ABSTRACT

The role of *CTR3* in the copper tolerance of nmd mutants of *Saccharomyces cerevisiae*

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The nonsense-mediated mRNA decay (NMD) pathway is a mechanism by which eukaryotic cells degrade mRNAs with nonsense codons and some natural mRNAs. *CTR3* mRNA is a natural mRNA degraded by the pathway and codes for a copper transporter. This study seeks to investigate the regulation of *CTR3* by the NMD pathway and the contributions this regulation has on the copper tolerance of nmd mutants of *Saccharomyces cerevisiae*. This is accomplished through creation of *CTR3* gene disruption fragments and transformation of these fragments into *S. cerevisiae* with an active and an inactive NMD pathway. This creates strains with an active and inactive *CTR3*. These strains will be grown on both copper-limiting and copper-rich media to assess the copper-tolerant nature of nmd mutants with and without *CTR3*. At the time of writing, *CTR3* gene fragments have been isolated and transformed into yeast strains. Unfortunately, none of the transformants showed *CTR3* disruption.

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THE ROLE OF *CTR3* IN THE COPPER TOLERANCE OF NMD MUTANTS OF *SACCHAROMYCES CEREVISIAE*

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By

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CHAPTER ONE

Introduction

Basic Principles and Saccharomyces cerevisiae

According to the central dogma of molecular biology, a portion of DNA known as a gene is transcribed into messenger RNA (or mRNA), which is in turn translated into a protein that goes on to perform specific functions in the cell [1]. The generalities of this idea were put forth by Francis Crick in 1958 as a result of contemporary evidence, and although following research has since modified his original claims, his central thesis is still an important concept in the understanding of molecular biology. Countless important cellular functions have their origins in this dogma, making it a fascinating and useful area of cell biology for research. A concept central to the following research is the investigation of the connection between genes, mRNA, proteins, and protein function, as well as the biological consequences of inactive genes. However, it is essential to have an organism that can be both easily and cheaply obtained, maintained, and manipulated in a laboratory environment to effectively investigate these phenomena, since many cellular functions are difficult to observe in more complex organisms.

Saccharomyces cerevisiae (baker's yeast) is the model organism used to stand in for the eukaryotic organisms, and it will fill this role in the context of this research. This is appropriate, considering the relative ease of genetic manipulation of yeast, the large volume of prior research surrounding the yeast genome, and the extent to which basic cellular processes are conserved between *S. cerevisiae* and other eukaryotic organisms, including humans.

S. cerevisiae has been shown to be effective in demonstrating the biological consequences resulting from disruption or manipulation of the central dogma processes due to the relative ease of inducing mutations or manipulating genes [2]. These manipulations (or transformations) can be accomplished by a myriad of methods, including the removal of the yeast cell wall to stimulate uptake of environmental genetic material (spheroplast method), the stressing of the yeast cells to encourage uptake of environmental DNA (the Lithium Acetate/single-stranded DNA/polyethylene glycol method, or LiAc/ssDNA/PEG), electoporation, the glass beads method, and biolistic transformation [3]. Many of these methods are low-cost, easy, and efficient ways to transform yeast cells, allowing for cost-effective and timely research [3]. The LiAC/ssDNA/PEG method is used in this experiment, and will be discussed later in this chapter and in “Chapter Two: Materials and Methods.”

In addition, the genome of *S. cerevisiae* was sequenced in 1996 and has been studied extensively for over a decade, which has led to the creation of the Saccharomyces Genome Database (SGD), an extensive database of all yeast genes, their functions, and research associated with them [2]. The SGD was used in this research to investigate the locations and properties of the genes of interest for the purposes of manipulation and study, which will also be discussed in more detail later.

Finally, the amino acid sequences and functions of many yeast proteins are highly conserved between yeast and many other organisms, meaning that discoveries regarding protein function in yeast are largely transferable to the same proteins in other organisms [2]. Discoveries made by this experiment in *S. cerevisiae* regarding the genes, proteins, and mechanisms in question can therefore be extended to other organisms as well, within reason. Thus, baker's yeast is a practical and efficient model organism for studying the interaction between DNA, RNA, proteins, and protein function.

The Nonsense-mediated mRNA Decay Pathway

Two potential errors (among others) arise from the practical use of the DNA-RNA-protein pathway by organisms such as *S. cerevisiae*: (a) the potential for transcription errors (miscoded mRNAs), which can lead to harmful accumulations of faulty proteins, and (b) the potential for inefficient use of cellular resources in the absence of controlled gene expression. In order to address these issues, the eukaryotic cell has mechanisms by which it can prevent the formation of faulty proteins and control the expression of unneeded mRNAs.

One such mechanism that potentially fills both of these roles is the nonsense-mediated mRNA decay pathway, or NMD pathway. This pathway is a mechanism by

which miscoded mRNAs, namely mRNAs with premature stop codons (also known as nonsense mutations), are targeted and destroyed [4]. These stop codons are specific sequences that appear in the genetic code at the end of a coding portion of mRNA. If they occur too early in the RNA, translation will stop prematurely and the protein itself will be truncated. These faulty proteins can accumulate and become detrimental to normal cell function [5]. The nonsense-mediated mRNA decay pathway targets and destroys mRNAs with premature stop codons, leading to a decrease in mRNA and consequently a decrease in truncated proteins being translated [4]. Three core trans-acting factors, namely Upf1p, Upf2p, and Upf3p, are necessary for NMD to occur in eukaryotes [5]. By selectively inhibiting the expression of one of these genes, the activity of the NMD pathway can be controlled and the effect of the absence of this pathway can be monitored.

Of more importance to the questions addressed in this research is the experimentally observed secondary function of the NMD pathway: the control of gene expression. Research indicates that the nonsense-mediated mRNA decay pathway will also target and destroy mRNAs that do not contain premature termination codons but instead exhibit features that cause them to be regulated by the pathway. One of these features is an atypically long 3' untranslated region (3' UTR) [6]. Typically ranging from 50 to 200 nucleotides in length, the 3'-UTR is located after the stop codon, and does not contain information that codes for specific genes, but instead contains regulatory information [6]. In *S. cerevisiae*, if this region of an mRNA is longer than 350 base pairs, the mRNA tends to be degraded by the pathway [6]. Expression of the protein will decrease if the mRNAs are destroyed, because less mRNAs will be available to be translated. Conversely, if the genes responsible for the proteins of the NMD pathway are

inactivated, phenotypic changes in cell structure and growth associated with mRNAs targeted by the pathway can be observed and measured, because more of these mRNA's will be present to be translated than in the wild-type (or unmanipulated) strain. This method of measurement has proven to be effective in assessing the extent of NMD control on the expression of multiple genes, including *PGA 1* [6] and *CTR2*, a vacuolar copper transporter [5]. Of importance to this experiment is the observation that nmd mutant cells have a higher level of copper tolerance than wild-type cells, which has been shown to be related to the increased expression of *CTR2* in nmd mutants [5].

The High-affinity Copper Transporter CTR3

One of many genes hypothesized to be targeted by the nonsense-mediated mRNA decay pathway is the gene encoding *CTR3*, a high-affinity copper transporter. This gene codes for a copper transporter located on the plasma membrane of cells and functions to transport copper across the membrane and into the cytosol [7]. Expression of this gene is up-regulated in low levels of copper, meaning that it is an important factor in ensuring the cell receives adequate copper when copper is in limited supply [7]. Using a 3'-end processing site predictor, the predicted 3'-UTR length of the mRNA associated with *CTR3* is 2367 base pairs, making it a likely candidate for degradation via the NMD pathway [8]. If this is the case, the removal of the NMD pathway will increase expression of *CTR3*, leading to increased growth in limiting levels of copper compared to the wild-type strain. This is advantageous, as it grants a readily measurable phenotype that can be used to determine both the extent of the influence of the NMD pathway on *CTR3* expression and the contribution of *CTR3* to the copper tolerance of nmd mutants.

Genetic Transformation

The selective inhibition of cellular expression of one or more proteins is necessary to negate the effects of the NMD pathway or *CTR3* and study the results of these changes. This selective inhibition is accomplished by genetic transformation. This process involves three steps: the acquisition of sufficient levels of genetic material to be taken up by the cell, the uptake of the genetic material by the cell, and the incorporation of the genetic material into the cell's genome.

The environmental genetic material to be taken up by the yeast cells is a small fragment of DNA containing the same gene as the gene targeted for inactivation. However, the environmental DNA contains a version of that gene that has been knocked out. To knock out a gene is to insert a genetic marker within the coding region of the gene to prevent its expression (**Figure 1**). The marker itself is also a gene, and usually encodes either a protein that allows the cell to create either an essential amino acid it otherwise could not or a protein that protects the cell from chemicals toxic to the wild-type strain. For the purposes of this experiment, the former will be most important. In order to obtain these small pieces of DNA, it is necessary to isolate them from yeast genomic DNA that already contains a knocked-out version of the gene. This small portion of the genome can be isolated and repeatedly duplicated to acceptable levels by a process called the polymerase chain reaction (or PCR). This process will be described in further detail in "Chapter Two: Materials and Methods."

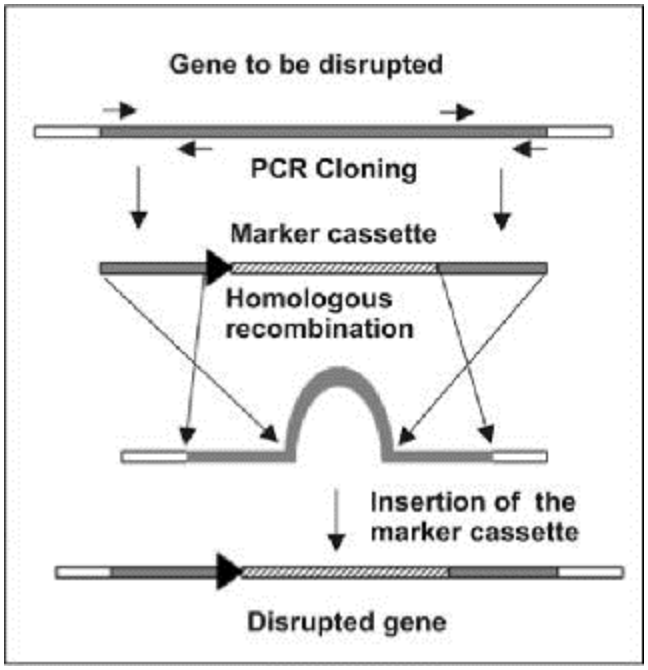

Figure 1 – Genetic transformation via PCR and homologous recombination. This graphic depicts the disruption of a gene with a marker, labeled "marker cassette." The marker will be inserted within the coding region of the disrupted gene via homologous recombination, inactivating the gene and causing expression of the marker cassette. [9]

In order to induce yeast cells to take up environmental DNA, one must place the cell under stress. In the case of this experiment, this is best accomplished via the presence of lithium acetate (LiAc), PEG, and a significant but non-lethal heat-shock, all of which are necessary to encourage transformation [3]. Although the exact mechanism is as of yet unknown, these factors will cause the yeast cells to take up the DNA.

Following the uptake of DNA by the cell, it is necessary for the environmental DNA to be incorporated into the genome of the cell. This is accomplished by a process called homologous recombination. If this process takes place, the cells will be unable to produce the proteins encoded for by the knocked-out genes because the DNA that was taken up has interrupted and replaced the normal gene. The transformed cells will also take on the phenotypic properties granted by the marker used to knock out the gene of interest. For instance, if the marker is a metabolic gene, the transformed cells will grow

on media that lacks the amino acid manufactured by the proteins associated with the marker gene, while the wild-type will not.

The genes of importance to this experiment are *CTR3* and *UPF1*, which code for the high-affinity copper transporter and a protein essential for the NMD pathway, respectively. Genetic transformation will be used to create four genetically distinct strains: a wild-type strain with functional *CTR3* and *UPF1* genes, a knockout strain with a functional *CTR3* gene and a knocked-out *UPF1* gene, and a knockout strain with a knocked-out *CTR3* gene and a functional *UPF1* gene, and a knockout strain with both nonfunctional *CTR3* and *UPF1* genes. With these four strains, the phenotypic changes in cell growth associated with knocking out either gene can be compared to a control strain to assess the extent of their respective effects on high and low-levels of copper. This process will be explored in greater detail in "Chapter 2:Materials and Methods".

The Role of Copper in Cell Growth

Copper is essential to the proper function of cellular processes. It acts as a cofactor for many important enzymes that would be non-functional in its absence [7]. However, it can be harmful to the cell in excessive amounts. Copper participates in Fenton-like reactions, which create hydroxyl radicals. These molecules are highly reactive and disrupt or inactivate important molecules and proteins in the cell, causing stunted growth and potentially death [7]. It is for this reason that cellular control of copper levels is essential for life and growth. If environmental levels of copper are too high, the cell will down-regulate the expression of *CTR1* and *CTR3* (the high-affinity copper transporters of the plasma membrane) [7]. This will decrease the presence of copper transporters on the cell surface, allowing less copper into the cell. If

environmental levels of copper are too low, the expression of genes such as *CTR1* and *CTR3* will be up-regulated, allowing more copper into the cell [7]. Other copper control methods include *CTR2*, a vacuolar membrane copper transporter. This protein has been shown to contribute to the copper tolerance of nmd mutants because it is hypothesized to be controlled by the NMD pathway [5]. A combination of all of these factors (and likely many more) allows for a graded response to the presence of copper and helps the cell maintain relatively constant cytoplasmic copper levels. A change in the careful balance of cytoplasmic copper levels will manifest itself to a greater or lesser extent in levels of cellular growth based on the extent of deviation from ideal copper levels, which in turn allows for the assessment of the activity of copper-control mechanisms such as *CTR1*, *CTR2*, and *CTR3*.

Summary of Questions of Interest

With all this in mind, we can begin to paint a clear picture of the goal of this experiment, namely: to determine whether or not, and to what extent, *CTR3* is controlled by the activity of the nonsense-mediated mRNA decay pathway. To look at it another way, this experiment seeks to determine the contribution of *CTR3* to the observed copper tolerance of nmd mutants. In order to do this, the gene coding for *CTR3* will be knocked out of both wild-type and nmd mutant strains. If the NMD pathway does control the expression of *CTR3*, there will be an increase in cell growth in limiting and possibly excess levels of copper for nmd mutants versus the wild type strain. Also, if *CTR3* contributes to the copper tolerance of nmd mutants, there will be an increase in growth for nmd mutants with active *CTR3* compared to nmd mutants with inactive *CTR3*. All genetic manipulations will be well controlled against unmanipulated strains to rule out possible confounding factors.

CHAPTER TWO

Materials and Methods

Yeast Strains and Media

The *S. cerevisiae* strains used in this study are shown in **Table 1.**

Name	Genotype	Reference
BKY8	*MAT**a** ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Wente et al.,1992 [10]
AAY320 (BKY43)	*MAT**a** ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1-Δ2 (URA3)*	Kebaara et al. 2003 [11]
AAY329 (BKY47)	*MAT**a** ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, UPF1::HIS3 (upf1-*Δ4)	Dr. Audrey Atkin
ctr1Δ/ctr2Δ/ctr3Δ	*MAT**a** ctr1::ura3::KanR ctr2::HIS3 ctr3::TRP1 lys 2-801*	Rees et al. 2004 and Pena et al. 1998 [12,13]

Table 1 – Saccharomyces strains. The nomenclature is derived from Dr. Bessie Kebaara's laboratory organization system.

Agarose Gel Electrophoresis

The majority of analysis of genetic material in this study was performed via agarose gel electrophoresis. This is a method by which DNA fragments can be separated by length and visualized using ethidium bromide.

First, an agarose gel is created, which will be the media through which the genetic material will pass. This gel is a solidified solution of agarose dissolved in 1 x TAE buffer, with the concentration of agar varied with respect to the expected base-pair length of the genetic entity under investigation. This is demonstrated in **Table 2**. For the purposes of this study, all agarose gels were at a concentration of 0.8%. 50 mL of TAE and 0.4 g of agarose were mixed, and agarose was completely dissolved by heating in a microwave for 1.5 minutes, stirring every 15 seconds. 2 µL of 10 mg/mL ethidium bromide solution was then added to the agarose solution and mixed well.

Agarose (%)	Effective range of resolution of linear DNA fragments (kb)
0.5	1 to 30
0.7	0.8 to 12
1.0	0.5 to 10
1.2	0.4 to 7
1.5	0.2 to 3

Table 2 – Effective resolution at varying Agarose concentrations. Because the expected length of the desired gene product is between 1850 and 2000 base pairs, a concentration of 0.8% agarose was used.

A casting tray was assembled and leveled, and a comb was placed at the head of the tray. Different comb widths were used for different portions of the study. The agarose gel solution was poured into the tray and allowed to cool for one hour. The tray with the cooled gel was then placed in an electrophoresis tank, which was filled with TAE buffer. This chamber was attached to a power source, with the negative lead closest to the wells/comb area of the tray, and positive lead on the opposite end. The comb was removed, leaving wells in which to load the DNA.

The DNA PCR product is combined with 6x DNA loading buffer, using approximately 4 μL of loading buffer per 20 μL of DNA sample solution. The loading buffer and DNA solution was added via pipette to the wells. The first well always contained a DNA ladder, which contains genetic material of predetermined size in order to give size estimates of the DNA samples under investigation. Once all samples have been loaded, the sample is run initially at 100 V, and then decreased to 60 V for approximately an hour, or until the bromophenol blue dye in the DNA buffer reaches the bottom of the gel. Finally, the gel is photographed via UV spectroscopy, and sizes of DNA fragments are determined by comparing the distance they travel to the distanced traveled by the fragments of known size in the DNA ladder.

Polymerase Chain Reaction

The purpose of the polymerase chain reaction (PCR) is to amplify a known section of DNA or genetic material to such an extent that it is useful for experimentation. This technique is important because it allows for the creation of a large number of copies of a specific gene or region of DNA with very little initial material.

The important components of PCR are the DNA template, the primers that specify the region to be amplified, the deoxynucleotide triphosphates (dNTPs), and the DNA polymerase. The DNA template provides the initial code that will be replicated to create the large number of copies of a gene. The primers are small pieces of DNA complimentary to the 3' regions of the sense and antisense strands of the gene targeted for replication. The DNA polymerase will begin DNA replication starting at the primers and moving in a 5' to 3' direction using dNTPs as raw material for the new DNA strands. Eventually, this will lead to the isolation of the gene of interest, as described in **Figure 2**.

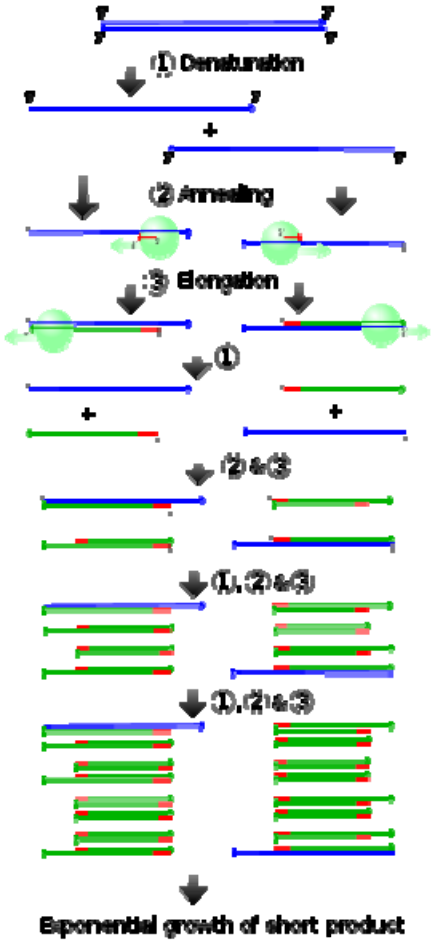

Figure 2 – Polymerase chain reaction. Blue lines represent template DNA, red lines represent primers, green spheres represent DNA polymerase, and the green lines represent replicated DNA. [14]

The components that must be added to the PCR mixture in order to elicit the desired reactions can be found in **Table 3**. All of these components are thawed in ice, combined in a 0.5 mL microcentrifuge tube, and spun briefly in a microcentrifuge. The volume totals to 50 µL.

Solution	Amount (µL)
ddH_2O	39.5
Klentaq buffer	5.0
10 mM dNTPs	2.0
Template DNA: *TRP1*: *ctr1Δ/ctr2Δ/ctr3Δ* *LEU2*: BKY8	1.0
Primers: *TRP1 :* OBK 224 (5'- TGATTTTTTCAGAACATCTC-3') *LEU2 :* OBK 232 (5'CACTTTGATCGAAGAAGAGGGATAACAACAGACGAAAAC ACATTTAAGGGCTATACAAAG**GGAGAAAAAGGAAAGGTGA GA**-3')	1.0
TRP1 : OBK 225 (5'- CGATAAAAACATTTAAACTA-3') *LEU2 :* OBK 233 (AAATGACGAAGGCGRCAATTTTTGAAACAAACCTCTCGGCT TTCCTCTATTCATTTTTGT**GGTTCAAGAAGGTATTGACTTA**-3')	1.0
Klentaq polymerase	0.5
TOTAL	50

Table 3 – Components of PCR by volume. Emboldened sequences indicate *LEU2* sequences.

PCR requires the usage of a thermocycler, which controls the drastic changes in temperature necessary for the reaction to occur. A table of these cycling parameters can be found in **Table 4**. After programming the thermocycler to follow these parameters, the centrifuge tube containing the PCR components is placed in the thermocycler and allowed to run until the end of segment 1. At this point the tube is temporarily removed, and the DNA polymerase is added to the mixture. The polymerase used for this study is KlenTaq DNA polymerase. After the entire program is completed, a 5 µL sample of DNA

solution can be analyzed via agarose gel electrophoresis to determine its content. This is a diagnostic gel to determine if the PCR reaction yielded a product. If there is product, the diagnostic gel will also display its size, allowing for the determination of whether it is the correct product or not.

Segment	Temperature (°C)	Time
1. Melting	93	5 minutes
2. Cycling (x35)	93	30 seconds
	50	45 seconds
	72	3 minutes
3. Amplifying	72	10 minutes
4. Hold	4	Hold

Table 4 – Cycling parameters for PCR

Each step in the PCR process (denaturation, annealing, and elongation) corresponds to changes that must take place in the DNA architecture and arrangement to bring about the chain reaction. First, the DNA is heated to denature it into single strands (denaturation). It is then cooled to allow the primers to bind to their complimentary portions (annealing). Then it is brought to a temperature ideal for DNA replication by DNA polymerase, allowing the polymerase to replicate the DNA strands starting from the primers (elongation). This is repeated multiple times to create sufficient product for analysis and experimentation.

LiAc-Mediated Yeast Transformation

Yeast transformation is the insertion of new DNA into yeast strains. This can be done for a variety of reasons. The purpose of transformation in this study was to disrupt the gene of interest that was amplified via PCR by inserting the amplified product into different strains of yeast. This involves the yeast cell taking up genetic material from its environment and incorporating it into its own genome. This is accomplished by making the yeast cells more "competent," that is, more likely to take up genetic material due to environmental stressors.

This study uses the LiAc-Mediated transformation of intact yeast cells to complete transformation, which involves the presence of lithium acetate (LiAc), polyethelyne glycol (PEG), and a significant but non-lethal heat-shock [15]. LiAc-Mediated transformation has been shown under previous investigation by Gietz R.D. *et al* to be effective in inducing transformation [15].

To perform this method of transformation, each yeast strain to be transformed is inoculated in 5 mL YAPD overnight to saturation at 30°C. The YAPD solution is then diluted to an optical density (OD_{600}) of 0.1. 20 mL of OD 0.1 media is made and re-incubated under the same conditions for approximately four hours, or until the OD_{600} is 0.4 to 0.6. The cells are then transferred to sterile 45 mL centrifuge tubes and pelleted by centrifugation at 4000 g for 5 minutes. Then, the cells are re-suspended in 10 mL LiAc solution and pelleted by centrifugation at 4000 g for 5 minutes. These final pellets are re-suspended in 100 μL LiAc solution to be used for transformations.

Carrier DNA is prepared by heating in a boiling water bath for 1 minute. Once it has cooled, the following components are combined in a 1.5 mL centrifuge tube: 10 μL

carrier DNA, 10 μL DNA to be transformed into the yeast cells, 100 μL of yeast cell solution, and 600 μL of PEG solution. For each strain of yeast, a control vial is created in which the 10 μL DNA to be taken up by the yeast cells is replaced by 10 μL of sterile deionized water. All of the mixtures are incubated at 30°C with agitation for 30 minutes, then heat-shocked in a 42°C water bath for 15 minutes, and finally pelleted by a 5 second spin in a microcentrifuge. The pellets are re-suspended in 1 mL of sterile water. 200 μL of the re-suspended solution for each strain, both transformed and control, are placed on selective plates, with another 200 μL of the control placed on YAPD plates to ensure viability. These plates are incubated at 30°C for 2 to 5 days until transformant colonies appear. Individual colonies are streaked for single colonies on selective plates for further experimentation.

Ideally, the transformed yeast cells will only grow on selective plates because of the genes it acquired via transformation. However, it is possible that a marker is "leaky," meaning that the absence of said marker does not always fully inhibit the growth of untransformed strains.

Isolation of Yeast Genomic DNA

To determine the genotype of transformed strains of yeast, or to determine if the desired transformation and disruption has occurred, genomic DNA must be isolated from transformants. Once the genomic DNA of a yeast strain has been isolated, PCR and agarose gel electrophoresis can be used to determine the length of the gene of interest, which will indicate whether a genetic marker has been inserted into the coding region of the gene to prevent its expression.

To isolate genomic DNA from a yeast colony, one colony is grown in 10 mL of YAPD overnight to saturation. The culture is transferred to sterile 45 mL centrifuge tubes. Cells are pelleted by centrifugation at 4000 g for 5 minutes. After the supernatant is discarded, the cells are re-suspended in 500 µL of water. This solution is transferred to a 1.5 mL centrifuge tube and pelleted by 5 second centrifugation in a microcentrifuge. The supernatant is discarded and the tube is briefly vortexed to re-suspend the pellet in the residual liquid. The following components are added in this order: 200 µL breaking buffer, 200 µL phenol-chloroform (25:25), and 0.3 g acid washed glass beads. The tube is then vortexed at top speed for 3 to 4 minutes. Then, 200 µL TE buffer (pH 8.0) is added, and the tube is vortexed briefly.

The tube is then spun for 5 minutes in a microcentrifuge at room temperature. The aqueous layer is transferred to a sterile 1.5 mL microcentrifuge tube. 1 mL of 100% ethanol is added to the new tube and mixed by inversion. This tube is then spun for 3 minutes at high speed in a microcentrifuge at room temperature. The supernatant is discarded, and the pellet is dried for 5 minutes at 37°C. The pellet is resuspended yet again in 400 µL of TE, after which 30 µL of 1 mg/mL RNase A solution is added. Then the tube is incubated for 10 minutes at 37°C. Next, 10 µL of 4M ammonium acetate and 1.0 mL of 100% ethanol is added to the tube, and the tube is inverted to mix the solutions. Finally, the tube is spun for 2 minutes in a microcentrifuge, the supernatant is discarded, and the pellet is dried for 5 minutes at 37°C. The pellet is resuspended in 50 µL of TE buffer. 5 µL of this suspension should contain 2 to 4 mg of DNA, which can be used for PCR or further experimentation as described previously.

The principle behind this procedure is to degrade and discard all cellular components that are not DNA and separate the DNA from the cellular debris. Many compounds must therefore be added to degrade the various cellular components and ensure their presence in the aqueous layer following centrifugation.

Serial Dilution Growth Test

A serial dilution growth test, also known as a drop test, is a series of dilutions of yeast cells plated one after the other in drops to determine the extent of cellular growth in standard and nonstandard conditions. Each successive dilution gives a more precise indication of cellular growth levels. This test can also be used to compare changes in growth between wild-type and mutant yeast strains. In the context of this study, the drop test is used to determine the extent of copper tolerance by nmd and *ctr3* mutants in high and limiting levels of copper.

To perform a drop test, each yeast strain to be grown is inoculated in 3 mL of YAPD and grown overnight to saturation. Then, multiple samples of 10 mL of fresh YAPD are inoculated with 5 µl, 10 µl, 50 µl and 100 µl of the overnight saturation and grown overnight again to an OD_{600} between 0.4 and 0.6. Using this overnight, four dilutions ranging from 10^0 to 10^3 are made, each in a sterile 1.5 mL microcentrifuge tube. This is done by pipetting 200 µl of overnight culture into the first tube, then pipetting 180 µl of sterile water into the next three tubes. 20 µl of the yeast cells grown in YAPD is transferred from the first tube into the second tube and mixed, then 20 µl from the second tube to the third, then 20 µl from the third to the fourth. This is done separately for each strain to prevent contamination. Then, 5 to 10 µl of solution from each tube is placed on either control media or limiting media in a grid formation. An example of this technique from a prior study is shown in **Figure 3** [5].

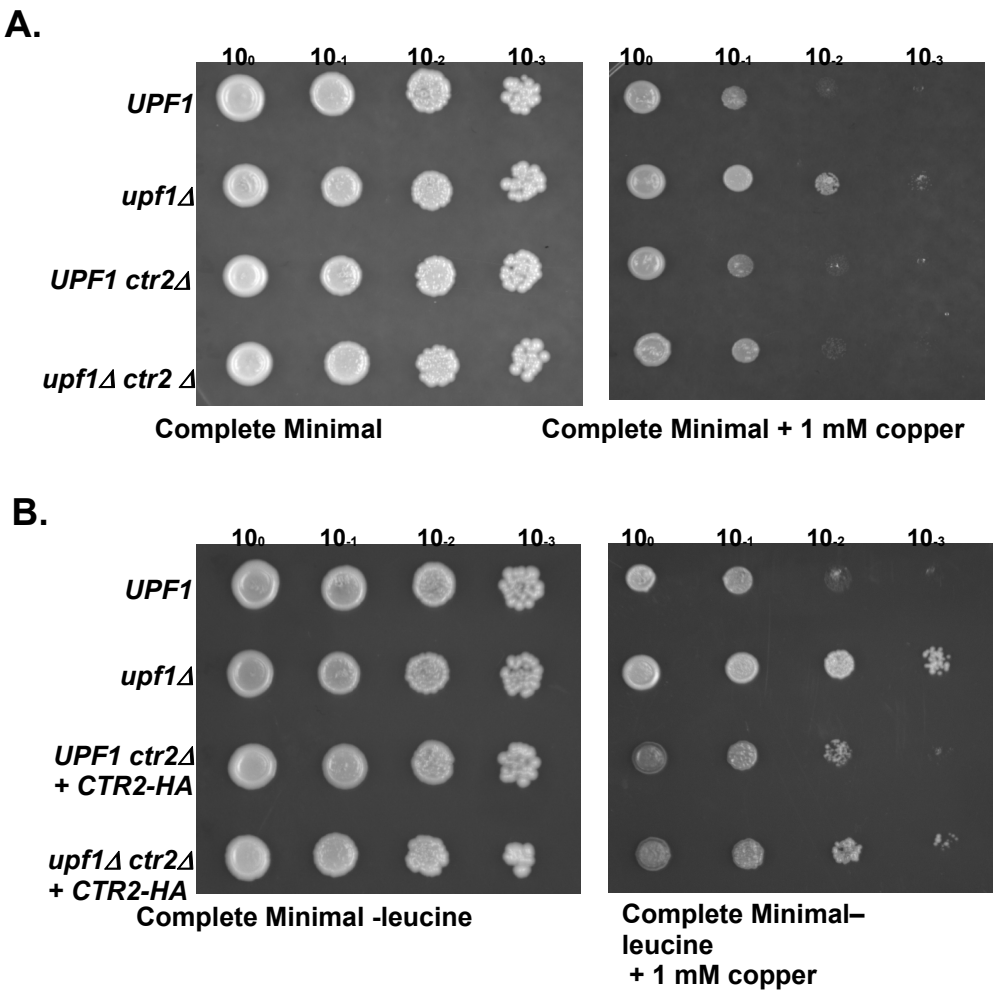

Figure 3 -- Serial dilution growth test. This particular test shows the changes in growth associated with deactivation of the NMD pathway, deactivation of the *CTR2* gene, and high levels of copper. **A** shows normal growth for all strains on complete minimal media, and demonstrates that the *upf1Δ* mutant shows increased growth in complete minimal leu^- +1 mM copper media. This growth is not observed in the strains with disrupted *CTR2* or active *UPF1*, indicating that *CTR2* contributes to the copper-tolerant phenotype of the *upf1Δ* mutant and is controlled by the NMD pathway. **B** shows that the copper-tolerant phenotype of the *upf1Δ* can be partially restored by the addition of a plasmid containing *CTR2*. [5]

Summary

In summary, the following is the overall procedure for the course of the study. First, an inactivated form of *CTR3* with a *TRP1* or *LEU2* selectable marker is isolated from BKY 8 or the *ctr1Δ/ctr2Δ/ctr3Δ* strain, respectively, and replicated via PCR. This is a gene disruption fragment that contains *CTR3* flanking sequences and a *TRP1* or *LEU2* selectable marker. The disruption fragment is then transformed into three strains of yeast: BKY8 (wild-type), AAY320 (a *upf1* mutant), and AAY329 (a *upf1* mutant with a different selectable marker) and grown on media lacking leucine or tryptophan, depending on which genetic marker is used to disrupt *CTR3*. The transformed yeast should show considerably more colonies on this media compared to non-transformed yeast strains. If a sufficient difference is observed between growth on the plates with transformed yeast and growth on the control plates, transformed colonies are streaked again on plates lacking tryptophan or leucine. Genomic DNA is isolated from four individual colonies per strain. PCR is performed on this genomic DNA to isolate copies of *CTR3* to verify that *CTR3* has been disrupted. This is determined by gel electrophoresis, the absence of a fragment indicates that the appropriate marker has been inserted into the coding region of *CTR3*. There will be six strains: a transformed strain and control strain for BKY8, BKY43, and BKY47. All six strains will be plated on standard media, on copper-limiting media, and on high-copper media via the drop test method. This will determine the extent to which *CTR3* contributes to the copper tolerance of nmd mutants.

CHAPTER THREE

Experimental Results

Overview of Methods and Expected Results

The following steps are taken to determine the influence of *CTR3* on copper tolerance in yeast: first, a gene disruption fragment is isolated from template DNA via polymerase chain reaction (PCR). Second, if the gene disruption fragment is the expected size, gel purification is performed to purify the fragment. Third, the concentration and total amount of purified gene disruption fragment is quantified. Fourth, the gene disruption fragment is transformed into wild-type and nmd mutant yeast strains to inactivate the *CTR3* gene. Fifth, yeast genomic DNA (YGD) is isolated from transformants that show evidence of gene disruption. Sixth, the disruption of *CTR3* is verified via PCR using the YGD isolated from the transformants as template DNA. Seventh, if *CTR3* disruption has occurred, a drop test is performed to assess copper tolerance.

Transformation Using the TRP1 *Marker*

Over the course of the research, two different methods were used in an attempt to transform the three different yeast strains under investigation (BKY 8, AAY320, and AAY329) with the *CTR3* DNA and disrupt the *CTR3* gene. The first approach was to create a gene fragment with a *TRP1* marker and *CTR3* flanking sequences and transform this fragment into all yeast strains. For this approach, the DNA that was used as the template for the PCR reaction was from a yeast strain in which *CTR3* had already been disrupted.

The first step was to generate a gene disruption fragment using PCR. This resulted in a gene fragment containing the *TRP1* gene with *CTR3* flanking sequences as depicted in **Figure 4.** The expected length of the *TRP1* disruption fragment is between 850 and 1,000 base pairs. The fragment shown in the figure is therefore the proper length, and thus gel purification was performed to ensure the purity of the product. The gel in **Figure 5** shows the purified *CTR3* gene disruption fragment, and as expected, the length of the product was between 850 and 1,000 base pairs. Next, the amount of product was quantified to be 5.1 ng/μL. This is lower than the recommended amount specified by the LiAc-mediated yeast transformation protocol, but the decision was made to proceed with the transformation.

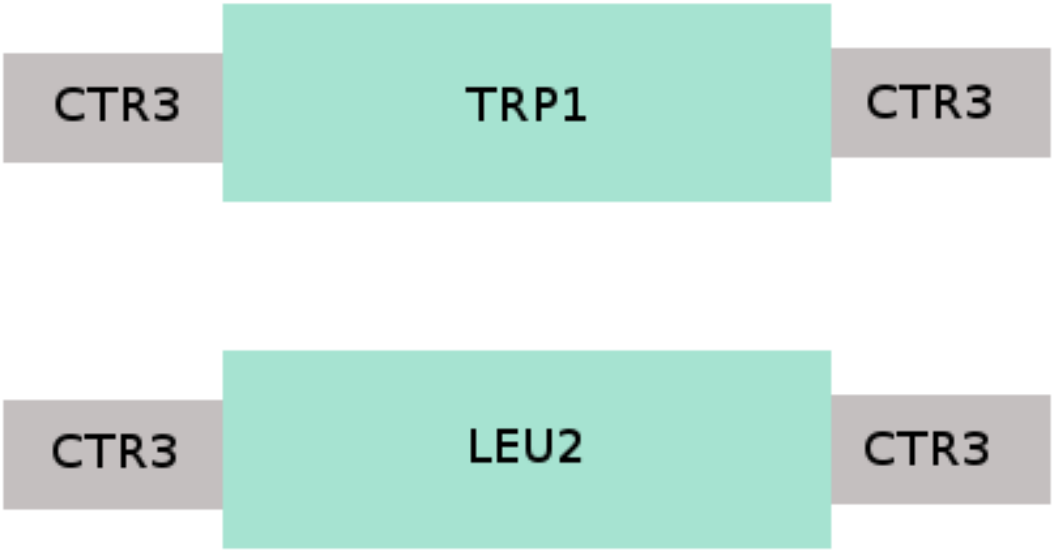

Figure 4 – *CTR3* disruption fragments with *TRP1* and *LEU2* markers. This schematic depicts the DNA fragments used to disrupt the *CTR3* gene. The green regions signify the marker cassettes, while the grey regions signify *CTR3* flanking sequences.

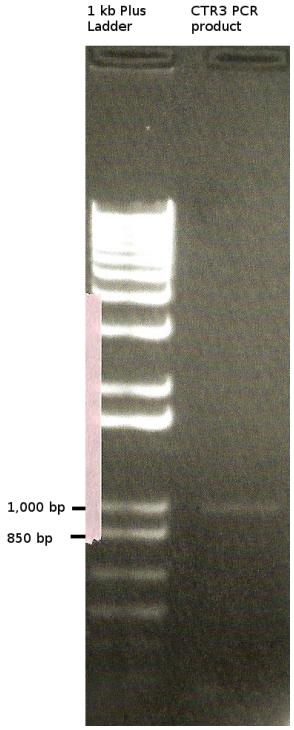

Figure 5 – Gel purified *TRP1* PCR Product. An agarose gel displaying the products of gel purification. . The left lane contains 1 kb Plus DNA ladder, while the right lane is a DNA fragment containing the *TRP1* genetic marker and *CTR3* flanking sequences. The length of the DNA fragment is between 850 and 1000 base pairs.

However, when transformation was attempted via the LiAc-mediated transformation protocol, it was unclear whether the transformed strains of BKY 8, AAY320, and AAY329 exhibited gene disruption. **Table 5** shows diagnostic growth on plates lacking tryptophan for both transformed and non-transformed strains. The level of growth on plates lacking tryptophan was identical for both strains transformed with the disruption fragment and strains left untransformed. This suggests that transformation did not occur. Transformation would be indicated by higher levels of growth for transformed strains, because the *TRP1* marker would be incorporated into the yeast genome and expressed, allowing the yeast to produce *TRP1* protein that would synthesize the essential tryptophan or leucine amino acids not present in the media. Evidence suggests that this did not occur with the gene disruption fragment.

Strain (media)	Growth Expected (y/n)	Number of colonies
BKY8 control (YAPD)	y	TNTC
BKY8 no DNA control (trp^-)	n	4
BKY8 transformed (trp^-)	y	4
BKY43 control (YAPD)	y	TNTC
BKY43 no DNA control (trp^-)	n	3
BKY43 transformed (trp^-)	y	contaminated
BKY47 control (YAPD)	y	TNTC
BKY47 no DNA control (trp^-)	n	5
BKY47 transformed (trp^-)	y	5

Table 5 – Table for *TRP1* transformation. This table represents the expected growth of wild-type (BKY 8) and nmd mutant (BKY 43, 47) strains of yeast on YAPD and media lacking tryptophan. The test is performed as the first step in determining whether transformation has occurred in strains that have undergone the LiAc-mediated yeast transformation protocol described in Chapter 2: Materials and Methods. Acronyms in parentheses indicate the media upon which each strain was grown. The no DNA control strains are used assess the selectivity of the marker. The YAPD control is used to assess the viability of the yeast strains used during the experiment. The table shows no difference in growth between transformed and untransformed strains, indicating that either transformation has not occurred or that the *TRP1* marker is not sufficiently selective to differentiate between untransformed and transformed strains. Further testing is necessary.

To determine whether the colonies that grew on the media lacking tryptophan were transformed with the *CTR3* disruption fragment, yeast genomic DNA (YGD) was isolated from transformants and analyzed using PCR. YGD was isolated from individual colonies of wild-type strains transformed with the *CTR3* disruption fragment and grown on media lacking tryptophan. This yeast genomic DNA can be seen on **Figure 6**. The lanes contain, from left to right, 1 kb Plus DNA ladder, *CTR3* normal gene product, and YGD from four different colonies of wild-type yeast strains with potential *CTR3* disruption.

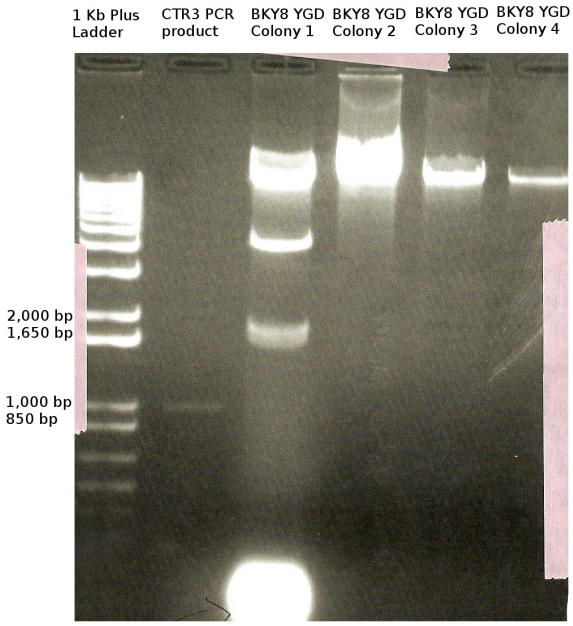

Figure 6 – YGD Isolation. This gel ensures the presence of the genomic DNA from four different colonies of wild-type yeast strains that were subjected to the LiAc-mediated transformation protocol and grew on trp^- media, indicating possible transformation. YGD was extracted from the wild-type strains transformed with the *CTR3* disruption via the yeast genomic DNA extraction protocol described in Chapter 2: Materials and Methods.

The *CTR3* gene (or *TRP1* marker with *CTR3* flanking sequences in transformed strains) was amplified from the genomic DNA of the different colonies via PCR, then run through an agarose gel, as seen in **Figure 7**. The *TRP1* marker will produce a gene fragment of a different length than a normal *CTR3* gene. The length of the DNA amplified by the PCR reaction from wild-type YGD was compared to gene products amplified by the same methods from both wild-type DNA (negative control) and DNA from *CTR3* knockout strains (positive control). If gene disruption had occurred, the DNA fragments isolated from the transformed colonies would resemble the positive control. Only one colony exhibited this trait: BKY8 colony 2. This indicates that the colony did in fact exhibit *CTR3* disruption. However, in order to generate experimental results, a *CTR3* disruption in either nmd mutant strain (BKY43 or BKY47) was also required. Since neither BKY43 nor BKY 47 showed any evidence of gene disruption, the the disruption of *CTR3* via the *LEU2* selectable marker was pursued.

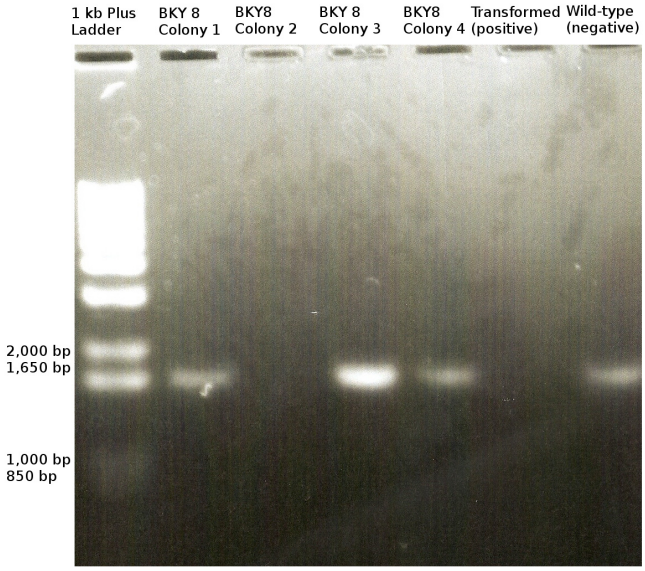

Figure 7 – PCR from YGD of wild-type strains transformed with the *CTR3* disruption fragment. This gel shows the product of a PCR reaction using the flanking sequences for the *CTR3* gene as primers and the genomic DNA from four different colonies of wild-type yeast that were subjected to *CTR3* gene disruption as template DNA. The gel contains a positive control in lane 6, which is the product of a PCR reaction using genomic DNA from a strain with a known deletion in *CTR3* with the *TRP1* genetic marker. However, there was no product observed in lane 6. The gel also contains a negative control in lane 7, which uses genomic DNA from a wild-type strain with a functional *CTR3* gene as the template DNA. From the test, it is shown that three of the four colonies (Colonies 1, 3 and 4) displayed DNA fragments the same length as the negative control, indicating that no gene disruption occurred in these colonies. The remaining colony (Colony 2) showed no fragment. Because this also occurred in the positive control, it is assumed that disruption has occurred in Colony 2.

Transformation Using the LEU2 *Marker*

Due to the incomplete results of the transformation attempted with the *TRP1* genetic marker, a second approach to transformation was pursued. The second approach employed a similar method to the *TRP1* transformation, except *LEU2* was used as a marker rather than *TRP1* and the DNA used as a template to generate the *CTR3* disruption fragment via PCR was from a yeast strain with functional *CTR3*. The disruption fragment, which contains the *LEU2* gene surrounded by *CTR3* flanking sequences, is between 1,650 and 2,000 base pairs in length. The initial product derived from PCR can be seen in **Figure 8.**

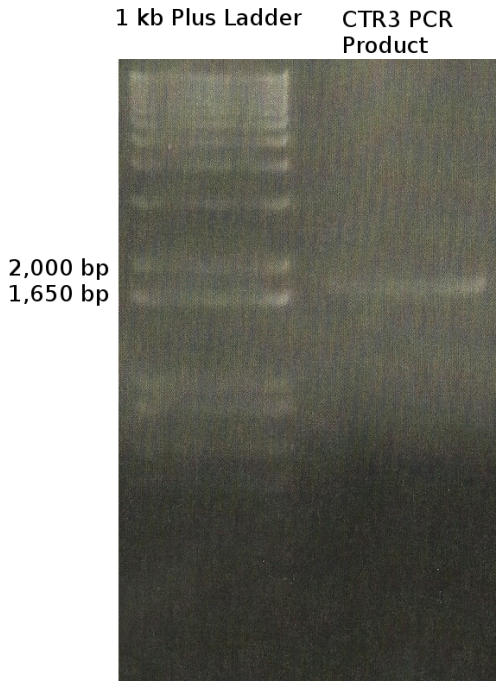

Figure 8 – An agarose gel of the *LEU2* PCR Product. The left well contains 1 kb plus DNA ladder, and the right well is a DNA fragment containing the *LEU2* genetic marker with *CTR3* flaking sequences. The DNA fragment is between 1,650 and 2,000 base pairs in length.

Because the PCR product was of the desired length, it was purified via gel purification, the results of which can be seen in **Figure 9**. Note that the product is within the 1,650 to 2,000 base pair range, indicating that this is the desired disruption fragment.

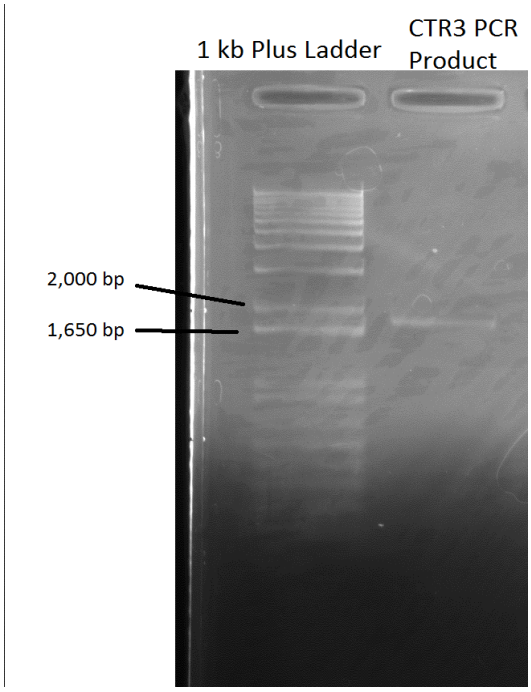

Figure 9 – *LEU2* PCR Product following gel purification. An agarose gel showing the DNA fragment with *LEU2* as a genetic marker surrounded by *CTR3* flanking sequences in the right well and 1 kb Plus DNA ladder in the left well. This is the fragment following gel purification, the process of which is described in Chapter 2: Materials and Methods.

Following purification, the nanodrop was used to quantify the amount of disruption fragment present in the solution. **Figure 10** is a graph from this procedure, and it indicates that the concentration of disruption fragment was far below the standards established by the LiAc-mediated transformation protocol (a minimum of 100 ng/µL) [3].

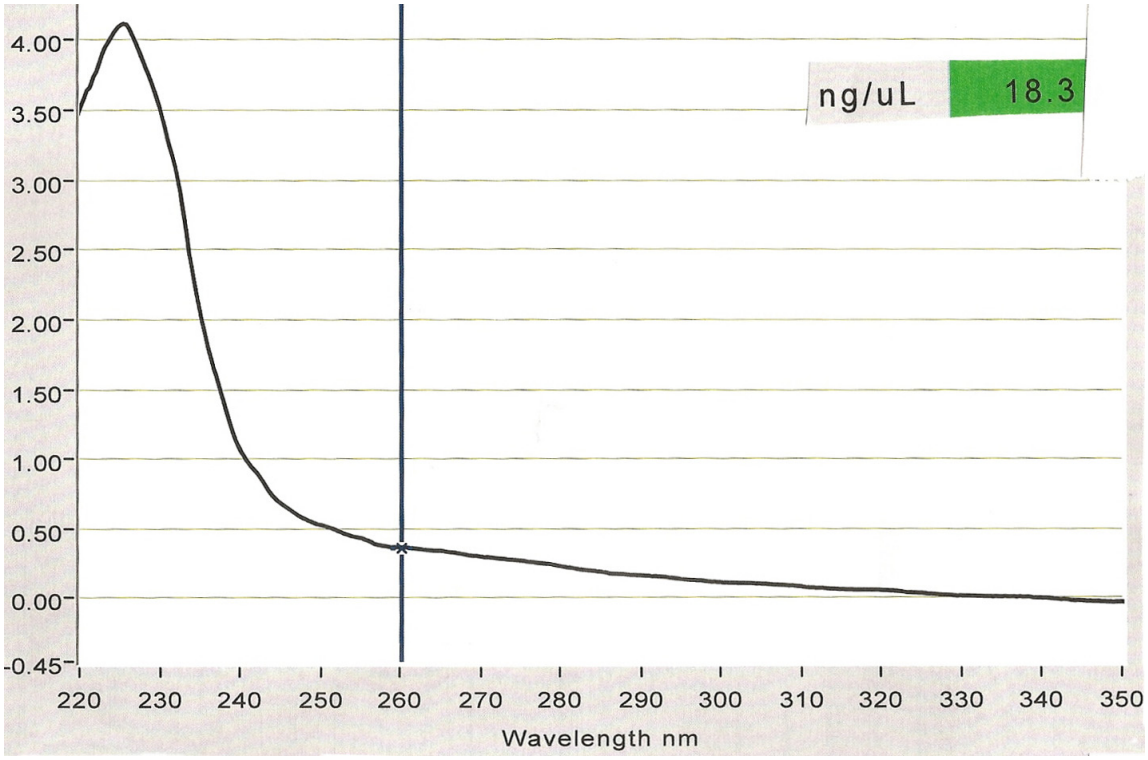

Figure 10 – Nanodrop quantification of *LEU2* PCR product. The nanodrop measures small concentrations of molecules based on wavelength. DNA absorbs light at a wavelength of 260 nm. Following gel purification, the nanodrop is used to assess concentration of PCR product. The concentration of the LEU2 disruption fragment was 18.3 ng/µL.

Unfortunately, when the *CTR3* disruption fragment with the *LEU2* marker was used in an attempt to transform wild-type and nmd mutant strains by *CTR3* disruption, all of the agar plates were contaminated and no yeast growth was observed. It was not possible to determine whether any transformed strain was created. Current research is focused on reisolating the *CTR3* disruption fragment with the *LEU2* marker in order to repeat this experiment.

The final step in this experiment will be a serial dilution drop test. If transformation occurs, the expected results will be as follows: all non-transformed strains would show the same level of growth on normal limiting media. On copper-limiting media (low levels of copper), any yeast strain that lacked *UPF1* function but retained

CTR3 function would exhibit increased levels of growth at lower concentrations compared to any strains that retained *UPF1* function or lacked *CTR3* function. Similarly, media with increased levels of copper would demonstrate increased growth for copper strains lacking *UPF1* function but retaining *CTR3* function, and strains with functional *UPF1* or non-functional *CTR3* would exhibit decreased growth.

CHAPTER FOUR

Discussion

Overview of Questions and Procedures

Saccharomyces cerevisiae expresses the gene *CTR3* as a high-affinity copper transporter. It also expresses the genes *UPF1, UPF2,* and *UPF3* to degrade mRNAs with premature stop codons, or nonsense codons, via the nonsense-mediated mRNA decay pathway (the NMD pathway). However, this pathway also controls gene expression by targeting and degrading mRNAs with long 3' untranslated regions (3'UTR) and other features. Previous studies suggest that the NMD pathway may play a role in regulation of *CTR3* expression [5]. Furthermore, it has been shown that yeast strains with an inactive NMD pathway are better able to grow in high levels of copper than strains with an active NMD pathway. The purpose of this study is to determine whether or not *CTR3* contributes to the copper tolerance of nmd mutants, and whether or not the expression of *CTR3* mRNA is controlled by the NMD pathway.

This is accomplished by creating or acquiring the following strains of yeast: one wild-type strain where all genes under investigation are functional, one strain in which *CTR3* is disrupted but *UPF1* is functional, one strain in which *CTR3* is functional and *UPF1* is disrupted, and one strain in which both *CTR3* and *UPF1* are disrupted. Any *CTR3* disruption would be accomplished by genetic transformation, using homologous recombination of a genetic marker into the coding region of the *CTR3* gene to interrupt its function. The genetic marker would also code for a protein to create essential nutrients

not normally synthesized by the parental yeast strain. By growing transformed strains on media lacking the nutrient synthesized by the protein coded for by the genetic marker, the presence of the genetic marker within the yeast genome is indicated. By extension, the disruption of *CTR3* is ensured.

Once created, the strains would be grown on media with normal copper, media with high levels of copper, and media with low levels of copper. By determining the level of growth of each strain, the effect of *CTR3* on copper tolerance in wild-type and nmd mutants can be determined. Strains with an inactivated NMD pathway should exhibit considerably more growth in high copper levels than wild-type strains. Strains with disrupted *CTR3* should exhibit less growth in high copper levels than wild-type strains as well. Meanwhile, disruption of *CTR3* in strains with an inactive NMD pathway should significantly decrease growth at high copper levels, indicating the contribution of *CTR3* to copper tolerance in nmd mutants.

Overview and Analysis of Experimental Results

First, the disruption of *CTR3* was attempted using *TRP1* as the genetic marker. This was accomplished by incorporating a gene fragment containing the *TRP1* marker gene with *CTR3* flanking sequences into the yeast genome. Gene fragments were isolated from the genomic DNA of yeast with *CTR3* already disrupted via polymerase chain reaction (PCR). This fragment was isolated, purified, and quantified to be approximately 5.1 ng/μL. The LiAc-mediated transformation protocol was pursued, and the *TRP1* fragment with *CTR3* flanking sequences was transformed into wild-type yeast strains and strains lacking NMD function. When grown on media lacking tryptophan, there was no significant difference in levels of growth between transformed strains and control strains

that were not transformed with the DNA. Analysis of the yeast genomic DNA of four different transformed colonies showed a single wild-type colony with evidence of gene disruption: a lack of *CTR3*. The same phenomenon occurred when amplification of the *TRP1* disruption fragment was attempted on the positive control, and thus the colony is assumed to express *CTR3* disruption. However, *CTR3* disruption did not occur in nmd mutant strains, and there was no basis for comparison between the phenotypic effects *CTR3* disruption of strains with active versus inactive NMD pathways. An alternative marker was used.

Transformation was next attempted on strains with active and inactive NMD using *LEU2* as the selectable marker. The gene fragment, a *LEU2* marker with *CTR3* flanking sequences, was isolated from strains with functional *CTR3*. Once the PCR product was purified, the final amount of product was quantified to be 18.3 ng/μL. This PCR product was transformed into strains using the LiAc-mediated transformation protocol and spread onto plates lacking leucine. Unfortunately, all plates were contaminated with mold and no growth was observed.

If the transformation had occurred, the *CTR3* gene had been disrupted, and the research had progressed to the point of doing a drop test, the results would have contributed to a better understanding of the *CTR3* gene and its role in the copper tolerance of nmd mutants. Results would have indicated whether or not the expression of *CTR3* is controlled by the NMD pathway, and whether or not *CTR3* contributes to the copper tolerance of nmd mutants. As it stands, the results still contribute to a better understanding of the *CTR3* gene. They show that the disruption of *CTR3* using standard LiAc-mediated yeast transformation methods is non-trivial. However, because there are other strains that exhibit this disruption, it is not impossible.

It is difficult to say with certainty the reason *CTR3* gene disruption did not occur using either *TRP1* or *LEU2* markers. However, there are a few factors that might have contributed to the apparent lack of gene disruption. One significant factor is the low concentration of disruption fragment following gel purification. The recommended amount of gene disruption fragment to be added during LiAc-mediated yeast transformation is 0.1-10 μg, and since 10 μL of disruption fragment solution are added, a concentration of at least 100 ng/μL is recommended [3]. However, concentrations of disruption fragment were consistently lower than this following purification. Low levels of disruption fragment would mean less fragments would be available to be transformed into the yeast cells. It is possible that there simply was not enough DNA to be taken into the yeast cell and homologously recombined to disrupt the *CTR3* gene to an observable level.

Another possible explanation for the observed lack of gene disruption is the "leakiness" of *TRP1* as a genetic marker. Untransformed strains are tryptophan

auxotrophs and do not have the *TRP1* gene so they cannot synthesize tryptophan. They and any yeast strain that is an auxotroph for tryptophan with an undisrupted *CTR3* gene should be unable to grow on media lacking tryptophan. However, when the untransformed strains were grown on media lacking tryptophan along with transformed strains, the untransformed strains showed the same level of growth as the transformed strains, even without the *TRP1* gene. This indicates that the *TRP1* marker, at least in this instance, was "leaky" and did not sufficiently select for colonies with disrupted *CTR3* with the *TRP1* marker. Thus, when colonies were selected for yeast genomic DNA analysis, it was not guaranteed that any given colony would be a transformant, and it is possible that a transformant was not selected. The yeast genomic DNA analysis would indicate that transformation did not occur, when in reality the transformed strain was simply not tested.

Future studies associated with *CTR3* expression in nmd mutants will require the isolation of PCR product with *LEU2* disruption of the *CTR3* gene, and then the transformation of this product into the appropriate yeast strains, as described previously. An alternative to this method would be to purchase strains with a *CTR3* gene that is already disrupted, and disrupt *UPF1* in those strains to create nmd mutants. Once the appropriate strains are created, the study could proceed as normal to a drop test, and a more satisfying conclusion could be reached concerning the interaction of *CTR3* and the NMD pathway.

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