ABSTRACT

CTR2 and the Nonsense-Mediated mRNA Decay Pathway in *Saccharomyces cerevisiae*

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The nonsense-mediated mRNA decay (NMD) pathway recognizes and degrades mRNA with premature termination codon and some natural mRNA as well. *CTR2* is a natural mRNA degraded by NMD in *Saccharomyces cerevisiae*. The goals of this research were to identify the sequence features that target the *CTR2* mRNA for NMD and the physiological consequences resulting from this degradation. These goals were addressed by making fusion constructs and determining total cellular, cytoplasmic and vacuolar copper levels in wild-type and nmd mutant yeast cells. Features contribute to the NMD-mediated degradation of *CTR2* were identified. When cultured in medium with excess copper, nmd mutants accumulated significantly higher vacuolar copper levels than wild-type yeast cells, however nmd mutants accumulated significantly less cytoplasmic copper levels than that in wild-type yeast cells. These results are consistent with the inference that nmd mutants tolerate excess copper as a result of ctr2p transporting excessive copper from cytoplasm into vacuole.

CTR2 and the Nonsense-Mediated mRNA Decay Pathway in *Saccharomyces cerevisiae*

by

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CHAPTER ONE

Introduction

Nonsense-Mediated mRNA Decay

The nonsense-mediated mRNA decay (NMD) is a conserved mRNA decay mechanism that has been identified in all eukaryotic organisms examined so far. NMD was first discovered in *Saccharomyces cerevisiae*, which is the unicellular model eukaryote. We use yeast because yeast has a well-defined genetic system. NMD eliminates mRNAs that undergo aberrant translation termination so that the errors from transcription are reduced. Premature translation termination is an aberrant event that may trigger NMD (Amrani, N. *et al.*, 2006, Nicholson, P. *et al.*, 2010). NMD gets its name because it was first identified on mRNAs that posses premature termination codons. A Premature termination codon is caused by a nonsense mutation within the mRNA. Such degradation of mRNAs by NMD avoids production of truncated proteins, which may bring about dominant negative effect to the organism (Fig.1.1).

Upf1p, Upf2p and Upf3p are three up-frameshift proteins which are required for the NMD pathway. These core trans-acting factors are required for NMD in all eukaryotes; they were originally discovered in the yeast, *Saccharomyces cerevisiae* and later found in multicellular eukaryotes. In yeast, the translation termination event involves the interaction between the ribosome and two release factors, namely Sup35p (eRF3) and Sup45p (eRF1) (Frolova, L. *et al*, 1994; Stansfield, I. *et al.*,1995; Zhouravleva, G. *et al.*,1995). The Upf1p, Upf2p and Upf3p can interact with Sup35p (eRF3), while only Upf1p can interact with Sup45p (eRF1) (Czaplinski, K. *et al*., 1998;

Wang, W. *et al.*, 2001). The mRNA targets for NMD become selectively stable if any one of the core trans-acting factors is nonfunctional (He, F. *et al.*, 2003), and combined nonfunctional core factors have nonadditive effect (He, F., Brown, A.H. and Jacobson, A., 1997).

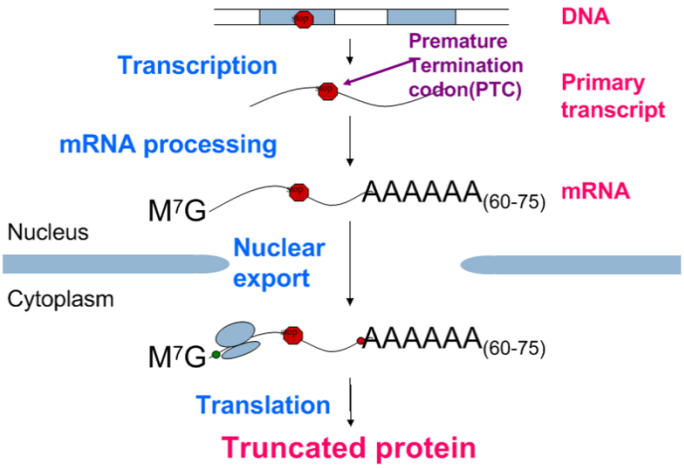


Fig. 1.1 Nonsense mediated mRNA decay. In some cases there can be a mutation in a gene leading to a premature termination codon on the mRNA. If the mRNA is transported to the cytoplasm and translated, it produces a truncated protein, which may bring about a dominant negative effect to the organisms.

In the process of normal translation termination, the termination codon binds with Sup35p (eRF3) and Sup45p (eRF1) which interact with the proximal Pab1p, these interactions signifies that there is a proper termination event and eventually leads to the release of the completed polypeptide and the ribosomal subunits. Normal translation termination is regarded as an efficient process, which is partially due to stimulation of Sup45p by the interaction of proximal Pab1 and Sup35p (Hoshino, S. *et al.*,1999; Cosson, B. *et al.*, 2002; Uchida, N. *et al.*, 2002; Uchida, N. *et al.*, 2003)(Fig. 1.2a). In the process of premature translation termination, the ribosome stalls at a premature termination codon, due to the absence of the proximal Pab1, it is thought to be an inefficient process and this allows the binding of Upf factors to interact with release factors, causing the

release of ribosomal subunits and the hydrolysis of uncompleted polypeptide. Once the Upf factors bind to the mRNA, they initiate the recruitment of Dcp1-Dcp2, the decapping enzyme complex and other factors, which facilitate the decapping and degradation of mRNAs (Maderazo, A.B. *et al.*, 2000; Weng, Y. *et al.*, 1996 (1) and (2)) (Fig. 1.2b).

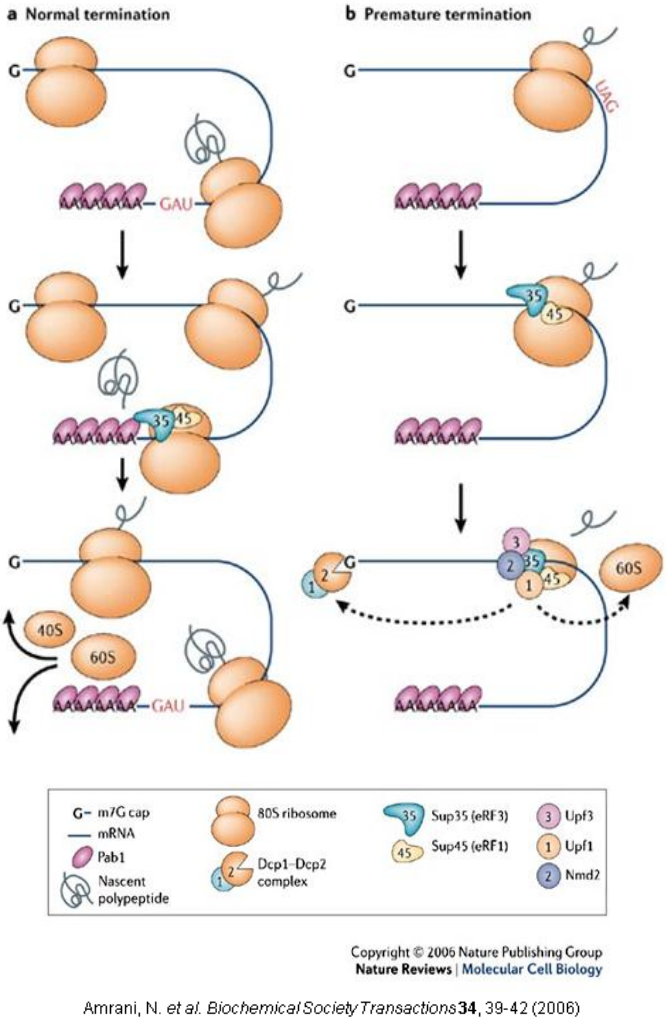


Fig. 1.2 The difference between normal termination and premature termination. (a) The normal termination event involves the interaction between proximal Pab1p, release factors Sup35p and Sup45p. (b) The faux 3'-UTR model. The premature termination lacks the proximal Pab1, making it possible for the Upf proteins to bind to the release factors, and the recruitment of Upf proteins induces the degradation of mRNAs by NMD.

The NMD mechanism widely affects organisms ranging from yeast to humans. Genome-wide analysis in *Saccharomyces cerevisiae, Drosophila melanogaster* and humans indicated that approximately 5-10% of the transcriptome is affected by NMD. Although not all, the accumulations of most natural mRNAs affected by NMD are increased in NMD mutants (He, F. *et al.*, 2003; Lelivelt, M.J. and Culbertson, M.R., 1999; Guan, Q. *et al.*, 2006; Johansson, M.J.O. *et al.*, 2007; Mendell, J.T. *et al.*, 2004; Rehwinkel, J. *et al.*, 2005). Similarly, orthologs of NMD genes (*UPF1, UPF2 and UPF3*) have been identified in *Caenorhabditis elegans* (Cali, B.M. *et al.*, 1999) and other organisms. It was demonstrated that there are *UPF1*, *UPF2* and *UPF3* orthologs in human similar to yeast Upf proteins based on sequence analysis (Perlick, H.A. *et al.*, 1996; Applequist, S.E. *et al.*, 1997), and the production of dominant negative hUpf proteins proved that Upf proteins are conserved in humans (Sun, X. *et al.*, 1998).

Natural mRNA Targeted by NMD

Natural mRNAs, lacking premature termination codons can also be potential targets for NMD (He, F. *et al.*, 2003; Lelivelt, M.J. and Culbertson, M.R., 1999; Guan, Q. *et al.*, 2006; Johansson, M.J.O. *et al.*, 2007; Mendell, J.T. *et al.*, 2004; Rehwinkel, J. *et al.*, 2005 Kebaara, B.W. and Atkin, A.L., 2009). Since these are natural mRNAs lacking premature termination codons and regulated by NMD, there are sequence features within these mRNAs that cause them to be degraded by NMD. In *S. cerevisiae*, there are five features that may trigger degradation of natural mRNAs by NMD. First, translation of upstream Open Reading Frames (uORFs) targets mRNAs for NMD (Guan, Q. *et al.*, 2006; Gaba, A. *et al.*, 2005). Second, mRNAs with out-of-frame AUG codons, which consequently lead to premature translational termination also referred to as leaky

scanning are targeted by NMD (Guan, Q. *et al.,* 2006; Welch, E.M. and Jacobson, A., 1999). Third, NMD targets inefficiently spliced pre-mRNAs that are transported to the cytoplasm (He, F. *et al.,* 1993). Fourth, endogenous ribosomal frameshift signals work as destabilizing elements and make mRNAs targets for NMD (Belew, A.T. *et al.,* 2011). Finally, *PGA1* mRNA is targeted to NMD due to the atypically long 3'-UTR of this mRNA (Kebaara, B.W. and Atkin, A.L., 2009). It has also been demonstrated that natural mRNAs with atypically long 3'-UTR are degraded by NMD in humans (Singh, G. *et al.,* 2008; Eberle, A.B. *et al.,* 2008; Yepiskoposyan, H. *et al.,* 2011). Therefore, this suggests that the mechanisms of degradation of mRNAs by NMD may be conserved amongst organisms. There might be some other unidentified features that target natural mRNAs for NMD.

A feature that targets natural mRNAs for NMD-mediated degradation mentioned above is mRNAs that have atypically long 3'-UTRs. *S. cerevisiae* mRNA 3'-UTRs tend to be short, they range in size from 50-200 nucleotides, with a median length of 121 nucleotides (Graber, J.H. *et al.,* 1999). A number of *S. cerevisiae* mRNAs are produced with different 3'-UTR lengths due to alternative 3'-end processing of the same pre-mRNA. *CTR2* is such an example, its protein product is involved in copper metabolism and its pre-mRNA undergoes alternative 3'-end processing. *CTR2* pre-mRNA undergoes alternative 3'-end processing producing two mRNA isoforms with 300 nt and 2 kb long 3'-UTRs. It was previously shown that if 3'-UTRs are longer than 350 nt, they can target mRNA for NMD-mediated degradation (Kebaara, B.W. and Atkin, A.L., 2009). In view of that fact *CTR2* mRNA isoforms have atypically long 3'-UTRs, which may target the *CTR2* mRNAs for NMD-mediated degradation.

Physiological Consequences that Result from the Regulation of Natural mRNAs by NMD

The regulation of natural mRNAs by NMD also has physiological effects. Deficiency in the NMD pathway is not lethal to *S. cerevisiae*, rather nmd mutant yeast cells show respiratory defects (Leeds, P. *et al.*, 1991; Altamura, N. *et al.*, 1992). In yeast, the telomere length and telomeric chromatin are affected by deleting *UPF* genes, since NMD regulates the levels of *STN1, TEN1* and *EST2* mRNA, which produce in vivo regulators of telomerase and telomerase catalytic subunits (Dahlseid, J.N. *et al.*, 2003; Dahlseid, J.N. *et al.*, 1993; Lew, J.E. *et al.*, 1998). The growth rate of nmd mutants is also decreased on lactate, this is partially due to over-expression of *ADR1*, which encodes a transcriptional factor involved in aerobic oxidation of non-fermentable carbon sources, including lactate (Taylor, R. *et al.*, 2005). Also, it has been shown that nmd mutants are sensitive to Calcoflour White, which disrupts the cell wall. This sensitivity to Calcoflour White is partly due to the degradation of *PGA1* mRNA by NMD (Kebaara, B.W. and Atkin, A.L., 2009). Addition of arginine has also been shown to induce degradation of *CPA1* mRNA in the wild-type, but not in the nmd mutants. This is due to arginine causing ribosomes to stall at an upstream open reading frame (uORF) termination codon, leading to NMD-mediated degradation of the *CPA1* mRNA (Gaba, A. *et al.*, 2005). Moreover, some alternative transcripts are regulated by thiamine starvation and degraded by the NMD mechanism (Johansson, M.J.O. *et al.*, 2007).

Besides *S. cerevisiae*, NMD also causes physiological consequences in other organisms. In *C. elegans* deleting genes that are required for NMD give rise to reduced offspring numbers and morphogenetic changes of genitalia (Hodgkin, J. *et al.*, 1989; Pulak, R. and Anderson, P., 1993). Previous research in *Arabidopsis* indicates that NMD

regulates plant development and survival (Arciga-Reyes, L. *et al.*, 2006). In *D. melanogaster*, *UPF1* and *UPF2* are required for proper expression of many wild type genes during development and larval viability (Metzstein, M.M. and Krasnow, M.A., 2006). Knockdown of genes that are required for NMD in *Drosopilia* or mammalian cells induce cell cycle arrest, which indicates that NMD plays a role in cell cycle progression (Rehwinkel, J. *et al.*, 2005; Azzalin, C.M. and Lingner, J., 2006). In mice, *UPF1* and *UPF2* are required for embryonic viability (Medghalchi, S.M. *et al.*, 2001; Weischenfeldt J. *et al.*, 2008). Furthermore, in zebrafish, it has been shown that NMD effectors are essential for embryonic development and survival (Wittkopp, N. *et al.*, 2009).

The natural mRNA we studied is the *CTR2* mRNA. *CTR2* encodes a copper transporter of vacuolar membrane that controls the flux of copper into the vacuole (Rees, E.M. *et al.*, 2004). *CTR2* mRNA undergoes alternative 3'-end processing and this alternative 3'-end processing is responsive to copper levels in the media (Guisbert, K.S.K. *et al.*, 2007). Besides *CTR2*, other genes like *CTR1* and *CTR3* are also involved in copper transport (Deliz-Aguirre R, *et al.*, 2011). *CTR1* and *CTR3* are copper transporters localized to the plasma membrane, whereas *CTR2* is localized to the vacuolar membrane (Rees, E.M. *et al.*, 2004). *CTR1* and *CTR3* encode high affinity transporters and they facilitate copper uptake from the environment (Eide, D.J., 1998; Peña, M.M. *et al.*, 1999). Before copper uptake, Cu^{2+} is reduced by a reductase located on the plasma membrane into Cu^{+}. If the levels of intracellular copper are elevated the copper in the cell may be sequestered in the vacuole in order to avoid copper toxicity (Eide DJ, Bridgham JT *et al.*, 1993). Molecules involved in copper uptake, distribution,

compartmentalization and sequestration have been identified. However, the mechanism of action and regulation of copper homeostasis remains to be elucidated (Lee, J. *et al.*, 2000).

Copper is an essential yet toxic micronutrient to yeast. Copper works as a transition metal in yeast since it can carry or release electrons (De Freitas, J. *et al.*, 2003). Copper is an important cofactor for a variety of enzymes, such as cytochrome C oxidase, ribonucleotide reductase, ceruloplasmin, lysyl oxidase and Cu, Zn superoxide dismutase (Karlin, K.D., 1993; Linder, C. and Hazegh-Azam, M., 1996; Lippard, S.J. and Berg, J.M., 1994). When cells are cultured in high copper levels in the medium, copper will readily participate in generating highly toxic superoxide radicals, which damages proteins, membranes and nucleic acids, and this makes excess copper toxic (Valentine, J.S. *et al.*,1998; Fridovich, I., 1999; Halliwell, B. and Gutteridge, J.M., 1984). Therefore, yeast cells have developed delicate, conserved mechanisms to regulate copper homeostasis, detoxify the reactive oxygen species (ROS), and keep copper levels in the yeast cells very low.

The alternative 3'-end processing of *CTR2* 3'-UTR is regulated by *HRP1/NAB4*. *NAB4* is an essential Heterogeneous nuclear RibonucleoProtein (hnRNP) that is part of the cleavage and polyadenylation complex. *NAB4* has also been implicated in NMD and in mRNA export (González, C.I. *et al.*, 2000). The transcripts produced by the *CTR2* pre-mRNA alternative 3'- end processing both have long 3'-UTRs and are both regulated by NMD. The transcripts sizes are about 2600nt and 900nt, and their 3'-UTR lengths are 2kb and 300nt respectively.

CTR2 mRNA is Degraded in an NMD-Dependent Manner

CTR2 mRNA encodes a protein that localized to the vacuole membrane, where it is reported to mobilize copper from vacuole to cytosolic copper chaperones. *CTR2* 3'-UTR undergoes alternative 3'-end processing in rich media (YPD), producing a longer and shorter mRNA isoform. This alternative 3'-end processing may be responsive to the copper levels in the medium. The longer isoform of *CTR2* is reported to have an atypically long 3'-UTR (Guisbert, K.S.K. *et al.*, 2007).

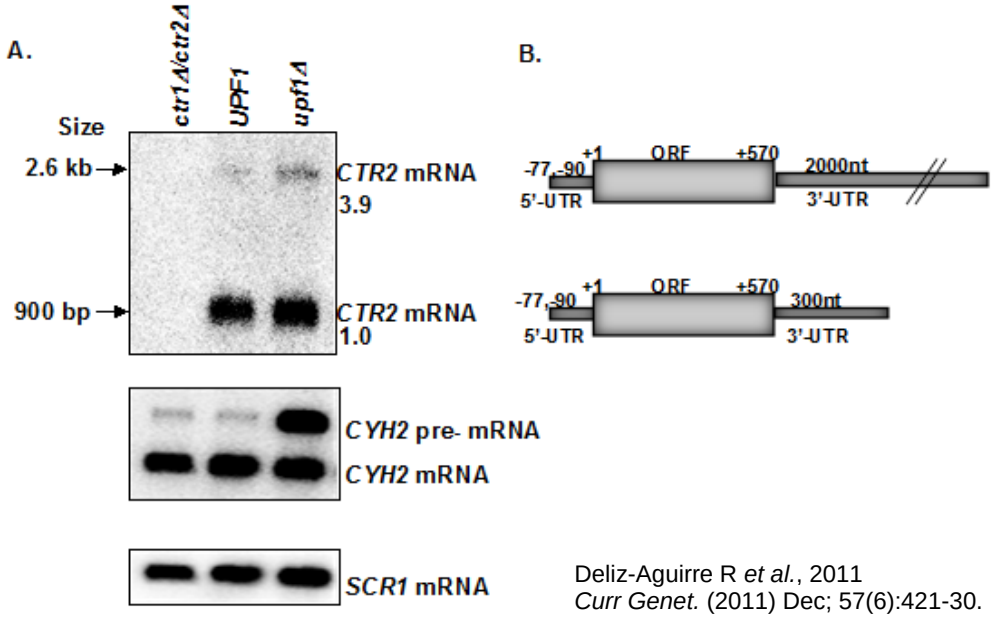


Fig.1.3 *CTR2* mRNAs accumulations in wild-type and nmd mutant yeast cells. (A) A northern blot of total RNA extracted from a *ctr1Δ/ctr2Δ* (lane 1), wild-type (*UPF1*) (lane 2) and nmd mutant (*upf1Δ*) (lane 3) yeast strain probed with oligolabeled *CTR2*, *CYH2*, and *SCR1* DNA. *CYH2* pre-mRNA is a target for NMD and is used to confirm the NMD phenotype of the yeast cells. *SCR1* is transcribed by RNA polymerase III and is not degraded by NMD; it is used as a loading control. (B) Schematic diagrams of *CTR2* mRNA, long and short isoforms.

Northern blotting was used to determine the extent to which NMD regulates the levels of the *CTR2* mRNAs. The accumulation of the longer isoform and shorter isoform in nmd mutant (*upf1Δ*) compared to wild-type (*UPF1*) yeast cells was 3.9 ± 0.8 (n=3)

fold and 1.0 ± 0.04 (n=3) fold respectively (Fig.1.3). The amount of the longer isoform was less than the shorter isoform in both nmd mutant (*upf1Δ*) and wild-type (*UPF1*). In wild-type (*UPF1*) yeast cells the shorter isoform accumulated 26 fold higher than the longer isoform. But in nmd mutant (*upf1Δ*), the shorter isoform only accumulated 7 fold higher than the longer isoform (Deliz-Aguirre R *et al.*, 2011).

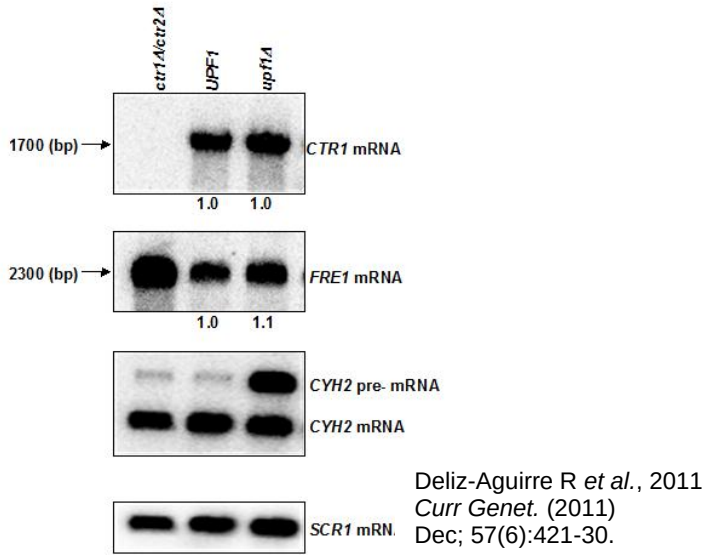


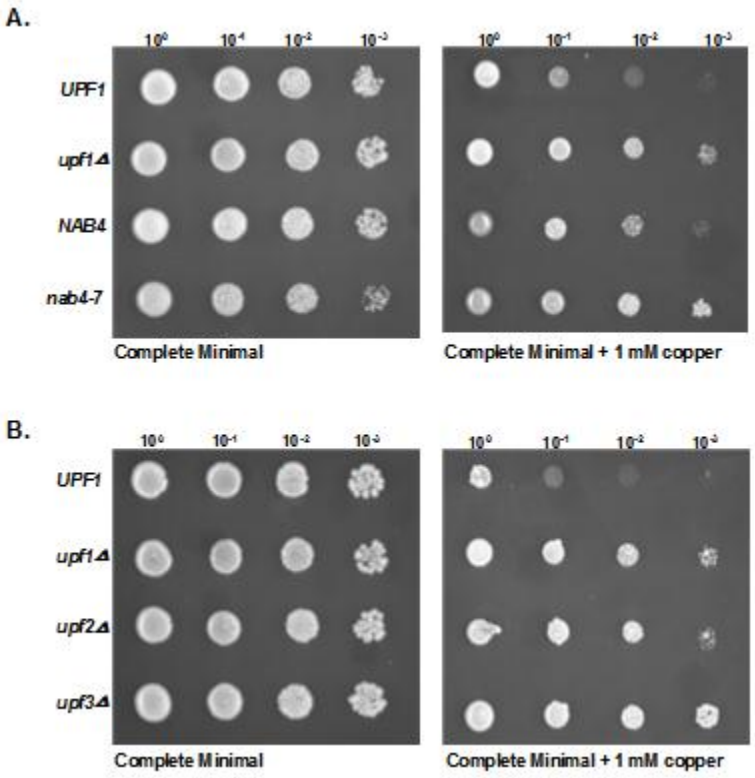
Fig.1.4 *CTR1* and *FRE1* mRNAs accumulations in wild-type *(UPF1)* and nmd mutant (*upf1Δ*) yeast cells. A northern blot of total RNA extracted from a *ctr1Δ/ctr2Δ* (lane 1), wild-type (*UPF1*) (lane 2) and nmd mutant (*upf1Δ*) (lane 3) yeast strain, was probed with oligolabeled *CTR1*, *FRE1*, *CYH2*, and *SCR1* DNA (Fig.1.3).

The accumulation of the longer isoform of *CTR2* mRNA is regulated in an NMD dependent manner. This accumulation is not specific to *CTR2* mRNA among the genes required for copper homeostasis, but is not a widespread phenomenon. The same northern blot was stripped and reprobed with *CTR1* and *FRE1* DNA. *CTR1* and *FRE1* mRNAs encode for proteins involve in copper metabolism. As previously mentioned, *CTR1* encodes a protein localized to plasma membrane, which is a high affinity copper transporter that is involved in copper uptake, while *FRE1* encodes a Cu^{2+}-Fe^{3+} reductase.

The accumulation of *CTR1* was 0.97 ± 0.02 (n=3) fold in nmd mutant (*upf1Δ*) compared to wild-type (*UPF1*) (Fig.1.4). There was no significant difference in accumulation of *CTR1* in nmd mutant (*upf1Δ*) and wild-type (*UPF1*). The accumulation of *FRE1* was 1.06 ± 0.10 (n=3) fold in nmd mutant (*upf1Δ*) compared to wild-type (*UPF1*) (Fig.1.4), also showed no difference in accumulation in nmd mutant (*upf1Δ*) and wild-type (*UPF1*) (Deliz-Aguirre R *et al.*, 2011).

Regulation of CTR2 mRNA by NMD is Physiologically Significant

In order to test the extent to which *CTR2* mRNA regulation by NMD is physiologically significant, drop tests were used to determine the growth rate of yeast cells on media with limiting amounts of copper or with excess copper. There was no observable difference in growth rate between nmd mutant (*upf1Δ*) and wild-type (*UPF1*) yeast cells on the media containing limiting amounts of copper. However, nmd mutant (*upf1Δ*) cells were more tolerant to 1.0 mM copper than wild-type (*UPF1*) cells when yeast cells are grown on Complete Minimal (CM) plates containing 1.0 mM copper for 4 days (Fig.1.5A). *nab4-7* is a positive control, since previous research showed *nab4-7* mutant cells are more tolerant to 1.0 mM copper than *NAB4* cells (wild type). Drop tests were also done with *upf2Δ* and *upf3Δ* nmd mutant yeast strains (Fig.1.5B). Wild-type (*UPF1*)*, upf1Δ, upf2Δ* and *upf3Δ* nmd mutant yeast cells were grown on CM media or CM media with 1.0 mM copper and incubated at 30°C for 3 days. *upf1Δ, upf2Δ* and *upf3Δ* nmd mutant yeast cells were all tolerant to 1.0 mM copper to a similar extent, which demonstrates this copper tolerance was not specific to *upf1Δ* nmd mutant yeast cells (Fig.1.5B), it was a common property of nmd mutants in general (Deliz-Aguirre R *et al.*, 2011).

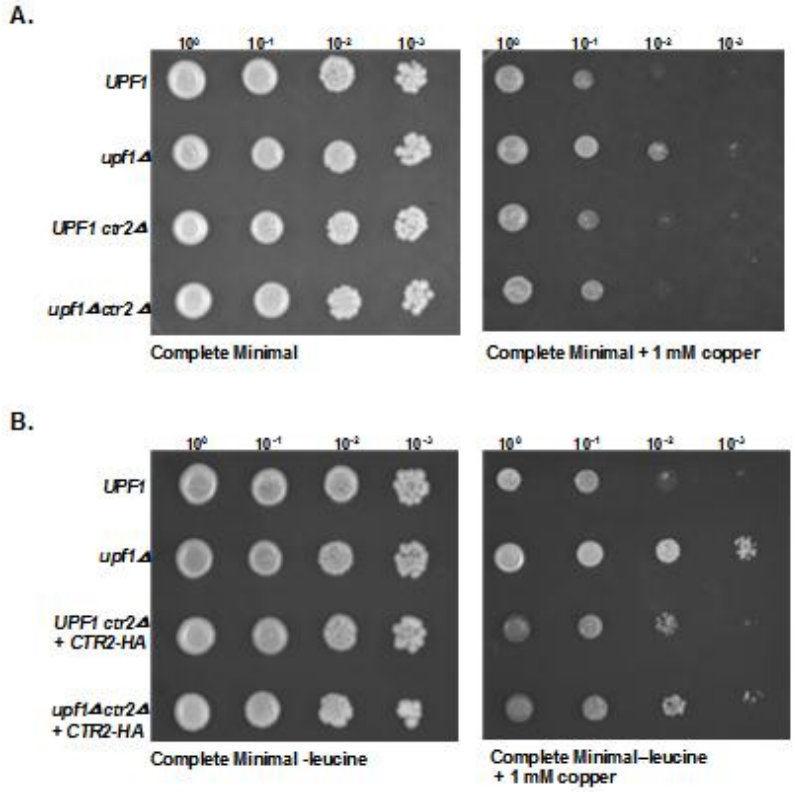


Deliz-Aguirre R *et al.*, 2011
Curr Genet. (2011) Dec; 57(6):421-30.

Fig.1.5 nmd mutant yeast cells are hypertolerant to 1.0 mM copper. (A) Wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type (*NAB4*) and *nab4-7* mutant yeast cells were grown to mid-log phase. Tenfold serial dilutions of the cells were spotted onto CM media or CM media containing 1.0 mM copper and incubated at room temperature for 4 days. *nab4-7* was used as positive control because a *nab4-7* mutant has been previously shown to grow faster than wild type *NAB4* cells on media containing excess copper (Guisbert, K.S.K. *et al.*, 2007). (B) The experiment in (A) was repeated with yeast strains wild-type (*UPF1*), *upf1Δ, upf2Δ and upf3Δ* nmd mutant and incubated at 30°C for 3 days.

Copper Tolerance of nmd Mutant (upf1Δ) is Dependent on the Presence of CTR2

Deletion of the *CTR2* gene was done in order to determine the extent to which the 1.0 mM copper tolerance of nmd mutant (*upf1Δ*) yeast cells was due to *CTR2.* Wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2 (UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2 (upf1Δctr2Δ)* were grown on either CM plates or CM plates with 1.0 mM copper and incubated at 30°C for 3 days. As showed in Fig.1.6A, wild-type yeast cells lacking *CTR2 (UPF1ctr2Δ)* and nmd mutant yeast cells lacking



Deliz-Aguirre R *et al.*, 2011
Curr Genet. (2011) Dec; 57(6):421-30.

Fig.1.6 nmd mutant (*upf1Δ*) yeast cells tolerance to 1.0 mM copper is dependent on the presence of the *CTR2* gene. (A) Wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2 (UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2 (upf1Δctr2Δ)* were grown to mid-log phase, serially diluted and spotted onto CM plate medium and CM medium containing 1.0 mM copper and incubated at 30°C for 3 days. (B) Wild-type (*UPF1*), nmd mutant (*upf1Δ*) transformed with *pRS315* (leucine vector) and wild-type yeast cells lacking *CTR2 (UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2 (upf1Δctr2Δ)* transformed with a HA-epitope-tagged *CTR2*. The transformants were grown as described in (A), spotted on selective medium lacking leucine containing 1.0 mM copper and incubated at 30°C for 3 days.

CTR2 (upf1Δctr2Δ) were both sensitive to 1.0 mM copper to the same extent as wild-type (*UPF1*) yeast cells; this phenomenon was caused by the deletion of *CTR2* gene. This data suggested that tolerance of nmd mutant (*upf1Δ*) yeast cells to excess copper in the media requires *CTR2.*

The wild-type yeast cells lacking *CTR2 (UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2 (upf1Δctr2Δ)* were transformed with a HA-epitope-tagged *CTR2*. This

restored the 1.0 mM copper tolerance of the nmd mutant (*upf1Δ*), although the copper tolerance phenotype was not to the same levels as the nmd mutant (*upf1Δ*) yeast cells (Fig.1.6B). This data further implied that 1.0 mM copper tolerance of nmd mutant (*upf1Δ*) yeast cells is dependent on presence of *CTR2* gene (Deliz-Aguirre R *et al.*, 2011).

Specific Aims

The *CTR2* mRNA isoforms do not contain nonsense codons and are both regulated in an NMD dependent manner. The molecular mechanism targeting both *CTR2* mRNA isoforms for NMD is not known. My first aim was to determine the features within the *CTR2* mRNA isoforms that target these mRNAs for NMD-mediated degradation. The underlying mechanism is hypothesized to be the atypically long 3'-UTRs. A construct was engineered and used to begin understanding the features within the *CTR2* mRNA that targeted it to NMD.

My second aim was on the physiological consequences associated with the NMD-mediated regulation of *CTR2*. Since nmd mutants were more tolerant to copper relative to wild-type yeast cells, this aim involved determining why nmd mutants were more tolerant to elevated copper levels compared to wild-type yeast cells. I hypothesized that copper is sequestered in nmd mutants. This aim was accomplished by measuring varying amounts of copper in wild-type and mutant yeast cells in different cellular compartments.

CHAPTER TWO

Materials and Methods

Cloning-Free PCR Method

Primers for the cloning free PCR method are listed in Fig.2.1 and Table 2.1. OBK190 and OBK191 have complementary sequences, 3'- ends of the OBK190 and OBK191 are homologous to each of the genes we used to fuse together. Primers OBK168 and OBK191 were used to amplify the *CYC1* 5'-UTR and ORF; primers OBK188 and OBK190 were used to amplify the *CTR2* 3'-UTR, and primers OBK168 and OBK188 were used to fuse *CYC1* 5'-UTR and ORF with *CTR2* 3'-UTR (Erdeniz, N. *et al.* 1997).

Table 2.1 Primes used for making the *CYC1-CTR2* 3'-UTR fusion construct.

Primer name	Sequence (5'-3')
OBK168	TAAATATTCTTTCCTTATACATTAG
OBK188	GGAATGATACATAGTAGTTC
OBK190	AAACGACTTAATTACCTACTTGAAAAAAGCCT GTGAGTAAGATGGTGACAAATATT
OBK191	AGATACAAATAGTATCAAATTTGTAATA TTTGTCACCATCTTACTCACAGGCTTTTT

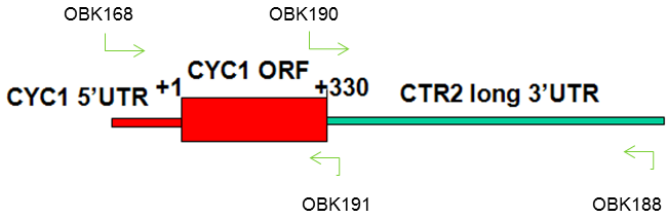


Fig. 2.1 Schematic diagram of *CYC1-CTR2* 3'-UTR and the location of each of the primers used.

Ligation of Insert DNA with TOPO TA Cloning Vector and Transformation

The *CYC1-CTR2* 3'-UTR fusion PCR product was inserted into the TOPO TA cloning vector. The PCR product containing the *CYC1* fused to the *CTR2* 3'-UTR was 3457 bp and it was inserted into the TOPO TA cloning vector according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). After insertion of the *CYC1-CTR2* 3'-UTR into the TOPO cloning vector, the plasmid was sent to be sequenced. Table 2.2 lists the plasmids used in this study.

Table 2.2 Plasmids used in this study. *pRS315, pRS425, pCUP1-lacZ, pCTR1-lacZ, YEp352* and *pCTR1lacZ* are yeast plasmids. TOPO TA cloning vector is an *E. coli* plasmid.

Plasmid	Description	Source
pRS315	*LEU2,CEN6,ARSH4, AmpR, lacZ*	Sikorski, R.S. and Hieter, P., 1989
pRS425	*LEU2,REP3,FRT,lacI,AmpR,lacZ*	Sikorski, R.S. and Hieter, P., 1989
pCUP1-lacZ	*pCUP1, lacZ*	Thorvaldsen, J.L. *et al.*, 1993
pCTR1-lacZ	*pCTR1, lacZ*	Dancis A. *et al.*, 1994
YEp352	*URA3, 2μ, AmpR, lacZ*	Hill, J.E. *et al.*, 1986
TOPO TA cloning vector	*KanR, AmpR, lacZ*	Invitrogen Corp., Carlsbad, CA

Insertion of CYC1-CTR2 3'-UTR into Yeast Plasmids

Restriction enzymes Not I and Sac I were used to cut *CYC1-CTR2* 3'-UTR from the TOPO cloning vector. For the double digests, the following components were added; 2 µl BSA, 1 µl 10Xbuffer K, 10 µl TOPO cloning vector with *CYC1-CTR2* 3'-UTR DNA, 4 µl water, 0.5 µl Not I and 0.5 µl Sac I to a 1.5 ml microcentrifuge tube. They were mixed well gently and then incubated in a 37°C water bath for 1 hour. The same restriction enzymes were used to digest *pRS315* and *pRS425* yeast plasmids, and incubated in a 37°C water bath for one hour. 1 unit of CIAP was added to the tubes and incubated at 37°C for 5 min, then incubated for an additional 15 min at 65°C water bath. *pRS315* and *pRS425* digested with Not I and Sac I were ligated to *CYC1-CTR2* 3'-UTR by adding the following components; 4 µl of $5XT_4$ DNA ligation buffer and 1 µl of T_4 DNA ligase to each 1.5 ml microcentrifuge tube, and added corresponding amount of TE (pH8.0), making the total amount 20 µl. The reaction was incubated at room temperature for one hour, and then transformed into the *E. coli* competent cells. The plasmid was then extracted from the *E. coli* cells and Not I and Sac I were used to map the plasmids to make sure they had the insert.

Transformation of Yeast Cells with the Yeast Plasmids

Yeast cells were transformed using the lithium acetate method (Gietz, D. *et al.*, 1992; Gietz, D. *et al.*, 1998). Wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type lacking *CTR2 (UPF1ctr2Δ)* and nmd mutant yeast strains lacking *CTR2 (upf1Δctr2Δ)* were grown to mid-log phase in YAPD media at 30°C. This can be done by two steps: inoculated the yeast cells in a 5 ml YAPD medium and incubated overnight in order to reach saturation, then diluted the overnight yeast cells to an OD_{600} of 0.1. The next day

the cells are incubated at 30°C until an OD_{600} of 0.4-0.6. Cells were transferred into 45ml centrifuged tube and centrifuged at 4000 rpm for 5 min. The pellet was washed with 10ml 1XLiAc, and then centrifuged again at 4000 rpm for 5 min. The pellet was then resuspended with 100 µl LiAc. The resuspended cells (100 µl), boiled carrier DNA (10 µl), the plasmid DNA (1 µg) and PEG (600 µl) solution were added to a 1.5 ml centrifuge tube in order. The tube was incubated with agitation at 30°C for 30 min and heat shocked at 42°C for 15 min. The cells were pelleted by a quick spin for 5 sec and resuspended in 1 ml of sterile water. 200 µl of the transformants ware plated on selective plates for the experimental group, and 200 µl of the transformants were plated on rich medium or selective medium for the no DNA control. The plates were incubated at 30°C for 2-5 days, depending on the yeast strain. Individual colonies were selected and streaked out on selective media plates.

RNA Extraction

Yeast total RNA used for mRNA steady-state levels was extracted by the hot phenol method from yeast cells harvested at mid-log phase (Kebaara, B. *et al.*, 2003 (1) and (2)). The yeast cells were grown to an OD_{600} value of 0.4 to 0.6 in a 5 ml rich medium or selective medium. This can be done in two steps: first, grow the yeast cells overnight to saturation in a 3 ml CM medium lacking leucine; second, inoculate multiple concentrations of the first overnight in a 5 ml CM medium lacking leucine, by doing this we ensured at least one of the cultures reached the correct OD_{600} value the next morning. The cells were cultured in 30°C incubator. The cells at the correct OD_{600} value were transferred to a 45 ml centrifuge tube and centrifuged at 4000 rpm for 5 min. They were then resuspended in 400 µl of DEPC treated water then transferred into an RNase-free 1.5

ml microcentrifuge tube and centrifuged for 20 sec at full speed. The supernatant was discarded and the cell pellet frozen in a dry ice / ethanol bath for 5 min. Then the frozen cell pellet was stored in the -80 °C freezer immediately. To extract the RNA, the protocol described in Kebaara, B. *et al.*, 2003 (1) and (2) was followed.

cDNA Synthesis

qScript™ cDNA Synthesis Kit (Quanta) was used to synthesize cDNA from mRNA. All the frozen components were thawed and briefly centrifuged before use. 200 µl PCR tubes from Axygen™ were used. For each tube the following components were added; 1 µg of RNA, 4 µl of qScript Reaction Mix (5X), 1 µl of qScript RT, variable amount of nuclease free water to a total volume of 20 µl. The PCR tubes were vortexed gently, and centrifuged to collect the contents. The cycling parameters used were; hold at 22°C for 5 min, then hold at 42°C for 30 min, then hold at 85°C for 5 min, and finally hold at 4°C. After synthesis the cDNA was diluted tenfold for future use.

Quantitative PCR Analysis

Quantitative real time PCR was used to measure the amount of mRNA transcribed from the cDNA. PerfeCTa® SYBR® Green SuperMix for iQ™ (Quanta) was used. qPCR was performed in a 36-well rotor in the Cobett qPCR Rotor-Gene™ 3000 (Qiagen, Velancia, CA). Primers for the experimental group were designed to amplify the fusion part of *CYC1-CTR2* 3′-UTR. The standard tubes were used for drawing standard curve by using the plasmid *pRS425* containing *CYC1-CTR2* 3′-UTR. The experimental groups were used to test accumulation of *CYC1-CTR2* 3′-UTR. Primers for the standard tubes were the same as the experimental group. Primers used for qPCR are all listed in Table

2.3. *SCR1* mRNA, a small cytoplasmic RNA, is not degraded by NMD or involved in copper metabolism and it was used as a loading control. As *CYH2* pre-mRNA, which encodes a ribosomal protein, is degraded by NMD, it was used as a positive control for NMD-mediated degradation. 200 µl PCR tubes from Axygen™ were used. Each of the standards contained 1µl of plasmid (concentration ranging from 10^7 to 10^9), 0.4 µl of forward primer, 0.4 µl of reverse primer, 8.2 µl of nuclease free water, and 10 µl of the PerfeCTa® SYBR® Green SuperMix. For each of the experimental or control tubes, they contained 2µl of template, 0.4 µl of forward primer, 0.4 µl of reverse primer, 7.2 µl of nuclease free water, and 10 µl of the PerfeCTa® SYBR® Green SuperMix. The expression levels of *CYC1-CTR2* 3′-UTR and *CYH2* were calculated against *SCR1*. The cycling parameters used for qPCR were: hold at 50 °C for 2 min, hold at 95 °C for 10 min, set 40 cycles for 95 °C 15 sec, and 60 °C 1 min. The melting curve condition was 65 °C to 95 °C with a rate of 10 sec/step. The data was analyzed with the assistance of Q-Gene Core Module file.

Table 2.3 Primers used for qPCR analysis.

Primer Name	Sequence (5'-3')
CYC1-CTR2 3'UTR Forward Primer	AGACACTCTGGTCAAGCTGAAGGG
CYC1-CTR2 3'UTR Reverse Primer	ATAACAACCCGCGCCCACCC
SCR1 Forward Primer	GGCTGTAATGGCTTTCTGGTGG
SCR1 Reverse Primer	GGTTCAGGACACACTCCATCC
CYH2 Forward Primer	CACTAAGACTAGAAAGCACAGAGG
CYH2 Reverse Primer	GTATTGGTCTCTCTTGTCTTCTGGG

Yeast Strains

The *S. cerevisiae* strains used in this study and their genotypes are listed in Table 2.4. Yeast strains were grown and maintained using standard techniques (Ausubel, F.M. *et al.*, 2003).

Table 2.4 *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
W303a	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Wente S. *et al.*,1992
AAY320	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1::URA3 (upf1-Δ2)*	Kebaara, B. *et al.*, 2003 (1) and (2)
AAY513	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, ctr2::HIS3*	Deliz-Aguirre R *et al.*, 2011
BKY92	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1:: TRP(upf1-Δ6)ctr2::HIS3*	This study
AAY514	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1::URA3 (upf1-Δ2), ctr2::HIS3*	Deliz-Aguirre R *et al.*, 2011
AAY329	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1::HIS3 (upf1-Δ4)*	Dr. Audrey Atkin

Determination of Total Cellular Copper Levels by Using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2 (upf1Δctr2Δ)* were grown to mid-log phase in complete minimal (CM) medium with increasing amounts of copper (200 - 1000 μM). This can be done by two steps: Grew the yeast cells to saturation overnight in

a 3 ml CM medium, then inoculated different amounts of yeast cells from the first overnight to a 10 ml CM medium with increasing amounts of copper at the end of the second day. This ensured at least one of the yeast cell culture concentrations would reach the correct OD_{600} the next day. Total cellular copper levels were measured using yeast cells that were grown for 2 overnights to OD_{600} of 1.0. 1 ml of the yeast cells was harvested by centrifuging for 2 min in a 1.5 ml microcentrifuge tube. The yeast cells pellets were washed three times with 1 mM EDTA to remove nonspecifically bound copper. Lastly the pellet was sent to the ICP-MS facility for analysis.

Determination of Cytosolic Copper Levels by Using β-galactosidase Assays

The wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ),* nmd mutant yeast strains lacking *CTR2* (*upf1Δctr2Δ)* were transformed with either *YEp352*, *pCUP1-lacZ* or *pCTR1-lacZ.* The transformants were grown in 5ml of CM media lacking uracil with increasing amounts of copper (200 - 1000 μM) at 30°C to an OD_{600} of 0.6-0.7. This can be done in two steps as mentioned previously. The cells were harvested and the pellet was resuspended in 1 ml of Z buffer. 75 μl chloroform and 50 μl 0.1% SDS was added, then vortexed for 10 sec. The sample was preincubated in a 28°C water bath for 5 min. The reaction was initiated by adding 200 μl ortho-Nitrophenyl-β-galactoside (ONPG) and incubated in a 28°C water bath for 8 min. The reaction was terminated by using 500 μl of 1 M Na_2CO_3. The cell debris was removed by centrifuging for 10 min. 200 μl of the supernatant was pipetted into a microtiter plate. OD_{450} of the reactions was measured using a microtiter plate reader (Amberg *et al*., 2005). The equation used to calculate the β-galactosidase units is:

$$\frac{OD_{450}}{(OD_{600} \text{ of assayed culture} * \text{Volume assayed} * \text{time})}$$

OD_{450} is the optical density of the product, o-nitrophenol.

OD_{600} is the optical density of the culture at the time of assay.

Volume is the amount of culture at the time of assay in ml.

Time is in minutes incubated at 28°C.

Growth Rate Determination Using Drop Test and Growth Curve

Yeast cells transformed with plasmid *YEp352*, *pCUP1-lacZ* or *pCTR1-lacZ* were grown to mid-log phase on CM dropout medium lacking uracil. This can be done in 2 steps as described above. The cells grew until they reached an OD_{600} of 0.4-0.6 in the morning of the second day. Tenfold serial dilutions of the cells were spotted onto CM medium lacking uracil with increasing amounts of copper (200 - 1000 μM) and incubated at 30°C for 3-4 days depending on the yeast strain used.

For the growth curves, wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2 (UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2 (upf1Δctr2Δ)* were cultured in 3 ml CM medium to saturation in the first overnight. The next morning the culture was diluted to an OD_{600} of 0.1 in 5 ml of CM media containing increasing amounts of copper (200 -1000 μM). The yeast cells were incubated at 30°C and the OD_{600} value was measured every two hours for 12 hours. For the higher levels of copper (600 μM and higher), the OD_{600} was also measured after 24 hours and 26 hours incubation at 30°C.

Determination of Vacuolar Copper Levels

Copper levels in the vacuoles of wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells were measured using yeast cells grown in CM medium or CM medium containing 1000 μM copper. The cells were converted to spheroplasts using zymolyase. The spheroplasts were lysed by adding DEAE-dextran. Vacuoles were resolved using a discontinuous Ficoll step gradient using the ultracentrifuge, and the protein were quantified using the BCA method. After quantification vacuolar copper levels were determined by ICP-MS analysis (Rees, E.M. *et al.*, 2004).

CHAPTER THREE

Results

CTR2 is Degraded by NMD Partly due to Its Atypically Long 3'-UTR

Previous research indicated that *CTR2* pre-mRNA produces two mRNA isoforms; both *CTR2* mRNA isoforms are degraded in an NMD dependent manner. *CTR2* is a direct substrate for NMD, which means the *CTR2* mRNAs have different decay rates when comparing nmd mutants *(upf1Δ)* with wild-type *(UPF1)* yeast cells, whereas indirect substrates have the same decay rate. The goal of this aim was to identify the sequence elements that target the *CTR2* mRNA for NMD. I hypothesized that the atypically long 3'-UTR led to degradation of *CTR2* mRNA by NMD.

The first construct examined contained the *CYC1* 5'-UTR, ORF and *CTR2* 3'-UTR (Fig.3.1). *CYC1* was used since *CYC1* is not a target for NMD and has been previously used to map NMD instability elements. I used PCR mediated ligation to synthesize *CYC1* 5'-UTR and ORF and *CTR2* 3'-UTR separately. After amplifying and purifying the separate DNA pieces, the *CYC1* 5'-UTR and ORF and *CTR2* 3'-UTR were fused using PCR. The *CYC1-CTR2* 3'-UTR PCR product was inserted into the TOPO cloning vector and transformed into the *E. coli* chemically competent cells (Invitrogen). To confirm the plasmid contained the *CYC1-CTR2* 3'-UTR, it was sent for sequencing (Eurofins, MWG Operon). The sequencing result indicated construction of *CYC1-CTR2* 3'-UTR fusion DNA sequence was successful. The insert, *CYC1-CTR2* 3'-UTR, was removed from the TOPO cloning vector and inserted into the yeast plasmid *pRS315* (low

copy number yeast plasmid) and *pRS425* (high copy number yeast plasmid) by using restriction digest and ligation. Then the yeast plasmid with the *CYC1-CTR2* 3'-UTR DNA was transformed into nmd mutant *(upf1Δ)* yeast cells and wild-type *(UPF1)* yeast cells. Quantitative PCR and northern blotting were used to measure the levels of the mRNA encoded by the *CYC1-CTR2* 3'-UTR construct using RNA extracted from wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells.

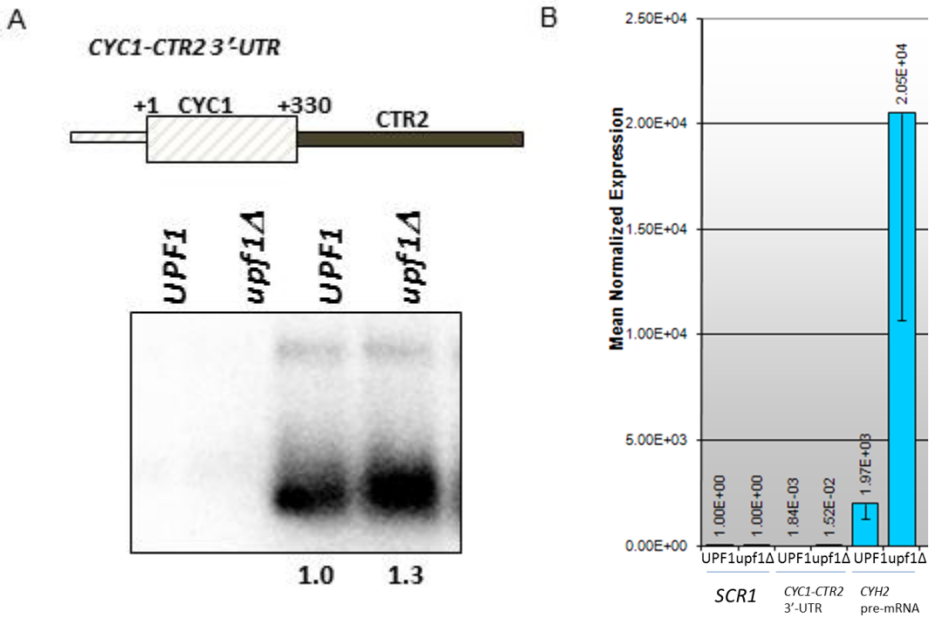


Fig.3.1 *CYC1-CTR2* 3'-UTR mRNA accumulations in wild type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells. (A) Top panel shows the schematic diagram of *CYC1-CTR2* 3'-UTR mRNA. Bottom panel shows a northern blot of total RNA extracted from a wild-type (*UPF1*) (lane1, 3) and nmd mutant (*upf1Δ*) (lane 2, 4) yeast strains. First two lanes were transformed with empty plasmids. (B) Mean normalized expression from qPCR. The first two columns show the expression levels for *SCR1* in wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells respectively. The third and fourth columns showed the expression levels for *CYC1-CTR2* 3'-UTR in wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells respectively. The fifth and sixth columns showed the expression levels for *CYH2* pre-mRNA in wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells respectively.

The accumulation of *CYC1-CTR2* 3'-UTR transcript in nmd mutant (*upf1Δ*) cells was higher than that in wild-type (*UPF1*) cells (Fig.3.1). According to the northern blot results, the accumulation in nmd mutant (*upf1Δ*) cells was 1.3 ± 0.2 fold higher than that

in wild-type (*UPF1*) cells. From the qPCR results, the difference in accumulation of *CYC1-CTR2* 3'-UTR between nmd mutant (*upf1Δ*) and wild-type (*UPF1*) cells was significant ($p<0.01$). These results supported our hypothesis that the atypically long 3'-UTR can lead to the degradation of *CYC1* mRNA, which normally is not a target for NMD.

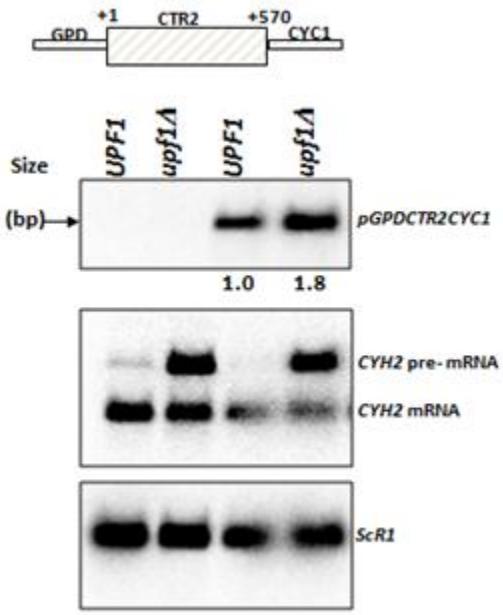


Fig.3.2 *pGPD-CTR2-CYC1* 3'-UTR mRNA accumulations in wild-type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells. Top panel shows the schematic diagram for *pGPD-CTR2-CYC1* 3'-UTR mRNA. Bottom panel shows a northern blot of total RNA extracted from a wild-type (*UPF1*) (lane1, 3) and nmd mutant (*upf1Δ*) (lane 2, 4) yeast strains. The northern blot was probed with oligolabeled *CTR2, CYH2,* and *SCR1* DNA. First two lanes were transformed with empty plasmids.

The second construct used contained the *GPD* promoter, *CTR2* ORF and *CYC1* 3'-UTR. The mRNA encoded by this construct was degraded by NMD (Fig.3.2). It accumulated 1.8 ± 0.3 (n=3) fold higher in nmd mutant (*upf1Δ*) yeast cells compared to wild-type (*UPF1)* yeast cells. Considering the results from both constructs, they point to the fact that the atypically long 3'-UTR contributes to NMD mediated degradation of

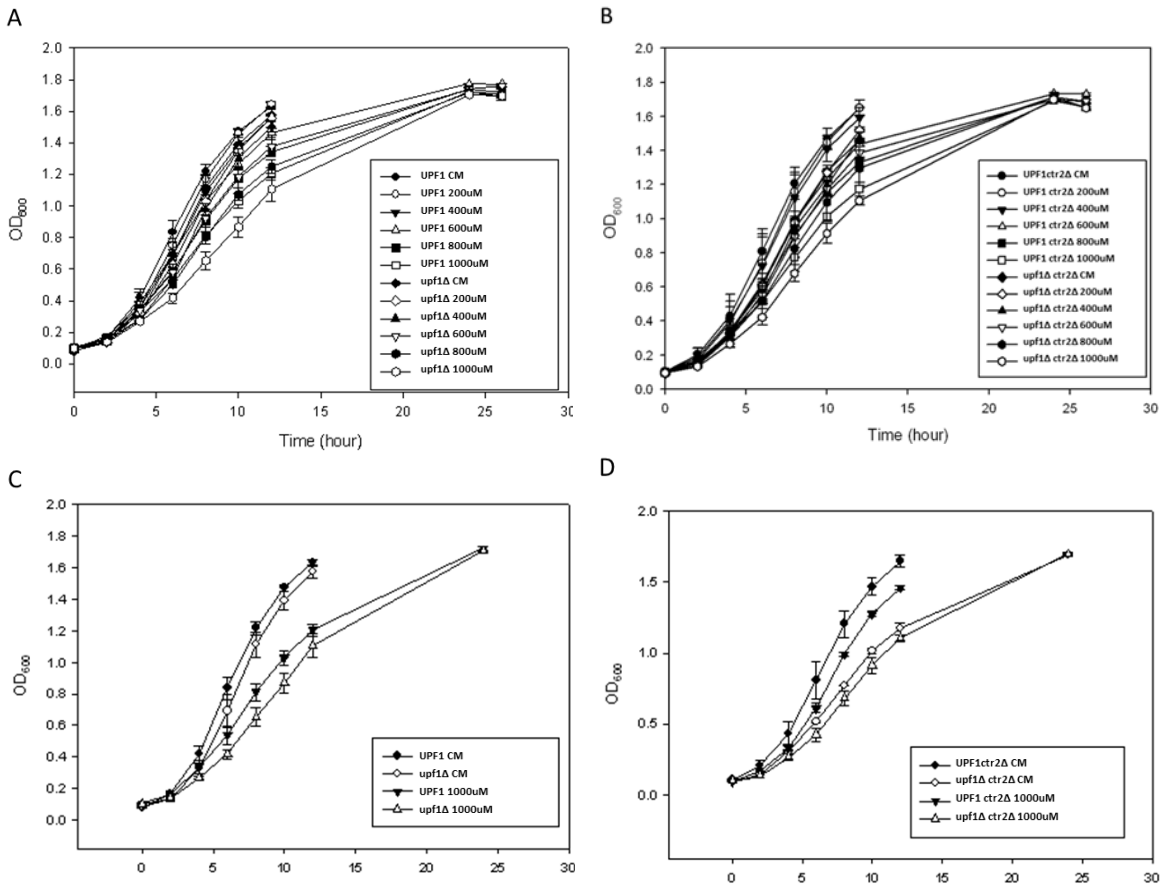


Fig.3.3 (A) Growth curve for wild-type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells grown in CM medium or CM medium with increasing amount of copper (200 - 1000 µM). (B) Growth curve for wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* grown in CM medium or CM medium with increasing amount of copper (200 - 1000 µM). (C) Comparison of the growth rate for wild-type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells grown in the medium without extra copper or with 1000 µM copper. (D) Comparison of the growth rate for wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* when they were grown in the medium without extra copper or with 1000 µM copper.

CTR2 but there are other undetermined NMD targeting elements present within the *CTR2* ORF that function cooperatively with the atypically long 3'-UTRs.

Growth Curves of Wild-type Yeast Cells (UPF1), nmd Mutant Yeast Cells (upf1Δ), Wild-type Yeast Cells Lacking CTR2 (UPF1ctr2Δ), and nmd Mutant Yeast Cells Lacking CTR2 (upf1Δctr2Δ)

To determine whether copper affected growth rate of the yeast cells in liquid media, growth curves were done for wild-type *(UPF1),* nmd mutant *(upf1Δ),* wild-type

yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ*). Yeast cells were grown in CM medium with increasing amounts of copper (200 - 1000 µM). Fig.3.3A shows the growth curves for wild-type *(UPF1)* and nmd mutants *(upf1Δ)* grown in CM medium with increasing amounts of copper (200-1000 µM). As the copper levels in the medium increased, the growth rate for the yeast cells became slower. Also there were no apparent difference in growth rate between wild-type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells grown in CM medium or in CM medium with 1000 µM Cu. Wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* in Fig.3.3B showed a similar tendency. When compared, the yeast cells grown in CM medium with no additional copper or with 1000 µM copper, there was a significant difference between their growth rates. The yeast cells cultured in CM medium with 1000 µM copper grew much slower than the yeast cells cultured in CM medium with regular amount of copper (Fig.3.3C, Fig.3.3D).

Total Cellular Copper Levels are Similar in Wild-type and nmd Mutant Yeast Cells

Previous research showed that nmd mutants were more tolerant to elevated copper levels and the reason for this copper tolerance is not known. To determine whether wild-type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells contain similar amounts of cellular copper levels, total cellular copper accumulation measurements were done by Inductively Coupled Mass Spectrometry (ICP-MS). ICP-MS was used to measure the total cellular copper levels of wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* which were grown in CM medium or CM medium with varying amounts of copper (200 - 1000 µM) incubated at 30°C to OD_{600} of 1.0.

When compared the yeast cells grown in CM medium with regular copper to CM medium with 1000 μM copper levels, yeast cells grown in CM medium with 1000 μM copper had significantly higher total cellular copper levels relative to yeast cells grown in CM medium (Fig.3.4A). Importantly, there was no significant difference in total cellular copper levels among wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ),* when cells were grown in CM medium with 1000 μM copper. This data suggested that 1000 μM copper tolerance of nmd mutant (*upf1Δ*) yeast cells was not due to reduced intracellular copper levels.

Total cellular copper levels were also measured in wild-type *(UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ),* grown in CM medium with empirically determined copper levels. When grown in CM medium containing 200, 400, 600, 800 or 1000 μM copper, wild-type *(UPF1*) and nmd mutant (*upf1Δ*) showed a similar trend. The cellular copper levels increased as the copper levels in the medium increased until the cells were exposed to 800 μM copper; at this copper concentration the levels of intracellular copper decreased (Fig.3.4B). Wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* showed a different trend from wild-type *(UPF1*) or nmd mutant (*upf1Δ*) cells. Both strains had lower total cellular copper levels. Wild--type *(UPF1ctr2Δ*), nmd mutant (*upf1Δctr2Δ*) yeast cells lacking *CTR2* showed an increase in intracellular copper levels as the copper levels in the medium increased up to and including 1000 uM copper.

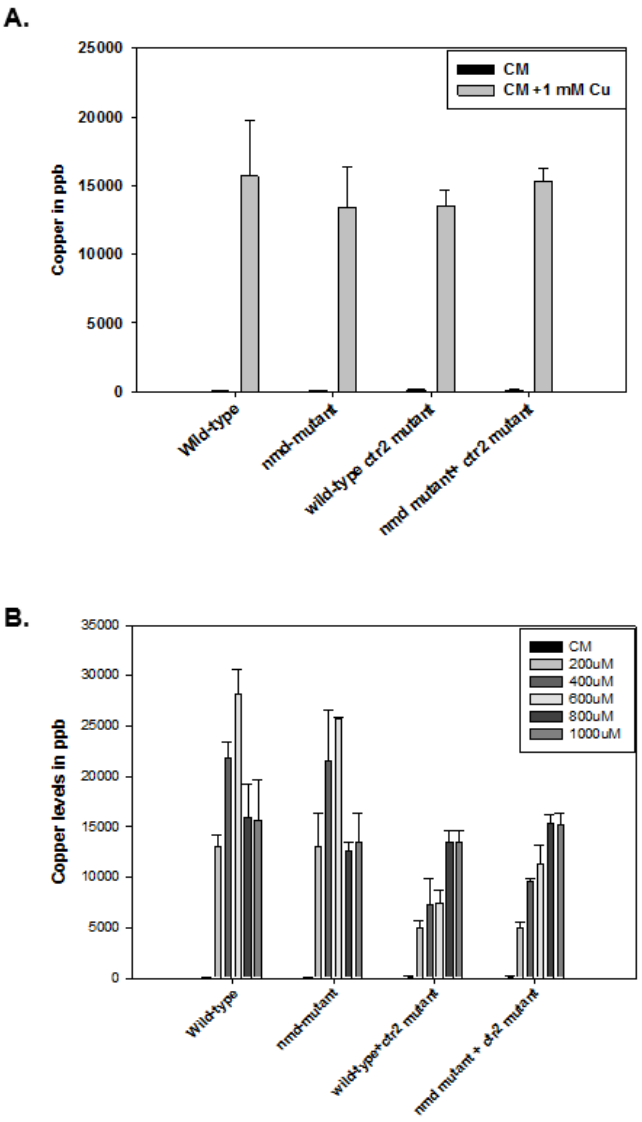


Fig.3.4 (A) Total cellular copper levels were measured in wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* grown in CM medium or CM medium with 1000 µM copper. (B) Total cellular copper levels were measured in wild-type (*UPF1*), nmd mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* grown in CM medium or CM medium with increasing amount of copper (200 - 1000 µM).

Measurements of Cytoplasmic Copper Levels in Wild-type (UPF1) and nmd Mutant (upf1Δ) Yeast Cells.

To determine whether NMD-mediated regulation of mRNA involved in copper homeostasis affects cytoplasmic copper levels, cytoplasmic copper levels of wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells was measured. The copper levels were

measured using the *pCUP1-lacZ* and the *pCTR1-lacZ* reporter plasmids (Thorvaldsen et al., 1993; Dancis et al., 1994). Cup1p is a metallothionein that binds copper. The expression of the *CUP1* gene is induced by *ACE1* metalloregulatory transcription factor when cells are exposed to high levels of copper; therefore the expression of *CUP1* is dependent on the cytosolic copper levels. *CUP1* is not regulated by NMD (He, F., *et al.*, 1993). *pCUP1-lacZ* plasmid contains the *CUP1* promoter fused with *lacZ* and can be used to measure cytosolic copper levels by using β-galactosidase assays. Increase in cytoplasmic copper levels leads to increased *CUP1* expression and increased β-galactosidase activity. *CTR1* encodes a protein that is a high affinity copper transporter needed for copper uptake, which is located at the cell membrane. *CTR1* mRNA is not regulated by NMD (Deliz-Aguirre R *et al.*, 2011). The expression of *CTR1* is sensitive to and negatively regulated by the copper levels in the cytoplasm (Dancis A. *et al.*, 1994). When the copper levels in the cytoplasm are very high, the expression of *CTR1* is repressed. The *pCTR1-lacZ* plasmid consists of *CTR1* gene fused with *lacZ* gene. So measurement of the cytoplasmic copper levels can also be achieved by using β-galactosidase assays.

First, we determined whether there was a phenotypic difference between wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells transformed with either the *pCUP1-lacZ* or *pCTR1-lacZ* reporter plasmids. Wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells were transformed with either *pCUP1-lacZ, pCTR1-lacZ* reporter plasmids or with a control plasmid *(YEp352)*. Transformants were grown on CM medium lacking uracil containing regular amount of copper or 1000 μM copper. We found that the *pCUP1-lacZ* plasmid did not affect the copper tolerance phenotypes of wild-type (*UPF1)* and nmd

mutant (*upf1Δ)* yeast cells (Fig.3.5A) as was observed previously (Deliz-Aguirre et al., 2011).

In contrast, transformation of the *pCTR1-lacZ* plasmid into wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells conferred a copper tolerance phenotype on both yeast strains, although the nmd mutant (*upf1Δ)* was slightly more copper tolerant than the wild-type (*UPF1)* yeast cells (Fig.3.5B).

Cytoplasmic copper levels in wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells transformed with either *YEp352* (control plasmid) or *pCUP1-lacZ* were measured using β-galactosidase assays when cells were grown in CM medium lacking uracil containing regular amount of copper (Fig.3.5C). This β- galactosidase assay was done in triplicate on cells grown to mid-log phase. The β-galactosidase activity in the wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells transformed with *pCUP1-lacZ* was higher than that in the wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells transformed with the control plasmid (*YEp352).*

Wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells transformed with *pCUP1-lacZ* were then grown in CM medium lacking uracil with regular amount of copper or with increasing amounts of copper (200 - 1000 μM) and their cytoplasmic copper levels were measured using β-galactosidase assays (Fig.3.5D). An increase in copper levels in the medium induced β-galactosidase activity up to a concentration of 600 μM for both the wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells (Fig.3.5D). As external copper levels increased above 600 μM, this resulted in decreased β-galactosidase activity in both yeast strains. The *pCUP1-lacZ* plasmid reported no significant difference in β-galactosidase activity between the wild-type (*UPF1)* and nmd mutant (*upf1Δ)* (Fig.3.5D)

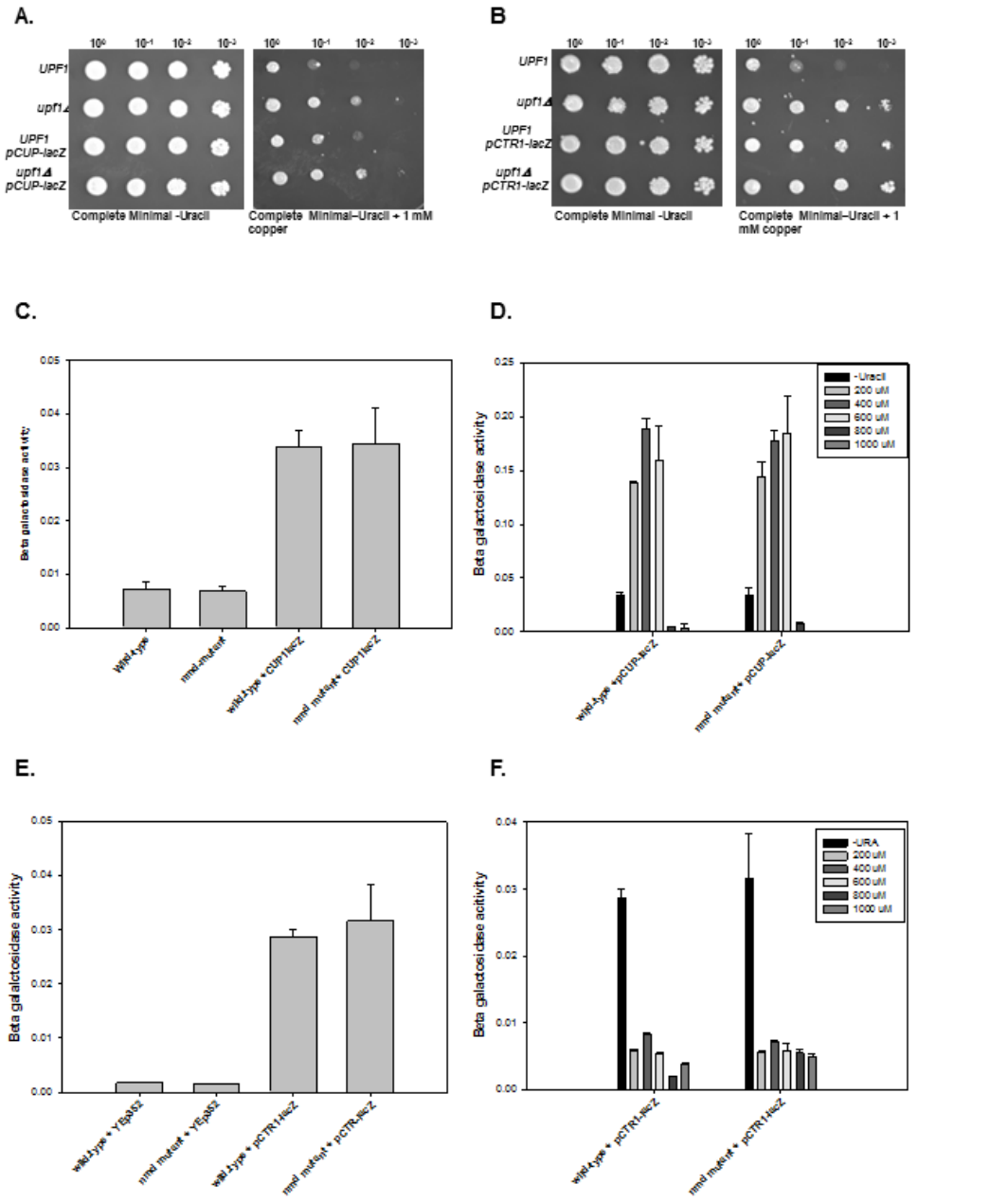


Fig.3.5 (A) Wild-type (*UPF1)* and nmd mutant *(upf1Δ)* yeast cells transformed with *YEp352* or *pCUP1-lacZ* were grown to mid-log phase, serially diluted and spotted onto CM medium lacking uracil and CM medium lacking uracil containing 1000 µM copper and incubated at 30°C for 3 days. (B) Wild-type (*UPF1)* and nmd mutant *(upf1Δ)* yeast cells transformed with *YEp352* or *pCTR1-lacZ* were grown to mid-log phase, serially diluted and spotted onto CM plate medium lacking uracil and CM medium lacking uracil containing 1000 µM copper and incubated at 30°C for 3 days. (C) Cytoplasmic cellular copper levels were measured in wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells transformed with *YEp352* or *pCUP1-lacZ.* Yeast cells were grown in CM medium lacking uracil. (D) Increasing amounts of copper (200 -1000 µM) were added to CM medium lacking uracil. The copper amounts in wild-type (*UPF1*) or nmd mutant (*upf1Δ*) transformed with *pCUP1-lacZ* yeast cells are shown. (E) Cytoplasmic copper levels were measured in wild-type (*UPF1*), nmd mutant (*upf1Δ*) transformed with *YEp352* or *pCTR1-lacZ.* (F) Increasing amount of copper (200 - 1000 µM) were added to CM medium lacking uracil. The β-galactosidase activity in wild-type (*UPF1*) or nmd mutant (*upf1Δ*) yeast cells transformed with *pCTR1-lacZ* is shown.

from CM with regular copper to CM with 600 µM copper. Unexpectedly, the *pCUP1-lacZ* plasmid failed to measure the β-galactosidase activity at copper levels above 800 µM in both wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells (Fig.3.5D).

Cytoplasmic copper levels in wild-type (*UPF1*), nmd mutant (*upf1Δ*) yeast cells transformed with either *YEp352* (control plasmid) or *pCTR1-lacZ* were also measured using β-galactosidase assays when cells were grown in CM medium lacking uracil containing regular amount of copper (Fig.3.5E). The β-galactosidase activity in the wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells transformed *pCTR1-lacZ* was higher than that in the wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells transformed with the control plasmid (*YEp352).* Wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells transformed with *pCTR1-lacZ* were then grown in CM medium lacking uracil with regular copper or with increasing amounts of copper (200 - 1000 µM) and their cytoplasmic copper levels were measured using β-galactosidase assays (Fig.3.5F). There was significantly higher copper induced β-galactosidase activity in wild-type *(UPF1)* compared to nmd mutant *(upf1Δ)* yeast cells above concentrations of 600 µM copper (Fig.3.5F). These data suggested that there were higher cytoplasmic copper levels in wild-type *(UPF1)* compared to nmd mutant *(upf1Δ)* yeast cells at higher concentrations of copper.

Measurements of Cytoplasmic Copper Levels in Wild-type (UPF1ctr2Δ) and nmd Mutant (upf1Δctr2Δ) Yeast Cells Lacking CTR2

To determine whether *CTR2* gene deletion affects cytoplasmic copper levels, cytoplasmic copper levels of wild-type (*UPF1)* and nmd mutant (*upf1Δ)* yeast cells lacking *CTR2* were measured. Wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* were transformed with either *pCUP1-*

lacZ, pCTR1-lacZ reporter plasmids or with a control plasmid (*YEp352)*. Transformants were grown on CM medium lacking uracil containing no additional copper or 1000 μM copper. Fig.3.6 (A) right panel shows that nmd mutants yeast cells lacking *CTR2* (*upf1Δctr2Δ)* have defects in the copper tolerance phenotype because of the *CTR2* gene deletion as was observed previously (Deliz-Aguirre et al., 2011). Transformation of the *pCUP1-lacZ* plasmid did not affect the phenotypes of the yeast cells. While transformation of the *pCTR1-lacZ* plasmid into wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* yielded a copper tolerance phenotype of both yeast strains (Fig. 3.6B).

Wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* transformed with *pCUP1-lacZ* were then grown in CM medium lacking uracil with regular copper or with increasing amounts of copper (200 - 1000 μM) and their cytosolic copper levels were measured using β-galactosidase assays (Fig.3.6D). Similar to the results from wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells transformed with *pCUP1-lacZ*, the β-galactosidase activity increased as copper level in the medium increased from CM with regular copper to CM with 600 μM copper (Fig.3.6D). As external copper levels increased above 600μM, β-galactosidase activity decreased in both yeast strains. Similarly, *pCUP1-lacZ* plasmid failed to measure the β-galactosidase activity at copper levels above 800 μM in both wild-type (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)*.

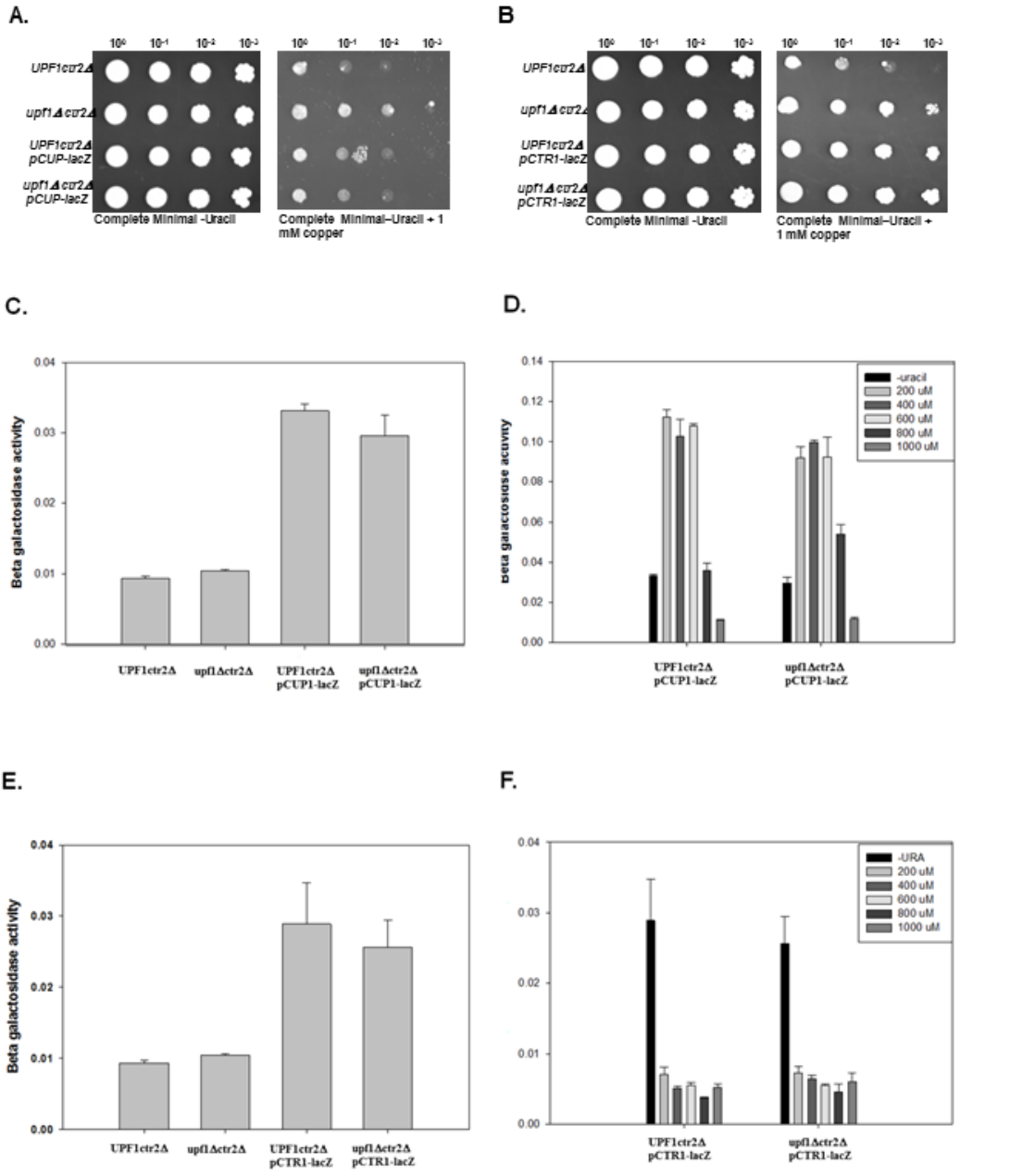


Fig.3.6 (A) Wild-type (*UPF1)* and nmd mutant *(upf1Δ)* yeast cells lacking *CTR2* transformed with *YEp352* or *pCUP1-lacZ* were grown to mid-log phase, serially diluted and spotted onto CM plate medium lacking uracil and CM medium lacking uracil containing 1000 µM copper and incubated at 30°C for 3 days. (B) The experiment described in A was done with wild-type (*UPF1)* and nmd mutant *(upf1Δ)* lacking *CTR2* yeast cells transformed with *YEp352* or *pCTR1-lacZ.* (C) Cytoplasmic copper levels were measured in wild-type (*UPF1*) and nmd mutant (*upf1Δ*) lacking *CTR2* transformed with *YEp352* or *pCUP1-lacZ.* Yeast cells were grown in CM medium lacking uracil. (D) Increasing amounts of copper (200 -1000 µM) were added to CM medium lacking uracil. The copper amounts in wild-type (*UPF1*) or nmd mutant (*upf1Δ*) yeast cells lacking *CTR2* transformed with *pCUP1-lacZ* yeast cells are shown. (E) Cytoplasmic copper levels were measured in wild-type (*UPF1*) or nmd mutant (*upf1Δ*) lacking *CTR2* transformed with *YEp352* or *pCTR1-lacZ.* (F) Increasing amount of copper (200 - 1000 µM) were added to CM medium lacking uracil. The β-galactosidase activity in wild-type (*UPF1*) or nmd mutant (*upf1Δ*) yeast cells lacking *CTR2* transformed with *pCTR1-lacZ* yeast cells is shown.

β-galactosidase assays were also used to measure cytoplasmic copper levels in wild-type (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* transformed with either *YEp352* (control plasmid) or *pCTR1-lacZ* grown in CM medium lacking uracil containing regular amount of copper (Fig.3.6E). Wild-type (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* transformed with *pCTR1-lacZ* were then grown in CM medium lacking uracil with regular copper or with increasing amounts of copper (200 - 1000 μM) and their cytoplasmic copper levels were measured with β-galactosidase assays (Fig.3.6F). There was no significant difference in β-galactosidase activity between wild-type (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* above concentrations of 600 μM copper (Fig.3.6F). These data suggested that the *CTR2* gene deletion affected the difference in β-galactosidase activity between wild-type and nmd mutant yeast cells at high copper levels in the medium.

Measurements of Vacuolar Copper Levels in Wild-type (UPF1) and nmd Mutant (upf1Δ) Yeast Cells

We then determined whether the nmd mutant (*upf1Δ*) yeast cells were sequestering copper in the vacuole, leading to less cytoplasmic copper levels and higher tolerance to elevated copper levels in the media. Yeast cells were grown in CM medium or CM medium containing 1000 μM Cu. According to Fig.3.7, there was no significant difference in vacuolar copper level between wild-type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells when they were grown in CM medium. However, when wild-type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells were grown in CM medium with 1000 μM copper levels, the vacuolar copper levels in nmd mutant (*upf1Δ*) was significantly higher than

that in the wild-type (*UPF1*) (Fig 3.7). This data suggested that the nmd mutants sequester copper in the vacuole when grown in elevated copper levels.

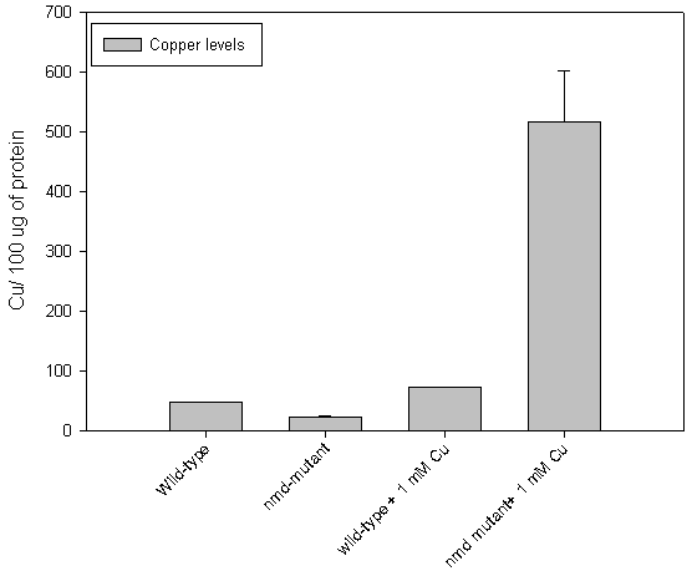


Fig.3.7 Vacuolar copper levels in wild-type *(UPF1)* and nmd mutant *(upf1Δ)* yeast cells grown in CM medium (column 1 and 2 respectively) or CM medium with 1000 µM copper levels (column 3 and 4 respectively).

CHAPTER FOUR

Discussion and Conclusions

In this study, the features targeting the *CTR2* mRNAs for degradation by NMD and the physiological effects resulting from this regulation were investigated. The atypically long *CTR2* 3'-UTR contributes to NMD mediated degradation of *CTR2* but additional features exist within the mRNA that contributes to its regulation by NMD. Regarding the physiological effects resulting from the regulation of *CTR2* by NMD, we found that the product of *CTR2* contributes to copper sequestration into the vacuole, allowing the nmd mutant yeast cells, which have higher levels of ctr2p to tolerate higher copper levels in the growth medium.

NMD got its name as it was first discovered in mRNAs that have premature termination codons. NMD also targets some natural mRNAs, which do not have premature termination codons. The natural mRNA that has been investigated in this study was the *CTR2* mRNA. *CTR2* was shown to be targeted by NMD-mediated degradation (Deliz-Aguirre R. *et al*., 2011). ctr2p is a low affinity copper transporter localized to the vacuolar membrane (Rees, E.M. *et al.*, 2004).

Previous studies showed that atypically long 3'-UTRs can be one of the features that targets natural mRNAs for NMD-mediated degradation (Kebaara, B.K. and Atkin, A.L., 2009). *CTR2* pre-mRNA undergoes alternative 3'-end processing and produces two mRNA isoforms (Guisbert, K.S.K. *et al.*, 2007). Examination of the *CTR2* mRNA isoforms showed that they do not contain a uORF; they do not contain an intron, are not

subject to -1 ribosomal frameshifting and are also not subject to leaky scanning. This evidence excludes these features as the likely features that target the *CTR2* transcripts for NMD-mediated degradation. Both the 900 and 2600 bp *CTR2* mRNA isoforms have longer than normal 3'-UTRs. Normal *S. cerevisiae* 3'-UTRs range in size from 50 to 200 nucleotides (nt). We hypothesized that the atypically long 3'-UTRs of *CTR2* mRNAs targets them for NMD-mediated degradation.

This hypothesis was tested using two fusion constructs, the *CYC1-CTR2* 3′-UTR and the *pGPD-CTR2-CYC1* 3'-UTR. If the long 3'-UTR of *CTR2* targets the transcripts for NMD-mediated degradation, we expected to see a significant difference in the accumulation of the *CYC1-CTR2* 3′-UTR construct between wild-type (*UPF1)* and nmd mutants (*upf1Δ*). There was a significant difference in the accumulation of the *CYC1-CTR2* 3′-UTR construct between wild-type (*UPF1)* and nmd mutant (*upf1Δ*). The *CYC1-CTR2* 3′-UTR accumulated higher in nmd mutants (*upf1Δ*), compared to wild-type *(UPF1)* yeast cells demonstrating that the *CTR2* 3'-UTR was sufficient to target an mRNA not normally regulated by NMD.

Analysis of the *pGPD-CTR2-CYC1* 3'-UTR mRNA in wild-type *(UPF1)* and nmd mutants (*upf1Δ*) yeast cells demonstrated that removal of the atypically long 3'-UTR from *CTR2* did not make the mRNA immune to NMD as anticipated. This experiment showed that the atypically long 3'-UTR contributed to NMD mediated degradation of the *CTR2* mRNA isoforms but there are other as yet undetermined NMD targeting features present within the *CTR2* ORF that worked cooperatively with the atypically long 3′-UTRs. The NMD targeting features within *CTR2* may be additive because the steady

state levels of the *pGPD-CTR2-CYC1* 3'-UTR mRNA in nmd mutants (*upf1Δ*) was lower than the 2600 bp *CTR2* mRNA isoform.

The element within the ORF of the *CTR2* mRNA that caused the degradation of the *pGPD-CTR2-CYC1* 3'-UTR is still unknown. In addition to the five features targeting natural mRNAs for degradation by NMD, the features associated with the degradation of the *pGPD-CTR2-CYC1* 3'-UTR may be novel.

Regulation of natural mRNAs by NMD can also have physiological consequences. Previous research showed that nmd mutant (*upf1Δ*) yeast cells were more resistant to 1000 µM copper when grown on plates. This copper tolerance was dependent on the presence of the *CTR2* gene (Deliz-Aguirre R *et al.*, 2011). Growth curves were done for wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells, wild-type (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)*. The growth curves showed that excess copper in the medium had a significant effect on the growth rate of the yeast cells, and it appeared that the growth rate decreased as the copper levels increased in the medium for both the wild-type *(UPF1)* and the nmd mutants (*upf1Δ*)(Fig.3.3). This further suggested that excess copper negatively affected the physiology of the yeast cells.

To determine why nmd mutant (*upf1Δ*) yeast cells showed tolerance to high copper levels, total cellular copper levels, cytoplasmic copper levels and vacuolar copper were measured. We hypothesized that copper was sequestered in the vacuole in nmd mutants *(upf1Δ)* in order to avoid copper toxicity when grown in the CM medium with elevated copper levels. The results showed that as the copper levels in the medium increased, the total cellular copper levels increased until a copper concentration of 600 µM, in both wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells. An increase in the

copper levels in the medium above 600 µM led to a decrease in total copper levels. Comparing wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells, there was no difference between their total cellular copper levels when the cells were grown in CM medium with increasing amounts of copper.

Correspondingly, wild-type (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* also had similar total cellular copper levels, although the total cellular copper levels increased as the copper levels in the medium increased until 1000 µM copper, and this trend was different from that in the wild-type *(UPF1)* and nmd mutants *(upf1Δ).* The total cellular copper levels in wild-type (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)* was clearly less than that in the wild-type (*UPF1*) with nmd mutant (*upf1Δ*) yeast cells, this was caused by the *CTR2* deletion. The cause of reduced total cellular copper levels in wild-type and nmd mutant yeast cells lacking *CTR2* is not known but could be due to three reasons. The first possible reason is that the ctr2p could be mislocalized to the plasma membrane in higher copper levels, so the deletion of *CTR2* leads to reduced copper uptake from the environment. The second possible reason is that when *CTR2* is deleted, the copper in the cytoplasm cannot be transported into the vacuole to avoid the resulting copper induced toxicity, so the plasma membrane copper transporters cannot take up more copper from the environment once the cytoplasmic copper levels reaches a certain threshold concentration. The third possibility is that the expression levels of *CTR1* and *CTR3*, the plasma membrane copper transporters is affected by deletion of *CTR2*. Importantly, comparing the yeast cells grown in CM medium or CM medium with 1000 µM shown in Fig.3.4A, there was no significant difference in total cellular copper levels among wild-type (*UPF1*), nmd

mutant (*upf1Δ*), wild-type yeast cells lacking *CTR2* (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ)*. These results indicated that the tolerance to 1000 μM copper of nmd mutant (*upf1Δ*) yeast cells was not due to its reduced cellular copper levels.

In order to further establish what caused the copper tolerance of nmd mutants (*upf1Δ*), the cytoplasmic copper levels were measured by using *pCUP1-lacZ* and *pCTR1-lacZ* plasmids using β-galactosidase assays (Fig.3.5, Fig.3.6). Transforming the *pCUP1-lacZ* into wild-type *(UPF1)* and nmd mutants *(upf1Δ)* did not affect the phenotypes of the yeast cells (Fig.3.5A, Fig.3.6A), while it could not measure the β-galactosidase activity at elevated copper levels (800 μM and higher) (Fig.3.5D, Fig.3.6D). In contrast, by using the *pCTR1-lacZ*, it affected the yeast cells phenotypes (Fig.3.5B, Fig.3.6B), but could measure the cytoplasmic copper levels from CM with regular copper to CM with 1000 μM copper (Fig.3.5F, Fig.3.6F). For these reasons, the cytoplasmic copper levels were analyzed using *pCTR1-lacZ.*

In general, the cytoplasmic copper levels increased as the copper level in the environment increased to 800 μM, and then decreased at 1000 μM for both wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells (Fig.3.5F). The changes in cytoplasmic copper levels probably were caused by the changes in total cellular copper levels. When compared wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells, there was a significant difference in their cytoplasmic copper levels when they were grown in CM medium with 400 μM or higher. Since NMD-mediated degradation was nonfunctional in the nmd mutant (*upf1Δ*) yeast cells, the accumulation of *CTR2* mRNA was more than that in the wild-type (*UPF1*). This increase in *CTR2* mRNA led to an increase in ctr2p, elevated

ctr2p can transport excess copper into the vacuole of the nmd mutant. This difference in cytoplasmic copper levels between wild-type (*UPF1*) and nmd mutant (*upf1Δ*) yeast cells was probably due to there being more *CTR2* mRNA in the nmd mutant (*upf1Δ*), and copper sequestration in the nmd mutant (*upf1Δ*) transported by ctr2p.

In the wild-type (*UPF1ctr2Δ)* and nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ),* the cytoplasmic copper levels went up until the copper levels in the medium was 800 µM, and then went down when the copper levels in the medium was 1000 µM. The increase in cytoplasmic copper levels was probably due to the increase in total copper levels. Comparing wild-type (*UPF1ctr2Δ)* with nmd mutant yeast cells lacking *CTR2* (*upf1Δctr2Δ),* they lost the significant difference in cytoplasmic copper levels when they were cultured in CM medium with 600 µM copper and above, and this may have been caused by *CTR2* gene deletion (Fig. 3.6F). This loss of significant difference in cytoplasmic copper levels supported the speculation that ctr2p transported the copper from cytoplasm to vacuole.

In order to verify whether copper tolerance of nmd mutant (*upf1Δ*) was due to sequestration of copper into the vacuole, vacuolar copper levels were measured. Vacuolar copper levels for wild-type (*UPF1*) and nmd mutant (*upf1Δ*) showed that there was no significant difference in their vacuolar copper levels when they were cultured in CM medium. However there was a significant difference between wild-type (*UPF1*) and nmd mutant (*upf1Δ*) when they were cultured in CM medium with 1000 µM. This was probably due to the higher accumulation of the *CTR2* mRNA in nmd mutant (*upf1Δ)* compared to the wild-type (*UPF1*). We speculated that the elevated levels of the ctr2p transported copper into the vacuole, resulting in there being more vacuolar copper in the

nmd mutant (*upf1Δ*). Taken together, these results suggest that the reason why nmd mutant (*upf1Δ*) yeast cells were more tolerant to 1000 µM copper than wild-type (*UPF1*) yeast cells was not due to reduced total cellular copper levels, but may be because of ctr2p transporting the excess amount of copper from cytoplasm to the vacuole.

Future Directions

The feature responsible for the degradation of the *CTR2* mRNA associated with its ORF is still not known. Therefore, future research will explore the cis-acting elements located within the *CTR2* ORF that target the *CTR2* transcripts for NMD-mediated degradation. The role that vacuole plays in copper tolerance is still not understood, so future research will explore its role by using vacuolar mutants.

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