ABSTRACT

The effect of copper on growth and the role of *PCA1* expression in *Saccharomyces cerevisiae* cells with and without a functional nonsense-mediated mRNA decay pathway

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The nonsense-mediated mRNA decay pathway (NMD) is a conserved physiological process in all tested eukaryotic organisms for recognizing and degrading mRNAs containing premature termination codons. NMD also regulates natural mRNAs encoding proteins that regulate copper concentrations inside the cell. As an essential micronutrient, copper has a role in many biological mechanisms as a cofactor but is also toxic at high concentrations inside the cell. NMD has shown to regulate mRNAs involved in copper homeostasis including *PCA1*. These mRNAs are sensitive to different environmental conditions such as high levels of copper. The degree of regulation continues to be investigated, but it is known that NMD is integral to the biological mechanisms as non-functional NMD *S. cerevisiae* cells portray increased rates of growth in the presence of high copper. Different yeast strains have also been shown to differentially confer copper resistance and mRNA expression in metal homeostatic proteins. To explore the degree of NMD regulation, I examined the growth rates and rate of mRNA expression when *S. cerevisiae* is grown in varying copper conditions with and without a functional NMD pathway. Through examination of *PCA1* gene expression, we can assess NMD's rate of regulation and *S. cerevisiae's* tolerance to copper.

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THE EFFECT OF COPPER ON GROWTH AND THE ROLE OF *PCA1* EXPRESSION IN *SACCHAROMYCES CEREVISIAE* CELLS WITH AND WITHOUT A FUNCTIONAL NONSENSE-MEDIATED MRNA DECAY PATHWAY

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CHAPTER ONE

Introduction

NMD Background

Biological significance

All organisms on earth have been shown to follow, what Francis Crick has come to name, the Central Dogma of Molecular Biology. In 1958, Crick described the keystone system of genomic replication and its expression. As presented in figure 1, it follows that the general flow or transfers of genetic information stems from DNA (Crick 561-562). RNA is transcribed from DNA and subsequently processed through translation into a protein. Specifically, transcription of DNA produces coding sequences of messenger RNAs (mRNAs). The mRNAs undergo translation through complementary anticodons on tRNAs. Each tRNAs carries an amino acid specific to their respective mRNA coding sequence. The amino acids form a polypeptide chain that makes up the primary structure of a protein.

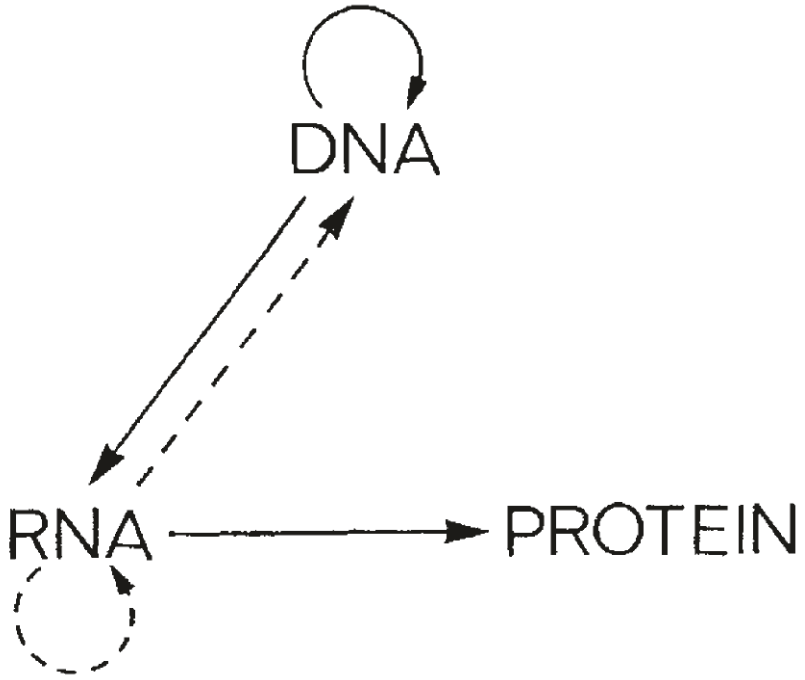

Figure 1 – Central Dogma of Molecular Biology. Francis Crick presented his third figure showing solid arrows to indicate "general transfers" that take place in all living cells. Dotted arrows represent "special transfers" that only occur in unique cells.

During the process of transcription, there are many mediators that participate in the transcription of DNA. Capable of making errors, there are various mechanisms regulating the continued synthesis and stability of mRNAs. By regulating mRNAs, the cell can control the production of various functional and nonfunctional proteins. The nonsense-mediated mRNA decay (NMD) pathway is a eukaryotic pathway that is responsible for the degradation of messenger RNAs (mRNAs) that prematurely terminate translation as well as some natural mRNAs. The mRNAs that prematurely terminate translation contain regions of a gene that encode stop codons in the wrong or incorrect position, called premature termination codons (PTCs). As shown in figure 2, the mRNAs containing PTCs can lead to truncated proteins if they are translated. PTCs are generated as a result of improper pre-mRNA processing or nonsense mutations. If translated, these

irregular proteins can lead to structural or biochemical pathway failures within eukaryotes. In response to these consequences, eukaryotes rely on the NMD to degrade these mRNAs, thus preventing the translation of the mRNAs into truncated proteins. Natural mRNAs that encode fully functional proteins are also degraded by the pathway through similar mechanisms, but they are induced by other NMD activating features. In addition, various stressors or environmental conditions can cause certain transcripts to be upregulated or downregulated through NMD (Peccarelli et al. 2016 and Peccarelli et al. 2019).

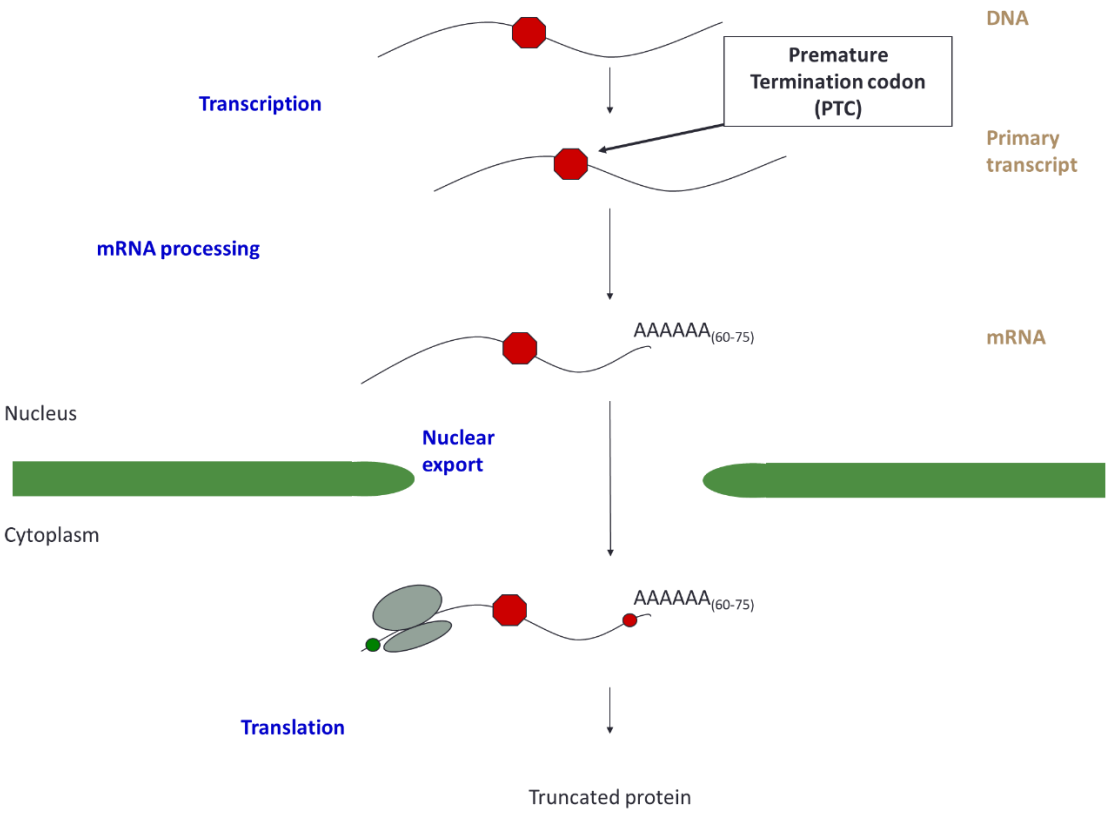

Figure 2 – NMD Pathway. Premature termination codons occur at the primary transcript signaling its degradation by the NMD pathway.

Core trans-acting factors

There are three core trans-acting factors that are necessary for NMD to function. Upf1p, Upf2p, Upf3p are the three upstream frameshift proteins that mark the mRNA for the degradation process. Upf1p has been shown to be a group 1 RNA helicase with ATPase activity and interacts with two release factors, eRF1 and eRF3. Upf2p and Upf3p are proteins that interact with Upf1p to regulate its function. Inactivation of any one of these factors can cause the pathway to be nonfunctional causing the selective stabilization of the mRNAs set to be regulated by NMD (Peccarelli et al. 2016).

NMD Targets

Physiological significance of natural mRNA regulation by NMD

Along with various other features in eukaryotes, the NMD pathway is a highly conserved mechanism across all tested eukaryotes due to the need for gene expression regulation. Organisms need a way to adapt to various environmental stressors. These stimuli can range from toxic environments, such as toxic metal conditions, to nutrient-deficient environments, such as low iron conditions. Eukaryotes use the NMD pathway to regulate the expression of various proteins through mRNA degradation. These mRNAs encode proteins that aid in the homeostatic process of the organisms by sequestering, exporting, or importing substances (Peccarelli et al. 2016). Remarkably, around 10% of the transcriptome in *S. cerevisiae* is affected with the inactivation of NMD suggesting how important this pathway is for cellular adaptation and responses to environmental stimuli. While the differences between the *S. cerevisiae* and mammalian NMD are apparent, both models are similar in many aspects. According to Peccarelli and Kebaara, the *faux*-UTR model provides a mechanism for the degradation of mRNAs through

NMD. A termination codon that is upstream to the mRNA's poly(A) tail increases the efficiency of that mRNA's degradation. According to the *faux*-UTR model, the farther the release factors that bind to the poly(A) tail are from each other, the harder it is for the release factors to interact with the terminating ribosome. Ultimately, this causes an inefficient terminating ribosomal context.

NMD-inducing features

In contrast to mRNAs containing PTCs, however, natural mRNAs contain NMD inducing features that target them for degradation (Peccarelli et al. 2016). Figure 3 separates the distinction between aberrant transcript and normal transcript degradation inducing features (Nickless et al. 2017). These features include translated upstream open reading frames (ORFs) (Gaba et al., 2005), ribosomal frameshifts (Belew et al., 2011), leaky ribosomal scanning (Welch and Jacobson, 1999), and inefficiently spliced pre-mRNAs (He et al., 1993) are all features that have been associated with the increased targeting by the NMD system. In addition, mRNAs containing long 3'-Untranslated Regions (UTRs) have been shown to be sensitive to regulation by NMD ((Deliz-Aguirre et al., 2011; Kebaara and Atkin, 2009; Parker, 2012; Peccarelli and Kebaara, 2014b; Rebbapragada and Lykke-Andersen, 2009; Schweingruber et al., 2013).

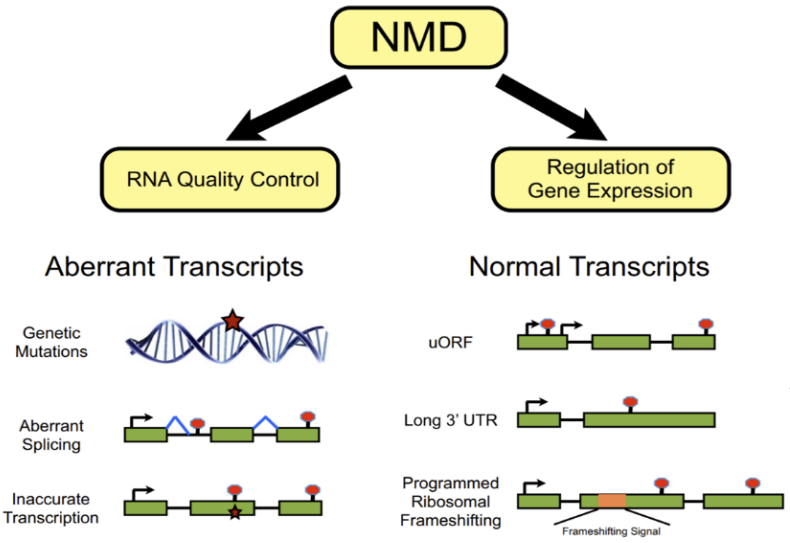

Figure 3: Natural transcript NMD regulation due to mRNA transcript targeting features. Normal transcripts contain NMD-targeting features that mark them for degradation by the NMD pathway.

Direct and indirect regulation of mRNAs

The regulation of natural mRNAs by NMD is either direct or indirect. Both direct and indirect NMD targets accumulate to higher levels in NMD mutants relative to wild-type strains. For mRNAs under direct regulation, half-life decay rates are significantly different between yeast strains with a functional and non-functional NMD pathway. In contrast, indirect regulation shows similar decay rates in both functional and non-functional NMD yeast strains. Transcripts with an indirect pathway for regulation by NMD tend to have no targeting features and therefore are subject to indirect regulation through control of other upstream NMD substrates. These upstream NMD substrates have been hypothesized to be directly regulated through interacting with Upf1 and thus influencing downstream targets such as *PCA1* (Peccarelli et al., 2016).

The yeast S. cerevisiae as a model organism

With the yeast *S. cerevisiae* complete genome being the first eukaryotic organism sequenced in 1996, the fields of functional genomics and systems biology have greatly expanded. As the primary driver of modeling all eukaryotic organisms, yeast serves as one of the most prominent model organisms to be used for advancing fields of eukaryotic research. Thanks to innovative advancements such as mutant libraries and genome databases, analysis over specific actions and consequences of various portions of eukaryotic organisms can be examined thoroughly. Mutant libraries allow scientists to assess individual gene alterations to an organism that allows us to investigate the workings of a specific gene. The results of gene mutations can be compared to the gene database to explore various necessities in gene functions within eukaryotes (Botsein and Fink 2011). Logistically, yeast is relatively easier to grow and is more cost efficient than alternative model organisms used in laboratories. Growth patterns are established throughout the lifespan as both haploid and diploid states are stable. Due to their ease of growth, cost, and stability, scientists are able to add temperature sensitive alleles to regulate various phases within the cell's growth cycle such as transcription or translation (Peccarelli and Kebaara, 2014).

Along with innovative advancements in the fields of genomics, yeast remains as an excellent model organism to explore for almost all eukaryotes due to the majority of its genome being conserved. Around 31 to 35% of the ORFs are homologous to mammalian sequences. Functions within the genome, such as the NMD pathway, are highly transferable to most eukaryotic organisms. With a high degree of genetic conservation, the consequences of gene interactions can be assessed through the study of

S. cerevisiae. For instance, inheritance traits or patterns can be examined through comparison of mutant and wild-type strains. Studies can demonstrate the likelihood of genetic diseases through this link. Yeast has already demonstrated its capabilities as a model organism with the functional characterization of their nonfunctional proteins and translation to their related human proteins (Botstein and Fink 2011). The potential for research in the field of NMD and its translation to human treatments remain high. Revealing the inner workings of mRNA expression in metal detoxification, for example in *S. cerevisiae*, can shed light into how human homologs of those mRNAs operate. As a result, more efficient treatments for diseases can be looked into as more information is known about physiological mechanisms.

Role of copper and metal toxicity

Copper is especially prevalent in the life processes of *S. cerevisiae* as it is needed as an essential micronutrient. It acts a cofactor for a variety of cellular processes such as biosynthesis of neurotransmitters and neuropeptides in humans, and mitochondrial aerobic metabolism (Kaplan et al. 2016). Essentially, copper is a cofactor for cytochrome C oxidase and copper, Zn-superoxide dismutase (Sod1) and is critical for the degradation of reactive oxygen species. At high concentrations, however, free copper molecules can be toxic to the cell and cause the production of superoxide radicals. These products are extremely reactive and disrupt the physiological processes within the cell. Thus, the regulation of the cell's copper homeostatic mechanisms is necessary to keep a healthy concentration of one free copper molecule per cell (Hodgins-Davis et al., 2012). Genes, such as *PCA1*, are translated into proteins to aid in metal detoxification. Figure 4,

provides other genes in *S. cerevisiae* that are regulated by NMD and their functions related to copper homeostasis.

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	-

Figure 4: Several of the S. cerevisiae genes that are involved in copper homeostasis. These are NMD-dependent genes and regulation is contingent on the displayed targeting features. Some features are not sensitive to NMD mediated degradation and requires further analysis. The *PCA1* gene highlighted I green is the focus of this study.
a) Presence or absence of an upstream open reading frame (uORF)
b) mRNA may be subject to -1 Ribosomal Frameshifting
c) 3'-UTR lengths predicted using the 3'-end processing site predictor
d) 3'-UTR lengths determined by 3'-RACE

Structure and Role of PCA1 as a metal transporting P-type ATPase

Size and composition of PCA1

PCA1 is a cadmium-exporting P-type ATPase known for its involvement in copper, iron, and cadmium regulation. The protein contains a degron, a region that is sufficient to trigger protein degradation, between 250-350 amino acids at the cytoplasmic N-terminus that is responsible for the majority of its processes. According to TASSER results in Figure 5, there are a few secondary structures with CXXC motifs that are integral for the operation of *PCA1*. Between amino acids 271-306, there is a long amphipathic helix, followed by an unstructured loop, and ended by two short β sheets. Within the helix, there are observed patches of hydrophobic residues that give rise to its amphipathic nature (Smith et al., 2006). *PCA1* is conserved in humans through the P_{1B}-type ATPase family. In human homologs ATP7a and ATLP7b, there are 1-6 CXXC motifs located in the N-terminal region. Within these motifs, there are two cysteine residues that are also responsible for the binding of copper in a linear bicoordinate manner (Adle et al., 2007).

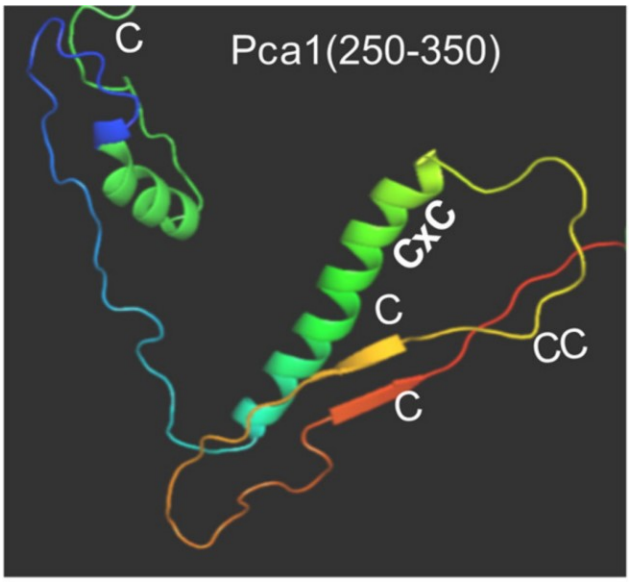

Figure 5 – PCA1 Secondary Structures and Motifs. *PCA1* contains a long amphipathic helix (CxC), an unstructured loop (CC), and two β sheets (C).

Additionally, *S. cerevisiae PCA1* has two alleles that vary in their function of cadmium transporting ATPase. Figure 6 identifies the genetic difference between *PCA1* from strain W303a (BKY8) and RM11-1a (BKY100). All compared yeast strains have multiple conserved regions in the gene that are integral to the function of the *PCA1* protein. PCA1 from W303a/S288c has an identified single nucleotide mutation, specifically the G970R substitution. The mutation is distinct from wild-type strains and confers a nonfunctional ATP-binding pocket. Due to this mutation, the protein is able to bind excess copper and cadmium but is unable to use its transporting ATPase mechanism for cellular translocation (Adle et al., 2007).

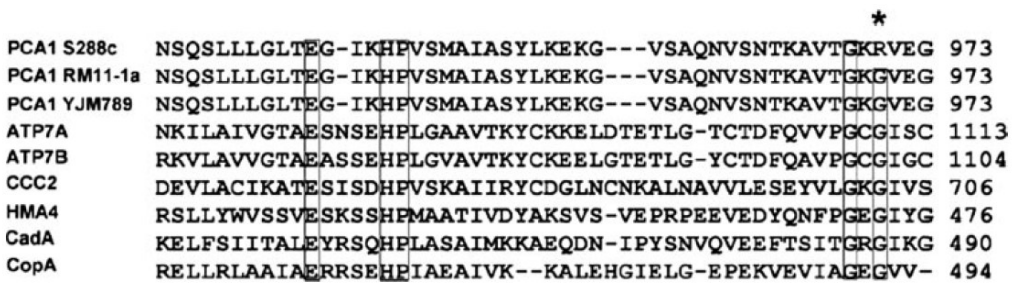

Figure 6 – Amino acid sequence of yeast strains. BKY100 (RM11-1a) and BKY8 (W303a/S288c) differ in their nucleotide binding domain (N-domain) for *PCA1*. The indicated G970R nucleotide mutation is indicated by the asterisk.

Cadmium and copper regulation

Increased cadmium and copper concentrations are believed to up-regulate *PCA1* to increase the exporting of toxic metals from the cell. Past studies have determined that up-regulation occurs through a degron-dependent manner. There are seven cysteine residues that are located within *PCA1*'s degron contributing to the protein's high affinity for cadmium and copper. While the mechanisms of binding to thiols for these two metals differ, they are both bound by the protein for translocation. Direct cadmium and copper sensing by the thiols can be regulated by *PCA1* through the alteration of the described secondary structures in the protein. For instance, decreasing the amount of exposure to the hydrophobic residues in the amphipathic helix has been shown to reduce the capability to bind the two metals (Smith et al., 2016). In *PCA1* human homologs, ATP7a and ATP7b are localized to the endoplasmic reticulum to transport copper across the Golgi membrane for secretion. Specifically, subcellular trafficking of copper through ATP7a is transported to the plasma membrane while ATP7b transports copper to cytoplasmic vesicles (Adle et al., 2007).

Past Studies and Results

Sensitivity of mRNA to environmental conditions

Various mRNAs involved in copper homeostasis are sensitive to different environmental conditions. Most of these mRNAs have been found to contain long 3'-UTRs and are thus directly targeted for degradation by the NMD pathway. Studies by Peccarelli and Kebaara in 2016, however, suggest that the sensitivity of these mRNAs to degradation changes with their environment. For example, *MAC1* mRNAs were shown to be less sensitive to NMD when the cells were grown in lower copper media. *MAC1* produced one predominant isoform in rich media compared to two isoforms in low copper conditions. Additionally, half-lives between these isoforms differed indicating stabilization of *MAC1* mRNAs through the evasion of NMD in low copper media. *PCA1* produced two mRNA isoforms, as shown in figure 7, when yeast cells were grown in rich media and regulation may also be dependent on the environmental conditions. These results have indicated some physiological significance as a decreased sensitivity to NMD allows the continued expression of copper homeostatic proteins. This mechanism hints to the conditional regulation of different mRNAs depending on the necessity of their proteins and functions in various environments (Peccarelli and Kebaara 2016).

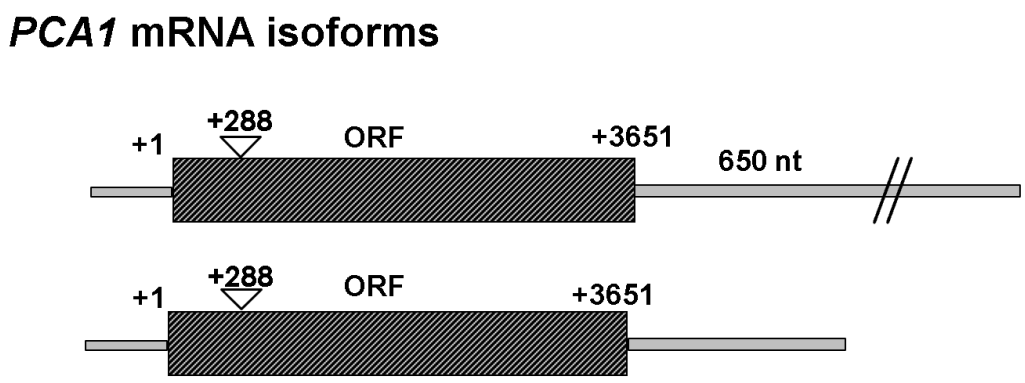

Figure 7 – PCA1 mRNA isoforms. In rich media, PCA1 produces two mRNA isoforms. Under low copper conditions, PCA1 mRNA are differentially regulated compared to rich media.

Research Objectives

To determine the tolerance of two genetically distinct yeast strains to varying amounts of copper

Various yeast strains may confer different resistance to copper toxicity due to their evolutionary divergence. Cellular responses between yeast strains are still being examined. Comparing the results of growth rates and mRNA expression in different strains will allow further research into the genetic and evolutionary background of eukaryotes.

To discover the degree of PCA1 regulation

NMD has been shown to regulate various mRNAs involved in copper homeostasis such as MAC1 and PCA1. These mRNAs have been shown to be sensitive to different environmental conditions such as high levels of copper. The extent and degree of regulation continues to be investigated, but it is known that NMD is integral to the biological function. Discovering how and why NMD regulates these mRNAs may

yield future clues to how the function of NMD may be dependent on other factors or differentially influenced.

To analyze how NMD regulation relates to copper tolerance

The degree of NMD regulation will give insight to the mechanisms for metal detoxification and homeostasis. Through examining the changing rate of mRNA expression when *S. cerevisiae* is grown in varying copper conditions, we can gather support for NMD's role of regulation and more information regarding *S. cerevisiae's* tolerance to copper.

CHAPTER TWO

Materials and Methods

Background

Table 1 - *Saccharomyces cerevisiae* strains used in this study.

Yeast Strain	Genotype	Source
W303a (BKY8)	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-101*	[19]
AAY320 (BKY43)	*a, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1::URA3 (upf-Δ2)*	[20]
RM11-1a (BKY100)	*MATa leu2Δ0, ura3Δ0 HO::KanMX6*	[14]
BKY111	*MATα leu2Δ0, ura3Δ0 HO::KanMX6, UPF1::URA3 (upf-Δ2)*	This study

For measurement of steady-state mRNA accumulation levels, several strains were used to compare the variation between strains with a functional and non-functional nonsense-mediated mRNA decay pathway. Originally, strains W303a/BKY8 (*UPF1*

wild-type) and AAY320/BKY43 (*upf1*Δ NMD mutant) were used to measure the general differences between growth in different environmental conditions and mRNA expression patterns in NMD wild-type and NMD mutant yeast strains. Strains RM11-1a (*UPF1* NMD wild-type) and BKY111 (*upf1*Δ *NMD* mutant) were also utilized to test growth and the change in *PCA1* mRNA expression. RM111-1a is a natural strain found in the environment and the NMD mutant was developed within this lab.

Complete minimal (CM) and CM with different concentrations of Copper

Standard sterile techniques were used when growing and storing yeast strains (Ausubel et al., 1998). To determine the influence of environmental copper levels on the growth of yeast cells and select mRNA expression, all strains were grown on solid YAPD agar plates. When inoculating the cells in media with a high copper concentration, cells were first cultured till saturation in complete minimal media and then subcultured into 600 μM copper containing CM media (high copper conditions). For growth in low copper conditions, the same procedure was used, but subcultured in CM containing 100 μM Bathocuproinedisulfonic acid (BCS) and yeast nitrogen base devoid of copper and iron (YNB-$CuSO_4$-$FeCl_3$). The glassware was soaked in 10% nitric acid overnight to remove any copper in the flasks and tubes.

Growth Curves

Growth curves were performed over a span of 24 hours. Yeast cells were cultured till saturation in complete minimal media overnight. The next day, 300 μL of each saturated yeast culture was pipetted into 10 mL of CM (approximately OD_{600} of 0.1 abs) and incubated for 12 hours at 30 °C. The absorbance was recorded every 2 hours using a spectrophotometer until 12 hours was reached. The results were graphed using Sigmaplot

2000, Version 6.10 software. A linear plot graphed the optical density (OD_{600}) versus time.

Harvesting yeast cells for the switch from CM to 600μM copper

For growth of yeast cells in liquid media, strains were saturated overnight in 3 mL of CM media at 30 °C and then diluted to an OD_{600} of 0.1 abs in 50 mL of CM. The cells were then grown to an OD_{600} of 0.4-0.6 abs (mid-log phase) and transferred to sterile centrifuge tubes. The culture was pelleted by centrifugation for 5 minutes at 4000g. The supernatant was removed, and the cells were washed by resuspending them in 10 mL of sterile autoclaved water. Pelleting was repeated for 5 minutes at 4000g in the centrifuge. Washing and pelleting was then repeated. Following growing the yeast cells, the pellet was resuspended in 30mL of 600 μM copper containing CM media (high copper conditions). Sampling began immediately by pipetting 3 mL of the cells into microcentrifuge tubes. The tubes were pelleted through centrifugation for 20 seconds at 2000g. The supernatant was removed, and the pellet was chilled in an ethanol bath containing dry ice and then frozen. The harvesting of each sampling time point was repeated following the first sampling for nine more time points. Time points were spaced 5 minutes apart from each other. Cell pellets were then stored at -70 °C.

Harvesting yeast cells for half-lives using thiolutin

Growth of the yeast cells was performed exactly as harvesting yeast cells for the switch from CM to 600μM copper was conducted. After yeast cells were grown, harvesting of cells for half-lives was conducted. The pellet was resuspended in 25 mL of CM media with 375 μL of Thiolutin (2 mg/mL) in dimethyl sulfoxide (DMSO). Sampling began immediately by taking 3 mL of the suspended cells in microcentrifuge

tubes. The tubes were pelleted through centrifugation for 20 seconds at 2000g. The supernatant was removed, and the pellet was chilled in an ethanol bath containing dry ice and then frozen. The harvesting of each sampling time point was repeated following the first sampling for seven more time points. Harvesting time points after the first collection were as followed: 3, 6, 9, 12, 18, 25, and 35 minutes. Pellets were then stored at -70 °C.

RNA extractions

RNA extraction from yeast cells was performed using the hot phenol method (Kebaara et al., 2003). RNA extractions were performed subsequent to harvesting for the switch and the RNA half-lives by first adding 500 µL of Complete Buffer A. The cells were resuspended by a brief vortex and 600 µL of Buffer A saturated phenol at 65 °C was added. Each tube was mixed by vortex for 10 seconds every 30 seconds. Tubes not in the process of mixing are placed in a water bath at 65 °C. After 6 minutes, the tubes were centrifuged for 30 seconds at 15000 rpm. The layer of phenol was then removed, and repetition of Buffer A saturated phenol addition and mixing was repeated. The samples were then centrifuged for 3 minutes at 15000 rpm and the aqueous layer of the sample was added to a new microcentrifuge tube containing 600 µL of 1:1 phenol buffered with TE:chloroform mixture. The samples were then vortexed for 20 seconds and then centrifuged for 3 minutes. The aqueous layer was subsequently moved to a new microcentrifuge tube containing 1 mL of absolute ethanol and 50 µL of 3 M NaOAc (pH 5.2).

After briefly vortexing the tubes, the samples were incubated for 15 minutes on ice. Afterwards, the cells were centrifuged at 15000 rpm for 10 minutes to form a pellet. The supernatant was aspirated and the tube was left open at 37 °C for 5 minutes. The

pellets were then resuspended in 400 µL of DEPC dH_2O along with 1 mL of absolute ethanol and 40 µL of 3 M NaOAc. Mixing, incubation in ice, and aspiration was repeated. The pellet was resuspended with 1 mL of 70% ethanol and mixed through vortexing for 20 seconds. Centrifugation for 5 minutes at 15000 rpm and aspiration followed. The resulting pellet was first left open at 37 °C for 5 minutes then dissolved in 50 µL of DEPC dH_2O and heated for 10 minutes at 65 °C. After vortexing and centrifuging briefly, the RNA absorbance at A_{260} and A_{280} was determined using a Thermo Scientific™ NanoDrop™ One^C, and the RNA concentration was diluted to 1 µg/µL. RNA samples were stored at -70 °C until RNA gels for northern blots were conducted.

Northern blotting

To measure mRNA half-lives and accumulation levels, yeast total RNA was used. Concentrations of total RNA equivalent to 15 µg was ran on a 1.0% agarose-formaldehyde gel. Following gel electrophoresis, the RNA was transferred to a GeneScreen Plus® (PerkinElmer, Boston, MA) nitrocellulose membrane. To conduct the protocol, the gel molds were first washed with RNase*Zap*® RNase Decontamination Solution and rinsed with dH_2O. To make the 1.0% agarose-formaldehyde gel, 1.5 g of agarose was added to 135 mL of DEPC treated dIH_2O in an Erlenmeyer flask and microwaved for 3 minutes. The flask was swirled every 15 seconds during the 3-minute heating process. The flask was then transferred to a 60 °C water bath. After 2 minutes in the water bath, 15 mL of 10X NorthernMax™ Denaturing Gel Buffer was added. After 4 more minutes, the liquid agarose was then poured into the gel molds and sat for 30 minutes.

For the samples, a total of 60 µL was ran for each well. The samples contained 15 µL of RNA and 45 µL of 3x loading dye. The RNA ladder used was the Ambion® RNA Millennium™ Markers and 3 µL was added to 12 µL of DEPC dH_2O along with 45 µL of 3x loading dye (REF). Each tube was incubated at 65 °C for 15 minutes and centrifuged briefly before loading on a gel and running the gel at 120V. After the most distal band from the wells traveled three fourths of the way across the gel, the lane with the ladder was removed and the membrane transfer was immediately performed. The ladder was then inoculated with 10 µL of ethidium bromide in 200 mL of dH_2O overnight.

The transfer of RNA to a membrane used the capillary blot transfer protocol from the NorthernMax™ Complete Northern Blotting kit (Life Technologies, Grand Island, NY). Figure 8 demonstrates the assembly of the transfer materials to perform the protocol. After a ~2-hour transfer, the transfer was disassembled and the RNA was cross linked to the membrane using a UV cross linker. The membrane was finally stored in glad wrapping after two hours of transferring the RNA.

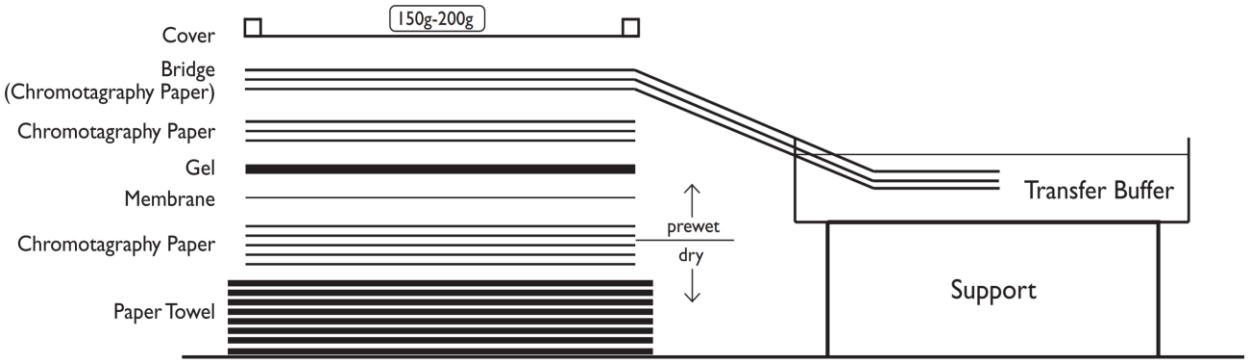

Figure 8 – Transfer material set up for NorthernMax™ Complete Northern Blotting kit protocol (REF). The RNA transfer set up onto a northern blot nylon membrane was left for two hours prior to probing with oligolabeled DNA probes.

Probing the membranes

Oligolabeled DNA probes were used to probe each northern blot. Labeling of the probe fragments used [α-^{32}P] dCTP using the RadPrime DNA Labeling System (Life Technologies, Grand Island, NY). All the membranes were probed with *PCA1, CYH2, SCR1*, and *CUP1* in that order. *PCA1* is a cadmium-transporting P1B-type ATPase associated with cadmium and copper regulation. This was the main mRNA being measured for the experiment. *SCR1* was a control to measure mRNA levels. Specifically, *SCR1* is an RNA polymerase III transcript that encodes for a subunit of the Signal recognition particle. *SCR1* is resistant to toxic copper concentrations and NMD degradation. This ensured that the concentration of RNA in each well of the gel was normalized. *CYH2* was another control for confirming the functionality of NMD within each sample as *CYH2* pre-mRNA is regulated by NMD (He et al., 1993). *CUP1* was the last control to confirm the high copper conditions. It produces a metallothionein copper-binding protein and is expressed in high copper concentrations by Ace1 transcription factor induction.

Each DNA probe was made using the RADPrime DNA Labeling System from Invitrogen Life Technologies. The membranes were pre-prehybridized for 2 hours in 24 mL of prehybridization/hybridization buffer at 42 °C (~10 mL of prehybridization/hybridization buffer was used for every 100 cm^2 of the membrane). The designated Open reading frame (ORF) probe was boiled for 5 minutes in a water bath and incubated on ice. In a microcentrifuge tube, 5 µL of the ORF probe, 16 µL of dH_2O, 1 µL of dATP, 1 µL of dGTP, 1 µL of dTTP, 20 µL of 2.5x rado prime buffer, 5 µL of [α-^{32}P] dCTP, and 1 µL of Klenow was added. The tube was then placed in a heat block at 37 °C for 1 hour. Afterwards, 50 µL of TE (pH 8.0) was added to the tube. To prepare a column

using a 1 mL disposable syringe, 0.1 mL of sterile glass wool was placed at the bottom of the syringe. The syringe was filled with Sephadex G-50 and then placed in a 15 mL corex tube.

Next, the tube was centrifuged at 1600 g for 4 minutes. Refilling of the syringe with Sephadex G-50 and centrifugation was continued until solid resin was built up until 9 mL. Following making the syringe, 0.1 mL of TE (pH 8.0) was added and centrifuged at 1600 g for 4 minutes. This step was repeated and 100 µL of the probe from the heated microcentrifuge tube was added to the syringe. A decapped microcentrifuge tube was added to the bottom of the 15 mL corex tube. The tube was centrifuged at 1600 g for 4 minutes and the effluent that collected in the decapped microcentrifuge tube was removed from the tube. The effluent was then used to calculate the specific activity of 1 µL of the probe using a liquid scintillation counter.

To prepare the probe for addition into the hybridization bottle with the membrane, a microcentrifuge tube with a screw on cap was placed in a 42 °C water bath with 1 mL of prehybridization/hybridization buffer. 1-5 X 10^6 cpm of the radiolabeled DNA probe per mL of hybridization buffer was then added to the microcentrifuge tube and placed on a heat block for 5 minutes at high temperature. The fluid heated probe in the hybridization solution from the microcentrifuge tube was then added to the hybridization bottle containing the membrane and left in the hybridization oven overnight.

After 16 hours of hybridization the membrane was washed twice for 15 minutes with 50 mL of 2X SSPE at room temperature and once with 50 mL of 2X SSPE/2% SDS for 15 minutes at 65 °C. The membranes were then wrapped in plastic wrap and placed on a PhosphorImaging screen after checking with the Geiger counter to estimate how

long to leave the membranes on the Phosporimaging screen. If the probe used was *PCA1*, the membranes were left on the screen for 10 days. The membrane was left for 2 days if using *CYH2*, and 2 hours for *SCR1* or *CUP1*. To strip the membrane of the labeled probe, the membranes were boiled in stripping solution (0.1% SDS/0.01 X SSC) for two minutes. The stripping solution was then poured out and replaced with new stripping solution. The membranes were boiled again a total of 5 times. The membranes were sequentially probed with *CYH2, CUP1*, and *SCR1*. After each probing session, they are scanned and stripped before continuing with the next probe.

Image analysis

Each Northern blot was phosphorImaged™ after probing using a Typhoon Phosphorimager (Amersham Pharmacia Biotech, Inc.) and quantified with ImageQuant software. In order to calculate the stead-states of each trial, Sigmaplot 2000, Version 6.10 software was used. *SCR1* was used as a loading control to normalize the amount of mRNA at each time point. The mRNA stead-states were calculated by finding the ratio of the amount of mRNA, after normalizing the amount to *SCR1*, to the amount of mRNA of the first time point (time point 0).

CHAPTER THREE

Experimental Results

Growth Curves

Growth of wild-type and NMD mutant yeast strains under normal growth conditions, high copper and low copper

To distinguish the copper tolerance of wild-type and NMD mutants, growth curves were conducted to assess the effects of copper conditions on *S. cerevisiae* with a functional and non-functional NMD pathway. Figure 9 portrays the growth rates of yeast strains wild-type (BKY8) and NMD mutants (BKY43) grown in regular media as well as lower copper conditions over a span of 24 hours. Both strains exhibit similar growth rates when grown in a complete minimal medium and low copper media but displayed a significant decrease in growth rates for low copper conditions compared to complete minimal media. The decreased growth rates in low copper conditions give support for copper as an essential micronutrient for eukaryotic processes.

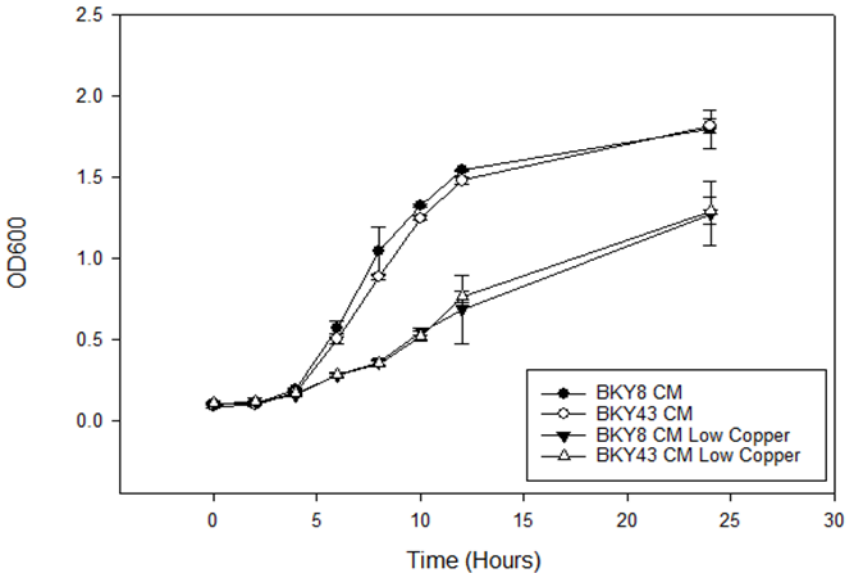

Figure 9 – Wild-type (BKY8) and NMD mutants (BKY43) grow at similar rates in Complete Minimal but have reduced growth rates under low copper media.

Moreover, Figure 10 below displays the growth rates of BKY8 and BKY43 in 600 µM copper (high copper conditions). Under high copper conditions both strains exhibited significantly lower growth rates compared to normal media. The decrease can most likely be attributed to the high toxicity of copper to the physiological processes of the cell. While not significant, the growth rate of the wild-type (BKY8) strain was slower than the NMD mutant (BKY43) yeast cells suggesting that yeast strains with an inactive NMD pathway are more capable of growing in high concentrated copper environments than strains with an active NMD pathway. These results support the notion that mRNAs for copper homeostasis are affected by the environmental conditions.

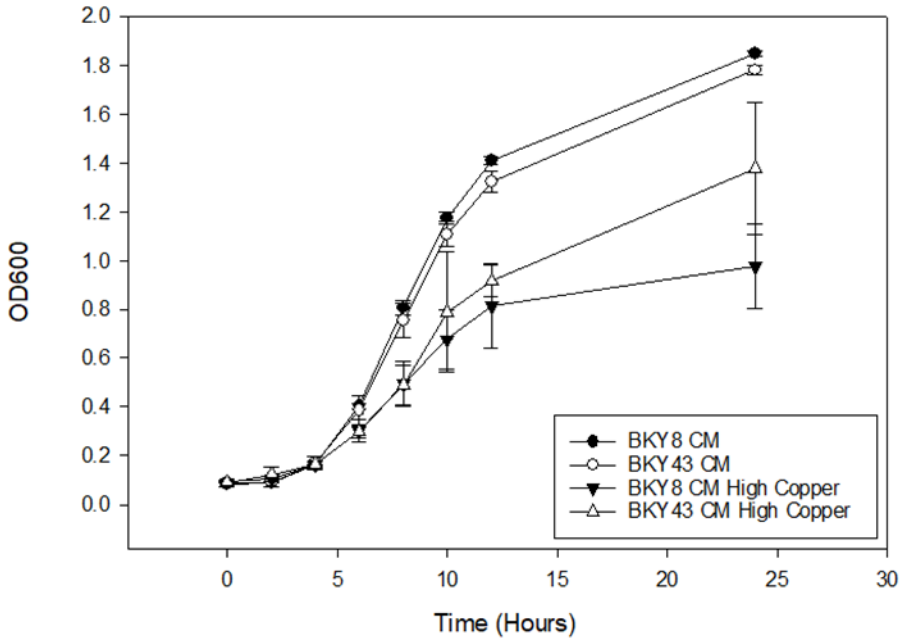

Figure 10 – Wild-type strains exhibit slower growth rates than NMD mutants in 600uM copper media compared to regular media.

Furthermore, growth curves were performed on RM11-1a *S. cerevisiae* wild-type (BKY100) and NMD mutant (BKY111) strains expressing the functional allele of *PCA1*. Figure 11 portrays the growth rates of BKY100 and BKY111 grown in complete minimal media as well as lower copper conditions over a span of 24 hours. Similar to the results in Figure 9 above, there were no significant differences between either strains. Low copper conditions, however, displayed no significant difference in growth compared to regular media.

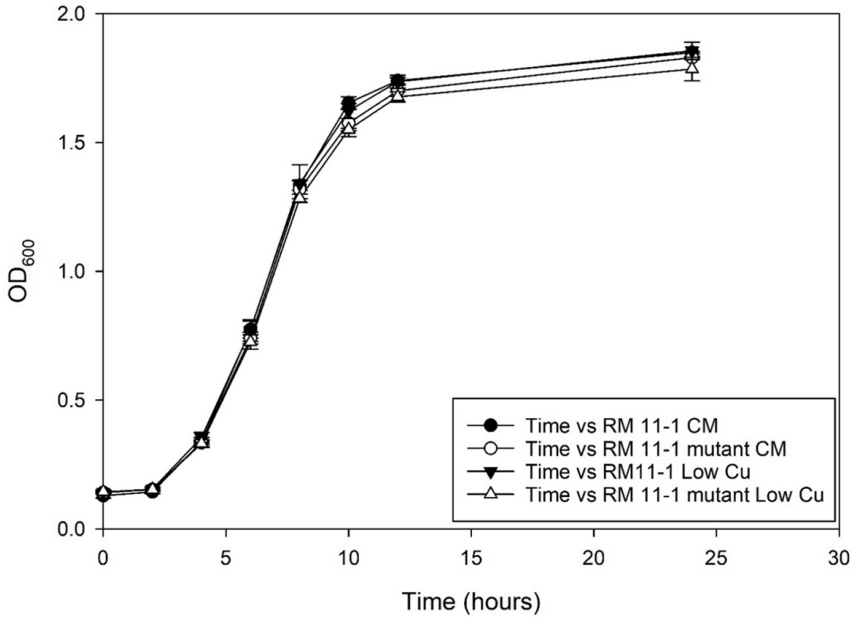

Figure 11 – Wild-type and NMD mutant RM11-1a grow at similar rates in Complete Minimal and Low copper media.

In a high copper environment, the growth rates of wild-type (BKY100) and NMD mutant (BKY111) performed similarly as shown in Figure 10. Both strains exhibited significantly lower growth rates when grown in high copper media compared to complete minimal media. In comparison to the same growth curves for W303a wild-type (BKY8) and W303a NMD mutant (BKY43), the wild-type strain was not slower than the mutant strain when grown in 600 μM copper. Figure 12 indicates that these differences may reflect either a role in *PCA1* or upf1 proteins for copper homeostasis. Ultimately, more in-depth analysis into the expression of these proteins was needed to reveal more about yeast's response to environmental stimuli.

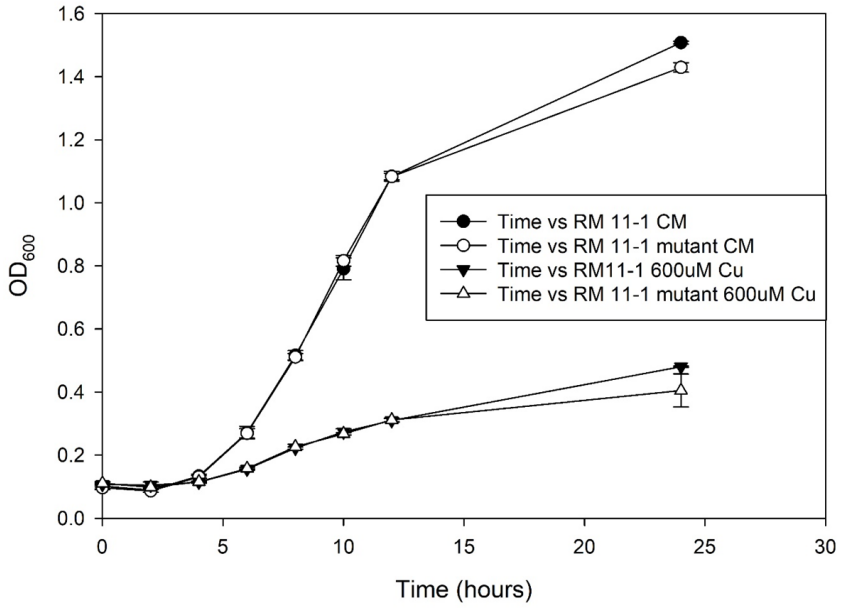

Figure 12 – Wild-type and NMD mutant RM11-1a grows at similar rates but is significantly lower in 600uM copper media relative to Complete Minimal media.

Accumulation of PCA1 mRNA Levels

PCA1 mRNA expression in W303a and RM11-1a yeast strains

Due to the differences observed in growth rates for wild-type and NMD mutant yeast strains. We sought to observe the *PCA1* mRNA accumulation levels in W303a and RM11-1a yeast strains that confer a difference in their *PCA1* gene. As stated in the introduction, the two strains encode different alleles of the *PCA1* gene (Figure 6). W303a has a similar *PCA1* allele as the S288c sequence from Figure 6, but a single nucleotide mutation in W303a confers a non-functional ATP-binding pocket. Table 2 shows the difference in *PCA1* mRNA accumulation levels between RM11-1a (natural PCA1) and W303a (lab PCA1) strains. This allows us to compare the differences in mRNA expression attributed to *PCA1* in NMD wild-type and NMD mutants.

We observed an increase of mRNA expression for W303a when inoculated in 100 µM copper and a decrease in expression in 600 µM copper compared to complete

minimal media. In contrast, the RM11-1a strain exhibited a decrease in both high copper conditions. For low copper conditions, the *PCA1* mRNA levels in W303a NMD mutant (BKY43) accumulated to higher levels compared to the wild-type strain (BKY8) but was lower than the results examined under complete minimal. These levels are similar to the *PCA1* mRNA levels that accumulated for the 600 µM copper condition suggesting expression levels are regulated by NMD depending on environmental copper concentrations.

Table 2. *PCA1* relative mRNA accumulation levels in *W303a* and *RM11-1a* under different growth conditions

Growth media	***W303*** **(*upf1Δ/UPF1*)**	***RM11-1*** **(*upf1Δ/UPF1*)**
Complete minimal	2.71 ± 0.3	10.9 (± 3.7)
100 uM copper	3.31 ± 0.3	8.0 (± 3.2)
600 uM copper	2.15 ± 1.2	1.63 (± 1.3)
100 uM cadmium	3.17 (± 1.3)	1.0 (± 0.3)
100 uM BCS*	1.73 ± 0.5	2.28 (± 1.0)

BCS* - (Bathocuproinedisulfonic acid) Low copper, ND- not determined. mRNA steady-state accumulation levels were done in triplicate and reported here as an average plus/minus the standard deviation (SD)

PCA1 mRNA expression in wild-type and NMD mutant W303a yeast strains after switch from complete minimal to 600 µM copper

Since there were noticeable, but not significant, growth rate differences between wild-type and NMD mutant W303a yeast strains (BKY8 and BKY43) as well as a different trend in *PCA1* mRNA expression for RM11-1a wild-type and NMD mutant

strains (BKY100 and BKY111), mRNA steady-state levels were evaluated at differing time points to evaluate temporal changes in *PCA1* gene expression. After switching yeast cells from complete minimal media to media containing 600 µM copper, the *PCA1* mRNA levels of wild-type (BKY8) accumulated to higher amounts at 15 minutes and then slowly decreased at 25 minutes as indicated through the fold changes in figure 13 below. Similar trends were identified for the NMD mutant (BKY43) but with a slower rate of accumulation in *PCA1* mRNA. These results suggest a decreased rate of *PCA1* expression in NMD mutants compared to wild-type strains in W303a yeast strains.

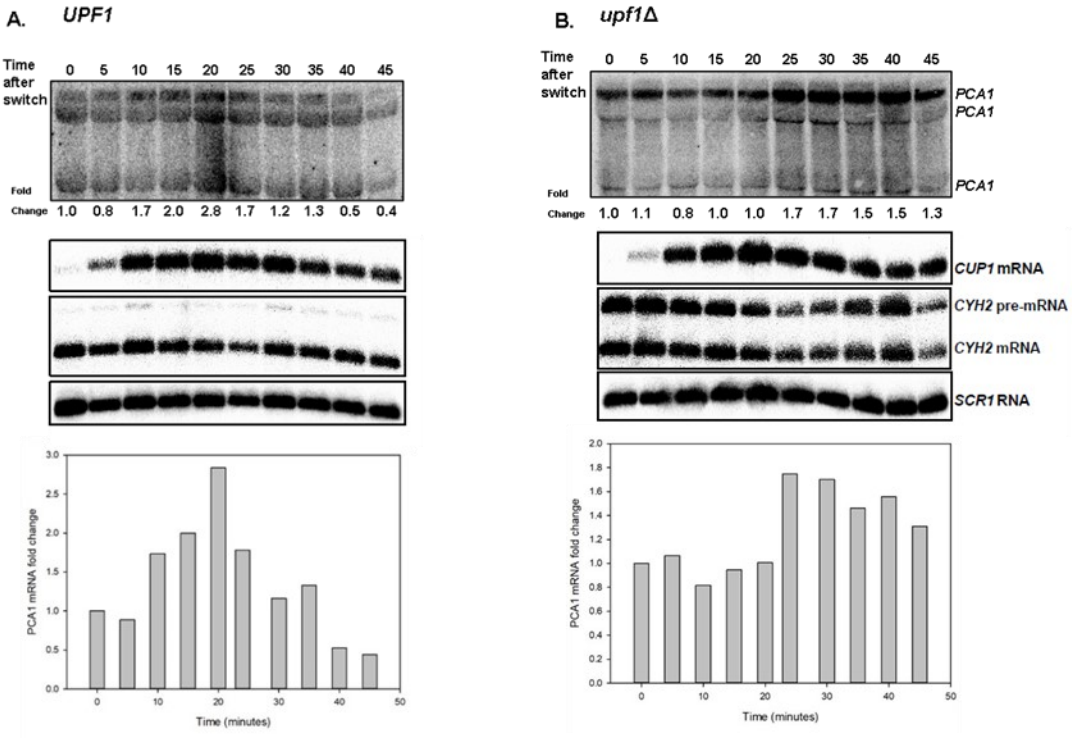

Figure 13 – W303a Wild-type and NMD mutant strains PCA1 mRNA expression rates when switching from Complete Minimal to 600uM copper media. Fold changes are graphically represented and indicate a delayed spike in expression rate for NMD mutants than the wild-type strain.

Interestingly, the *PCA1* mRNAs were stabilized during periods of high copper concentrations and subsequently destabilized. This phenomenon may explain the cellular response to copper for *PCA1* gene expression for proteins involved in metal toxicity regulation. As indicated by similar comparisons between *CUP1* and *PCA1* mRNA levels. *PCA1* mRNA expression are regulated by copper levels within the cell. Probing the same northern blots with *CUP1* mRNA probes revealed increases in *CUP1* mRNA expression with the addition of copper to the media and subsequent decreases. Thus, expression of genes involved with metal detoxification specifically remain dependent on copper levels and can temporally change based on metal concentrations over time.

PCA1 mRNA expression in wild-type and NMD mutant RM111-1 yeast strains after switch from complete minimal to 600 μM copper

The switch from complete minimal media to 600 μM copper was also conducted for RM11-1a wild-type and NMD mutant strains (BKY100 and BKY111). After switching RM11-1a yeast cells from complete minimal media to media containing 600 μM copper, the *PCA1* mRNA levels from both wild-type (BKY100) and NMD mutant (BKY111) remained constant throughout the entirety of the experiment (Figure 14). The differences in mRNA accumulation trends between W303a and RM111-1a are most likely attributed to the single nucleotide mutation for the *PCA1* gene. Paired with the differences observed in growth rates, cellular responses to copper environments between both strains are significantly distinct. As W303a strains exhibited an increase followed by a decrease in *PCA1* mRNA expression, RM11-1a strains did not show a change in *PCA1* mRNA expression. The growth curves for RM11-1a NMD strains also did not show any differences when grown in high copper media, but W303a NMD strains showed variance in growth rates between the wild-type and NMD mutant strains.

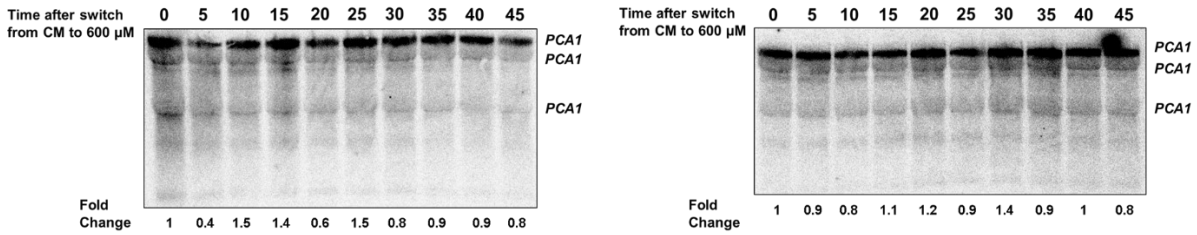

Figure 14 – RM11-1a Wild-type and NMD mutant strain PCA1 mRNA expression rates when switching from Complete Minimal to 600uM copper media. Fold changes stay consistent throughout the length of the experiment with no significant difference between NMD mutant and wild-type strain.

Comparison of RNA expression trends

In comparison to the W303a strain, the RM11-1a *PCA1* mRNAs were stabilized following the introduction of high copper concentrations but did not portray any significant deviation from the initial level of mRNA accumulation. The W303a strains exhibited an increase in expression followed by destabilization of the *PCA1* mRNAs. This trend may allude to either the difference in cellular response to copper for *PCA1* gene expression or the difference in copper sequestration efficiency for RM11-1a compared to W303a. Ultimately, the results from RM11-1a and W303a mRNA accumulation levels allude to the variable expression of genes involved with metal detoxification.

CHAPTER FOUR

Discussion

Differences in growth rates in different conditions

Among the differences in growth rates between high and low copper conditions, the RM11-1a and W303a strains showed similar trends when comparing high copper conditions to complete minimal. Both the RM11-1a and W303a NMD wild-type and NMD mutant strains exhibited a decreased rate of growth when inoculated in high copper concentrated media. These support the prognosis that copper at high levels can be toxic to the cell by introducing superoxide radicals that disrupt cellular processes. Under low copper conditions, both strains were predicted to exhibit similar trends of decreased growth due to the requirement of copper as an essential micronutrient. The RM11-1a strain, however, showed similar growth rates between NMD wild-types and mutants when grown in low copper concentrations compared to complete minimal.

The RM11-1a strain may have a particular tolerance for low copper concentrations given its genetic difference and the *PCA1* gene. In comparison to the W303a strain, the RM11-1a strain contains a functional ATP-binding pocket for copper. This change supports the belief that a functional binding pocket may induce a more efficient sequestration of copper for cellular use within *Saccharomyces cerevisiae* allowing an increased resistance to low copper conditions.

For comparisons between the wild-type and NMD mutants for both strains, the growth curves showed that there were no significant differences in growth. For W303a high copper conditions, however, the NMD mutant (BKY43) exhibited slightly higher rates of growth than the NMD wild-type (BKY8). This result supports the belief that

NMD mutants exhibit higher levels of copper tolerance compared to NMD wild-types. This can be explained by the increased rate of expression for copper homeostatic mRNAs that are involved in regulating the copper concentration within the cell. Due to their nonfunctional NMD pathway, these mRNAs are regulated at a lesser extent in NMD mutants allowing more copper homeostatic proteins to resist the higher levels of copper in the media. While not significant, the same trend did not hold for the RM11-1a strain and suggests that the RM11-1a strain cannot tolerate high levels of copper to the same degree as W303a. The result may be explained again by the genetic difference in *PCA1* composition for W303a compared to RM11-1a.

NMD regulation of W303a PCA1 correlated with copper concentrations

In contrast to the RM11-1a strain, W303a wild-type and NMD mutant expression of *PCA1* mRNA varies depending on the concentration of copper in the media. Figure 13 portrays the correlation between mRNA accumulation levels with copper levels over time. There is a temporal accumulation pattern that can be correlated between *CUP1* and *PCA1* mRNA. As more copper is transported into the cell (indicated by *CUP1* mRNA accumulation), more *PCA1* mRNA is stabilized for the production of the pca1 protein. The *CUP1* gene encodes for a copper-binding metallothionein that is stimulated by an Ace1 transcription factor in the presence of high copper concentrations. These findings support the hypothesis that an increase in copper requires the production of metal homeostatic proteins to regulate the amount of copper that is present inside. With an increase in the amount of proteins that sequester copper inside of the cell, the amount of superoxide radicals that are produced from high levels of copper is minimized thereby protecting the cell from damage.

NMD regulation of RM11-1a PCA1 does not correlated with copper concentrations

The same cannot be supported for the RM11-1a strain since mRNA accumulation levels remained unchanged throughout the experiment indicated (Figure 14). Additionally, the copper concentrations were not able to be evaluated due to COVID-19 obstructing the necessary materials for *CUP1* northern blots. It is likely, however, that the copper levels increased to a significant level and remained unchanged subsequently. This can be explained by the results in Figure 12 with both the RM11-1a wild-type and NMD mutant growing at slower rates than W303a strains (Figure 10). Comparison between the high copper growth rates of RM11-1a and W303a strains after 24 hours indicate that W303a strains grew more than twice the rate of RM11-1a strains. These support the conclusion that W303a strains exhibit increased tolerance to copper at high levels.

Variations between strains may indicate altered decay rates

The differences in RNA steady state levels between the RM11-1a and W303a strains may be attributed to the divergence in genetic backgrounds. Whether the difference is solely due to *PCA1* variation remains to be investigated, but the variance in results may be caused by the lack of a functional binding pocket in pca1p from the W303a strains. Further explanations may be caused by evolutionary divergence. RM11-1a strains may not show a temporal cellular response to copper because the lack of prior exposure to copper over its timeline. W303a strains might be more tolerant to copper because it was exposed to higher concentrations of copper during its evolutionary timeline. With both explanations being plausible, the same diverging trend in degrees of regulation can be seen in the conducted growth curves shown in figures 10 and 12. Differences in growth rates between wild-type and NMD mutants remain unchanged for

the RM11-1a strain. Similarly, no change was observed in the mRNA steady state levels. For the W303a strain, the growth curves showed a difference between wild-type and NMD mutant growth rates that similarly correlate with the change in mRNA accumulation. These trends may give rise to more considerations on altered decay rates between both the W303a and RM11-1a strain. Further evaluation of the cellular response that these strains have to high levels of copper will be necessary to review the full effect that *PCA1* has on both strains.

Research implications and translational considerations

This study aims to research the effects of metal on eukaryotic organisms as well as their conserved mechanisms in humans. Specifically, *PCA1* is an evolutionarily conserved gene in humans as well and consequences of a genetic defect can be examined through various diseases. Menkes disease, attributed to a copper deficiency within cells, and Wilson's disease, caused by an excess of copper in cells, both affect a significant portion of our population. With copper being an important micronutrient that is also toxic at high levels, this research will help aid our understanding on how humans respond to metals on a broader scale and pinpoint the focus on cellular mechanisms that are crucial to metal homeostasis. Potential treatments for these diseases cannot be specifically characterized, but these results ought to aid in their development for the near future.

Future analysis

Further experimentation to accompany this paper will be conducting similar experiments with cadmium. This metal, while not an essential micronutrient, has been shown to express higher levels of toxicity as a carcinogen and is prevalent in the day-to-day lives of humans. Many potential concerns of cadmium have been seen in electronic

and electrical waste disposal of batteries, plastic, and jewelry. Table 2 indicates that there is a difference in steady state accumulation levels of *PCA1* mRNAs in W303a and RM11-1a wild-type and NMD mutant cells grown in the presence of cadmium. Similar to copper, *PCA1* in W303a strains showed a difference between wild-type and NMD mutants. The NMD mutants exhibited an increased rate of *PCA1* expression compared to the wild-type strains. The difference in expression was also shown to be more significant than the same experiment with 600 μM copper. To examine the rate of cadmium regulation in eukaryotes, a similar analysis of mRNA steady state levels will be conducted by switching *S. cerevisiae* cells from complete minimal to cadmium concentrated media. Growth curves for the same strains may also be conducted in order to support the results displayed for mRNA accumulation. The experiments can also include examining the differences in wild-type and NMD mutant strains in order to show how regulation is affected within the NMD pathway.

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