ABSTRACT

Characterization of a Nonsense-mediated mRNA Decay (NMD) Copper Regulon

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The nonsense-mediated mRNA decay (NMD) pathway was originally identified as a pathway that degrades mRNAs harboring premature termination codons. NMD is now also recognized as a pathway that degrades natural mRNAs. In *Saccharomyces cerevisiae*, five features are known to target natural mRNAs to NMD. The extent to which natural mRNAs from the same functional group are regulated by this pathway is not widely known. Here, we investigated eight mRNAs involved in copper homeostasis that are predicted to be sensitive to NMD. We found that the majority of these mRNAs have atypically long 3'-Untranslated Regions (UTRs) that could potentially target them for NMD-mediated degradation. We investigated the sequence elements that target a subset of these mRNAs to NMD and found that the long *CTR2* 3'-UTR and the *COX23* 3'-UTR are sufficient to target an NMD-insensitive mRNA to NMD. We also found that the *COX19* and *FRE2* 3'-UTRs contribute to the degradation of the transcripts by the pathway. Additional studies involving sequence elements demonstrated that lengthening the open reading frame of *CTR2* abrogates NMD, preventing degradation of the mRNA. Moreover, we found that transcription of *CTR2* mRNAs driven by the *GPD* promoter

causes altered NMD sensitivity when compared to *CTR2* driven by the *CTR2* promoter. Lastly, we found that low copper growth conditions affect NMD sensitivity of the *MAC1* mRNA; demonstrating that NMD mediated regulation can be influenced by environmental conditions.

The studies presented here are novel in that they investigate the regulation of functionally related, natural mRNAs by NMD. We show that the regulation of these mRNAs is transcript specific, and that regulation can be influenced by sequence elements as well as the environmental conditions.

Characterization of a Nonsense-mediated mRNA Decay (NMD) Copper Regulon

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LIST OF ABBREVIATIONS

-1 Ribosomal frameshift (-1RFS)

Eukaryotic initiation factor (e1F4E)

Eukaryotic release factor (eRF)

Exon-junction complex (EJC)

Messenger RNA (mRNA)

No-go decay (NGD)

Non-coding RNA (ncRNA)

Nonsense Mediated mRNA Decay (NMD)

Non-stop decay (NSD)

Open reading frame (ORF)

Poly(A) binding protein (PABP)

Premature-termination codon (PTC)

Ribosomal RNA (rRNA)

Transfer RNA (tRNA)

Untranslated region (UTR)

Up-frame shift (UPF)

Upstream open reading frame (uORF)

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CHAPTER ONE

Introduction and Background

Saccharomyces cerevisiae *Organism Overview*

Saccharomyces cerevisiae, or baker's yeast, is an extensively studied, eukaryotic model organism. The *S. cerevisiae* life cycle is rather simple and consists of a prolonged period of cell growth followed by mitosis. Yeast can complete the cell cycle in either a haploid or a diploid state. Haploid cells reproduce asexually through a process known as budding, in which a daughter cell buds from the parent cell. In detail, the parent cell's nucleus divides and a portion of the nucleus enters the newly forming daughter cell. The daughter cell continues to grow on the parent cell for the duration of the cell cycle until it is fully mature and can function independently. Alternatively, diploid yeast cells can reproduce sexually via sporulation. During meiosis, four haploid spores are produced: two "a" spores and two "α" spores. The spores can then germinate and produce yeast strains. Sexual reproduction occurs when an "a" spore mates with an "α" spore. Mating between the two spore types can introduce genetic recombination.

The *S. cerevisiae* genome was completely sequenced in 1996, and became the first fully sequenced eukaryotic genome (Botstein and others 1997). The genome is approximately 12.1 MB in size; making it significantly smaller than the human genome. The small size of the yeast genome can be attributed to the limited numbers of non-coding RNA (ncRNA) and repetitive sequences. Additionally, open reading frames (ORFs) account for over 72% of the entire *S. cerevisiae* genome (Dujon 1996).

Since the initial sequencing, numerous homologs have been identified between *S. cerevisiae* and humans. For example, early studies identified two homologs of the mammalian *ras* oncogene in yeast (*RAS1* and *RAS2*, respectively). Yeast cells lacking both *RAS* genes are not viable; however, introduction of mammalian H-*ras* in *RAS* deficient yeast strains restored viability (Kataoka and others 1985). These results indicate conservation of both the sequence and cellular function (Kataoka and others 1985).

Additional studies have also revealed that yeast and humans share similar cell cycles as well as similar regulatory mechanisms. The homology and similar cell cycle makes *S. cerevisiae* a suitable eukaryotic model organism (Botstein and others 1997). Furthermore, *S. cerevisiae* is easy to maintain and manipulate in a laboratory setting. More specifically, *S. cerevisiae* has a short generation time of approximately one-hundred minutes with an average lifespan of about twenty-six total cellular divisions. Many strains are also stable in both the haploid and diploid state, making the accumulation of recessive mutants feasible for researchers. Additionally, temperature sensitive mutations can be created.

mRNA Quality Control and Decay

In all cells, different regulatory machinery exists to control the turnover of messenger RNAs (mRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and non-coding RNAs (ncRNAs). Quality control mechanisms are important for all RNA species; however, targeted degradation of mRNAs is of the utmost importance. mRNAs exist within the cell to form proteins; therefore, mRNA levels must be strictly regulated to allow for controlled production of proteins depending on intracellular conditions.

Furthermore, aberrant mRNAs must be quickly destroyed to prevent the formation of faulty proteins (Parker 2012).

Currently, three pathways are considered responsible for mRNA quality control and decay in yeast: non-stop decay (NSD), no-go decay (NGD), and nonsense-mediated mRNA decay (NMD) (Kervestin and Jacobson 2012; Parker 2012). These pathways function during translation and target, as well as degrade, aberrant mRNAs. mRNAs can become aberrant through improper pre-mRNA splicing, polyadenylation, or methylation. The NSD pathway is responsible for degrading mRNAs that lack a termination codon (Frischmeyer and others 2002; van Hoof and others 2002). mRNAs without a termination codon can occur simply as a result of mutations or through the utilization of polyadenylation sites within open reading frames (ORFs). When no stop codon is present, translation cannot be terminated. As a result, the ribosomes become stalled at the 3' end of the mRNA and fail to dissociate (Parker 2012). NGD leads to the destruction of mRNAs in which ribosomes stall during translation elongation (Doma and Parker 2006). The presence of stem loop structures, rare codons, frameshift sites, depurination sites, and polyLys or polyArg runs can all cause translation stalls. Additionally, these aberrant mRNAs can develop as a result of exposure to ultraviolet light or as a result of chemically induced mutagenesis (Doma and Parker 2006).

Alternatively, the NMD pathway is responsible for degrading mRNAs that prematurely terminate translation. This includes mRNAs with premature termination codons (PTC). PTCs can arise as a result of improper splicing or random mutation. Remarkably, NMD also plays a role in regulating gene expression by degrading many natural transcripts (Deliz-Aguirre and others 2011; Guan and others 2006; He and others

2003; Kebaara and Atkin 2009; Lelivelt and Culbertson 1999). The extent and reason for the targeting of most natural mRNAs is generally unknown. A study by Guan *et al.* suggests that the expression of over 600 naturally occurring *S. cerevisiae* mRNAs is controlled by NMD (Guan and others 2006). The NMD pathway is highly conserved in all tested eukaryotic species from yeast to humans, and appears to serve as an important regulator of gene expression and cellular function. In *S. cerevisiae* nmd mutants (possessing an inactive NMD pathway), 5-10% of the transcriptome is affected (Guan and others 2006; He and others 2003). Further research in *Drosophila melanogaster* and humans indicates that similar percentages of the respective transcriptomes are affected when NMD is absent or mutated (Mendell and others 2004; Rehwinkel and others 2006; Wittmann and others 2006; Yepiskoposyan and others 2011). Additionally, in yeast, nmd mutants show severely altered chromosome structure. The altered structure can present itself as a reduced number of hexameric repeats at telomeres, altered kinetochore function, or through the absence of gene silencing in silent regions of telomeric DNA (Altamura and others 1992; Dahlseid and others 2003; Dahlseid and others 1998; Guan and others 2006; Lew and others 1998).

In humans, NMD has additional physiological significance due to its close relationship with genetic disorders and inherited forms of cancer. More specifically, one third of genetic diseases and cancers result from translation termination mutations; which, in healthy systems, trigger NMD (Frischmeyer and Dietz 1999). Under circumstances in which mRNAs with a PTC are able to evade NMD, truncated proteins are produced. These truncated proteins may be completely functionless, or alternatively, may be partially functional. Partially functional proteins can be a danger to the cell because these

proteins may acquire new functions or may possess a dominant-negative function, possibly leading to disease. Alternatively, partially functional proteins may work well within the cellular system and only perform their natural function. The location of the PTC can control functionality of the protein and contribute to the severity of disease manifestation (Nicholson and others 2010). More specifically, mRNAs with a PTC near the 3’ end are more likely to maintain proper function than mRNAs with a PTC near the 5’ end (Gonzalez-Hilarion and others 2012). However, because NMD targets any mRNA coding for a truncated protein with a PTC, the proteins with lingering, natural function are often degraded (Nicholson and others 2010). Additionally, in mammalian systems, studies have shown that NMD is regulated during myogenesis (Gong and others 2009), hypoxia (Gardner 2008), and neurogenesis (Lou and others 2014).

NMD Machinery

Due to the high level of conservation among eukaryotes, and the physiological significance, the factors that regulate NMD have been thoroughly studied. Numerous factors are necessary for NMD to occur; however, the up-frame shift (UPF) proteins are the most important. The UPF family of proteins consists of Upf1p, Upf2p, and Upf3p. In *Caenorhabditis elegans*, the UPF proteins are referred to as *smg-2*, *smg-3,* and *smg-4*, respectively. The UPF family of proteins is so important for NMD, that silencing or mutations in any of the UPF genes has stabilizing effects on mRNAs that would typically be degraded by the NMD pathway (He and others 1997). The three proteins interact with one another; however, Upf1p is the central regulator of the pathway and is the most conserved of the UPF proteins (Culbertson and Leeds 2003; Deliz-Aguirre and others

2011; Nicholson and others 2010; Page and others 1999). *UPF2* and *UPF3* serve as specific regulators of *UPF1* (Ghosh and others 2010; Kervestin and Jacobson 2012).

Upf1p is a rather large protein that is capable of binding mRNA and the other UPF proteins. Upf1p is 109 kDa in yeast, and 130 kDa in humans (Kervestin and Jacobson 2012). Immunolocalization studies in human cells revealed that Upf1p is primarily a cytoplasmic protein. Although it is typically found in the cytoplasm, biochemical analyses indicate that Upf1p has shuttling capabilities between the cytoplasm and nucleus in order to carry out all of its cellular functions (Lykke-Andersen and others 2000; Mendell and others 2002; Serin and others 2001). Upf1p is structurally composed of two primary domains. The first domain is a CH domain that is located on the amino terminal end. The CH domain contains an array of three zinc-binding motifs and is able to interact with Upf2p (Altamura and others 1992; Nicholson and others 2010). Upf2p is approximately 127 kDa in size and contains three MIF4G domains, one of which is capable of binding Upf3p (He and Jacobson 1995). Upf3p is much smaller than Upf1p and Upf2p at only 45kDa in size (He and others 1997). Upf3p is also more basic in structure, and only contains a RNA-binding domain (RBD). The second Upf1p domain is a linker region composed of superfamily I (SFI) helicase motifs (Altamura and others 1992). This domain contains two recombinase A-like domains and two UPF1-specific domains (Cheng and others 2007). The ATPase activity of Upf1p is found within the SF1 helicase motifs, and plays a role in 5' to 3' helicase activity (Bhattacharya and others 2000; Czaplinski and others 1995). The activities of both Upf1p domains require ATP input in order to function properly (Kervestin and Jacobson 2012). In addition to interacting with Upf2p and Upf3p, Upf1p directly interacts with translation

release factors in eukaryotes. These release factors are known as eukaryotic release factor 1 (eRF1) and eukaryotic release factor 3 (eRF3) (Czaplinski and others 1998; Ivanov and others 2008).

Because of their direct involvement with NMD, upf^{-} mutants are often used to identify mRNA targets of NMD. NMD targets can be narrowed down to two categories: direct and indirect. In an NMD mutant (upf^{-}), the accumulation of mRNA transcripts, that are NMD targets, can easily be observed experimentally. More specifically, a change in decay rate, based on half-life experiments, can be observed when comparing yeast cells with a functional NMD pathway (wild-type strains) and cells with a non-functional NMD pathway (nmd mutant yeast strains). In detail, the mRNA levels are shown to decrease steadily over a designated time period after transcription has been inhibited. If a statistically significant difference in half-lives is observed between the wild-type and nmd mutant strains, then the mRNA is considered to be a direct target, and the steady-state accumulation is due to the stabilization of the mRNA in nmd mutants. On the other hand, if the half-lives of a transcript in wild-type and nmd mutant strains are the same, then the transcript is considered an indirect target. The steady-state accumulation visualized with indirect targets is a result of increased transcription that is stimulated due to an increased level of a direct mRNA target upstream (Guan and others 2006).

The Regulation of Natural mRNAs by the NMD Pathway

Originally, it was thought that all direct targets of the NMD pathway contain a PTC. However, with the growing list of natural mRNAs that are regulated by the pathway, it became apparent that other targeting mechanisms must exist. Previous

studies in *S. cerevisiae* have identified five features that target natural mRNAs for NMD. These features include an atypically long 3'-untranslated regions (UTRs), a translated upstream open reading frame (uORF), -1 ribosomal frameshifts (into an alternative reading frame), inefficiently or alternatively spliced pre-mRNAs, and leaky ribosomal scanning (Parker 2012; Schweingruber and others 2013). These features are discussed at length in Chapter Two.

One specific targeting mechanism of interest to us is the presence of a long 3'-UTR on an mRNA (Deliz-Aguirre and others 2011; Kebaara and Atkin 2009; Rebbapragada and Lykke-Andersen 2009). The presence of a long 3'-UTR has been shown to be sufficient to target mRNAs for NMD in multiple eukaryotic species. In *S. cerevisiae*, the majority of the mRNA 3'-UTRs range from 50-200 nucleotides in length, with a median length of 121 nts (Graber and others 2002). The majority of transcripts with 3'-UTRs of 350 nucleotides or longer are degraded by the NMD pathway (Deliz-Aguirre and others 2011; Kebaara and Atkin 2009).

Previous studies have shown that a long 3'-UTR is sufficient to induce NMD. In *in vitro* studies, translation termination events are distinctly different in transcripts with a stop codon followed by a normal versus a long 3'-UTR. In *S. cerevisiae*, it appears that the length of the 3'-UTR, and not the specific sequence of the 3'-UTR, determines which mRNAs are degraded by the pathway due to the presence of an atypically long 3'-UTR. More specifically, the distance between the stop codon and the proteins bound to the 3'-end of the mRNA is an important determinant as to whether or not a translation termination is seen as normal or aberrant (Amrani and others 2004; Hagan and others 1995). These results are confirmed experimentally through rescue experiments. For

example, rescue of an aberrant mRNA can be achieved by adding a poly(A) binding protein in close proximity with the stop codon; thus, shortening the distance between the stop codon and the poly(A) binding protein. This idea is known as the *faux*-UTR model. The *faux*-UTR model has been tested further. A naturally occurring, long 3'-UTR was removed from the natural *PGA1* mRNA. *PGA1* encodes a protein involved with cell wall biosynthesis, and is vulnerable to degradation via NMD. The replacement of the *PGA1* 3'-UTR with a 3'-UTR that does not trigger NMD lead to the stabilization of the *PGA1* mRNA (lacking the endogenous 3'-UTR) in yeast strains with a functional NMD pathway (Kebaara and Atkin 2009).

Copper Toxicity and Homeostasis

As a mechanism controlling gene expression, NMD is a suitable model for studying the level of variation in mRNA abundance. This variation can arise due to genetic or environmental influences. Furthermore, different mRNA populations show varying levels of plasticity; however, the mechanisms that underlie plasticity and the polymorphisms themselves are still unclear. One way to study the relationship between plasticity and gene expression is by exposing cells to an environmental stressor. Many different stressors can be used, including copper (Hodgins-Davis and others 2012). Copper is a micro-nutrient that is essential for a number of cellular functions (Deliz-Aguirre and others 2011; Hodgins-Davis and others 2012). More specifically, copper serves as a cofactor in superoxide anion detoxification, iron metabolism, and mitochondrial oxidative phosphorylation. Even though copper is required for normal cellular function, copper in its free form is extremely toxic to the cell. In detail, the copper concentration inside of the cell must remain around one free copper molecule per

individual cell to avoid toxicity (Hodgins-Davis and others 2012). In order to avoid cell death due to copper induced toxicity, cells have developed a number of mechanisms to maintain copper homeostasis. These mechanisms include copper compartmentalization and sequestration. Specific proteins involved in these mechanisms have been identified; however, the actual mechanisms involved in copper regulation are still under investigation (Deliz-Aguirre and others 2011). Interestingly enough, cells with an inactive NMD pathway are more tolerant of high copper concentrations. These results indicate that nmd mutants may be utilizing copper homeostatic mechanisms better than wild type cells. Specifically, nmd mutants are capable of sequestering higher amounts of copper into the vacuole (Deliz-Aguirre and others 2011; Wang and others 2013).

In *S. cerevisiae*, the *CTR2* mRNA has been extensively studied. *CTR2* is directly involved in copper homeostasis. More specifically, *CTR2* encodes a copper transporter in the membrane of the yeast vacuole. This transporter regulates the flow of copper into, and out of, the vacuole. *CTR2* is a natural transcript that undergoes alternative 3'-end processing, producing two mRNA isoforms of different lengths. The isoforms differ dramatically, with one having a 300 nt 3'-UTR and the other having a 3'-UTR that is 2-kb in length. Experimental analysis revealed that both isoforms are regulated by the NMD pathway, but to varying degrees (Figure 4.1). These results are interesting because *CTR2* is a natural mRNA that lacks a PTC. We found that the long 3'-UTR contributes to the degradation of the *CTR2* mRNA by NMD (Peccarelli and others 2014). Additionally, *CTR2* was found to be required for the copper tolerance phenotype in nmd mutants. In other words, when *CTR2* is removed, the nmd mutant cells are unable to withstand higher

copper concentrations relative to wild-type cells (Deliz-Aguirre and others 2011), demonstrating that the regulation of *CTR2* mRNA by NMD is physiologically significant.

Summary of Research Objectives

Further study is needed to evaluate how NMD regulates natural mRNAs involved in cellular processes, including copper homeostasis, and how this regulation is influenced by environmental conditions. As previously stated, copper can become toxic to cells at high concentrations. Defects in copper homeostasis have been linked to cancer, organ damage, degenerative diseases, and growth and development issues. For this reason, it is important to understand which mRNAs involved in copper homeostasis within the cell are regulated by the NMD pathway, as well as how they are regulated. Global expression profiling studies identifying mRNAs regulated by the NMD pathway identified *CTR2, CTR3, MAC1, COX23, CRS5, PCA1, FRE2*, and *COX19* mRNAs as potential NMD substrates (Guan and others 2006; Johansson and others 2007) (Table 5.1). Six of the mRNAs (*MAC1, CTR3, CTR2, COX23, COX19*, and *FRE2*) were predicted to have an atypically long 3'-UTR using the mRNA 3'-end Processing Site Predictor (Graber and others 2002). The presence of an atypically long 3'-UTR is a known NMD targeting feature. This dissertation identifies which mRNAs are direct NMD targets, the features that target some of these mRNAs to the pathway, and the influence environmental copper levels have on the regulation of one of the mRNAs. More specifically, Chapter Four describes the specific features that target the *CTR2* mRNA for NMD-mediated degradation. Chapter Five investigates regulation of *MAC1, CTR3, COX23, COX19, FRE2, PCA1* and *CRS5* mRNAs by the NMD pathway, as well as the specific features that target *COX19* for degradation. Chapter Five also examines regulation of *MAC1*

mRNA under low copper growth conditions and determines that *S. cerevisiae* mRNAs can be regulated differently depending on the environmental conditions. The low copper study also demonstrated that 3'-end processing can vary based on cellular growth conditions. Chapter Six describes the specific features that target *COX23* and *FRE2* mRNAs for NMD through the study of fusion constructs. Last, Chapter Seven details the conclusions that can be drawn from the research presented in the previous chapters as well as the future studies.

General Methodology

Measurement of mRNA Stability and Half-lives

In steady-state mRNA accumulation experiments, wild-type (BKY8) and nmd mutant (BKY43) strains were used (Table 1.1). Alternatively, wild-type and nmd mutant yeast strains with a *rpb1-1* genetic background were used in mRNA half-life experiments (BKY49 and BKY50). *rpb1-1* is a temperature-sensitive allele of RNA polymerase II (Nonet and others 1987). When *rpb1-1* yeast cells are subjected to a non-permissive temperature of 39°C, RNA polymerase II is not functional and mRNAs are no longer synthesized. One can then measure the disappearance of the mRNA at different time points. Standard techniques were used to grow and maintain all of the yeast strains (Ausubel and others 1998). Overnight growth and cell harvesting are detailed in Chapter Three.

Table 1.1: Yeast strains used in this study.

Yeast Strain	Genotype	Source
W303 (BKY8)	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-101*	Wente *et al*. 1992
AAY320 (BKY43)	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1::URA3 (upf1-Δ2)*	Kebaara *et al*., 2003
AAY334 (BKY49)	*a, ADE2, ura3-1 or ura3-52, his3-52, his3-11,15; trp1-1, leu2-3,112, rpb1-1*	Kebaara *et al*., 2003
AAY335 (BKY50)	*a, ADE2, ura3-1 or ura3-52, his3-52, his3-11,15; trp1-1, leu2-3,112, rpb1-1, upf1-Δ2 (URA3)*	Kebaara *et al*., 2003

RNA extraction from yeast. Harvested cells were thawed, and the yeast cell wall was lysed through the hot phenol method. The phenol was later separated from the aqueous phase and removed through centrifugation, and 1 ml of absolute ethanol was added to the aqueous layer containing RNA. The RNA was then ethanol precipitated. After precipitation, the RNA was pelleted, and the ethanol was removed via aspiration. After ethanol removal, the pellets were dried in an open tube at 37°C for five minutes. The RNA was then ethanol precipitated, pelleted, aspirated and dried a second time. The pellets were washed with 1 ml of 70% ethanol. The 70% ethanol was removed via aspiration and the pellets were dried in an open tube at 37°C for five minutes. The RNA was re-suspended in 50μl of dH_2O. Tubes were placed in a 65°C water bath for 10 minutes to assist suspension. The concentration of each sample was checked on the NanoDrop, and all RNA was then diluted to 1μg/μl. Following dilution, the samples were stored at -80°C.

Quantitative northern blotting. Equivalent concentrations of the extracted RNA (15μg) were run on a 1.0% agarose-formaldehyde gel for both steady-state and mRNA half-life northerns. The RNA was transferred to a GeneScreen Plus® (PerkinElmer,

Boston, MA) nitrocellulose membrane. Transfers were completed using the capillary blot transfer protocol from the NorthernMax™ Complete Northern Blotting kit (Ambion, Inc., Austin, TX). Following the transfer, the RNA was cross-linked to the membrane using the UVC500 UV Cross-linker (Hoefer, Holliston, MA).

Preparation of oligolabeled probes. To create a probe fragment, PCR of the gene of interest was performed. The PCR product was run on an agarose gel, and the product was cut out of the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Venlo, Limburg). After purification, the oligolabeled probe fragment was prepared by combining 25 ng of the purified DNA probe product with [α-^{32}P]dCTP using the RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA).

Hybridization of RNA blots. Membranes were placed in hybridization bottles and were pre-hybridized with pre-hybridization buffer at 42°C for 2-4 hours. The pre-hybridization buffer was removed and hybridization buffer and the oligolabeled probe were added. The membrane was allowed to hybridize for 16-24 hours at 42°C. In cases where the mRNAs were present at low levels in the cell, a combination Prehyb/hyb buffer (Ambion, Austin, TX) was used. After hybridization, the hybridization buffer containing the probe was removed, and the membranes were washed with two washes. The first wash is 2X SSPE, and the second and third washes are composed of 2 X SSPE and 2% SDS. Following washing, the membranes are individually wrapped in plastic wrap and were exposed to a phosphorimaging screen for varying amounts of time. The membranes were then visualized using the Typhoon Phosphorimager (GE Healthcare). This process is further explained in Chapter Three.

Quantitative analysis. Northerns were quantified using ImageQuant software. Sigmaplot software was used to calculate half-lives in the mRNA half-life northerns. Quantitative analysis is discussed in Chapter Three.

3'-RACE

cDNA is created from total RNA using RNase-free techniques. The cDNA was amplified using the adapter primer from the 3'RACE System for Rapid Amplification of cDNA Ends (3'RACE) kit. Primary PCR reactions were used as a template for nested PCR reactions. Secondary PCR reactions were run on 1.5% agarose gels (Kebaara and Atkin 2009).

3'-RACE was performed using the 3'-RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen, Carlsbad, CA). More specifically, 2 μl of cDNA was combined with the components from the 3'RACE kit. The 3'RACE program was run on the thermocycler. PCR conditions are specified in Table 1.2.

Table 1.2: PCR conditions for 3'-RACE.

Step	Temperature	Time
Initial Denaturation	94°C	3 minutes
*Denaturation	92°C	45 seconds
*Annealing	56°C	1 minute
*Extension	72°C	2 minutes
Final Extension	72°C	10 minutes
Hold	4°C	hold

*These steps were repeated 35 times.

Creation of Fusion Constructs

Polymerase chain reaction (PCR). To create a fusion construct for an mRNA with an atypically long 3'-UTR, the long 3'-UTRs from *COX23, FRE2,* and *COX19* were amplified by PCR. The PCR conditions used to create all fusions constructs are denoted

in Table 1.3. The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Next, the 5'-UTR and ORF of a second gene, *CYC1*, was amplified by PCR. *CYC1* was used because it is not an NMD target. The PCR product was run on a 0.8% agarose gel, and the product was cut out and purified using the QIAquick Gel Extraction Kit (Qiagen,Valencia, CA). Third, ligation mediated PCR of the two products was performed in order to make a fused gene (Figure 1.1). The ligation mediated PCR product was run on a 0.8% agarose gel, and the product was cut out and purified using the QIAquick Gel Extraction Kit (Qiagen,Valencia, CA). The fusion construct (PCR product) was then inserted into the TOPO-TA cloning vector according to manufacturer's instructions (Life Technologies, Grands Island, NY), and sent for sequencing to verify that the correct fusion construct was created. This protocol was repeated to create additional constructs in which the *CYC1* mRNA 3'-UTR was fused with *COX23, FRE2*, and *COX19* ORFs and 5'-UTRs.

Table 1.3: PCR conditions used to create fusion constructs.

Step	Temperature	Time
Initial Denaturation	93°C	5 minutes
*Denaturation	93°C	30 seconds
*Annealing	45°C	45 seconds
*Extension	72°C	4.5 minutes
Final Extension	72°C	10 minutes
Hold	4°C	hold

*These steps were repeated for 35 cycles.

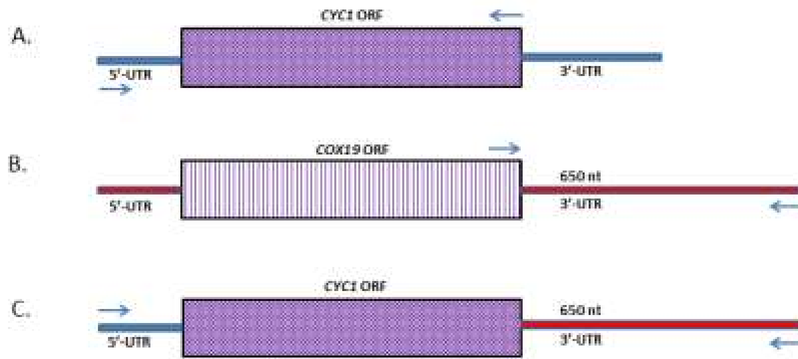

Figure 1.1: Creation of fusion constructs using *COX19* as an example. Arrows indicate direction of the primers. (A) Endogenous *CYC1*. (B) Endogenous *COX19*. (C) Fusion construct containing the *CYC1* 5'-UTR and ORF and the *COX19* 3'-UTR.

Plasmid mapping and transfer of fusion construct to the yeast vector. The TOPO-TA cloning vectors (Life Technologies, Grand Island, NY) containing the fusion DNA were mapped with restriction enzymes *BamHI*, *NotI,* and *XbaI*. Single digests with each of the restriction enzymes was performed, followed by a double digest. The fragment of expected size, from the double digest, was then moved into either a pRS315 or pRS425 yeast vector so that they could then be transformed into yeast strains.

Transformation. Yeast cells are grown in nutrient rich media (YAPD) overnight to an OD_{600} of 0.4-0.6. The fusion constructs were transformed into the yeast strains using Lithium Acetate mediated transformation (Gietz and others 1992; Gietz and Woods 1998). Approximately 1µg of DNA was added to each transformation. The cells were suspended in 1.0 ml of sterile water, and 200µl were plated on selective (-leucine) plates. A no DNA control was used and plated on both YAPD and selective plates. All plates were incubated at 30°C for 2-5 days until transformants appeared. Transformants were selected, then the previous protocols described were also used to extract RNA, run

formaldehyde gels, transfer the RNA to nitrocellulose membranes, and probe with [α-^{32}P]dCTP.

Low copper methods. For all low copper northerns, complete minimal media was used to grow the required yeast cells. The media incorporated yeast nitrogen base without copper (YNB-CuSO4-FeCl3) and 100 μM Bathocuproinedisulfonic acid (Sigma-Aldrich). Glassware used in these experiments was soaked in 10% nitric acid overnight to remove any trace amounts of copper. All of the cells used for low copper northern blots were grown in the copper deficient media described above as well as in acid washed glassware.

CHAPTER TWO

Regulation of Natural mRNAs by the Nonsense-Mediated mRNA Decay Pathway

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Abstract

The nonsense-mediated mRNA decay (NMD) pathway is a specialized mRNA degradation pathway that degrades select mRNAs. This pathway is conserved in all eukaryotes examined so far and it triggers the degradation of mRNAs that prematurely terminate translation. Originally identified as a pathway that degrades mRNAs with premature termination codons as a result of errors during transcription, splicing, or damage to the mRNA, NMD is now also recognized as a pathway that degrades some natural mRNAs. The degradation of natural mRNAs by NMD has been identified in multiple eukaryotes including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Arabidopsis*, and humans. *S. cerevisiae* is used extensively as a model to study natural mRNA regulation by NMD. Inactivation of the NMD pathway in *S. cerevisiae* affects approximately 10% of the transcriptome. A similar percentage of natural mRNAs in *D. melanogaster* and human transcriptomes are also sensitive to the pathway; indicating that NMD is important for regulation of gene expression in multiple organisms. NMD can either directly or indirectly regulate the decay rate of natural mRNAs. Direct NMD targets possess NMD inducing features. This minireview focuses on the regulation of natural mRNAs by the NMD pathway, as well as the features demonstrated to target

these mRNAs for decay by the pathway in *S. cerevisiae*. We also compare NMD targeting features identified in *S. cerevisiae* with known NMD targeting features in other eukaryotic organisms.

The amount of a particular mRNA present at a specific time in a cell is dependent on the rate of synthesis and decay. The nonsense-mediated mRNA decay (NMD) pathway is a translation-dependent mRNA degradation pathway that recognizes and elicits the rapid degradation of select mRNAs. This pathway is highly conserved in all examined eukaryotes from yeast to humans. NMD targets mRNAs that prematurely terminate translation. Such mRNAs can be produced due to genomic mutations or errors in gene expression. In cases where the mRNAs have a premature termination codon (PTC), degradation of these mRNAs by NMD prevents the accumulation of potentially harmful, truncated proteins.

The NMD pathway also regulates the expression of specific genes by degrading natural mRNAs (1-7). Natural mRNAs regulated by NMD are normal, cellular mRNAs that largely code for functional proteins. Importantly, this subset of mRNAs has features that can induce the translating ribosome to prematurely terminate translation, leading to degradation of the transcript by NMD. The regulation of natural mRNAs by NMD is fairly widespread. Genome-wide studies have shown that 5-10% of the *Saccharomyces cerevisiae* transcriptome is affected when NMD is inactivated (1). A similar percentage of the nematode *Caenorhabditis elegans* (8), the fruit fly *Drosophila melanogaster* (9), and the human transcriptomes are sensitive to NMD (10, 11). Natural mRNAs regulated by the pathway have also been identified in other eukaryotic organisms including the fungi *Yarrowia lipolytica, Aspergillus nidulans*, *Schizosaccharomyces pombe,* and several plant species (12-15).

Three core trans acting factors are required for a functional NMD pathway in all eukaryotes. These core NMD factors are the up frameshift proteins Upf1, Upf2, and

Upf3. The Upf proteins were originally identified in *S. cerevisiae* (16, 17). Mutations or silencing of any one of these three factors selectively stabilizes mRNAs that are regulated by NMD (18). Upf1p is the central regulator of the degradation pathway and is the most conserved of the Upf proteins (19-23). Upf2p and Upf3p are responsible for regulating Upf1p function. Upf1p is a group 1 RNA helicase with ATPase activity (24-26). *S. cerevisiae* Upf1p has been shown to interact with Upf2p, which in turn interacts with Upf3p (18, 27). Upf1p also associates with additional factors including the eukaryotic translational release factors, eRF1 and eRF3 (28). It is important to note that Upf1p plays additional roles distinct from NMD. These cellular processes include staufen 1-mediated mRNA decay (SMD), telomere maintenance, histone mRNA decay, genome stability and advancement of the cell cycle (29-32).

The NMD mechanism in S. cerevisiae

NMD in *S. cerevisiae* is known to be triggered by the messenger ribonucleoprotein (mRNP) context surrounding the translation termination event. Models exist that explain the mechanism that causes a termination codon to be recognized as premature. In the most widely accepted model, premature translation termination is perceived to be an inefficient event because it is in an improper context compared to a normal translation termination event. Specifically, there is evidence in *S. cerevisiae* and other organisms that NMD targets can be recognized as targets due to the lack of factors bound downstream of the termination codon (23). In this model, known as the *faux*-UTR model, a ribosome terminating translation at a termination codon substantially upstream of the poly(A) tail terminates translation inefficiently. The *faux*-UTR model posits that NMD occurs because the Pab1p, or other factors bound to the poly(A) tail, are not in

close proximity to the terminating ribosome to enable interaction of the Pab1p with release factor 3 (eRF3p), which is bound to the terminating ribosome, thus establishing the correct mRNP context for a normal translation termination event (33). In the absence of correct translation termination, the Upf factors interact with the release factors, eRF1p and eRF3p (28), resulting in an aberrant translation termination event and NMD activation (23, 34-36). NMD activation leads to the decapping of the mRNA by the Dcp1-Dcp2p complex followed by 5' to 3' degradation of the mRNA by the exoribonuclease Xrn1p (37). The *faux*-UTR explains how some natural mRNAs with known NMD inducing features are targeted for degradation by the pathway, however, it does not explain the observation that the presence of Pab1p is not required to distinguish a normal termination event from a premature translation termination event in *S. cerevisiae* (38).

The NMD mechanism in mammals

In metazoans, additional factors are required for NMD to operate normally. These factors include the Suppressors with Morphological effect on Genitalia or SMGs in *C. elegans*. SMG proteins are also found in other multicellular organisms, and perform a variety of functions. SMG-1, SMG-5 and SMG-7 regulate the phosphorylation and dephosphorylation of SMG-2, the *C. elegans* homolog of Upf1p, while SMG-6 is an endonuclease. Additionally, the exon-junction complex (EJC) is a multi-protein complex that enhances NMD of mRNAs that undergo splicing. The EJC is deposited 20-24 nucleotides upstream of exon-exon junctions. The core components of the EJC are eIF4AIII, Y14, MAGOH, barentsz, and additional effector proteins such as Upf3p (39).

Upf3p is reported to associate with the EJC during splicing, and subsequently, Upf2p associates with the EJC bound Upf3p within the cytoplasm (40).

The initial model to explain mammalian NMD proposed that the round of translation an mRNA is undergoing determines whether an mRNA is an NMD target. In newly synthesized mRNAs, the 5' cap structure is bound by the cap-binding complex consisting of the proteins CBP80 and CBP20. While still bound by CBP80/20, mRNAs undergo the first or “pioneer” round of translation. mRNAs undergoing the “pioneer” round of translation are subject to NMD (41). These mRNAs are targeted to the pathway if the translating ribosomes terminate translation 50-55 nucleotides upstream of an EJC. In most mRNAs, the natural stop codon is found in the last exon, and all EJCs would be displaced from the mRNA before the translating ribosome encounters the natural stop codon. In contrast, a ribosome stalled at a PTC associates with the SURF complex which is composed of the SMG-1 kinase, Upf1p, and release factors, eRF1 and eRF3. The SURF complex associated Upf1p interacts with the downstream EJC which is still bound to the mRNA. This association occurs via interactions between Upf2p and the EJC bound Upf3p, consequently targeting the mRNA to NMD.

It is becoming apparent that NMD in mammalian cells is not always restricted to spliced transcripts undergoing the first round of translation. In fact, there are reports of intronless mammalian mRNAs undergoing NMD, demonstrating that mammalian mRNAs are subject to NMD in the absence of an EJC downstream of a stop codon (36, 42, 43). Furthermore, recent work showing that mRNAs bound by the cap-binding protein, eukaryotic initiation factor 4E (eIF4E), are subject to NMD suggests that mammalian mRNAs undergoing bulk translation can also be subject to NMD (44, 45).

The *S. cerevisiae* NMD model is applicable to mammalian NMD to some extent; however, apparent differences exist. *S. cerevisiae* may be a good model for EJC-independent NMD. The EJC mark deposited on mammalian mRNAs during splicing adds an additional layer of regulation and is absent in *S. cerevisiae*. In *S. cerevisiae,* NMD is splicing independent. Nevertheless, EJC-independent NMD has been demonstrated in mammalian cells akin to NMD in *S. cerevisiae.* Moreover, there have been reports of proteins such as the RNA helicase hrp1p, marking *S. cerevisiae* mRNAs downstream of a premature stop codon and targeting the mRNAs to the pathway analogous to the EJC targeting mechanism in mammals (48, 49).

As we already noted, the core NMD factors are conserved in all eukaryotes examined so far. In addition, recent studies demonstrating that mammalian mRNAs are subject to NMD while undergoing bulk translation suggests that mammalian mRNAs targeted to the pathway may not be restricted to the first round of translation and that mRNAs can be targeted to the pathway at each round of translation, similar to *S. cerevisiae* (46). However, mRNAs that are subject to NMD in *S. cerevisiae* are primarily degraded by Xrn1p from the 5' ends of the mRNAs after removal of the 5' cap, while mammalian NMD is initiated by endonucleolytic cleavage of the target transcript followed by exonucleolytic degradation of the 5' and 3' RNA fragments (47).

Natural mRNAs regulation by NMD

As previously stated, natural mRNAs regulated by the NMD pathway have been identified in multiple organisms. These natural NMD targets have been most extensively studied in *S. cerevisiae,* and can be classified as either direct or indirect NMD targets. Direct NMD targets typically have significantly altered decay rates in cells with a

functional NMD pathway relative to cells with a non-functional pathway. In most cases, indirect NMD targets have comparable decay rates in cells with a functional or non-functional NMD pathway. Indirect NMD targets may accumulate as a result of increased transcription, whereby a transcription factor that regulates the expression of a specific mRNA is the direct NMD target. An example of an indirect NMD target is the *S. cerevisiae URA3* mRNA. The *URA3* mRNA accumulates to higher levels in NMD mutants due to increased levels of its transcription activator Ppr1p (16). *PPR1* mRNA is a direct NMD target. In *S. cerevisiae,* it is estimated that ~ 48% of the natural NMD targets are direct targets with significantly altered decay rates between wild-type and NMD mutants (2). mRNAs that are direct NMD targets typically contain NMD inducing features that can trigger their degradation by the pathway.

NMD inducing features in *S. cerevisiae*

Initially, it was perceived that all targets of the NMD pathway contain a PTC. However, with the growing list of endogenous, error free, natural mRNAs that are regulated by the pathway, it is becoming apparent that sequence features exist that can trigger the regulation of natural mRNAs by NMD. These NMD inducing features have been identified in *S. cerevisiae* and in other organisms and activate NMD by directing translation to terminate prematurely. Here, we discuss each of the recognized NMD inducing features in *S. cerevisiae* and their conservation in other eukaryotic species. These features are depicted schematically in figure 2.1 and include inefficiently or alternatively spliced pre-mRNAs, mRNAs containing atypically long 3'-untranslated regions (UTRs), mRNAs containing upstream open reading frames (uORFs), mRNAs subject to programmed ribosomal frameshifting, and mRNAs subject to out-of-frame

translation initiation caused by leaky ribosomal scanning (2, 4, 50-54). Although these features typically trigger degradation, the presence of these NMD-inducing features on an mRNA does not always activate the pathway. Some mRNAs with these features are immune to degradation by NMD (4, 34, 55). For example, the *S. cerevisiae GCN4* and *YAP1* mRNAs contain uORFs, but evade degradation by NMD (55). Furthermore, these NMD targeting features are not known to cause the degradation of all natural mRNAs that are regulated by NMD in *S. cerevisiae,* indicating that additional NMD targeting features remain to be identified (2).

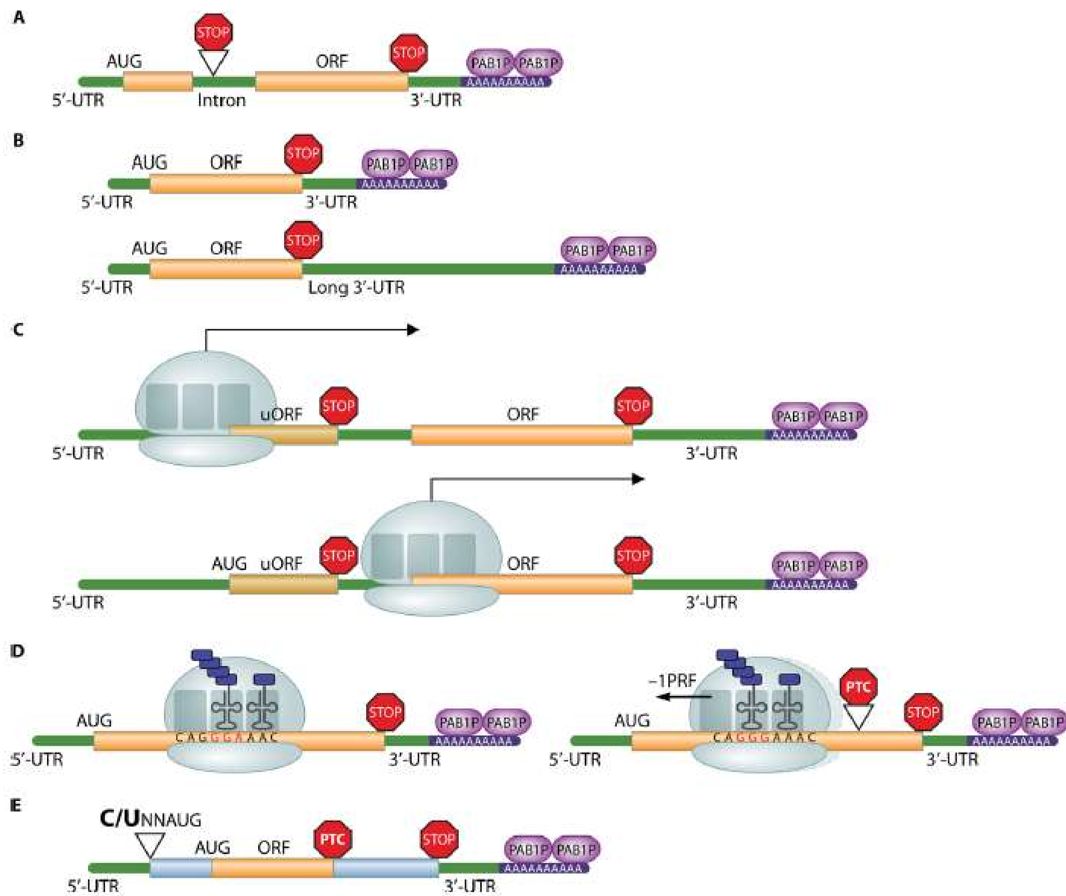

Figure 2.1. Schematic representation of NMD-targeting features in *S. cerevisiae*. (A) Intron containing pre-mRNA containing a stop codon. (B) mRNA with an atypically long 3' UTR. (C) mRNA with an upstream open reading frame (uORF) in the 5' UTR that can induce NMD. (D) mRNAs with sequence features that can induce a -1 ribosomal frameshift. (E) mRNA with a suboptimal start codon context that may be subject to out-of-frame translation initiation in an alternate AUG codon within the ORF, which can lead to a PTC and NMD activation. ORFs are illustrated by thick orange lines, and untranslated regions (UTRs) and introns are shown by thin green lines. The poly(A) tail is shown in blue, and ribosomes are illustrated in gray. AUG, start codon; PTC, premature termination codon; PRF, programmed (-1) ribosomal frameshift; PAB1P, poly(A)-binding protein.

Some intron containing pre-mRNAs are NMD targets

Generation of a functional mRNA depends on precise processing of the pre-mRNA. Pre-mRNAs containing introns undergo splicing prior to export to the cytoplasm. In some cases, pre-mRNA processing events result in retention of an inefficiently spliced intron. The majority of intron containing pre-mRNAs that are exported to the cytoplasm are degraded by the NMD pathway in *S. cerevisiae* because these pre-mRNAs are likely

to have an in frame stop codon (figure 2.1A) (51, 56). This regulation prevents the accumulation of incompletely processed transcripts (57). Alternatively, if a retained intron does not contain a stop codon but is out of frame with the main open reading frame, it can cause a frameshift to a downstream exon. This can result in the introduction of a PTC, and consequently, degradation of the transcript by NMD. Several studies in *S. cerevisiae* have shown that inefficiently spliced pre-mRNAs accumulate in NMD mutants (51, 56), albeit only ~5% of the genes in *S. cerevisiae* contain introns (58). For example, the *S. cerevisiae CYH2* pre-mRNA is inefficiently spliced, transported to the cytoplasm and degraded by NMD. Similar results have been observed in the hemiascomycete fungi, *Yarrowia lipolytica,* which contains four times as many introns as *S. cerevisiae* (12). In some cases, the accumulation of unspliced pre-mRNAs in *S. cerevisiae* NMD mutants was found to occur under stress conditions (56). In addition, localization experiments demonstrated that the unspliced pre-mRNAs accumulate in cytoplasmic processing-bodies (P-bodies) in the absence of a functional NMD pathway (56).

Some non-productive alternatively spliced transcripts are NMD targets

Pre-mRNA processing can also result in alternative splicing events, where exons are spliced together differentially to generate proteome diversity. Aberrant alternative splicing can generate NMD sensitive transcripts by introducing PTCs. This mechanism of NMD targeting is often referred to as “alternative splicing coupled to NMD”. A recent study in *S. cerevisiae* demonstrated that non-productive alternatively spliced transcripts that contain a PTC or produce non-functional proteins are regulated by NMD (59). In addition, genome-wide studies have shown that aberrantly spliced transcripts are regulated by the NMD pathway in *C. elegans* (8) and in *D. melanogaster* (60).

Interestingly, in *C. elegans,* alternatively spliced transcripts of ribosomal protein genes are also regulated by the NMD pathway linking the pathway to splicing regulation (61).

The NMD pathway is also linked to splicing regulation in mammalian systems. Studies using mouse cell lines found that down-regulating levels of the NMD factor *UPF2* affected splicing, resulting in aberrant alternatively spliced mRNA isoforms that were sensitive to NMD (62). Consistent with these findings, Saltzman *et al*., 2008 found that in mammalian cells, pre-mRNAs that undergo alternative splicing coupled to NMD were enriched for core splicesomal proteins, further linking the NMD pathway to regulation of splicing (63). Collectively, these studies demonstrate that NMD controls splicing regulators and aberrant alternatively spliced transcripts in mammalian systems.

In other systems such as plants, NMD has also been found to regulate the expression of pre-mRNAs that retain introns due to alternative splicing. It has been reported that retention of introns accounts for ~41% of alternative splicing events in plants (64). In addition, the presence of an intron in the 3'-UTR has been shown to activate NMD, although only ~ 3.6% of *Arabidopsis* genes contain introns in the 3'-UTR (65-67). The regulation of these intron containing pre-mRNAs by NMD is reported to occur in a position dependent manner (67). Specifically, if the intron is located at least 50 nt downstream of the stop codon, NMD is triggered. Furthermore, efficiency of NMD was found to increase with increasing distance from the stop codon (67).

Consistent with the findings in *S. cerevisiae*, plants, and mammals, non-productive alternatively spliced transcripts are regulated by the NMD pathway in other systems such as Zebrafish and *Paramecium tetraurelia* (68). Collectively, these studies show that pre-mRNA processing events that result in the generation of intron containing

pre-mRNAs as well as aberrantly spliced transcripts generate NMD targets in a wide range of organisms. Moreover, in some species, NMD has been found to have an additional level of regulation by directly affecting splicing factors.

Atypically long 3'-UTRs can target mRNAs to the NMD pathway

The presence of a long 3'-UTR has been shown to be sufficient to target mRNAs for NMD. The mechanism by which atypically long 3'-UTRs induce NMD has been extensively studied in *S. cerevisiae*. In *S. cerevisiae*, mRNA 3'-UTRs range in size from 50-200 nts in length, with a median length of 121nts (69). The majority of the examined transcripts with 3'-UTRs of 350 nts or longer are degraded by the NMD pathway (4, 70). Interestingly, some mRNAs produce different isoforms of the same transcript that vary in their 3'-UTR lengths. Transcripts with varying 3'-UTR lengths can be produced as a result of alternative 3'-end processing, which can be sensitive to growth conditions (71).

Pre-mRNAs that undergo alternative 3'-end processing producing multiple mRNA isoforms with different 3'-UTR lengths can be differentially regulated. This differential regulation can be observed through mRNA stabilization or destabilization. Surprisingly, in some mRNAs, half-lives can vary significantly between mRNA isoforms separated by <3 nt at the 3'-end (72). Furthermore, some mRNA isoforms produced by alternative 3'-end processing may be regulated by NMD while others may not. For example, the *S. cerevisiae MAK31* mRNA has two mRNA isoforms with varying 3'-UTR lengths; a short (200 nt) and long (920 nt) 3'-UTR. The short, 200 nt 3'-UTR mRNA isoform is immune to degradation by NMD; however, the long, 920 nt 3'-UTR mRNA isoform is degraded by the pathway (4). On the other hand, *MPA43* mRNA also produces two mRNA isoforms with varying 3'-UTR lengths of 300 nt and 600 nt. In this case, both 3'-UTRs

lead to NMD induced degradation (4). In *S. cerevisiae,* it appears that natural mRNAs targeted to the pathway by a long 3'-UTR are regulated by NMD due to the length of the 3'-UTR and not due to the specific sequence of the 3'-UTR. This is consistent with the *faux*-UTR model which states that increasing the 3'-UTR length increases the distance between the terminating ribosome and the Pab1p (figure 2.1B) (23).

Similar to *S. cerevisiae*, *C. elegans*, *Drosophila*, and mammalian mRNAs with long 3'-UTRs are targeted for degradation by NMD. For example, in murine embryonic stem cells (mESCs), increasing mRNA 3'-UTR length was found to correlate with increasing sensitivity to the NMD pathway (73). Likewise, human transcripts with long 3'-UTRs are subject to degradation by the pathway. These transcripts were found to be up regulated in *UPF1*, *SMG-6* and *SMG-7* down-regulated cells (34). Interestingly, the NMD factors *UPF1*, *SMG-5* and *SMG-7* mRNAs were also found to contain long 3'-UTRs that induce degradation by NMD. These studies point to a feedback loop where components of the NMD machinery are also regulated by the pathway (34). The regulation of NMD factors by the pathway has also been observed in zebrafish and *C. elegans*, further supporting the notion of a conserved NMD feedback loop (74).

A long 3'-UTR can also induce NMD in plants and provides additional support for the *faux*-UTR model (65, 66). The average length of plant 3'-UTRs is reported to be 241nt. A 3'-UTR of >350 nt has been shown to consistently elicit NMD in plants (75). Additionally, the efficiency of NMD in plants has been reported to increase with increasing length of the 3'-UTR (65).

Collectively, these studies demonstrate that a long 3'-UTR is a conserved NMD inducing feature in multiple organisms. Furthermore, it appears that in several organisms

increasing the length of the 3'-UTR increases NMD efficiency, suggesting that the distance between the stop codon and the features downstream of the stop codon is the NMD determining characteristic. If the distance between the stop codon and the poly (A) tail is the essential NMD determinant, then it follows that the length of the 3'-UTR itself, and not the 3'-UTR sequence, is the important NMD-inducing feature. In addition, transcripts that undergo alternative 3'-end processing may generate multiple mRNA isoforms that are differentially regulated by NMD. This would enable cells to selectively regulate mRNAs transcribed from the same gene. An important question for future studies is to determine whether physiological conditions can affect alternative 3'-end processing, resulting in the generation of transcripts with altered NMD sensitivity.

A subset of upstream open reading frame (uORF) containing mRNAs is sensitive to the NMD pathway

An upstream open reading frame, or uORF, is an open reading frame that is located in the 5'-UTR of a transcript (Figure 2.1C). mRNAs can have one or more uORFs, some of which overlap with the main protein coding region. These uORFs can play regulatory roles by affecting mRNA stability and protein synthesis (76). When actively translated, a uORF can interfere with the expression of the main ORF. Furthermore, if the uORF of an mRNA is translated, the stop codon of the uORF may be recognized as a PTC, resulting in degradation of the mRNA by NMD. The presence of a uORF has been shown to target mRNAs for degradation by NMD in *S. cerevisiae* and in the fission yeast, *S. pombe*. RNA-Seq analysis of the *S. cerevisiae* transcriptome predicted that 321 transcripts contain uORFs (77). A subset of these transcripts is expected to be regulated by NMD. An additional *S. cerevisiae* study identifying direct

and indirect NMD targets estimated that ~ 35% of direct NMD targets are sensitive to the pathway due to the presence of uORFs (2). An example of a *S. cerevisiae* mRNA targeted by NMD due to the presence of a uORF in the 5'-UTR is the *CPA1* mRNA. *CPA1* encodes the small subunit of arginine-specific carbamoyl phosphate synthetase (50). The sensitivity of the *CPA1* mRNA to the NMD pathway is responsive to the levels of arginine in the medium. Addition of arginine causes ribosomal stalling at the uORF termination codon, resulting in destabilization and NMD-mediated degradation of the *CPA1* mRNA(50). The *S. cerevisiae ALR1* mRNA is also targeted to NMD due to the presence of a uORF (78). *ALR1* encodes for the major magnesium transporter in *S. cerevisiae.* Inactivation of the NMD pathway results in stabilization of the *ALR1* mRNA and increased intracellular magnesium levels. This increase in intracellular magnesium levels promotes reduced translational termination fidelity and leads to an increase in read-through at PTCs. This study established a link between the NMD pathway with magnesium homeostasis and translational termination fidelity (78). Consistent with studies in *S. cerevisiae*, a genome-wide study of *S. pombe* transcripts regulated by the NMD pathway also found that the majority of the transcripts that were up-regulated upon NMD inactivated contained uORFs, suggesting that this is also major NMD targeting feature in *S. pombe* (14).

In addition, uORFs can trigger NMD in *C. elegans* (8) and mammals (10, 11, 73). In *C. elegans,* a genome-wide study of NMD inducing features found that natural mRNAs regulated by the pathway are likely to have uORFs (8). In mammalian cells, it is estimated that ~50% of transcripts contain uORFs (79). Many of these uORF containing mRNAs are not regulated by NMD, demonstrating that some uORF containing

transcripts are capable of evading degradation by the pathway (80). This discrepancy is thought to be a result of varying translation efficiency. This view is supported by a study utilizing mESCs that found that translated uORFs were likely to target mRNAs to NMD while non-translated uORFs did not (73). The same study also found that translated NMD targets containing uORFs were enriched for transcriptional regulators, suggesting that genes regulated by these transcription factors would be indirect NMD targets (73).

Consistent with the above studies, plant mRNAs containing uORFs are also regulated by NMD. An estimated 20-30% of plant genes have been reported to contain uORFs (65, 81, 82). In plants, it appears that the likelihood of a uORF inducing NMD depends on several factors. These factors include the length of the uORF and the distance between the stop codon of the uORF and the main start codon of the protein coding region. Therefore, it has been proposed that longer plant uORFs induce NMD, while shorter uORFs do not (81).

Collectively, these studies establish that uORFs are also conserved NMD inducing features in all of the organisms that have been examined so far. These studies of uORFs also point to the fact that not all uORF containing mRNAs are subject to NMD, and that other factors such as the level of translation the uORF is undergoing, the length of the uORF, and the distance between the uORF stop codon and the main ORF start codon can influence NMD targeting of these mRNAs. It is also apparent that some physiological conditions can affect the NMD targeting of uORF containing transcripts by influencing the efficiency by which the uORF is translated.

mRNAs subject to -1 ribosomal frameshift signals may be regulated by NMD

Programmed ribosomal frameshifting (PRF) has been observed in multiple organisms. Analyses of multiple genomes indicated that ~ 8-12% of genes contain a probable -1 ribosomal frameshift signal (83). A study showed that -1 ribosomal frameshifting plays a role in mRNA stability in *S. cerevisiae* through utilization of the NMD pathway (83). Specifically, -1 PRF signals were reported to cause destabilization of mRNAs by driving translating ribosomes into an alternate reading frame. In mRNAs undergoing -1 PRF, the ribosome moves toward the 5' end of the mRNA, shifting the reading frame by one nucleotide (figure 2.1D). As a result of the -1 frameshift, the translating ribosome is out of frame and may encounter a PTC, thus triggering NMD.

The majority of the PRF signals contain three features: a 'slippery site', spacer sequence, and an mRNA pseudoknot (83). Currently, -1 ribosomal frameshift signals have not been reported as an NMD inducing feature in eukaryotic organisms other than in *S. cerevisiae*. These signals appear to be common in *S. cerevisiae*, and as much as 11% of genes have a likely -1 PRF (83). Although PRF signals are present across a wide range of yeast species, specific signals are not entirely conserved. For example, in multiple yeast species strong candidate -1 ribosomal frameshift signals are present in many gene orthologs, but not across all species examined (83). It remains to be determined whether -1 ribosomal frameshift signals target natural transcripts to the NMD pathway in other eukaryotic organisms.

mRNAs subject to out-of-frame translation initiation caused by leaky ribosomal scanning may be NMD targets

A number of *S. cerevisiae* mRNAs are targeted to the NMD pathway due to out-of-frame initiation of translation within the open reading frame. This mechanism is referred to as leaky scanning. During leaky scanning, ribosomes scan past the main ORF start codon, which is in a suboptimal context, and initiate translation with an alternate start codon within the ORF (Figure 2.1E). This alternate start codon can be out of frame and lead the translating ribosome to a stop codon, causing NMD induced degradation of the transcript. Whether a translation initiation codon is considered to be in an optimal or a suboptimal context is based on the sequence context surrounding the initiator AUG (84). Nucleotides at the -6 to +6 positions in relation to the initiator AUG are important for translation initiation. Of particular importance is the -3 position where an A or a G at that position results in more efficient translation initiation than a C or a U (2, 52, 84).

Presently, leaky scanning has only been recognized as an NMD inducing feature in *S. cerevisiae,* and an estimated 3% of *S. cerevisiae* mRNAs may be subject to leaky scanning (2, 52). One such mRNA is the *S. cerevisiae SPT10* mRNA which encodes a putative histone acetylase and has a role in transcriptional silencing. The *SPT10* start codon is in a suboptimal context, resulting in degradation of the mRNA by the pathway (52). Additional *S. cerevisiae* mRNAs targeted by NMD due to leaky scanning were also identified by Guan *et al* 2006 (2). However, how widely distributed this NMD inducing feature is among other eukaryotic organisms remains to be determined.

Conservation of the NMD-inducing features

Out of the five identified NMD-inducing features discussed here, three appear to be broadly distributed across a wide range of eukaryotic organisms. Intron containing and non-productive alternatively spliced transcripts have been shown to be NMD targets in multiple yeast species as well as in *C. elegans*, *D. melanogaster*, plants, and mammalian systems. Atypically long 3'-UTRs also induce NMD in multiple eukaryotic organisms, suggesting that this mechanism is conserved as well. Similarly, a subset of natural transcripts containing uORFs is regulated by NMD in multiple eukaryotes.

It is also apparent that the presence of known NMD-inducing features on transcripts does not always activate NMD. Some transcripts containing NMD-inducing features escape degradation by the pathway. For example, multiple transcripts containing uORFs and atypically long 3'-UTRs appear to be immune to the NMD pathway. This observation raises the question as to whether the functionality of NMD-inducing features is condition specific. Specifically, are these NMD-inducing features only functional under particular physiological conditions and not in others?

The conservation of specific NMD-inducing features across eukaryotic ancestors has been debated. These ancestors, often referred to as stem eukaryotes, are believed to have had a functional NMD pathway. However, some suggest that the NMD pathway in stem eukaryotes degraded mRNAs with a long 3'-UTR centered approach (85). This notion is supported by the observation that long 3'-UTRs as NMD-inducing features is observed in most eukaryotes. Furthermore, this model supports the idea that an intron centered approach developed in NMD in vertebrates (85). Alternatively, a second model advocates the idea that both a long 3'-UTR centered approach and an intron based

approach were present in eukaryotic ancestors (65, 66). This model is supported by the study of plant NMD. In plants, both long 3'-UTR based and intron based NMD occur (65, 66). Specifically, long 3'-UTR based NMD in plants shows similarities to yeast and *Drosophila*, while intron-based NMD shows similarities to mammalian NMD (66). Since plants are distantly related to both animals and fungi, these results support the presence of both types of NMD in eukaryotic ancestors.

Physiological consequences resulting from the regulation of specific natural mRNAs by NMD

The regulation of natural mRNAs by NMD raises several questions. Why are specific transcripts regulated by NMD? Is this regulation physiologically significant? Furthermore, is the regulation of specific transcripts condition specific? There are reports showing that the regulation of specific natural mRNAs by the pathway can be of functional significance. In *S. cerevisiae* NMD mutants have reduced growth rates on non-fermentable carbon sources (86). The growth defect on non-fermentable carbon sources like lactate is partly due to overexpression of *ADR1* mRNA (87). In addition, *S. cerevisiae* NMD mutants are sensitive to Calcofluor White, a cell wall disruptor. This sensitivity is partially attributed to the regulation of the *PGA1* mRNA by NMD (4). *PGA1* encodes for an essential component involved in GPI anchor synthesis. Pga1p functions in association with Gpi18p to add the second mannose residue in the GPI anchor synthesis. It appears that a functional NMD pathway maintains the *PGA1* mRNA at appropriate levels for GPI anchor synthesis (2). Moreover, NMD mutants have recently been shown to be more tolerant of toxic levels of copper partly due to the regulation of *CTR2* mRNA by the pathway. *CTR2* encodes for a copper transporter of the vacuolar

membrane that controls the flux of copper into the vacuole (88, 89). As previously stated, NMD has also been found to regulate magnesium homeostasis. Thus, *S. cerevisiae* NMD is involved in both copper and magnesium homeostasis by regulation of specific mRNAs (2, 3, 50, 88).

In addition, regulation of natural mRNAs by NMD or perturbation of NMD factors has physiological consequences in other organisms. For example, in *C. elegans,* mutations in the genes required for NMD result in morphogenetic effects on the genitalia and reduced number of offspring (90). In *Arabidopsis*, upf1 and upf3 mutants have multiple developmental phenotypes linking the NMD pathway to the regulation of genes during normal plant development and responses to pathogens (91, 92). A functional NMD pathway is also required for cell growth and differentiation in *Drosophila* (9, 93). In fact, NMD is essential for normal development in mice. Mice with defects in *UPF1* and *UPF2* die during embryogenesis (94, 95). Similarly, it has been shown that NMD effectors are essential for zebrafish embryonic development and survival (13). As previously stated, *UPF1* plays additional roles in cells distinct from NMD and down-regulation of *UPF1* could affect other cellular processes. Nevertheless, these studies show that although a functional NMD pathway is not required for viability of *S. cerevisiae,* a functional pathway is required for the development and viability of multiple organisms.

Studies have shown that NMD has physiological consequences in humans as well. It is estimated that ~30% of disease-associated mutations lead to the production of transcripts containing PTCs (96-98). The majority of these PTC containing transcripts would not be categorized as natural mRNAs. Nevertheless, these transcripts are subject to

regulation by the NMD pathway. Thus, NMD can play a critical role in whether or not these diseases manifest themselves in patients. In instances where the location of the PTC would result in the generation of a non-functional truncated protein, NMD is beneficial to the patients. In other cases where the location of the PTC would result in the generation of a protein with partial function, NMD worsens the diseases by degrading the transcripts. Hence, NMD can be beneficial or detrimental depending on the specific mutations and the genes mutated (99). Additionally, mutations in the pathway or in any of the factors involved can have serious consequences. For example, in humans, mutated *UPF3B* has been linked to mental retardation, autism and schizophrenia (100-102).

Conclusion

The NMD pathway exits as both a quality control mechanism and a mechanism to fine tune gene expression. As noted in this review, a subset of natural mRNAs with specific NMD-inducing features are recognized and rapidly degraded by the NMD pathway, demonstrating that the pathway plays an important role in the regulation of gene expression. A significant study discussed here that demonstrates the consequences of NMD regulating specific natural transcripts is the *ALR1* study. In *S. cerevisiae* NMD regulates intracellular magnesium levels and consequently translational fidelity (78). NMD mutants have elevated *ALR1* mRNA levels and an as a result increased intracellular magnesium levels which leads to reduced translational fidelity and read-through at PTCs.

Much is known about the factors required for NMD in *S. cerevisiae* and other eukaryotes. The repertoire of NMD targeted transcripts is also beginning to be recognized. As discussed here, natural mRNAs regulated by NMD have been identified in multiple eukaryotes, and the NMD-inducing features for a subset of these natural

mRNAs have been identified. These NMD-inducing features appear to be recognized in the same way in different organisms. Several studies suggest that the recognition of NMD-inducing features is conserved to some extent. In most cases, the presence of the NMD targeting feature induces a premature translation termination event and activates NMD. However, there are apparent differences in the recognition of mRNA targets and the identity of the NMD targets themselves across species.

Two of the NMD-inducing features discussed have only been recognized in *S. cerevisiae*. These features are the -1 ribosomal frameshift signals and out-of-frame initiation of translation. Future studies are needed to determine the functionality of these features in other eukaryotic organisms. In addition, not all natural mRNAs regulated by the pathway possess recognizable NMD-inducing features, indicating that other NMD targeting features exist. Furthermore, some natural mRNAs regulated by the pathway possess multiple NMD-inducing features. It is possible that these transcripts are subject to differential levels of NMD. It remains to be determined whether the presence of multiple NMD targeting features on an mRNA could have an additive effect. If so, then these transcripts would be more efficiently degraded by the NMD pathway.

In summary, further investigation into natural mRNAs regulation by NMD will identify novel NMD-inducing features and enhance our understanding of how the pathway regulates transcripts with multiple NMD-inducing features. In addition, further investigation of the consequences that result from NMD regulating specific transcripts will enhance our understanding on the extent to which NMD regulates particular cellular processes.

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CHAPTER THREE

Measurement of mRNA decay rates in *Saccharomyces cerevisiae* using *rpb1-1* strains

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KEYWORDS: *Saccharomyces cerevisiae*; mRNA decay; mRNA stability; nonsense-mediated mRNA decay; mRNA half-life; transcription inhibition

SHORT ABSTRACT: The steady-state level of specific mRNAs is determined by the rate of synthesis and decay of the mRNA. Genome-wide mRNA degradation rates or the decay rates of specific mRNAs can be measured by determining mRNA half-lives. This protocol focuses on measurement of mRNA decay rates in *Saccharomyces cerevisiae*.

LONG ABSTRACT: mRNA steady-state levels vary depending on environmental conditions. Regulation of the steady-state accumulation levels of an mRNA ensures that the correct amount of protein is synthesized for the cell's specific growth conditions. One approach for measuring mRNA decay rates is inhibiting transcription and subsequently monitoring the disappearance of the already present mRNA. The rate of mRNA decay can then be quantified, and an accurate half-life can be determined utilizing several techniques. In *S. cerevisiae*, protocols that measure mRNA half-lives have been developed and include inhibiting transcription of mRNA using strains that harbor a temperature sensitive allele of RNA polymerase II, *rpb1-1*. Other techniques for measuring mRNA half-lives include inhibiting transcription with transcriptional

inhibitors such as thiolutin or 1,10-Phenanthroline, or alternatively, by utilizing mRNAs that are under the control of a regulatable promoter such as the galactose inducible promoter and the TET-off system. Here, we describe measurement of *S. cerevisiae* mRNA decay rates using the temperature sensitive allele of RNA polymerase II. This technique can be used to measure mRNA decay rates of individual mRNAs or genome-wide.

INTRODUCTION: The transcription and decay of specific mRNA are crucial determinants of gene expression. The rate of synthesis and decay of specific mRNAs determines the steady-state level of that particular mRNA. The steady-state levels of mRNAs govern the abundance of mRNAs and determine how much of each mRNA is available for protein synthesis. Measurements of mRNA half-lives are used extensively to determine the decay rate of mRNAs. Specific mRNAs decay at different rates that are related to features of the mRNA, the function of the protein encoded by the mRNA and the environmental conditions. Depending on the technique utilized to determine mRNA decay rates, decay rate measurements can be determined either globally or for individual transcripts. In the yeast *S. cerevisiae*, the techniques that are most commonly used to measure global mRNA decay rates include utilizing a yeast strain harboring the temperature sensitive allele of RNA polymerase II and chemical transcriptional inhibitors such as thiolutin and 1,10-Phenanthroline [1-5]. These methods can also be utilized to measure individual mRNA decay rates [4]. Other methods can also be utilized to measure mRNA decay rates. These methods include approach to steady-state labeling or utilization of mRNA molecules that are expressed from a regulated promoter that is

expressed only in select conditions. Each of these techniques has certain advantages and limitations. The technique described here utilizes the temperature sensitive allele of RNA polymerase II. This method uses *S. cerevisiae* as the model, but can been modified and utilized in other systems using specific transcriptional inhibition techniques [6].

mRNA half-life measurements using the temperature sensitive allele of RNA polymerase II are extensively used for both genome-wide and individual measurements of mRNA decay [4,5]. This technique requires the use of a specific yeast strain that harbors a temperature sensitive allele of RNA polymerase II, *rpb1-1* [1]. The rationale for this technique is that exposure of the temperature sensitive yeast strain to the non-permissive temperature inhibits mRNA synthesis. Subsequently, decay of the preexisting mRNA is monitored at different time points after transcription has been inhibited. The disappearance of the preexisting mRNA is monitored by extracting RNA from the yeast cells at different time points after transcription has been turned off. The time points at which the yeast cells are harvested are predetermined by a pilot experiment, and depend on the transcripts and system being investigated. Quicker time points are used for short-lived transcripts, while longer time points are used for longer lived transcripts. Afterwards, the decay of the mRNA is monitored by either northern blot analysis, quantitative PCR or RNAseq.

Measurement of mRNA half-lives using a temperature sensitive allele of RNA polymerase II has its advantages. First, this technique is easy and straight forward. Second, once the yeast strain is acquired or generated in the laboratory the mRNA half-

life measurements can be determined in different growth conditions; enabling determination of environmental influence on mRNA decay. Third, mRNA decay rates can be monitored genome-wide. Use of other transcription inhibition techniques also has advantages and limitations. For example, use of an inducible promoter requires subcloning to generate an mRNA that is under the control of the regulated promoter. Thiolutin is not readily available and is expensive when available. In addition, thiolutin's mode of action is not completely understood and it has been reported to affect other cellular processes including inhibiting mRNA decay [7]. Alternatively, 1,10-Phenanthroline, is more readily available. Furthermore, all of the techniques used to inhibit transcription can perturb cellular function and can affect different mRNAs in distinct ways. An investigator needs to determine the most appropriate method to use in their experimental conditions to attain the most reliable results. To determine which method is most suitable for their application, a researcher needs to identify the transcripts and function of the proteins encoded by the transcripts being investigated. The most reliable mRNA decay rate measurements are those that are determined using multiple techniques and show the same decay rate. No single technique is always the best, and the most appropriate technique depends on the specific situation.

Numerous studies in *S. cerevisiae* have measured mRNA decay rates in various conditions and genetic backgrounds. The conditions that mRNA decay rates are measured in depend on the specific experiment being investigated. Measuring mRNA decay rates in different cellular environments determines whether the conditions being examined preferentially affect the decay rates of specific mRNAs. The decay rates of mRNAs can

also vary depending on the yeast strain being used. For example, mRNA decay rates can be determined in wild-type yeast cells and yeast cells with a non-functional nonsense-mediated mRNA degradation (NMD) pathway. This mRNA degradation pathway is found in all eukaryotic organisms that have been examined so far and it triggers the degradation of mRNAs that prematurely terminate translation [8]. NMD was initially identified as a pathway that degrades mRNAs with premature termination codons or nonsense codons, but is now recognized as a pathway that also regulates the expression of non-nonsense containing natural mRNAs. mRNAs that are targets of the pathway are rapidly degraded in yeast cells with a functional NMD pathway and stabilized in yeast cells with a non-functional NMD pathway. Thus, the half-lives of mRNAs that are direct targets of this pathway are shorter in wild-type yeast cells compared to yeast cells with a non-functional NMD pathway.

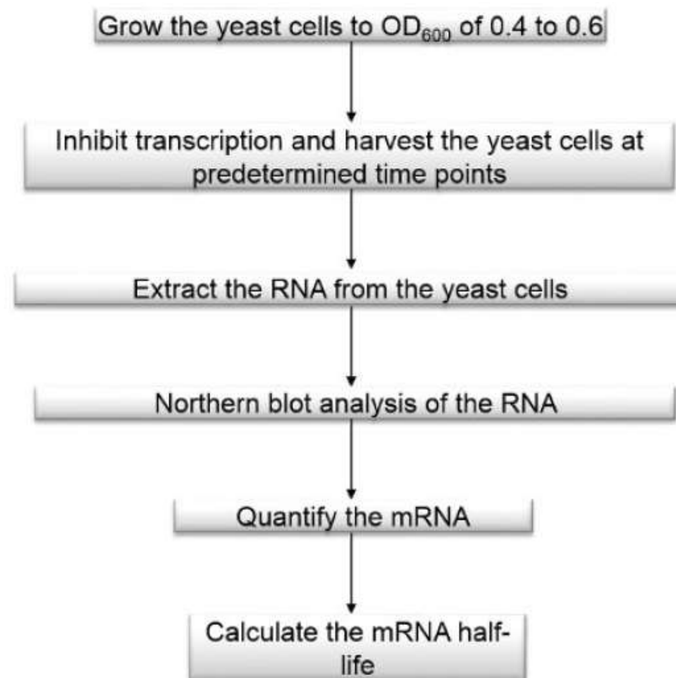

Figure 3.1: Method flowchart. Measurement of mRNA decay rate flowchart

PROTOCOL:

1. Growth of yeast cells

1.1) Select the appropriate yeast strains to be utilized for the mRNA decay rate measurements. To inhibit transcription using the temperature sensitive allele of RNA polymerase II, use yeast strains harboring the *rpb1-1* mutation [1]. Obtain this yeast strain from a laboratory that already has one, or generate it in the laboratory using standard techniques if a specific genetic background is required [9].

1.2) Using sterile technique, prepare yeast media using standard procedures [10]. If no selection is required, prepare rich media such as YPD. Alternatively, prepare selective media if the yeast cells are transformed with plasmids and selection is required. Autoclave the media.

1.3) Grow the yeast cells at 28 °C, which is the permissive temperature for this yeast strain. Do this in two steps:

1.3.1) For the first overnight, grow the yeast cells in 5 ml of growth media to saturation.

1.3.2) Set up the second overnight at the end of the second day. For the second overnight, inoculate different amounts of yeast cells from the first overnight into 100 to 150 ml of growth media (ranging from 100 µl to 1 ml, 2 ml or more, depending on when the yeast

cells need to be harvested the following day). Do this step to ensure that one of the cultures is at the correct OD_{600} the following day.

2. Harvest the yeast cells.

2.1) Prepare to harvest the yeast cells**.** The timing for this step is critical; label all the required tubes and gather all required equipment and materials in one place. Preheat a water bath to 39 °C and preheat two 15 ml test tubes of the growth media at 28 °C and at 60 °C. Note: The 15 ml growth media volume can be varied depending on the number of time points the cells are to be harvested.

2.2) Harvest the yeast cells when they reach an OD_{600} of 0.4 to 0.7. Transfer the cells to 4-6 sterile 50 ml screw cap bottles. Centrifuge at 7500 x g in a high speed centrifuge for 5 mins at room temperature.

2.3) Discard the supernatant and resuspend the pellets in the 15 ml growth media that was equilibrating at 28 °C. Pool the yeast cells in one sterile 250 ml flask and equilibrate them for ~5 mins at the 28 °C growth temperature.

2.4) After the yeast cells have equilibrated at the growth temperature, immediately add the 15 ml of the growth media equilibrated at 60 °C to raise the temperature to 39 °C (the non-permissive temperature) and immediately place the flask in the 39 °C water bath.

Make sure that the culture medium remains in the 39 °C water bath throughout the remaining cell harvesting steps to ensure that transcription is turned off.

2.5) Harvest the cells at different times after placing the flask in the 39 °C water bath. For the first time point, harvest the cells immediately after placing the flask at 39 °C. Harvest a 3 ml aliquot and distribute the 3 mls into two, 1.5 ml microcentrifuge tubes. Pellet the cells for 10 secs in a mini centrifuge or picofuge with rapid deceleration and pour out the supernatant.

2.6) Immediately freeze the pellet in a dry ice/ethanol bath or in liquid nitrogen. Designate the first time point the cells are harvested at as the 0 time point.

2.7) Experimentally determine the time points the cells are harvested at thereafter by a pilot experiment, depending on the mRNAs of interest. Normally, harvest yeast cells at the following time points: 0, 3, 6, 9, 12, 18, 25 and 35 min (Figure 3.2B). If the mRNA half-life cannot be determined using the above-mentioned time points, adjust these time points. For example, for mRNAs with anticipated short half-lives, use shorter times points, (*i.e.* 0, 1, 2, 3, 4, 6, 9, 12 and 18 min) and for mRNAs with anticipated long half-lives, extend the time points.

2.8) After the cells are harvested, store them in a -80°C freezer until RNA extraction is carried out.

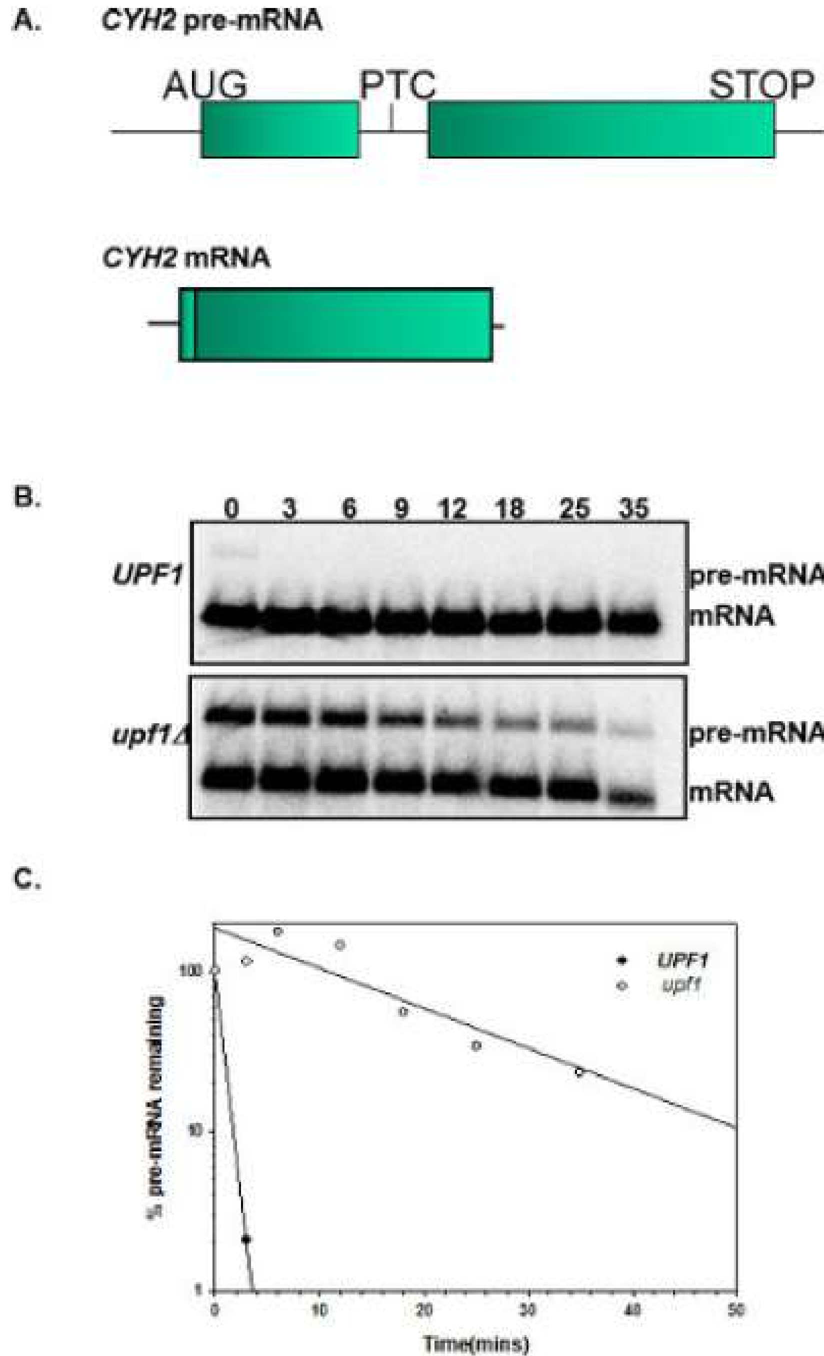

Figure 3.2: mRNA half-life of *CYH2*-pre-mRNA and mRNA. A) Schematic representation of the *CYH2* pre-mRNA and *CYH2* mRNA. *CYH2* pre-mRNA is inefficiently spliced and transported to the cytoplasm where it is degraded by the NMD pathway. The *CYH2* mRNA is not degraded by the NMD pathway because it lacks the intron containing the premature termination codon (PTC). B) Half-life northern blots of RNA extracted from wild-type (*UPF1*) and nmd mutant strains (*upf1Δ*). The time points after transcription inhibition are listed above the northern blots. The blots were probed with radiolabelled *CYH2* DNA. C) A graph of % *CYH2* pre-mRNA remaining versus time in wild-type (*UPF1*) and nmd mutant strains (*upf1Δ*). This graph shows that the *CYH2* pre-mRNA is degraded at a faster rate in wild-type (*UPF1*) than in nmd mutant yeast strains (*upf1Δ*).

3. Extract RNA from the yeast cells

3.1) For the RNA extraction portion of the protocol, use RNase free techniques to prevent degradation of the RNA by RNases. Prepare RNase free solutions, glassware and plastic ware to be used in the extraction of the RNA.

3.2) Extract the RNA from the yeast cells according to standard protocols. Typically, use the hot phenol method [11]. Alternatively, use kits that can be used to extract RNA from yeast cells.

3.3) Determine the quantity and purity of the RNA, normally by measuring the absorbance at A_{260} and A_{280} of 2μl of RNA using a Nanodrop. Determine the concentration of the RNA from the $A_{260.}$ Based on the concentration, dilute the RNA to 1 μg/μl using DEPC treated water. Note: The ratio of the A_{260}/A_{280} provides information on the purity of the RNA. An RNA sample is pure if the A_{260}/A_{280} ratio is 2.0 ± 0.1.

4. Northern blot analysis.

Use northern blots to quantify mRNA levels and obtain information on the size of the transcripts. In addition, use northern blots to detect mRNAs that produce multiple isoforms of the same mRNA.

4.1) Prepare a 1.0% agarose-formaldehyde gel. Run equal amounts of the sample RNA on the agarose formaldehyde gel by electrophoresis according to standard protocols [12].

Run a RNA ladder alongside the RNA samples and use it to determine the sizes of the RNA that are detected on the northern blot. Before transferring the RNA separated on the agarose formaldehyde gel to a membrane, cut the lane with the RNA ladder from the gel and visualize using ethidium bromide. Note: Alternatively, the whole gel can be stained with ethidium bromide before transfer of the RNA.

4.2) After the RNA samples have migrated to an appropriate distance on the gel, transfer the RNA to a membrane using RNase free techniques. Use one of several protocols available to transfer RNA to membranes [12,13]. To transfer the RNA to a membrane, cut the membrane to approximately the same size as the gel. Transfer the RNA according to standard protocols [12].

4.3) UV cross-link the RNA to the membrane using a UV crosslinker following the manufacturer's instructions. Alternatively, bake the membrane in an oven set to 80°C for 1 hour. Store the membrane at -20 °C in a plastic bag indefinitely until it is hybridized to specific probes. Note: The dry membrane can also be stored at room temperature between filter papers.

5. Hybridize probes complementary to the RNA of interest to the membrane. One way to detect mRNA on the membrane is to hybridize ^{32}P labeled DNA probes. CAUTION: Researchers working with ^{32}P need to use protective procedures to prevent contamination. Follow institutional guidelines on use of radioactive material.

5.1) Prepare the DNA probe by labeling 25-50 ng of the DNA to be hybridized to the membrane according to standard protocols [12]. Generate the DNA fragment by PCR or plasmid digestion. Determine the specific activity of the DNA probe by counting 1 µl of the radiolabelled DNA probe after the unincorporated nucleotides are removed [12].

5.2) Preheat prehybridization/hybridization buffer at 42 °C. Use ~10 ml of the prehybridization/hybridization buffer per 100 cm^2 of the membrane [12].

5.3) Hybridize 1-5 X 10^6 cpm of radiolabeled DNA probe per ml of hybridization buffer to the membrane overnight in a hybridization oven to detect the mRNA of interest [12]. Make sure that during the hybridization the hybridization oven rotation is in a direction that causes the entire membrane to be exposed to the hybridization solution, to ensure proper hybridization.

5.4) Wash the membrane two times at room temperature for 15 mins with 50 ml of 2X SSPE and one time at 65°C with 50 ml of 2X SSPE/2% SDS for 15 mins [12]. Wrap the membrane in plastic wrap to ensure that it does not dry out or contaminate the Phosphor screen.

5.5) Using a Geiger counter, estimate the intensity of the radioactivity on the membrane. Note: This estimate ensures that the membrane is exposed to the Phosphor screen for the appropriate amount of time. Use longer exposure times for membranes with low amounts of radioactivity.

5.6) For northern blots, use a loading control to confirm that equal amounts of RNA are loaded on each spot. To do this, strip the membrane and reprobe it with an RNA that is not affected by the process being examined. Strip the membrane by placing it in a glass tray containing 200 ml of boiling stripping solution (0.1% SDS/0.01 X SSC) for 2 min. Pour out the stripping solution and repeat the procedure five times [12]. Note: An example of an RNA used as a loading control is *SCR1*. *SCR1* is an RNA polymerase III transcript that is stable and abundant. *SCR1* RNA abundance should not change significantly during short periods of RNA polymerase II inhibition.

6. Quantify the RNA that is bound to the membrane. To quantify the amount of radioactivity on membrane, expose the membrane to a Phosphor screen. After the appropriate exposure time, scan the Phosphor screen using a phosophorimager.

6.1) Scan the Phosphor screen using the instructions provided by the manufacturer of the phosophorimager.

6.2) Quantify the mRNA at each time point. Quantify mRNA levels using ImageQuant software or related software. Normalize the mRNA at each time point to the loading control. If *SCR1* was used as the loading control, normalize the half-life northerns to *SCR1*.

6.3) The half-life of the mRNA is calculated by dividing the amount of mRNA remaining at each time point by the amount of mRNA present at time point 0 (the initial time point).

Graph the percent mRNA remaining versus time on a semilogarithmic plot (Figure 3.2C). Calculate mRNA half-lives by the following equation:

$$t_{1/2}=0.693/k$$

k is the slope of the best fit line and $t_{1/2}$ is the mRNA half-life.

REPRESENTATIVE RESULTS:

The ability of this protocol to accurately measure mRNA decay rates depends on inhibition of transcription, the harvesting of yeast cells at the appropriate time points, and utilization of RNase free techniques while extracting RNA and northern blotting. Probing for two control mRNAs known to be unstable and stable, respectively, provides confidence that the experiment worked. For example, this can be accomplished by probing with a probe that detects both the *CYH2* pre-mRNA and mRNA. Figure 3.2B shows the disappearance of the *CYH2* pre-mRNA in wild-type and nmd mutant yeast strains at different time points after transcription inhibition. The *CYH2* pre-mRNA is degraded faster in yeast cells with a functional NMD pathway (*UPF1*) relative to yeast cells with a non-functional NMD pathway (*upf1Δ*).

DISCUSSION:

Inhibition of mRNA synthesis and monitoring mRNA turnover in the absence of new synthesis is a method that is frequently used to measure mRNA decay rates. In *S. cerevisiae,* measurement of mRNA decay rates by inhibiting transcription using the temperature sensitive allele of RNA polymerase II is one of the most frequently used methods. This method specifically inhibits RNA polymerase II. The most critical steps for determination of mRNA decay rates using this technique are: 1) Prior to harvesting

the yeast cells, ensure that transcription has been shut off by maintaining the culture at 39°C; 2) During the RNA extraction and RNA gel electrophoresis steps of the protocol ensure that RNase free techniques are used; 3) Use a control RNA for normalization to ensure that equal amounts of RNA were loaded and that the experimental treatments are working as expected; 4) repeat the mRNA decay rate measurements at least three times to ensure reproducibility and accuracy of the half-life measurements.

rpb1-1 yeast strains are normally transferred to 37°C to inhibit transcription. However, we have found that at 37 °C inhibition of transcription occurs ~3 mins after the temperature shift. At 39 °C transcription inhibition is immediate [4,11]. However, the use of this technique to determine mRNA decay rates has some limitations. The primary limitation is that a special yeast strain is required. As previously stated, this yeast strain can either be obtained from other laboratories or generated in the laboratory if a specific yeast genetic background is required. Once the yeast strain is obtained, this technique is simple and straight forward. A second limitation is that the method entails exposing the yeast cells to heat shock to inhibit transcription. Heat stress can affect cellular processes including the decay rate of particular mRNAs. For example, the decay rate of those mRNAs that encode for proteins that are involved in stress response may be affected. Lastly, the utilization of a yeast strain with a mutation in RNA polymerase II can result in the production of alternative transcripts that behave differently from the normal transcripts.

As discussed in the introduction, other techniques are utilized to measure mRNA decay rates in *S. cerevisiae*. This includes inhibition of transcription using chemicals such as thiolutin and 1-10-Phenanthroline. These techniques are advantageous in that they can be done using any yeast strain and mRNA decay rates can also be determined genome-wide or for individual endogenous transcripts. In addition, mRNA decay rate measurements can be done in various physiological conditions. The utility of these drugs is limited by the fact that they can also affect cellular processes and influence the decay rates of mRNA differentially. Additionally, thiolutin is not readily available and when available is expensive.

After mastering this technique one will be able to determine mRNA decay rates of individual mRNAs or genome-wide. In addition, mRNA decay rates can be measured in different physiological conditions to examine whether different conditions affect mRNA decay rates differentially.

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CHAPTER FOUR

Regulation of CTR2 mRNA by the nonsense-mediated mRNA decay pathway

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Abstract

The nonsense-mediated mRNA decay (NMD) pathway was originally identified as a pathway that degrades mRNAs with premature termination codons; however, NMD is now known to regulate natural mRNAs as well. Natural mRNAs are degraded by NMD due to the presence of specific NMD targeting features. An atypically long 3'-UTR is one of the features that has been shown to induce the rapid degradation of mRNAs by NMD in *Saccharomyces cerevisiae* and other organisms. *S. cerevisiae CTR2* mRNAs have long 3'-UTRs and are sensitive to NMD, although the extent by which these long 3'-UTRs target the *CTR2* mRNAs to the pathway is unknown. Here, we investigated the sequence elements that induce NMD of the *CTR2* mRNAs and determined that the long *CTR2* 3'-UTR is sufficient to target an NMD-insensitive mRNA to the pathway. We also found that, although the *CTR2* 3'-UTR contributes to NMD-induced degradation, *CTR2* mRNAs contain additional NMD-inducing features that function cooperatively with the atypically long 3'-UTR to trigger mRNA degradation. Lengthening the *CTR2* ORF abrogates NMD and renders the mRNAs immune to the NMD pathway. Moreover, we found that transcription of *CTR2* driven by the *GPD* promoter, which is not identical to the *CTR2* promoter, affects degradation of the transcripts by NMD.

1.0 Introduction

The nonsense-mediated mRNA decay pathway (NMD) is a specialized mRNA degradation pathway that was initially identified as a pathway that degraded nonsense-containing mRNAs. NMD is now recognized as a pathway that also regulates natural mRNAs that largely code for functional proteins. mRNAs regulated by this pathway have been identified in multiple organisms including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and humans; inactivation of the NMD pathway affects 5-10% of the *S. cerevisiae* transcriptome (1, 2). A similar percentage of the *Drosophila* and human transcriptomes are also affected when NMD is inactivated (3-6). Three core trans-acting factors are required for a functional NMD pathway in *S. cerevisiae*, the up frameshift proteins, Upf1p, Upf2p, and Upf3p. Inactivation of any one of these three proteins selectively stabilizes mRNAs that are regulated by the NMD pathway.

In *S. cerevisiae,* a number of features have been shown to induce the rapid degradation of mRNAs by NMD. Several mRNAs are targeted to the pathway through translation of an upstream open reading frame (uORF) in the 5'-UTR or overlapping with the protein-coding region (1, 2, 7, 8). Other mRNAs are targeted to NMD by out-of-frame initiation of translation followed by premature translation termination, also referred to as leaky scanning, which can occur when the initiator AUG is in a suboptimal context for translation initiation (2, 9). Inefficiently spliced pre-mRNAs transported to the cytoplasm have also been found to be degraded by the pathway (2, 10). Furthermore, endogenous ribosomal frameshift signals have been reported to target mRNAs to the NMD pathway in *S. cerevisiae* (11). An additional NMD targeting feature is an atypically

long 3'-UTR; long 3'-UTRs have been shown to target *S. cerevisiae, D. melanogaster*, plant, and human mRNAs for NMD-mediated degradation (12-17). Nevertheless, the presence of an NMD inducing feature on an mRNA does not always activate NMD, and a number of mRNAs containing NMD targeting features are immune to degradation by the pathway in *S. cerevisiae* (13, 18).

In *S. cerevisiae,* mRNA 3'-UTRs tend to be short and range in size from 50-200 nts with a median size of ~121 nts. mRNAs with 3'-UTRs longer than 350 nts are likely to be regulated by the NMD pathway (13). A number of *S. cerevisiae* pre-mRNAs undergo alternative 3'-end processing, resulting in the production of multiple mRNAs with differing 3'-UTR lengths (13, 19, 20). Some of these alternatively processed mRNAs have atypically long 3'-UTRs and may be targets for NMD. *S. cerevisiae CTR2* mRNA is an example of such an mRNA; *CTR2* encodes a copper transporter of the vacuolar membrane that controls the flux of copper into the vacuole (21). Transcription of *CTR2* produces two mRNA isoforms of 900 and 2600 nt that have atypically long 3'-UTRs of 300 nt and 2000 nt, respectively, and are regulated in an NMD-dependent manner (20). The *CTR2* gene does not contain an intron and the mRNAs lack a uORF; therefore, these elements cannot be the NMD-inducing features. However, the *CTR2* mRNA isoforms may be subject to NMD due to out-of-frame initiation of translation followed by premature termination of translation because the *CTR2* initiation codon is in a suboptimal context.

Here, we investigated the features that target the *CTR2* mRNA isoforms to the NMD pathway. We show that the long *CTR2* 3'-UTR is sufficient to target an NMD-insensitive mRNA to this pathway. We also found that although the atypically long *CTR2*

3'-UTR contributes to the NMD-induced degradation of *CTR2*, an additional NMD targeting feature functions cooperatively with the long 3'-UTRs to target the mRNAs to the pathway, suggesting that the presence of multiple NMD targeting features within an mRNA may be additive. Furthermore, the lengthening of the *CTR2* open reading frame (ORF) by addition of GFP sequences eliminated NMD of the mRNA in the absence of the *CTR2* 3'-UTR. These findings demonstrate that the length of the *CTR2* ORF is important in determining whether these mRNAs are regulated by NMD due to long 3'-UTRs. Moreover, we found that while chromosomally encoded *CTR2* produces two mRNA isoforms, *CTR2* driven by the *GPD* promoter produces one *CTR2* transcript with altered sensitivity to NMD, indicating that the promoter may be an important determinant of mRNA decay.

2.0 Materials and methods

2.1, Yeast strains.

Saccharomyces cerevisiae strains used in this study and their genotypes are listed in Table 4.1. Yeast strains were grown and maintained using standard techniques (22). Plasmids containing the constructs were transformed into yeast strains using lithium acetate-mediated transformation (23).

Table 4.1. *S. cerevisiae* strains used in this study.

Strain	Genotype	Source
W303a	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	(42)
AAY320	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1-Δ2 (URA3)*	(33)
AAY334	*MATα ura3Δ his3-11,15 trp1-1 leu2-3,112 rpb1-1*	(33)
AAY335	*MATα ura3Δ his3-11,15 trp1-1 leu2-3,112 rpb1-1 upf1-Δ2 (URA3)*	(33)
AAY513	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ctr2::His3*	(20)
AAY514	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1-Δ2 (URA3) ctr2::HIS3*	(20)
AAY274	*MATα ura3-52 trp1-Δ1 his4-38 leu2-1 rpb1-1*	(33)
AAY275	*MATα ura3-52 trp1-Δ1 his4-38 leu2-1 rpb1-1 upf1::hisG*	(33)

2.2, DNA methods.

Plasmids used in this study were maintained in *E. coli* DH5α and generated as described here. *CYC1-CTR2* 3'-UTR was generated using cloning free PCR by fusing PCR products containing the *CYC1* 5' UTR and ORF to 3000 nts from the *CTR2* 3'-UTR to generate the *CYC1-CTR2* 3'-UTR fusion PCR product. The fusion DNA fragments were then inserted into the TOPO-TA cloning vector according to the manufacturer's instructions (Life Technologies, Grand Island, NY). The *CYC1-CTR2* 3'-UTR fusion PCR product was then excised from the TOPO-TA cloning vector and inserted into the high copy yeast vector pRS425 (24, 25). P_{GPD}*CTR2* was generated by modifying P_{GPD}*CTR2CYC1*, previously utilized to investigate the function of *CTR2* (21). P_{GPD}*CTR2* was generated by replacing the *CYC1* 3'-UTR in P_{GPD}*CTR2CYC1* with either 2286 or 3000 nts from the *CTR2* 3'-UTR using cloning free PCR. P_{GPD}*CTR2GFPCYC1* was

generated previously (21). All plasmids were sequenced to verify the sequences as well as to ensure that precise fusions were generated in the constructs.

2.3, RNA methods.

Yeast total RNA used to measure mRNA steady-state levels and decay rates was extracted by the hot phenol method from yeast strains harvested at mid-log phase (26). 10-15 μg of total RNA were resolved on 1.0% agarose-formaldehyde gels and transferred to GeneScreen Plus® membranes (PerkinElmer, Boston, MA) using the NorthernMax™ Complete Northern Blotting Kit (Life Technologies, Grand Island, NY). The northern blots were probed with oligo-labeled DNA probes prepared by digesting DNA fragments from plasmids or by PCR. The probe fragments used for each northern blot are listed in the figure legends. The probes were labeled with ^{32}P using an Oligolabeling Kit (Life Technologies, Grand Island, NY). The northern blots were phosphorimaged using a typhoon phosphorimager. The amount of mRNA on the northern blots was quantified using ImageQuant TL software (Amersham Pharmacia Biotech, Inc.). All mRNA levels were normalized to *SCR1* RNA; *SCR1* is an RNA polymerase III transcript that is insensitive to the NMD pathway. *CYH2* pre-mRNA was used as an NMD control. *CYH2* pre-mRNA is a target for NMD and was used to confirm the NMD phenotype of our yeast cells (10). To measure the half-lives of the mRNAs, the percent mRNA remaining for each time point was divided by the mRNA at time zero. The mRNA decay rates were then determined by graphing $\log_{10}$ of the percent mRNA remaining versus time using SigmaPlot 2000, Version 6.10 (SPSS Science, Chicago, IL).

2.4, 3′-RACE analysis.

3′-Rapid Amplification of cDNA Ends (RACE) analysis was done as described in Kebaara *et al*, 2012 (27). Specifically, 5 μg of yeast total RNA from wild-type or nmd mutants transformed with the plasmids that were used for quantitative northern analysis was also utilized to make cDNA using SuperScript™ II RT (Life Technologies, Grand Island, NY). The cDNA was then used as template DNA for the primary PCR reactions using the Abridged Universal Amplification Primer (AUAP) provided with the 3′-RACE kit and a gene-specific primer to *CYC1* (5'-TGAAAAAAGCCTGTGAGTAA-3'), *CTR2* (5'-TGGGGCAATATGGGGTAATTACA-3') or *GFP* (5'-TAGTTCTAGAGCGGGCCGCTAA-3'). After the primary PCR reactions, a nested PCR reaction was done using the initial PCR product (primary reaction) as the template, and a nested *CYC1* or *CTR2* primer. The PCR products were run on 1.5% agarose gels for visualization of the 3′-RACE PCR products.

2.5, 5′-RACE analysis.

5′ Rapid Amplification of cDNA Ends (RACE) was done as described previously (27). Briefly, 5μg of yeast total RNA extracted from wild-type (W303), *UPF1 ctr2Δ* (AAY513), *UPF1 ctr2Δ* expressing P_{GPD}*CTR2*, *UPF1 rpb1-1* (AAY334), and *UPF1 rpb1-1* (AAY334) expressing P_{GPD}*CTR2CYC1* was used to synthesize cDNA utilizing a primer that anneals to sequences 300 nt from the *CTR2* 5'-end (5'-TGGACACAGTACTTTAAGTA-3'). The cDNA was then purified using S.N.A.P column purification (Life Technologies, Grand Island, NY). Tdt tailing of the purified cDNA was done prior to PCR amplification. The cDNA was then used as template DNA using the Abridged Anchor Primer provided with the 5'-RACE kit and a gene-specific

primer to *CTR2* (5'-CTCATAGAGGTAGGCTAGA-3'). This *CTR2* gene specific primer was internal to the *CTR2* primer utilized for cDNA synthesis. After the primary PCR reactions, a nested PCR reaction was done using the initial PCR product (primary reaction) as the template, and a nested *CTR2* primer (5'CAAAAATTGCTAAACAACTT 3'). The PCR products were run on 1.5% agarose gels for visualization of the 5′-RACE PCR products.

3.0 Results and Discussion

3.1 The *CTR2* 3'-UTR is sufficient to target an NMD insensitive mRNA to NMD-mediated degradation.

We previously showed that transcription of the chromosomally encoded *CTR2* generates two mRNA isoforms of 2.6 kb and 900bp (Fig 4.1A). Both *CTR2* mRNA isoforms have atypically long 3′-UTRs of 300 nt for the shorter isoform and 2 kb for the longer isoform and are sensitive to the NMD pathway (20)Fig. 4.1A and 4.1B]. As previously stated, mRNAs with long 3'-UTRs are potential NMD targets in *S. cerevisiae* (12, 13), and these long 3′-UTRs may target the *CTR2* mRNAs to NMD. To determine the role the long 3'-UTRs play in NMD-mediated degradation of the *CTR2* mRNAs, we first determined the extent to which these *CTR2* 3'-UTRs target an NMD insensitive mRNA to the pathway. To do this, we replaced the *CYC1* 3'-UTR with 3000 nt from the *CTR2* 3'-UTR so as not to omit any regulatory sequences. The *CYC1* mRNA, which encodes for iso-1-cytochrome C, was selected because it has previously been utilized to investigate mRNA instability elements and it is insensitive to the NMD pathway (28). 3′-RACE was utilized to measure the 3′-UTR length of the *CYC1-CTR2* 3′-UTR mRNA and confirmed that the 3′-UTR of the *CYC1* mRNA was precisely replaced with the *CTR2* 3′-

UTR (Fig 4.2B). 3'-RACE analysis using cDNA from an nmd mutant yeast strain lacking the *CYC1-CTR2* 3′-UTR mRNA showed two bands, one of ~ 250nt which corresponds to the *CYC1* 3'-UTR and a nonspecific band of ~ 450 nt (Fig 4.2B lane 1). However, 3'-RACE analysis utilizing cDNA from an nmd mutant yeast strain expressing the *CYC1-CTR2* 3′-UTR mRNA from a high copy vector showed one major band of ~300nt, corresponding to the short *CTR2* 3'-UTR, and minor bands of ~450nt and 250nt (Fig 4.2B lane 2). We anticipated that the *CYC1-CTR2* 3'-UTR mRNA would be subject to NMD if the *CTR2* 3'-UTR was sufficient to target the *CYC1* mRNA for NMD.

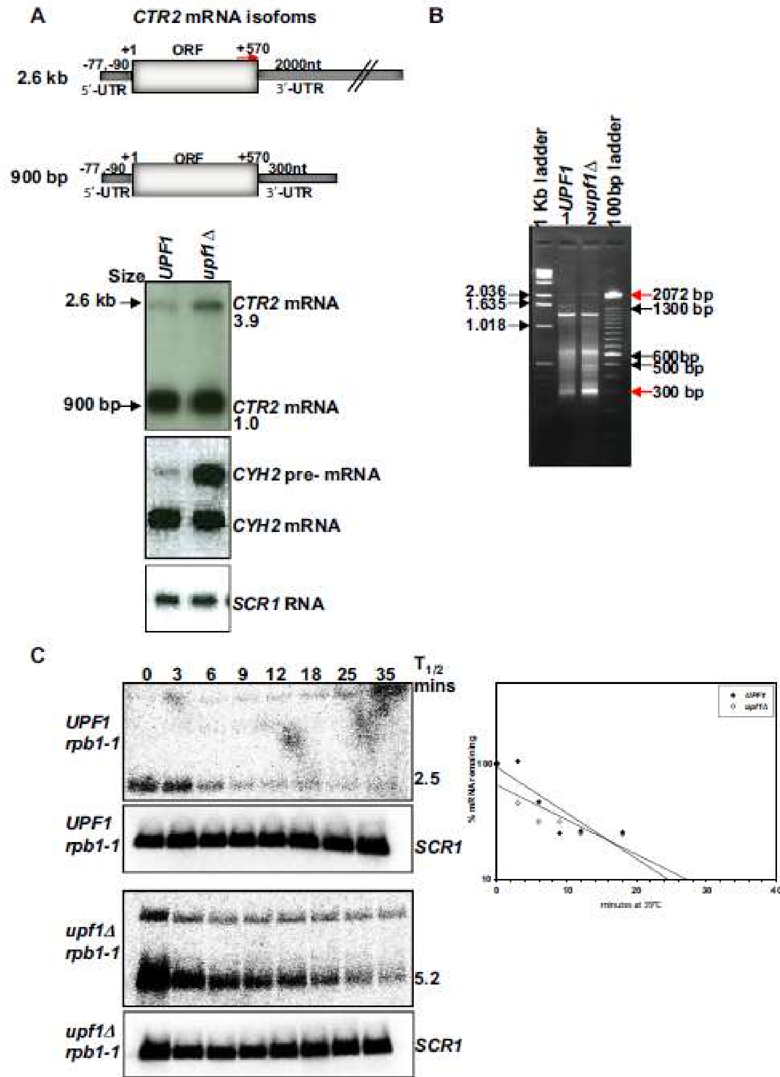

Figure 4.1: The *CTR2* mRNAs are regulated by the NMD pathway. Schematic representations of both *CTR2* mRNA isoforms are shown above the steady-state northern blot (A). The steady-state northern blots are loaded with RNA from *UPF1* (W303a (42)) and *upf1Δ* (AAY320 (26)) yeast strains (A). The northern blots were probed with radiolabeled DNA from the *CTR2* ORF (A). The relative *CTR2* mRNA levels are shown to the right of the northern blot (A). *CYH2* and *SCR1* are used as controls. *CYH2* pre-mRNA is degraded by the NMD pathway and is used to check the NMD phenotype of the yeast strains. *SCR1* is transcribed by RNA polymerase III and is used as a loading control. A 1.5% agarose gel of primary 3'-RACE PCR products from *UPF1* (W303a) and *upf1Δ* (AAY320) yeast strains (lanes labelled 1 and 2 respectively). The primer used for the 3'-RACE PCR reactions is illustrated by a red arrow on the *CTR2* mRNA schematic and is specific to the last 20 nucleotides of the *CTR2* ORF. The red arrows on the right of the agarose gel points to the ~2000 and ~300 nt 3'-RACE PCR products (B). Half-lives were measured with RNA from *UPF1 rpb1-1* (AAY334 [33]) and *upf1Δ rpb1-1* (AAY335 [33]). Time points after inhibition of transcription are listed above the northern blots and the half-lives of the short *CTR2* mRNA isoform are shown to the right of the northern blots (C). Percent mRNA remaining at each time point after inhibiting transcription was divided by the mRNA at time zero. The mRNA decay rates were then determined by graphing the $\log_{10}$ of the percent mRNA remaining versus time with SigmaPlot 2000. A representative graph showing the decay rate of the short *CTR2* mRNA isoform in *UPF1* and *upf1Δ* yeast strains is shown to the right of the half-life northern blots (C). Steady-state and half-life measurements are an average of at least three independent experiments.

The *CYC1-CTR2* 3'-UTR mRNA steady-state levels in isogenic wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast strains were measured by quantitative northern blotting. Replacement of the *CYC1* 3'-UTR with the *CTR2* 3'-UTR resulted in the production of one transcript that accumulated 1.3 ± 0.2 fold higher in the nmd mutant strain (*upf1Δ*) relative to wild-type (*UPF1*) (Fig 4.2A). Although the mRNA accumulation in the nmd mutant was not much higher than the wild-type yeast strain, the half-life measurements showed stabilization of the *CYC1-CTR2* 3'-UTR mRNA in the nmd mutant relative to wild-type (Fig 4.2C and Table 4.2). The half-life of the *CYC1-CTR2* 3'-UTR mRNA was determined by inhibiting transcription using the temperature sensitive allele of RNA polymerase II, *rpb1-1* (29). The half-life of the *CYC1-CTR2* 3'-UTR mRNA was 12 ± 3.6 min in the wild-type (*UPF1*) strain. In contrast, the half-life of the *CYC1-CTR2* 3'-UTR mRNA in the nmd mutant strain *(upf1Δ)* was biphasic with rapid decay in the first 0 to 9 min followed by a slower decay rate up to 35 min. The half-life of the transcript in the nmd mutant strain *(upf1Δ)* was 19 ± 3.7 min (Fig 4.2C and Table 4.2). These biphasic decay results suggest that when the *CYC1-CTR2* 3'-UTR mRNA in the nmd mutants reaches a specific steady-state level, the degradation rate changes. This pattern of decay is not unique to the *CYC1-CTR2* 3'-UTR mRNA and biphasic decay curves are regularly observed in NMD target mRNAs (2). These observations demonstrate that replacement of the *CYC1* 3'-UTR with the *CTR2* 3'-UTR results in the production of one NMD-sensitive transcript. Thus, the long *CTR2* 3'-UTR is sufficient to target an NMD insensitive mRNA to the NMD pathway.

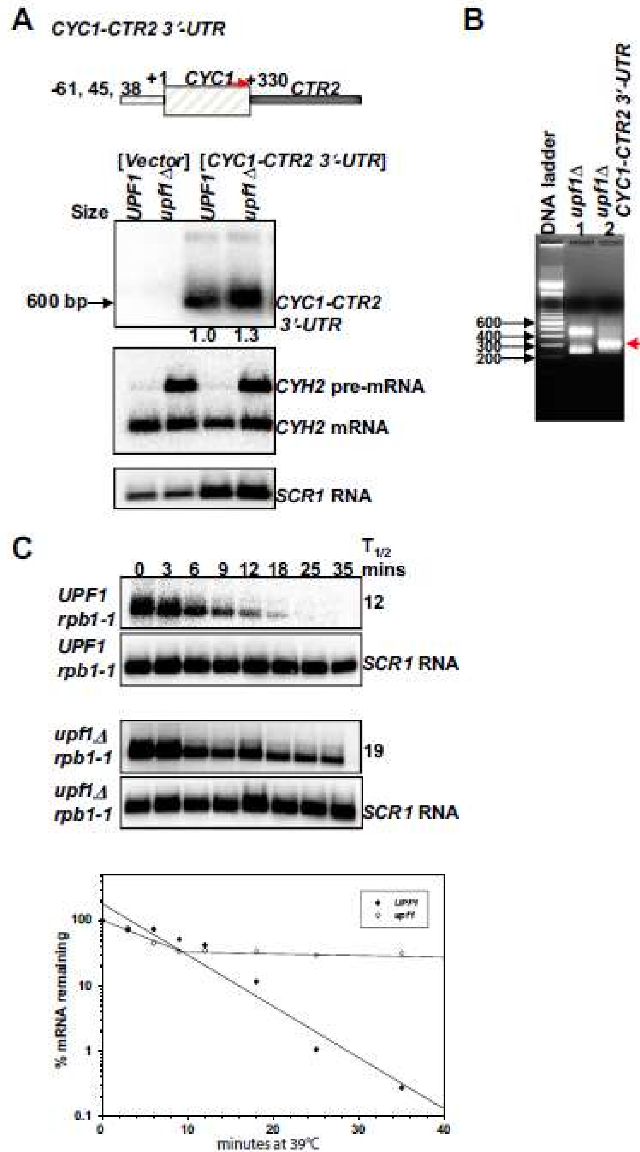

Figure 4.2: The atypically long *CTR2* 3′-UTR is sufficient to target an NMD-insensitive mRNA to the NMD pathway. Schematic representation of the *CYC1-CTR2* 3'-UTR mRNA is shown above the representative steady-state northern blots, (A). The steady-state northern blots are loaded with RNA from *UPF1* (W303a) and *upf1Δ* (AAY320) yeast strains (A). The first two lanes of the steady-state northern blot (A) are loaded with RNA from a yeast strain lacking the *CYC1-CTR2 3′*-UTR construct and transformed with pRS315 (Vector control). The northern blots were probed with radiolabeled DNA from the *CTR2* 3'-UTR (A). The relative *CYC1-CTR2* 3'-UTR mRNA levels are shown below the northern blot (A). *CYH2* and *SCR1* are used as controls as described in Figure 4.1. A 1.5% agarose gel of primary 3'-RACE PCR products from *upf1Δ* (AAY320) yeast strains lacking the *CYC1-CTR2* 3'-UTR mRNA (lane 1) and strains expressing the *CYC1-CTR2* 3'-UTR mRNA (lane 2) is shown. The primer used for the 3'-RACE PCR reactions is illustrated by a red arrow on the *CYC1-CTR2* 3'-UTR mRNA schematic and is specific to the last 20 nucleotides of the *CYC1* ORF. The red arrow on the right of the gel shows the ~300 nt PCR product in yeast cells expression the *CYC1-CTR2* 3'-UTR mRNA (B). The mRNA half-lives were measured as described in Figure 4.1 and are shown to the right of the northern blots (C). A representative graph showing *CYC1-CTR2* 3'-UTR mRNA decay in *UPF1* and *upf1Δ* yeast strains is shown below the half-life northern blots. Steady-state and half-life measurements are an average of at least three independent experiments.

In our previous studies with *CTR2* no significant difference in the steady-state accumulation of the shorter 900 bp *CTR2* transcript was detected between wild-type and nmd mutant strains (Fig 4.1A). However, we observed a significant difference in the decay of the 900 bp *CTR2* transcript between wild-type and nmd mutants (Fig 4.1C) (20). As we reported previously, the decay of the 2.6 kb *CTR2* transcript was complex in both the wild-type (*UPF1*) and the nmd mutant strain (*upf1Δ*) (20). This complex decay pattern is not due to continuing transcription of *CTR2* because expression of the shorter 900 bp *CTR2* transcript is highest at the 0 time point followed by a gradual decline (Fig 4.1C). This decay pattern could be due to stress that is induced when using the temperature-sensitive allele of RNA polymerase II to inhibit transcription. A previous study on global heat stress response in *S. cerevisiae* found that *CTR2* transcripts respond to heat stress (30). After heat stress, some mRNAs such as the 2.6 kb *CTR2* transcript are protected from NMD (20, 30). The reason for the differential protection of one, but not both *CTR2* mRNA isoforms is not clear.

Table 4.2. mRNA 3'-UTR lengths, steady-state accumulation and half-life levels.

mRNA	~3'-UTR length (nt)	Relative mRNA levels (*upf1Δ/UPF1*)	mRNA half-life (min) *UPF1*	mRNA half-life (min) *upf1Δ*
CTR2 900 bp	300	1.0chap±0.04	2.5±1.3	5.2±0.8
CTR2 2.6 kb	2000	3.9±0.8	ND	ND
CYC1CTR2 3'-UTR	300	1.3±0.2	12±3.6	19±3.7
P_{GPD}*CTR2*	350	5.3±1.1	2.3±0.6	12±1.7
P_{GPD}*CTR2CYC1*	250	2.1±0.3	12±1.0	20±5.2
P_{GPD}*CTR2GFPCYC1*	250	1.0±0.3	17.3±4.4	16.6±3.7
GPD	ND	0.9±0.2	>65	>65

The steady-state and half-life results seen with the shorter *CTR2* transcript are comparable to what we observed here for the *CYC1-CTR2* 3'-UTR transcript, confirming that only the 300 nt *CTR2* 3'-UTR was present within the *CYC1-CTR2* 3'-UTR transcript. This conclusion is supported by two observations. First, the *CYC1-CTR2* 3'-UTR transcript was ~600 nt long. If the 2000 nt *CTR2* 3'-UTR was present in the *CYC1-CTR2* 3'-UTR mRNA, the expected size of the transcript would be ~2340 nt. No transcript of ~2340 nt was visualized on the steady-state or half-life northern blots (Fig 4.2A and 4.2C). Second, 3'-RACE analysis showed a PCR product of 300 nt in the nmd mutant strain (*upf1Δ*) expressing the *CYC1-CTR2* 3'-UTR mRNA. This PCR product was absent from the same strain lacking the *CYC1-CTR2* 3'-UTR mRNA (Fig 4.2B).

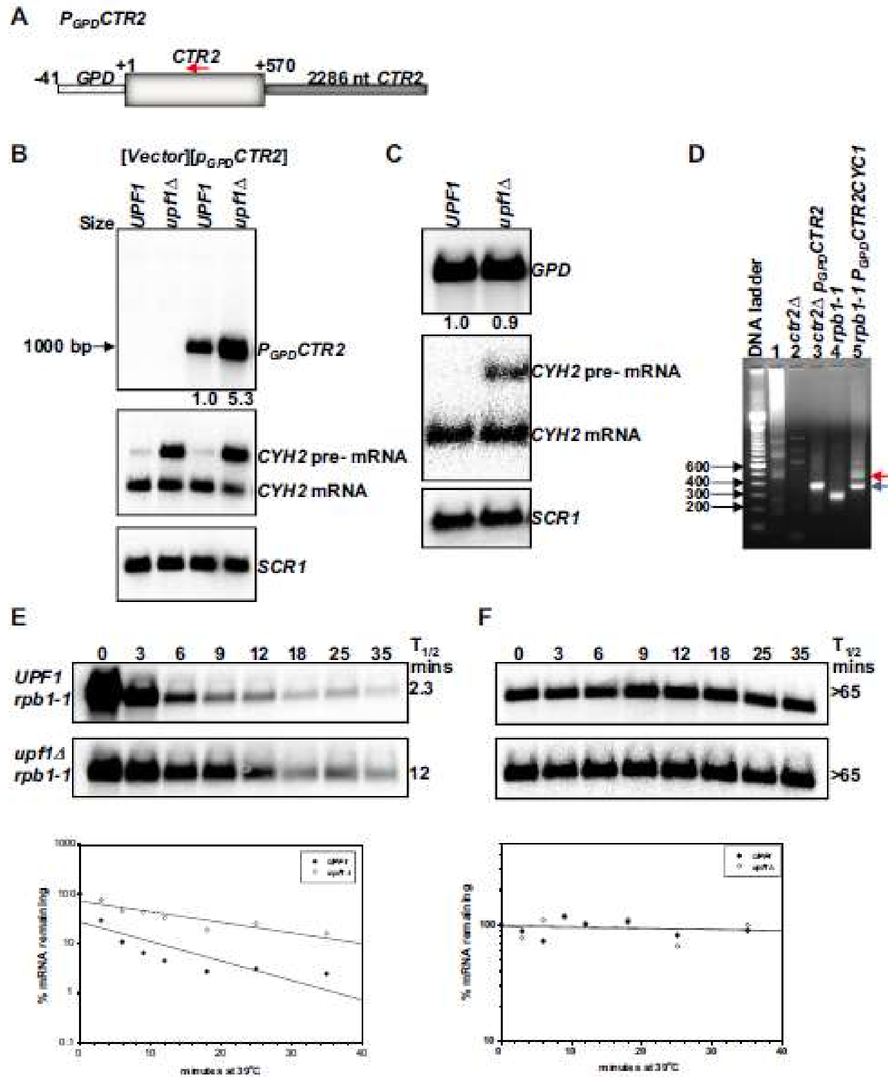

Figure 4.3. The atypically long *CTR2* 3′-UTR contributes to the NMD-mediated regulation of *CTR2*. A schematic diagram of the *P_{GPD}CTR2* mRNA is shown above the northern blot (A). *P_{GPD}CTR2* and *GPD* mRNA steady-state levels (B and C) and mRNA half-lives (E and F) in wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast strains. The first two lanes of the steady-state northern blot are loaded with RNA from the *UPF1 ctr2Δ* (AAY513, [20]) and *upf1Δ ctr2Δ* (AAY514, [20]) yeast strains lacking the *P_{GPD}CTR2* construct and are transformed with pRS315 (vector control), the last two lanes are transformed with *P_{GPD}CTR2* (B). The steady-state northern blots were probed with DNA from the *CTR2* ORF or *GPD* and the relative mRNA levels are shown below the blots. *CYH2* and *SCR1* are used as controls as described in Figure 4.1. A 1.5% agarose gel of 5'-RACE PCR products from wild-type *UPF1* (W303), *UPF1 ctr2Δ* (AAY513), *UPF1 ctr2Δ* expressing *P_{GPD}CTR2*, *UPF1 rpb1-1* (AAY334), *UPF1 rpb1-1* (AAY334) expressing *P_{GPD}CTR2CYC1*, from lanes labeled 1-5 respectively. The primer used for the 5'-RACE PCR reactions is illustrated by a red arrow on the *P_{GPD}CTR2* mRNA and anneals to sequences 300 nt from the *CTR2* 5'-end. The blue arrow on the right of the agarose gel points to the ~350 nt PCR product from yeast cells expressing the *P_{GPD}CTR2* and *P_{GPD}CTR2CYC1* (D). The red arrow points to the ~400 nt PCR product generated from yeast cells expressing *CTR2*. Half-lives were measured as described in Figure 4.1 and are shown to the right of the northern blots (E and F). Representative graphs showing *P_{GPD}CTR2* mRNA (E) and *GPD* mRNA (F) decay rates in *UPF1* and *upf1Δ* yeast strains are shown below the half-life northern blots. Steady-state and half-life measurements are an average of at least three independent experiments.

3.2 *CTR2* driven by the *GPD* promoter produces one transcript with increased NMD sensitivity.

Because the 300 nt long *CTR2* 3'-UTR is sufficient to target the NMD-insensitive *CYC1* mRNA to the NMD pathway, we evaluated the contribution that the *CTR2* 3'-UTR has on the degradation of the *CTR2* mRNAs. To do this, we modified an existing fusion construct containing the *GPD* promoter (glyceraldehyde-3-phosphate dehydrogenase), the *CTR2* open reading frame, and the *CYC1* 3'-UTR by replacing the *CYC1* 3'-UTR with 2286 nt from the *CTR2* 3'-end to generate the P_{GPD}*CTR2* fusion construct (Fig 4.3A (21)). 3′-RACE was utilized to measure the 3′-UTR length of the P_{GPD}*CTR2* and P_{GPD}*CTR2CYC1* mRNAs. 3′-RACE confirmed that the 3′-UTR of the *CYC1* mRNA was precisely replaced with the *CTR2* 3′-UTR (Fig. 4.4C). A 3'-RACE PCR product of ~350 nt was observed from yeast strains expressing the P_{GPD}*CTR2* mRNA. In contrast, a PCR product of ~250 nt was observed from strains expressing P_{GPD}*CTR2CYC1.* This 3'-UTR length corresponds to the *CYC1* 3'-UTR (Fig. 4.4C, lanes 2 and 3). Expression of the P_{GPD}*CTR2* construct was driven by the strong *GPD* promoter; thus, the P_{GPD}*CTR2* mRNA was more abundant than the endogenous *CTR2* mRNAs. 5′-RACE analysis was utilized to measure the 5′-UTR lengths of the *CTR2* mRNA and the P_{GPD}*CTR2* mRNA. 5'-RACE results showed that the endogenous *CTR2* 5′-UTR was ~ 100 nt while the *GPD* 5'-UTR was ~50 nt (Fig 4.3D). Zhang and Dietrich, (31) reported the *CTR2* 5'-UTR to be 94 nt, while Miura et al., (32) measured it to be 77 and 90 nt, which is approximately what we measured. In addition, Nagalakshmi et al., (33) determined the *GPD* 5'-UTR to be 41 nt, which is comparable to what we measured. The 5'-RACE results also confirmed

that the P_{GPD}*CTR2* mRNA was more abundant than the endogenous *CTR2* mRNA (Fig 4.3D).

Transcription of the P_{GPD}*CTR2* construct resulted in the production of a single transcript as opposed to the two transcripts previously observed from chromosomally encoded *CTR2* (Figs 4.1A, 4.3B and (20)). As previously stated, earlier studies with *CTR2* showed that the longer mRNA isoform accumulates to higher levels in the nmd mutant, while the shorter isoform does not [Fig 4.1A, Table 4.2], (20). The P_{GPD}*CTR2* transcript was ~1kb in length, comparable to the shorter, 900 bp *CTR2* mRNA isoform. However, unlike the shorter *CTR2* mRNA isoform, which did not show significant accumulation in the nmd mutant strains, P_{GPD}*CTR2* mRNA accumulated 5.3 ±1.1 (n=3) fold higher in the nmd mutants (*upf1Δ*) than in the wild-type (*UPF1*) yeast strains (Fig 4.3B and Table 4.2). Measurement of the *GPD* mRNA steady-state levels in wild-type (*UPF1*) and nmd mutants (*upf1Δ*) showed that the *GPD* mRNA does not accumulate to higher levels in nmd mutants. Furthermore, determination of the *GPD* mRNA half-life showed that the mRNA is not stabilized in nmd mutants (Fig 4.3F and Table 4.2). Utilizing the 0-35 minute time intervals we typically use to measure mRNA half-lives, the *GPD* mRNA half-lives were determined to be longer than 65 minutes in both wild-type and nmd mutants (Fig 4.3F and Table 4.2). These half-life measurements in *UPF1* strains are consistent with previous genome-wide measurements of mRNA stability that found the half-life of the of *GPD* mRNA ranges from ~59 to 60 minutes (34, 35). Thus, the *GPD* mRNA is not regulated by the NMD pathway and could not be contributing to the degradation of the P_{GPD}*CTR2* transcript by NMD (Table 4.2). Additionally, we generated an identical P_{GPD}*CTR2* construct that contained 3000 nt from the *CTR2* 3'-UTR

instead of the 2286 nt to ensure that we were not omitting any regulatory sequences. Similarly, this P_{GPD}*CTR2* construct did not encode the longer *CTR2* mRNA isoform and produced one transcript of ~1kb in length that accumulated to higher levels in nmd mutants than in wild-type strains (results not shown). If the P_{GPD}*CTR2* construct contained the longer *CTR2* 3'-UTR, the expected transcript size would be ~2.6 Kb. No transcript of that size was detected on northern blots (Fig 4.3B). These results demonstrate that the production of a single P_{GPD}*CTR2* transcript with increased NMD sensitivity was not due to the lack of *CTR2* 3'-end processing signals but was most likely due to the change in promoter and/or transcript expression level.

The half-life of P_{GPD}*CTR2* mRNA was determined to be 2.3 ± 0.6 min in the wild-type (*UPF1*) strain and 12 ± 1.7 min in the nmd mutant (*upf1Δ*) (Fig 4.3E and Table 4.2). Hence, the P_{GPD}*CTR2* mRNA is stabilized in yeast strains with a non-functional NMD pathway. Interestingly, the 2.3 min half-life of the P_{GPD}*CTR2* mRNA in the wild-type yeast strains is comparable to the half-life measured for the short *CTR2* mRNA isoform (Table 4.1), which was determined to be 2.5 min in the wild-type yeast strains (Fig 4.1C) (20). Additionally, the 12 min half-life measured for the P_{GPD}*CTR2* mRNA in nmd mutants is approximately two times longer than the half-life of the short *CTR2* mRNA isoform in nmd mutants (Table 4.2), which was determined to be 5.2 min (Fig 4.1C and Table 4.2) (20). The difference in the half-lives between this *CTR2* mRNA isoform and the P_{GPD}*CTR2* mRNA in the nmd mutants could account for the difference in steady-state accumulation levels observed between the two mRNAs. This difference could be a result of faster degradation of the shorter *CTR2* mRNA isoform in the nmd mutants compared to the P_{GPD}*CTR2* mRNA, resulting in greater accumulation of

P_{GPD}CTR2 mRNA in the mutants. The higher level of accumulation of *P_{GPD}CTR2* mRNA would result in a significant difference in the steady-state levels of *P_{GPD}CTR2* mRNA in wild-type strains relative to the nmd mutants.

These results suggest that replacing the *CTR2* promoter with the strong *GPD* promoter may affect both the processing and degradation of *CTR2* mRNA by NMD. The finding that *CTR2* driven by the *GPD* promoter results in the production of one NMD-sensitive transcript suggests that the promoter affects the processing of *P_{GPD}CTR2*. Second, the sensitivity of *CTR2* transcripts to the NMD pathway may also be affected by the promoter driving expression of that transcript. This conclusion is based on the observation that in previous studies, the short 900 bp *CTR2* mRNA isoform was stabilized in the nmd mutants (*upf1Δ*) but did not accumulate to significantly higher levels in the mutant strains (Fig 4.1A, 4.1C and Table 4.2) (20). In contrast, the 1Kb *P_{GPD}CTR2* transcript accumulated to higher levels and was also stabilized in nmd mutants (*upf1Δ*) (Fig 4.3B, 4.3E and Table 4.2). We hypothesize that replacement of the *CTR2* promoter with the *GPD* promoter may have affected 3'-end processing resulting in the loss of the longer transcript and increased accumulation of the *P_{GPD}CTR2* mRNA in the nmd mutants (Fig 4.3B).

Previous studies have shown that promoter elements can independently regulate cytoplasmic mRNA decay in yeast and mammalian cells (36-38). In mammalian cells, changing the promoter of an mRNA resulted in elevated expression of a nonsense containing β-globin mRNA (36). Here, we suggest a link between a transcript's sensitivity to the NMD pathway and the promoter that drives expression of the transcript. The *P_{GPD}CTR2* transcript was transcribed from a different promoter, the *GPD* promoter,

and displayed altered sensitivity to the NMD pathway. Thus, it appears that in *S. cerevisiae* events in the nucleus can affect the degree of transcript sensitivity to the NMD pathway. Of significance to this study was the finding that the P_{GPD}*CTR2* mRNA contained features that induce degradation of the *CTR2* mRNA by NMD. This allowed us to utilize the P_{GPD}*CTR2* construct to further evaluate the features within *CTR2* that target the mRNAs to the NMD pathway.

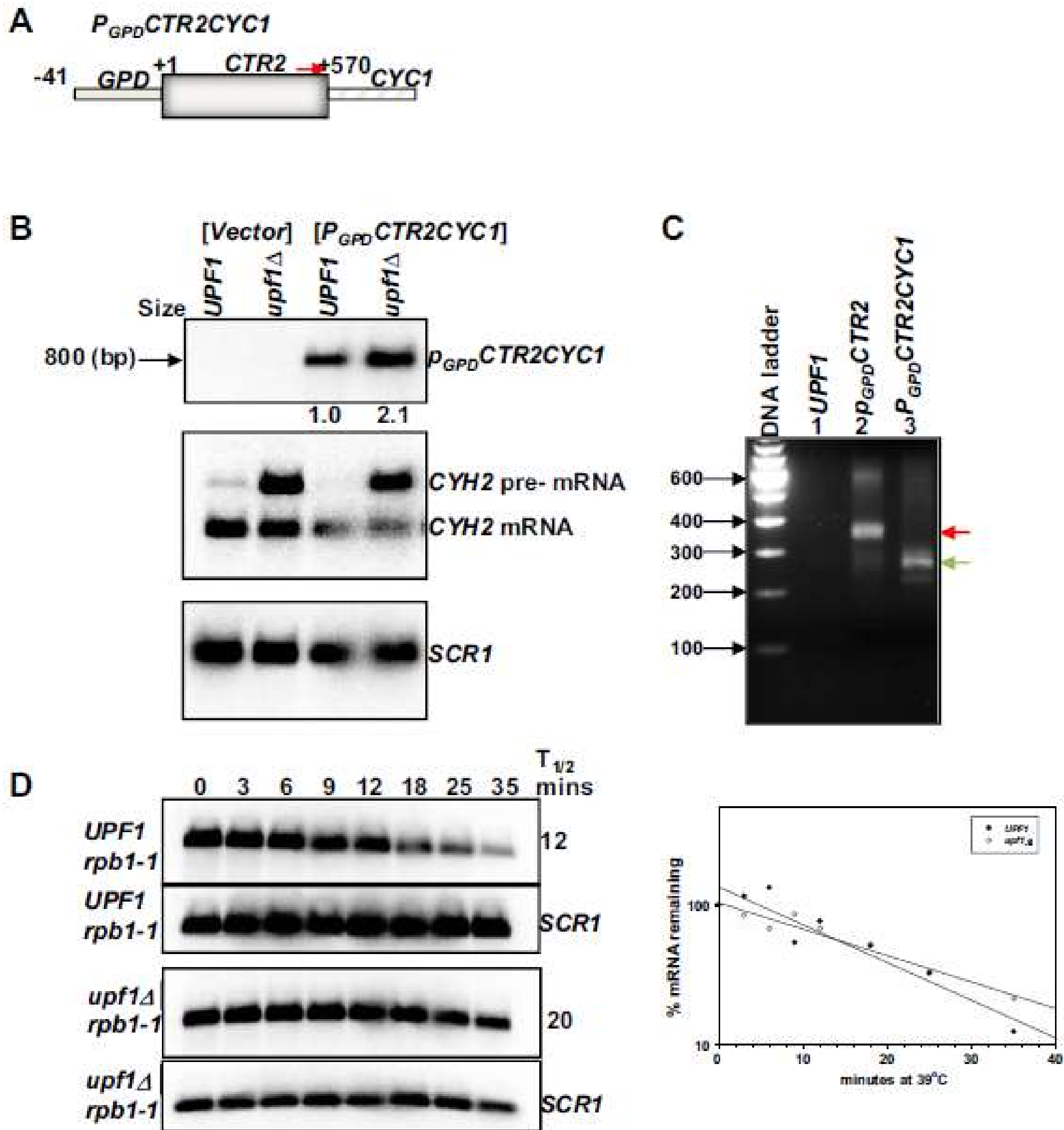

Figure 4.4. Regulation of *CTR2* mRNA by NMD is not exclusively dependent on the atypically long 3′-UTR. A schematic representation of the P_{GPD}*CTR2CYC1* mRNA (A). P_{GPD}*CTR2CYC1* steady-state levels in *UPF1 ctr2Δ* (AAY513) and *upf1Δ ctr2Δ* (AAY514) yeast strains (B). The first two lanes were loaded with RNA from the yeast strains lacking the P_{GPD}*CTR2CYC1* construct as described in Figure 4.3. The relative mRNA levels of the P_{GPD}*CTR2CYC1* mRNA in *UPF1 ctr2Δ* (AAY513) and *upf1Δ ctr2Δ* (AAY514) are shown below the steady-state northern blot. The northern blot was probed with DNA from the *CTR2* ORF, *CYH2*, and *SCR1*. *CYH2* and *SCR1* are used as controls as described in Figure 4.1. A 1.5% agarose gel of primary 3'-RACE PCR products from *UPF1* (W303) yeast strains expressing a control vector (lane 1) and strains expressing P_{GPD}*CTR2* (lane 2) or P_{GPD}*CTR2CYC1* mRNA (lane 3) is shown. The primer used for the 3'-RACE PCR reactions is illustrated by a red arrow on the P_{GPD}*CTR2CYC1* mRNA schematic and is specific to the last 20 nucleotides of the *CTR2* ORF. The red arrow on the right side of the gel shows the ~350 nt PCR product from yeast cells expressing the P_{GPD}*CTR2* mRNA (C), while the green arrow points to the ~250 nt PCR product generated from yeast strains expressing the P_{GPD}*CTR2CYC1* mRNA (C). Half-lives were measured as described in Figure 4.1 and are shown to the right of the northern blots (D). A representative graph showing P_{GPD}*CTR2CYC1* mRNA decay rates in *UPF1* and *upf1Δ* yeast strains is shown to the right of the half-life northern blots (D). Steady-state and half-life measurements are an average of at least three independent experiments.

3.3 Degradation of the *CTR2* mRNA isoforms by NMD is not exclusively dependent on the atypically long 3′-UTR.

We showed that the *CTR2* 3'-UTR was sufficient to target an NMD-insensitive mRNA to the NMD pathway and that the P_{GPD}*CTR2* transcript containing the *CTR2* 3'-UTR was also regulated by the NMD pathway. These results led us to investigate whether the long *CTR2* 3'-UTR was necessary to target the *CTR2* mRNA for NMD. We utilized the previously generated construct P_{GPD}*CTR2CYC1* for this analysis (21). This construct is identical to the P_{GPD}*CTR2* except that the *CTR2* 3′-UTR is replaced with the *CYC1* 3'-UTR, and this mRNA contains the *CTR2* stop codon (Fig 4.4A and 4.4C). As mentioned above, 3'-RACE analysis showed a 250 nt PCR product from strains expressing P_{GPD}*CTR2CYC1,* which corresponds to the *CYC1* 3'-UTR (Fig. 4.4C, lane 4.2). The *CYC1* 3'-UTR has been used previously to map instability elements and is not known to target mRNAs to the NMD pathway (12). We hypothesized that if the *CTR2* 3'-UTR is necessary to target the *CTR2* mRNAs to NMD, the P_{GPD}*CTR2CYC1* mRNA would be stabilized in wild-type yeast strains and would no longer be regulated by NMD. 5′-RACE analysis of the P_{GPD}*CTR2CYC1* mRNA results showed that the 5'-UTR was ~ 50 nt, which is identical to the P_{GPD}*CTR2* mRNA 5'-UTR (Fig. 4.3D, lanes 3 and 5).

Transcription of the P_{GPD}*CTR2CYC1* construct also resulted in the production of one transcript. We referred to this transcript as P_{GPD}*CTR2CYC1* mRNA (Fig. 4.4B). The P_{GPD}*CTR2CYC1* mRNA lacks the atypically long *CTR2* 3′-UTR, but was nevertheless subject to NMD (Fig. 4.4B). This transcript accumulated 2.1 ± 0.3 (n=3) fold higher in the nmd mutants (*upf1Δ*) relative to wild-type *(UPF1*) yeast strains (Fig. 4.4B and Table 4.2). The level of accumulation of the P_{GPD}*CTR2CYC1* mRNA was lower than that of the

P_{GPD}CTR2 mRNA, which accumulated 5.3 ±1.1 (n=3) fold higher in the nmd mutants relative to wild-type yeast strains (Table 4.2). Because the only difference between *P_{GPD}CTR2CYC1* and *P_{GPD}CTR2* mRNA was the 3'-UTR, these results demonstrate that the presence of the *CTR2* 3'-UTR contributes to the increased sensitivity of the *P_{GPD}CTR2* mRNA to NMD, and that replacement of the long *CTR2* 3'-UTR with the *CYC1* 3'-UTR results in a lower steady-state level of *P_{GPD}CTR2CYC1* mRNA in the nmd mutants relative to wild-type strains.

Half-life measurements of the *P_{GPD}CTR2CYC1* mRNA showed that the mRNA was stabilized in the nmd mutants (*upf1Δ)* relative to wild-type (*UPF1)* strains (Fig 4.4D). The half-life of the *P_{GPD}CTR2CYC1* transcript was 12 ± 1.0 min in the wild-type strain (*UPF1*) and 20 ± 5.2 min in the nmd mutants (*upf1Δ*) (Fig 4.4D and Table 4.2). The half-lives for both the wild-type and nmd mutant strains are longer than the half-lives determined for the *P_{GPD}CTR2* mRNA (Table 4.2 and compare figure 4.3E to 4.4D), demonstrating that the *CTR2* 3'-UTR on *P_{GPD}CTR2* mRNA has a general destabilizing effect. Although the *P_{GPD}CTR2* transcript was unstable in both wild-type and nmd mutant strains relative to the *P_{GPD}CTR2CYC1* transcript, the *P_{GPD}CTR2* transcript was stabilized in nmd mutants relative to wild-type yeast strains. The most significant finding from examining the *P_{GPD}CTR2CYC1* transcript was that, although the *CTR2* 3'-UTR contributes to the NMD-mediated degradation of *CTR2*, it is not the only NMD targeting feature.

The observation that replacement of the *CTR2* 3'-UTR with the *CYC1* 3'-UTR did not eliminate degradation of the *P_{GPD}CTR2CYC1* mRNA by NMD was unexpected. In previous studies, replacement of an atypically long 3'-UTR from an NMD-regulated

mRNA with a 3'-UTR from an NMD-insensitive transcript resulted in stabilization of the mRNA in wild-type yeast strains. For example, replacement of the natural *PGA1* 3'-UTR with the NMD-insensitive *ACT1* 3'-UTR resulted in expression of an NMD-insensitive *PGA1-ACT1* 3'-UTR mRNA (13). Our data show that the long *CTR2* 3′-UTR enhanced degradation of the *CTR2* transcripts, but it is not the exclusive NMD-inducing feature. Thus, an additional NMD targeting feature, or features, must be present within the *CTR2* mRNA and appears to function cooperatively with the long 3′-UTR.

As previously stated, both the P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* fusion mRNAs contain the glyceraldehyde-3-phosphate dehydrogenase (GPD) 5'-UTR (Figs 4.3A and 4.4A). However, *GPD* is not regulated by the NMD pathway and therefore would not be expected to contribute to the NMD-induced degradation of either transcript (Fig 4.3C and 4.3F). Furthermore, because both the P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* fusion mRNAs lack the *CTR2* 5'-UTR, they can no longer be subject to NMD due to leaky scanning, and neither fusion mRNA contains introns or is subject to -1 ribosomal frameshifting. These are features that have been shown to target mRNAs to the NMD pathway in *S. cerevisiae*. Therefore, neither P_{GPD}*CTR2CYC1* nor P_{GPD}*CTR2* mRNAs contain any recognizable NMD-inducing features.

3.4 The presence of *GFP* in the 3'-end of the P_{GPD}*CTR2CYC1* open reading frame abrogates NMD.

The observation that the P_{GPD}*CTR2CYC1* mRNA was sensitive to NMD lead us to hypothesize that the *CTR2* ORF contains an NMD-targeting feature or features. Two previous studies have reported a correlation between ORF length and sensitivity to NMD. A genome-wide study identifying alternatively spliced mRNA isoforms down regulated

by NMD in *D. melanogaster* found that NMD targets had shorter coding sequences (39). In addition, a recent study in *S. cerevisiae* reported that mRNAs subject to NMD due to the presence of a long 3'-UTR had short ORFs and that increasing the ORF length of these mRNAs had a stabilizing effect (40). To evaluate whether the length of the *CTR2* ORF affected degradation of *CTR2* mRNAs by NMD, we utilized a previously-generated construct that contains a modified *CTR2* ORF. The P_{GPD}*CTR2GFPCYC1* fusion is identical to the P_{GPD}*CTR2CYC1* construct except for the addition of the *GFP* open reading frame immediately upstream of the *CTR2* stop codon (21), adding 753 nucleotides to the *CTR2* ORF and more than doubling the length (Fig 4.5A). 3′-RACE analysis of the P_{GPD}*CTR2GFPCYC1* mRNA results showed that the 3'-UTR was ~250 nt, which corresponds to the *CYC1* 3'-UTR. This ~250 nt PCR product was absent from a yeast strain lacking the P_{GPD}*CTR2GFPCYC1* mRNA (Fig. 4.5B, lanes 4.2). If the shorter *CTR2* ORF is associated with sensitivity to the NMD pathway, we anticipated that lengthening of the *CTR2* ORF would stabilize the transcript in wild-type yeast strains.

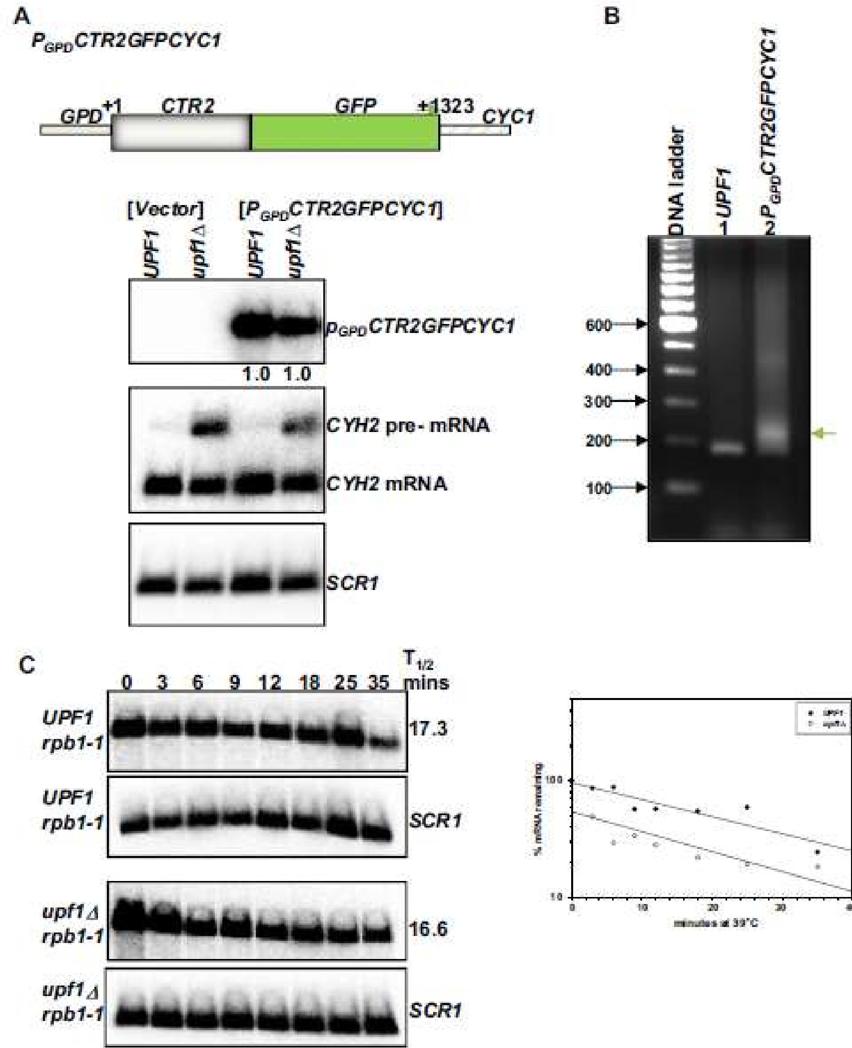

Figure 4.5. Lengthening the *CTR2* open reading frame abrogates NMD of the P_{GPD}*CTR2GFPCYC1* transcript.

Schematic representation of the P_{GPD}*CTR2GFPCYC1* mRNA and representative steady-state northern blots of total RNA extracted from *UPF1 ctr2Δ* (AAY513) and *upf1Δ ctr2Δ* (AAY514) yeast strains. The first two lanes were loaded with RNA from strains lacking the P_{GPD}*CTR2GFPCYC1* construct and transformed with a control vector as described in Figure 4.3. The northern blot was probed with DNA from the *CTR2* ORF, *CYH2* and *SCR1* as described in Figure 4.1. The P_{GPD}*CTR2GFPCYC1* mRNA fold change levels are shown below the northern blot (A). A 1.5% agarose gel of nested 3'-RACE PCR products from *UPF1* (W303) yeast strains lacking the P_{GPD}*CTR2GFPCYC1* mRNA (B, lane 1) and strains expressing the P_{GPD}*CTR2GFPCYC1* mRNA (B, lane 2). The primer used for the 3'-RACE PCR reactions is illustrated by a green arrow on the P_{GPD}*CTR2GFPCYC1* mRNA schematic and is specific to the last 20 nucleotides of the *GFP* ORF. The green arrow on the right side of the gel shows the ~250 nt PCR product in yeast cells expressing the P_{GPD}*CTR2GFPCYC1* mRNA (B). Half-lives were measured with RNA from *UPF1 rpb1-1* (AAY274 (26)) and *upf1Δ rpb1-1* (AAY275 (26)) transformed with the P_{GPD}*CTR2GFPCYC1* construct as described in Figure 4.1 and are shown to the right of the northern blots (C). A representative graph showing P_{GPD}*CTR2GFPCYC1* mRNA decay rates in *UPF1* and *upf1Δ* yeast strains is shown to the right of the half-life northern blots (C). The steady-state and half-life measurements are an average of at least three independent experiments, except for the half-life in the *UPF1* strain which is an average of two independent experiments.

Northern blot analysis of the *P_{GPD}CTR2GFPCYC1* mRNA showed that the *P_{GPD}CTR2GFPCYC1* construct generated one mRNA species that did not accumulate to higher levels in nmd mutants (*upf1Δ*) relative to wild-type (*UPF1*) yeast strains (Fig. 4.5A). The mRNA accumulated 1.0 ± 0.3 (n=3) fold in nmd mutants (*upf1Δ*) relative to wild-type *(UPF1*) strains (Fig 4.5A and Table 4.2). The reason the *P_{GPD}CTR2GFPCYC1* mRNA did not accumulate to higher levels in the nmd mutant strain is unlikely to be due to stabilizing effects of the *GFP* ORF itself. This conclusion is based on a previous study that showed that a *GFP* reporter comprising the *GFP* ORF followed by the hammerhead ribozyme and a premature termination codon was degraded by the NMD pathway, demonstrating that an mRNA containing the *GFP* ORF was regulated by NMD (41). However, it is possible that the *GFP* ORF could have a stabilizing effect on the *CTR2* ORF in this context. Overall, these results demonstrate that lengthening the *CTR2* ORF results in loss of steady-state accumulation of the mRNA in nmd mutants.

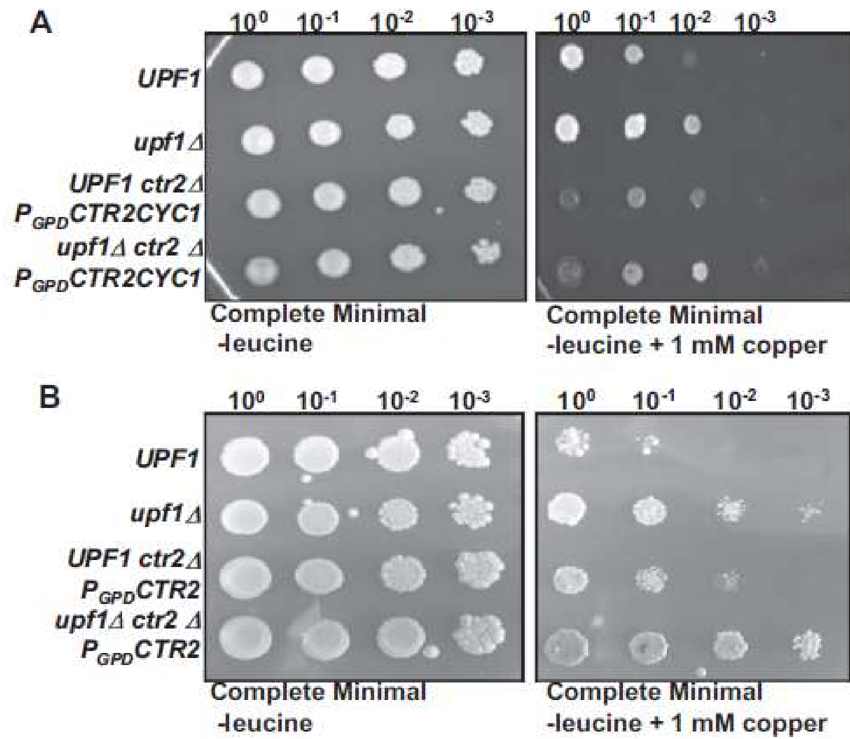

Figure 4.6. Products of the P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* constructs can confer copper tolerance to nmd mutant strains lacking *CTR2*.
UPF1 (W303) and *upf1Δ* (AAY320) yeast strains were transformed with pRS315 (leucine vector), and *UPF1 ctr2Δ* (AAY513) and *upf1Δ ctr2Δ* (AAY514) yeast strains were transformed with P_{GPD}*CTR2CYC1* or P_{GPD}*CTR2.* Tenfold serial dilutions of the transformants were spotted onto complete minimal medium lacking leucine or complete minimal medium lacking leucine and containing 1 mM copper (right panel) (A and B). The plates were incubated at 30°C for 4-5 days before photographing.

Measurement of the P_{GPD}*CTR2GFPCYC1* mRNA half-life showed that the mRNA was degraded at comparable rates in the nmd mutants (*upf1Δ)* relative to wild-type (*UPF1)* strains (Fig. 4.5C). The half-life of the P_{GPD}*CTR2GFPCYC1* transcript was 17.3 ± 4.4 min in the wild-type strain (*UPF1*) and was 16.6 ± 3.7 min in the nmd mutants (*upf1Δ*) (Fig. 4.5C and Table 4.2). These results are in contrast to what we observed for both the short *CTR2* mRNA isoform (Fig. 4.1A and 4.1C) and the *CYC1-CTR2* 3'-UTR transcript (Fig. 4.2A and 4.2C). For both transcripts there was no detectable difference in mRNA steady-state accumulation levels in wild-type and nmd mutant strains, but the transcripts were stabilized in nmd mutants (Fig. 4.7). This pattern of steady-state mRNA

accumulation and decay is not typically observed with mRNAs that are targets of the NMD pathway. Typically, NMD targets accumulate to higher levels and are also stabilized in nmd mutants as was observed with the P_{GPD}*CTR2* and P_{GPD}*CTR2CYC1* mRNAs (1, 2).

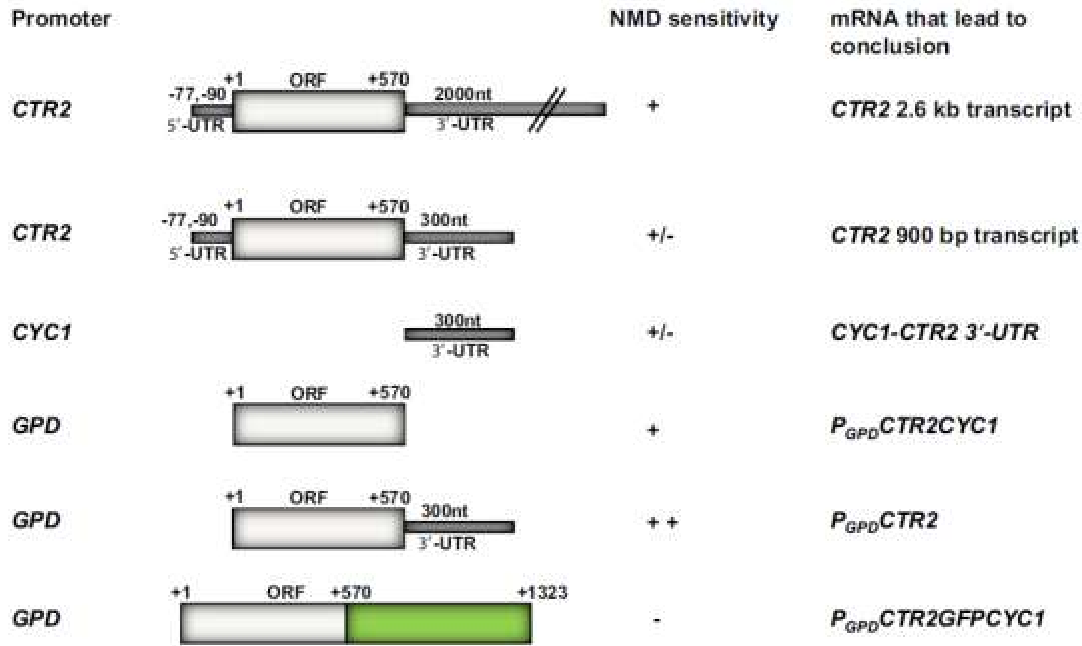

Figure 4.7. Schematic summary of the *CTR2* mRNA analysis.
A schematic representation of the fragments of the *CTR2* mRNAs that are found in each of the fusion mRNAs, the promoter driving the expression of each mRNA, and the mRNA that lead to the conclusion on the level of NMD sensitivity is shown. NMD sensitivity of + indicates that the mRNA containing that fragment of *CTR2* was sensitive to NMD. ++ indicates the mRNA containing that fragment of *CTR2* had elevated sensitivity to NMD. +/- indicates that the fragment of *CTR2* was sensitive to NMD in half-life measurements but there was no detectable difference in mRNA steady-state accumulation levels in wild-type and nmd mutants. – indicates that the mRNA was insensitive to the NMD pathway. The numbers on the schematic images are based on the *CTR2* ORF start point at +1.

The finding that the P_{GPD}*CTR2GFPCYC1* is insensitive to the NMD pathway suggests that ORF length in addition to 3'-UTR length can regulate the sensitivity of a natural mRNA to NMD, consistent with the report by Decourty et al., 2014 (40). In our previous study examining natural mRNAs with atypically long 3'-UTRs that are sensitive to NMD, we found one mRNA out of eleven with long 3'-UTRs that was insensitive to

the NMD pathway (13). This particular mRNA had the longest ORF, suggesting that ORF length may also play a role in the escape of this mRNA from decay by the NMD pathway.

In *S. cerevisiae*, it appears that when the 3'-UTR length is the NMD-targeting feature, the ORF length is also important. If ORF length is an important determinant of sensitivity of an mRNA to the NMD pathway, then the efficiency with which an mRNA is translated may play a role in its susceptibility to NMD. This suggestion is based on the fact that NMD is dependent upon ongoing translation.

3.5 Both the P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* constructs can confer copper tolerance to nmd mutant strains lacking *CTR2*.

Our previous studies showed that regulation of *CTR2* mRNA by NMD is physiologically significant. We found nmd mutants to be more tolerant of toxic copper levels (20), a phenotype dependent on the presence of the *CTR2* gene. Deletion of *CTR2* resulted in a similar copper tolerance phenotype for both wild-type and nmd mutant strains. To determine the extent to which the products of both the P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* constructs, Ctr2p, conferred copper tolerance to an nmd mutant lacking *CTR2* (*upf1Δctr2Δ*), P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* constructs were transformed into *UPF1ctr2Δ* and *upf1Δctr2Δ* yeast strains. Transformants were spotted onto either complete minimal medium lacking leucine or complete minimal medium lacking leucine and containing 1mM copper. As observed with *CTR2*, the transformation of P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* into *UPF1ctr2Δ* and *upf1Δctr2Δ* yeast strains produced yeast strains with an increased copper tolerance phenotype (Fig 6A and 6B), a phenotype previously observed with nmd mutant strains with a functional Ctr2p (20). Thus, both

P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* expressed a functional Ctr2p. This observation supports the conclusion that the long *CTR2* mRNA isoform is not essential for the tolerance to toxic copper of nmd mutants.

4.0 Conclusions

In summary, the results presented here demonstrate that the regulation of the *CTR2* mRNAs by the NMD pathway is complex and depends on the length of both the 3'-UTR and the ORF (Fig. 4.7 and Table 4.2). The 300 nt *CTR2* 3'-UTR is sufficient to target an NMD-insensitive mRNA to the pathway, and the 570 nt size of the *CTR2* ORF is required for the mRNAs to be regulated by NMD in the context of the *GPD* promoter. Furthermore, both mRNA features appear to determine the degree to which the *CTR2* mRNAs are sensitive to the NMD pathway (Fig. 4.7). It appears that both the *CTR2* 3'-UTR and ORF length function cooperatively to regulate degradation of the mRNAs by the NMD pathway. This conclusion is based on the observation that replacement of the *CTR2* 3'-UTR partially stabilizes P_{GPD}*CTR2CYC1* mRNA, and lengthening the *CTR2* ORF fully stabilizes the mRNA and renders it insensitive to NMD (Table 4.2 and Fig 4.7). Previous studies on NMD instability elements have identified individual NMD-inducing features that elicit degradation of natural mRNAs. This study provides insights into how multiple features within mRNAs can affect the regulation of specific mRNAs by this pathway. We postulate that multiple features within an mRNA can function cooperatively to control whether an mRNA is regulated by the NMD pathway and to what extent the mRNA is sensitive to the pathway. Specifically, mRNAs with multiple NMD-inducing features would be likely to have elevated sensitivity to NMD.

Furthermore, we found that mRNA sensitivity to the NMD pathway could also be affected by its promoter. Expression of the *CTR2* mRNA driven by the *GPD* promoter resulted in the expression of one transcript and affected both the processing and the NMD-mediated regulation of *CTR2* mRNA. The highly-expressed P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* transcripts showed increased sensitivity to the NMD pathway relative to the endogenous *CTR2* transcript of comparable size (Table 4.2). The basis for this increased sensitivity to NMD is not completely understood. One explanation could be the elevated expression of the transcripts and a second explanation could be that the *GPD* 5'-UTR may have a stabilizing effect on the transcripts. Further investigations on the role promoter elements play in the regulation of mRNA by NMD will provide insights into the mechanism.

The protein products of P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* were found to restore the copper tolerance phenotype of nmd mutants. This copper tolerance phenotype depends on the presence of a functional Ctr2p. This demonstrates that the expression of both *CTR2* mRNA isoforms is not essential for the copper tolerance phenotype of the nmd mutant and suggests that the product of the *CTR2* ORF is sufficient to restore copper tolerance in this strain. In conclusion, further studies investigating the role promoter elements and ORF length plays in degradation of natural NMD targets will enhance our understanding of how the NMD pathway regulates natural mRNAs and subsequently gene expression.

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CHAPTER FIVE

mRNAs Involved in Copper Homeostasis are Regulated by the Nonsense-mediated mRNA Decay Pathway Depending on Environmental Conditions.

ABSTRACT

The nonsense-mediated mRNA decay pathway (NMD) is an mRNA degradation pathway that degrades mRNAs that prematurely terminate translation. These mRNAs include mRNAs with premature termination codons as well as many natural mRNAs. In *Saccharomyces cerevisiae*, a number of features have been shown to target natural mRNAs to NMD. However, the extent to which natural mRNAs from the same functional group are regulated by NMD, and how environmental conditions influence this regulation, is not widely known. Here, we examined mRNAs involved in copper homeostasis and are predicted to be sensitive to NMD. We found that the majority of these mRNAs have long 3'-UTRs that could target them for degradation by NMD. Analysis of one of these mRNAs, *COX19,* found that the long 3'-UTR contributes to NMD-mediated degradation of *COX19*. Furthermore, we examined an additional mRNA, *MAC1,* under low copper conditions. We found that low copper growth conditions affect 3'-UTR length and NMD sensitivity of the *MAC1* mRNA, demonstrating that sensitivity to NMD can be altered by environmental conditions. *MAC1* is a copper sensitive transcription factor that regulates genes involved with high affinity copper transport. Our results expand our understanding on how NMD regulates mRNAs from the same functional group and how the environment influences this regulation.

Keywords: *Saccharomyces cerevisiae*; mRNA; mRNA decay; copper; copper homeostasis; Nonsense-mediated mRNA decay

1.1 INTRODUCTION

The nonsense-mediated mRNA decay pathway (NMD) is an mRNA degradation pathway that degrades select mRNAs. Specifically, NMD degrades mRNAs that prematurely terminate translation due to premature termination codons (PTC) or other features. PTCs can arise as a result of improper pre-mRNA processing or random mutations. NMD also has an additional role and has been shown to degrade many natural transcripts (Deliz-Aguirre et al., 2011; Guan et al., 2006; He et al., 2003; Kebaara and Atkin, 2009; Lelivelt and Culbertson, 1999). The extent and reason for the targeting of most of these natural mRNAs is generally unknown.

The NMD pathway is highly conserved in all tested eukaryotes from yeast to humans, and serves as an important regulator of gene expression and cellular function. In *S. cerevisiae* nmd mutants, 5-10% of the transcriptome is affected (Guan et al., 2006; He et al., 2003). Further research in the fruit fly *Drosophila melanogaster* and humans indicates that similar percentages of the respective transcriptomes are affected when NMD is inactivated (Mendell et al., 2004; Rehwinkel et al., 2005; Wittmann et al., 2006). Three core trans-acting factors are required for a functional NMD pathway in all eukaryotes. These are the up-frameshift proteins Upf1p, Upf2p and Upf3p. Inactivation of any one of these three proteins selectively stabilizes mRNAs that are regulated by the pathway.

NMD targets can be regulated by the pathway directly or indirectly. Direct NMD targets have significantly altered decay rates in cells with a functional NMD pathway

compared to their decay rates in cells with a nonfunctional NMD pathway. On the other hand, indirect NMD targets have similar decay rates in cells with a functional or non-functional NMD pathway. Initially, it was perceived that all direct targets of the NMD pathway contain a PTC. However, with the ever increasing number of natural mRNAs that are degraded by the pathway, it is apparent that other targeting mechanisms exist. Previous studies in *S. cerevisiae* have identified features that target natural mRNAs to the pathway. These features include atypically long 3'-untranslated regions (UTRs), a translated upstream open reading frame (uORF), -1 ribosomal frameshifts (into an alternative reading frame), inefficiently spliced pre-mRNAs, and mRNAs subject to leaky ribosomal scanning (Parker, 2012; Peccarelli and Kebaara, 2014; Schweingruber et al., 2013). One specific targeting mechanism of interest is the presence of a long 3'-UTR on an mRNA (Deliz-Aguirre et al., 2011; Kebaara and Atkin, 2009; Rebbapragada and Lykke-Andersen, 2009). In *S. cerevisiae*, mRNA 3'-UTRs are fairly short and typically range in size from 50-200 nts with a median size of ~121 nts(Graber et al., 2002). In general, mRNAs with 3'-UTRs that are 350 nt or longer are considered atypically long and are likely to be regulated by NMD(Deliz-Aguirre et al., 2011; Kebaara and Atkin, 2009).

Interestingly enough, some mRNAs produce different isoforms of the same mRNA that vary in their 3'-UTR lengths. Transcripts that vary in the 3'-UTR length can be produced as a result of alternative 3'-end processing. Additionally, mRNA 3'-end processing is sometimes sensitive to growth conditions, and differing conditions can produce mRNA isoforms of different lengths (Kim Guisbert et al., 2007). Thus, genes that produce multiple transcripts that vary in their 3'-UTR lengths may generate one form

of the transcript that is degraded by NMD while the other may not be regulated by the pathway.

NMD may regulate gene expression by controlling mRNA levels in response to environmental stimuli and stressors. Many different stressors exist including copper (Hodgins-Davis et al., 2012). Copper is a micro-nutrient that is essential for a number of cellular functions (Deliz-Aguirre et al., 2011; Hodgins-Davis et al., 2012). Specifically, copper serves as a cofactor in superoxide anion detoxification, iron metabolism, and mitochondrial oxidative phosphorylation. Even though copper is required for normal cellular function, copper in its free form is extremely toxic to the cell. Thus, the copper concentration inside of the cell must remain around one free copper molecule per individual cell to avoid toxicity (Hodgins-Davis et al., 2012). In order to avoid cell death due to copper induced toxicity, cells have developed a number of mechanisms to maintain copper homeostasis. These mechanisms include copper compartmentalization and sequestration. Specific proteins involved in these mechanisms have been identified; however, the actual mechanisms involved in copper homeostasis are still under investigation (Deliz-Aguirre et al., 2011). Interestingly enough, yeast cells with an inactive NMD pathway are more tolerant to high copper levels. Further analysis of this copper tolerance phenotype revealed that nmd mutants tolerate toxic copper levels better than wild-type yeast strains, partly due to the sequestration of copper in the vacuole (Deliz-Aguirre et al., 2011; Wang et al., 2013).

It is important to understand how NMD regulates natural mRNAs involved in specific cellular processes and how this regulation is altered in changing environmental conditions. Here, we used copper homeostasis in *S. cerevisiae* as a model. Global

expression profiling studies identified *CTR2, CTR3, MAC1, COX23, CRS5, PCA1, FRE2*, and *COX19* mRNAs as potential NMD targets (Table 5.1) (Guan et al., 2006; Johansson et al., 2007). The proteins encoded by these eight mRNAs are involved in various aspects of copper homeostasis. Here, we examined the regulation of these mRNAs by the NMD pathway. The majority of these mRNAs (*MAC1, PCA1, CTR2, COX23, COX19*, and *FRE2*) were found to have atypically long 3'-UTRs that could contribute to their degradation by NMD (Graber et al., 2002; Peccarelli et al., 2014). This study expands our understanding of how NMD regulates mRNAs from the same functional group, and also demonstrates that the presence of multiple NMD inducing features may increase the sensitivity of mRNAs to the pathway. Lastly we found that low copper growth conditions lead to alternative forms of the *MAC1* mRNA that vary in the length of the 3'-UTR. These alternative forms of the mRNA have altered sensitivity to NMD. This result demonstrates that environmental conditions can influence alternative 3'-end processing, and also NMD sensitivity of specific mRNAs.

Table 5.1. Genes involved in copper homeostasis in *S. cerevisiae* and regulated in an NMD-dependent manner. The potential NMD targeting features are shown. Bold genes encode for mRNAs that may be potentially regulated by NMD through multiple features. The green highlighted gene *(CRS5)* has no identifiable NMD targeting feature. The tan highlighted column represents experimentally determined 3'-UTR lengths.

Gene name	Function	[a] uORF	[b] -1 PRF	[c] 3'-UTR nt (predicted)	[d] 3'-UTR nt (3' RACE)	[e] Leaky scanning
CRS5	Copper-binding metallothionein	-	-	79	75	-
COX23	Mitochondrial intermembrane space protein that functions in mitochondrial copper homeostasis	-	-	184, 2222	300	-
PCA1	Cadmium transporting P-type ATPase; may also have a role in copper and iron homeostasis	-	+ (882)	86	200, 650	-
MAC1	Copper-sensing transcription factor involved in regulation of genes required for high affinity copper transport	-	-	2143	300	-
CTR2	Copper transporter of the vacuolar membrane	-	-	2203	300, 2000*	+
FRE2	Ferric and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters	+	+ (288)	1440	700, 1400	-
COX19	Protein required for cytochrome c oxidase assembly	-	-	484	650	+
CTR3	High-affinity copper transporter of the plasma membrane	-	-	2367	ND	-

*) 3' RACE measured 3'-UTR length of the *CTR2* mRNA was previously reported (Peccarelli et al., 2014).
[a] Presence or absence of an upstream open reading frame (uORF)
[b] mRNA maybe subject to -1 Programmed Ribosomal Frameshifting, (Belew et al., 2011).
[c] 3′-UTR lengths predicted using the 3′-end processing site predictor, (Graber et al., 2002).
[d] 3'-UTR lengths determined by 3' RACE.
[e] Start codon is in a suboptimal context, mRNA may be targeted to NMD through leaky scanning.

1.2 MATERIALS AND METHODS

1.2.1 Yeast Strains

The strains and genotypes of *Saccharomyces cerevisiae* used in this study are listed in Table 5.2. Standard techniques were used to grow and maintain all of the yeast strains (Ausubel et al., 1998).

Table 5.2. *Saccharomyces cerevisiae* strains used in this study.

Yeast Strain	Genotype	Source
W303	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-101*	(Wente et al., 1992)
AAY320	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1::URA3 (upf1-Δ2)*	(Kebaara et al., 2003)
AAY334	*a, ADE2, ura3-1 or ura3-52, his3-52, his3-11,15; trp1-1, leu2-3,112, rpb1-1*	(Kebaara et al., 2003)
AAY335	*a, ADE2, ura3-1 or ura3-52, his3-52, his3-11,15; trp1-1, leu2-3,112, rpb1-1, upf1-Δ2 (URA3)*	(Kebaara et al., 2003)

1.2.2 DNA methods

Plasmids used in this study were maintained in *E. coli* DH5α. *CYC1-COX19 3'UTR* fusion construct was generated using cloning free PCR by fusing PCR products containing the *CYC1* 5'-UTR and ORF to 700 nts from the *COX19 3'-UTR*. *COX19-CYC1 3'UTR* fusion construct was generated by fusing PCR products containing the *COX19* 5'-UTR and ORF to 350 nts from the *CYC1* 3'-UTR. The *CYC1-COX19 3'UTR* and *COX19-CYC1 3'UTR* fusion DNA products were inserted into TOPO-TA cloning vector according to the manufacturer's instructions. *CYC1-COX19* 3'-UTR was digested with *BamHI* and *NotI* before ligation to the yeast vector pRS425. *COX19-CYC1* 3'-UTR was digested with *BamHI* and *XhoI* before ligation into pRS425.

1.2.3 RNA methods

Yeast total RNA was used for accumulation and half-life northerns. The total RNA was extracted using the hot phenol method from yeast strains harvested at mid-log phase (Kebaara et al., 2003). Equivalent concentrations of the total RNA (15μg) were run on a

1.0% agarose-formaldehyde gel for both accumulation and half-life northerns. The RNA was then transferred to a GeneScreen Plus® (PerkinElmer, Boston, MA) nitrocellulose membrane. Transfers were done using the capillary blot transfer protocol from the NorthernMax™ Complete Northern Blotting kit (Life Technologies, Grand Island, NY). All northern blots were probed with oligolabeled DNA probes. Each probe was prepared by PCR or through the digestion of DNA fragments from plasmids. The oligolabeled DNA probe fragments were labeled with [α-^{32}P] dCTP using the RadPrime DNA Labeling System (Life Technologies, Grand Island, NY). The northern blots were phosphorImaged™ using a Typhoon Phosphorimager (Amersham Pharmacia Biotech, Inc.). *SCR1* RNA was used as a control to normalize all mRNA levels. *SCR1* is an RNA polymerase III transcript and is NMD insensitive. *CYH2* pre-mRNA was also used as a control. *CYH2* pre-mRNA is a known target for NMD-mediated degradation and was used to confirm the NMD phenotype of the yeast strains (He et al., 1993). Northerns were quantified using ImageQuant software. Sigmaplot 2000, Version 6.10 software was used to calculate half-lives by graphing $\log_{10}$ of the percent mRNA remaining versus time (SPSS Science, Chicago, IL).

1.2.4 3'RACE

3' RACE was performed using the 3' RACE System for Rapid Amplification of cDNA Ends kit (Life Technologies, Grand Island, NY) and was done as described in Kebaara *et al*., 2012 (Kebaara et al., 2012). In detail, 5 µg of yeast total RNA from wild-type or nmd mutants was used to make cDNA using SuperScript™ II RT (Life Technologies, Grand Island, NY). The cDNA was used as the template DNA for all of the primary PCR

reactions. Primary PCR reactions utilized the Abridged Universal Amplification Primer (AUAP) that was provided with the 3' RACE kit and a variety of gene specific primers (Table 5.3). Next, a nested PCR reaction was done using the primary PCR reaction product as template DNA. Nested PCR reactions utilized nested gene specific primers (Table 5.4). All PCR products were run on 1.5% agarose gels for visualization.

Table 5.3. Primary and nested gene-specific primers used in 3' RACE analysis.

Gene	Primary Gene-specific Primer	Nested Gene-specific Primer
MAC1	5'-GCGATGCCACCACTTCATAA-3'	5'-AATCACGTAAATACAGATAG-3'
PCA1	5'-TGCGTTATGCAAAGATTTAG-3'	5'-ACTGCTGCTATTTGATTGA-3'
FRE2	5'-AAGAGTATCAATGCTGGTGA-3'	5'-TCATTTTTTACTTAAAACTA-3'
CRS5	5'-GCAATTGTGAAAAATGTTAG-3'	5'-GTGACCCCTACTCTTTACTT-3'
COX19	5'-ACGCCACCGATAATAAATAG-3'	5'-CCGCCCACATCGGAATATCG-3'
COX23	5'-ACAGACAGCAGTGGGAATGA-3'	5'-GGCAGCCTTTTCTTCTTGTT-3'

Table 5.4. Transcription factors upstream of *PCA1*, *CRS5*, and *COX23* which are potential NMD targets.

Standard Name	**Systematic Name**	**Function**	**Ref**
INO4	YOL108C	Transcription factor involved in phospholipid synthesis	3
SKN7	YHR206W	Nuclear response regulator and transcription factor	16
PAF1	YBR279W	Component of the Paf1p complex involved in transcription elongation	16
UGA3	YDL170W	Transcriptional activator for GABA-dependent induction of GABA genes	3, 16
CBF1	YJR060W	Basic helix-loop-helix (bHLH) protein	16
AFT1	YGL071W	Transcription factor involved in iron utilization and homeostasis	16
STB1	YNL309W	Protein with role in regulation of MBF-specific transcription at Start	16
MET32	YDR253C	Zinc-finger DNA-binding transcription factor	16
RPD3	YNL330C	Histone deacetylase, component of both the Rpd3S and Rpd3L complexes	16
SIN4	YNL236W	Subunit of the RNA polymerase II mediator complex	16
STE12	YHR084W	Transcription factor that is activated by a MAPK signaling cascade	16
SUM1	YDR310C	Transcriptional repressor that regulates middle-sporulation genes	16
AFT2	YPL202C	Iron-regulated transcriptional activator	16
ZAP1	YJL056C	Zinc-regulated transcription factor	16
SOK2	YMR016C	Nuclear protein that negatively regulates pseudohyphal differentiation	16

1.2.5 Low copper methods

For low copper northerns, complete minimal media was used to grow yeast cells. This media contained yeast nitrogen base without copper (YNB-$CuSO_4$-$FeCl_3$) and 100 μM Bathocuproinedisulfonic acid (Sigma-Aldrich). Glassware used in these experiments was soaked in 10% nitric acid overnight to remove trace amounts of copper. All yeast cells used for low copper northerns were grown in the copper deficient media described above in acid washed glassware.

1.3 RESULTS

1.3.1 Analysis of transcripts involved in copper homeostasis for potential NMD targeting features.

We previously demonstrated that the *CTR2* mRNA, which encodes for a copper transporter of the vacuolar membrane, is regulated in an NMD dependent manner (Deliz-Aguirre et al., 2011). We also found that this mRNA is sensitive to NMD due to both the length of the 3'-UTR and the ORF(Peccarelli et al., 2014). In order to determine whether additional mRNAs involved in copper homeostasis are regulated by NMD, data generated from global expression profiling studies identifying mRNAs regulated by NMD was examined. This data was utilized as described below to identify additional natural transcripts involved in copper homeostasis that could potentially be regulated by NMD (Deliz-Aguirre et al., 2011).

Johansson *et al.* (2007) identified NMD targets through their association with Upf1p, a core NMD factor. The *Saccharomyces* Genome Database (SGD) classifies 41 mRNAs as mRNAs encoding proteins involved in copper metabolism. Out of these mRNAs, only *CTR2, CTR3, MAC1, COX23* and *CRS5* were identified as potential NMD

targets by Johansson *et al.* (2007). In addition, Johansson *et al.* (2007) used the NMD reactivation system to identify NMD sensitive transcripts. Under this system, the expression of a factor required for NMD (*UPF2*) is controlled by a regulated promoter. The yeast strains used have an inactive NMD pathway which is activated under NMD inducing conditions. When NMD is activated, mRNAs regulated by the pathway are down-regulated as a result (Johansson et al., 2007). Johansson *et al.* (2007) identified *COX19, FRE2,* and *PCA1* as NMD targets using this method. Additionally, a previous study by Guan *et al.* (2006) found similar results. Specifically, Guan *et al.* (2006) identified *COX19* as a direct NMD target, and *PCA1* and *FRE2* as indirect targets (Guan et al., 2006).

Examination of these eight mRNAs for potential NMD inducing features found that each mRNA contains at least one known NMD targeting feature with the exception of *CRS5* (Table 5.1). *CRS5* mRNA may be an indirect NMD target, or it may be regulated by the pathway through a novel mechanism. *CTR2*, *FRE2* and *COX19* mRNAs contain multiple potential NMD targeting features (Table 5.1). Interestingly, the majority of these mRNAs are predicted to have atypically long 3'-UTRs, suggesting that it could be the NMD targeting feature (Table 5.1). The atypically long 3'-UTRs of the 900 and 2600 nt *CTR2* mRNA isoforms were found to play a role in the degradation of the mRNA by NMD (Peccarelli et al., 2014). We first investigated the extent to which these mRNAs are directly regulated by NMD.

1.3.2 *COX23, CRS5, PCA1, FRE2*, and *COX19* mRNAs are regulated in an NMD dependent manner.

As we stated above, seven of the eight mRNAs have known NMD targeting features. Six of these mRNAs were predicted to have atypically long 3'-UTRs (Table 5.1) (17). The 3'-UTR lengths of these mRNAs were experimentally verified using 3' RACE (Table 5.1). Six of the mRNAs were found to have 3'-UTRs that are longer than the average length of *S. cerevisiae* 3'-UTRs. Thus, we expected that each of these mRNAs would be degraded by the NMD pathway as a result of the long 3'-UTR. In addition, *PCA1* and *FRE2* are predicted to be subject to -1 Ribosomal Frameshifting, which has also been shown to induce degradation of mRNA by NMD (Belew et al., 2011; Belew et al., 2014). While *PCA1* mRNA was not predicted to have a long 3'-UTR, 3'-RACE and northern analyses showed that *PCA1* produces two mRNA isoforms, one with an atypically long 3'-UTR (Table 5.1).

Steady-state mRNA accumulation levels of the mRNAs were compared in wild-type and nmd mutants. We anticipated that *COX23, PCA1*, *MAC1, FRE2,* and *COX19* mRNAs would accumulate to higher levels in the nmd mutant strains. *CRS5* was not expected to accumulate to higher levels in nmd mutants due to the lack of any identified NMD targeting features. Additionally, northerns would allow us to identify genes that produced multiple mRNA isoforms in the conditions utilized. Under similar growth conditions, we found that chromosomally encoded *CTR2* generates two transcripts with varying 3'-UTR lengths (5). The two *CTR2* mRNA isoforms were 2.6 nt and 900 nt, respectively (Peccarelli et al., 2014).

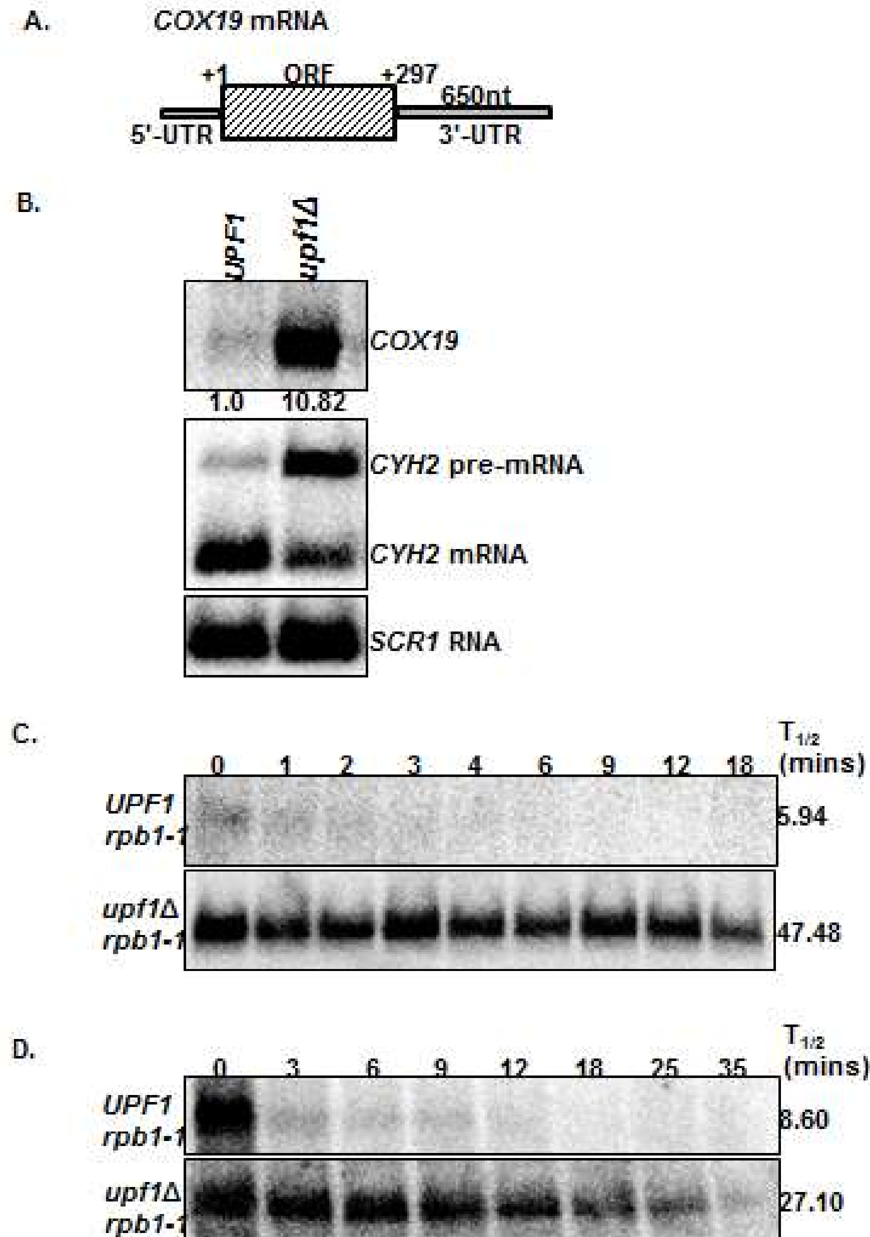


Figure 5.1. *COX19* mRNA is a direct NMD target in two conditions. A Schematic representation of *COX19* mRNA (A). Representative *COX19* mRNA steady-state accumulations in wild type (*UPF1* (Wente et al., 1992)) and nmd mutants (*upf1Δ* (Kebaara et al., 2003)) (B). Representative *COX19* mRNA half-life northerns in wild-type strain AAY334 (*UPF1 rpb1-1* (Kebaara et al., 2003)) and nmd mutant strains AAY335 (*upf1Δ rpb1-1* (Kebaara et al., 2003)) of yeast strains grown in YAPD (C) or Synthetic complete media lacking leucine (D). Yeast cells were harvested at nine time points over an eighteen minute period after transcription inhibition. The time points are indicated above the half-life northerns. The half-lives were determined using SigmaPlot and are shown to the right of the northern blots. *CYH2* pre-mRNA is degraded by NMD and is used as a control for the NMD phenotype of the yeast strains. *SCR1* is an RNA polymerase III transcript that is not regulated by NMD and was used as a loading control. Steady-state and half-life measurements are an average of at least three independent experiments.

In rich media, *COX23, MAC1, CRS5* and *COX19* all produced one transcript, while *PCA1* and *FRE2* produced two transcripts. The two *PCA1* and *FRE2* mRNA isoforms differed in size due to the lengths of the 3'-UTRs (Tables 5.1 and 5.2). *MAC1* and the short *PCA1* mRNA isoform did not accumulate to significantly higher levels in the nmd mutant relative to the wild-type strain (Table 5.5). Additionally, *COX23, CRS5, COX19,* both isoforms of *FRE2* and the long *PCA1* mRNA accumulated to significantly higher levels in the nmd mutant relative to the wild-type strain (Table 5.5). The relative accumulation levels for these mRNAs in nmd mutants varied (Table 5.5). For example, the relative accumulation level for *MAC1* mRNA was 0.98 (± 0.34) (Table 5.5). Interestingly, *COX19* mRNA which has two potential NMD targeting features, accumulated to significantly higher levels in the nmd mutant relative to the wild-type strain. *COX19* accumulated 10.82 (± 1.18) fold higher in nmd mutants (Figure 5.1 and Table 5.5). The *COX19* mRNA was barely detectable in the wild-type yeast strain (Figure 5.1). Furthermore, *FRE2,* like *COX19,* has multiple, potential NMD targeting features and both the long and short isoforms of *FRE2* mRNA accumulated to significantly higher levels in the nmd mutant (Table 5.5). These results suggest that the presence of multiple NMD targeting features on the *FRE2* and *COX19* mRNAs may be increasing the sensitivity of these mRNAs to NMD (Tables 5.1 and 5.2). We recognize that other variables may also account for the difference in NMD efficiency.

Table 5.5. Isogenic wild-type (W303) and nmd mutants (AAY320) were used to measure mRNA steady-state accumulation levels, while AAY334 and AAY335 were used to determine mRNA half-lives. All yeast strains used were grown under standard conditions in nutrient rich YAPD media (18).

Standard Name	3'-UTR nt (3' RACE)	Relative mRNA accumulation (*upf1Δ/UPF1*)	mRNA half-life (mins) *UPF1*	mRNA half-life (mins) *upf1Δ*
CRS5	75	1.67 ± 0.46	11.67 ± 4.20	10.63 ± 3.59
COX23	300	1.58 ± 0.17	16.19 ± 2.43	12.20 ± 2.05
PCA1	650	1.96 ± 0.68	22.02 ± 7.45	15.92 ± 3.29
	200	1.35 ± 0.41	ND	ND
MAC1	300	0.98 ± 0.34	2.43 ± 0.2	4.13 ± 0.55
*CTR2**	2000	3.9 ± 0.8	ND	ND
	300	1.0 ± 0.04	2.5 ± 1.3	5.2 ± 0.8
FRE2	1400	6.44 ± 0.52	7.55 ± 3.23	84.17 ± 21.00
	700	13.55 ± 3.47	10.84 ± 2.67	31.20 ± 10.33
COX19	650	10.82 ± 1.18	5.94 ± 1.44	47.48 ± 22.51
CTR3	ND	ND	ND	ND

ND, not determined
*3' RACE measured 3'-UTR length, steady-state accumulation levels, and half-lives for this mRNA were reported previously (18).

We were unable to detect the *CTR3* transcript under the conditions utilized here. When expressed, *CTR3* encodes for a high affinity Copper (I) transporter. However, many commonly used *S. cerevisiae* laboratory strains do not encode for a functional Ctr3p due to the presence of a Ty2 transposable element in the promoter region in these

strains (Knight et al., 1996; Pena et al., 2000). The strains utilized here (Table 5.2) most likely contain a disrupted *CTR3,* and thus do not produce a *CTR3* transcript.

Taken together, these results show that *CRS5, COX23, PCA1, FRE2*, and *COX19* are regulated by NMD. This regulation may be direct or indirect. As previously stated, mRNAs that are direct NMD targets are stabilized in nmd mutant strains. Conversely, indirect regulation is due to an upstream transcription factor that is subject to NMD-mediated degradation. Therefore, the stabilization of an upstream transcription factor causes downstream mRNAs to accumulate to higher levels in nmd mutants. Because of the presence of known NMD targeting features, we anticipated that *COX23*, *PCA1, FRE2* and *COX19* would be directly regulated by NMD. On the other hand, we expected that *CRS5* would be an indirect NMD target.

1.3.3 *COX19* and *FRE2* are directly regulated by NMD.

To determine which of the five mRNAs are direct NMD targets, we measured mRNA decay rates using the temperature sensitive allele of RNA polymerase II to inhibit transcription (Nonet et al., 1987). *COX19* and *FRE2* mRNAs had significantly longer half-lives in the nmd mutants relative to wild-type strains grown in rich media (Figures 2C and 4B). We found that both *FRE2* transcripts had significantly longer half-lives in the nmd mutant relative to the wild-type strain and thus *FRE2* is directly regulated by NMD (Table 5.5 and Figures 5.2C and 5.4B).

In wild-type strains grown in rich media, the half-life for *COX19* mRNA was 5.94 (± 1.44) mins relative to 47.48 (± 22.51) mins in the nmd mutant strain (Table 5.5, Figure 5.1C). In addition, we measured the *COX19* mRNA half-life in yeast strains grown in synthetic complete media lacking leucine (SC-leucine). In these conditions the half-life

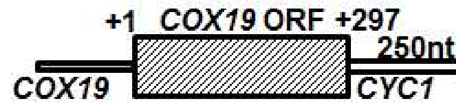
for *COX19* mRNA was 8.6 (± 1.1) mins relative to 27.1 (± 6.4) mins in the nmd mutant. Thus, the *COX19* mRNA was slightly stabilized in wild-type yeast strains grown in SC-leucine relative to rich media. Notably, *COX19* mRNA was stabilized significantly in nmd mutants grown in both conditions relative to wild-type strains (Figure 5.1C and 5.1D). This stabilization in the mutants indicates that the *COX19* mRNA is directly regulated by NMD, and that the accumulation in nmd mutants was due to stabilization of the mRNA. Interestingly, *COX19*, *FRE2* and *CTR2* mRNAs are the only three mRNAs out of eight involved in copper homeostasis that could potentially be targeted to NMD due to multiple targeting features (Table 5.1). Furthermore, two features that target the *CTR2* mRNAs to NMD have been described previously and function cooperatively to regulate the degradation of the mRNA (Peccarelli et al., 2014).

1.3.4 The *COX19* 3'-UTR influences regulation of the mRNA by NMD.

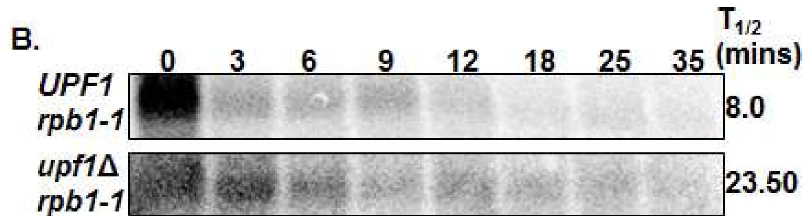
Because the *CTR2* 3'-UTR plays a role in the degradation of the mRNA by NMD we wanted to determine the role the *COX19* 3'-UTR plays in the degradation of the mRNA by NMD. To do this we generated two fusion mRNAs. The first was used to determine if the long 3'-UTR is sufficient to target *COX19* mRNA to NMD. The *COX19* 3'-UTR was replaced with the short *CYC1* 3'-UTR, creating the *COX19-CYC1 3'-UTR* fusion mRNA (Figure 5.2A). The *CYC1* 3'-UTR is not known to target mRNAs to NMD. Unlike the endogenous *COX19* mRNA which accumulated to significantly higher levels in nmd mutants (Figure 5.1B), the *COX19-CYC1 3'-UTR* fusion mRNA's relative accumulation level was 0.77(± 0.33). Although this mRNA did not accumulate to higher levels in nmd mutants it was stabilized in the mutants relative to wild-type strains (Figure 5.2B). *COX19-CYC1 3'-UTR* fusion mRNA had a half-life of 8.0 (± 1.41) mins in the wild-type

strain relative to 23.50 (± 2.12) mins in the nmd mutants. Thus, the half-life of the *COX19-CYC1 3'-UTR* fusion mRNA in wild-type strains and nmd mutants was almost identical to the half-life of *COX19* mRNA in the same growth conditions (Figure 5.1D and 5.2B). Thus, replacement of the *COX19* 3'-UTR with the *CYC1* 3'-UTR affected the steady-state accumulation levels of the *COX19-CYC1 3'-UTR* mRNA, but it did not affect the decay rate of the mRNA. These results suggest that the *COX19 3'-UTR* does not function independently in targeting the mRNA to NMD similar to the *CTR2 3'-UTR.*

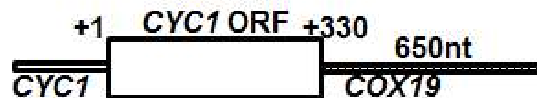
A. *COX19-CYC1* 3'-UTR mRNA



B.



C. *CYC1-COX19* 3'-UTR mRNA



D.

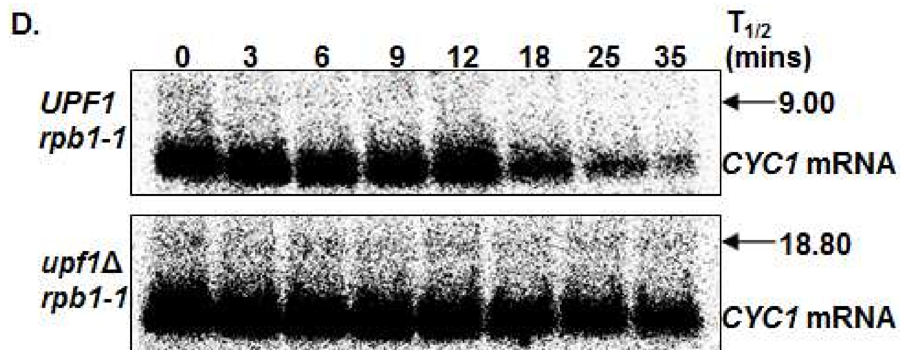


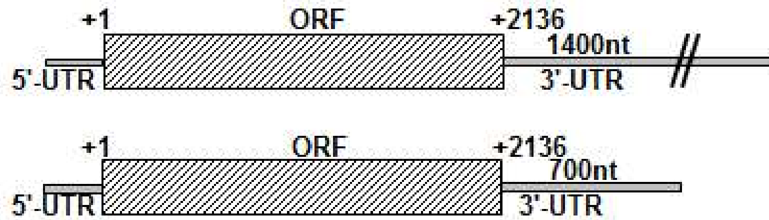
Figure 5.2. The *COX19* 3'-UTR impacts the regulation of *COX19* mRNA by NMD. The mRNA schematics and half-life northern analysis of *COX19-CYC1 3'-UTR* mRNA (A, B) and *CYC1-COX19 3'-UTR* mRNA (C, D). Half-lives were measured with total RNA extracted from wild-type strain AAY334 (*UPF1 rpb1-1* (Kebaara et al., 2003)) and nmd mutant strain AAY335 (*upf1Δ rpb1-1* (Kebaara et al., 2003)) transformed with a plasmid expressing *COX19-CYC1 3'-UTR* (B) and *CYC1- COX19 3'-UTR* (D). Time points after transcription inhibition are indicated above the northern blots and the mRNA half-lives are shown to the right of the northerns. *COX19-CYC1* 3'-*UTR* northerns were probed with *CYC1* 3'UTR and *CYC1-COX19* 3'-UTR northerns was probed with *CYC1* 5'-UTR and ORF. Half-lives for *COX19-CYC1* 3'-*UTR* mRNA are from two independent experiments while half-lives for *CYC1-COX19 3'-UTR* mRNA are an average of three independent experiments. The bottom bands in 3D is the endogenous *CYC1* mRNA, which is much more abundant than the *CYC1-COX19 3'-UTR* mRNA.

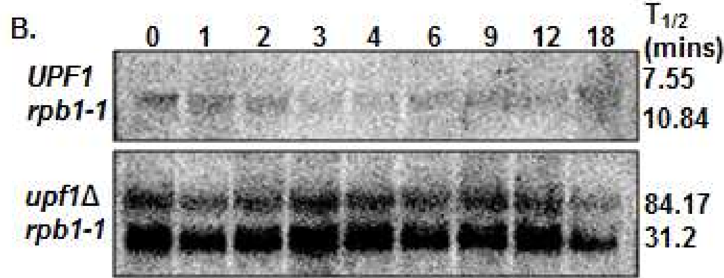
To determine whether the *COX19* 3'-UTR is sufficient to target an NMD insensitive mRNA to the pathway, we replaced the *CYC1* 3'-UTR with the *COX19* 3'-UTR, creating the *CYC1-COX19* 3'-UTR fusion mRNA (Figure 5.2C). *CYC1* was utilized because we and others have successfully used it previously to map NMD instability elements (Peccarelli et al., 2014). Unlike the *COX19-CYC1 3'-UTR* mRNA which did not accumulate to higher levels in nmd mutants, the *CYC1-COX19* 3'-UTR mRNA accumulated to significantly higher levels in nmd mutants and was also stabilized. *CYC1-COX19 3'-UTR* fusion mRNA had a half-life of 9.00 (± 0.53) mins in wild-type strains relative to 18.80 (± 4.60) mins in nmd mutants (Figure 5.2D). These results demonstrate that replacing the 3'-UTR of the NMD-insensitive *CYC1* with the *COX19* 3'-UTR is sufficient to target the fusion mRNA to the NMD pathway.

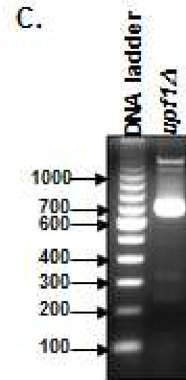
1.3.5 *CRS5, COX23* and *PCA1* are indirectly regulated by the NMD pathway.

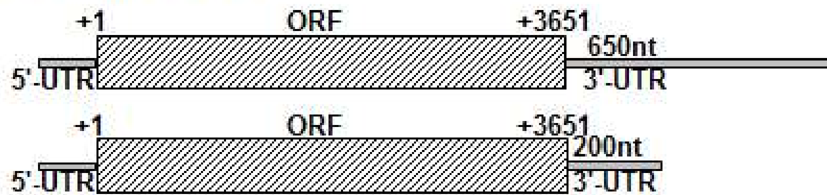
We demonstrated above that *CRS5, COX23* and the long *PCA1* transcripts accumulate to significantly higher levels in nmd mutants relative to wild-type strains (Table 5.5). We anticipated that *PCA1* mRNA would be directly regulated by NMD since it is predicted to be subject to -1 ribosomal frame shifting and has a long 3'-UTR (Table 5.1). Interestingly, in the conditions tested, the half-lives for both *PCA1* transcripts in wild-type and mutants were comparable. In wild-type strains, the half-life for the longer transcript was determined to be 22.02 (± 7.45) mins (Table 5.5 and Figure 5.3E). In the nmd mutant strain, the half-life was 15.92 (± 3.29) mins (Table 5.5 and Figure 5.3E). Although it appears that the half-life is slightly longer in the wild-type strain, the difference was not determined to be statistically significant. Half-lives of the shorter *PCA1* transcript showed that it is also not stabilized in nmd mutants relative to the wild-

type strain in the 0-35 minute time points we typically use to measure half-lives. In order to obtain a specific half-life measurement, longer time points could be used. However, since the decay rate in both the wild-type and nmd mutants is comparable, the shorter *PCA1* mRNA isoform is most likely not directly regulated by NMD.









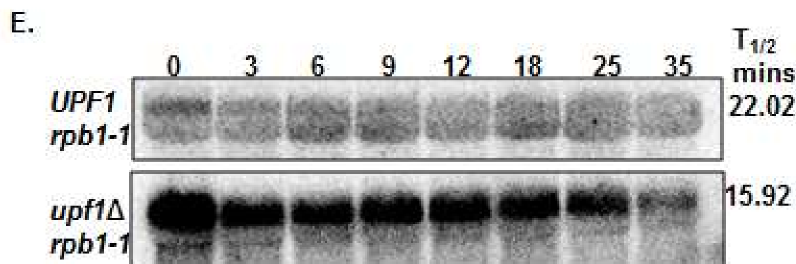


Figure 5.3. Both *FRE2* and *PCA1* encode two mRNA isoforms, although both *FRE2* mRNA isoforms are direct NMD targets, both *PCA1* mRNA isoforms are indirect targets. Schematic representations of *FRE2* and *PCA1* mRNA isoforms (A and D). Representative *FRE2* mRNA (B) and *PCA1* mRNA (E) half-life northerns in wild-type strain AAY334 (*UPF1 rpb1-1* (Kebaara et al., 2003)) and nmd mutant strains AAY335 (*upf1Δ rpb1-1* (Kebaara et al., 2003)). A 1.5% agarose gel of primary 3' RACE PCR products of *FRE2* mRNA in nmd mutants (*upf1Δ*) (C). Cells were harvested at nine time points over eighteen minutes for *FRE2* mRNA. Alternatively, cells were harvested over eight time points over thirty-five minutes for *PCA1* mRNA. Respective time points are indicated above the half-life northerns. The half-lives were determined using SigmaPlot. *CYH2* and *SCR1* are used as controls as stated in Figure 5.1. The half-life measurements are an average of at least three independent experiments.

Similarly, *COX23* was found to be an indirect NMD target despite having an atypically long 3'-UTR (Table 5.1 and 2) and accumulating to higher levels in nmd mutants. As stated previously, *CRS5* was not expected to be directly regulated by NMD due to the lack of a recognizable NMD targeting feature. *CRS5* mRNA half-lives clearly show this is in fact the case. Specifically, the half-lives of *CRS5* mRNA were comparable in wild-type and nmd mutant strains (Table 5.5 and Figure 5.4).

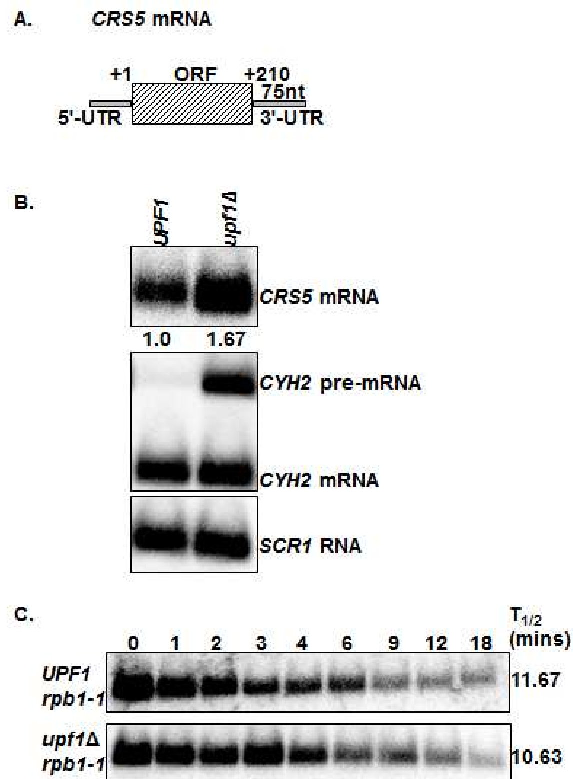


Figure 5.4. *CRS5* mRNA is an indirect NMD target. Schematic representation of the *CRS5* mRNA (A). Representative northern blot of the *CRS5* mRNA steady-state accumulation levels (B) and half-lives (C). The steady-state mRNA levels were measured with total RNA from wild-type strain W303 (*UPF1* (Wente et al., 1992)) and the nmd mutant strain AAY320 (*upf1Δ* (Kebaara et al., 2003)) (B). mRNA half-lives were measured with total RNA extracted from wild-type strain AAY334 (*UPF1 rpb1-1* (Kebaara et al., 2003)) and nmd mutant strain AAY335 (*upf1Δ rpb1-1* (Kebaara et al., 2003)) (C). Yeast cells were harvested at nine time points over an eighteen minute period after transcription inhibition. The time points are indicated above the half-life northerns. The half-lives were determined using SigmaPlot and are shown to the right of the northern blots. *CYH2* and *SCR1* are used as controls as stated in Figure 5.1. The half-life measurements are an average of at least three independent experiments.

Taken together, these results demonstrate that *CRS5, COX23* and *PCA1* mRNAs are indirect NMD targets in the conditions tested. *CRS5, COX23* and *PCA1* mRNAs were previously categorized as direct NMD targets by Johansson *et al.* (Johansson et al., 2007). In addition, both *COX23* and *PCA1* mRNAs have identifiable NMD targeting features, suggesting that they could be directly regulated by the pathway. However, we did not find that to be the case in the conditions utilized here.

1.3.6 *MAC1* mRNA does not accumulate to significantly higher levels in nmd mutants but is directly regulated by the pathway.

As stated above, there was no detectable difference in *MAC1* mRNA steady-state accumulation levels between wild-type and nmd mutants grown in rich media (Table 5.5). However, measurement of *MAC1* mRNA half-lives in wild-type and nmd mutants showed that there was a statistically significant difference in the mRNA half-lives between the two strains. Specifically, the half-life of major *MAC1* mRNA in the wild-type strains was 2.43 (± 0.20) mins relative to 4.13 (± 0.55) mins in the nmd mutant (*upf1*Δ). This observation demonstrates that *MAC1* mRNA is directly regulated by NMD in this condition (Table 5.5 and Figure 5.5B). These results are not typically observed for mRNAs that are direct NMD targets, but are not unique to *MAC1* mRNA. Normally, transcripts that are direct NMD targets have increased steady-state accumulation levels as well as significantly longer half-lives in nmd mutants. However, previous studies have described examples of transcripts that do not accumulate to significantly higher levels, but are stabilized in the nmd mutants; and thus, are directly regulated by the pathway (Peccarelli et al., 2014). For example, we found here that the *COX19-CYC1 3'-UTR* fusion mRNA did not accumulate to significantly higher levels in nmd mutants but was

stabilized, similar to what we previously found with the shorter *CTR2* mRNA isoform (Deliz-Aguirre et al., 2011; Peccarelli et al., 2014).

1.3.7 Yeast strains grown under copper deplete conditions produce two NMD-insensitive *MAC1* transcripts.

MAC1 is a transcription factor that senses copper availability and regulates the expression of genes involved in high affinity copper transport. It was the only transcription factor examined in this study. Because *MAC1* is activated in low copper conditions, we investigated whether regulation of this mRNA by NMD was affected by copper availability. We measured the half-life of *MAC1* mRNA in wild-type and nmd mutants grown in low copper conditions. As stated above, *MAC1* mRNA produced one predominant transcript of ~1.6 nt when yeast cells were grown in rich media. Additionally, a weak second band of 2.9 nt was detected (Figure 5.5B). However, growth of both strains in low copper conditions resulted in the production of two predominant *MAC1* transcripts of the same size detected in YAPD, 1.6 nt and 2.9 nt (compare Figures 5.4B and 5.4D). The two transcripts vary in the length of their 3'-UTRs, as corroborated by 3' RACE (Figure 5.5C). The 1.6 nt *MAC1* transcript that is predominant in rich media has a 3'-UTR of ~300nt, while the 2.9 nt *MAC1* transcript has a 3'-UTR of ~1400 nt (Figure 5.5C lanes 2 and 3). The 3' RACE PCR product shows that the ~1400 nt PCR product is only detectable under low copper conditions. Furthermore, the gel shows that the ~300nt 3' RACE product was more abundant in yeast strains grown in rich media (YAPD), relative to yeast strains grown in low copper conditions, consistent with the northern blot analysis (Figure 5.5B and 5.5D). An additional 3' RACE PCR product of ~520 nt was detected on the gel. This is most likely a nonspecific band because in both

the rich media (YAPD) and low copper conditions, a transcript of 1.8 nt, which would correspond to a 3'-UTR length of ~ 520 nt, was not detected.

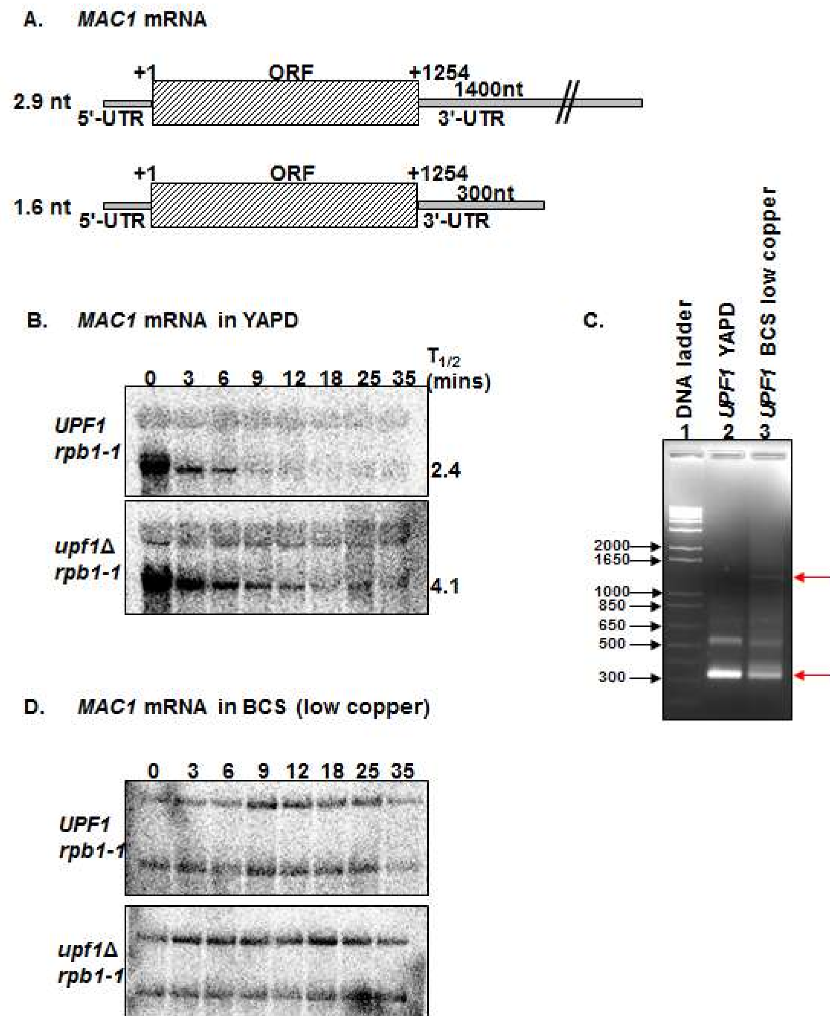


Figure 5.5. NMD-mediated regulation of *MAC1* is dependent on the environmental conditions. A schematic representation of *MAC1* mRNA isoforms (A). Representative half-life northerns of *MAC1* mRNAs with total RNA isolated from wild-type strain AAY334 (*UPF1 rpb1-1* (Kebaara et al., 2003)) and nmd mutant strains AAY335 (*upf1Δ rpb1-1* (Kebaara et al., 2003)). Total RNA was isolated as described in figure 5.1 above from yeast cells grown in YAPD (B) or yeast strains grown in media containing Bathocuproinedisulfonic acid (low copper) (D). The low copper half-life membranes were also probed with *CYH2* as an NMD control and also to confirm that transcription was inhibited. A 1.5% agarose gel of primary 3' RACE PCR products of the *MAC1* mRNA isoforms in wild-type stains grown in YAPD or low copper medium (C). Red arrows indicate the size of the 3' RACE PCR products expected to be present in the two *MAC1* mRNA isoforms. The additional bands seen on the 3' RACE PCR gel were considered non-specific since there were no corresponding bands on the respective northern blot.

The half-lives of the *MAC1* transcripts in low copper were measured using the same time points used to measure the half-lives in rich media (YAPD). These time points were utilized in order to accurately compare the results. The decay rate of the two *MAC1* mRNA isoforms in low copper varied greatly from the decay rate of the mRNA in rich media. Specifically, both *MAC1* transcripts did not appear to be degraded within the 35 minutes time period (Figure 5.5D). Longer time points are needed to accurately determine specific half-lives in these conditions. However, these results demonstrate that *MAC1* mRNA, despite having an atypically long 3'-UTR, is insensitive to NMD when yeast strains are grown in low copper conditions. Alternatively, the predominant *MAC1* mRNA isoform detected in rich media was much more abundant and directly regulated by NMD (Figure 5.5B). These results suggest that *MAC1* stability is very complex and is possibly controlled by multiple factors. However, the findings support the conclusion that NMD-mediated regulation of transcripts involved in specific cellular processes can be altered due to environmental conditions.

1.3.8 Several transcription factors upstream of *PCA1*, *CRS5*, and *COX23* are likely NMD targets.

Because we identified *CRS5*, *COX23* and *PCA1* mRNAs as indirect NMD targets, we hypothesized that there is/are transcription factors upstream of these genes which could be direct NMD targets. Using data from the Saccharomyces Genome Database we constructed a regulatory network of the genes acting upstream of *PCA1*, *CRS5*, and *COX23*. In brief, using a recursive search we identified transcription factors with binding sites in the promoter regions of *PCA1*, *CRS5*, *COX23*, or an upstream transcription factor identified in a previous round of the search. For simplicity, we excluded transcriptional

repressors from the search. We then created a directed graph of these genes. This graph contains 96 nodes and 456 edges, with each node representing a gene and each edge indicating that the source gene activates the target gene. The majority (88/96) of the transcription factors we identified are part of a strongly connected subgraph. In this case, activation of any of these transcription factors could potentially activate all of the transcription factors in the subgraph. The adjacency matrix for the graph can be found in supplementary table S1.

We found that fourteen genes in this network encoded for mRNAs that were found to associate with Upf1p by Johansson *et al.* (2007) (Johansson et al., 2007). One mRNA, *INO4*, was identified as a direct NMD target while another mRNA, *UGA3*, was identified as an indirect target by Guan *et al*. (2006) (Guan et al., 2006). These transcription factors and their functions are listed in Table 5.5. We also calculated the shortest path length to *COX23, CRS5*, and *PCA1* from *INO4*. We created a diagram showing the shortest path from *INO4* to each of *COX23, CRS5*, and *PCA1* (Figure 5.6). As depicted in the regulatory network, *INO4* can act through *SWI5* to activate *XBP1*. *XBP1* may then activate *COX23* directly or activate various factors which induce transcription of *CRS5*, and *PCA1*.

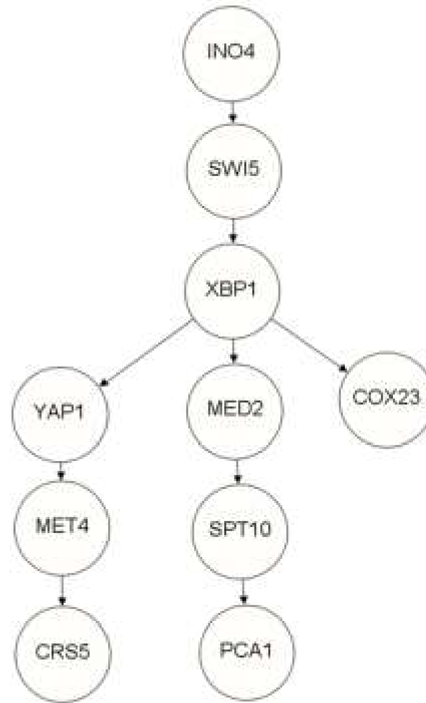


Figure 5.6. Model of a transcriptional regulatory network for *CRS5*, *PCA1* and *COX23*. A transcriptional regulatory network showing the shortest and most direct paths from *INO4* to *COX23, CRS5, and PCA1*. As shown, *INO4* could activate *COX23, CRS5, and PCA1* through the activation of *SWI5* and *XBP1*.

1.4 DISCUSSION

It is apparent that the NMD pathway is involved in the regulation of various aspects of copper homeostasis. NMD is involved in regulating mRNAs encoding protein required for sensing copper availability and transport through regulation of *MAC1* and possibly *CTR3* mRNAs. NMD also plays a role in mitochondrial and vacuolar copper homeostasis through regulation of *COX19*, *COX23*, and *CTR2*(Peccarelli et al., 2014). We postulate that because these mRNAs belong to the same functional group and are regulated by the NMD pathway, this regulation is physiologically significant. In fact, we previously found that nmd mutants are more tolerant of toxic copper levels (Deliz-Aguirre et al., 2011). In addition, six of the mRNAs have an atypically long 3'-UTR. We found that the long 3'-UTRs of the *CTR2*(Peccarelli et al., 2014) and *COX19* mRNAs contribute to the

regulation of these mRNAs by this pathway. These findings suggest that these functionally related mRNAs could be targeted for degradation by NMD through similar mechanisms depending on the environmental conditions.

We found that the relative accumulation levels of these mRNAs varied greatly. Interestingly, both *FRE2* transcripts and *COX19* accumulated to much higher levels and were more stable in nmd mutants relative to the other mRNAs in this study (Table 5.5). These two mRNAs and *CTR2* were the only mRNAs that have more than one known NMD targeting feature. Because we are looking at the total pool of the mRNAs, we hypothesize that the presence of multiple NMD targeting features on an mRNA may cause these mRNAs to be more sensitive to degradation by NMD. That is, if the multiple NMD targeting features are functional, they may have an additive effect on the degradation of these mRNAs. The additive effect may cause these select transcripts to be further stabilized in nmd mutants, leading to the increased steady-state accumulation levels. Observations made with *COX19* and *CTR2* mRNAs support this hypothesis. We found that the *COX19* 3'-UTR affects regulation of the mRNA by NMD. In addition, we previously showed that *CTR2* mRNAs are regulated by NMD due to the length of both the ORF and 3'-UTR. These two features were found to have an additive effect (Peccarelli et al., 2014). Four additional mRNAs involved in copper homeostasis and regulated in an NMD-dependent manner have atypically long 3'-UTRs that could trigger their degradation by the pathway (Table 5.1). These observations raise the possibility that the long 3'-UTRs of the additional mRNAs play a role in their regulation by NMD. We propose that mRNAs from the same functional group that are sensitive to the NMD pathway may be targeted for degradation by similar mechanisms.

CRS5, *COX23* and *PCA1* mRNAs have previously been reported as direct NMD targets. However, in the conditions utilized here, we found these three mRNAs to be indirect NMD targets. *PCA1* mRNA was also previously reported as an indirect NMD target by Guan *et al*. (2006)(Guan et al., 2006), which is consistent with our study. It is possible that sensitivity of these mRNAs to NMD is condition specific. 3' RACE and northern analysis showed that the *PCA1* mRNA encodes two mRNA isoforms that vary in the 3'-UTR length. We expected the longer *PCA1* isoform with the 650 nt 3'-UTR to be directly regulated by NMD due to the atypically long 3'-UTR. However, this was not the case. A contributing factor to this result may be the *PCA1* mRNA ORF length which is 3651nt. This is the longest ORF of the mRNAs examined in this study. In our previous study, we found that lengthening the ORF of the *CTR2* mRNA, which is targeted to NMD due to an atypically long 3'-UTR, caused the mRNA to be insensitive to NMD (Peccarelli et al., 2014). In addition, Decourty *et al*., (Decourty et al., 2014) reported that in *S. cerevisiae* mRNAs targeted to NMD due to the presence of a long 3'-UTR had short ORFs and that lengthening the ORFs of these mRNAs had a stabilizing effect. Thus, it is likely that the length of the *PCA1* ORF is stabilizing the longer isoform.

COX23 mRNA was also found to be an indirect NMD target despite the presence of a 300nt 3'-UTR. This 3'-UTR length may not be long enough to directly target the mRNA to NMD, or *COX23* may possess features that facilitate stabilization in the presence of NMD. In humans, it has been shown that a large fraction of mRNAs with long 3'-UTRs evade degradation by NMD due to NMD-inhibiting cis-elements (Toma et al., 2015). Additionally, *CRS5* mRNA was determined to be an indirect target. This was not unexpected since *CRS5* does not have a known NMD inducing feature. It is likely

that an mRNA, or mRNAs, upstream of *COX23* and/or *CRS5* is a direct NMD target as discussed below.

We identified 14 transcription factors that could influence the NMD sensitivity of *CRS5*, *COX23* and *PCA1* mRNAs to NMD. These transcription factors encode for mRNAs shown to associate with Upf1p but were not found to decrease upon reactivation of NMD (Johansson et al., 2007). This may indicate that these transcription factors are indirect NMD targets. Alternatively, the correlation between direct targets identified by Johansson *et al.* and Guan *et al*. is low, which suggests that the NMD reactivation method may not identify all direct NMD targets. Consequently, one or more of these transcription factors may be a direct NMD target. Additional studies on the mRNAs encoded by the transcription factors are necessary to determine if any are direct NMD targets and also if these genes are responsible for the increased steady-state levels of *COX23*, *CRS5*, and *PCA1*.

We identified one gene, *INO4*, which was reported to be a direct NMD target by Guan *et al.* (2006)(Guan et al., 2006). *INO4* encodes a transcriptional upregulator of phospholipid, sterol and fatty acid biosynthetic genes(Santiago and Mamoun, 2003). Interestingly, copper toxicity has been linked to a decline in plasma membrane integrity. Furthermore, transcription of *OPI3*, a gene regulated by *INO4*, is induced in high copper environments(Yasokawa et al., 2008). The likely regulatory network shows that *INO4* could regulate *COX23, CRS5*, and *PCA1* through increasing expression of *SWI5* and *XBP1* (Figure 6). Expression of these genes is known to occur during acute stress response. Similarly, *COX23*, *CRS5*, and *PCA1* are involved in copper homeostasis (Table

5.1), and overexpression of *CRS5*, which encodes for a copper binding metallothionein suppress copper toxicity

(Adle et al., 2007; Culotta et al., 1994). Further studies are required to determine if *INO4* has a role in the cellular response to copper toxicity and whether the potential targeting of *INO4* mRNA by NMD affects steady-state accumulation of *COX23*, *CRS5*, and *PCA1*.

We also found that the 3'-end processing and NMD sensitivity can be regulated by the environmental conditions. In rich media (YAPD), both *PCA1* and *FRE2* produced two mRNA isoforms that varied in their 3'-UTR lengths. As described above, both *PCA1* mRNA isoforms were found to be indirectly regulated by NMD, while both *FRE2* mRNA isoforms were directly regulated by the pathway. *FRE1* and *FRE2* are two widely studied reductases. *FRE2* encodes a plasma membrane ferric and cupric reductase that reduces siderophore-bound iron and oxidized copper. *FRE1* also encodes both a ferric and cupric reductase of the plasma membrane and is regulated by Mac1p. In our previous studies, we found that the *FRE1* mRNA did not accumulate to higher levels in nmd mutants (Deliz-Aguirre et al., 2011). These results suggest that regulation of *FRE2* mRNAs by NMD is specific and is possibly physiologically significant.

Additionally, *MAC1* mRNA undergoes condition specific 3'-end processing and NMD-mediated degradation. In rich media *MAC1* produced one predominant transcript which did not accumulate to higher levels in nmd mutant strains, but was stabilized (Table 5.5 and Figure 5.5B). However, under low copper conditions, two predominant *MAC1* transcripts were detected (Figure 5.5D). Interestingly, both transcripts vary in their half-lives when compared to the half-life of *MAC1* mRNA in rich media (Figure 5.5D). Thus, under low copper conditions, both *MAC1* isoforms evade NMD-mediated

degradation over the 35 minute time period we normally use to determine mRNAs half-lives despite having the atypically long 3'-UTRs (Figure 5.5D). Stabilization of *MAC1* mRNAs in low copper conditions could be due to formation of mRNA secondary structure or binding of stabilizing trans- acting factors expressed in these conditions. As stated previously, *MAC1* is activated in low copper conditions. Therefore, we would anticipate the *MAC1* mRNA would be stabilized under this specific growth condition, when higher levels of *MAC1* are required. These results indicate that 3'-end processing and, also NMD regulation, can be condition specific. Thus, it is necessary for natural mRNAs regulated by NMD to have NMD targeting features and be in the correct environmental conditions to be regulated by the pathway. The environmental conditions that affect regulation of specific mRNAs by NMD may be related to the protein encoded by the mRNA. In this case since we are investigating mRNAs involved in copper homeostasis, copper levels in the media were found to influence regulation of mRNAs by NMD.

NMD-mediated regulation of a fraction of natural mRNAs involved in the same cellular process occurs as demonstrated here. Further research categorizing natural mRNAs regulated by NMD into functional categories will provide insight into the functional relationships of mRNAs regulated by the pathway. In addition, identification of the features that trigger the degradation of functionally related mRNAs will enable understanding of whether there is coordination in the regulation of specific mRNAs by NMD. Furthermore, determination of the environmental influence on the degradation of functionally related mRNAs by the pathway will reveal the physiological consequences associated with the regulation of specific mRNAs.

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CHAPTER SIX

NMD Targeting Mechanisms of Functionally Related mRNAs

ABSTRACT

The nonsense-mediated mRNA decay (NMD) pathway is a pathway that regulates mRNAs harboring premature termination codons as well as some natural mRNAs. Natural mRNAs are degraded by NMD due to the presence of NMD targeting features. One of these features is an atypically long 3'-UTR. A long 3'-UTR has been shown to trigger NMD in eukaryotic organisms including *Saccharomyces cerevisiae*. Analysis of a subset of functionally related mRNAs that are regulated by the pathway found that some of these mRNAs are direct targets, while others are indirect (Chapter Five). *S. cerevisiae FRE2* mRNAs are directly regulated by NMD and contain long 3'-UTRs. Alternatively, *COX23* mRNA contains a long 3'-UTR but is indirectly regulated by NMD. Here, we investigated the sequence elements that target the *FRE2* transcripts to NMD. We found that the *FRE2* 3'-UTR contributes to the NMD-mediated degradation of the mRNAs, but is not sufficient to trigger NMD independently. Additionally, we determined that the *COX23* 3'-UTR is sufficient to trigger NMD. This finding suggests that a sequence element within the *COX23* mRNA is preventing the mRNA from NMD.

INTRODUCTION

The nonsense-mediated mRNA decay (NMD) pathway is a highly conserved mRNA degradation pathway. NMD is conserved in all tested eukaryotes from yeast to humans and functions as an important regulator of gene expression. In *Saccharomyces cerevisiae*, 5-10% of the transcriptome is affected if nmd is non-functional [1, 2]. Similar results were observed in studies involving *Drosophila melanogaster* and humans [3-5].

NMD was first identified as a pathway that degraded premature termination codon (PTC) containing mRNAs to prevent the synthesis of truncated proteins. NMD is also recognized as a pathway that degrades natural mRNAs that encode fully functional proteins. NMD-mediated degradation of natural mRNAs has been observed in a variety of organisms including *S. cerevisiae*, *D. melanogaster*, *Caenorhabditis elegans*, and humans. A study by Guan *et al*. suggested that over 600 naturally occurring transcripts are regulated by the NMD pathway in *S. cerevisiae* [2].

Natural mRNAs are targeted by NMD through a variety of documented mechanisms in eukaryotes. In *S. cerevisiae*, there are five known NMD targeting features: translation of an upstream open reading frame (uORF) [1, 2, 6, 7], out of frame initiation of translation (also known as "leaky scanning") [2, 8], inefficiently sliced pre-mRNAs [2, 9], endogenous ribosomal frameshift signals [10], and an atypically long 3'-untranslated region (UTR) [11-13]. An atypically long 3'-UTR has been shown to target an mRNA to NMD-mediated degradation in yeast, *D. melanogaster*, plants and humans. Thus, it is a conserved targeting feature. In *S. cerevisiae*, 3'-UTRs range from 50-200nts in length with a median size of 121nts. mRNA with 3'-UTRs that are 350nts or longer are expected to be degraded by NMD [11]; however, it is important to note that the

presence of a long 3'-UTR, or any other known NMD targeting feature, does not always activate NMD [11, 14].

Previous studies have shown that the NMD pathway may differentially regulate gene expression in response to environmental stressors. For example, phenotypic studies in *S. cerevisiae* revealed that cells with an inactive NMD pathway are more tolerant to high copper concentrations [13]. This finding prompted us to evaluate mRNAs involved in copper homeostasis to determine if they were directly regulated by the NMD pathway. Out of the eight mRNAs involved in that study, *MAC1, CTR2, COX19* and *FRE2* were determined to be direct NMD targets (Chapter Five). mRNAs that have a statistically significant difference in half-life between wild-type and nmd mutant strains are classified as direct NMD targets. On the other hand, indirect NMD targets accumulate in nmd mutants in steady-state experiments, but do not have a statistically significant difference between half-lives of wild-type and nmd mutants. In our previous study, we found that the long 3'-UTRs of *CTR2* and *COX19* contribute to the regulation of these mRNAs by NMD (Chapter Five)[15]. Therefore, we expect that *FRE2* is also targeted to NMD by the long 3'-UTRs. *FRE2* mRNA produces two transcripts under standard growth conditions, with varying 3'-UTR lengths (700nt and 1400nt, respectively). *FRE2* is also predicted to be subject to -1 PRF, which could additionally target the mRNAs to the NMD pathway. Our previous study also determined that a subset of mRNAs involved in copper homeostasis and regulated in an NMD-dependent manner are indirect targets. One of these mRNAs was the *COX23* mRNA. We identified *COX23* mRNA as an indirect NMD target despite the presence of an atypically long 3'-UTR of 300 nt. (Chapter Five).

Here, we determined the extent to which each of the long 3'-UTRs is sufficient to target *FRE2* and *COX23* to NMD by replacing them with the short *CYC1* 3'-UTR that is not known to trigger NMD. We also aimed to determine if the *FRE2* 3'-UTR is sufficient to target *FRE2* to NMD or, alternatively, demonstrate that other, additional targeting features are involved. This study examined the extent to which the *COX23* 3'-UTR is sufficient to target mRNAs for NMD-mediated degradation, despite the observation that the mRNA is an indirect NMD target in rich media (Chapter Five). This would allow us to understand if the *COX23* mRNA 3'-UTR was too short to target the mRNA for NMD, whether it is in the incorrect context in *COX23* or whether *COX23* contains features that enable the mRNA to escape degradation by NMD in the conditions tested.

MATERIALS AND METHODS

Yeast Strains

All *Saccharomyces cerevisiae* strains and genotypes used in this study are listed in Table 6.1. All yeast strains were maintained and grown using standard techniques [16].

Table 6.1. *Saccharomyces cerevisiae* strains used in this study.

Yeast Strain	Genotype	Source
W303	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-101*	[17]
AAY320	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1::URA3 (upf-Δ2)*	[18]
AAY334	*a, ADE2, ura3-1 or ura3-52, his3-52, his3-11,15, trp1-1, leu2-3,112, rpb1-1*	[18]
AAY335	*a, ADE2, ura3-1 or ura3-52, his3-52, his3-11,15, trp1-1, leu2-3,112, rpb1-1, upf1-Δ2, (URA3)*	[18]

RNA Methods

S. cerevisiae total RNA was utilized for all accumulation and half-life experiments. The yeast cells were harvested at mid-log phase as described in *Peccarelli and Kebaara, 2014* [19]. RNA was extracted from harvested cells via the hot phenol method. Equal concentrations of RNA (15 μg) were run on 1.0% agarose-formaldehyde gels for all accumulation and half-life northerns. Total RNA was transferred to GeneScreen Plus® (PerkinElmer, Boston, MA) nitrocellulose membranes using the NorthernMax™ Complete Northern Blotting kit (Life Technologies, Grand Island, NY) transfer protocol. Northern blots were probed with oligolabeled DNA probes that were labeled with [α-^{32}P]dCTP using the RadPrime DNA Labeling System (Life Technologies, Grand Island, NY). All probes were prepared through digestion of DNA fragments from plasmids or by PCR. All northern blots were phosphorImaged™ using a Typhoon Phosphorimager (Amersham Pharmacia Biotech, Inc.). In every case, *CYH2* pre-mRNA was used as an NMD control to confirm the NMD phenotype of all of the yeast strains. *CYH2* pre-mRNA is a known NMD target, while *CYH2* mRNA is not [9]. *SCR1* RNA was used as a loading control to normalize all mRNA levels. *SCR1* is not sensitive to NMD and is an RNA polymerase III transcript. All northerns were quantified using ImageQuant software. Sigmaplot 2000, Version 6.10 software was used to calculate half-lives as described in *Peccarelli and Kebaara, 2014* [19].

3'RACE

3'RACE was done as described in *Kebaara et al., 2012* [20] using the 3'RACE System for Rapid Amplification of cDNA Ends kit (Life Technologies, Grand Island, NY). Yeast total RNA was employed to make cDNA using SuperScript™II RT (Life

Technologies, Grand Island, NY). The cDNA was utilized as the template for all primary PCR reactions. Primary reactions used the Abridged Universal Amplification Primer (AUAP) from the 3'RACE kit in combination with gene specific primers. Primary PCR product served as a template for the nested PCR reactions. All nested PCR reactions utilized gene specific primers (Table 6.2). PCR products for both primary and nested reactions were run on 1.5% agarose gels.

Table 6.2. Primary and nested gene-specific primers used in 3'RACE analysis.

Construct	Primary Gene-specific Primer	Nested Gene-specific Primer
CYC1FRE2 3'-UTR	5’- TGAAAAAAGCCTGTGAGTAA -3’	5’-TCATTTTTTACTTAAAACTA -3’
FRE2CYC1 3'-UTR	5’-AAGAGTATCAATGCTGGTGA-3’	5’- CTGTGAGTAAACAGGCCCCT -3’
CYC1COX23 3'-UTR	5’-TGAAAAAAGCCTGTGAGTAA -3’	5’-GGCAGCCTTTTCTTCTTGTT -3’
COX23CYC1 3'-UTR	5’-ACAGACAGCAGTGGGAATGA-3’	5’-CTGTGAGTAAACAGGCCCCT -3’

DNA Methods

To create a fusion construct for an mRNA with an atypically long 3'-UTR, the long 3'-UTRs from *COX23* and *FRE2* were amplified by PCR. Next, the 5'-UTR and ORF of a second gene, *CYC1*, was amplified by PCR. *CYC1* is not an NMD target. Third, ligation mediated PCR of the two products was performed in order to make fused genes. To create *CYC1FRE2* 3'-UTR, the *CYC1* 5'-UTR and ORF were fused to 1450nt from the *FRE2* 3'-UTR. Alternatively, to create *FRE2CYC1* 3'-UTR, the 5'-UTR and ORF of *FRE2* were fused to 350 nt from the *CYC1* 3'-UTR. To create *CYC1COX23* 3'-UTR, the *CYC1* 5'-UTR and ORF were fused to 350 nt from the *COX23* 3'-UTR. The

COX23CYC1 3'-UTR was created by fusing the *COX23* 5'-UTR and ORF to 350 nt from the *CYC1* 3'-UTR. The fusion constructs were then inserted into the TOPO cloning vector according to manufacturer's instructions, and sent for sequencing to verify that the correct fusion construct was created. *CYC1FRE2* 3'-UTR and *FRE2CYC1* 3'-UTR in TOPO were digested with *BamHI* and *NotI* prior to ligation to the yeast vector pRS425. *CYC1COX23* 3'-UTR and *COX23CYC1* 3'-UTR were digested with *BamHI* and *SacI* before ligation into pRS425.

RESULTS AND DISCUSSION

A long 3'-UTR is not sufficient to target an mRNA to NMD, but contributes to the degradation of the *FRE2* mRNAs

We hypothesized that both *FRE2* transcripts are degraded by NMD due to the presence of atypically long 3'-UTRs (700 and 1400 nt, respectively). This prediction was tested through the formation of two fusion constructs. Previous studies have demonstrated that the replacement of an NMD-insensitive mRNA's short 3'-UTR with an atypically long 3'-UTR can cause the mRNA to become subject to NMD [11, 15]. In order to determine the mechanism behind the NMD-mediated degradation of the *FRE2* transcripts, we replaced the 3'-UTR of *CYC1* with 1450nt from the *FRE2* 3'-UTR, creating the *CYC1FRE2* 3'-UTR construct (Figure 6.1A).

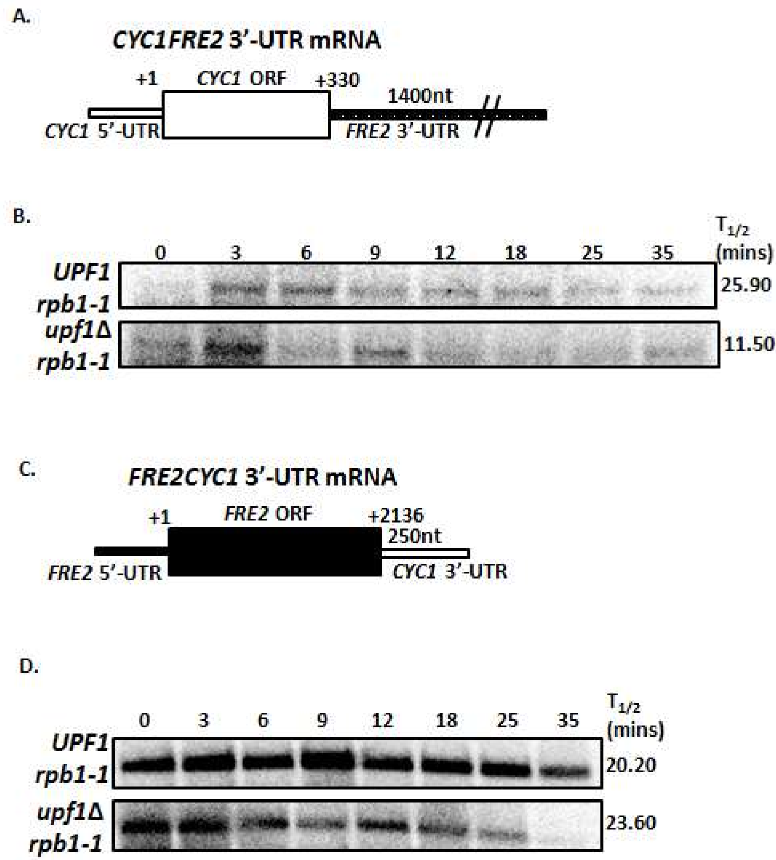

Figure 6.1. The *FRE2* 3'-UTR is not sufficient to target an mRNA to NMD, but contributes to the degradation of the *FRE2* mRNAs. Schematic representation of the *FRE2CYC1* 3'-UTR mRNA (A). Representative northern blot of the *FRE2CYC1* 3'-UTR half-lives (B). Schematic representation of the *CYC1FRE2* 3'-UTR mRNA (C). Representative northern blot of the *CYC1FRE2* 3'UTR mRNA half-lives (D). mRNA half-lives were measured with total RNA extracted from wild-type strain AAY334 (*UPF1 rpb1-1*)[18] and nmd mutant strain (AAY335) (*upf1Δ rpb1-1*)[18]. All yeast cells were grown in Synthetic complete media lacking leucine, and were harvested at eight time points over thirty-five minutes after transcription inhibition. Specific time points are indicated above the half-life northerns. The half-lives were determined using SigmaPlot and are shown to the right of the northern blots. All half-life measurements are an average of at least three independent experiments. *CYH2* and *SCR1* were used as controls. *CYH2* pre-mRNA was used as an NMD phenotype control because *CYH2* pre-mRNA is degraded by NMD. *SCR1* was used as a loading control for all northern blots. *SCR1* is an RNA polymerase III transcript that is not regulated by NMD.

We anticipated that *CYC1FRE2* 3'-UTR mRNA would be stabilized in nmd mutants relative to wild-type yeast strains and thus, would be directly regulated by NMD. *CYC1FRE2* 3'-UTR mRNA did not show a statistically significant difference in half-life in the wild type strain (25.90±8.2 minutes) compared to the nmd mutant strain (11.5±5.0 minutes) (Figure 6.1B, Table 6.3). These results suggest that *CYC1FRE2* 3'-UTR mRNA

is not regulated by NMD under the conditions tested. Although the *FRE2* 3'-UTR used in this study was very long, there are a few possibilities as to why it was not sufficient to cause *CYC1* mRNA to be degraded by NMD. First, the ORF lengths for these mRNAs vary greatly. Specifically, *CYC1* has a relatively short ORF of 330 nts. Alternatively, *FRE2* has a very long ORF of 2,136nt in length. We have previously shown that a long ORF can act cooperatively to inhibit NMD [15]. It is possible that the long ORF of *FRE2* is further stabilizing the transcript in nmd mutants. It is also possible that elements within the endogenous *FRE2* ORF or 5'-UTR may be required for NMD to occur, and that the long 3'-UTR is not sufficient to trigger degradation alone. Additionally, endogenous *FRE2* and *CYC1FRE2* 3'-UTR utilize different promoters. Promoter regions can greatly influence how a gene is expressed. For example, the *CTR2* mRNA driven by the *GPD* promoter showed altered NMD sensitivity and mRNA processing in comparison to *CTR2* driven by the *CTR2* promoter [15]. It is possible that the *CYC1* promoter found on the *CYC1FRE2* 3'-UTR mRNA is regulating transcript expression and potentially preventing degradation despite the presence of an atypically long 3'-UTR.

Table 6.3. Steady-state mRNA accumulation and half-lives of the *FRE2* and *COX23* mRNA fusion. Wild-type (W303) and nmd mutants (AAY320) were used in mRNA steady-state accumulation measurements, while AAY334 and AAY335 transformed with the respective constructs were used to determine half-lives. All yeast strains used were grown under standard conditions in complete minimal media lacking leucine [16]. The steady-state and half-life experiments were done in triplicate.

Standard Name	Relative mRNA accumulation (*upf1*Δ/*UPF1*)	mRNA half-life (mins) *UPF1*	mRNA half-life (mins) *upf1*Δ
CYC1FRE2 3'-UTR	ND	25.90±8.20	11.50±5.00
FRE2CYC1 3'-UTR	1.03±0.26	20.20±10.40	23.60±15.50
CYC1COX23 3'-UTR	2.35±0.68	14.20±5.50	20.70±3.50
COX23CYC1 3'-UTR	0.6±0.44	8.00±2.83	ND

ND, not determined

Although the 1400nt *FRE2* 3'-UTR was not sufficient to target *CYC1* mRNA for NMD, we wanted to observe whether or not the *FRE2* transcripts would be regulated by NMD in the absence of long 3'-UTRs. We predicted that *FRE2* would still be regulated by NMD despite the removal of the 3'-UTR. As mentioned previously, *FRE2* has a second, potential NMD targeting mechanism and may be subject to -1 programmed ribosomal frameshifting. If active, the second nmd targeting mechanism may cause the *FRE2* transcripts to continue to be degraded by NMD, albeit at lower levels than before. To test this prediction, we created the *FRE2CYC1* 3'-UTR fusion construct, which contains the 5'-UTR and ORF of *FRE2* along with the 250nt *CYC1* 3'-UTR (Figure 6.1C).

The *FRE2CYC1* 3'-UTR fusion mRNA did not accumulate to significantly higher levels in nmd mutants (1.03±0.26) (Table 6.3). Likewise, no statistically significant difference was observed between the *FRE2CYC1* 3'-UTR fusion mRNA half-lives. In detail, the half-life for the wild-type strain was 20.20±10.40 minutes compared to 23.60±15.50 minutes in the nmd mutant strain (Figure 6.1D and Table 6.3). This data suggests that *FRE2CYC1* 3'-UTR is not regulated by NMD under these conditions. Additionally, these results suggest that the 3'-UTR is required for *FRE2* mRNAs to be degraded by NMD. Removal of the 3'-UTR causes the mRNAs to escape degradation by NMD; however, this 3'-UTR is not sufficient to target *CYC1* to NMD. These results indicate that another feature must be present within the *FRE2* 5'-UTR and/or ORF that functions cooperatively with the 3'-UTR to target *FRE2* mRNA to NMD.

The *COX23* long 3'-UTR is sufficient to target an NMD-insensitive transcript for NMD

Despite having an atypically long 3'-UTR of 300nt, we found that the *COX23* mRNA is indirectly regulated by the NMD pathway when yeast cells are grown in rich media (Chapter Five and Figure 6.2). It is possible that the *COX23* mRNA is being stabilized by an additional feature that is preventing its degradation or, alternatively, that the 3'-UTR is not long enough or is in the incorrect context to induce NMD. To test this hypothesis, we first determined if the *COX23* 3'-UTR is sufficient to target an NMD insensitive mRNA to the pathway. To do this, we generated the *CYC1COX23* 3'-UTR fusion construct, which contains the 5'-UTR and ORF from *CYC1* and the *COX23* 3'-UTR (Figure 6.3A).

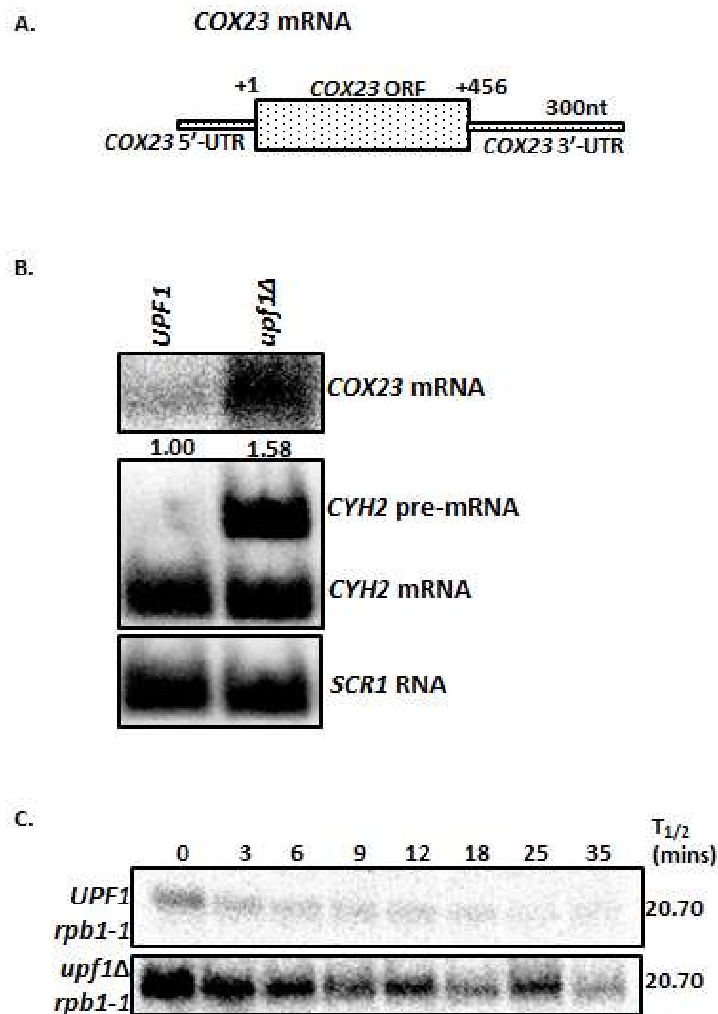

Figure 6.2. ***COX23*** **mRNA is indirectly regulated by the NMD pathway.** Schematic representation of the *COX23* mRNA (A). Representative northern blot of the *COX23* mRNA steady-state accumulation levels (B) and half-lives (C). All yeast cells for (B) and (C) were grown in YAPD and harvested as described in Figure 6.1. Steady-state and half-life measurements are an average of at least three independent experiments. The half-lives were determined using SigmaPlot. *CYH2* and *SCR1* are used as controls as described in Figure 6.1.

CYC1COX23 3'-UTR mRNA accumulated to significantly higher levels in nmd mutants relative to wild-type strains. Specifically, the transcript accumulated 2.35±0.68 fold in the mutant strain (Figure 6.3B and Table 6.3). In addition, the half-life of *CYC1COX23* 3'-UTR mRNA in the wild-type strain was 14.20±5.50 minutes (Figure 6.3C and Table 6.3). Alternatively, the half-life in the mutant strain was 20.70±3.50

minutes (Figure 6.3C and Table 6.3). Although the values were somewhat close, the difference between the two half-lives is statistically significant, indicating that *CYC1COX23* 3'-UTR is directly regulated by NMD. These results suggest that the *COX23* 3'-UTR is sufficient to target a NMD-insensitive transcript to NMD. This observation supports the idea that the *COX23* mRNA evades degradation by NMD. It is possible that the 3'-UTR may be in the incorrect context within the *COX23* mRNA, potentially causing this evasion. Further studies are needed in order to determine what feature(s) is potentially stabilizing this mRNA.

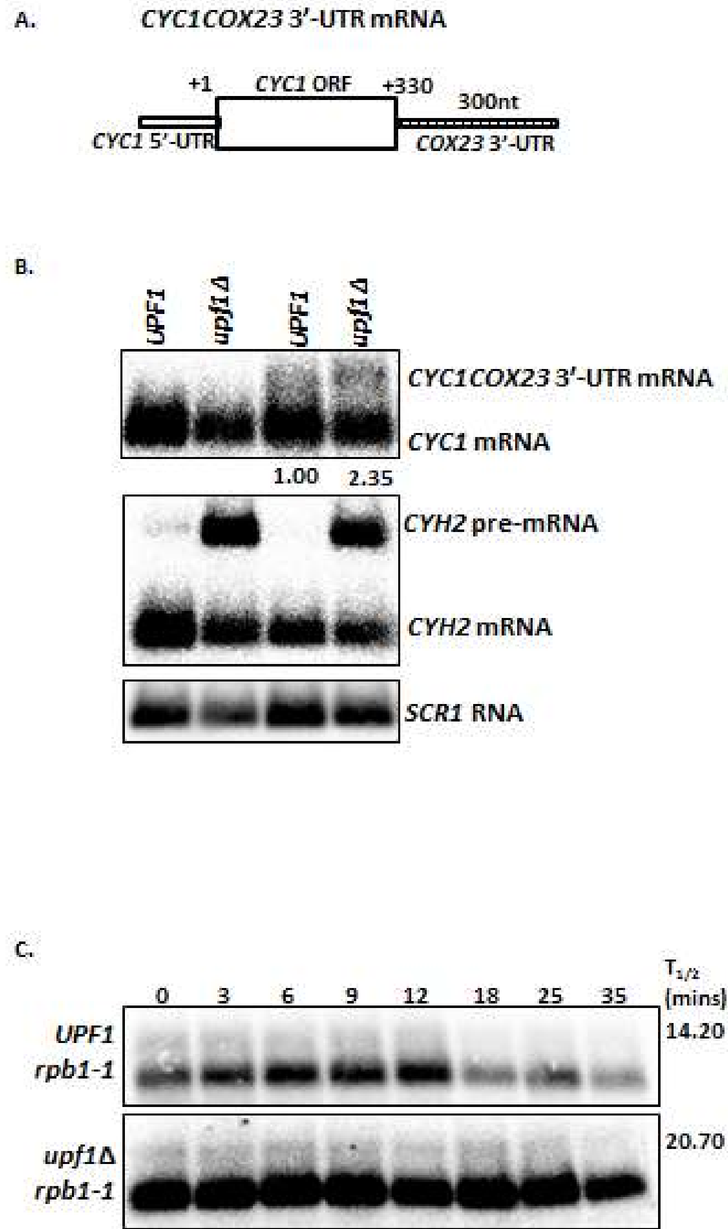

Figure 6.3. The *COX23* 3'-UTR is sufficient to target *CYC1* mRNA to NMD. Schematic representation of *CYC1COX23* 3'-UTR mRNA (A). Representative northern blot of the *CYC1COX23* 3'-UTR steady-state accumulation levels (B) and half-lives (C). All yeast cells for (B) and (C) were grown in Synthetic media lacking leucine, and were harvested as described in Figure 6.1. Steady-state and half-life measurements are an average of at least three independent experiments. The half-lives were determined using SigmaPlot. *CYH2* and *SCR1* are used as controls as described in Figure 6.1.

In order to determine the effects removing the 3'-UTR from *COX23* mRNA would have on the regulation of the mRNA by NMD, the *COX23CYC1* 3'-UTR construct was generated. This construct contains the 5'-UTR and ORF of *COX23* fused with 350 nt 3'-UTR from *CYC1* (Figure 6.4A).

Because endogenous *COX23* was not directly regulated by NMD under the conditions tested (Chapter Five and Figure 6.2), we did not expect *COX23CYC1* 3'-UTR to be regulated by NMD. This hypothesis was supported by our steady-state accumulation and half-life experiments. In the steady-state accumulation, *COX23CYC1* 3'-UTR did not accumulate to significantly higher levels in nmd mutants (0.6 ± 0.44) (Table 6.3). The half-life of *COX23CYC1* 3'-UTR mRNA was 8.00 ± 0.42 minutes in the wild-type yeast strains (Figure 6.4B and Table 6.3). We were unable to detect *COX23CYC1* 3'-UTR in the nmd mutant strain; however, endogenous *CYC1* could be detected. These results suggest that *COX23CYC1* 3'-UTR is much more abundant and more stable in the wild-type strain; therefore, we conclude that *COX23CYC1* 3'-UTR is not regulated by the NMD pathway under these conditions. If *COX23CYC1* 3'-UTR were directly regulated by the pathway, we would expect a statistically significant difference in half-life between the wild-type and nmd mutant yeast strains. Furthermore, we would expect the nmd mutant yeast strains transformed with *COX23CYC1* 3'-UTR to be stabilized in nmd mutants and to have a longer half-life than the wild-type strains. The results seen here were not unexpected since endogenous *COX23* was not found to be directly regulated by NMD under standard conditions (Chapter Five).

A. ***COX23CYC1*** **3'-UTR mRNA**

B.

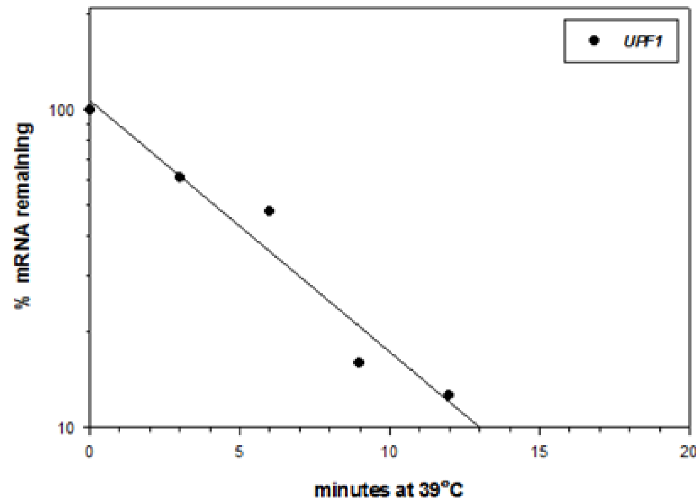

Figure 6.4. *COX23CYC1* 3'-UTR fusion mRNA is not regulated by NMD. Schematic representation of *COX23CYC1* 3'-UTR mRNA (A). A representative graph showing *COX23CYC1* 3'-UTR mRNA decay in the wild-type *(UPF1)* yeast strain (B).

Conclusions

In summary, the results presented here reveal that the regulation of two mRNAs involved in copper homeostasis by NMD is highly complex. Specifically, we found that the *FRE2* transcripts are directly regulated by NMD due to the presence of both an atypically long 3'-UTR and an additional feature. Removal of the endogenous 3'-UTR rendered *FRE2* immune to NMD, and suggests that the 3'-UTR functions cooperatively with another feature within the FRE2 mRNA. Furthermore, we anticipated that the *FRE2* 3'-UTR would be sufficient to target other mRNAs to NMD as well; however, this was

not the case. The *CYC1FRE2* 3'-UTR fusion mRNA was not regulated by NMD. This result indicates that the FRE2 3'-UTR is not sufficient to target an mRNA for NMD.

Additionally, we found that the *COX23* 3'-UTR is sufficient to target an NMD-insensitive transcript to NMD. This result was surprising since endogenous *COX23* is not directly regulated by NMD in the conditions tested (Chapter Five). We anticipate that a feature within the promoter or ORF of *COX23* is stabilizing the transcript in wild-type strains. Further investigation into the roles the promoter and ORF play in this regulation is needed to explain what is causing *COX23* to be immune to NMD.

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CHAPTER SEVEN

Conclusion

Collectively, the goal of these studies was to contribute to the overall understanding of how NMD regulates natural mRNAs using *S.cerevisiae* as a model to investigate mRNAs involved in copper homeostasis. A subset of these natural mRNAs was categorized as direct and indirect NMD targets, the targeting mechanisms were investigated, and the influence that the environmental conditions have on the regulation of one mRNA was examined. The subset of mRNAs included *CTR2, CTR3, MAC1, COX23, CRS5, PCA1, FRE2*, and *COX19*. These mRNAs were previously identified as potential NMD substrates (Guan and others 2006; Johansson and others 2007). The eight mRNAs all encode proteins that have various roles in copper homeostasis.

Ctr2p encodes for a copper transporter of the vacuolar membrane. Continued studies on *CTR2* demonstrated that the regulation of this mRNA by NMD is highly complex. This regulation depends on the lengths of both the ORF and the 3'-UTR (Peccarelli and others 2014). Additionally, the *CTR2* studies suggest that the presence of multiple NMD targeting features may be additive. Specifically, when the long *CTR2* 3'-UTR is removed, the *CTR2* fusion mRNA was only partially stabilized, indicating that the 3'-UTR contributes to the degradation of *CTR2* mRNA by NMD (Peccarelli and others 2014). However, lengthening of the *CTR2* ORF in addition to removing the 3'-UTR rendered the mRNA insensitive to NMD (Peccarelli and others 2014). These results

suggest that the two targeting features are functioning cooperatively to control regulation and NMD sensitivity of the *CTR2* mRNA.

The *CTR2* studies described in Chapter Four also provide insight into how mRNA sensitivity to the NMD pathway is affected by the promoter region. The P_{GPD}*CTR2CYC1* and P_{GPD}*CTR2* transcripts were highly-expressed in comparison to endogenous *CTR2* with the *CTR2* promoter (Peccarelli and others 2014). Furthermore, the constructs with the *GPD* promoter showed increased sensitivity to NMD in comparison to endogenous *CTR2* (Peccarelli and others 2014). This sensitivity could be due to a stabilizing element in the *GPD* 5'-UTR, or due to the elevated expression of *CTR2* mRNA from the promoter. Additionally, additional *CTR2* fusion constructs driven by the *GPD* promoter produce only one transcript, whereas *CTR2* mRNA expressed from the *CTR2* promoter produces two transcripts of varying 3'-UTR lengths (Peccarelli and others 2014). This study shows that the promoter can affect NMD sensitivity as well as processing of an mRNA.

Next, in Chapter Five, the other seven mRNAs involved in copper homeostasis were evaluated. Out of the seven mRNAs, three were found to be direct targets of the NMD pathway. *COX19* and *FRE2* were found to be directly regulated by NMD. Both mRNAs accumulated in nmd mutant strains, and showed a statistically significant difference in half-lives. Through the creation of fusion constructs, the *COX19* 3'-UTR was shown to be sufficient to target an NMD insensitive transcript to NMD. The *COX19CYC1* 3'-UTR mRNA was directly regulated by NMD, but showed decreased sensitivity in comparison to endogenous *COX19* mRNA. These results indicate that the *COX19* 3'-UTR contributes to the degradation of *COX19* by NMD, but that a second

targeting mechanism must be present. *COX19* is predicted to be subject to leaky scanning, which may be the targeting mechanism responsible for the degradation of *COX19CYC1* 3'-UTR. The long 3'-UTR appears to function cooperatively with leaky scanning and/or an additional targeting mechanism.

MAC1 mRNA encodes for a copper-sensing transcription factor that regulates the genes required for high affinity copper transport. *MAC1* was also found to be directly regulated by the NMD pathway. Despite not accumulating to significantly higher levels in nmd mutants, half-life experiments revealed stabilization in nmd mutant strains. *MAC1* was further analyzed under low copper growth conditions. Under these conditions, alternative forms of *MAC1* mRNA with altered NMD sensitivity were observed. *MAC1* grown in low copper also showed increased stabilization in comparison to *MAC1* grown in standard conditions. This study shows that environmental conditions can affect NMD sensitivity as well as alternative 3'-UTR processing.

Out of the subset of mRNAs examined here, *COX23, PCA1*, and *CRS5* were all found to be indirectly regulated by NMD in the conditions tested. All three mRNAs accumulated to higher levels in nmd mutants, but showed no statistically significant difference in half-lives between wild-type and nmd mutant strains. *COX23* and *PCA1* were found to have atypically long 3'-UTRs, and alternatively, *CRS5* has no known NMD targeting feature. It is important to note that the presence of a long 3'-UTR does not guarantee NMD activation. Lastly, *CTR3* could not be detected by northern blot analysis. Although *COX23, PCA1,* and *CRS5* were all found to be indirect NMD targets under the conditions tested here, it is possible that these mRNAs may be directly regulated by NMD under different environmental conditions.

Chapter six includes additional studies involving *FRE2* and *COX23* mRNAs. This chapter aims to determine the targeting mechanism for the *FRE2* mRNAs. Specifically, the *FRE2* 3'-UTR was demonstrated to be insufficient to trigger NMD under the conditions tested. However, upon removal of the endogenous 3'-UTR of *FRE2*, the transcript was no longer regulated. These results indicate that the *FRE2* 3'-UTR contributes to the NMD phenotype seen in endogenous *FRE2*. However, there must be a sequence element or additional feature within the 5'-UTR and/or ORF of *FRE2* that works cooperatively with the 3'-UTR to prompt degradation. It is possible that the 3'-UTR can only trigger NMD when associated with a long ORF, or, that the *FRE2* promoter is required for degradation.

COX23 was not determined to be a direct NMD target under the conditions tested despite the presence of an atypically long 3'-UTR. As expected, *COX23* was not regulated upon removal of the endogenous 3'-UTR. Interestingly, the *COX23* 3'-UTR was found to be sufficient to target an NMD-insensitive transcript to the pathway. Taken together, these results indicate that *COX23* mRNA evades NMD-mediated degradation. It is possible that the *COX23* 3'-UTR may be in the incorrect context within the *COX23* mRNA, causing the transcript to avoid NMD.

Taken together, these studies provide new information about NMD-regulated gene expression. Additionally, this study is novel because it investigates the regulation of natural mRNAs that are functionally related. Previous studies examining natural mRNAs regulated by NMD investigated subsets of mRNAs that were not related by function. Specifically, the studies show that structural elements within an mRNA can greatly impact whether an mRNA is regulated by NMD. Both the promoter region and

the ORF length were shown to greatly impact NMD sensitivity of transcripts. Additionally, these studies demonstrate that multiple NMD targeting features can act cooperatively to affect NMD sensitivity. Removing one targeting mechanism may not render an mRNA insensitive to the pathway; however, it is likely that the transcript will display decreased sensitivity. Furthermore, these studies show that NMD sensitivity can also be condition specific. The *MAC1* studies provide an example of how changing environmental conditions can alter both NMD sensitivity and 3'-end processing.

The chapters presented in this dissertation provide information regarding copper homeostasis mRNAs. Some of the selected mRNAs involved in copper homeostasis were directly regulated by NMD, while others were indirectly regulated, indicating that regulation is often both transcript and condition specific. However, since multiple copper homeostasis mRNAs are directly regulated by NMD, one can conclude that NMD plays an important role in maintaining copper homeostasis.

Clinical Implications

The NMD regulation presented here indicates possible clinical implications for both NMD and copper related diseases. Numerous disorders and diseases are suggested to be caused by nonsense mutations. This causation appears to be relatively widespread. Specifically, previous studies suggest that over 2400 genetic disorders have at least one causative nonsense allele (Peltz and others 2013), and that around 20% of disease-associated basepair substitutions are a result of a nonsense allele (Mort and others 2008). It is likely that up-regulation or inefficient NMD of specific mRNAs is responsible for human diseases and disorders as well. As previously stated in Chapter One, NMD can enhance or reduce a clinical phenotype of specific diseases. The findings presented here

increase the general understanding of how NMD regulates natural transcripts as well as different ways the regulation can be affected, which is potentially useful when treating NMD-related disorders. Specifically, understanding the specific conditions that enhance or inhibit NMD can potentially allow physicians to alter the cellular environment in such a way that NMD activity is altered with the goal of favorably reducing the clinical phenotype of a disorder. Although this may be possible at some point in time, it is important to note that altering NMD activity is not transcript specific, and would instead alter NMD of all transcripts. This could potentially cause additional problems or produce undesirable consequences.

In humans, both copper toxicity and copper deficiency are known to cause disease. Wilson's disease is of genetic origin and is caused by copper toxicity. Additionally, elevated copper levels have been seen in various forms of cancers including prostate, breast, colon, and brain cancer. Alternatively, Menkes disease is a result of copper deficiency. At this point in time, most treatment options for both Wilson's and Menkes diseases involve the use of copper chelators or copper supplements (Tisato and others 2010). Copper chelators are used to remove excess copper within a cellular system. Copper chelation therapies are also being studied as a technique to treat neurodegenerative diseases including, but not limited to, Alzheimer's disease, Parkinson's, and CreutzfeldtJakob (Tisato and others 2010). Additionally, copper appears to be a required co-factor for angiogenesis (Tisato and others 2010).

The work presented here provides further insight into how copper excess or deficiency can occur at the molecular level. Specifically, if there are problems within the NMD pathway, copper homeostasis mRNAs that are typically regulated by NMD will be

present at higher than normal levels. This could potentially lead to copper toxicity or copper excess depending on the function of the mRNA in question. Furthermore, previous studies have shown that nmd mutants are more tolerant to copper toxicity than cells with a functional NMD pathway (Wang and others 2013). Specifically, *CTR2* is required for this copper tolerance phenotype (Wang and others 2013). It is possible that potential treatments involving up-regulating or inhibiting the NMD pathway, or treatments controlling the regulation of the specific copper homeostasis genes studied here, could be developed in the future to help treat a variety of diseases and cancers caused by atypical copper levels.

Future Studies

The studies described here provide ample opportunity for continued research. For example, it would be interesting to continue the work regarding the relationship between ORF length and atypically long 3'-UTRs. Future studies could determine the minimum ORF length necessary to elicit NMD, as well as the mechanism behind the stabilization observed in *CTR2* when the ORF was lengthened.

There is also opportunity for additional study utilizing the same eight mRNAs used here. These mRNAs do not encode essential genes; therefore, knock-out strains can be created. After creating knock-out strains, any physiological consequences from removing the genes can be observed by performing drop tests. *PCA1, COX23* and *CRS5* were all determined to be indirect targets under the conditions tested. Similar studies involving upstream transcription factors of these mRNAs could possibly determine the upstream, direct NMD target(s). Finding a direct target upstream would explain the steady-state accumulation seen for *PCA1, COX23* and *CRS5*. Another option for these

mRNAs would be to test them in low or high copper conditions. It is possible that these transcripts are directly regulated by NMD under different environmental conditions. Other direct targets such as *FRE2* could also be tested under different environmental conditions. As stated previously, *FRE2* produced two transcripts of varying 3'-UTR lengths. Changing the conditions may lead to alternative 3'-end processing and/or altered NMD sensitivity. Lastly, the *COX23* studies presented in Chapter Six show that the *COX23* 3'-UTR is sufficient to trigger NMD; however, endogenous *COX23* is not directly regulated by the pathway. Future studies could be conducted to determine the stabilizing element of *COX23* mRNA.

The observable relationship between genes involved in copper homeostasis and the NMD pathway opens the door for future study as well. Similar studies involving genes involved in iron homeostasis could be conducted to determine if the link between metal ion regulation and NMD is somewhat widespread. Iron homeostasis is a sensible choice because of its close association and regulation with copper. Specifically, there is some overlap in the genes involved in regulating copper and iron levels. For example, *PCA1* and *FRE2* mRNAs are thought to be involved in both copper and iron homeostasis.

Addendum

Since the publication of Chapter Two in *Eukaryotic Cell* in 2014, new information regarding -1 ribosomal frameshift signals was published. Chapter Two states that some mRNAs subject to -1 ribosomal frameshifting are regulated by NMD, and that this regulation had only been seen in yeast. Recently published data demonstrates that -1 ribosomal frameshifting can also trigger NMD in mammalian cells, indicating that this targeting mechanism is conserved across species (Belew and others 2014).

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