MAINTENANCE OF COPPER HOMEOSTASIS: EFFECTS OF *CUP1* OVEREXPRESSION, *MAC1*-DEPENDENT GENE EXPRESSION AND SURVIVAL OF *SACCHAROMYCES CEREVISIAE*

A

Thesis

By

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Abstract

Copper is essential for life in all cells, but high levels of this cofactor can become toxic. Therefore, cells must maintain a balance between copper uptake and copper export/sequestration. Genetic defects in copper metabolism can be lethal and may cause diseases such as Menkes disease and Wilson's disease. Metallothioneins are involved in copper homeostasis; these small, sulfur-rich proteins bind copper (or other metals) and deliver excess copper to transporters that can export it from the cell. In the yeast *Saccharomyces cerevisiae*, the *CUP1* gene encodes the copper metallothionein Cup1. Within minutes of copper exposure, the *CUP1* gene is transcribed to make *CUP1* mRNA, which is then translated into Cup1 protein. This results in rapid sequestration of copper by Cup1. Within 20-30 minutes, transcription of the *CUP1* gene is normally shut down to prevent synthesis of too much metallothionein. However, strains of yeast with mutations in both *SPT10* and histone H2A have been described that fail to downregulate *CUP1*. I have studied these mutant strains and determined how *CUP1* overexpression affects their growth. I predicted that these mutants would exhibit poor growth when exposed to toxic levels of copper; however, certain H2A mutants containing a serine to alanine mutation at position 122 (*htaS122A*) exhibited almost normal growth when exposed to toxic copper levels. I am currently studying the expression of other genes involved in copper metabolism, such as Mac1-dependent genes. Mac1 is a transcription factor that is normally involved in responding to copper starvation, controlling the expression of genes whose functions are to transport copper into the cell. I am measuring Mac1-dependent gene expression in mutant strains of *S. cerevisiae* that over express

CUP1 to determine whether these cells enter copper starvation due to Cup1 overexpression.

Introduction

Minimum levels of copper are essential for survival of all cells in all species; however, at high levels copper becomes toxic. Cells must therefore maintain a careful balance between copper uptake and copper export/sequestration. Human genetic defects in copper metabolism demonstrate the mechanisms that mammals have developed to maintain this balance (Shim and Harris 2003). The defects that upset this balance can be lethal. Two serious diseases in humans, Menkes disease and Wilson's disease, both result from disruption of copper homeostasis (Daniel et al. 2004). Menkes disease is caused by the loss of a protein involved in copper export from cells. In this disease, copper in the diet is absorbed normally into intestinal cells, but these cells then fail to export this copper for delivery throughout the body, so that most tissues are starved for copper. This disease is generally fatal by 3-4 years if untreated. Similarly, Wilson's disease is also caused by a defect of copper transport. Normally, excess copper in the body is sent to the liver, which then transports the copper into bile for excretion. In Wilson's disease, the liver fails to secrete copper into the bile. This leads to toxic levels of copper in liver cells, causing liver damage, and release of excess copper back into the bloodstream, damaging the eyes, brain and kidneys. Untreated, this results in liver failure, severe brain damage, and death.

The yeast *Saccharomyces cerevisiae* is a powerful model system in which to study copper homeostasis. The proteins involved in regulating copper levels within the cell have been conserved throughout evolution and are therefore very similar between

yeast and human. Yeast cells are easy to grow in the laboratory, and there are many genetic and molecular tools available for working with yeast which make it possible to address the roles of individual genes in copper regulation. Yeast cells, like all other living cells, must constantly monitor copper concentrations. Cells respond to either copper starvation or copper overload by rapidly expressing genes that encode the proteins needed to increase or decrease copper levels (Winge et al., 1998; Gross et al., 2000).

High copper levels induce *metallothioneins* in all species. Metallothioneins are small, sulfur-rich proteins that bind copper (or other metals) to prevent the toxic effects of free copper in the cell, and can also deliver excess copper to other proteins that can export it from the cell (Theocharis *et al.* 2003). In yeast, the *CUP1* gene encodes the copper metallothionein Cup1. The *CUP1* gene is activated by the transcription factor Ace1, which senses the level of copper in the cell (Buchman et al, 1989). Within minutes of copper exposure, Ace1 activates the *CUP1* gene, leading to synthesis of *CUP1* mRNA, which is then translated into Cup1 protein. The Cup1 protein immediately binds the excess copper in the cell. Within 20-30 minutes, transcription of the *CUP1* gene is normally shut down to prevent synthesis of too much metallothionein. It has been demonstrated that *CUP1* transcriptional shutdown is an active process (Kuo et al. 2005), but it is not yet understood how this shutdown is controlled.

In contrast to the Ace1-dependent response to toxic copper levels, cells must also be able to cope with copper starvation. Mac1 is a transcription factor involved in this process. Mac1 senses when copper levels in the cell drop too low, and in response activates transcription of the *CTR1* gene (as well as other related genes). The Ctr1

product is a copper transporter, which binds copper outside the cell and imports it into the cell (Dancis et al., 1994; Gross et al., 2000).

Gene transcription must occur within the context of chromatin, the compact form of DNA which consists of approximately two turns of DNA wrapped around histone octamers to form nucleosomes. A histone octamer consists of a pair each of histones H2A, H2B, H3, and H4. Each histone contains an N-terminal tail that extends from the histone core, and H2A contains an additional C-terminal tail. These N- and C-terminal tails are targets of the majority of post-translational modifications to the core histones. Our lab has developed strains of *S. cerevisiae* with N- and C- terminal tail mutations in H2A that have different *CUP1* expression patterns (Kuo *et al.* 2005). The genes that code for histone H2A (*HTA*) and histone H2B (*HTB*) have been deleted from the genome in these strains, and a single copy of each of these essential genes is provided on a plasmid in the yeast cells. In the wtH2A strain (control), *HTA* and *HTB* were left unaltered in the plasmid. In the *htaS2A* mutant the second residue of H2A was changed from a serine to an alanine and in the *htaS122A* mutant, the 122nd residue of H2A was changed from a serine to an alanine. Our lab has also developed mutants that fail to downregulate *CUP1* expression: *spt10Δ-htaS2A* and *spt10Δ-htaS122A* (Kuo *et al.* 2005). These strains have *SPT10* deleted from their genomes in addition to the altered *H2A* gene. Spt10 is thought to be involved in chromatin maintenance and transcriptional regulation (Denis *et al.* 1994) and is required for expression of some but not all of the histone genes (Dollard *et al.* 1994). It is not an essential gene, but deletion of *SPT10* causes very slow growth (Natsoulis *et al.* 1994). It has been proposed that Spt10 acts primarily at the histone gene promoters which it activates and that any global effects observed in *spt10Δ* mutants are

indirectly the result of reduced histone synthesis resulting in incomplete assembly of chromatin (Eriksson *et al.* 2005). This hypothesis was supported by results that indicate a similar set of genes is affected by H4 depletion, and the fact that the *spt10Δ* growth phenotype can be rescued by supplying extra histone genes. The nucleosomal organization of chromatin is generally defective in *spt10Δ* cells. Spt10 functions through binding specifically, and cooperatively as a dimer (Mendiratta *et al.* 2007) to pairs of UAS elements in the core histone promoters not found elsewhere in the genome, thus selectively activating histone gene expression (Eriksson *et al.* 2005). Another mutant our lab has studied is *swi1Δ*. Swi1 is part of the SWI/SNF complex, which is an ATP-dependent chromatin remodeling complex that allows transcription factors to gain access to DNA (Wang, 2003). The *swi1Δ* mutant does not downregulate *CUP1* normally (Kuo *et al.* 2005).

H2A S122 is important for survival in the presence of DNA-damaging agents (Harvey *et al.* 2005) and it is therefore worthwhile to study the effect this residue has on different functions in the cell. H2A S122 has been found to facilitate survival in the presence of DNA damage and may mediate homologous recombination. The mutation of this residue does not affect DNA damage-dependent Rad53 phosphorylation or G2/M checkpoint responses, as both of these processes remain intact in an *hta1*-S122A mutant strain (Harvey *et al.* 2005).

I have studied these mutant strains that fail to downregulate *CUP1* and have determined how *CUP1* overexpression affects their growth. I have shown that most of the mutants have copper response profiles that are very similar to the wild type response. However, the *htaS122A* and *spt10Δ-htaS122A* strains show a remarkable indifference to

copper stress, and grow at their normal growth rate even in the presence of high copper levels, which normally reduce the growth rate even of wild type cells. These results demonstrate there is more affecting cell growth upon exposure to toxic amounts of copper than levels of *CUP1* expression alone.

Results

After our lab observed the failure of the mutant strains *spt10Δ-htaS2A*, *spt10Δ-htaS122A* and *swi1Δ* to downregulate *CUP1,* I wished to test the hypothesis that overexpression of Cup1 in response to high copper levels actually results in copper starvation within the cell. When *CUP1* is over-expressed, copper may be sequestered extensively, putting the cell into a state of copper starvation. If this is the case, the cell will respond by inducing the expression of Mac1-dependent genes such as *CTR1*. Furthermore, if the excess Cup1 binds all the copper in the environment of the cell, the cell may be forced to slow or stop its growth (or may perish). Both of these outcomes can be measured directly by measuring the growth of normal cells (wild type) and cells that produce excess *CUP1* mRNA, as well as by detecting the presence of Mac1-dependent *CTR1* transcription in the same cell populations.

S122A *mutants tolerate higher than expected Cu levels.* Growth curves were conducted for wild type, *spt10Δ*, *htaS2A*, *spt10Δ-htaS2A*, *swi1Δ*, *htaS122A*, and *spt10Δ-htaS122A* cells, with and without 1.5 mM $CuSO_4$ treatment. OD_{600} values were recorded at 0 hours, 16 hours, 19 hours and 22 hours after $CuSO_4$ treatment and plotted (Fig. 1). Although growth curves were conducted for a total of 7 strains, only 4 strains were studied at a time. Each group of 4 strains includes wild type. The growth curves in Figure

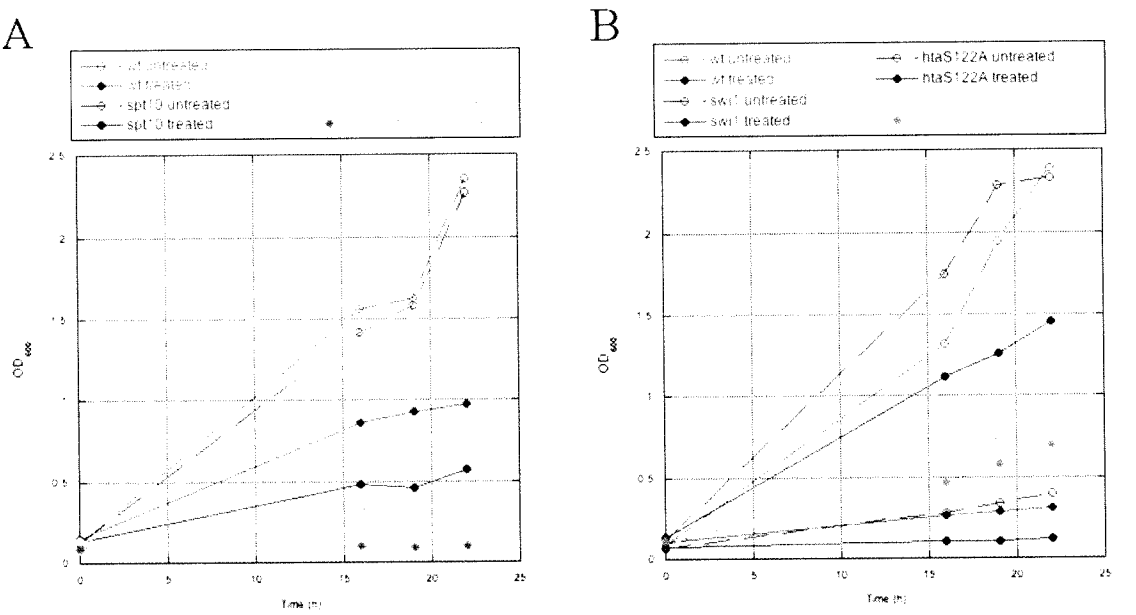

FIG. 1. ***htaS122A* and *spt10Δ-htaS122A* mutants unexpectedly tolerate high Cu levels.** Growth curves are from two independent experiments and represent cell growth in response to 1.5 mM CuSO4 treatment. Untreated cells are represented by dashed lines with empty circles at time points and treated cells are represented by solid lines with filled circles at time points. A, 22-hour growth curve comparing wild type (blue lines), *spt10Δ* (red lines), *htaS2A* (tan lines) and *spt10Δ-htaS2A* (green lines) cell growth. B, 22-hour growth curve comparing wild type (blue lines), *swi1Δ* (brown lines), *htaS122A* (purple lines), and *spt10Δ-htaS122A* (orange lines) cell growth.

1 serve to demonstrate how data was recorded and represent only 2 individual experiments. Because some strains grow slower than others, average data is better represented as a ratio of treated cell growth to untreated cell growth for each strain at the final time point, 22 hours (Fig. 2). As seen in Figure 2, only *htaS122A* and *spt10Δ-htaS122A* treated/untreated growth ratios at 22 hours differ significantly from wild type. Table 1 summarizes the data for the 22 hour growth ratios. Additional growth curves will be conducted for *spt10Δ*, *htaS2A*, and *spt10Δ-htaS2A* to obtain 3 or more independent experiments for each of these strains.

Detection of expression of genes involved in copper starvation. After observing the failure of the mutant strains *spt10Δ-htaS2A*, *spt10Δ-htaS122A* and *swi1Δ* to

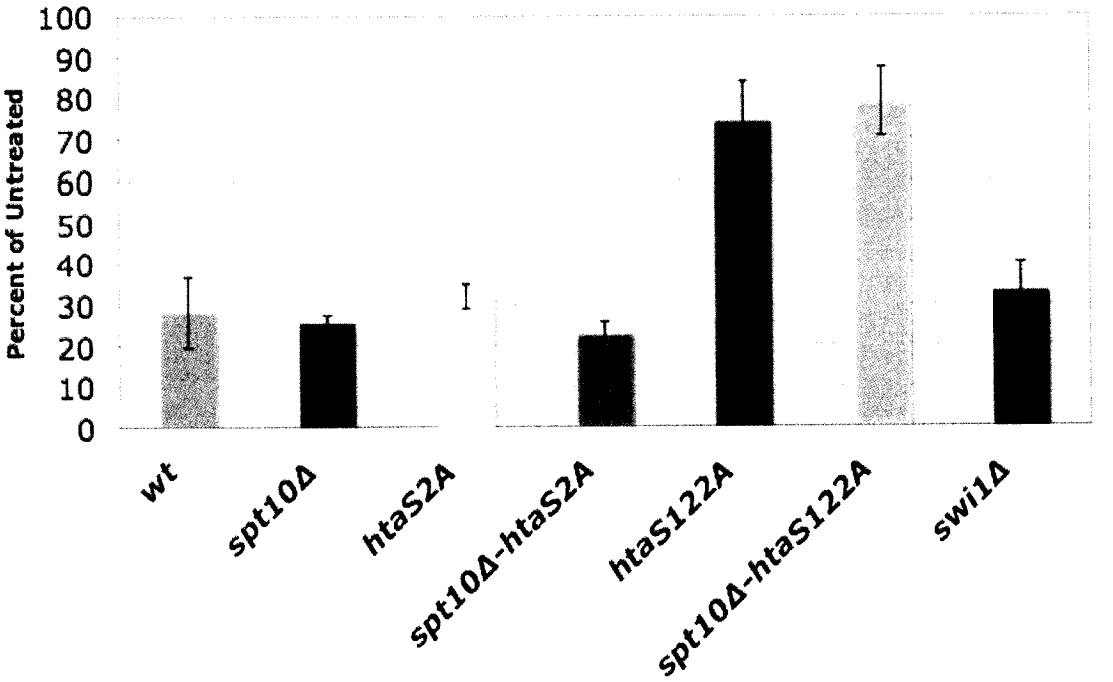

FIG. 2. **Treated/untreated growth ratios at 22 hours show *htaS122A* mutants are impervious to copper stress.** Treated OD_{600} values at 22 hours were divided by respective untreated OD_{600} values at 22 hours to obtain treated/untreated growth ratios for wild type (blue bar), *spt10Δ* (red bar), *htaS2A* (tan bar), *spt10Δ-htaS2A* (green bar), *htaS122A* (purple bar), *spt10Δ-htaS122A* (orange bar) and *swi1Δ* (brown bar).

downregulate *CUP1*, I wished to test whether or not overexpression of Cup1 in response to high copper levels actually results in copper starvation within the cell. We are currently performing real-time reverse transcriptase polymerase chain reaction (RT-RT-PCR) analysis (see description of procedure below) to detect if there is expression of copper starvation genes in the mutant strains that fail to downregulate *CUP1*. This technique involves isolating mRNA from cell cultures and reverse transcribing it into its DNA complement using reverse transcriptase, an RNA-dependent DNA polymerase. Sequence specific primers (discussed below) were used for detection of copper starvation genes. After the mRNA has been reverse-transcribed into DNA, the DNA is amplified by the polymerase chain reaction (PCR). Product formation was detected using SYBR Green, a fluorescent dye that intercalates into double-stranded DNA. This method allows

	Treated/untreated growth ratio	Standard deviation	n
WT	28	8.6	n=7
spt10Δ	26	1.8	n=2
htaS2A	32	2.9	n=2
spt10Δ-htaS2A	22	3.3	n=2
htaS122A	74	10.2	n=5
spt10Δ-htaS122A	79	8.4	n=5
swi1Δ	33	7.1	n=5

TABLE 1. **Treated/untreated growth ratio data.** Data representing $CuSO_4$ treated/untreated growth ratios, standard deviations, and number of independent experiments (n) for wild type, *spt10Δ*, *htaS2A*, *spt10Δ-htaS2A*, *htaS122A*, *spt10Δ-htaS122A, and swi1Δ*. Additional growth curves for *spt10Δ*, *htaS2A*, *spt10Δ-htaS2A* will be conducted to obtain 3 or more independent experiments for each of those strains.

for detection of relative gene expression at a particular time, such as time points during a growth curve.

In order to perform RT-RTPCR analysis, I designed forward and reverse primers for *CTR1* and *ACT1* (loading control); our lab had already designed *CUP1* primers. I have developed successful *CTR1* and *ACT1* primers. I generated standard curves for each primer that are shown in Figure 3. Figure 3A-D depicts PCR reactions using the *CTR1* primers and shows the fluorescence reading from PCR (A), the melt curve: the single melting temperature meaning there was only one product formed (B), the standard curve generated using serial dilutions of *S. cerevisiae* genomic DNA (C) and the *CTR1* PCR products which are at approximately 195 base pairs (D). Figure 3E-H depicts *ACT1* PCRs and shows the fluorescence reading from PCR (E), the single melting temperature

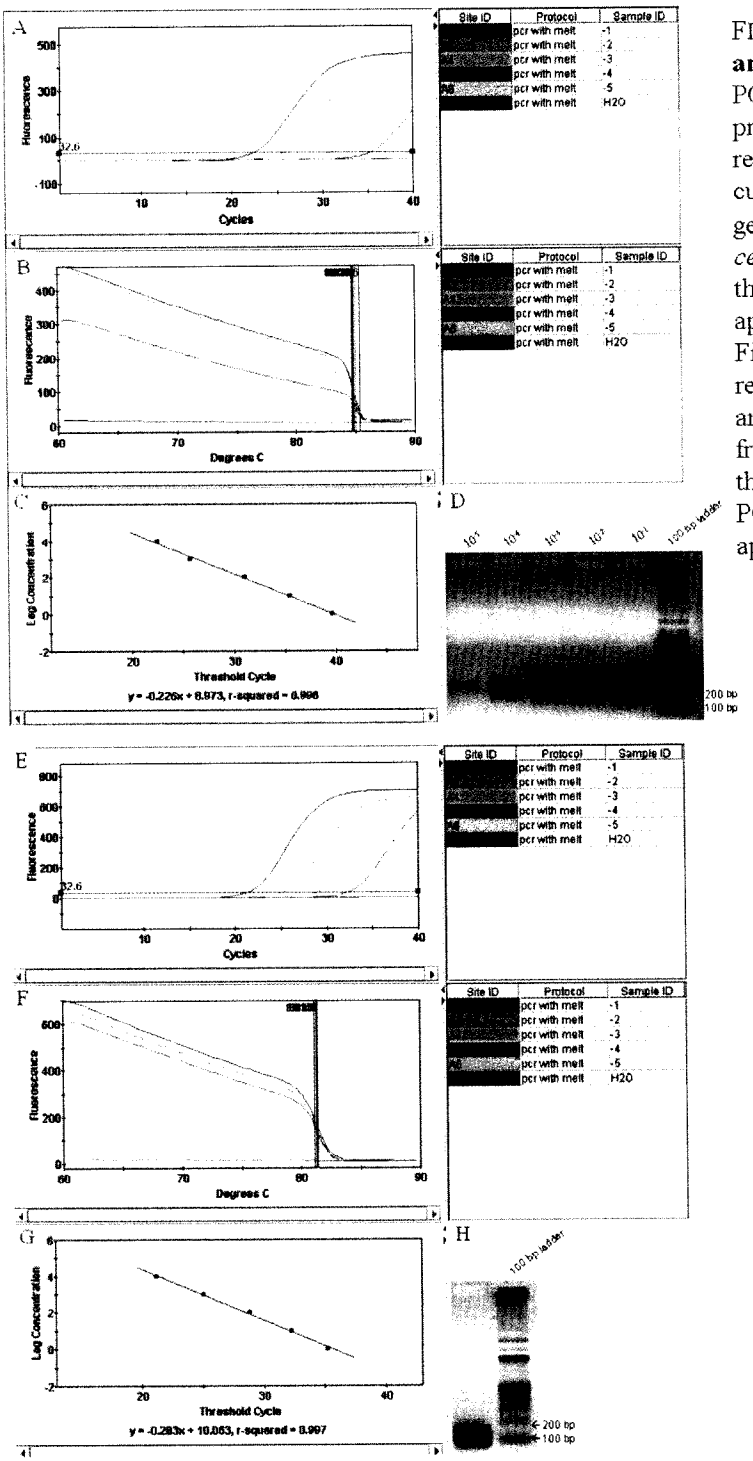

FIG. 3. **Standard curves for *CTR1* and *ACT1*.** Figure 3A-D depicts PCR reactions using the *CTR1* primers and shows the fluorescence reading from PCR (A), the melt curve (B), the standard curve generated using serial dilutions of *S. cerevisiae* genomic DNA (C) and the *CTR1* PCR products which are at approximately 195 base pairs (D). Figure 3E-H depicts *ACT1* PCR reactions using the *ACT1* primers and shows the fluorescence reading from PCR (E), the melt curve (F), the standard curve (G) and the *ACT1* PCR product which is at approximately 110 base pairs (H).

meaning there was only one product formed (F), the standard curve (G) and the *ACT1* PCR product generated using serial dilutions of *S. cerevisiae* genomic DNA, which is at approximately 110 base pairs (H). I am beginning to conduct PCR analysis on RNA samples isolated during growth curve analyses.

Discussion

My work was based on the observation that *S. cerevisiae* strains with certain H2A C- and N- terminal tail mutations combined with *SPT10* deletions fail to downregulate *CUP1* expression (Kuo *et al.* 2005). I have demonstrated that these mutants, despite similar levels of *CUP1* mRNA levels, exhibit different growth rates in response to exposure to toxic levels of copper.

I predicted that overexpression of *CUP1* would be detrimental to *S. cerevisiae.* Therefore, copper treated double mutants (*spt10Δ-htaS2A* and *spt10Δ-htaS122A*) that do not downregulate *CUP1* expression should have growth rates lower than their respective untreated growth rates. However, my data only partially support this hypothesis because the double mutants behave differently from one another when treated with copper, as seen in the growth curves shown in Figure 1. When treated with 1.5 mM $CuSO_4$, wild type cells show reduced growth rates compared to their untreated counterparts (blue lines). Of the copper-treated mutants that fail to downregulate *CUP1*, *swi1Δ* (brown lines) and *spt10Δ-htaS2A* (green lines) mutants also show reduced growth compared to their untreated counterparts. However, the copper-treated *spt10Δ-htaS122A* (orange lines) double mutant grew almost as well as its untreated counterpart. It is interesting to note that the copper-treated *htaS2A* (tan lines) single mutant grew poorly in the presence of copper while the copper-treated *htaS122A* (purple lines) single mutant grew well, just like *spt10Δ-htaS122A.* Both *htaS2A* and *htaS122A* show proper shutdown of *CUP1* similar to wild type cells (Kuo *et al.* 2005). These data, which are discussed in depth

below, suggest that there is more affecting the growth of the copper-treated mutants than the levels of *CUP1* being expressed.

An important future direction of this research will be to try to understand why control of *CUP1* regulation is lost in these particular mutants, and why the *htaS122A* mutants behave differently from other mutants in response to toxic levels of copper, despite similar *CUP1* expression phenotypes.

Loss of CUP1 *regulation.* It has been demonstrated that *CUP1* shutdown is an active process and that it may be dependent on Spt10 and/or H2A (Kuo *et al.* 2005). It is not yet understood how *CUP1* shutdown occurs and our lab is currently investigating this process.

Why are htaS122A *mutants impervious to copper stress?* It is unlikely that *CUP1* transcription plays a role in the survival of *htaS122A* mutants when exposed to toxic levels of copper. This is because *spt10Δ-htaS122A* and *spt10Δ-htaS2A* mutants produce similar amounts of *CUP1* mRNA when exposed to copper yet exhibit very different growth rates. It is possible that *CUP1* mRNA levels do not reflect Cup1 metallothionein levels in these mutants. Previous work found no general translational defect in these mutants (with or without copper) when total proteins were run on SDS-PAGE (Kuo *et al.* 2005); however, levels of Cup1 protein itself have not been monitored in these mutants. In order to rule out the possibility of a translational defect, it would be necessary to determine whether or not *CUP1* mRNA accumulation in the cell is followed by a concurrent accumulation of Cup1 levels in the cell. Without this knowledge, we cannot be certain that all *CUP1* mRNAs are undergoing translation to yield Cup1

metallothionein. Currently, no antibody exists against Cup1; future work will therefore depend on tagging the Cup1 protein for detection.

I have demonstrated differences in growth rates between *CUP1*-overexpressing mutants exposed to toxic levels of copper. However, a reduction in growth rate in a culture can be caused either by slowing of the cell cycle, or by loss of viability of some cells in the culture. I have not determined cell viability of these strains when exposed to copper, which can be tested by plating known numbers of cells from a sample and determining what percent of these cells can form colonies. Viability assays need to be performed for each of these mutants, with and without copper, at each time point of my growth curves.

H2A S122 may play a critical role in cell cycle regulation. The *htaS122A* and *spt10*Δ-*htaS122A* mutants and the *htaS2A* and *spt10*Δ-*htaS2A* mutants have similar growth phenotypes (slow growth) but the *htaS122A* and *spt10*Δ-*htaS122A* copper-treated mutants have much higher growth rates (when compared to their non-treated counterparts) than the *htaS2A* and *spt10*Δ-*htaS2A* copper-treated mutants. After further evaluation, this observation may provide insight regarding the role played by H2A S122 in the cell cycle. The sensitivity of yeast cells to copper toxicity depends on the cell cycle stage the cell occupies; G_2/M is more sensitive to lethality by copper than G_1/S (Howlett and Avery, 1999). Though this sensitivity has been linked to variations in basal levels of Cu, Zn-superoxide dismutase (Sod1) activity throughout the cell cycle (Sumner *et al.* 2003), an alternative explanation for the differences in *CUP1* expression of our mutant strains could be differences in cell cycle rates or different distributions of cell cycle stage occupancies (Kuo *et al.* 2005). Our lab has monitored cell cycle progression of *spt10*Δ-

htaS2A and observed that a larger percentage of logarithmically growing cells in this strain occupy G_2/M, the most copper sensitive stage, compared with wild type or *spt10*Δ alone (Kuo *et al.* 2005). We predict that toxic levels of copper may be too stressful for *spt10*Δ-*htaS2A* and this may trigger a specific bypass of the *CUP1* autoregulation process. The stress caused by toxic levels of copper may then activate non-Ace1-dependent pathways, such as the oxidative stress response, which could maintain *CUP1* expression. Future experiments will test whether alternate stress pathways are involved in response to copper in these strains. While we have monitored cell cycle progression in *spt10*Δ-*htaS2A*, this has not been done for *spt10*Δ-*htaS122A*. It will be interesting to see what cell cycle stage the majority of logarithmically growing cells occupy since growth of *spt10*Δ-*htaS122A* cells seem largely unaffected by toxic levels of copper. Previous work has shown that G_2/M checkpoint responses remain intact in an *htaS122A* mutant strain (Harvey *et al.* 2005).

Other components of metal trafficking pathways, in addition to Ace1-dependent and Mac1-dependent pathways, may be involved in the different behaviors of our *CUP1*-overexpressing mutants. Recent work has underscored the intimate links between iron and copper metabolism and mitochondrial and vacuolar function in metal trafficking, as well as a small regulatory iron pool that links copper and iron responses (Harm *et al* 2005). The copper regulatory apparatus is complex and involves more than the two pathways we have studied.

By better understanding the mechanisms by which *CUP1* is shut down and why our mutant strains respond to copper stress differently, we may open the way to greater

insights involving copper homeostasis. This, in turn, will help us better understand copper homeostasis diseases of the human.

Materials and Methods

Growth curves. Each strain of yeast was plated from a glycerol stock derived from a single yeast cell colony. After 2 or 3 days of growth at 30°C, 4 mL of complete synthetic defined media was inoculated with several colonies for each strain and incubated overnight in a shaker at 30°C. One day later, each 4 mL culture was added to 100 mL of synthetic defined media and shaken at 30°C for 3 hours. When the cells were at OD_{600} of ~0.1 (early log phase), half of each culture (50 mL) was added to another flask and $CuSO_4$ was added to a final concentration of 1.5 mM. The OD_{600} values of $CuSO_4$-treated samples and untreated samples were recorded at this time (Time 0). Cells were incubated at 30°C with shaking. Sixteen hours after Time 0, and every 3 hours after that until 22 hours, OD_{600} values were recorded and yeast cells were harvested for RNA preparation.

Preparation of RNA from yeast cells. Yeast cells were grown as described above. Samples were harvested at each timepoint in amounts equivalent to 2 ml at OD_{600}= 0.5 in order to collect similar numbers of cells at each timepoint. Samples were spun down at 4°C in a tabletop centrifuge at 3,000 rpm for 4 minutes. Supernatant was discarded and the cell pellets were either frozen and stored at -80°C to undergo RNA isolation at a later time, or resuspended in 400 µl of TES (10 mM Tris-HCL pH 7.5, 10 mM EDTA, 0.5% SDS). The TES suspension could be frozen and stored at -20°C for up to a month. Acid phenol (400 µl) was added to the TES suspension and vortexed vigorously for 10

seconds. The mixture was incubated for 30-60 minutes at 65°C and vortexed occasionally during this time. The mixture was placed on ice for 5 minutes then spun in a microfuge for 5 minutes at 13,000 rpm at 4°C. The upper aqueous layer, containing the RNA, was transferred to a new tube and re-extracted with 400 µl of acid phenol, vortexed vigorously and left on ice for 5 minutes. The mixture was microfuged for 5 minutes at 13,000 rpm at 4°C. 200 µl of the upper aqueous phase was transferred to a new tube. 5M NaCl (20 µl) and 500 µl 100% EtOH were added and mixed by inverting the tubes 5-6 times. RNA was precipitated by placing the tubes at -20°C for half an hour to overnight. The mixture was spun in a microfuge for 15 minutes at 13,000 rpm at 4°C and the supernatant was discarded with care in order not to dislodge the pellet. 200 µl of 70% ethanol was added onto the pellet and briefly vortexed and spun for 2 minutes at 13,000 rpm at 4°C. The supernatant was discarded and the pellet was air dried at 65°C. Distilled H_2O (50-100 µl) was added to the dry pellet and placed at 65°C for 10-20 minutes to resuspend the RNA. The RNA samples were stored at -80°C until ready to perform real time RT-PCR.

Real Time RT-PCR analysis. RNA was kept on ice at all times. Genomic DNA contamination was removed using a DNase treatment: Distilled H_2O (6.5 µl), 1 µl 10X PCR buffer, 0.5 µl Turbo DNase (Ambion), and 2 µl RNA were incubated at 37°C for 15 minutes and then 75°C for 6 minutes to inactivate the DNase. Real time RT-PCR was performed, combining the reverse transcription and PCR steps. RT+ and RT- master mixes were made for multiple reactions. Each RT+ master mix contained 10.5 µl H_2O, 12.5 µl 2X SYBR green mix and 0.1 µl reverse transcriptase (eAMV). Each RT- master mix used 0.1 µl H_2O instead of reverse transcriptase. The master mixes were divided into

individual 0.5 ml microfuge tubes (23.1 µl per tube) and 1 µl (0.5 µl 5'primer + 0.5 µl 3'primer) of 10 µM PCR primers and 1 µl of DNase treated RNA were added. The reactions used the 3' PCR primers as the RT primers. The purpose of the RT- control reactions was to check background levels of product resulting from remaining genomic DNA in the preps.

After each PCR reaction was prepared, the reaction mixtures were transferred to real-time PCR tubes and run for 30 minutes at 46°C for the reverse transcriptase reaction and 94°C for 2 minutes. This was followed by 40 cycles of 15 seconds at 95°C, 15 seconds at 57°C, and 30 seconds at 72°C. PCR products were run on gels to ensure desired product lengths were obtained.

The following primers were used to amplify *CTR1*: 5'- GGTAGCAGCATG AATATGGACGC -3' and 5'- CGACATACTTGCCATTGACGACATAG -3'. The product size is 195 base pairs. The following primer sequences were used to amplify *CUP1*: 5'- CAGCGAATTAATTAACTTCCA-3' and 5'- TTTCCCAGAGCAGCATGACTT-3'. The following primers were used to amplify *ACT1* (control): 5'- CCTACGAACTTCCAGATGGTCAAGTCA -3' and 5'- GTCAATACCGGCAGATTCCAAACCC -3'. The product size is 110 base pairs.

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