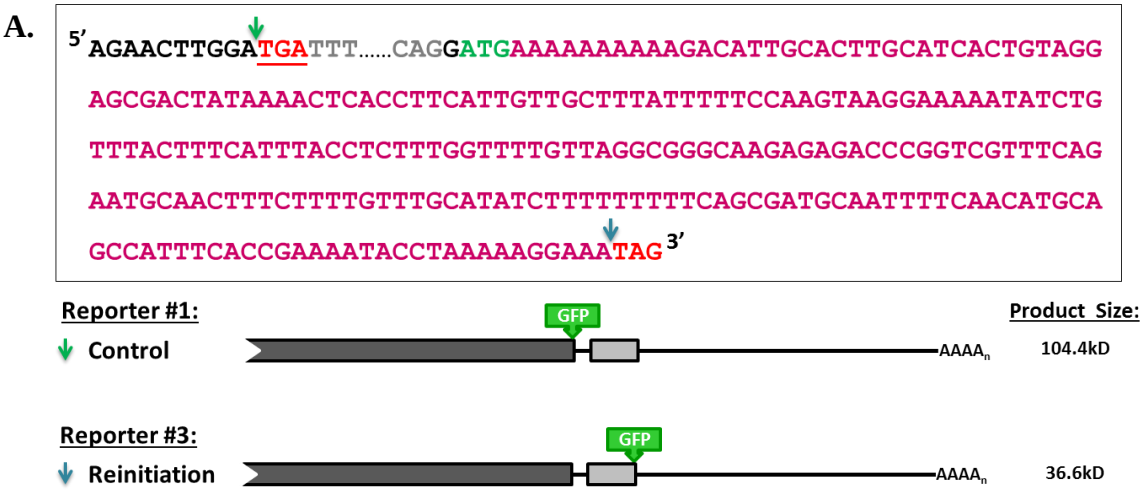
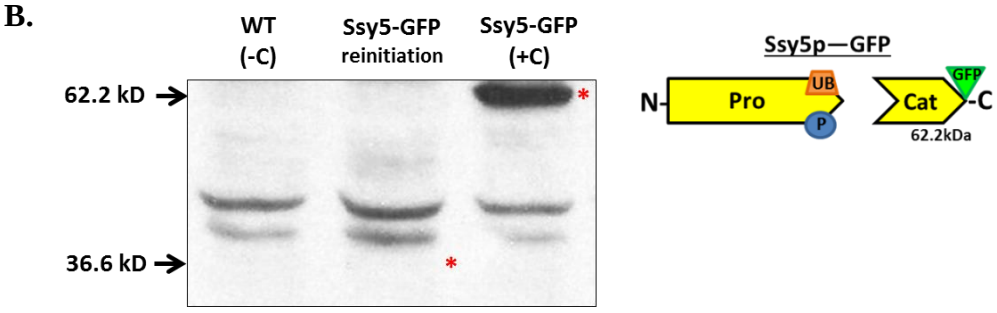


Figure 2-9





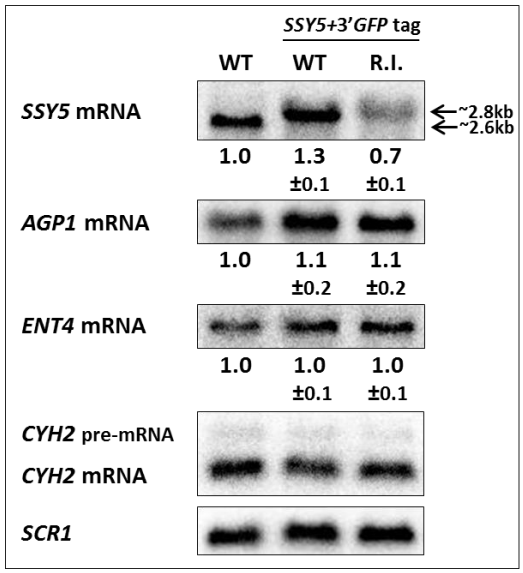


Figure 2-10

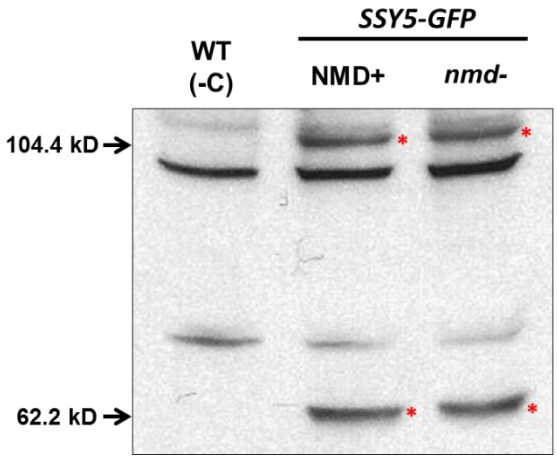


Figure 2-11

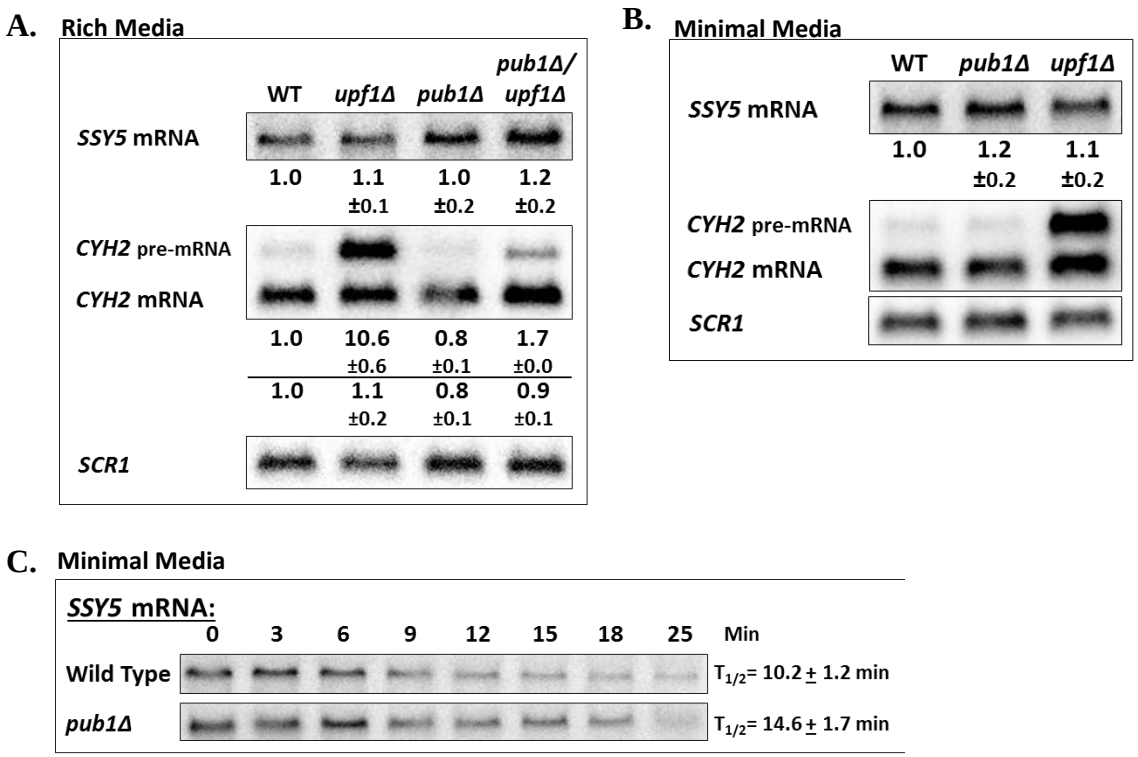


Table 2-1. Strains used in this study

Strain	Parent Strain	Genotype	Source
W303		*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Ralser *et al.*, 2012
BY4741		*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winston *et al.*, 1998
AAY320	W303	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1::URA3*	Atkin lab
AAY363	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 upf1::URA3*	Atkin lab
BY4743		*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 lys2Δ0/LYS2 [suc2Δ/suc2Δ]*	Winston *et al.*, 1998
AAY538	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pub1Δ*	Open Biosystems
AAY561*	BY4743	*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 lys2Δ0/LYS2 [suc2Δ/suc2Δ] pGAL-GFP-HISMX6-SSY5/SSY5*	Atkin lab
AAY568*	AAY561*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pGAL-GFP-HISMX6-SSY5*	Atkin lab
AAY572*	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GFP-HISMX6-SSY5 before ORF stop codon*	Atkin lab
AAY576*	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SSY5 3'UTR::ADH1 3'UTR(HIS3)*	Atkin lab
AAY581*	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GFP-HISMX6-SSY5 before reinitiation stop codon*	Atkin lab
AAY585*	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GFP-HISMX6-SSY5 before readthrough stop codon*	Atkin lab
AAY590*	AAY538	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pub1Δ upf1::URA3*	Atkin lab
AAY601*	AAY576*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 upf1::URA3 SSY5 3'UTR::ADH1 3'UTR(HIS3)*	Atkin lab
AAY623*	AAY572*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 upf1::URA3 GFP-HISMX6-SSY5 before ORF stop codon*	Atkin lab
AAY625*	AAY568*	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 upf1::URA3 pGAL-GFP-HISMX6-SSY5*	Atkin lab
AAY630*	BY4743	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pGAL-HISMX6-SSY5*	Atkin lab
AAY632*	AAY630*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 upf1::URA3 pGAL-HISMX6-SSY5*	Atkin lab
* = new strain created for this study			

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CHAPTER 3

SSY5 mRNA is decay predominantly 5' to 3'

Abstract

The degradation of mRNAs contributes significantly to the regulation of gene expression as all mRNAs are subject to turnover. In *Saccharomyces cerevisiae*, most cytoplasmic wild-type mRNAs are decapped in a deadenylation-dependent manner and subsequently degraded by the 5'→3' exonuclease Xrn1. Alternatively, mRNAs can be degraded 3'→5' by the exosome complex, albeit at a much slower rate. mRNAs that are targeted for degradation by nonsense-mediated mRNA decay (NMD) are distinguished from wild-type mRNAs at the early stages of mRNA decay. NMD substrates are rapidly decapped in a deadenylation-independent manner and degraded 5'→3' by Xrn1 as well as being rapidly deadenylated. The *SSY5* mRNA is a wild-type mRNA in *S. cerevisiae* with multiple NMD-targeting signals but is not degraded by NMD. We hypothesized that investigating the genetic requirements for *SSY5* mRNA decay would provide insight into how this mRNA is degraded and when protection from NMD is incurred. Here we show that the *SSY5* mRNA is predominantly degraded 5'→3' because blocking Dcp1-mediated decapping and Xrn1-mediated 5'→3' degradation results in increased accumulation and stability of the *SSY5* mRNA. In contrast, blocking deadenylation does not cause a significant change in *SSY5* mRNA accumulation. We also show that the 3' end of the *SSY5* mRNA is longer in wild-type and *upf1Δ* strains but is shortened in strains where decapping or 5'→3' exonucloelytic degradation is blocked suggesting that *SSY5* mRNA is deadenylated prior to 5'→3' degradation.

Introduction

In all organisms the fidelity of gene expression is a crucial aspect of cell health and survival. Aberrant gene expression often result in deleterious consequences for the organism. There are several mechanisms that act coordinately to ensure proper gene expression. Two of these mechanisms are the opposing processes of mRNA transcription and mRNA degradation.

In yeast, wild-type mRNAs are typically degraded in the cytoplasm by a deadenylation-dependent mechanism that begins with shortening of the 3' poly(A) tail to an oligo(A) length of 10-12 nt by the Ccr4/Pop2/Not and Pan2/Pan3 deadenylase complexes (Brown and Sachs, 1998; Decker and Parker, 1993; Tucker et al., 2002; Tucker et al., 2001; Wahle and Winkler, 2013). Deadenylation is the rate-limiting step in wild-type mRNA decay (Decker and Parker, 1993). Deadenylation of the mRNA disrupts the circularization of the mRNP and exposes the 7-methylguanosine 5' cap of the mRNA. The coupling of translation termination to deadenylation is suggested by the observation that translation termination factor eRF3 directly interacts poly(A) binding protein, Pab1 (Cosson et al., 2002). Additionally, disruption of the eRF3-Pab1 interaction leads to defective deadenylation (Funakoshi et al., 2007; Kobayashi et al., 2004). However, it has also been shown that mRNAs that never initiate translation are also deadenylated, so translation termination does not appear to be a prerequisite for deadenylation (Beelman and Parker, 1994; Muhlrad et al., 1995).

For wild-type mRNAs, deadenylation subsequently leads to rapid decapping of the mRNA by the Dcp1/Dcp2 holoenzyme complex (Coller and Parker, 2004).

Additionally, wild-type mRNAs with poly(A) tails are resistant to decapping, and this resistance depends on the presence of Pab1 (Beilharz and Preiss, 2007; Gallie, 1991; Wilusz et al., 2001). Removal of the 5' cap generates a 5' monophosphate on the mRNA end allowing the rapid 5'→3' mRNA degradation by the exonuclease Xrn1, which is the predominant pathway for mRNA degradation in yeast (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1995; Muhlrad and Parker, 1994). Alternatively, mRNAs can also be degraded 3'→5' by the exosome complex (Fig.1; Anderson and Parker, 1998; Mangus and van Hoof, 2003; Mitchell and Tollervey, 2003).

In addition to wild-type mRNA turnover, eukaryotes have a conserved surveillance mechanism known as the nonsense-mediated mRNA decay pathway (NMD) that also plays a large role in the fidelity of gene expression (Bedwell et al., 1997; Conti and Izaurralde, 2005; Hall and Thein, 1994; Hentze and Kulozik, 1999; Maquat and Carmichael, 2001; Maquat and Serin, 2001; Pulak and Anderson, 1993). This mRNA decay pathway is responsible for removing mRNAs that contain premature termination codons (PTCs) from the translation pool before they lead to the buildup of truncated proteins which could have deleterious effects (Gonzalez et al., 2001; Isken and Maquat, 2008; Muhlemann et al., 2008). Importantly, in addition to degrading transcripts with PTCs the NMD pathway is also responsible for the regulation of a large portion of wild-type mRNAs as well. Previous work shows that ~5-20% of the yeast, *Drosophila,* and human transcriptomes are affected upon inactivation of NMD (Guan et al., 2006; He et al., 2003; Johansson et al., 2007; Lelivelt and Culbertson, 1999; Mendell et al., 2004). Several mechanisms have been identified that target a wild-type mRNA for degradation

by NMD. These include: 1) a long 3' UTR (Amrani et al., 2004; Kebaara and Atkin, 2009; Muhlrad and Parker, 1999), 2) translation of an upstream open reading frame (uORF; Amrani et al., 2006; Barbosa et al., 2013; Guan et al., 2006; Nyiko et al., 2009), 3) a start codon in a suboptimal context which can lead to leaky scanning and out of frame initiation of translation (Welch and Jacobson, 1999), and 4) the presence of programmed ribosome frameshift (PRF) sites (Plant et al., 2004). The execution of NMD for both PTC-containing substrates and wild-type substrates requires the three *trans*-acting factors Upf1, Upf2 and Upf3. Mutations or deletions in any one of the genes encoding these factors causes significant accumulation (≥2.0 fold) of NMD substrates (Cui et al., 1995; He et al., 1997; He and Jacobson, 1995; Lee and Culbertson, 1995; Lelivelt and Culbertson, 1999; Maderazo et al., 2000).

Degradation of NMD substrates is different from that of wild-type mRNAs in that NMD-substrates are rapidly decapped by Dcp1/Dcp2 without prior removal of the poly(A) tail (Beelman et al., 1996; Cao and Parker, 2003; Hagan et al., 1995). This deadenylation-independent decapping is likely due to improper termination of translation in which the ribosome terminates too far from the poly(A) tail to interact with Pab1. This is supported by the observation that tethering Pab1 close to a prematurely terminating ribosome is able to block decapping (Coller et al., 1998). Additionally, wild-type mRNAs with long 3' UTRs are substrates for degradation by NMD (Amrani et al., 2004; Kebaara and Atkin, 2009; Muhlrad and Parker, 1999), and the presence a long 3' UTR would mimic the context of a prematurely terminating ribosome placing the ribosome too far from the poly(A) tail to interact with Pab1 (Amrani et al., 2004). After decapping the

mRNA is subjected to rapid 5'→3' degradation by Xrn1 and accelerated deadenylation by the deadenylase complexes (Cao and Parker, 2003). However, if decapping or 5'→3' decay is blocked NMD substrates can also undergo 3'→5' decay via the exosome, but this occurs at a much slower rate (Fig. 1; Cao and Parker, 2003; Mitchell and Tollervey, 2003; Muhlrad and Parker, 1994).

Previously, we showed that an mRNA in *Saccharomyces cerevisiae*, the *SSY5* mRNA, has multiple NMD-targeting signals but is not degraded by the NMD pathway (Kebaara and Atkin, 2009; Obenoskey et al., 2014; Chapter 2). We have shown that the protection of the *SSY5* mRNA from NMD is not explained by any of the known mechanisms of protection. Thus, this mRNA is likely protected by a novel mechanism. Determining the mechanism of protection of the *SSY5* mRNA from NMD will add valuable insight into the underlying gene regulatory mechanisms by NMD.

Since we have previously ruled out the known mechanisms of protection from NMD for the *SSY5* mRNA we have sought to determine how the *SSY5* mRNA is degraded. The comparison of *SSY5* mRNA decay with decay patterns of known NMD substrates (*ENT4* mRNA and *CYH2* pre-mRNA) and non-NMD substrates (*PGK1* mRNA and *CYH2* mRNA) will provide clues as to how protection from NMD occurs. Here we show that the *SSY5* mRNA decay is predominately 5'→3' because mutations in the genes encoding the enzymes required for decapping (Dcp1) and 5'→3' mRNA decay (Xrn1) result in the accumulation and stabilization of the *SSY5* mRNA. In contrast, mutations in genes encoding components of the deadenylation complexes have no effect on *SSY5* mRNA accumulation. We also show that the *SSY5* mRNA is likely deadenylated prior to

decapping because the predominant 3' ends of the *SSY5* mRNA in *dcp1Δ* and *xrn1Δ* strains are significantly shorter than in the wild-type and *upf1Δ* strains. This is the same pattern observed for the predominant 3' ends of the *PGK1* mRNA, which is not degraded by NMD. Alternatively, the predominant 3' ends of the *ENT4* mRNA, which is degraded by NMD, are longer in the *dcp1Δ* and *xrn1Δ* strains than in the wild-type and *upf1Δ* strains. Finally, we compare the susceptibility of the *SSY5* mRNA to NMD in different genetic backgrounds and show that protection of the *SSY5* mRNA from NMD may be background-dependent. In the commonly used BY4741 and W303 genetic backgrounds *SSY5* mRNA shows no difference in accumulation between wild-type and *upf1Δ* strains. However, in a different genetic background, similar to W303, the *SSY5* mRNA actually has a FCR of 1.9±0.2 between wild-type and *upf1Δ* strains. This suggests that slight changes in genetic background may influence the protection of the *SSY5* mRNA from NMD.

Materials and Methods

Yeast strains

The yeast strains used in this study are listed in Table 3-1. All yeast transformations were done using Lithium Acetate-Mediated transformation as previously described (Gietz and Woods, 2002). AAY589 was constructed by transforming AAY360 with the *upf1Δ2* fragment from pAA70 using primers oAA48 and oAA79. AAY594, AAY595, AAY596, AAY609, AAY610, AAY611 and AAY621 were constructed by

transforming the corresponding parent strain (listed in Table 3-1) with the *upf1Δ6* fragment from pAA167 using primers oAA48 and oAA79.

Growth conditions

Unless otherwise noted yeast cells were grown using standard techniques with mild agitation equivalent to 225rpm at 30˚C. All strains were grown in YAPD media, which consists of: 1% yeast extract, 2% Bacto-Peptone, 2% dextrose, and 100mg/L Adenine hemisulfate salt. Strains were maintained on YAPD from initial plating from frozen stocks and all through subsequent liquid cultures.

RNA Extractions

Yeast strains were grown in 10mL cultures to an OD_{600} of 0.4-0.6. Cells were harvested by centrifugation, washing in DEPC-ddH_20, and flash-freezing in dry ice/ethanol or liquid nitrogen. Cell pellets were stored at -70˚C until used for RNA extractions. RNA extractions were performed as previously described (Kebaara et al., 2012). RNA samples were diluted to 1µg/µl in DEPC-ddH_20 and stored at -70˚C. RNA quality check gels are performed for every RNA sample (1µl of 1µg/µl Total RNA is run through a 0.8% agarose gel to check for degradation).

Quantitative Northern Analysis

10µg of Total RNA mixed with 3µl Formaldehyde loading dye (Ambion, cat. no. 8552) was separated through a 1.0% agarose gel containing 5.6% Formaldehyde and 1%

MOPS (10X MOPS: 0.2 M sodium morpholinopropanesulfonic acid (MOPS), pH 7.0, 0.05 M sodium acetate, 0.01 M EDTA; adjust pH to 7.0 with 10 M NaOH, do not autoclave, store at room temperature in the dark.) RNA was transferred onto a GeneScreen Plus membrane (PerkinElmer) using NorthernMax transfer buffer (Ambion, cat. no. 8672) following the manufacturer's protocol for downward transfer. The lane with the RNA ladder was cut off of the gel before transfer and stained overnight in 0.5µg/ml ethidium bromide. Membranes were rinsed in 2X SSC and dried for 15 minutes at 80˚C.

Membranes were hybridized with NorthernMax prehybridization/hybridization buffer (Ambion # 8677). ^{32}P-labelled probes were synthesized using ~25ng of PCR product corresponding to the gene of interest, the RadPrime DNA Labeling System® (Invitrogen #18428-011), and ~50 µCi [α-32P]dCTP (3000 Ci/mmol, 10 mCi/ml) (Perkin Elmer) following the manufacturer's protocol. Probes are purified through a Sephadex G-50 column equilibrated with TE pH 8.0. Membranes were hybridized overnight (12-24 hours) and then washed once at room temperature with 2X SSPE and once at 65˚C with 2X SSPE/2% SDS. Membranes were PhosphorImaged™ (GE Healthcare, Typhoon FLA 9500) and quantified using the ImageQuant™ software. All membranes were also autoradiographed using a phosphorescent ruler to determine the size of the bands by comparison to the RNA ladder. Membranes are stripped and stored at -20˚C for re-probing. Detailed protocol for Northern analysis can be found in Kebaara *et al.* (2012).

Poly(A) tail length analysis

This experiment was performed as described in the protocol provided with the Poly(A) Tail-Length Analysis Kit® (Affymetrix 76455). Prior DNase treatment of RNA samples was done using the TURBO DNA-*free* kit® (AM1907). Primers were designed using the information provided in the Saccharomyces Genome Database for predicted polyadenylation sites. Since all of the mRNAs we were interested in contained multiple predicted poly(A) sites, the primers pairs were designed to anneal upstream of the polyadenylation site that is predicted to be most 3' of the other predicted sites. PCR products were resolved on a 2.5% TAE agarose gel.

Results

***SSY5* mRNA accumulation and half-life are significantly increased in *xrn1Δ* cells**

The predominant decay route for both wild-type mRNAs and NMD substrates is through Xrn1-mediated 5'→3' exonucloelytic degradation following removal of the 5' mRNA cap, although this process tends to occur more rapidly for NMD substrates (Cao and Parker, 2003). Given this, we hypothesized that the *SSY5* mRNA is likely degraded 5'→3' by Xrn1, so *SSY5* mRNA levels should significantly increase in an *xrn1Δ* strain, but we are unsure as to what extent since we do not know the contribution of 3'→5' decay for the *SSY5* mRNA. If the *SSY5* mRNA accumulates to significantly high levels (comparable to a known NMD substrate such as the *ENT4* mRNA) then it is likely that 3'→5' decay rate is significantly slower.

When 5'→3' decay in blocked by deletion of the Xrn1 exonuclease (*xrn1Δ*) quantitative Northern analysis of steady-state mRNA levels reveals a significant increase in *SSY5* mRNA accumulation compared to wild-type with a fold-change ratio (FCR) of *xrn1Δ*/wild-type=7.8±0.6, n=3 (Fig. 2A). The half-life ($T_{1/2}$) of the *SSY5* mRNA in *xrn1Δ* cells is also significantly longer at 33.9 minutes compared to 12.2 minutes in wild-type cells, n=1 (Fig. 2B). The *ENT4* mRNA is shown for comparison as this mRNA is a wild-type mRNA that is a substrate for NMD likely due to the presence of its long 3' UTR (Kebaara and Atkin, 2009; Chapter 2). The *CYH2* pre-mRNA is also shown for comparison as a wild-type NMD substrate, whereas the *CYH2* mRNA and *PGK1* mRNA are not NMD substrates (Figure 2A). We show that *xrn1Δ* results in a significant increase in *ENT4* mRNA accumulation indicating the 5'→3' decay mechanism is the predominant route of decay and that exosome-mediated 3'→5' decay is not sufficient to compensate for loss of Xrn1 (likely due to the slower rate of 3'→5' for NMD substrates; Muhlrad and Parker, 1994). The *PGK1* mRNA and *CYH2* mRNA have been used extensively in mRNA decay studies and these wild-type mRNAs are degraded by the canonical deadenylation-dependent mRNA decay pathway (Cao and Parker, 2003; Mitchell and Tollervey, 2003; Muhlrad and Parker, 1994; Tucker et al., 2002; Tucker et al., 2001). Our results show that *xrn1Δ* has no significant impact on the accumulation of the *PGK1* mRNA indicating a much more significant role of 3'→5' decay (Fig. 2A-B; Muhlrad and Parker, 1994). In comparison, the *SSY5* mRNA yields FCRs that are much closer to the pattern exhibited by the *ENT4* and *CYH2* mRNAs than the *PGK1* mRNA. These results indicate that the predominant route of degradation for *SSY5* mRNA is

through Xrn1-mediated 5'→3' decay. This resembles the pattern for known NMD substrates (*ENT4* mRNA and *CYH2* pre-mRNA) and is different than the effect of *xrn1Δ* on non-NMD substrates (*PGK1* and *CYH2* mRNAs).

Additionally, the accumulation of *SSY5* mRNA in the double mutant *xrn1Δupf1Δ* is also significantly higher compared to wild-type cells with a FCR of *xrn1Δupf1Δ*/wild-type=9.7±0.6, n=3 (Fig. 2A). Likewise, *SSY5* mRNA $T_{1/2}$ in *xrn1Δupf1Δ* cells is significantly longer at 40.3 minutes compared to 12.2 minutes in wild-type cells, n=1 (Fig. 2B). We do recognize that both the steady-state accumulation and $T_{1/2}$ of the *SSY5* mRNA in the double mutant are slightly increased from the *xrn1Δ* cells (FCR of *xrn1Δupf1Δ*/ *xrn1Δ*=1.2±0.6, n=3, and difference in $T_{1/2}$ of +6.4 minutes, n=1; Fig. 2A-B). However, this can be accounted for by considering the slight increase in accumulation and $T_{1/2}$ of *SSY5* mRNA between wild-type and *upf1Δ* strains alone (Fig. 2A-B). This difference is addressed in greater detail below (Fig. 6). This same pattern is also observed for the *ENT4* mRNA and *CYH2* pre-mRNA.

SSY5 mRNA accumulation is modestly increased in _dcp1Δ_ cells

During mRNA decay, Dcp1/2-mediated decapping immediately precedes 5'→3' degradation by Xrn1 (Hsu and Stevens, 1993; Muhlrad and Parker, 1994). If the mRNA is not decapped, 5'→3' decay cannot occur because the 5' end of the mRNA is not exposed for exonucloelytic degradation (Stevens, 2001). Because these two events occur in sequence, we hypothesized that blocking decapping of the mRNA by deletion of *DCP1* (*dcp1Δ*) might yield similar results to blocking 5'→3' degradation by Xrn1 (*xrn1Δ*).

Alternatively, we might see different results because in the *dcp1Δ* strains the mRNAs are capped whereas in the *xrn1Δ* strains the population of mRNAs includes both newly synthesized capped mRNAs and decapped mRNAs unable to undergo 5'→3' decay (He and Jacobson, 2001). Decapped mRNAs accumulate in p-bodies and p-bodies lack 3'→5' decay enzymes (Balagopal and Parker, 2009; Sheth and Parker, 2003). This may lead to higher accumulation levels observed in *xrn1Δ* strains.

Our results show that *dcp1Δ* had a modest effect on the steady-state accumulation of *SSY5* mRNA with a FCR of *dcp1Δ*/wild-type=2.3±0.4, n=3 (Fig. 3A). This is significantly less than the *SSY5* mRNA accumulation that resulted from *xrn1Δ* (FCR of *dcp1Δ*/wild-type=7.8±0.6, n=3; Fig. 2A). However, the $T_{1/2}$ of *SSY5* mRNA in *dcp1Δ* cells increased to 35.9 minutes compared to 12.2 minutes in wild-type cells (n=1), which is a similar difference to that observed with the *xrn1Δ* vs wild-type cells (Fig. 3B). Similar to *SSY5* mRNA, the *ENT4* mRNA and *CYH2* pre-mRNA also show a modest increase in accumulation upon *dcp1Δ* (FCR of *dcp1Δ*/wild-type=1.8±0.4 and 6.4±0.5, respectively, n=3; Fig. 3A). Interestingly, the *ENT4* mRNA shows a reduction in mRNA stability in the *dcp1Δ* cells compared to *upf1Δ* cells, although the fold change in mRNA accumulation between the two is not quite 2-fold (Fig. 3A). The double mutant *dcp1Δupf1Δ* strains also showed a different trend in accumulation between the *SSY5* mRNA and the *ENT4* mRNA. In contrast, accumulation of the non-NMD substrates *PGK1* and *CYH2* mRNAs, as well as the half-life of the *PGK1* mRNA, was the same in wild-type, *upf1Δ*, *dcp1Δ*, and *dcp1Δupf1Δ* cells (Fig. 3A-B).

Moreover, the trend observed for the accumulation of the *PGK1* mRNA is quite similar to the trend seen in accumulation of the *CYH2* mRNA in the *dcp1Δ* cells and *dcp1Δupf1Δ* cells, which is consistent with both mRNAs being non-NMD substrates (Fig. 3A). The trend observed for the *ENT4* mRNA is quite similar to the trend seen in accumulation of the *CYH2* pre-mRNA in the *dcp1Δ* cells and *dcp1Δupf1Δ*, which is consistent with both being NMD substrates (Fig. 3A). Accumulation of the *SSY5* mRNA in the *dcp1Δ* cells and *dcp1Δupf1Δ* cells, however, does not fit the pattern of either the *CYH2* mRNA or pre-mRNA. Together these results lead us to the conclusion that *SSY5* mRNA degradation is different from wild-type mRNA degradation and NMD-substrate degradation at the step of decapping.

SSY5 mRNA accumulation is not significantly impacted by deletion of different deadenylation components

In *S. cerevisiae* newly synthesized mRNAs contain poly(A) tails approximately 60-80nt in length (Beilharz and Preiss, 2007). The length of the mRNA poly(A) tail functions, in combination with the 5' mRNA cap, to regulate both translational efficiency and mRNA decay (Anderson and Parker, 1998; Gallie, 1991; Muhlrad et al., 1995; Munroe and Jacobson, 1990). The degradation of an mRNA involves a deadenylation step regardless of the mRNA being an NMD substrate or a wild-type mRNA degraded through the canonical pathway (Norbury, 2013). The difference is the prerequisite of deadenylation to precede decapping for wild-type mRNA degradation where it is the rate-limiting step of mRNA decay (Cao and Parker, 2003; Decker and Parker, 1993; Muhlrad

and Parker, 1994). While deadenylation is not a prerequisite for decapping of NMD substrates, the mRNAs do undergo rapid deadenylation (Cao and Parker, 2003; Muhlrad and Parker, 1994).

Deadenylation of an mRNA occurs through the combined efforts of the Pan2/Pan3 and the Ccr4/Pop2/Not deadenylase complexes (Wahle and Winkler, 2013; Wiederhold and Passmore, 2010). The Pan2/Pan3 deadenylation complex was the first to be discovered in yeast (Boeck et al., 1996; Lowell et al., 1992; Sachs and Deardorff, 1992). The Pan2 subunit is responsible for the catalytic activity of the complex (Wahle and Winkler, 2013). The Ccr4/Pop2/Not complex is a multisubunit complex containing at least one definitive catalytic subunit, Ccr4 (Chen et al., 2002; Tucker et al., 2002; Tucker et al., 2001). There are conflicting reports on whether or not Caf1 is a catalytic subunit as well in *S. cerevisiae*, although the catalytic activity of this subunit has been verified in other organisms (Andersen et al., 2009; Daugeron et al., 2001; Jonstrup et al., 2007; Liang et al., 2009; Thore et al., 2003; Tucker et al., 2002; Viswanathan et al., 2004). It was originally shown that the Pan2/Pan3 complex acted first in the initial trimming of the poly(A) tail in a Pab1-dependent manner (Brown and Sachs, 1998). However, later it was observed that *pan2Δ* and *pan3Δ* strains have few defects in mRNA deadenylation (Boeck et al., 1996; Brown et al., 1996). Together these observations suggest that in the absence of the Pan2/Pan3 complex, the Ccr4/Pop2/Not complex is capable of handling the full range of mRNA deadenylation (Tucker et al., 2001). Considering these observations in the context of our previous results (Figs. 2 and 3) we

hypothesized that deadenylation would likely play a pertinent role in the degradation of the *SSY5* mRNA.

To test this hypothesis we analyzed the *SSY5* mRNA steady-state accumulation in several deadenylation mutant strains. Fold-change ratios between the wild-type strain and those lacking Ccr4 (*ccr4Δ* strains) or Caf1 (*caf1Δ* strains) subunits, or both (*ccr4Δcaf1Δ* strains) showed very little effect on *SSY5* mRNA levels, n=3 (Fig. 4). Likewise, deletion of the Pan2 (*pan2Δ* strains) catalytic subunit of the Pan2/Pan3 complex showed very little effect on *SSY5* mRNA levels as well, n=3 (Fig 4). These results are not surprising as deletion of the catalytic activity of one complex can be compensated for by the catalytic activity of the other complex (Tucker et al., 2001). However, the *SSY5* mRNA levels showed little change in strains in which both complexes were rendered inactive (*pan2Δccr4Δ*) compared to wild-type strains, n=3 (Fig. 4). A previous study showed that the *MFA2* mRNA contains the longest poly(A) tail in the *pan2Δccr4Δ* strain indicating a lack of deadenylation in this strain as expected. However, decay intermediates of the *MFA2* mRNA were observed in the *pan2Δccr4Δ* strain indicating that at least some mRNAs in this strain can still be degraded due to a slow rate of deadenylation-independent decapping and 5'→3' decay (Tucker et al., 2001). Alternatively, this observation can also be explained by the possibility that the Caf1 subunit is able to compensate for the loss of both Ccr4 and Pan2, which would support the studies claiming Caf1 does indeed have deadenylase activity. There is also no previous data that would support the exosome being able to compensate for loss of deadenylase activity. Additionally, we observed the effect of NMD on the accumulation

of the *SSY5* mRNA in the deadenylation mutant strains by deletion of Upf1 (*upf1Δ* strains). We observed no significant fold changes upon Upf1 deletion in these strains compared to strains with a wild-type Upf1 allowing us to conclude that the effect observed for *SSY5* mRNA accumulation is independent of NMD (Fig. 4). We do note that band intensity of the *upf1Δ* strains does appear to be darker, however, fold change values do not reflect a difference in *SSY5* mRNA accumulation for these strains. Originally, this was surprising, but upon closer analysis we determined this is most likely due to the bands in the *upf1Δ* strains actually being thicker in width, which reflects a slightly wider variation in mRNA lengths, but not necessarily more intense in pixel density.

We also analyzed steady-state accumulation of NMD substrates (*ENT4* mRNA and *CYH2* pre-mRNA) as well as non-NMD substrates (*PGK1* and *CYH2* mRNAs) for comparison. Similar to the *SSY5* mRNA, none of these mRNAs appear to be largely effected by perturbation of deadenylation (Fig. 4). The only difference can be seen in the *upf1Δ* strains for the *ENT4* mRNA and *CYH2* pre-mRNA, which is expected since these are NMD substrates.

Based on these results we can conclude that blocking deadenylation as little effect on *SSY5* mRNA steady-state accumulation. However, this result is consistent with that of both an NMD-substrate (*ENT4* mRNA) and a wild-type mRNA (*PGK1* mRNA).

SSY5 mRNA has a shortened 3' end in _xrn1Δ_ and _dcp1Δ_ strains

Following the results in Figures 2-4 we wanted to determine if the *SSY5* mRNA was deadenylated and, if so, if we could determine at which step the mRNA is deadenylated. We hypothesized that the mRNA would be deadenylated as both wild-type mRNAs and NMD-substrates are deadenylated, but we were unsure if deadenylation was occurring as a prerequisite to decapping. To do this we used the Poly(A) Tail-Length Assay Kit from Affymetrix (Materials and Methods). Briefly, a poly(A) polymerase first adds a short series of guanosine and inosine residues (G/I) to the 3' ends of polyadenylated mRNAs. The G/I tails become the priming site for reverse transcription of the mRNAs to cDNA molecules. Two pairs of primers are then used to amplify the 3' ends of target mRNAs. The first primer pair, the gene-specific (G.S.) primer pair, consists of a forward and reverse primer that anneal to the 3' end of the mRNA both of which are upstream of the polyadenylation site(s). The second primer pair, the poly(A) tail ("Tail") primer pair, consists of the same upstream primer from the G.S. primer pair and a universal reverse primer that anneals to the G/I tail (Fig. 5A). PCR products obtained from the use of these two primer pairs are then compared in order to determine the length of the 3' poly(A) tail. It should be noted that because the poly(A) sites for the mRNAs we used are not definitive (as there are multiple predicted sites) we designed the G.S. primer pair to anneal upstream (3') of all predicted poly(A) sites (SGD).

The Poly(A) Tail-Length Assay Kit was used to determine the length of the 3' end, which includes the poly(A) tail, of steady-state *SSY5*, *ENT4*, and *PGK1* mRNAs in wild-type, *upf1Δ*, *xrn1Δ*, and *dcp1Δ* strains, n=2. However, an RNase H control was not

done so we are unable to positively confirm that the results observed are strictly due to differences in poly(A) tail length. We can, however, use the results to analyze differences in the 3' end length of the mRNAs which allows us to make a preliminary assumption about the poly(A) tail length of the mRNAs. As shown in Figure 5B, the 3' end of the *SSY5* mRNA is the same in both wild-type and *upf1Δ* strains (lane with the "Tail" primers product). In contrast, the 3' end of the *SSY5* mRNA is much shorter in both *xrn1Δ* and *dcp1Δ* strains. This indicates that in the absence of decapping or 5'→3' nucleolytic degradation the *SSY5* mRNA can be shortened from the 3' end. This same pattern is observed for the 3' end of the *PGK1* mRNA among all four strains (Fig.5 B). In contrast, the *ENT4* mRNA shows the same 3' end length in both wild-type and *upf1Δ* strains, but in these strains the 3' end is much shorter than in the *xrn1Δ* and *dcp1Δ* strains (Fig.5 B). This leads to the preliminary conclusion that when the *ENT4* mRNA, which is an NMD substrate, cannot be degraded by the 5' end then shortening of the 3' end of the mRNA is perturbed as well.

From these results we can conclude that the 3' end of the *SSY5* mRNA is behaving just like wild-type mRNA (*PGK1* mRNA) in contrast to an NMD substrate (*ENT4* mRNA) when comparing wild-type, *upf1Δ*, *xrn1Δ*, and *dcp1Δ* strains.

SSY5 mRNA has different stability in different strain backgrounds

In order to examine the behavior of the *SSY5* mRNA in the decapping and 5'→3' decay mutants discussed above (Figs. 2 and 3) we utilized previously constructed strains which are of a different genetic background (He et al., 1997; He and Jacobson, 1995). It

was previously shown that relative mRNA accumulation in wild-type and *upf1Δ* strains can differ depending on strain background (Kebaara et al., 2003). With this knowledge we originally analyzed *SSY5* mRNA levels in both commonly used BY4741 and W303 genetic backgrounds and found no difference in susceptibility of *SSY5* mRNA to NMD (Chapter 2). However, we unexpectedly found a slight difference in the susceptibility of the *SSY5* mRNA to NMD in the decay mutants utilized in Figures 2 and 3. Figure 6 shows a direct comparison of the *SSY5* mRNA steady-state accumulation in the BY4741 and W303 backgrounds along with the wild-type and *upf1Δ* strains from the same background used for construction of the *dcp1Δ* and *xrn1Δ* strains (He et al., 1997; He and Jacobson, 1995, 2001). Here we show there is no difference in *SSY5* mRNA accumulation between wild-type and *upf1Δ* strains in either the BY4741 or the W303 backgrounds. However, in the background used for the *dcp1Δ* and *xrn1Δ* strains, *SSY5* mRNA accumulation shows a FCR of *upf1Δ*/wild-type =1.9±0.2, (n=3; Fig 6). Likewise, *ENT4* mRNA accumulation shows a similar FCR between wild-type and *upf1Δ* strains in both the BY4741 background and the W303 background, but in the strain background used to create the *dcp1Δ* and *xrn1Δ* strains this FCR is higher, (n=3; Fig. 6; He et al., 1997; He and Jacobson, 1995, 2001). We created the isogenic *upf1Δ* strain used with this set of strains, but the strain was constructed by transformation of the wild-type strain (AAY360) of this genetic background with the *upf1Δ2* cassette. The strain background of this set wild-type, *upf1Δ*, *xrn1Δ* and *dcp1Δ* strains is published as having the same auxotrophic markers as the W303 background that we originally tested (He et al., 1997; He and Jacobson, 1995). However, there is clearly a difference in the accumulation of

the *SSY5* mRNA between these strains and our original W303 strains. From these observations we conclude that the protection of the *SSY5* mRNA from NMD is not entirely robust in all strain backgrounds. However, this can be used to our advantage in determining what protects the *SSY5* mRNA from NMD, discussed below.

Discussion

In this study we began to dissect the mechanism of *SSY5* mRNA decay. The *SSY5* mRNA is a wild-type mRNA with multiple NMD-targeting signals but is not degraded by the NMD pathway (Kebaara and Atkin, 2009; Obenoskey et al., 2014; Chapter 2). We have previously presented the case that the *SSY5* mRNA is likely protected from NMD by a novel mechanism (Chapter 2). The goal in studying the mechanism of *SSY5* mRNA decay was to determine if the mRNA was being degraded via the wild-type deadenylation-dependent decapping pathway or by the deadenylation-independent decapping NMD pathway. As a consequence we were hoping to be able to identify if protection of the *SSY5* mRNA from NMD is incurred before recognition as an NMD substrate, in which we would see evidence of degradation by the deadenylation-dependent decapping pathway, or if protection is incurred at a point after recognition as an NMD substrate, in which we would potentially see evidence of deadenylation-independent decapping and decay. However, our data presented here does not provide a clear-cut answer. Instead, we learn something quite intriguing about *SSY5* mRNA decay. When Xrn1-mediated 5'→3' decay is blocked in *xrn1Δ* strains, *SSY5* mRNA accumulates to significantly higher levels compared to wild-type cells (Fig. 2). However, when Dcp1-

mediated decapping is blocked in *dcp1Δ* strains, *SSY5* mRNA accumulates at much lower levels than in the *xrn1Δ* cells. Previous work has shown that a significant fraction of the mRNAs in *xrn1Δ* cells are in fact decapped (He and Jacobson, 2001). This indicates that the presence of the 5' mRNA cap (in *dcp1Δ* cells) is able to increase the rate of exosome-mediated 3'→5' degradation, while this process is much slower when the cap is removed (in *xrn1Δ* cells) allowing for the greater accumulation of *SSY5* mRNA (Figs. 2A and 3A). This exact same trend is not consistent for either an NMD substrate (*ENT4* mRNA) or a wild-type mRNA (*PGK1*; Figs. 2A and 3A). Further, blocking deadenylation through deletion of the catalytic subunits of either the Pan2/Pan3 deadenylase complex (*pan2Δ* strains) or the Ccr4/Pop2/Not complex (*ccr4Δ*, *caf1Δ*, *ccr4Δcaf1Δ* strains) or both complexes (*ccr4Δpan2Δ* strains) had very little effect on the steady-state accumulation of *SSY5* mRNA (Fig. 4). Together these results suggest an incredibly tight window in the regulation of *SSY5* mRNA levels as there is a significant difference in mRNA accumulation between the *dcp1Δ* strains and the *xrn1Δ* strains, two steps that occur in immediate sequence during 5'→3' mRNA decay (Figs. 2A and 3A).

Moreover, a previous study was done to identify direct targets of the NMD pathway in *S. cerevisiae* by virtue of association with core NMD factor Upf1 (Johansson et al., 2007). Interestingly, in this study Upf1 was found to specifically associate with the *SSY5* mRNA. An mRNA was considered to be enriched for association with Upf1 if the signal intensity from the mRNAs co-purifying with TAP-Upf1 differed from the negative control by at least two-fold. The *SSY5* mRNA showed an enrichment of 4.76 fold over the negative control. If enriched association of an mRNA with Upf1 is indicative of the

mRNA being a direct NMD substrate, as was hypothesized in this study, the *SSY5* mRNA is being recognized by the NMD machinery as an NMD substrate. According to this interpretation, protection of the *SSY5* mRNA from NMD should be incurred at a point after substrate recognition. It is also possible that protection is incurred even earlier but I a way that does not interfere with stable Upf1 binding.

Additionally, we found that in working with a new strain background *SSY5* mRNA susceptibility to NMD was slightly different than in the BY4741 and W303 backgrounds used previously (Fig 6). Upon further contemplation, this initially surprising and somewhat frustrating observation can actually be used to our advantage. Previously, we determined that *trans*-acting factor Pub1 is not responsible for the protection of *SSY5* mRNA from NMD. We had thought this may be the case based on an earlier study showing Pub1 is responsible for the protection of *GCN4* and *YAP1* mRNAs from NMD (Ruiz-Echevarria and Peltz, 2000). This leaves us in search of potential *trans*-acting factors that may be involved in the protection of SSY5 mRNA from NMD. We have developed the strains to be able to test RNA binding protein (RBP) mutants for the effect on *SSY5* mRNA levels (Chapter 2), but taking on the entire yeast RBP collection at once is a cumbersome task. By comparing differences between the BY4741 and W303 backgrounds with those of the *dcp1Δ* and *xrn1Δ* strain background we are presented with a much more realistic starting point for determining which factors may be involved in *SSY5* protection from NMD.

Figure legends

Fig. 3-1. Wild-type mRNAs and NMD substrates are degraded by different mechanisms. Most wild-type mRNAs are first deadenylated by the Pan2/Pan2 and Ccr4/Pop2/Not deadenylase complexes. Shortening of the poly(A) tail leads to deadenylation-dependent decapping by the Dcp1/Dcp2 decapping complex. Once decapped the mRNA is subjected to 5'→3' degradation by the Xrn1 exonuclease or 3'→5' degradation by the exosome complex. NMD substrates are rapidly decapped in a deadenylation-independent manner, followed by rapid deadenylation and 5'→3' degradation by Xrn1. In the absence of decapping or 5'→3' degradation NMD substrates can also undergo 3'→5' degradation, but at a much slower rate.

Fig. 3-2. *SSY5* mRNA steady-state accumulation is significantly increased and it has a significantly longer half-life in an *xrn1Δ* strain. **A)** Northern blot analysis of the steady-state accumulation of the *SSY5*, *ENT4* (a wild-type NMD substrate), and *PGK1* (a wild-type mRNA degraded by the canonical pathway) mRNAs in wild-type (AAY360), *upf1Δ* (AAY589), *xrn1Δ* (AAY389), and *xrn1Δupf1Δ* (AAY611) strains grown in YAPD to mid-log phase. *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials. **B**) mRNA half-life analysis of the *SSY5* and *PGK1* mRNAs in the same strains used in part A. Strains were grown in YAPD and 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations

were determined using a graph of percent mRNA remaining at each time point during the exponential decay.

Fig. 3-3. *SSY5* mRNA steady-state accumulation is moderately increased in a *dcp1Δ* strain while half-lives are significantly stabilized in a *dcp1Δ* strain. **A**) Northern blot analysis of the steady-state accumulation of the *SSY5*, *ENT4*, and *PGK1* mRNAs in wild-type (AAY360), *upf1Δ* (AAY589), *dcp1Δ* (AAY390), and *dcp1Δupf1Δ* (AAY621) strains grown in YAPD to mid-log phase. *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials. **B**) mRNA half-life analysis of the *SSY5* and *PGK1* mRNAs in the same strains used in part A. Strains were grown in YAPD. 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay.

Fig. 3-4. Mutations altering the deadenylase complexes do not have a significant influence on *SSY5* mRNA stability. Northern blot analysis of the steady-state accumulation of the *SSY5*, *ENT4*, and *PGK1* mRNAs in wild-type (AAY391), *upf1Δ* (AAY594), *ccr4Δ* (AAY393), and *ccr4Δupf1Δ* (AAY596), *ccr4Δcaf1Δ* (AAY394), *ccr4Δcaf1Δupf1Δ* (AAY609), *pan2Δ* (AAY395), *pan2Δupf1Δ* (AAY595), *pan2Δccr4Δ* (AAY396), and *pan2Δccr4Δupf1Δ* (AAY610) strains grown in YAPD. *CYH2* pre-

mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials.

Fig. 3-5. *SSY5* mRNA has a shortened 3' end in *xrn1Δ* and *dcp1Δ* strains. **A**) Schematic of how the Poly(A) Tail Length Analysis works. This analysis was done using the "Poly(A) Tail-Length Assay Kit" from Affymetrix (#76455). In this assay, a G/I tail is added to the end of mRNAs containing a Poly(A) tail. These G/I-tailed mRNAs are then reverse transcribed to cDNA, which provides a template for subsequent PCR. PCR is performed using two sets of primers independently. The Gene Specific (G.S.; teal) primer pair is comprised of a forward primer that binds anywhere within the open reading frame (ORF) or 3' UTR and a reverse primer that binds immediately upstream (5') of the Poly(A) start site. *It is important to note that the exact Poly(A) start site of the mRNAs in this figure were unknown so the reverse primer was designed just upstream of the first *predicted* Poly(A) start site according to the *Saccharomyces* Genome Database (SGD). The Poly(A) Tail (Tail; pink) primer pair uses the same forward primer from the G.S. pair and a reverse primer that anneals to the G/I tail (provided with the kit). **B**) Products from the PCR reactions using the above primer pairs for the *SSY5*, *ENT4*, and *PGK1* mRNAs in the wild-type (AAY360), *upf1Δ* (AAY589), *xrn1Δ* (AAY389), and *dcp1Δ* (AAY390) strains were resolved on a 2.5% agarose TAE gel. Strains were grown in

YAPD and the total RNA samples used for the reactions were the same RNA samples that were used for Northern analysis in figures 2A and 3A.

Fig. 3-6. *SSY5* mRNA has different stability in different strain backgrounds. Northern blot analysis of the steady-state accumulation of the *SSY5* and *ENT4* mRNAs in wild-type (AAY277) and *upf1Δ* (AAY363) in the BY4741 background, wild-type (AAY187) and *upf1Δ* (AAY320) in the W303 background, and in wild-type (AAY360) and *upf1Δ* (AAY589) in the "W303" background that originated from the wild-type strain in He and Jacobson, 1995. Strains were grown in YAPD. *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials.

Figure 3-1

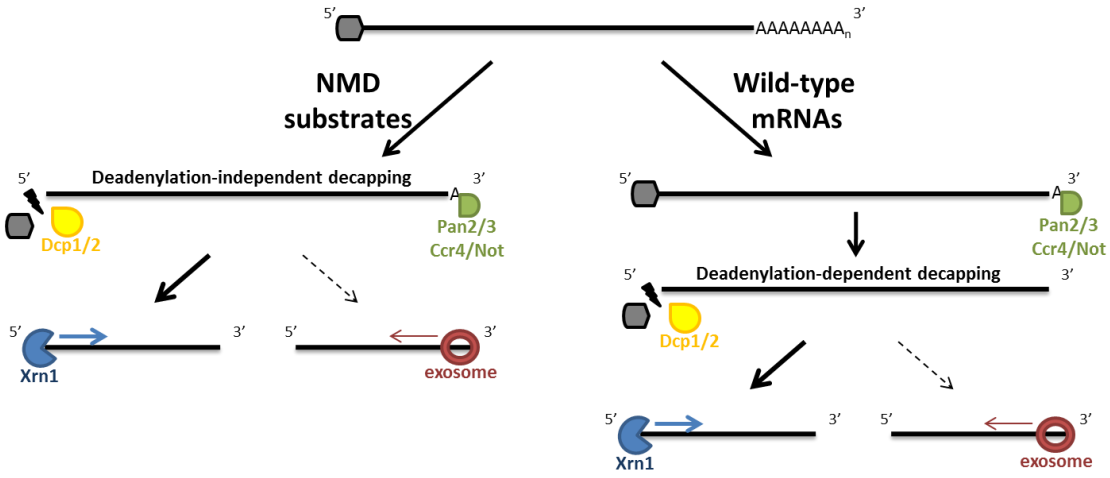
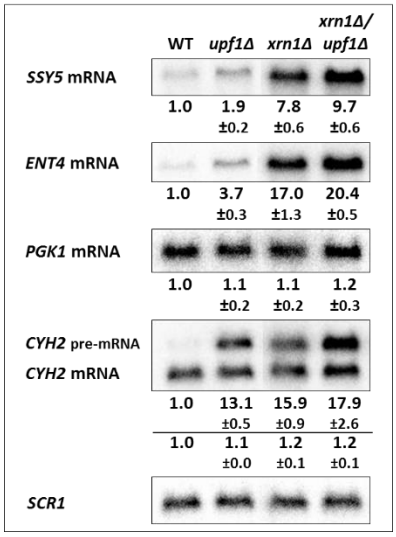


Figure 3-2

A.



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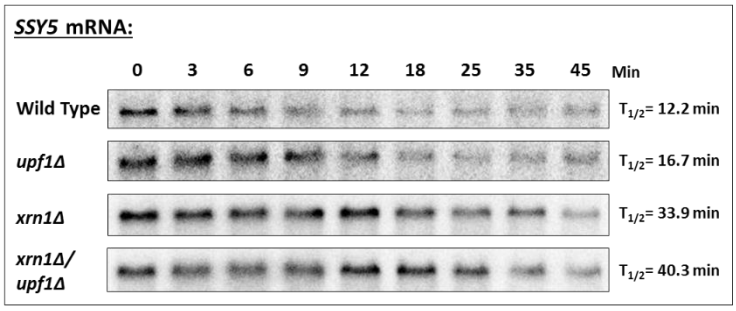

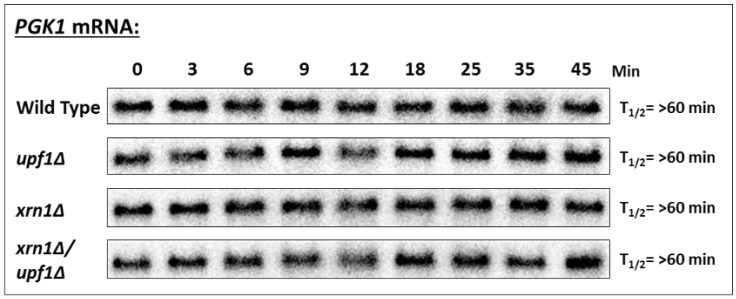

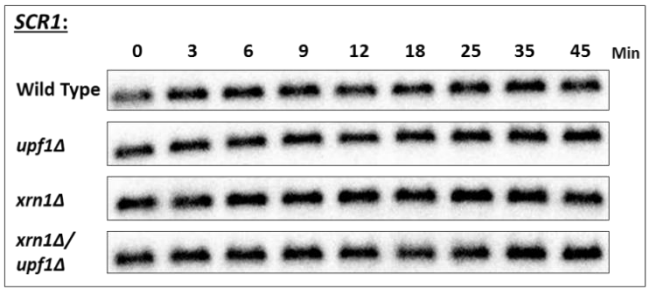

Figure 3-3

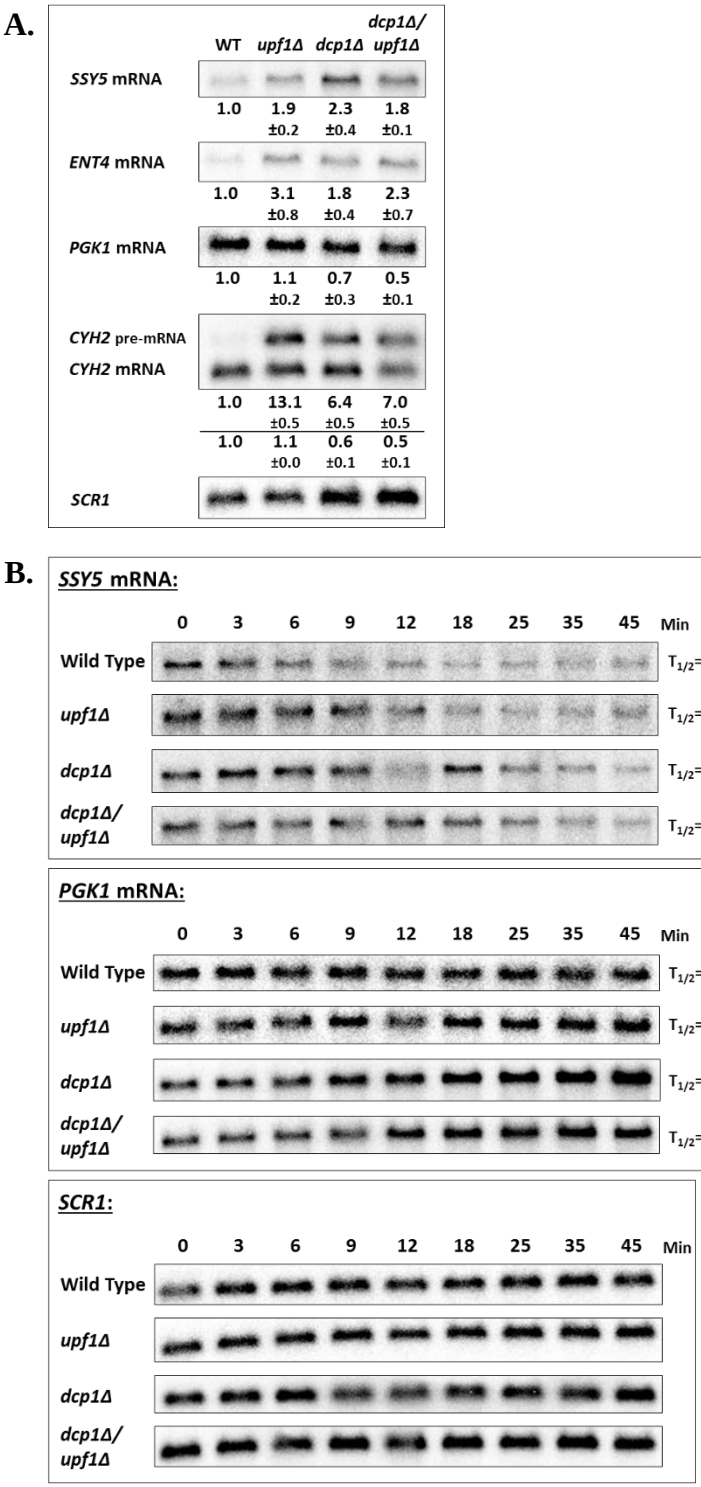

Figure 3-4

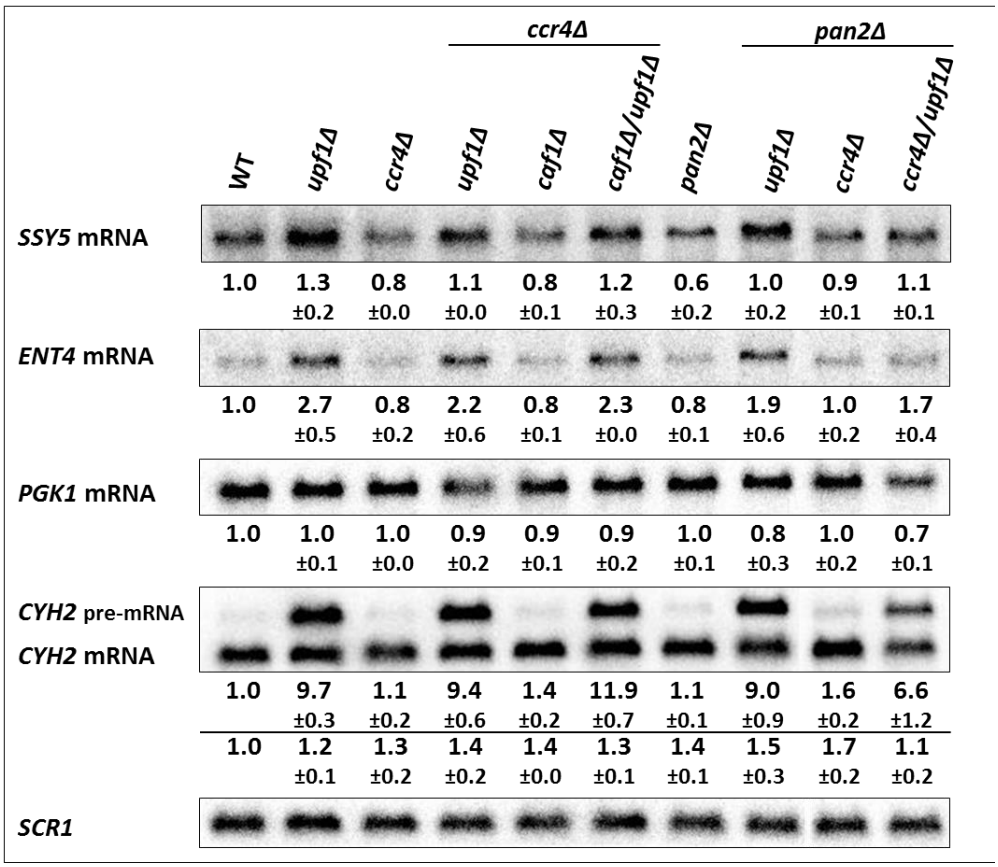

Figure 3-5

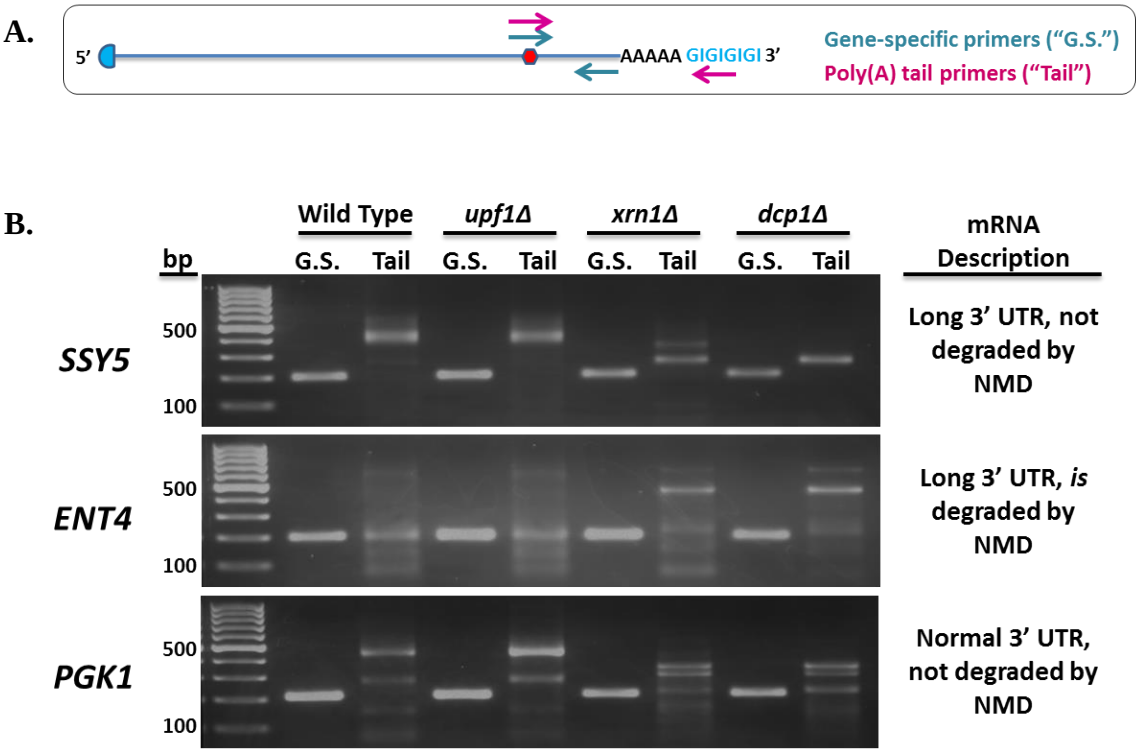

Figure 3-6

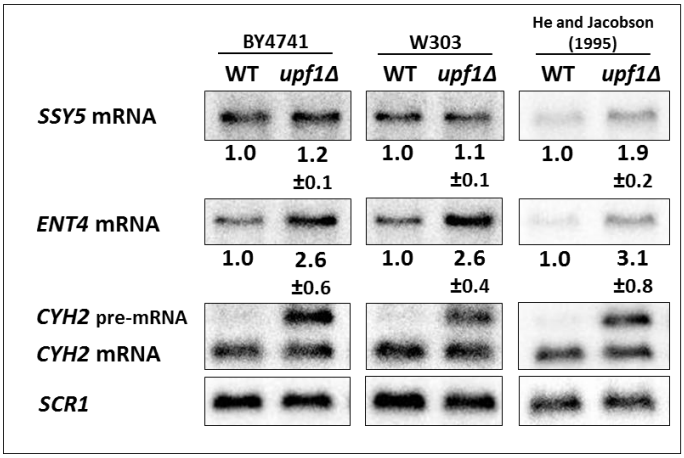

Table 3-1. Strains used in this study

Strain	Parent Strain	Genotype	Source
W303		*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Ralser *et al.*, 2012
BY4741		*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winston *et al.*, 1998
AAY320	W303	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1::URA3*	Atkin lab
AAY360		*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	He and Jacobson, 1995
AAY363	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 upf1::URA3*	Atkin lab
AAY389	AAY360	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 xrn1::ADE2*	He and Jacobson, 2001
AAY390	AAY360	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 dcp1::URA3*	He and Jacobson, 2001
AAY391		*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM*	Tucker *et al.*, 2001
AAY393	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO*	Tucker *et al.*, 2001
AAY394	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO caf1::URA3*	Tucker *et al.*, 2001
AAY395	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM pan2::URA3*	Tucker *et al.*, 2001
AAY396	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO pan2::URA3*	Tucker *et al.*, 2001
AAY589*	AAY360	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1::URA3*	Atkin lab
AAY594*	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM upf1::TRP1*	Atkin lab
AAY595*	AAY395	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM pan2::URA3 upf1::TRP1*	Atkin lab
AAY596*	AAY393	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO upf1::TRP1*	Atkin lab
AAY609*	AAY394	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO caf1::URA3 upf1::TRP1*	Atkin lab
AAY610*	AAY396	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO pan2::URA3 upf1::TRP1*	Atkin lab
AAY611*	AAY389	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 xrn1::ADE2 upf1::TRP1*	Atkin lab
AAY621*	AAY390	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 dcp1::URA3 upf1::TRP1*	Atkin lab
* = new strain created for this study			

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CHAPTER 4

The protection of the *Saccharomyces cerevisiae* wild-type *GCN4* and *YAP1* mRNAs from degradation by NMD

Abstract

Nonsense-mediated mRNA decay was first discovered because of its role in the rapid degradation of mRNAs that harbor premature termination codons (PTCs). It is now recognized that a significant fraction of wild-type mRNAs are regulated by NMD as well. Importantly, not all of the underlying mechanisms of the NMD pathway are understood. Because as many as 1/3 of all genetic diseases and cancers link to NMD the development of safe and effective therapies to treat these diseases is a pressing area of research. Given this it is absolutely critical that we continue to elucidate the underlying molecular mechanisms of NMD. Here we investigate two wild-type mRNAs, *YAP1* and *GCN4*, which both contain at least one NMD-targeting signal but neither mRNA is degraded by NMD. Previously, it was reported that both mRNAs are protected by *trans*-acting factor Pub1; however, we were unable to confirm these results. We show that both mRNAs cofractionate with polyribosomes so are likely to be translated. We also show that blocking 5'→3' exonucloelytic degradation results in moderate accumulation of both mRNAs while blocking decapping has little effect on the accumulation of either mRNA. Additionally, blocking deadenylation has little effect on the accumulation of either mRNA. Together these results show that both *YAP1* mRNA and *GCN4* mRNA likely have a unique method of degradation that is tightly regulated.

Introduction

The regulation of gene expression in all cells is critical for the proper functioning of the organism. Many things contribute to the fidelity of gene expression but among the most influential are the biogenesis of mRNA molecules through transcription and the decay of mRNA molecules through mRNA degradation. In yeast, wild-type mRNAs are typically degraded in the cytoplasm by a process that is initiated by removal of the 3' poly(A) tail by the Pan2/Pan3 and Ccr4/Pop2/Not deadenylase complexes. Once the poly(A) tail reaches a length of 10-12 adenine residues the circularization of the mRNP is disrupted due to the lack of poly(A) binding protein (Pab1), which was originally bound to the poly(A) tail (Brown and Sachs, 1998; Decker and Parker, 1993; Tucker et al., 2002; Tucker et al., 2001; Wahle and Winkler, 2013). Disruption of the mRNP circularization results in the exposure of the 5' 7-methylguanosine to the decapping complex Dcp1/2, which rapidly decaps the mRNA leaving a 5' monophosphate exposed (Coller and Parker, 2004). This 5' monophosphate becomes the substrate for the exoribonuclease Xrn1, which then degrades the mRNA in a 5'→3' direction. This appears to be the primary pathway for the degradation of most yeast mRNAs (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1995; Muhlrad and Parker, 1994). Alternatively, mRNAs can also be degraded 3'→5' by the exosome complex following deadenylation (Anderson and Parker, 1998; Mangus et al., 2003; Mangus and van Hoof, 2003; Mitchell and Tollervey, 2003).

Another part of the fidelity of gene expression is the presence of several quality control pathways, which are responsible for the rapid degradation of aberrant mRNA

transcripts (Behm-Ansmant et al., 2007; Hilleren et al., 2001; Isken and Maquat, 2007, 2008; Lykke-Andersen et al., 2001). One of these quality control pathways is the nonsense-mediated mRNA decay pathway (NMD), which is responsible for the rapid degradation of mRNAs that harbor premature termination codons (PTCs). If mRNAs with PTCs were not rapidly removed from the translational pool of the cell they would lead to the build-up of truncated proteins which could have dominant negative effects (Gonzalez et al., 2001; Isken and Maquat, 2007; Muhlemann et al., 2008). NMD is a conserved mRNA decay pathway which is present in all eukaryotes that have been examined (Bedwell et al., 1997; Conti and Izaurralde, 2005; Hall and Thein, 1994; Hentze and Kulozik, 1999; Maquat and Carmichael, 2001; Maquat and Serin, 2001; Pulak and Anderson, 1993). Importantly, it is now recognized the NMD plays a vital role in wild-type gene regulation as well. It has been observed that ~5-20% of the yeast, *Drosophila*, and human transcriptomes are affected upon inactivation of NMD (Guan et al., 2006; He et al., 2003; Johansson et al., 2007; Lelivelt and Culbertson, 1999; Mendell et al., 2004). NMD requires three *trans*-acting factors: Upf1, Upf2 and Upf3. Mutations or deletions in one or more of the genes encoding these factors stabilizes NMD substrates (Cui et al., 1995; He et al., 1997; He and Jacobson, 1995; Lee and Culbertson, 1995; Lelivelt and Culbertson, 1999; Maderazo et al., 2000).

Several mechanisms have been identified which can be responsible for targeting wild-type mRNAs for degradation by NMD including: 1) a long 3' UTR (Amrani et al., 2004; Kebaara and Atkin, 2009; Muhlrad and Parker, 1999), 2) translation of an upstream open reading frame (uORF; Amrani et al., 2006; Barbosa et al., 2013; Nyiko et al., 2009),

3) a start codon in a suboptimal context which can lead to leaky scanning and out of frame initiation of translation (Welch and Jacobson, 1999), 4) the presence of programmed ribosome frameshift (PRF) sites (Plant et al., 2004), and 5) the presence of pre-mRNA introns and regulated alternative splicing resulting in PTCs (He et al., 1993; Lewis et al., 2003; McGlincy and Smith, 2008; Ni et al., 2007).

The degradation of NMD substrates is different from that of most wild-type mRNAs in that NMD substrates are rapidly decapped in a deadenylation-independent process (Beelman et al., 1996; Cao and Parker, 2003; Hagan et al., 1995). NMD substrates are also rapidly deadenylated but the deadenylation does not need to occur as a prerequisite for mRNA decapping (Cao and Parker, 2003). After decapping NMD substrates are rapidly degraded 5'→3' by Xrn1 (He and Jacobson, 2001; He et al., 2003). NMD substrates can also be degraded 3'→5' by the exosome complex following deadenylation, but this degradation takes place at a much slower rate (Cao and Parker, 2003; Mitchell and Tollervey, 2003; Muhlrad and Parker, 1994).

It was previously shown that two wild-type mRNAs in *Saccharomyces cerevisiae*, *YAP1* and *GCN4*, which have translated uORFs, are protected from degradation by NMD (Ruiz-Echevarria et al., 1998; Ruiz-Echevarria and Peltz, 2000; Vilela et al., 1998). In this study we further characterize the mechanism of protection of these two wild-type mRNAs from NMD by determining that they both co-fractionate as expected with polyribosomes, and also by determining their stability and accumulation in various decay and deadenylation mutants. We also show that trans-acting factor, Pub1, is not likely responsible for the protection of these two mRNAs from NMD in contrast to what was

previously reported (Ruiz-Echevarria and Peltz, 2000).

Materials and Methods

Yeast strains

The yeast strains used in this study are listed in Table 4-1. All yeast transformations were done using Lithium Acetate-Mediated transformation as previously described (Gietz and Woods, 2002). AAY590 was constructed by transforming AAY538 with the *upf1Δ2* fragment from pAA70 using primers oAA48 and oAA79. AAY589 was constructed by transforming AAY360 with the *upf1Δ2* fragment from pAA70 using primers oAA48 and oAA79. AAY594, AAY595, AAY596, AAY609, AAY610, AAY611 and AAY621 were constructed by transforming the corresponding parent strain (listed in Table 4-1) with the *upf1Δ6* fragment from pAA167 using primers oAA48 and oAA79.

Growth conditions

Unless otherwise noted yeast cells were grown using standard techniques with mild agitation equivalent to 225rpm at 30˚C. When cells are stated as being grown in a certain type of media, the same media was used to grow plate cultures from frozen stock and all subsequent liquid cultures. In this study, YAPD media consists of: 1% yeast extract, 2% Bacto-Peptone, 2% dextrose, and 100mg/L Adenine hemisulfate salt; and minimal media (SD+amino acids) consists of: 0.67% yeast nitrogen base without amino acids, 2% dextrose, 20 mg/L L-Histidine, 30 mg/L L-Leucine, 20 mg/L L-Methionine, 20

mg/L Uracil, and 30 mg/L L-Lysine.

RNA Extractions

Yeast strains were grown in 10mL cultures to an OD_{600} of 0.4-0.6. Cells were harvested by centrifugation, washing in DEPC-ddH_20, and flash-freezing in dry ice/ethanol or liquid nitrogen. Cell pellets were stored at -70˚C until used for RNA extractions. RNA extractions were performed as previously described (Kebaara et al., 2012). RNA samples were diluted to 1µg/µl in DEPC-ddH_20 and stored at -70˚C. RNA quality check gels are performed for every RNA sample (1µl of 1µg/µl Total RNA is run through a 0.8% agarose gel to check for degradation).

Quantitative Northern Analysis

10µg of Total RNA mixed with 3µl Formaldehyde loading dye (Ambion, cat. no. 8552) was separated through a 1.0% agarose gel containing 5.6% Formaldehyde and 1% MOPS (10X MOPS: 0.2 M sodium morpholinopropanesulfonic acid (MOPS), pH 7.0, 0.05 M sodium acetate, 0.01 M EDTA; adjust pH to 7.0 with 10 M NaOH, do not autoclave, store at room temperature in the dark.) RNA was transferred onto a GeneScreen Plus membrane (PerkinElmer) using NorthernMax transfer buffer (Ambion, cat. no. 8672) following the manufacturer's protocol for downward transfer. The lane with the RNA ladder was cut off of the gel before transfer and stained overnight in 0.5µg/ml ethidium bromide. Membranes were rinsed in 2X SSC and dried for 15 minutes at 80˚C.

Membranes were hybridized with NorthernMax prehybridization/hybridization buffer (Ambion # 8677). ^{32}P-labelled probes were synthesized using ~25ng of PCR product corresponding to the gene of interest, the RadPrime DNA Labeling system (Invitrogen #18428-011), and ~50 μCi [α-32P]dCTP (3000 Ci/mmol, 10 mCi/ml) (Perkin Elmer) following the manufacturer's protocol. Probes are purified through a Sephadex G-50 column equilibrated with TE pH 8.0. Membranes were hybridized overnight (12-24 hours) and then washed once at room temperature with 2X SSPE and once at 65˚C with 2X SSPE/2% SDS. Membranes were PhosphorImaged™ (GE Healthcare, Typhoon FLA 9500) and quantified using the ImageQuant™ software. All membranes were also autoradiographed using a phosphorescent ruler to determine the size of the bands by comparison to the RNA ladder. Membranes are stripped and stored at -20˚C for re-probing. Detailed protocol for Northern analysis can be found in Kebaara *et al.* (2012).

Polyribosome Analysis

Yeast polyribosome analysis was performed as previously described (Atkin et al., 1995). Lysis buffer was composed of 20mM Tris-HCl pH 8.0, 140mM KCl, 1.5 mM $MgCl_2$, 1% Triton X, 0.1 mg/ml Cycloheximide, and 1.0 mg/ml Heparin; Cycloheximide and Heparin were made fresh and added just before use. Gradient buffer (50% and 15% sucrose) was composed of 20mM Tris-HCl pH 8.0, 140mM KCl, 5.0 mM $MgCl_2$, 0.1 mg/ml Cycloheximide, 0.5 mg/ml Heparin, and 0.5mM DTT; Cycloheximide, Heparin, and DTT were made fresh and added just before use. 15%-50% sucrose gradients were made by hand, frozen at -70˚C and thawed at 4˚C overnight just before use. After

fractionation RNA was extracted from each fraction using acid phenol/chloroform extraction. Total RNA from each fraction collected was loaded onto an RNA Northern gel and transferred to a GeneScreen Plus membrane as described above. Membranes were hybridized with a ^{32}P-labelled probe as described above.

Poly(A) tail length analysis

This experiment was performed as described in the protocol provided with the Poly(A) Tail-Length Analysis Kit® (Affymetrix 76455). Prior DNase treatment of RNA samples was done using the TURBO DNA-*free* kit® (AM1907). Primers were designed using the information provided in the Saccharomyces Genome Database for predicted polyadenylation sites. Since all of the mRNAs we were interested in contained multiple predicted poly(A) sites, the primers pairs were designed to anneal upstream of the polyadenylation site that is predicted to be most 3' of the other predicted sites. PCR products were resolved on a 2.5% TAE agarose gel.

Results

***YAP1* mRNA is protected from degradation by NMD while *GCN4* mRNA is partially protected from degradation by NMD**

A previous study showed that the uORF-containing *GCN4* and *YAP1* mRNAs are not degraded by the NMD pathway despite presence of an NMD-targeting signal (Ruiz-Echevarria et al., 1998). Because we recently identified the *SSY5* mRNA as a wild-type mRNA that is also protected from NMD we were hoping to include the *GCN4* and *YAP1*

mRNAs in our studies as well (Kebaara and Atkin, 2009; Chapter 2).

In order to validate these results we grew *S.cerevisiae* cells in rich growth medium (YAPD) and confirmed by quantitative Northern analysis that steady-state accumulation of *YAP1* and *GCN4* mRNAs show no significant fold change (FC) between wild-type and *upf1Δ* strains. Additionally, because it has been shown that different genetic backgrounds of yeast strains can result in varying sensitivity of mRNAs to NMD we tested the steady-state accumulation of these mRNAs in two of the most commonly used genetic backgrounds, BY4741 and W303 (Kebaara et al., 2003). We found that the *YAP1* mRNA is protected from significant degradation by NMD in both the BY4741 and W303 genetic backgrounds with a FCR of *upf1Δ*/wild-type=1.4±0.1 and 1.3±0.2, respectively (Fig. 1A). Interestingly, in the *upf1Δ* strains we consistently notice a slower migrating band for the *YAP1* mRNA, which appears at a much lower intensity (Fig. 1A). For the *GCN4* mRNA we do not see ≥2.0 fold change in accumulation between wild-type and *upf1Δ* strains that would be indicative of an NMD substrate. However, the stead-state accumulation of the *GCN4* mRNA is consistently elevated in the *upf1Δ* strains in both the BY4741 and W303 genetic backgrounds with a FCR of *upf1Δ*/wild-type =1.9±0.1 and 1.6±0.1, respectively (Fig. 1A). The *ENT4* mRNA is shown because it is a wild-type mRNA in *S. cerevisiae* that has a long 3' UTR and is an NMD substrate (Chapter 2). Additionally, the *CYH2* pre-mRNA is an NMD substrate while the mature *CYH2* mRNA is not. *SCR1* is shown for the loading control.

Further, in rich media the half-life ($T_{1/2}$) of the *YAP1* mRNA exceeds 60 minutes in both the wild-type and *upf1Δ* strains (Fig. 1B). The $T_{1/2}$ of the *GCN4* mRNA is also

not significantly different between wild-type ($T_{1/2}$=47.0min) and *upf1Δ* strains ($T_{1/2}$=47.5min; Fig. 1B). Based on the $T_{1/2}$ analysis of the *YAP1* and *GCN4* mRNAs, neither mRNA appears to be a substrate for NMD.

The *YAP1* and *GCN4* mRNAs do not appear to be substrates for the NMD pathway when cells are grown in rich media (Fig. 1A-B). However, the *GCN4* mRNA codes for a transcriptional activator of amino acid biosynthetic genes, thus we hypothesized that the *GCN4* mRNA might have varying stability in different growth media that had decreased availability of extracellular amino acids. To test this hypothesis we grew the cells in minimal media which contained only the amino acids necessary to support the auxotrophies of the strains (Materials and Methods). Consistent with results in rich media, the steady-state accumulation of the *GCN4* mRNA was only moderately increased and not significantly different between wild-type and *upf1Δ* strains (FCR=1.5±0.3; Fig. 1C). Additionally, the $T_{1/2}$ of the *GCN4* mRNA was not significantly different between wild-type ($T_{1/2}$=21.2±3.7 min) and *upf1Δ* strains ($T_{1/2}$=20.7±1.0 min) when cells were grown in minimal media (Fig. 1D). However, the $T_{1/2}$ in both the wild-type and *upf1Δ* strains is much longer when the cells are grown in rich media compared to when the cells are grown in minimal media (Figs. 1B and 1D). So, the difference in growth media does influence *GCN4* mRNA stability but not as a result of NMD. This same pattern was observed for the *SSY5* mRNA in Chapter 2.
In yeast, the *YAP1* mRNA codes for a transcription factor required for oxidative stress tolerance. However, since we already had the membranes prepared we also analyzed the *YAP1* mRNA when cells were grown in minimal media. Consistent with the results from

cells grown in rich media, the *YAP1* mRNA steady-state accumulation was not significantly different between wild-type and *upf1Δ* strains when cells were grown in minimal media (FCR of *upf1Δ*/wild-type=1.1±0.2; Fig. 1C). Likewise, the $T_{1/2}$ of the *YAP1* mRNA was not significantly different between wild-type ($T_{1/2}$=15.3±1.5 min) and *upf1Δ* strains ($T_{1/2}$=14.0±0.9 min) when cells were grown in minimal media (Fig. 1D). But again, just like the *GCN4* and *SSY5* mRNAs, the $T_{1/2}$ for the *YAP1* mRNA in both the wild-type and *upf1Δ* strains is much longer when the cells are grown in rich media compared to when the cells are grown in minimal media (Figs. 1B and 1D).

Together, these results allow us to confirm that both the *GCN4* and *YAP1* mRNAs are wild-type mRNAs that are not likely substrates for the NMD pathway. However, the regulation of the *GCN4* mRNA is not quite as clear as previously shown (Ruiz-Echevarria et al., 1998; Ruiz-Echevarria and Peltz, 2000; Vilela et al., 1998).

YAP1 and _GCN4_ mRNAs are not protected from NMD by _trans_-acting factor Pub1

A follow up study to the one that originally showed *YAP1* and *GCN4* mRNAs were protected from NMD identified poly(U) binding protein, Pub1, as being the *trans*-acting factor responsible for this protection from NMD (Ruiz-Echevarria and Peltz, 2000). In light of our previous work which identified the *SSY5* mRNA as a wild-type mRNA that is protected from NMD these results piqued our interest (Kebaara and Atkin, 2009; Chapter 2). We hypothesized that if Pub1p was involved in protection of the *YAP1* and *GCN4* mRNAs from NMD then it could also be involved in *SSY5* mRNA protection from NMD. We did analyze *SSY5* mRNA steady-state accumulation in wild-type, *upf1Δ*,

pub1Δ, and *upf1Δpub1Δ* strains and did not find any influence of Pub1 on *SSY5* mRNA susceptibility to NMD (Chapter 2). However, during this analysis the *YAP1* and *GCN4* mRNAs were included as positive controls since it was previously shown that Pub1 does protect these mRNAs from NMD (Ruiz-Echevarria and Peltz, 2000). In contrast to the previous study we do not show a Pub1-dependent protection of the *YAP1* and *GCN4* mRNAs from NMD (Fig. 2).

When cells are grown in either rich or minimal media we do not see a significant increase in *YAP1* mRNA stability upon deletion of Pub1 with a FCR of *pub1Δ*/wild-type=0.9±0.2 in rich media and a FCR of *pub1Δ*/wild-type=0.9±0.0 in minimal media (Fig. 1A-B). Likewise, we do not see a significant increase in *GCN4* mRNA stability upon deletion of Pub1 with a FCR of *pub1Δ*/wild-type=0.8±0.0 in rich media and a FCR of *pub1Δ*/wild-type=0.8±0.1 in minimal media (Fig. 1A-B). Further, $T_{1/2}$ analysis in *pub1Δ* strains showed no significant difference in $T_{1/2}$ of the *YAP1* mRNA in wild-type ($T_{1/2}$=13.2±0.4 min) versus *pub1Δ* strains ($T_{1/2}$=16.3±2.5 min; Fig. 2C). The *GCN4* mRNA also showed no difference in $T_{1/2}$ between wild-type ($T_{1/2}$=20.5±5.1 min) and *pub1Δ* strains ($T_{1/2}$=24.1±4.2 min; Fig. 2C).

It should be noted that we were unable to obtain the exact strains used in the original publication as the lab is no longer in operation (Ruiz-Echevarria and Peltz, 2000). However, we analyzed steady-state mRNA accumulation in multiple strain backgrounds including the parent strains for those used in the publication and were still unable to see any effect of *pub1Δ* on mRNA stability (data not shown).

From this we conclude that *trans*-acting factor Pub1 is not responsible for *YAP1* or *GCN4*

mRNA protection from NMD in the strain backgrounds and conditions that we used. Thus, there is likely another mechanism of protection that is stabilizing these two mRNAs.

YAP1 and *GCN4* mRNAs co-fractionate with polyribosomes

Since we have confirmed that the *YAP1* and *GCN4* mRNAs are wild-type mRNAs that are not degraded by NMD (Fig. 1), and we are unable to confirm that *trans*-acting factor Pub1 is responsible for this protection (Fig. 2), we wanted to try to identify the mechanism of protection of these two mRNAs. One way in which mRNAs can be protected from NMD is by inhibition of translation (Oliveira and McCarthy, 1995; Shoemaker and Green, 2012). We hypothesize that both *YAP1* and *GCN4* mRNAs are translated because of the vital functions of their protein products within the cell.

To confirm whether or not the *YAP1* and *GCN4* mRNAs are likely translated we performed a polyribosome analysis for each of these mRNAs. As expected, the *YAP1* mRNA is present in the polyribosome fractions (Fig. 3A). There is no significant difference in the *YAP1* mRNA co-fractionation with polyribosomes between wild-type and *upf1Δ* strains other than the presence of the larger migrating band in the *upf1Δ* strain, which is also consistently present in the *upf1Δ* strain in the steady-state and $T_{1/2}$ analysis (Fig. 3A; Fig. 1A-B). Interestingly, this slower migrating band of the *YAP1* mRNA behaves just like the pre-mRNA of *CYH2*, which is an NMD substrate, in the polyribosome analysis with the larger migrating band accumulating to higher levels in the lighter fractions in the *upf1Δ* strain (Chapter 2, Fig. 6). The pre-mRNA of *CYH2*

contains an intron, but the sequence analysis for *YAP1* mRNA does not indicate the presence of an intron, which would generate a pre-mRNA (SGD). This larger migrating band could be a longer form of the mRNA generated by translation initiation at an alternate upstream AUG, the one of the uORF for instance. In this case the ribosome would translate a significant portion of the 5' leader of the *YAP1* mRNA and proceed to translate in an alternate reading frame which ultimately results in the introduction of a PTC. This would explain why the longer mRNA is a substrate for NMD and is not detected in wild-type strains. Alternatively, the ribosome may readthrough the ORF stop codon and terminate downstream leading to the generation of a longer mRNA, however, this does not readily explain the sensitivity of the larger migrating band to NMD.

The *GCN4* mRNA also associates with polyribosomes as expected. The *GCN4* mRNA contains four short uORFs preceding the ORF. Translation of these uORFs and the scanning of the small ribosomal subunit along the 5' leader is what controls translation of the *GCN4* ORF where translation of the uORFs generally represses *GCN4* ORF translation (Grant and Hinnebusch, 1994; Gunisova et al., 2016; Hinnebusch, 2005). Because these short uORFs are continually translated more actively than the *GCN4* ORF it is expected that the *GCN4* mRNA will co-fractionate in the lighter fractions as the mRNA is associated with fewer ribosomes which occupy the short (2-3 codons) uORFs (Hinnebusch, 2005). Consistent with this, we do see the *GCN4* mRNA heavily present in the lighter fractions and more faintly present in the heavier fractions (Fig. 3B). Thus, although the *GCN4* ORF may not be actively translated at all times, the mRNA is still co-fractionating in a manner indicating that the mRNA is associating with ribosomes.

Importantly, the polyribosome profile of the *GCN4* mRNA is not significantly different between wild-type and *upf1Δ* strains (Fig. 3B).

From this we can conclude that both *YAP1* and *GCN4* mRNAs are likely to associate with polyribosomes as they co-fractionate in a pattern that is indicative of polyribosome association. However, further studies are needed to confirm the production of Yap1 and Gcn4 protein products.

YAP1 and *GCN4* mRNAs accumulate significantly in *xrn1Δ* strains

The predominant decay route for both wild-type mRNAs and NMD substrates is through Xrn1-mediated 5'→3' exonucloelytic degradation, although this process tends to occur more rapidly for NMD substrates (Cao and Parker, 2003). Given this, we hypothesized that the *YAP1* and *GCN4* mRNAs are likely degraded 5'→3' by Xrn1. However, the levels to which these mRNAs accumulate in an *xrn1Δ* strain will give us an indication of the contribution of 3'→5' mRNA decay by the exosome.

Upon deletion of the cytoplasmic 5'→3' exonuclease, Xrn1, both *YAP1* and *GCN4* mRNAs accumulate to significantly higher levels compared to wild-type strains. The *YAP1* mRNA had a FCR of *xrn1Δ*/wild-type=6.7±0.9 and *GCN4* mRNA had a FCR of *xrn1Δ*/wild-type=3.8±0.3 (Fig. 4A). It is interesting to note that though the fold changes are significantly increased in *xrn1Δ* strain for the *YAP1* and *GCN4* mRNAs accumulation is not nearly as high as what is seen for the *ENT4* mRNA (a wild-type NMD substrate), but also differ from the *PGK1* mRNA (a wild-type mRNA degraded by the deadenylation-dependent decapping pathway) which shows no significant difference in

fold change (Fig. 4A). Additionally, the fold changes for the *YAP1* and *GCN4* mRNAs in the *xrn1Δ* strain are less than what was seen for the *SSY5* mRNA (Chapter 2).

Consistently, the $T_{1/2}$ analysis of the *YAP1* and *GCN4* mRNAs both indicate significant stabilization of the mRNAs in the *xrn1Δ* strain (Fig. 4B). The *YAP1* mRNA has a $T_{1/2}$ of 19.2 minutes in wild-type strains compared to a $T_{1/2}$ of 31.3 minutes in the *xrn1Δ* strain. Likewise, the *GCN4* mRNA has a $T_{1/2}$ of 28.1 minutes in wild-type strains compared to a $T_{1/2}$ of 44.9 minutes in the *xrn1Δ* strain.

From this we can conclude that 5'→3' exonucloelytic degradation by Xrn1 is a significant contributor to the degradation of both the *YAP1* and *GCN4* mRNAs. However, because the steady-state mRNA accumulation fold changes in the *xrn1Δ* strain are not quite as significant as for other mRNAs we have looked at (e.g. *SSY5* and *ENT4*) the 3'→5' decay is likely more efficient for the *YAP1* and *GCN4* mRNAs.

YAP1 and _GCN4_ mRNAs do not accumulate in _dcp1Δ_ strains, but half-life analysis shows that _dcp1Δ_ stabilizes the mRNAs albeit to different extents

When degradation of an mRNA is initiated the Dcp1/2 decapping complex removes the 5' 7-methylguanosine cap to expose a 5' monophosphate which becomes the immediate substrate of the Xrn1 5'→3' exonuclease (Coller and Parker, 2004). If the mRNA is not decapped, 5'→3' decay cannot occur because the 5' end of the mRNA is not exposed for exonucloelytic degradation by Xrn1 (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1995; Muhlrad and Parker, 1994). Given this, we hypothesized that blocking decapping of the mRNA by deletion of Dcp1 (*dcp1Δ*) would

yield similar results to blocking 5'→3' degradation by Xrn1 (*xrn1Δ*) as these two events occur in immediate sequence.

Surprisingly, when the mRNA decapping complex Dcp1/2 is rendered inactive by deletion of the Dcp1 catalytic subunit the steady-state accumulations of the *YAP1* and *GCN4* mRNAs are not significantly affected compared to the wild-type strain. The *YAP1* mRNA only shows a FCR of *dcp1Δ*/wild-type=1.7±0.1, which is equivalent to the accumulation seen in the *upf1Δ* strain (FCR of *dcp1Δ*/wild-type=1.8±0.0) and is not considered to be significant (Fig. 5A). Similarly, the *GCN4* mRNA shows a FCR of *dcp1Δ*/wild-type=1.2±0.0, which is even less than the accumulation seen in the *upf1Δ* strain (FCR of *dcp1Δ*/wild-type=2.0±0.2; Fig. 5A). Both the *YAP1* and *GCN4* mRNAs exhibit a pattern that is similar to the wild-type NMD substrate *ENT4* mRNA.

However, the $T_{1/2}$ analysis in these strains reveals a bit of a different story, especially for the *YAP1* mRNA. Compared to the wild-type strain, in which the *YAP1* mRNA has a $T_{1/2}$=19.2 minutes, the $T_{1/2}$ of the *YAP1* mRNA in the *dcp1Δ* strain is more than double that in the wild-type strain at 51.2 minutes (Fig 5B). For the *GCN4* mRNA the difference is not quite as significant but is still increased compared to the steady-state mRNA accumulations. In the wild-type strain the *GCN4* mRNA has a $T_{1/2}$=28.1 minutes compared to 39.2 minutes in the *dcp1Δ* strain (Fig 5B).

Although mRNA stability measurements are typically more indicative of what is actually happening to the mRNA, the trend seen in the mRNA steady-state accumulations for the *YAP1* and *GCN4* mRNAs cannot simply be ignored. From these results we can conclude that at steady-state levels in the cell the *YAP1* and *GCN4* mRNAs are able to be

efficiently degraded 3'→5' by the exosome when decapping is blocked. When considering the $T_{1/2}$ analysis of the *YAP1* mRNA, blocking of decapping actually stabilizes the mRNA even more than in the *xrn1Δ* strain (Fig. 4B and Fig. 5B). The same is not true for the *GCN4* mRNA, however, where blocking decapping has a relatively similar effect to deletion of Xrn1 (Fig. 4B and Fig. 5B). Up until now our analysis has shown similar behavior of the *YAP1* and *GCN4* mRNAs. However, the decay of these two mRNAs seems to be slightly different in the influence of decapping of the mRNAs.

Some strains show greater *YAP1* and *GCN4* mRNA susceptibility to NMD

As was noticed with the *SSY5* mRNA, the *YAP1* and *GCN4* mRNAs appear to have differential susceptibility to NMD in the different strain backgrounds used in these studies. We originally only tested the susceptibility of the *YAP1* and *GCN4* mRNAs to NMD in the commonly used BY4741 and W303 backgrounds. Using these strains we saw no difference in *YAP1* or *GCN4* mRNA susceptibility to NMD (Fig. 1). However, when we began using the *dcp1Δ* and *xrn1Δ* strains we noticed a difference in both *YAP1* and *GCN4* mRNA accumulation in the wild-type versus *upf1Δ* strains from the same background (Figs. 4A and 5A). The *YAP1* and *GCN4* mRNAs show a steady-state FCR of *upf1Δ*/wild-type=1.8±0.0 and 2.0±0.2, respectively (Figs. 4A and 5A). Likewise, *ENT4* mRNA accumulation shows a similar FCR between wild-type and *upf1Δ* strains in both the BY4741 background and the W303 background, but in the strain background used to create the *dcp1Δ* and *xrn1Δ* strains this FCR is increased (compare *ENT4* mRNA in Fig. 1A with *ENT4* mRNA in Figs. 4A and 5A). The wild-type and *upf1Δ* strains from

the same background as the *xrn1Δ* and *dcp1Δ* strains were genetically constructed in the W303 background and the auxotrophic markers are published as being the same as the W303 strains that we originally tested (He et al., 1997; He and Jacobson, 1995, 2001). However, there is clearly a difference in the accumulation of the *YAP1* and *GCN4* mRNAs between these strains and our original W303 strains. Identifying the difference(s) between the strains from our earlier studies and the strains showing increased NMD susceptibility will be very beneficial in determining how the *YAP1* and *GCN4* mRNAs are protected from NMD.

Deadenylation mutants show little effect on *YAP1* and *GCN4* mRNA stability

The length of the mRNA poly(A) tail (~60-80 nt at steady state in *S. cerevisiae*) functions, in combination with the 5' mRNA cap, to regulate both the translational efficiency of the mRNA and the regulation of mRNA decay (Anderson and Parker, 1998; Beilharz and Preiss, 2007; Gallie, 1991; Muhlrad et al., 1995; Munroe and Jacobson, 1990). All mRNAs undergo a deadenylation step regardless of whether or not the mRNA is an NMD substrate (Norbury, 2013). The difference is that wild-type mRNA degradation is initiated by deadenylation of the mRNA poly(A) tail, which is a prerequisite for 5' decapping of the mRNA (Decker and Parker, 1993; Muhlrad and Parker, 1994). NMD substrates on the other hand undergo rapid 5' decapping and rapid deadenylation, but deadenylation is not required prior to decapping (Cao and Parker, 2003; Muhlrad et al., 1994; Muhlrad and Parker, 1994).

Deadenylation of an mRNA occurs through the combined efforts of the

Pan2/Pan3 and the Ccr4/Pop2/Not deadenylase complexes (Wahle and Winkler, 2013; Wiederhold and Passmore, 2010). The Pan2 subunit of the Pan2/Pan3 complex exhibits specific deadenylase activity and the Ccr4 subunit of the Ccr4/Pop2/Not is the catalytic subunit (Chen et al., 2002; Daugeron et al., 2001; Tucker et al., 2002; Tucker et al., 2001). There are conflicting reports on whether or not Caf1 is a catalytic subunit as well in *S. cerevisiae*, although the catalytic activity of this subunit has been verified in other organisms (Andersen et al., 2009; Daugeron et al., 2001; Jonstrup et al., 2007; Liang et al., 2009; Thore et al., 2003; Tucker et al., 2002; Viswanathan et al., 2004). Previous observations indicate that in the absence of the Pan2/Pan3 complex the Ccr4/Pop2/Not complex is capable of full mRNA deadenylation (Tucker et al., 2001). Because deadenylation is an important step in mRNA decay we hypothesized that deadenylation would likely play a prominent role in the degradation of the *YAP1* and *GCN4* mRNAs. To test this hypothesis we analyzed the *YAP1* and *GCN4* mRNA steady-state accumulation in several deadenylation mutant strains. To our surprise, the various deadenylation mutants did not cause a significant increase or decrease in the steady-state accumulation of either the *YAP1* or *GCN4* mRNAs (Fig. 6). Because the Pan2/Pan3 and Ccr4/Pop2/Not complexes can compensate for loss of the other it was not totally surprising that single deletions which only effected one of the complexes did not result in drastic changes in mRNA accumulation. However, deletions that render both complexes inactive (*ccr4Δpan2Δ*) did not result in significant accumulation either (*YAP1* mRNA FCR *ccr4Δpan2Δ*/wild-type=1.1±0.1 and *GCN4* mRNA FCR *ccr4Δpan2Δ*/wild-type=1.3±0.2; Fig. 6). The only strain in which there were slight notable differences,

although not considered significant according to the ≥ 2.0 FCR threshold, was the *ccr4Δcaf1Δ* strain for the *YAP1* mRNA (FCR *ccr4Δcaf1Δ*/wild-type=1.6±0.2) and in the same strain for the *GCN4* mRNA where the double deletion seemed to have the opposite effect (FCR *ccr4Δcaf1Δ*/wild-type=0.6±0.0; Fig. 6). It is possible based on this observation that the Pan2/Pan3 deadenylase complex is more efficient in compensating for loss of Ccr4/Pop2/Not activity on the *GCN4* mRNA than on the *YAP1* mRNA. We also analyzed steady-state accumulation of both the *ENT4* mRNA and the *PGK1* mRNA for comparison. Similar to the *YAP1* and *GCN4* mRNAs, neither the *ENT4* mRNA nor the *PGK1* mRNA seem to be largely effected by loss of deadenylation (Fig. 4). The only difference can be seen in the *upf1Δ* strains for the *ENT4* mRNA, which is expected since this mRNA is an NMD substrate.

From this we can conclude that blocking deadenylation through the Pan2/Pan3 complex or the Ccr4/Pop2/Not complex or both has little effect on *YAP1* and *GCN4* mRNA steady-state accumulation in cells. This can be explained by: 1) efficient decapping and 5'→3' mRNA decay without prior deadenylation, and/or 2) another mechanism of deadenylation aside from the Pan2/Pan3 and Ccr4/Pop2/Not deadenylase complexes.

The *YAP1* mRNA 3' end is significantly shorter in *xrn1Δ* and *dcp1Δ* strains compared to wild-type and *upf1Δ* strains

Finally, we wanted to determine at which step the *YAP1* and *GCN4* mRNAs are deadenylated. We hypothesized that the mRNA would be likely deadenylated as both

wild-type mRNAs and NMD-substrates are deadenylated, but we were unsure if deadenylation was occurring as a prerequisite to decapping. To do this we used the Poly(A) Tail-Length Assay Kit from Affymetrix (Materials and Methods). Using this kit a poly(A) polymerase first adds a short series of guanosine and inosine residues (G/I) to the 3' ends of polyadenylated mRNAs. The G/I tails become the priming site for reverse transcription of the mRNAs to cDNA molecules. Two pairs of primers are then used to amplify the 3' ends of the desired mRNAs. The first primer pair is the gene-specific (G.S.) primer pair, which consists of a forward and reverse primer that anneal to the 3' end of the mRNA both of which are upstream of the polyadenylation site(s). It is important to note that because the poly(A) sites for the mRNAs we used are not definitive as there are multiple predicted poly(A) sites we designed the G.S. primer pair to anneal upstream (3') of all predicted poly(A) sites (SGD). The second primer pair is the poly(A) tail ("Tail") primer pair, which consists of the same upstream primer from the G.S. primer pair and a universal reverse primer that anneals to the G/I tail (Fig. 7A). PCR products obtained from the use of these two primer pairs are then compared in order to determine the length of the 3' poly(A) tail.

The Poly(A) Tail-Length Assay Kit was used to determine the poly(A) tail length of steady-state *YAP1*, *ENT4*, and *PGK1* mRNAs in wild-type, *upf1Δ*, *xrn1Δ*, and *dcp1Δ* strains. Due to the cost of the kit and the number of reactions provided in each kit we did not analyze the 3' end of the *GCN4* mRNA. An RNase H control was not done so we are unable to positively confirm that the results observed are strictly due to differences in poly(A) tail length. We can, however, use the results to analyze differences in the 3' end

length of the mRNAs.

The results of this assay are shown in Figure 7B. In wild-type and *upf1Δ* strains the *YAP1* mRNA has a longer 3' end compared to the *xrn1Δ* and *dcp1Δ* strains. This pattern is consistent to what is seen for the *PGK1* mRNA (not an NMD substrate) and also the *SSY5* mRNA (Chapter 3, Figure 5), which is a wild-type mRNA that is protected from NMD (Fig. 7B; Kebaara and Atkin, 2009; Obenoskey et al., 2014; Chapter 2). Additionally, the pattern of 3' end length for the *YAP1* mRNA is opposite of the pattern for the *ENT4* mRNA, which is a wild-type NMD substrate, in the tested strains. However, it is important to note that the product resulting from use of the "Tail" primers is smaller than the product for the G.S. primers, which should not be the case if the primers were designed accurately. This indicates that the primers for the *YAP1* mRNA need redesigned for a more accurate analysis.

From this we can conclude that the *YAP1* mRNA is able to be shortened at the 3' end when either decapping or 5'→3 decay are blocked. This also shows that the 3' end shortening is occurring much more slowly or not at all when both decapping and 5'→3' decay are functioning normally as in the wild-type and *upf1Δ* strains.

Discussion

Previously we presented the case of the *SSY5* mRNA, which is a wild-type mRNA in *S. cerevisiae* that contains multiple NMD-targeting signals but is not degraded by NMD (Chapter 2). Here we investigate two additional wild-type mRNAs in *S. cerevisiae*, the *YAP1* and *GCN4* mRNAs that also have at least one NMD-targeting

signal—a translated uORF—and are protected from degradation by NMD (Fig. 1). The discovery of the *YAP1* and *GCN4* mRNAs as wild-type mRNAs protected from NMD was not novel as this was previously shown (Ruiz-Echevarria et al., 1998; Vilela et al., 1998). However, a follow-up study identified a simple mechanism of protection of these two mRNAs from NMD by which *trans*-acting factor Pub1 binds to a stabilizer element in the 5' leader region of the mRNAs. In this study the binding of Pub1 to the STE was convincingly shown to be solely responsible for the protection of both *YAP1* and *GCN4* mRNAs from NMD (Ruiz-Echevarria and Peltz, 2000). These results were of great interest to us as we searched for the mechanism by which the *SSY5* mRNA is protected from degradation by NMD. The *SSY5* mRNA also has a translated uORF just like the *YAP1* and *GCN4* mRNAs, and it seemed like an appropriate hypothesis that Pub1 could also be responsible for the protection of the *SSY5* mRNA from NMD. However, when we began the experiments to see if Pub1 was responsible for the protection of *SSY5* mRNA from NMD (Chapter 2) we were unable to reproduce the results showing Pub1 was responsible for the protection of *YAP1* and *GCN4* mRNAs from NMD, which had been included in the experiment as positive controls (Fig. 2). Cells were grown in both rich and minimal media (Fig. 2) and we tested as many different strain backgrounds as we could obtain in order to rule out all known variables (data not shown).

We were able to confirm that both the *YAP1* and *GCN4* mRNAs are likely protected from degradation by NMD, but this stability is not quite as clear as previously reported and is likely strain dependent (Figs. 1, 4-5). The $T_{1/2}$ analysis of both mRNAs in wild-type and *upf1Δ* strains is more indicative of protection from NMD than is the

analysis of the steady-state accumulation of the mRNAs in wild-type and *upf1Δ* strains (Fig. 1). Further, we were able to show that *YAP1* mRNA does cofractionate with heavy fractions of the polyribosomes, which is an indication of association with polyribosomes (Fig 3A). Likewise, the *GCN4* mRNA is also found cofractionating where we would expect although this pattern looks rather different from a normally translated mRNA (Fig. 3B). Translation of the *GCN4* mRNA is regulated by the translation of its four short uORFs, which are continually translated in order to repress the translation of the main ORF until the gene product, Gcn4, is needed (Grant and Hinnebusch, 1994; Gunisova et al., 2016; Hinnebusch, 2005). Thus, the *GCN4* mRNA is expected to be present in the lighter fractions since the continual translation of its four short uORFs means the mRNA is associated with fewer ribosomes as these uORFs are only 2-3 codons in length. Importantly, neither the *YAP1* mRNA nor the *GCN4* mRNA show differences in fractionation between the wild-type and *upf1Δ* strains (Fig. 3A-B). Association with polyribosomes (in the case of the *YAP1* mRNA) or the few uORF occupying ribosomes (in the case of the *GCN4* mRNA) leads to the likely hypothesis that the mRNA is being actively translated, although Westerns would need to be done in order to positively confirm this. If the mRNA is being actively translated then it rules out the possibility that the mRNA is protected from degradation by NMD by inhibition of translation.

We also looked at the stability of the *YAP1* and *GCN4* mRNAs in decapping, 5'→3' decay, and various deadenylation mutants. When 5'→3' decay is blocked in the *xrn1Δ* strains, both the *YAP1* and *GCN4* mRNAs behave similarly and accumulate to significantly higher levels compared to the wild-type strain. However, this accumulation

is not as great as what we saw for the *SSY5* mRNA (Chapter 2) or for the *ENT4* mRNA (a wild-type NMD substrate; Fig. 4). The two most obvious explanations for this are 1) the exosome-mediated 3'→5' decay of the *YAP1* and *GCN4* mRNAs is more efficient (i.e. faster) than for the *SSY5* or *ENT4* mRNAs, or 2) Rat1 (also known as Kem1) digestion, which was identified as the only other enzyme in yeast with 5'→3' exonuclease activity, is able to work more efficiently on the *YAP1* and *GCN4* mRNAs than on the *SSY5* or *ENT4* mRNAs (He and Jacobson, 2001).

A previous study also showed that Upf1 can actually stimulate the degradation of decapped transcripts in an *xrn1Δ* strain (He and Jacobson, 2001). This could be one of the reasons that we see higher steady-state accumulations and slightly longer $T_{1/2}$ in the *xrn1Δupf1Δ* strains. Another reason could also be that in this background the *YAP1* and *GCN4* mRNAs are slightly more susceptible to NMD as can be seen comparing the wild-type and *upf1Δ* strains (Fig. 4).

Interestingly, blocking decapping in the *dcp1Δ* strain, which we expected to yield a similar result as seen in the *xrn1Δ* strain, had very little influence on the steady-state accumulation of either the *YAP1* mRNA or the *GCN4* mRNA (Fig. 5A). This indicates that the exosome-mediated 3'→5' degradation of both mRNAs is much more efficient when the 5' mRNA cap is present since mRNAs in the *xrn1Δ* strain are decapped (He and Jacobson, 2001). However, the $T_{1/2}$ analysis for the *YAP1* and *GCN4* mRNAs in the *dcp1Δ* strain tells a bit of a different story. Both mRNAs are more stable in the *dcp1Δ* strain compared to the wild-type strain, and this increase in stability is significant for the *YAP1* mRNA (Fig. 5B). Taking into consideration the $T_{1/2}$ analysis of both mRNAs in

the wild-type, *xrn1Δ*, and *upf1Δ* strains it does appear that blocking both decapping and 5'→3' degradation causes significant mRNA stabilization indicating that the 3'→5' degradation is not as efficient as the steady-state analysis in the *dcp1Δ* strain leads us to believe. This also leads us to point out that steady-state accumulations do not always tell the whole story and a $T_{1/2}$ analysis is an important piece of the puzzle, which we also saw in Figure 1.

When blocking deadenylation through deletion of the catalytic subunits of either the Pan2/Pan3 complex or the Ccr4/Pop2/Not complex, or both we saw little influence on steady-state mRNA accumulation of either the *YAP1* mRNA or the *GCN4* mRNA (Fig. 6). However, as pointed out previously, a $T_{1/2}$ analysis of both mRNAs in these strains would need to be done in order to make any definitive conclusions. If we make the assumption that the $T_{1/2}$ analysis would agree with the steady-state accumulations, then we can say that blocking deadenylation has little effect on the degradation of the *YAP1* or *GCN4* mRNAs. This tells us that deadenylation-independent decapping and subsequent 5'→3' decay is able to proceed in the absence of deadenylation. Although the Pan2/Pan3 complex and the Ccr4/Pop2/Not complex are able to compensate for loss of one another, the fold changes in the double mutant *ccr4Δpan2Δ* are the most revealing about how lack of deadenylation influences the stability of these two mRNAs (Fig. 6). To make this analysis more complete it would also be helpful to look at the steady-state mRNA accumulation and half-lives in 3'→5' decay (exosome) mutants.

When we used the Poly(A) Tail-Length Analysis kit to determine what was happening on the 3' end of the mRNA we found that the *YAP1* mRNA behaved more

similarly to the *PGK1* mRNA, which is not an NMD substrate and is typically degraded 5'→3' by the deadenylation-dependent decapping mechanism. However, the *PGK1* mRNA can also undergo 3'→5' degradation when 5'→3' degradation is blocked, but this process occurs more slowly (Muhlrad and Parker, 1994). This appears to be what is happening for both the *PGK1* mRNA and the *YAP1* mRNA according to our results in Figure 7. Both the *PGK1* and *YAP1* mRNAs show longer 3' ends in the wild-type and *upf1Δ* strains and shorter 3' ends in the *xrn1Δ* and *dcp1Δ* strains. In contrast, the known wild-type NMD substrate *ENT4* mRNA shows the opposite pattern with longer 3' ends in the *xrn1Δ* and *dcp1Δ* strains than in the wild-type and *upf1Δ* strains (Fig. 7).

Going forward, it will be important to identify if the NMD mRNP, which ultimately triggers the degradation of an mRNA through the NMD pathway, is assembling on the *YAP1* mRNA or the *GCN4* mRNA. If the NMD mRNP is forming on these mRNAs then protection from NMD is most likely incurred at a point downstream of substrate recognition. Conversely, if the NMD mRNP complex never forms on the mRNA, then protection is likely incurred before substrate recognition.

Figure Legends

Fig. 4-1. *YAP1* mRNA is protected from degradation by NMD while *GCN4* mRNA is partially protected from degradation by NMD. **A**) Northern blot analysis of the steady-state accumulation of the *YAP1*, *GCN4* and *ENT4* (a wild-type NMD substrate) mRNAs in wild-type (AAY277) and *upf1Δ* (AAY363) strains in the BY4741 background and in wild-type (AAY187) and *upf1Δ* (AAY320) strains in the W303 background. Strains were grown in rich media (YAPD). *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials. **B**) Northern blot analysis of the *YAP1* and *GCN4* mRNA half-lives in wild-type (AAY277) and *upf1Δ* (AAY363) strains from the BY4741 background. Strains were grown in rich media (YAPD). 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay. **C**) Northern blot analysis of the steady-state accumulation of the *YAP1*, *GCN4* and *ENT4* mRNAs in wild-type (AAY277) and *upf1Δ* (AAY363) strains in the BY4741 background. Strains were grown in minimal media (SD + his, leu, met, ura, lys). **D**) Northern blot analysis of the *YAP1* and *GCN4* mRNA half-lives in wild-type (AAY277) and *upf1Δ* (AAY363) strains from the BY4741 background. Strains were grown in minimal media (SD + his, leu, met, ura, lys). 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential

decay and are the average of three independent trials.

Fig. 4-2. *YAP1* and *GCN4* mRNAs are not protected from NMD by *trans*-acting factor Pub1. **A**) Northern blot analysis of the steady-state accumulation of *YAP1* and *GCN4* mRNAs in wild-type (AAY277), *upf1Δ* (AAY363), *pub1Δ* (AAY538), and *pub1Δupf1Δ* (AAY590) strains in the BY4741 background. Strains were grown in rich media (YAPD). *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials. **B**) Northern blot analysis of the steady-state accumulation of *YAP1* and *GCN4* mRNAs in wild-type (AAY277), *upf1Δ* (AAY363), and *pub1Δ* (AAY538) strains in the BY4741 background. Strains were grown in minimal media (SD + his, leu, met, ura, lys). **C**) Northern blot analysis of *YAP1* and *GCN4* mRNA half-lives in wild-type (AAY277) and *pub1Δ* (AAY538) strains in the BY4741 background. Strains were grown in minimal media (SD + his, leu, met, ura, lys). 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay and are the average of three independent trials.

Fig. 4-3. *YAP1* and *GCN4* mRNAs co-fractionate with polyribosomes. Polyribosome analysis of *YAP1* (**A**) and *GCN4* (**B**) mRNAs in wild-type (AAY187) and *upf1Δ* (AAY320) strains. Strains were grown in YAPD. Whole cell lysate was centrifuged

through a 15-50% sucrose gradient. Total RNA was extracted from each fraction collected and total RNA from fractions 2-19 was transferred to a membrane for Northern analysis. Fractions corresponding to the 40S ribosomal peak are in lanes 4-5, fractions corresponding to the 60S ribosomal peak are in lanes 6-7, the fraction corresponding to the 80S ribosomal peak is in lane 8 and polyribosome fractions are in lanes 9-15. The graph represents the distribution of the mRNA in each fraction based on the relative corrected volume of the mRNA present in each lane. In lanes where two bands were present only the band corresponding to the size of the expected band for the mRNA was quantified (~2.0 kb for the *YAP1* mRNA). Polysome analysis was repeated twice with similar results.

Fig. 4-4. *YAP1* and *GCN4* mRNAs accumulate significantly in *xrn1Δ* strains. Northern blot analysis of the steady-state accumulation of the *YAP1*, *GCN4*, *ENT4*, and *PGK1* mRNAs in wild-type (AAY360), *upf1Δ* (AAY589), *xrn1Δ* (AAY389), and *xrn1Δupf1Δ* (AAY611) strains grown in YAPD. *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials. **B**) mRNA half-life analysis of the *YAP1* and *GCN4* mRNAs in the same strains used in part A. Strains were grown in YAPD. 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay.

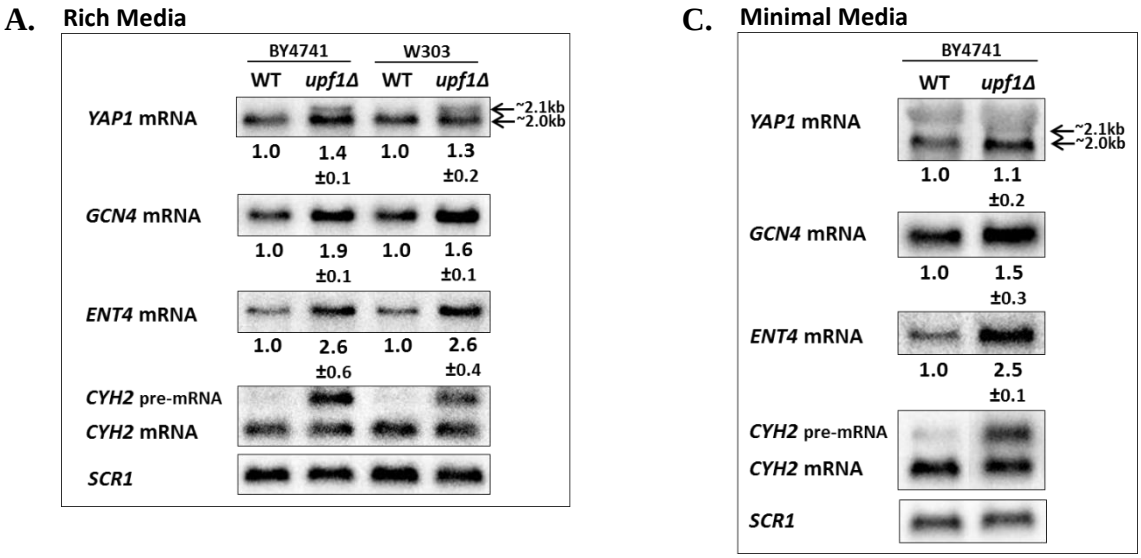
Fig. 4-5. *YAP1* and *GCN4* mRNAs do not accumulate in *dcp1Δ* strains, but half-life analysis shows that *dcp1Δ* stabilizes the mRNAs albeit to different extents. **A**) Northern blot analysis of the steady-state accumulation of the *YAP1*, *GCN4*, *ENT4* and *PGK1* mRNAs in wild-type (AAY360), *upf1Δ* (AAY589), *dcp1Δ* (AAY390), and *dcp1Δupf1Δ* (AAY621) strains grown in YAPD. *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio from wild-type; values are an average of three independent trials. **B**) mRNA half-life analysis of the *YAP1* and *GCN4* mRNAs in the same strains used in part A. Strains were grown in YAPD. 10µg/ml Thiolutin was added to mid-log cultures at time=0 min. Half-life calculations were determined using a graph of percent mRNA remaining at each time point during the exponential decay.

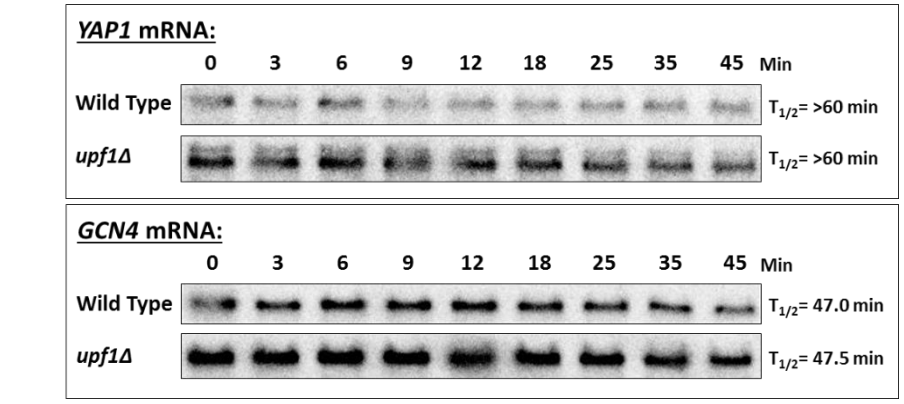
Fig. 4-6. Deadenylation mutants show little effect on *YAP1* and *GCN4* mRNA stability. Northern blot analysis of the steady-state accumulation of the *YAP1*, *GCN4*, *ENT4*, and *PGK1* mRNAs in wild-type (AAY391), *upf1Δ* (AAY594), *ccr4Δ* (AAY393), and *ccr4Δupf1Δ* (AAY596), *ccr4Δcaf1Δ* (AAY394), *ccr4Δcaf1Δupf1Δ* (AAY609), *pan2Δ* (AAY395), *pan2Δupf1Δ* (AAY595), *pan2Δccr4Δ* (AAY396), and *pan2Δccr4Δupf1Δ* (AAY610) strains grown in YAPD. *CYH2* pre-mRNA is an NMD control (*CYH2* pre-mRNA is an NMD substrate, *CYH2* mRNA is not an NMD substrate) and *SCR1* is shown for loading. Fold change values are normalized and determined as a fold change ratio

from wild-type; values are an average of three independent trials.

Fig. 4-7. The *YAP1* mRNA 3' end is significantly shorter in *xrn1Δ* and *dcp1Δ* strains compared to wild-type and *upf1Δ* strains. **A**) Schematic of how the Poly(A) Tail Length Analysis works. This analysis was done using the "Poly(A) Tail-Length Assay Kit" from Affymetrix (#76455). In this assay, a G/I tail is added to the end of mRNAs containing a Poly(A) tail. These G/I-tailed mRNAs are then reverse transcribed to cDNA, which provides a template for subsequent PCR. PCR is performed using two sets of primers independently. The Gene Specific (G.S.; teal) primer pair is comprised of a forward primer that binds anywhere within the open reading frame (ORF) or 3' UTR and a reverse primer that binds immediately upstream (5') of the Poly(A) start site. *It is important to note that the exact Poly(A) start site of the mRNAs in this figure were unknown so the reverse primer was designed just upstream of the first *predicted* Poly(A) start site according to the *Saccharomyces* Genome Database (SGD). The Poly(A) Tail (Tail; pink) primer pair uses the same forward primer from the G.S. pair and a reverse primer that anneals to the G/I tail (provided with the kit). **B**) Products from the PCR reactions using the above primer pairs for the *YAP1*, *ENT4*, and *PGK1* mRNAs in the wild-type (AAY360), *upf1Δ* (AAY589), *xrn1Δ* (AAY389), and *dcp1Δ* (AAY390) strains were resolved on a 2.5% agarose TAE gel. Strains were grown in YAPD and the total RNA samples used for the reactions were the same RNA samples that were used for Northern analysis in figures 2A and 3A.

Figure 4-1





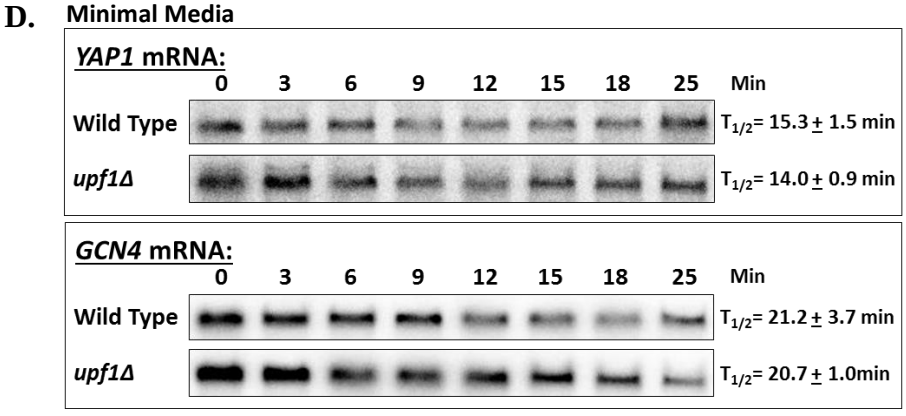


Figure 4-2

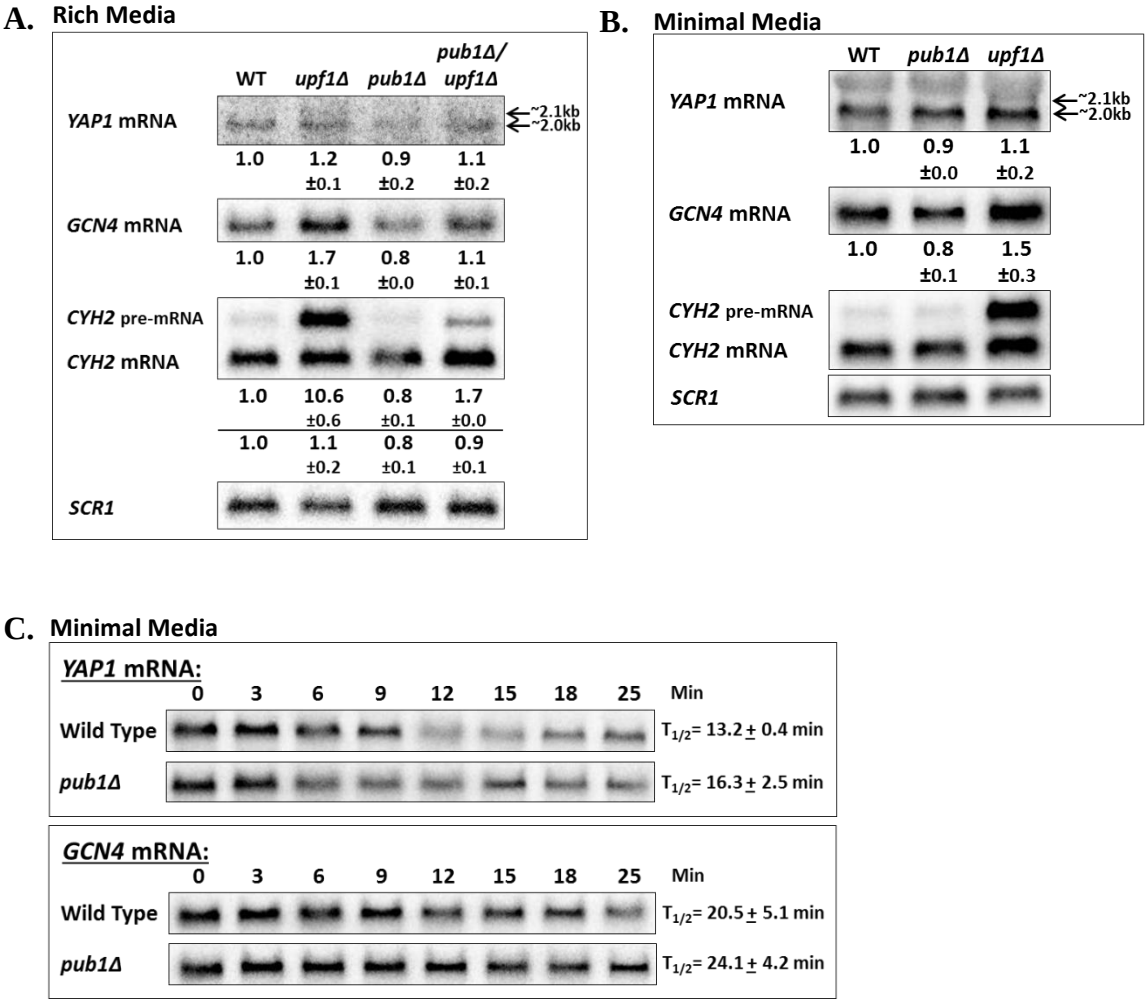
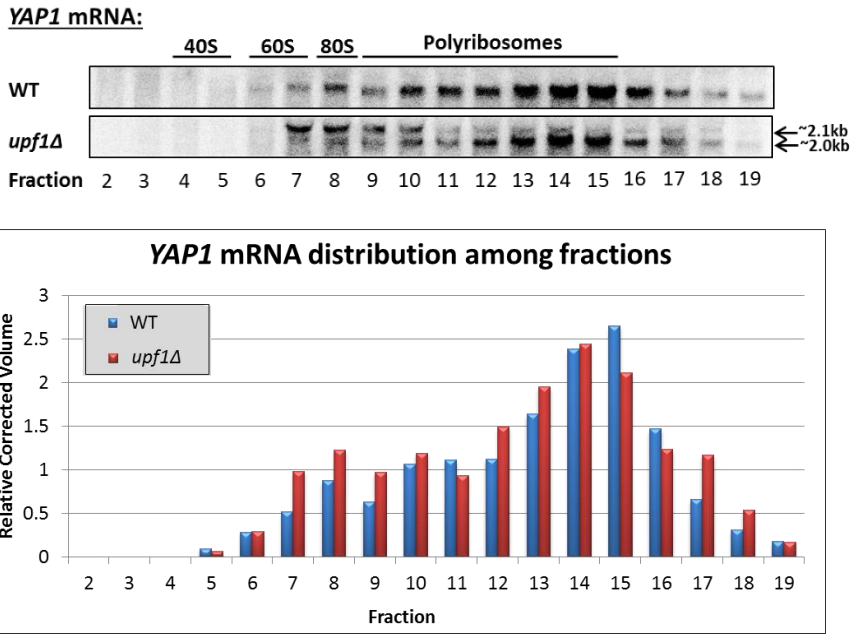


Figure 4-3

A.



B.

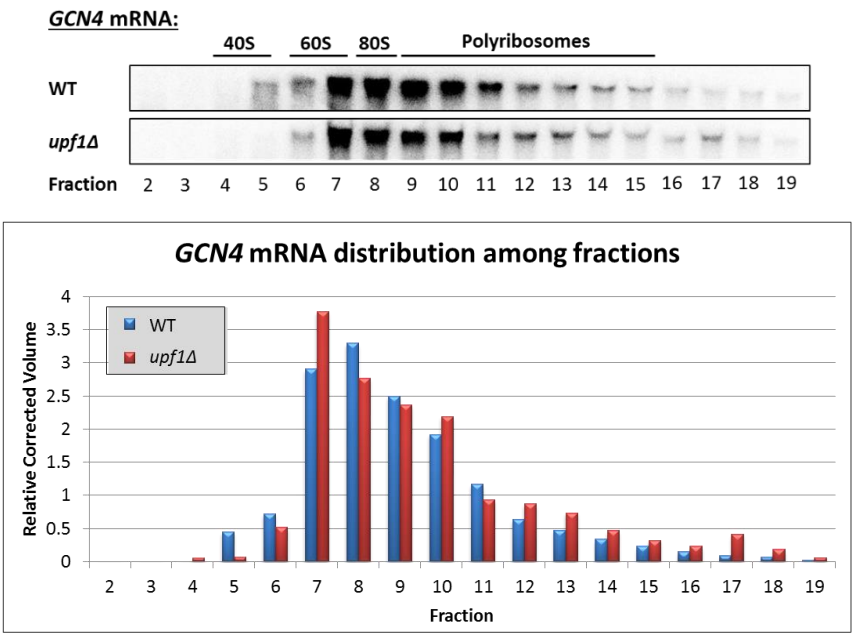
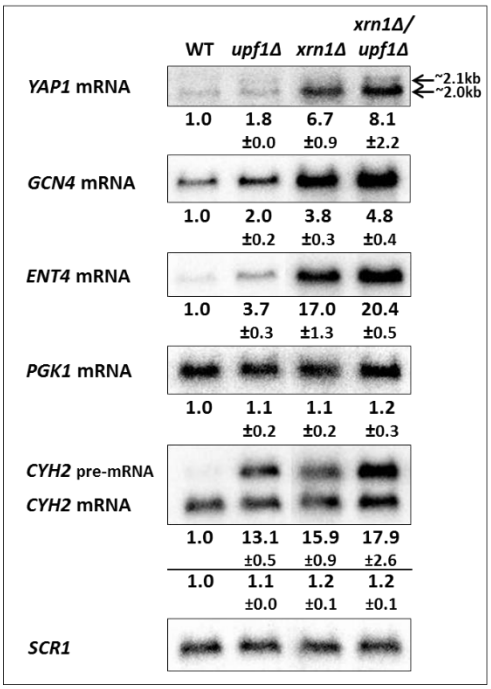


Figure 4-4

A.



B.

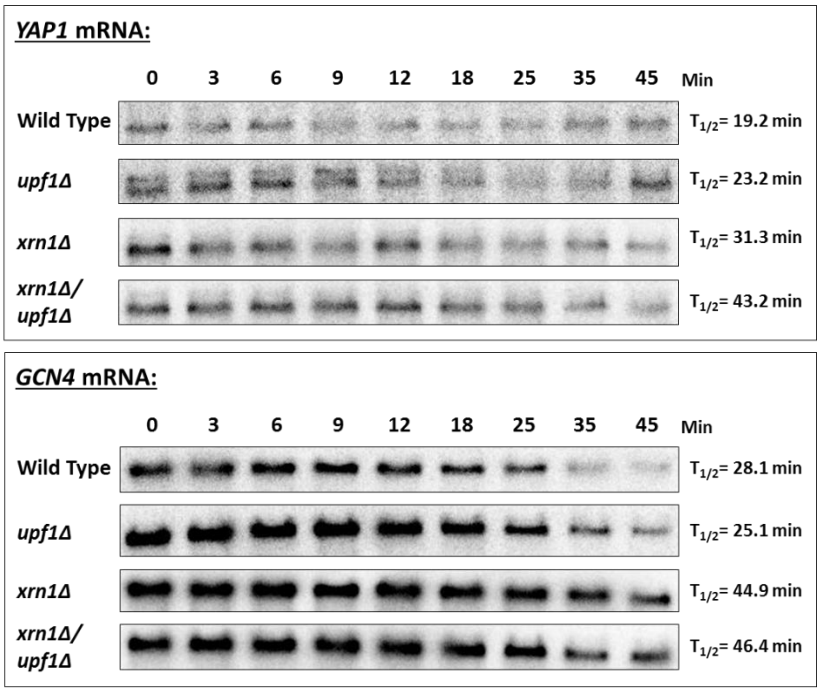
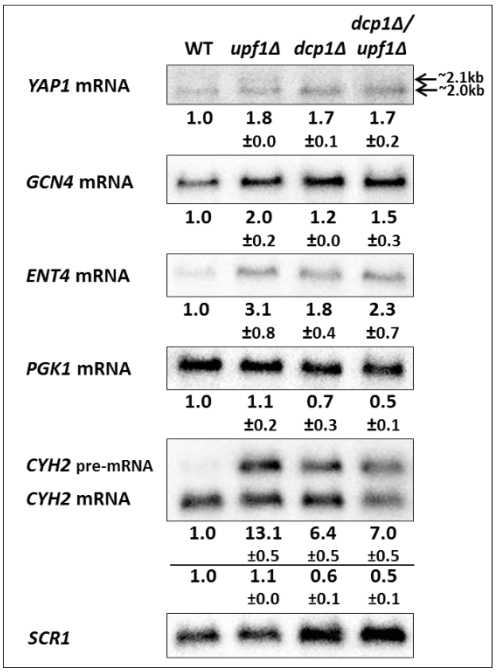


Figure 4-5

A.



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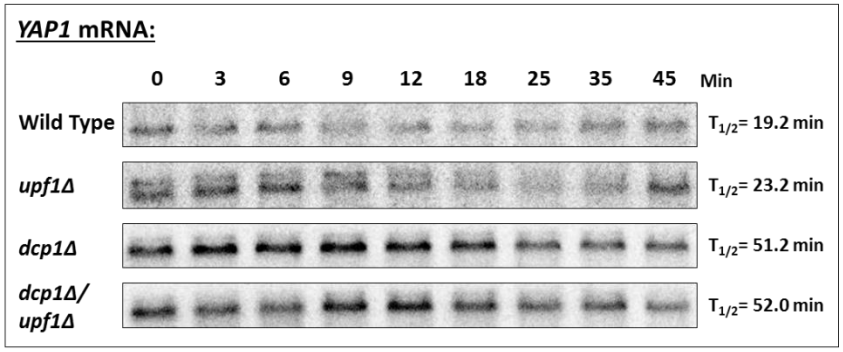

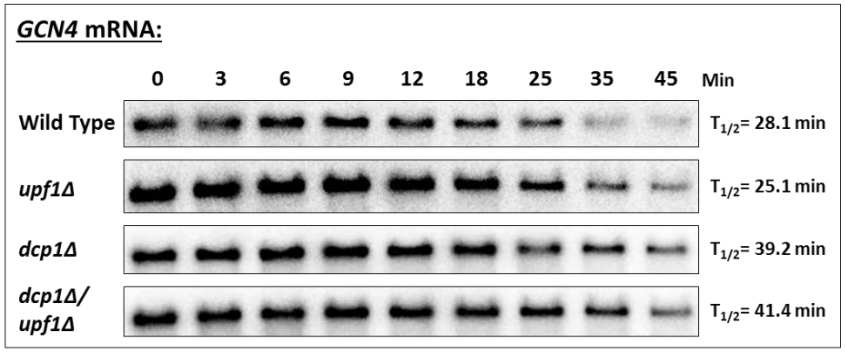

Figure 4-6

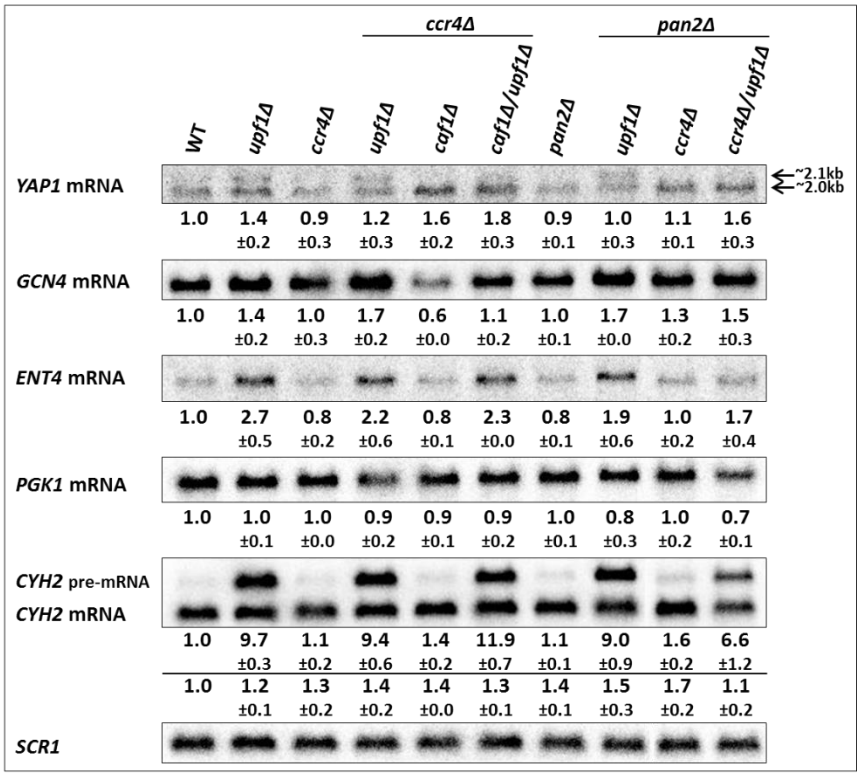

Figure 4-7

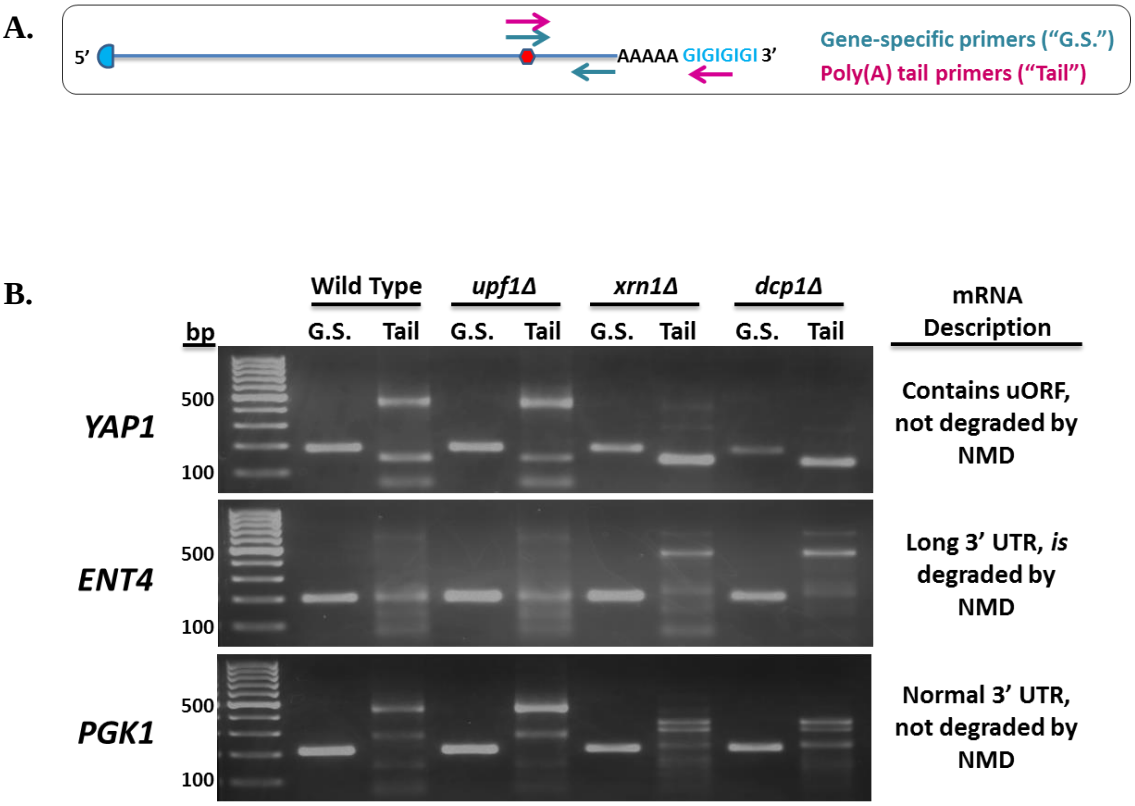

Table 4-1. Strains used in this study

Strain	Parent Strain	Genotype	Source
W303		*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Ralser *et al.*, 2012
BY4741		*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winston *et al.*, 1998
AAY320	W303	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1::URA3*	Atkin lab
AAY360		*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	He and Jacobson, 1995
AAY363	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 upf1::URA3*	Atkin lab
AAY389	AAY360	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 xrn1::ADE2*	He and Jacobson, 2001
AAY390	AAY360	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 dcp1::URA3*	He and Jacobson, 2001
AAY391		*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM*	Tucker *et al.*, 2001
AAY393	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO*	Tucker *et al.*, 2001
AAY394	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO caf1::URA3*	Tucker *et al.*, 2001
AAY395	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM pan2::URA3*	Tucker *et al.*, 2001
AAY396	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO pan2::URA3*	Tucker *et al.*, 2001
AAY538	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pub1Δ*	Open Biosystems
AAY589*	AAY360	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1::URA3*	Atkin lab
AAY590*	AAY538	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pub1Δ upf1::URA3*	Atkin lab
AAY594*	AAY391	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM upf1::TRP1*	Atkin lab
AAY595*	AAY395	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM pan2::URA3 upf1::TRP1*	Atkin lab
AAY596*	AAY393	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO upf1::TRP1*	Atkin lab
AAY609*	AAY394	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO caf1::URA3 upf1::TRP1*	Atkin lab
AAY610*	AAY396	*MATa his4-539 trp1 ura3-52 leu2-3,112 cup1Δ::LEU2PM ccr4::NEO pan2::URA3 upf1::TRP1*	Atkin lab
AAY611*	AAY389	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 xrn1::ADE2 upf1::TRP1*	Atkin lab
AAY621*	AAY390	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 dcp1::URA3 upf1::TRP1*	Atkin lab
* = new strain created for this study			

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CHAPTER 5

N^5-Phosphonoacetyl-$_L$-ornithine (PALO): influence on regulation of amino acid biosynthetic genes in *Saccharomyces cerevisiae*

Reference:

Johnson, B., Steadman, R., Patefield, K.D., Bunker, J.J., Atkin, A.L., Dussalt, P. (2011) N^5-Phosphonoacetyl-$_L$-ornithine (PALO): A convenient synthesis and investigation of influence on regulation of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. *Bioinorganic and Med. Chem. Letters*. **21:** 2351-2353.

Abstract

A scalable four-step synthesis of the ornithine transcarbamylase inhibitor N^5-phosphonoacetyl-L-ornithine (PALO) is reported based upon selective protection of the amino acid of ornithine as a boroxazolidinone. Investigations in the model organism *Saccharomyces cerevisiae* found PALO did not influence growth rate or expression of genes involved in arginine metabolism.

Introduction

N^5-Phosphonoacetyl-$_L$-ornithine (PALO) is a bisubstrate transition-state analog which competitively inhibits ornithine transcarbamylase (OTC), shutting down the biosynthesis of citrulline in both the urea and the arginine biosynthetic pathways (Mori et al., 1977; Penninckx and Gigot, 1978). Competitive inhibitors of amino acid metabolism have been key components in studies ranging from arginine starvation in fungi and bacteria to studies on OTC deficiency (Alewood et al., 1984; Kinney and Lusty, 1989).

Earlier work by Kinney and Lusty suggested that PALO would be an effective tool for the study of arginine metabolism in the yeast *Saccharomyces cerevisiae* (Kinney and Lusty, 1989). Arginine is used by all organisms for protein synthesis, and is also used by yeast as a nitrogen source (Hoogenraad, 1978; Penninckx and Gigot, 1978, 1979). The biosynthesis and metabolism of arginine are carefully regulated (Wu and Morris, 1998). Arginine metabolism is required for the escape of the human pathogenic yeast *Candida albicans* from macrophages (Lorenz et al., 2004). We were specifically interested in applying PALO as a tool to determine whether both arginine biosynthesis and metabolism are required for this evasion of the immune system, which is presumed critical to the success of *C. albicans* as an invasive opportunistic pathogen. We now report a short and scalable synthesis of PALO, as well as results of investigations into the influence of PALO on growth and arginine metabolism in the model organism *S. cerevisiae.*

Materials and Methods

Yeast strains and growth conditions

Saccharomyces cerevisiae strains BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*) (Ref 1, 2, 7), YSC1178-7500224 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 ARG5,6-TAP*), YSC1178-7502950 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0, CPA1-TAP*) and YSC1178-7500415 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0, TRP5-TAP*) were used. BY4741 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The YSC1178 strains were purchased from Open Biosystems Products (Huntsville, AL). *S. cerevisiae* strains were inoculated from a saturated culture incubated at 30°C in YPD in a tube roller in minimal medium supplemented with leucine, methionine, uracil and histidine. Cultures were treated with 15 µM PALO in a 50 mM potassium phosphate pH 6.8 buffer, potassium phosphate buffer pH 6.8 alone (untreated), 1 mg/mL arginine, or both 15 µM PALO and 1 mg/mL arginine at the time of inoculation. Cells were incubated at 30°C and then harvested at mid-log growth phase (OD_{600} 0.4-0.6). Cells were observed for effects on growth via incubation at 30°C in nitrogen-limiting medium containing 2% dextrose, 1 mM ammonium sulfate, 100 µM potassium phosphate buffer pH 6.8, and 10 µg/mL leucine, methionine, uracil and histidine.

Quantitative northern analysis of mRNA levels

Steady-state mRNA levels were measured as previously described. Oligolabeled DNA probes were used to probe the northern blots. DNA probes were generated using primer sets for amplifying yeast open reading frames based on the sequences available from the Saccharomyces Genome Database (http://www.yeastgenome.org/).

Western blot analysis

Western blots were prepared as previously described (Atkin et al., 1995). TAP-tagged Arg5,6p, Cpa1p and Trp5p were detected with Supersignal West Pico chemiluminescent substrate, using the manufacturers protocol (Pierce, Rockford, IL). Rabbit anti-TAP polyclonal antibodies were purchased from Thermo Fisher Scientific Inc. (Rockford, IL) and goat anti-rabbit IgG polyclonal antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Results

A study by Kinney and Lusty reported that PALO increased expression of *CPA1*, *CPA2*, *HIS3*, and *TRP5* reporter constructs in the yeast *S. cerevisiae* (Kinney and Lusty, 1989). The data were consistent with limitation of arginine leading to specific up regulation of arginine biosynthetic genes (*CPA1* and *CPA2*), and a general amino acid starvation response typified by up regulation of *HIS3* and *TRP5*.

We tested whether treatment with PALO resulted in changes in mRNA accumulation and/or translation of genes involved in amino acid biosynthesis. We examined expression of two genes required for arginine biosynthesis; *ARG5,6,* which encodes protein involved in the second and third steps of arginine biosynthesis from glutamate, and *CPA1*, which encodes a subunit of arginine-specific carbamyl-phosphate synthetase. Both of these genes are up regulated specifically in response to arginine limitation. Expression of *TRP5,* which encodes an enzyme that catalyzes the final step in tryptophan biosynthesis, was also examined as an indicator of the general amino acid

starvation response. Quantitative northern analysis was performed to measure mRNA abundance, a measure of the combined rates of transcription and mRNA decay. Western analysis was performed to measure mRNA abundance, a measure of the combined rates of transcription and MRNA decay. Western Analysis was used to look at the level of protein synthesis for these genes. This combined approach enabled direct evaluation of effects on transcription, mRNA stability, and translation.

Although the yeast strains from the earlier studies were not available, (Kinney and Lusty, 1989) they were derived from the W303 genetic background, which usually carry the *can1-100* mutation (Thomas and Rothstein, 1989). As *CAN1* encodes an arginine permease required for efficient uptake of arginine, the *can1-100* mutation is not well suited for the current experiments. As a compromise, and to assess if PALO will be generally useful as a metabolic tool in *S. cerevisiae,* the current data was obtained using the BY4741 strain of *S. cerevisiae* (Brachmann et al., 1998). This strain is derived from S288C, the strain used in the systematic sequencing project, and it is the most commonly used genetic background for genome-wide functional analyses.

If PALO affects transcription or mRNA stability, we expected to observe an increase in expression of *ARG5,6 CPA1,* and *TRP5* mRNA transcripts in the presence of PALO. We also expected that addition of arginine would relieve the arginine limitation induced by PALO. Cells were grown and treated with either 15 μM of the synthesized PALO, 1 mg/mL L-arginine, or 15 μM of the synthesized PALO and 1 mg/mL L-arginine as described by Kinney and Lusty (Kinney and Lusty, 1989). An untreated culture was grown in the same media as a control. The abundance of *ARG5,6, CPA1,* and *TRP5*

mRNAs was measured by quantitative northern analysis (Fig. 1A). The abundance of *ARG5,6*, *CPA1*, and *TRP5* mRNAs in the PALO treated cells was not significantly different from the untreated cells. The abundance of *ARG5,6* and *CPA1*, but not *TRP5* mRNAs was lower in cells treated with arginine regardless of whether the cells were treated with PALO or not. Additionally, the abundance of the *TRP5* transcript did not show any variation in response to addition of PALO, arginine, or both. In summary, treatment with PALO had no effect on the abundance of *ARG5,6*, *CPA1* or *TRP5* mRNAs.

The possible effect of PALO on translation was investigated by western analysis (Fig. 1B). If PALO affects translation, we expected to see an increase in the production of Arg5,6p, Cpa1p, and Trp5p in the presence of PALO. We also expected that addition of L-arginine would reduce protein levels of Arg5,6p and Cpa1p, but not Trp5p. We used cell extracts from strains (YSC1178-7500224, YSC1178-7502950 and YSC1178-7500415, respectively) carrying TAP-tagged alleles of *ARG5,6*, *CPA1*, and *TRP5* for the Western analysis. These strains produce a TAP-fusion protein for each gene, which can be detected on western blots with anti-TAP antibodies. BY4741 was used as an untagged control because it is the parent strain for the TAP-tagged strains. Cells were grown and treated in the same manner as for the northern analysis. Measurement of the signal intensity of the bands was detected by western blotting revealed no difference in the production of Arg5,6, Cpa1, or Trp5 between strains treated with 15 μM PALO and the control. As expected, addition of L-arginine reduced production of Arg5,6 and Cpa1p, but not Trp5p regardless of whether PALO was added or not. In summary, PALO had no

effect on Arg5,6, Cpa1, and Trp5 protein levels.

PALO did not affect the growth rate of BY4741 in minimal media or nitrogen-limiting medium (data not shown), used in an attempt to increase sensitivity to PALO by lowering intracellular arginine concentrations. Growth in nitrogen-limiting medium induces cells to use amino acids, including arginine, as a source of nitrogen. These findings were consistent with the observations reported by Kinney and Lusty (Kinney and Lusty, 1989).

Conclusions

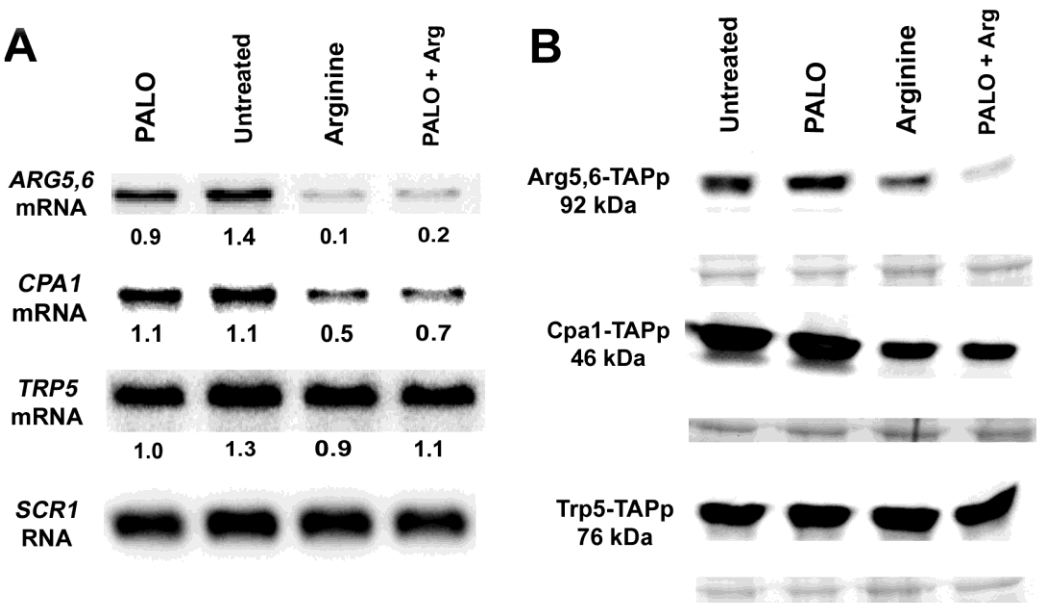
We have developed an efficient synthesis of very pure PALO by a route suitable for both analytical and preparative applications. In contrast to a previous report describing PALO-induced arginine starvation, (Kinney and Lusty, 1989) we observed no response in *S. cerevisiae* strain BY4741 to treatment with PALO (Fig. 1). The discrepancy suggests either that not all yeast strains are sensitive to PALO or that the earlier indications of arginine starvation resulted from impurities in the samples of PALO employed. The current results are consistent with observations that PALO had no effect in isolated rat mitochondria, intact rat hepatocytes, or *E. coli* (Hoogenraad, 1978; Penninckx and Gigot, 1979).

Figure Legend

Fig. 5-1. PALO has no effect on expression of the *ARG5,6, CPA1*, and *TRP5* genes in *Saccharomyces cerevisiae. S. cerevisiae strains* (Brachmann et al., 1998) were inoculated from a saturated culture into minimal medium supplemented with leucine, methionine, uracil, and histidine. Cultures were treated with 15 μM PALO in a potassium phosphate pH 6.8 buffer, potassium phosphate buffer pH 6.8 alone (untreated), 1 mg/mL L-Arginine, or both 15 μM PALO and 1 mg/mL L-Arginine at the time of inoculation. Cells were incubated at 30°C and then harvested at mid-log growth phase (OD_{600} 0.4-0.6). **A**) Northern blots were prepared with total RNA extracted from BY4741 (Brachmann et al., 1998) and probed with oligolabeled DNA probes (Kebaara et al., 2003). DNA probes were generated using primer sets for amplifying yeast open reading frames based on the sequences available from the Saccharomyces Genome Database. Shown are representative phosphor-images of a northern blot probed with radioactive *ARG5,6, CPA1, TRP5*, and *SCR1* DNAs. *SCR1* was used as a loading control. Quantitative results depicted are the average of three replicate trials and are normalized to the *SCR1* loading control. **B**) Western blots were prepared using protein extracts from *S. cerevisiae* YSC1178-7500224, YSC1178-7502950 and YSC1178-7500415 strains expressing TAP-fusion proteins Arg5,6-TAP, Cpa1-TAP, and Trp5-TAP, respectively, as well as BY4741 (untagged control; Atkin et al., 1995). The TAP-tagged proteins were detected using an anti-TAP antibody. No proteins bound the anti-TAP antibody in the untagged control. Duplicate polyacrylamide gels were stained with Coomassie blue for use as loading controls. The corresponding loading controls are shown beneath the

western blots.

Figure 5-1



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CHAPTER 6:

Summary and Future Directions

Summary and Future Directions

The regulation of gene expression in eukaryotes is a very delicate process regulated at multiple levels. A mishap at any point in the regulatory process can be detrimental to organisms. On the same token, as long as all of the cellular processes that contribute to the regulation of gene expression are in-check, then organisms are able to maintain cellular homeostasis and overall health. The phrase "gene expression" is often most closely associated with the regulation of translation. While this is not incorrect, we must be aware that the regulation of gene expression begins on a much deeper level. Even before the translation machinery begins translating an mRNA the mRNA itself is subject to extensive regulation through the regulation of mRNA transcription and mRNA degradation. Further, there are cellular quality control processes that ensure improperly transcribed mRNAs are not further translated into deleterious or toxic proteins. These quality control processes include: 1) no-go decay (NGD), which is responsible for releasing ribosomes that have stalled on the mRNA and degrading the mRNA (Harigaya and Parker, 2010), 2) nonstop decay (NSD), which degrades transcripts that lack a stop codon allowing the ribosome to translate through the poly(A) tail adding a string of poly-lysines to the end of the protein (Frischmeyer et al., 2002), and 3) nonsense-mediated mRNA decay (NMD), which is responsible for the rapid degradation of mRNAs that harbor premature termination codons (PTCs; Akimitsu, 2008; Baker and Parker, 2004; Behm-Ansmant et al., 2007; Chang et al., 2007; Frischmeyer and Dietz, 1999; Hentze and Kulozik, 1999; Hilleren and Parker, 1999; Muhlemann et al., 2008; Schweingruber et al., 2013; Shyu et al., 2008). While NMD was first discovered as an mRNA surveillance

mechanism it is now recognized that NMD plays a much broader role in the regulation of wild-type mRNAs as well (Guan et al., 2006; He et al., 2003; Johansson et al., 2007; Kalyna et al., 2012; Lelivelt and Culbertson, 1999; Mendell et al., 2004; Mitrovich and Anderson, 2005; Peccarelli and Kebaara, 2014; Rayson et al., 2012; Rehwinkel et al., 2005).

The known features that have been shown to target a wild-type mRNA for degradation by NMD include: 1) a long 3' UTR (Amrani et al., 2004; Kebaara and Atkin, 2009; Muhlrad and Parker, 1999), 2) translation of an upstream open reading frame (uORF; Amrani et al., 2006; Barbosa et al., 2013; Gaba et al., 2005; Nyiko et al., 2009), 3) a start codon in a suboptimal context which can lead to leaky scanning and out of frame initiation of translation (Welch and Jacobson, 1999), 4) the presence of programmed ribosome frameshift (PRF) sites (Plant et al., 2004) and 5) the presence of pre-mRNA introns and regulated alternative splicing resulting in PTCs (He et al., 1993; Lewis et al., 2003; McGlincy and Smith, 2008; Ni et al., 2007).

Further, not all mRNAs with NMD-targeting signals are degraded by NMD. The mechanisms of protection of mRNAs from degradation by NMD that have been identified include: 1) inhibition of translation (Bertram et al., 2001; Czaplinski et al., 1998; Gozalbo and Hohmann, 1990; He et al., 1993; Herrick et al., 1990; Keeling et al., 2004; Kisselev et al., 2003; Peltz et al., 1992; Rospert et al., 2005; Tholstrup et al., 2012; Zhang et al., 1997; Zuk and Jacobson, 1998), 2) stop codon readthrough (Bonetti et al., 1995; Keeling et al., 2004; Namy et al., 2001), 3) translation reinitiation after a ribosome encounters a stop codon (Hall and Thein, 1994; Neu-Yilik et al., 2011; Thein et al.,

1990), and, 4) *trans*-acting factor Pub1 (Ruiz-Echevarria and Peltz, 2000).

Although the NMD pathway has been studied extensively over the past decade, and in multiple eukaryotes including *Saccharomyces cerevisiae*, *Drosophila*, *C. elegans*, *Arabidopsis*, and mammalian cells, the exact molecular mechanism of the NMD pathway remains obscure (Bedwell et al., 1997; Grimson et al., 2004; Hall and Thein, 1994; He et al., 2003; Hentze and Kulozik, 1999; Kalyna et al., 2012; Kurihara et al., 2009; Maquat and Carmichael, 2001; Maquat and Serin, 2001; Mendell et al., 2004; Pulak and Anderson, 1993; Rayson et al., 2012; Rehwinkel et al., 2005). Because as many as one third of all genetic diseases and cancers are linked to NMD, manipulation of the NMD pathway has been a targeted area of study for the development of disease therapies (Culbertson, 1999; Frischmeyer and Dietz, 1999; Kuzmiak and Maquat, 2006; Peltz et al., 2013). However, the development of safe and effective therapies is hindered by the obscurity of the exact molecular mechanisms of the NMD pathway. Thus, a thorough understanding of how the cellular machinery distinguishes and wild-type mRNA from an NMD substrate is a vital piece of being able to effectively treat NMD-related diseases.

Here we have provided a detailed investigation of a unique mRNA in *Saccharomyces cerevisiae*, the *SSY5* mRNA, which contains multiple NMD-targeting signals but is not degraded by the NMD pathway. We show that many of the known mechanisms that are responsible for the protection of mRNAs from NMD do not apply to the *SSY5* mRNA, thus, this mRNA is protected from NMD through a novel mechanism (Chapter 2). We also show that the *SSY5* mRNA is very tightly regulated during the mRNA decay process (Chapter 3). Additionally, we also begin to characterize the

stability of two additional wild-type mRNAs in *S. cerevisiae*, the *YAP1* and *GCN4* mRNAs, which are protected from degradation by NMD despite containing translated uORFs—an NMD-targeting signal (Chapter 4). Characterization of the stability of the *SSY5*, *YAP1* and *GCN4* mRNAs provides valuable clues to understanding the molecular mechanisms that control wild-type gene regulation by NMD.

Chapter 1: The *S. cerevisiae SSY5* mRNA is not degraded by NMD despite multiple NMD-targeting signals

The *SSY5* mRNA was originally identified as a wild-type mRNA that should be degraded by NMD in a screen for mRNAs in *S. cerevisiae* which contained long 3' UTRs (Kebaara and Atkin, 2009). Initial data from this study indicated that the *SSY5* mRNA was the only mRNA identified in the screen that contained a long 3' UTR but showed no difference in mRNA accumulation or half-life between wild-type and *upf1Δ* strains. Upon closer analysis of the *SSY5* mRNA we discovered that there were actually multiple NMD-targeting signals present including: 1) a translated uORF, 2) a start codon in a poor context that could lead to leaky scanning and out-of-frame initiation of translation, and 3) the presence of five predicted ribosome frameshift sites. The long 3' UTR of the *SSY5* mRNA was previously characterized as a positive NMD-targeting signal (Obenoskey et al., 2014). The translation of the uORF is shown with ribosome footprint data from genome-wide studies that has been compiled in the GWIPS-viz genome browser (Michel et al., 2014). However, further studies are needed to determine if leaky scanning or programmed ribosome frameshifting is occurring.

Our studies confirm that the *SSY5* mRNA shows similar accumulation and half-life in both wild-type and *upf1Δ* strains in two different *S. cerevisiae* genetic backgrounds, BY4741 and W303. Additionally, differences in amino acid composition of growth media does not affect *SSY5* mRNA stability. The amino acid composition of the growth media was taken into consideration because the *SSY5* mRNA codes for an essential proteolytic component of the SPS amino-acid-sensing complex in *S. cerevisiae* (Conrad et al., 2014; Ljungdahl, 2009).

A previous study showed that when the long 3' UTR of the *SSY5* mRNA is placed on an NMD-insensitive mRNA and the construct becomes a substrate for NMD (Obenoskey et al., 2014). This data provides evidence that the long 3' UTR of the *SSY5* mRNA is sufficient to target an mRNA for degradation by NMD. Thus, there is a mechanism that is protecting the *SSY5* mRNA from being degraded by NMD. We further showed that replacing the long 3' UTR of the *SSY5* mRNA with the short 3' UTR of the *ADH1* mRNA does not affect the stability of the construct. So, the mechanism of protection from NMD is not contained within the *SSY5* mRNA 3' UTR. We also removed the *SSY5* mRNA uORF by replacing the 5' leader of the *SSY5* mRNA with the galactose promoter sequence. This construct did not have altered mRNA accumulation or half-life in wild-type vs *upf1Δ* strains showing that the *SSY5* mRNA uORF is not required for protection from NMD.

The *SSY5* mRNA is also not protected from NMD by known mechanism of protection. We showed that the *SSY5* mRNA co-fractionates with polyribosomes and is actively translated. Additionally, although the *SSY5* mRNA 3' UTR is favorable for both

stop codon readthrough and reinitiation of translation, we do not find evidence of either.

Together, these studies show that the *SSY5* mRNA is a very likely candidate for degradation by the NMD pathway, and the protection of this mRNA from NMD occurs through a novel mechanism. Future studies should investigate whether the NMD mRNP is forming, either partially or completely, on the *SSY5* mRNA. This would provide information as to whether or not the *SSY5* mRNA is recognized by the NMD machinery as a substrate for NMD. Preliminary studies of this have begun but the experimental procedures and controls need further optimization.

Additionally, we characterized the *ENT4* mRNA in *S. cerevisiae* as a wild-type mRNA that has a long 3' UTR and is a substrate for NMD. This provides a positive control for a wild-type substrate of NMD. Moreover, the current function of the gene product of the *ENT4* mRNA is unknown. Determining the function of the product of the *ENT4* mRNA will provide further insight into the physiological significance of wild-type gene regulation by NMD in *S. cerevisiae*.

Further, the SPS-sensing pathway, which Ssy5 is an essential component of, is critical for amino-acid sensing of the human fungal commensal and pathogen *Candida albicans*. Importantly, the SPS pathway also controls the expression of virulence factors in *C. albicans* (Davis et al., 2011). Further investigation of the stability of the *SSY5* mRNA in *C. albicans* could provide important clues for understanding both the pathogenicity of *C. albicans* in humans as well as *SSY5* mRNA regulation by NMD.

Chapter 3: The *SSY5* mRNA is very tightly regulated during mRNA degradation

Further characterization of the degradation of the *SSY5* mRNA shows several unique characteristics. First, the *SSY5* mRNA does not follow a pattern representative of wild-type mRNA decay through a deadenylation-dependent mechanism nor does the mRNA follow a pattern of deadenylation-independent degradation representative of an NMD substrate. Second, *SSY5* mRNA accumulation is significantly increased when the primary 5'→3' exonuclease Xrn1 is deleted. However, when decapping is blocked by deletion of decapping component Dcp1, *SSY5* mRNA accumulation is very modestly increased. So, when the *SSY5* mRNA is capped (in the *dcp1Δ* cells) the mRNA can still be rapidly degraded 3'→5' by the exosome, but when the mRNA is decapped and 5'→3' degradation is blocked (in the *xrn1Δ* cells), 3'→5' degradation is not able to adequately compensate. Further, deletion of any of the deadenylation components does not impact *SSY5* mRNA stability. Together these results show that *SSY5* mRNA stability is very tightly regulated at a step between mRNA decapping and 5'→3' exonucloelytic degradation. This is fascinating because these processes occur subsequently and very rapidly during mRNA decay. For completeness, future experiments should include an analysis of the *SSY5* mRNA in mutants of the components of the cytoplasmic exosome, such as the catalytic component Dis3.

Additionally, analysis of the 3' end of the *SSY5* mRNA shows a pattern of 3' end shortening that is similar to what is seen for a wild-type mRNA that is deadenylated before being decapped (*PGK1* mRNA). Both the *PGK1* and *SSY5* mRNAs have longer 3' ends in wild-type and *upf1Δ* strains and shorter 3' ends in *xrn1Δ* and *dcp1Δ* strains.

This is in contrast to the *ENT4* mRNA—a wild-type substrate degraded NMD in S. cerevisiae—which shows the opposite pattern with a shorter 3' end in wild-type and *upf1Δ* strains and a longer 3' end in *xrn1Δ* and *dcp1Δ* strains. This observation is likely the result of the rate at which the 3' end is degraded in wild-type versus NMD substrates where 3' end degradation occurs very rapidly following decapping for an NMD substrate (Cao and Parker, 2003). However, to be able to conclude a change in length of the 3' end is the result of deadenylation we need to repeat the experiment with an RNase H control, which removes the poly(A) tail.

An unexpected result of the studies in this section was that in a certain strain background (not BY4741 or W303) the *SSY5* mRNA does appear to be slightly stabilized in the *upf1Δ* mutant. Initially, this was a troubling observation. However, upon further consideration we have realized this can be used to our advantage. By determining the difference(s) between this strain background and the BY4741 and W303 strains we can drastically narrow down potential NMD-protecting mechanisms. Further characterization of this strain background will be required. Once differences in the strain background are identified we can begin studies to determine what might be, at least partially, responsible for the protection of the *SSY5* mRNA from NMD. This mechanism of protection may then be further studied for other wild-type mRNAs that have NMD-targeting signals but are not degraded by NMD, such as the *YAP1* and *GCN4* mRNAs.

Chapter 4: The *S. cerevisiae* wild-type mRNAs *YAP1* and *GCN4* also contain NMD-targeting signals but are not degraded by NMD

The *YAP1* and *GCN4* mRNAs were previously identified as wild-type mRNAs in *S. cerevisiae* that have translated uORFS—an NMD-targeting signal—but are not degraded by NMD (Ruiz-Echevarria et al., 1998; Vilela et al., 1998). A subsequent study showed that both the *YAP1* and *GCN4* mRNAs are protected from degradation by NMD due to the *trans*-acting factor Pub1 binding to a stabilizer element (STE) in the 5' leader of the mRNA downstream of the uORF stop codon (Ruiz-Echevarria and Peltz, 2000).

During our initial investigation into the protection of the *SSY5* mRNA from NMD we made the hypothesis that, based on the previous results, Pub1 could also be involved in the protection of the *SSY5* mRNA from NMD. Because Pub1 had already been clearly implicated in the protection of both *YAP1* and *GCN4* mRNAs from NMD these were included as controls. However, in our hands we were unable to reproduce the previously published results despite extensive efforts. This means that we now have two additional mRNAs in *S. cerevisiae* that have at least one NMD-targeting signal (we have not looked for others) but are protected from degradation by NMD through an unknown mechanism.

We confirmed that both *YAP1* and *GCN4* mRNAs are protected from degradation by NMD. However, half-life analysis in wild-type and *upf1Δ* strains provided stronger confirmation of protection from NMD than steady-state mRNA accumulations. Because the product of the *GCN4* mRNA, Gcn4, is also involved in amino acid regulation we again looked to see if the amino acid composition of the media had any influence on mRNA stability of either of these mRNAs. We found that neither strain background nor

amino acid composition of the media influenced the stability of either mRNA. However, as was the case when looking at *SSY5* mRNA stability, the same holds true here where we could not use a true amino-acid starvation media due to the auxotrophies of the strains used. We could only provide fewer amino acids than are present in rich YAPD media. True amino acid starvation or complete nitrogen starvation may influence mRNA stability, but we were unable to test this.

We were unable to show that *trans*-acting factor Pub1 is solely responsible for the protection of *GCN4* and *YAP1* mRNAs from NMD. However, we have preliminary data that suggests, at least for the *YAP1* mRNA, that Pub1 may be involved in the stability of the mRNA but its role is more complex than previously indicated. Additionally, in looking at the decay of the *YAP1* and *GCN4* mRNAs we find a unique pattern that does not mimic that of a known NMD substrate (*ENT4* mRNA), a wild-type mRNA degraded by the deadenylation-dependent decapping pathway (*PGK1* mRNA), or the *SSY5* mRNA. Unlike the *SSY5* and *ENT4* mRNAs, the *YAP1* and *GCN4* mRNAs show only a slight increase in accumulation in an *xrn1Δ* strain, but still more accumulation than what is observed for the *PGK1* mRNA. Further, the *YAP1* and *GCN4* mRNAs show no significant difference in accumulation in a *dcp1Δ* strain. This is again in contrast to the *SSY5* mRNA, which shows a slight increase in accumulation in a *dcp1Δ* strain. However, it is similar to what is observed for the *PGK1* mRNA, which shows no significant difference in accumulation in a *dcp1Δ* strain. This does point to a tight window of regulation between decapping and 5'→3' mRNA decay for both *YAP1* and *GCN4* mRNAs, but the fold changes are less than what are observed for the *SSY5* mRNA, which

could suggest a larger role of 3'→5' mRNA decay for *YAP1* and *GCN4* mRNAs. The deadenylation mutants also did not influence the accumulation of the *YAP1* or *GCN4* mRNAs consistent with what we observed for the *SSY5*, *ENT4* and *PGK1* mRNAs.

We also looked at the behavior of the 3' end of the *YAP1* mRNA in the wild-type, *upf1Δ*, *xrn1Δ* and *dcp1Δ* strains. The data from this experiment shows that the 3' end of the *YAP1* mRNA behaves similarly to the 3' end of the *PGK1* mRNA in all of the mutants. The 3' end of both *YAP1* and *PGK1* mRNAs is longer in wild-type and *upf1Δ* strains and shorter in *xrn1Δ* and *dcp1Δ* strains. Again, to be able to conclude this change in length is the result of deadenylation we need to repeat the experiment with an RNase H control. This assay also needs to be performed for the *GCN4* mRNA, which was not originally included due to the cost of the kit and the minimal number of reactions provided.

Final Thoughts

Although manipulation of the NMD pathway as a means of disease treatment seems logical, and is currently being pursued all the way into clinical trials, this is troubling given how much we still do not know about the underlying mechanisms of the NMD pathway and wild-type gene regulation by NMD. Clinical trials of drugs, such as Ataluren, are optimistically reporting no significant side effects of such therapies. However, no long-term study data is available as these are newly developed therapies. It is quite possible that side-effects may become apparent several years down the line. Thus, it is absolutely critical that we continue attempts to elucidate the underlying

mechanisms of the NMD pathway in order to make treatments safer and more effective. As with the development of any disease therapy, NMD-disease therapies will continue to be modified as we learn more about the mechanisms of NMD. However, clinical trials of NMD-related therapies at this point seems troublingly premature.

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