Differential Effects of Acute Cadmium, Copper, and Chromium Assault on Glutathione and Transcription Profiles in Saccharomyces cerevisiae

DIFFERENTIAL EFFECTS OF ACUTE CADMIUM, COPPER, AND CHROMIUM ASSAULT ON GLUTATHIONE AND TRANSCRIPTION PROFILES IN *SACCHAROMYCES CEREVISIAE*

by

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Under the Direction of John E. Houghton, PhD

ABSTRACT

Heavy metal assault in *Saccharomyces cerevisiae* results in increased oxidative stress, resulting in direct damage to proteins, lipid membranes and nucleic acids; even after relatively short (>1hr) exposures. Such effects are mitigated and potentially exacerbated by changes in intracellular metabolic pathways, causing a variety of responses. Synthesis and recycling of glutathione within the cell (a major metabolic cycle which minimizes oxidative stress) is especially influenced by exposure to heavy metals depending on the type. Redox active metals (copper and chromium (VI)), influence the oxidative stress of the cell by affecting GSH/GSSG pools, while the redox inactive metal (cadmium) appears to lead to a major increase in glutathione production. Supplementation of cells with mixed amino acids (FFAAP) provides an exogenous source of the building blocks for glutathione, has a recuperative effect on all cells, leading to a decrease in oxidative stress and a lowering of total glutathione and/or normalizing GSH/GSSG pools.

INDEX WORDS: *Saccharomyces cerevisiae*, Glutathione, Reactive oxygen species (ROS), GSH/GSSG, Free form amino acid precursors (FFAAP), cadmium, copper, chromium (IV).

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DEDICATION

This paper is dedicated to my wonderful parents Jerry and Beth Poling who have supported me and without whom I would not be writing this paper. I could not have done this without the support of the love of my life Angel who has been by my side the whole time. I owe my degree to these wonderful people who have done nothing but support me throughout this whole experience.

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I would like to acknowledge Dr. Houghton for guiding me through my graduate school career. Dr. Houghton has always been there to help me get through hardships as well as better understand any questions I may have about science whether it be big or small. I would like to also acknowledge Dr. Hilliard and Dr. Eichenbaum who have helped me through anything I have asked of them. I would also like to thank the core facility at GSU who have worked tirelessly with me to ensure that all my work could be completed. This work could not have been completed without the help of my wonderful lab mates who have stuck with me through all the difficult times. I owe everyone related to the lab at GSU for helping me get through everything. Leaving GSU feels like leaving a family behind who I have grown to love and cherish through my time at the school.

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1 INTRODUCTION

Saccharomyces cerevisiae is a eukaryotic model organism that is used by a number of researchers to better understand some of the more fundamental features of eukaryotic cellular function. The use of *S. cerevisiae* in this role is appropriate for many reasons, primarily because this single-celled eukaryote has been extensively studied, with respect to a series of relatively simple metabolic/cellular functional activities that often mirrors that of its more advanced, metazoic counterparts. Indeed, it has provided a suitable clonal host to characterize several higher eukaryotic protein function (*Hawkins, 1999*). The entire genome of *Saccharomyces cerevisiae* has been sequenced and a full complement of mutant libraries have been created, which together with its alternative potential chromosomal haploidy and diploid capacity has allowed for its use to complement intrinsic gene activity as well as extrinsic gene function. Such attributes, as well as its relatively simple, single celled metabolism, make *Saccharomyces cerevisiae* an exemplary model organism to test and understand first response mechanisms within lower eukaryotes to various environmental influences that higher eukaryotes may encounter.

To this point, previous researchers have shown that *Saccharomyces cerevisiae* has a single caspase gene, YCA1, that is actually a metacaspase (*Madeo et al., 2002*) and which provides one of the more specific triggers of apoptosis in response to cellular, metabolic stress. This somewhat unique characteristic of yeast simplifies analyses of the apoptotic response(s) in yeast because most other eukaryotes possess multiple caspase genes. Humans, for example, have twelve caspase genes [*Spanos et al., 2002; Nagata, 2000*], which lead to a far more complicated series of caspase-mediated responses to exogenous and endogenous changes that trigger their activation. When activated by the increase of environmental H_2O_2 at low levels, the product of

YCA1 (yeast caspase) induces a cascade of specific protein cleavage reactions that eventually leads to the cell entering a caspase-dependent apoptosis [*Carmona-Gutierrez et al., 2010; Madeo et al., 1999*]. This pathway of programmed cell death (PCD) can be initiated in response to the presence of external and internal stressors, allowing cells to commit to a type of cellular steps that ultimately result in the minimal release of cytoplasmic contents to the surroundings, which, in turn, limits the stress response of surrounding cells (*Farrugia, 2012*). The environmental factors that can induce apoptosis in yeast are highly varied in nature and response mechanisms but tend to range between H_20_2 (through assorted other reactive oxygen species (ROS)), cellular aging, UV irradiation, heat stress, pharmacological drugs (such as aspirin), and heavy metals (such as cadmium and iron) [*Madeo et al., 2009; Cabiscol et al., 2005; Almeida et al., 2008*].

The pathway of yeast apoptosis is complicated and contains many different proteins and steps which leads to eventual caspase activation and response. Under environmental stress, yeast cells undergo confirmational changes and protein translocations which leads to induction of YCA1 and apoptosis. One key factor in the induction of ROS and YCA1 within the cell is AIF1P, which is an apoptosis-induction factor protein as shown in Figure 1 below (*Madeo et al., 2009*). AIF1P is initially translocated from the mitochondrial to the nucleus where it stimulates chromatin condensation and DNA degradation in response to apoptotic stimuli [*Wissing et al., 2004; Susin et al., 2019*]. This process is supplemented by mitochondrial fragmentation and the release of cytochrome c from the mitochondria. Together, each of these processes leads to the stimulation of ROS buildup and eventually YCA1 induction in the cell (*Madeo et al., 2009*).

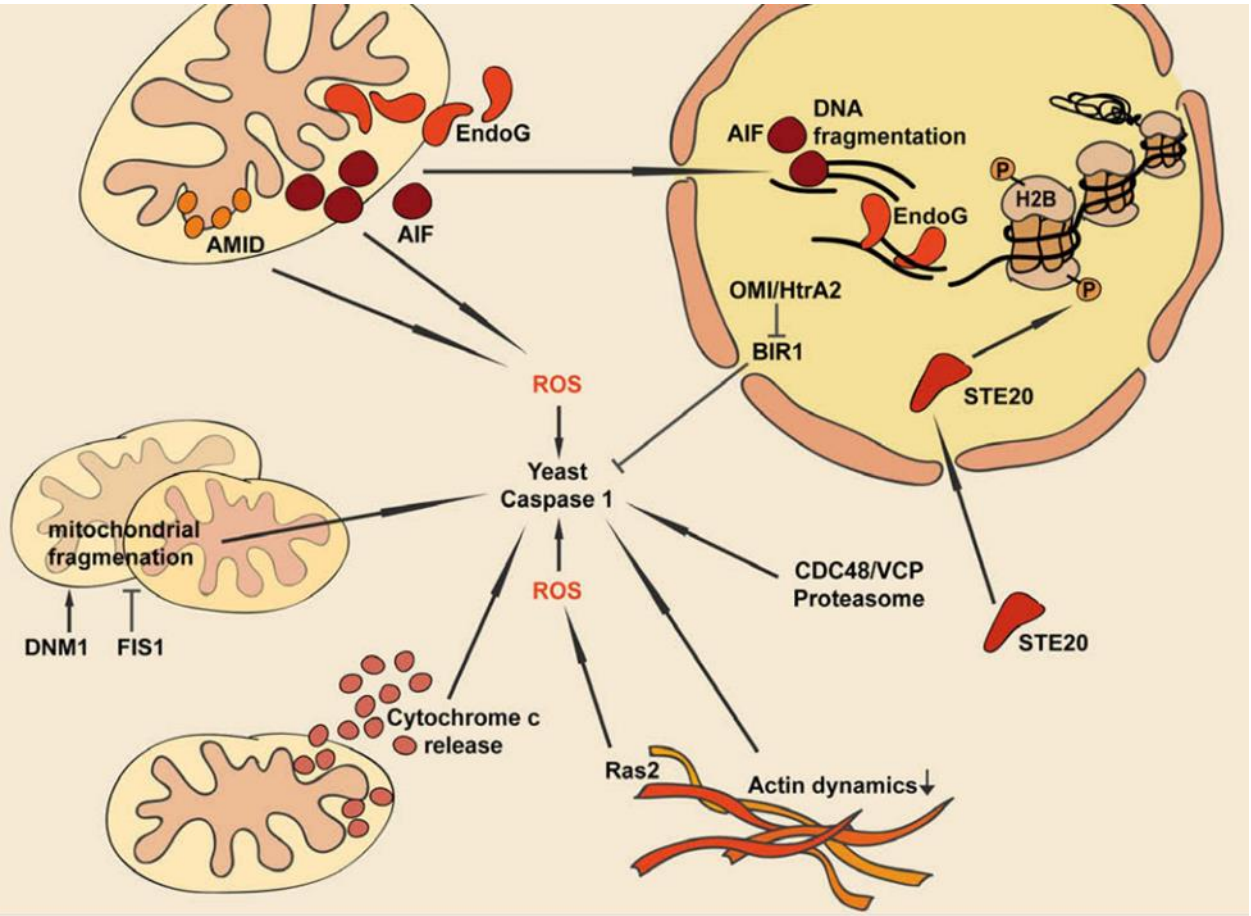


Figure 1. Apoptotic machinery and regulation in yeast.
Different intercellular triggers in S. cerevisiae which leads to YCA1 stimulation and activation of apoptosis. (Madeo et al., 2009)

Previous research has also shown that the pathway for cell death is not simply apoptosis, which is demonstrated in Figure 2 below (*Carmona-Gutierrez et al., 2018*). Yeast cells can undergo multiple different pathways that lead to cell death via necrosis or apoptosis. The pathway that is chosen is mostly relative to the environmental factors that lead to death (for example, high concentrations of H_2O_2 can lead to necrosis rather than induction of YCA1 and apoptosis) (*Carmona-Gutierrez et al., 2010*). This demonstrates that it is critical to understand the appropriate levels of various treatments to conduct to induce apoptosis in yeast populations specifically. Understanding how acute heavy metal exposure can lead to apoptosis along with

other cellular responses is critical to gaining a better understanding of how yeast cells choose the various death pathways presented. Previous research in the lab has demonstrated that exposure to heavy metals such as cadmium or copper at the acute level is capable to inducing this apoptotic pathway of programmed cell death [*Shanmuganathan, 2008; Nargund, 2008*].

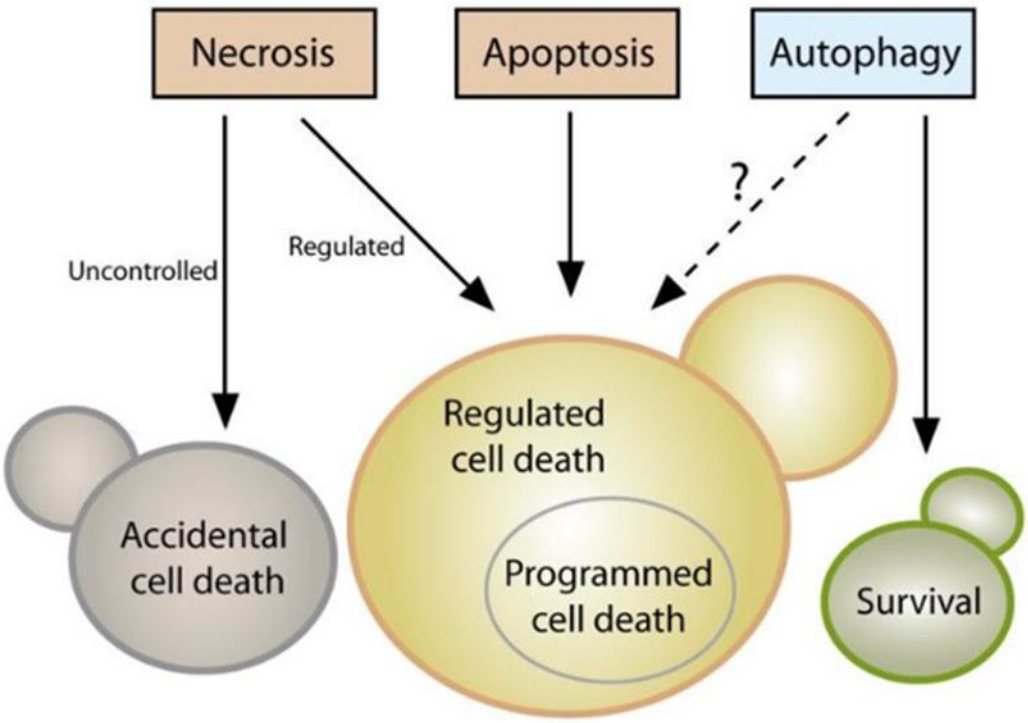


Figure 2. Possible pathways of cell death in S. cerevisiae.
Schematic demonstrating the various pathways of yeast cell death. Yeast cells may undergo uncontrolled cell death via necrosis when exposed to harsh environmental factors. Preferentially, yeast cells undergo regulated cell death, or programmed cell death (PCD), via the necrosis or apoptosis pathways allowing for better regulation of the process. Finally, the cells may undergo autophagy, which is relatively not well understood currently. (Carmona-Gutierrez et al., 2018)

Exposure of cells to heavy metals has been shown to cause an increase in the presence of any number of reactive oxygen species (ROS), within yeast and other cells, which (at high enough levels) can lead to significant damage to the metabolic and cellular infrastructure (*Halliwell, 2007*). These reactive oxygen species are negatively charged free radicals that then

interfere with basic cellular processes (*Bouveris, 1982*). If their production and damaging effects are left unchecked, the buildup will cause major issues, such as targeted and miscellaneous DNA, cell membrane and protein damage. ROS molecules preferentially target molecules that contain free thiol groups. This can specifically lead to proteolysis by targeting aromatic amino acids and cysteine groups by causing fragmentation, cross linking, or even protein unfolding (*Perrone, 2008*). A simple diagram of how metals may exert their effect within yeast is shown in Figure 3 below (*Shanmuganathan, 2008*) through one of two potentially discrete pathways: the one through direct induction of ROS, and an alternative mechanism(s) by which the presence of the metal indirectly leads to cell death depending on how the cell responds to the presence of the metal. Understanding how and why *S. cerevisiae* respond to increased heavy metal toxicity, therefore, is important to determine what pathways are influenced by ROS and/or other molecules. As such, a clearer understanding of how each type of heavy metal initiates metal-induced stress in the cell and thereby potentially triggers an acute, programmed response to their presence.

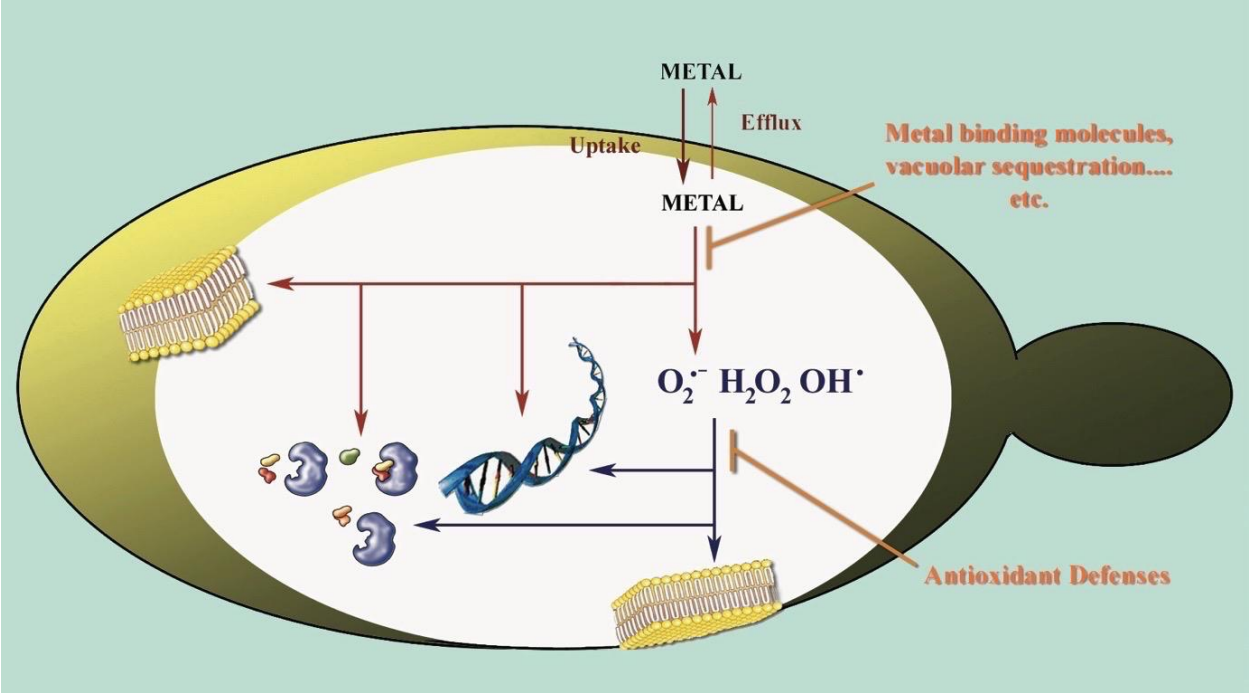


Figure 3. Heavy Metal Effects on Yeast.
Schematic of heavy metal toxicity within Saccharomyces cerevisiae which leads to intracellular damage and buildup of reactive oxygen species within the cell.

Saccharomyces cerevisiae has several mechanisms through which it can counteract heavy metal toxicity, and thereby mitigate much of the damage that either the metal, or associated ROS free radical production can cause. One of the major pathways to reduce the overt production of ROS in the cells that may be caused by the presence of heavy metals is the production of glutathione within the cell. This antioxidant can be used either to reduce ROS produced by metals such as copper or chromium directly into more innocuous forms, or remove some of the offending metals, cadmium specifically, from the cell through glutathione-mediated sequestration of the metal within vacuoles [*Gomes at al., 2002; Li et al., 1997*]. Each of these metals induces YCA1 activation and apoptosis within the cell, which in some cases is linked to ROS generation, which is demonstrated in Figure 4 below (*Perrone, 2008*).

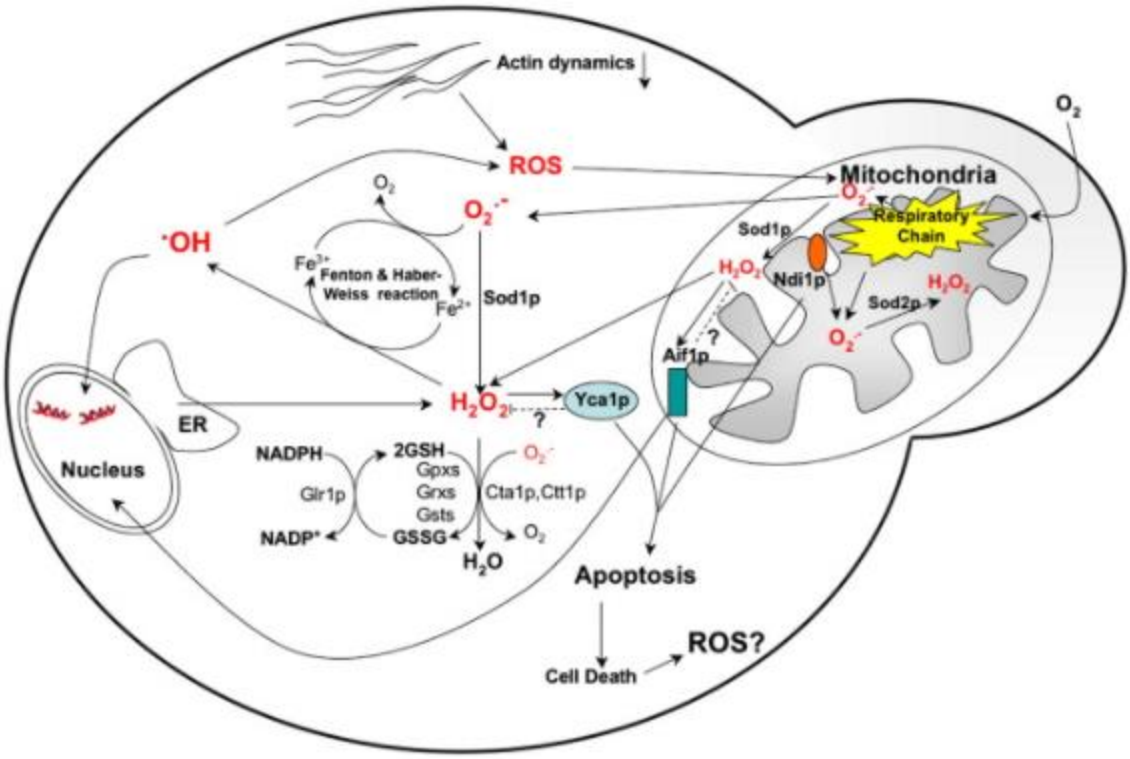


Figure 4. Sources of ROS and how they are linked to apoptotic machinery in yeast. *ROS buildup (indicated in red) induces mitochondrial and cytosolic reactions leading to apoptosis. These pathways include the regulation of glutathione to reduce ROS levels within the cell. (Perrone, 2008).*

Glutathione itself is a tripeptide consisting of glutamate, cysteine, and glycine, that is present in two contrasting forms within the cell: an oxidized glutathione (GSSG) form and a reduced (GSH) form. It is synthesized as its reduced form, in which the sulfur group on the cysteine moiety is simply bound to a hydrogen (GSH) (*Gomes et al., 2002*). The oxidized form is generated from two reduced glutathione moieties bound at the sulfur groups of the molecules (Figure 5). Reduced glutathione is the "reactive form" of the molecule that can respond to ROS buildup as it can directly reduce these ROS free radicals by donating its hydrogens bound to the sulfur on the thiol groups (Figure 5A). This reductive activity leads to an increase in oxidized glutathione (GSSG) in the cell, which in turn, must eventually be converted back to its reduced

form (via the oxidation of $NAPH_2$) to restore the GSH/GSSG ratio in the cell or reuse (Figure 5A).

In yeast, there is a conserved pathway that allows for the conversion between oxidized and reduced forms of glutathione within the cell to maintain a homeostatic balance of the two molecules -as shown in Figure 5A, below (*Rubino et al., 2015*). In this pathway, glutathione peroxidase converts two reduced glutathione molecules into one oxidized glutathione with the help of hydrogen peroxidase that acts as an electron acceptor. In the reverse reaction, one oxidized glutathione molecule is cleaved into two reduced glutathione molecules by glutathione reductase that utilizes NADPH as an electron donor. The interplay of these two pathways effectively help to maintain the levels of glutathione at a basal level within the cell at any given time.

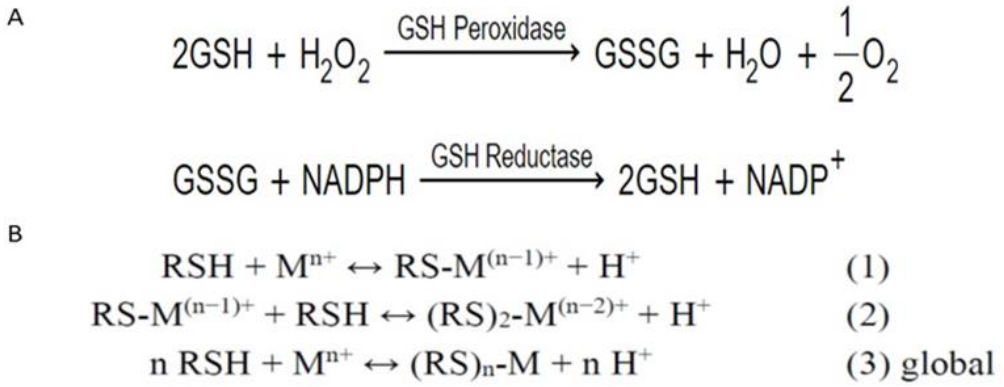


Figure 5. Recycling of Glutathione.

Illustration of glutathione interactions within the cell. (A) This pathway illustrates the process of converting the two forms of glutathione between one another to remain at a basal level. (B) This is the pathway of how glutathione directly reacts with heavy metals within the cell to combat toxicity. In this reaction, a reduced molecule of glutathione (RSH) binds to a free heavy metal (M) such as cadmium direction (step 1). A second reduced form of glutathione then binds to the RS-M moiety to create a heavy molecule where one heavy metal is flanked by two glutathione molecules (step 2). Step 3 shows the global reaction that occurs between steps 1 and 2 within the cell. (Rubino et al., 2015)

Not too surprisingly, the levels of glutathione are highly regulated within the cell, potentially at both the transcriptional expression level of the enzymes that promote its synthesis as well as the functional, enzymatic level that maintains the metabolic flow of the associated substrates within its synthetic pathway. In the absence of any variation in transcriptional expression of the appropriate genes, Noctor et al. and others have shown that the concentration of glutathione in the cell is heavily controlled through a strongly competitive feedback inhibition of its own synthesis (*Noctor et al., 2002*), which maintains levels of reduced glutathione that reach a homeostatic point and inhibit the first enzyme in the synthesis pathway, γ-ECS (Figure 6).

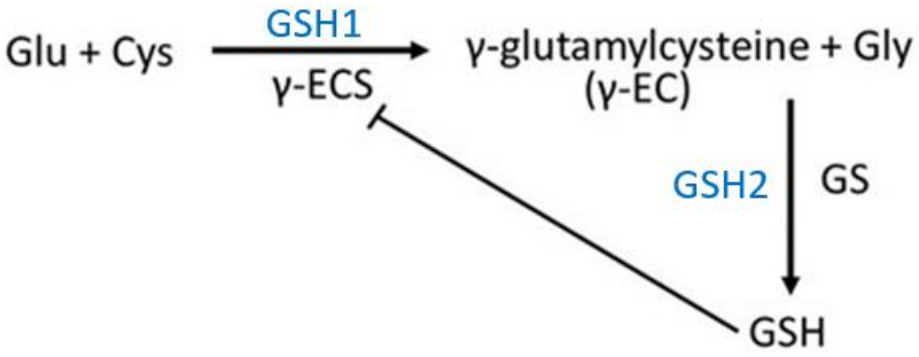


Figure 6. Synthesis of glutathione.
Diagram illustrating the production of glutathione coupled with feedback inhibition on the γ-ECS enzyme via competitive interactions of reduced glutathione (GSH). Genes related to the proteins involved in the process are indicated in blue. Modified from Noctor, 2002.

Given the nature of how different metals potentially affect cellular activity, they are often referenced in one of two forms, “redox active” and “redox inactive”, which speaks to the inherent ability of the heavy metal in question to directly cause the production and buildup of

ROS within the cell. This is not to say that redox inactive metals cannot induce ROS within living cells. They can, but the mechanism(s) by which they do so are, of necessity, indirect, causing a latent ROS buildup, for example, by potentially displacing other more "redox active" metals that are present within the cell (*Sharma et al., 2014*). Previous research in the lab has demonstrated that *S. cerevisiae* presents a quite different acute response when transiently exposed to these two types of heavy metals. Cadmium, a "redox inactive" metal, has been shown to induce only negligible levels of ROS buildup in the cell within the first hour of heavy metal treatment. Even so, this metal has still been shown to create a rapid and quite dramatic apoptotic response in the cell [*Shanmuganatahan, 2008; Nargund, 2008*], along with a significant increase in glutathione levels [*Gardner, 2019, Poling and Gardner, unpublished observations*]. Previous data acquired by Rubino et al. have also indicated that cadmium has a direct interaction with glutathione, and that it can apparently lead to a rapid increase in total glutathione levels (*Rubino et al., 2015*). As shown in Figure 5, cadmium ions, along with other "redox inactive" heavy metals, have been shown to bind directly to glutathione molecules within the cell (*Rubino et al., 2015*). Conversely, Copper, a "redox active" metal, has been shown to promote a pronounced increase in ROS in the cell even under acute conditions of exposure (within an hour), but (curiously) fails to elicit any significant increase in the overall glutathione levels within the cell [*Gardner, 2019, Poling and Gardner, unpublished observations*]. Given that copper has been shown to produce ROS within the cell under the Haber-Weiss reaction by acting as a catalyst (*Kehrer, 2000*), it is apparent that the immediate effect of copper exposure in the cell is principally an increase in the formation of ROS, although additional means of immediate (acute) effects of metal-induced metabolic stress within yeast have not been ruled out. Studies conducted by Nargund were conducted under acute metal toxicity of one-hour treatment,

which was sufficient time of exposure to elicit an apoptotic response in the sub populations of exposed cells (*Nargund, 2008*). While Nargund further demonstrated a similar, but distinct apoptotic response of cells to copper (*Nargund, PhD Dissertation, 2014*), no in-depth metabolic appreciation as to how different the two responses of the two metals were have been addressed. Thus, it appears that acute exposure of *S. cerevisiae* to these two metals presents quite distinct and contrasting cellular responses, both of which appear to be mitigated by the action(s) of glutathione.

Synthesis of glutahione within *S. cerevisiae* and its effective homeostatic maintenance of redox potential in the cell has proven to be of great importance for research in recent years (*Noctor, 2002*). One specific goal of the research presented in this thesis is to demonstrate, quickly and clearly, the different ways by which *S. cerevisiae* responds to the acute exposure of different heavy metals, with respect to their ability to elicit high levels of ROS. To help in this endeavour, Proimmune™ is used, a dietary supplement of freeform amino acids (FFAAP) to assess the importance of changing pools of glutathione. FFAAP is a mixture of the three amino acids that make up glutathione; glutamic acid, cysteine, and glycine (as well as a small amount of selenium) that is designed to provide a "preventative measure" against ROS levels in the cell. This supplementation allowing for cells to sustain increased pools of amino acids availablility, that can be utilized in production of the anti oxidant, glutathione (*The ProImmune™ Company, NY, USA*). Results presented here will indicate how this particular mixture of free form amino acids does indeed affect the metal induced imbalance of redox potential within the cells (under different conditions of heavy metal exposure). As such the presence of FFAAP provides an important counterpoint to investigate how the different heavy metals used affect the critical

GSH/GSSH pools in the cell, and potentially relate to the degree of programmed cell death that they initiate.

2 MATERIALS AND METHODS

2.1 Strains and Growth Conditions

The BY4741 (MATα, his3D1, leu2D0, met15D0, ura3D0) strain of *Saccharomyces cerevisiae*, purchased from Euroscarf was used in this study. Cells were initially cultured on YEPD agar plates and rotated every month to ensure proper growth of cell cultures. Cells were grown in fresh YEPD (yeast extract with 2% dextrose) media that was buffered with phosphates to ensure a pH of 6.8 for all tests. Flasks were inoculated with 10µL of starter culture and grown overnight at 30°C, 250 rpm in a water bath to ensure even heating. When cells reached an OD_{600} value of ~2.2-2.5 each flask was treated with varying heavy metals for one hour. Four heavy metals were used, cadmium (II) nitrate [$Cd(NO_3)_2$] (80µM), copper (II) nitrate [$Cu(NO_3)_2$] (8mM), chromium (III) nitrate [$Cr(NO_3)_3$] (2.4mM), and chromium (VI) oxide [CrO_3] (2.4mM), as determined by previous growth data. These concentrations were used because they do not impact cell growth but do have an impact on the apoptotic response to the heavy metal for an acute toxicity treatment. A duplicate flask was grown for each experiment containing FFAAP (2mM) to test the changes made by introducing more amino acids to the culture.

2.2 Buffers and Solutions

Buffers and solutions used for experiments outlined in this document were created as follows. All growth media used for these experiments were created by initially making YEPD media and buffering with phosphates to retain a pH ~ 6.8 for all growth conditions. This was generated by initially creating a sterile potassium phosphate buffer (1M) by mixing 0.5-mole potassium phosphate monobasic [KH_2PO_4] (Fisher Chemical, Cat # 7778-77-0) and 0.5-mole potassium phosphate dibasic [K_2HPO_4] (Fisher Chemical, Cat # 7758-11-4) in 1L diH_2O. YEPD media was created following Cold Spring Harbor protocol in 1L of diH_2O (*Cold Springs*

Harbor). Buffered YEPD media was then created by adding 100mL of phosphate buffer (PB) to 1L YEPD media to generate a final concentration of 100mM. All media was then further sterilized by autoclave for at least 45 minutes and stored at room temperature until further use.

Each heavy metal stock solution was created in diH_2O as follows: cadmium (II) nitrate solution (100 mM) (Alfa Aesar, Cat # 10663) for 80 µM treatment, copper (II) nitrate (1 M) (Aldrich Chemistry, Cat # 229636-25G) for 8 mM treatment, chromium (III) nitrate (1 M) (Acros Organics, Cat # 7789-02-8) for 2.4 mM treatment, and chromium (VI) oxide (1 M) (Alfa Aesar, Cat # 12522) for 2.4 mM treatment. All heavy metal solutions were created and stored at 4°C until further use. Lysis buffer solutions were created the day of experimentation by mixing 3% glycerol and 1x Tris HCl in DI H_2O, pH ~ 8.8. Further addition of 5% Metaphosphoric Acid (MPA) (Acros Organics, Cat # 37267-86-0) was added when using the Enzo Life Sciences glutathione kit (Enzo, Cat # ADI-900-160). Lysis buffer solutions were created and stored at 4°C each day they were used to keep the solution fresh. Fresh 5% 5-sulfo-salicylic acid (SSA) (Fisher Chemical, Cat # A297-100) was created for use in the Invitrogen glutathione kit (Thermo Fisher, Cat # EIAGSHC) each day of experimentation.

Fresh FFAAP stock solutions (200 mM) were created the day of inoculation at least 30 minutes prior to addition to buffered YEPD media. Initially, 5 mL diH_2O was added to 5.5 mL NaOH (12N) in a 15 mL conical tube and 1g of fresh FFAAP was added. The solution was mixed thoroughly by vortex and inversion and 3.5 mL HCL (10N) was added to the solution carefully. This solution was then inverted to mix and stored at room temperature to ensure proper cooling of the mixture. The final FFAAP mixture (200 mM) was then stored at room temperature until added to buffered YEPD media. Fresh FFAAP was created each day of inoculation as the solution is unstable and yellows over time.

2.3 Determination of Heavy Metal Treatment Concentrations

Previous studies in the lab had established proper treatment of each heavy metal (*Nargund, 2008*), however further checks on growth rate effects of copper and chromium were conducted. Cells were inoculated as previously stated and were assayed to determine cellular density at OD_{600}. At an OD_{600} ~2.2, the cell cultures were treated with varying heavy metal treatments as follows: untreated, copper (II) nitrate (8 mM), copper (II) nitrate (10 mM), chromium (III) nitrate (2.4 mM), chromium (III) nitrate (3 mM), chromium (VI) nitrate (2.4 mM), and chromium (VI) nitrate (3 mM). Each treatment was analyzed for cellular growth every 30 minutes for 6 hours to determine the effect on growth rate of each treatment. Doubling times of each treatment were calculated by selecting two time points during exponential growth phase.

2.4 Cellular Apoptosis Levels

Saccharomyces cerevisiae cells were grown as explained above and treated with each heavy metal, cadmium nitrate [$Cd(NO_3)_2$] (80µM), copper nitrate [$Cu(NO_3)_2$] (8mM), chromium(III) nitrate [$Cr(NO_3)_3$] (2.4mM), and chromium(VI) nitrate [CrO_3] (2.4mM), for one-hour acute treatment. Post heavy metal treatment, the cells were washed using warm saline solution. To accomplish this, the cells were harvested and spun down in a centrifuge at 5000rpm for one minute. The supernatant was decanted and then washed with saline twice. Post wash, the cells were resuspended in fresh buffered YEPD media and further tested for cell density at OD_{600}. Each treatment was diluted to an OD_{600} ~ 1 to allow for proper cell density for staining. The cells were then placed in a covered, dark microcentrifuge tube containing Fam-FLICA (Fluorochrome-Labeled Inhibitors of Caspase Activity) caspase dye to incubate for three hours. This three-hour rest allows for initial signs of apoptosis to begin within the cell cultures and the

dye to bind to cleaved metacaspase within the cell population. Once the three-hour rest period was over, cultures were washed to remove excess dye and culture tubes were made for each treatment for flow cytometry: cell cultures containing FAM-FLICA dye were diluted by adding 50µL of cell culture to 450µL fresh YEPD. Flow cytometry was conducted using BD LSRFortessa (*BD Biosciences, USA*) by reading each cell treatment under 10,000 events. FAM-FLICA is a dye that has an excitation of 520 nm and emission of 490 nm and was analyzed using the FITC laser settings on the cytometer. Once processed, total apoptotic levels were generated by gating cells outside of the untreated control sample.

2.5 Intracellular Glutathione Analysis

Cells were grown and harvested as explained above, without allowing them to rest for three hours post metal treatment. Once ready, the cells were collected and pelleted at 4°C, 5000rpm and resuspended in 3mL of lysis buffer. 1.5mL of this resuspended solution was added to tubes that contained 0.5mm silica beads and then lysed using a bead beater set to maximum for 1 minute, then allowed to rest on ice for 30 seconds. This process was repeated 4 times to ensure for proper lysis of the cells. Once sufficiently lysed, the samples were pelleted at maximum rpm for 5 minutes. The supernatant was collected and placed into fresh microcentrifuge tubes. Serial dilutions (1:50 and 1:100) were then conducted on each cell lysate sample. This was done in duplicate to determine the difference between oxidized and reduced forms of glutathione within the cell. For the oxidized glutathione, the cell lysate was treated with 4-vinyl pyridine for one hour to block any free thiols of the reduced glutathione using the Enzo Life Sciences kit (2-vinyl pyridine was used in the Invitrogen kit). Serial dilutions were also created for these samples at 1:20. Extra cell samples were also collected in tandem to assay for appropriate apoptotic effect using the FAM-FLICA protocol above. Once the serial dilutions

were created, a 96 well plate was loaded with 50µL of each sample in triplicate along with a standard of glutathione. The plate was then loaded and run on the Enspire Plate Reader which measured absorbance every minute for ten minutes total. For the Enzo Life Sciences kit, the active reagent was added automatically at 150µL aliquots to each well by the machine. For the Invitrogen kit, the active reagent was added by hand at 50µL aliquots and placed onto the plate reader for analysis.

To analyze the data acquired by the plate reader, it was important to first determine the number of cells that were present in each sample before lysis. This was conducted using the cell counter. The collected data from the glutathione assay was then analyzed using the cell density of each sample to determine the per cell amounts of glutathione under each treatment. This was conducted because cell density can vary between treatments which can lead to false readings.

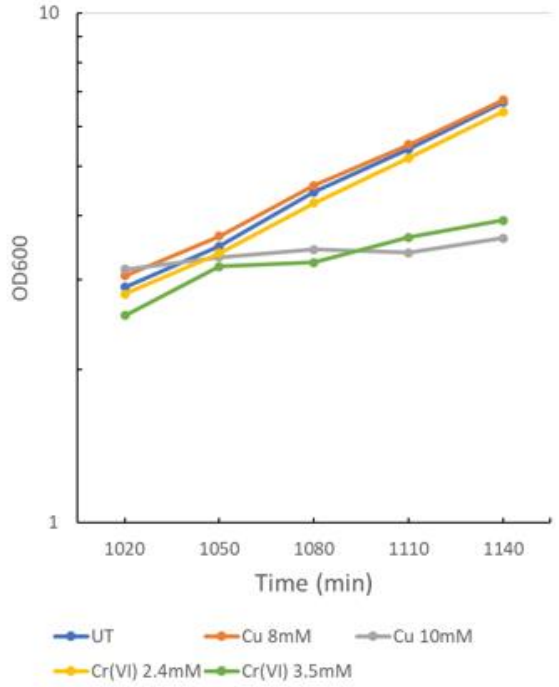
2.6 Transcriptional Expression Impact

Cells were grown and treated following the protocols listed above. In this experiment, cells were collected at varying time points within the one-hour treatment window to show the transcriptional impact of acute heavy metal assault (0 min, 5 min, 10 min, 15 min, 30 min, and 60 min). One 5mL aliquot was collected and washed for each time interval. The cells collected for RNA were frozen at -80°C until RNA extraction. The RNA was extracted using the protocol explained in the Invitrogen extraction kit (Thermo Fisher, Cat # 12183018A) and then cell purity and concentrations were analyzed. The RNA samples were then diluted and hybridized with the two custom tags supplied by Nanostring for 16 hours. Post hybridization, the samples were loaded onto the 16 well plate supplied by the company and ran on the Nanostring machine. The data was then collected and analyzed for transcriptional gene expression levels.

3 RESULTS

3.1 Optimal heavy metal treatment determination

The optimal concentration of cadmium that could be used to ascertain metabolic changes in yeast, while not affecting growth rate, has proven to lie within a range of 30µM – 80µM [*Nargund, 2008; Gardner 2019*]. It was considered prudent to confirm the equivalent concentrations of copper and chromium (VI) that would yield comparable levels of metal induced stress [*Shanmuganathan 2008; Nargund 2008*]. Treatments with 8mM copper and 2.4mM chromium (VI) resulted in similar doubling times of 91 and 92 minutes, respectively, while higher concentrations of each heavy metal lowered overall doubling times significantly. Treatments at 10mM copper and 3.5mM chromium (VI) resulted in lowered overall doubling times of 586 and 183 minutes, respectively (Fig. 7). The optimal concentration of subtoxic heavy metal treatment, therefore, was determined to be 8mM for copper and that of chromium (VI) to be 2.4mM, which are in excellent agreement to previously published concentrations [*Shanmuganathan 2008; Nargund 2008*].



Treatment:	Doubling Time (min):
UT	84.8
Cu 8mM	90.7
Cu 10mM	585.6
Cr(VI) 2.4mM	91.8
Cr(VI) 3.5mM	182.9

Figure 7. Growth curve analysis of heavy metal treatments in S. cerevisiae.
Yeast cell cultures were grown as normal in YEPD media until an OD_{600} value of 2.2. Cells were treated with 50µM PO_4 (UT), 8mM copper, 10mM copper, 2.4mM chromium (VI), and 3.5mM chromium (VI) and returned to incubate. OD_{600} values were collected every 30 minutes during exponential growth to determine doubling time. Higher concentrations of metal treatments inhibit cellular growth, so lower concentrations are used for further study.

3.2 Induction of apoptosis, as a result of acute heavy metal toxicity

It has previously been shown that yeast cells, when exposed even to subtoxic levels of cadmium, initiate a caspase-specific response to the presence of the heavy metal (*Nargund, 2008*). While little has been shown as to the apoptotic effects of similarly acute exposure to either copper or chromium in response, the ability for copper to initiate a caspase-specific response has been ruled out (*Liang and Zhou, 2007*). The response to ROS buildup within the cell has been shown to be metal specific in previous, chronic exposure studies. To demonstrate the disparate cellular response(s) of yeast to subtoxic levels of all four heavy metals, the levels of activated yeast caspase were monitored. Intriguingly, and in contrast to all published evidence,

each metal treatment appeared to give some discernable activation of the yeast caspase (Yca1), relative to background activity levels, indicating that each metal does elicit some level of caspase response, and potential apoptotic programmed cell death. Caspase activity was determined by analyzing the amount of FAM-FLICA stained cells that were excited by the FITC-A laser, as shown in Figure 8 below. Basal levels of apoptotic activity of *S. cerevisiae* were determined to be greater than 1.0% via the untreated culture (Fig. 8A). Acute treatment of the redox metals 8mM copper (Fig. 8B) and 2.4mM chromium (VI) (Fig. 8E) led to a significant increase in metacaspase activity of 9.0% and 10%, respectively. Acute treatment of the redox inactive metal, cadmium (Fig. 8C), led to smaller metacaspase activation compared to the redox active metals at 4.4%. Similarly, the less toxic metal, chromium (III), gave rise to a slight increase in metacaspase activity at 3.8% (Fig. 8D).

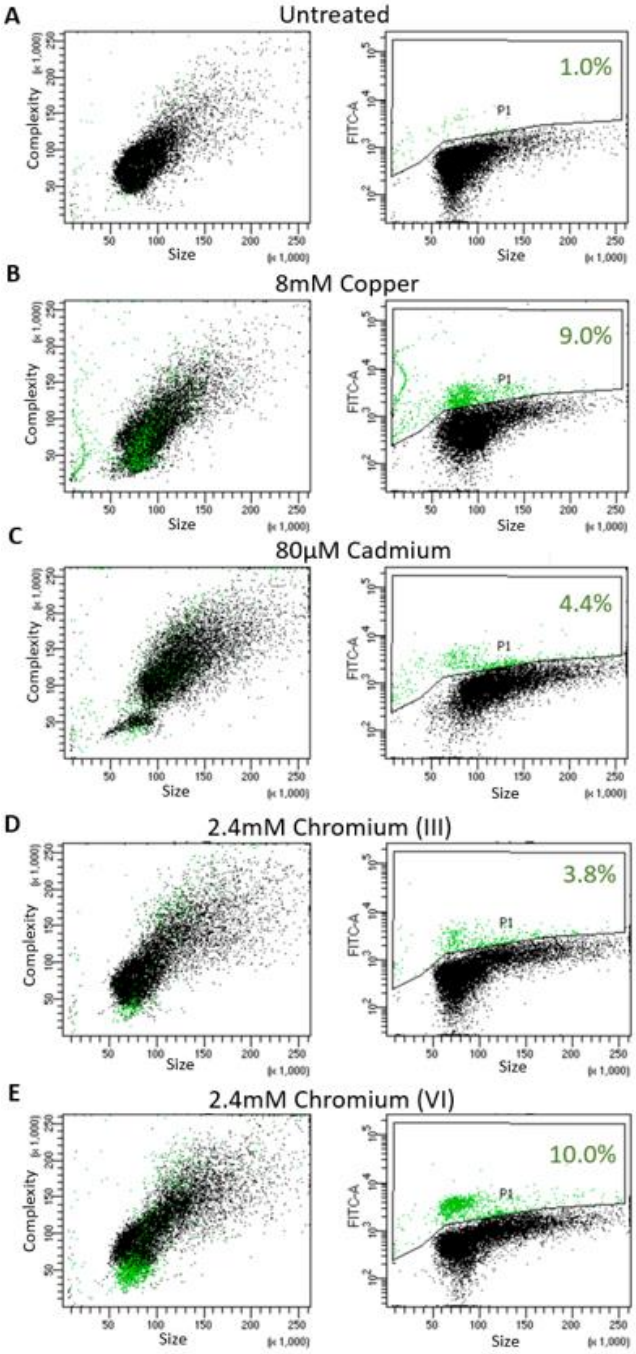


Figure 8. Basal metacaspase activity levels of S. cerevisiae under acute heavy metal exposure.

Data representing metacaspase activity of cells via FAM-FLICA dye treated with (A) 50µM PO_4 for control, (B) 8mM copper, (C) 80µM cadmium, (D) 2.4mM chromium(III), and (E) chromium(VI) for one hour. All cell cultures were analyzed by comparing complexity (side scatter) with cell size (forward scatter) as well as comparing FAM-FLICA activation (FITC-A) vs cell size (side scatter). Value of caspase activation is denoted by the % values for each treatment.

3.3 Glutathione biosynthesis and regulation is changed due to acute heavy metal toxicity

The effects of acute exposure to heavy metals cadmium, copper, chromium (III) and chromium (VI) were investigated to determine by analyzing changes in total glutathione levels as well as GSH and GSSG. As discussed previously, two types of heavy metals were used in for these analyses, copper, chromium (III), chromium (VI) (redox active), and cadmium (redox inactive). It was thought that these two types of heavy metals would have potentially different effects on glutathione and the associated GSH/GSSG balance as they would potentially elicit differing metabolic effects on the oxidation states of the different cells. Data obtained for glutathione levels (using the Invitrogen glutathione kit), after one hour of metal treatment are shown in Figure 9. Cell cultures treated with phosphate buffer for one hour provides a basal level of total glutathione, approximately 190nM per cell, as well as a basal concentration of oxidized GSSG (12nM per cell; Fig, 9). As a result, a basal ratio of GSH/GSSG were found to be 14, which denotes and defines the average cellular GSH/GSSG ratio of untreated cells grown in rich medium (see Materials and Methods Section) . Treatment of cells with any of the redox-active metals tested appeared to have no impact on the total levels of glutathione but did decrease the GSH/GSSG. This would reflect cells under oxidation stress, as indicated by a significant increase in the levels of GSSG, most clearly demonstrated by the cells exposed to copper (Fig 9). In the case of copper, the increase in GSSG within the cell population leads to a significant decrease in the GSH/GSSG ratio to 5. In marked contrast, treatment of cells with the redox-inactive metal, cadmium, leads to a significant increase in total glutathione levels, but maintains the GSH/GSSG at 14, indicating an absence of any oxidative stress in these cells (Fig. 9). Further data on glutathione levels (using the Enzo Life Sciences glutathione kit) was obtained previously for chromium (III) due to the kit being discontinued. These data were obtained and

normalized against the values of the new kit for comparison (Fig. 9). Treatment of chromium (III) did not lead to an increase in overall glutathione, nor did it cause a significant change to the GSH/GSSG as shown with the redox-active metals, indicating no oxidative stress to the cell under exposure.

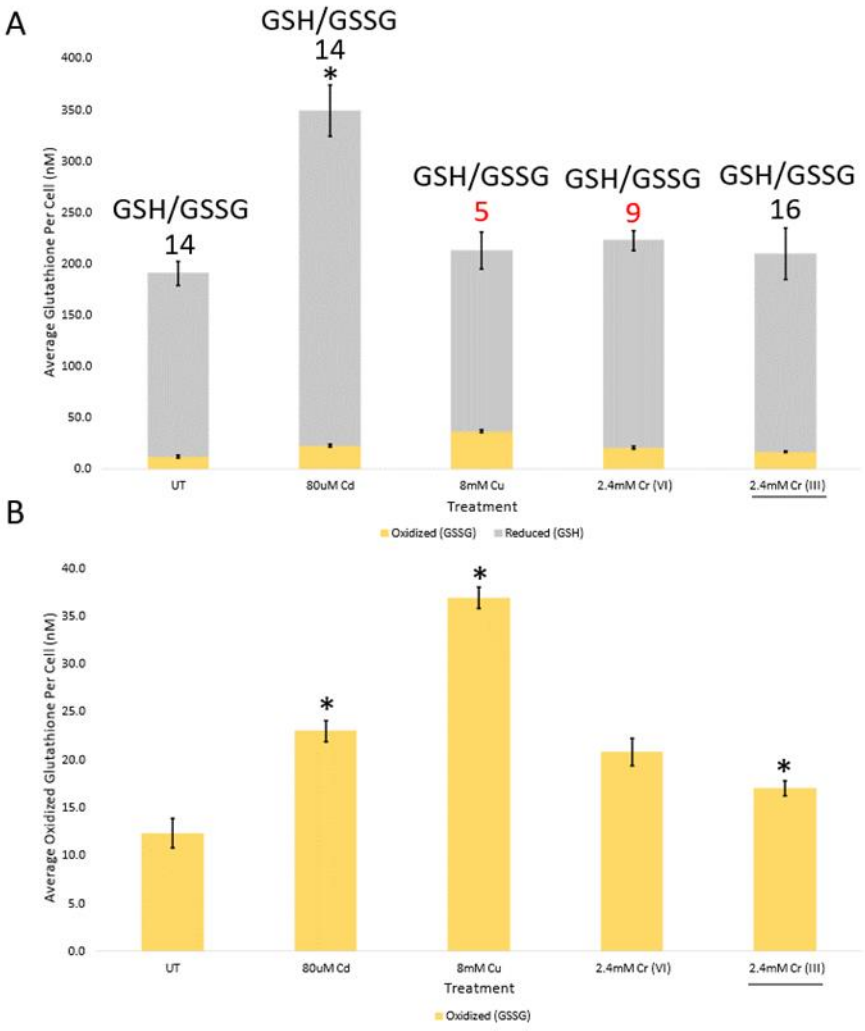


Figure 9. Treatment of heavy metals impacts glutathione levels within the cell. *Glutathione levels within the cell remain at a basal level when not under any oxidative stress. Treating the cells with 8mM copper or 2.4mM chromium (VI) shifts the ratio of GSH/GSSG towards the oxidized form of glutathione, indicating high oxidative stress within the cells. Treating the cells with 80µM cadmium does not have an impact on the oxidative stress of the cell (indicated by a basal level of GSH/GSSG) however the metal does increase the total volume of glutathione within the cell. 2.4mM chromium (III) treatment did not cause a change in either total levels or GSH/GSSG ratios. Significant change from untreated sample group denoted by *($p<0.05$). Samples analyzed with newly discontinued Enzo Life assay and normalized to UT values are denoted with a gray underline.*

3.4 Effect of FFAAP supplementation on apoptotic induction of heavy metal assault

FFAAP is a dietary supplement that has been developed to supply the necessary amino acids that make up glutathione, in an attempt to exogenously boost glutathione pools. The supplement is designed to be able to assist the cells in combatting oxidative stress and any concomitant buildup of ROS within the cell. Given that the supplement is made up of the essential precursors of glutathione, its direct role in glutathione homeostasis as well as any residual, potentially latent effect on the apoptotic response of cells to the presence of each metal was investigated. Preliminary experiments were performed to determine an effective concentration of FFAAP at which concentration the mixture remained in solution (*Gardner, 2019*). This concentration of 2mM FFAAP supplement was therefore used to demonstrate the effect of excess glutathione constituents on the cells capacity to synthesize glutathione and potentially offset some of the more detrimental oxidative stress resulting from metal exposure. Somewhat surprisingly, the presence of FFAAP in the growth media appeared to have little, if any affect up on yeast's apoptotic response to the presence of any of the heavy metals (Fig. 10).

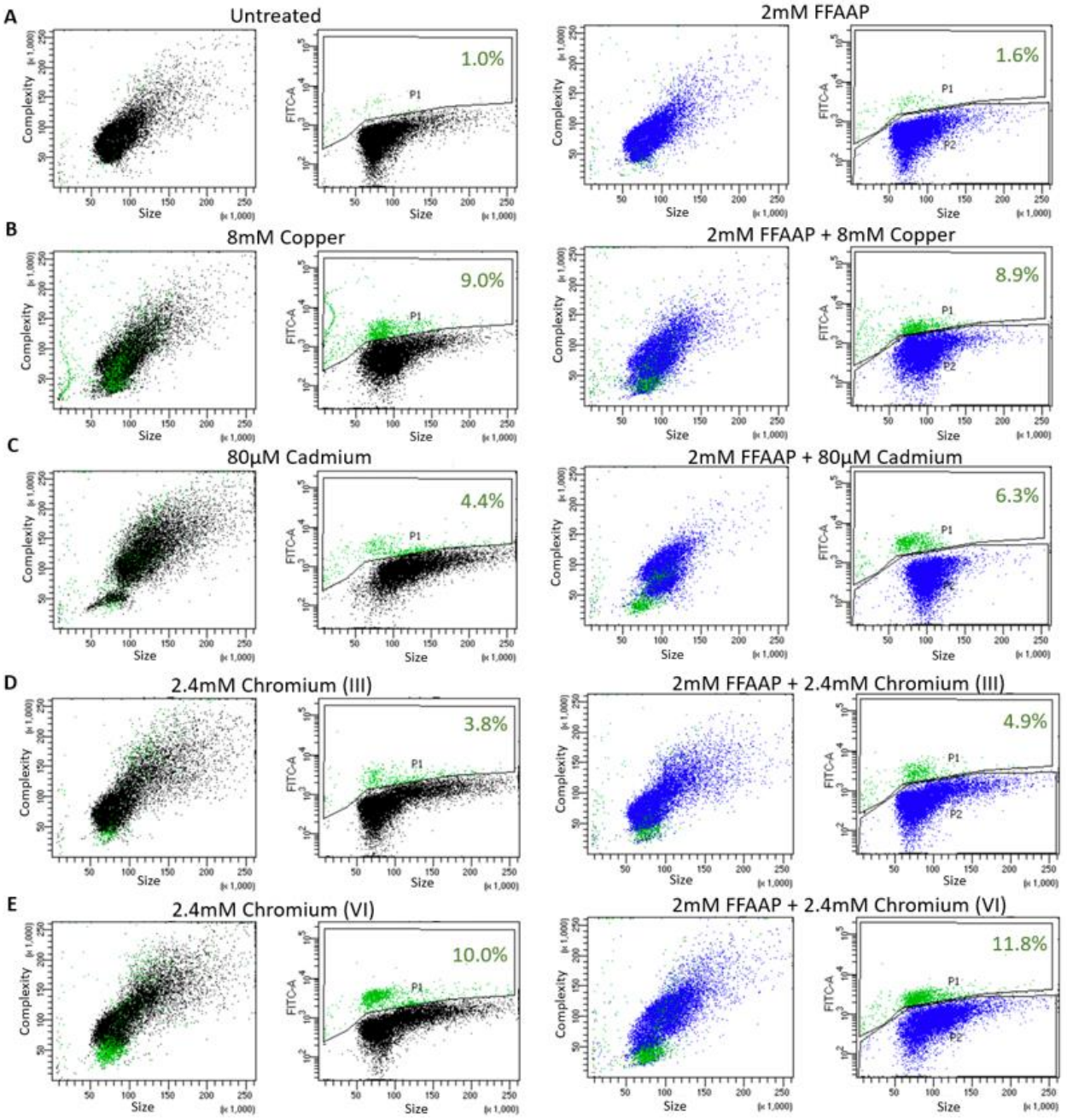


Figure 10. The addition of FFAAP treatment does not impact apoptosis levels.
Data representing the comparison of metacaspase activity of 2mM FFAAP supplemented and non-supplemented cell cultures via FAM-FLICA dye treated with (A) 50µM PO_4 for control, (B) 8mM copper, (C) 80µM cadmium, (D) 2.4mM chromium(III), and (E) chromium(VI) for one hour. All cell cultures were analyzed by comparing complexity (side scatter) with cell size (forward scatter) as well as comparing FAM-FLICA activation (FITC-A) vs cell size (side scatter). Value of caspase activation is denoted by the % values for each treatment.

3.5 Supplementation of FFAAP on glutathione biosynthesis in *S. cerevisiae*

In the absence of any effect on any programmed cell death, verification that the supplement could get into the cells and affect the glutathione pools was conducted via additional glutathione studies on cells grown in media supplemented with 2mM FFAAP prior to heavy metal treatment, as described in the Materials and Methods section. In the absence of any metal treatment, the presence of the FFAAP failed to alter the total amount of glutathione in the cell, but did reduce the cellular conditions, as determined by an increase in the GSH/GSSG ratio from 15 to 23 (Fig. 11). The most significant influence of FFAAP on glutathione pools, however, was demonstrated in cells that were subsequently exposed to heavy metals. Regardless of the nature of the heavy metal, the presence of FFAAP appeared to reduce the total presence of glutathione in the cell by 20 - 25 %, while at the same time lowering any potential oxidative stress in the cell to approximate normal levels, as demonstrated by an increase in the GSH/GSSG ratios relative to cells in the presence of redox-active metals. These findings were not consistent with the addition of chromium (III), however, as the supplementation of FFAAP did not lower the overall levels of glutathione but did result in an increase of GSH/GSSG from 16 to 18 (Fig. 11). As stated above, these values were obtained using the discontinued assay and were normalized to the newer Invitrogen results for analysis.

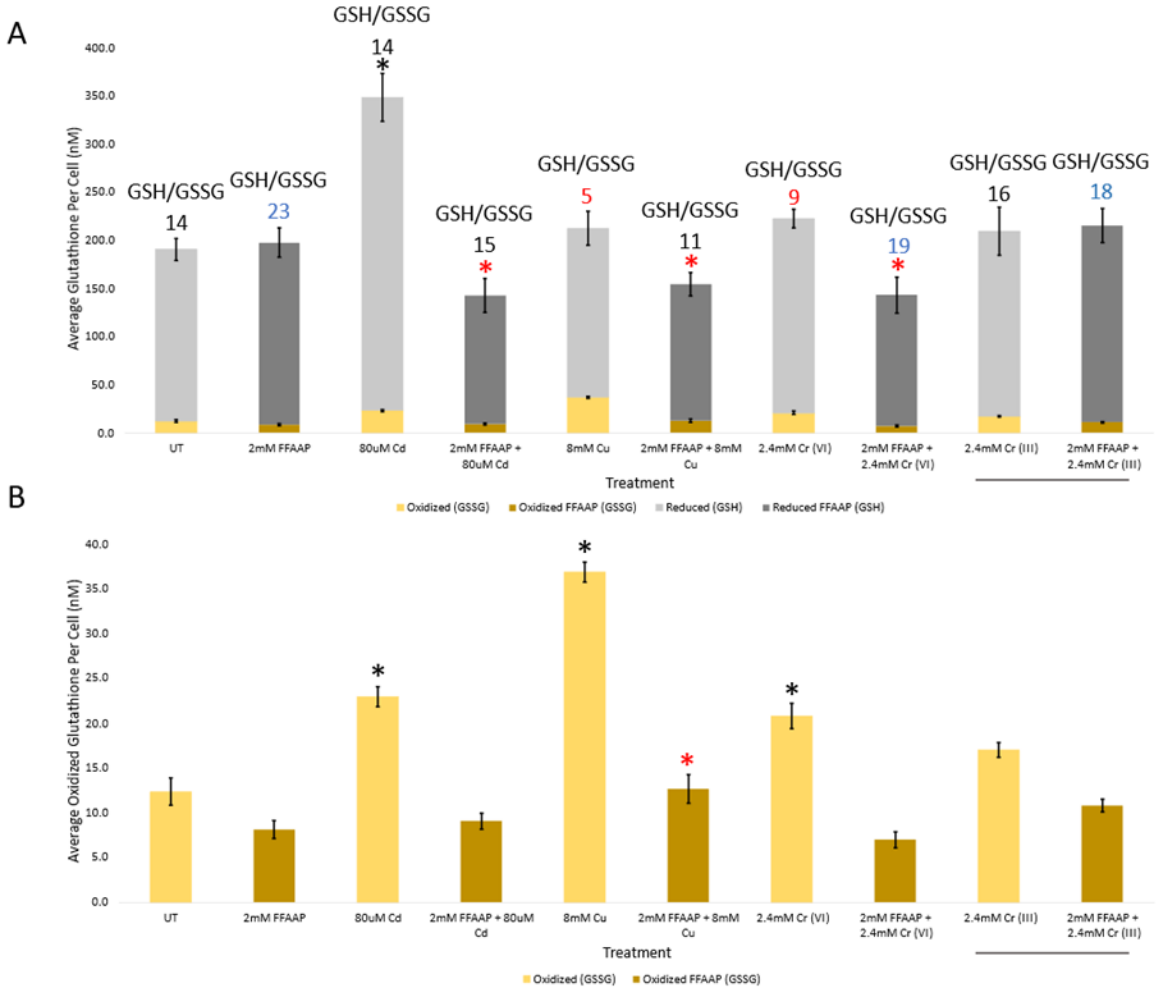


Figure 11. Supplementation of FFAAP leads to variation in glutathione biosynthesis and regulation.

*Glutathione levels within the cell remain at a basal level when not under any oxidative stress. Supplementation of 2mM FFAAP during initial growth leads to variation in glutathione biosynthesis and regulation. Treating the cells with 8mM copper or 2.4mM chromium (VI) shifts the ratio of GSH/GSSG towards the oxidized form of glutathione, indicating high oxidative stress within the cells, however FFAAP has a recuperative effect by increasing these ratios. Treating the cells with 80µM cadmium does not have an impact on the oxidative stress of the cell (indicated by a basal level of GSH/GSSG) however the metal does increase the total volume of glutathione within the cell. Supplementation of FFAAP to the cadmium treated culture leads to an overall decrease in total glutathione levels while not having an impact on the GSH/GSSG ratios. Supplementation of FFAAP to chromium (III) did not reduce the overall levels of glutathione however it did slightly increase the GSH/GSSG ratios. Significant change from untreated sample group denoted by *($p<0.05$). Significant change from untreated sample group supplemented with FFAAP denoted by *($p<0.05$). Samples analyzed with newly discontinued Enzo Life assay and normalized to UT values are denoted with a gray underline.*

3.6 Acute heavy metal treatment leads to temporal gene expression variations in *S. cerevisiae*

In addition to overt apoptotic effects and changes in glutathione activity, *S. cerevisiae* cultures were treated with cadmium, copper, and chromium (VI) for one hour to determine the impact on transcriptional regulation of a select 72 genes on a customized panel of targeted fluorescent probes. As specified in the Materials and Methods section, transcriptional expression of these genes was analyzed at 5 min, 10 min, 15 min, 30 min, and 60 min time points post initial exposure to each metal, to temporally profile expression of these targeted genes. Purified RNA extracted from cells at these times were analyzed using Nanostring technology to determine the mRNA transcriptional profile of these targeted genes. The genes analyzed were broken down into subgroups and their relative expressions analyzed in more detail. Each of the cells that had undergone various metal treatments were found to have different expression profiles that indicate differing metal-specific genetic responses to exposure of each metal, which were grouped according to their generalized function.

The first set of genes to be analyzed were ones involved in the biosynthesis and regulation of glutathione within the cell (Fig. 12). The two genes involved in glutathione biosynthesis, GSH1 (gamma glutamylcysteine synthetase) and GSH2 (glutathione synthetase), showed a 6 to 8-fold upregulation within the first hour of treatment under cadmium assault, while GSH1 only was upregulated under chromium (VI) treatment which showed a decremental trend following an initial burst of activity after 5’ of exposure. Conversely, copper exposure led to a significant downregulation of both GSH1 and GSH2 expression profiles, mostly within the first 30 minutes of exposure before trending back to normal levels by 60 minutes. Expression of GLR1 (glutathione oxidoreductase) followed the same trend as GSH1, with upregulation in

cadmium and chromium (VI) exposed cells and downregulation from copper. Regulation of GRX2 (glutathione-dependent disulfide oxidoreductase) was significantly upregulated under all three metal treatments within the hour, with a higher response due to cadmium treatment. Finally, regulation of GRX5 (glutathione-dependent oxidoreductase) was downregulated due to acute copper exposure and was not affected by treatment with cadmium or chromium (VI).

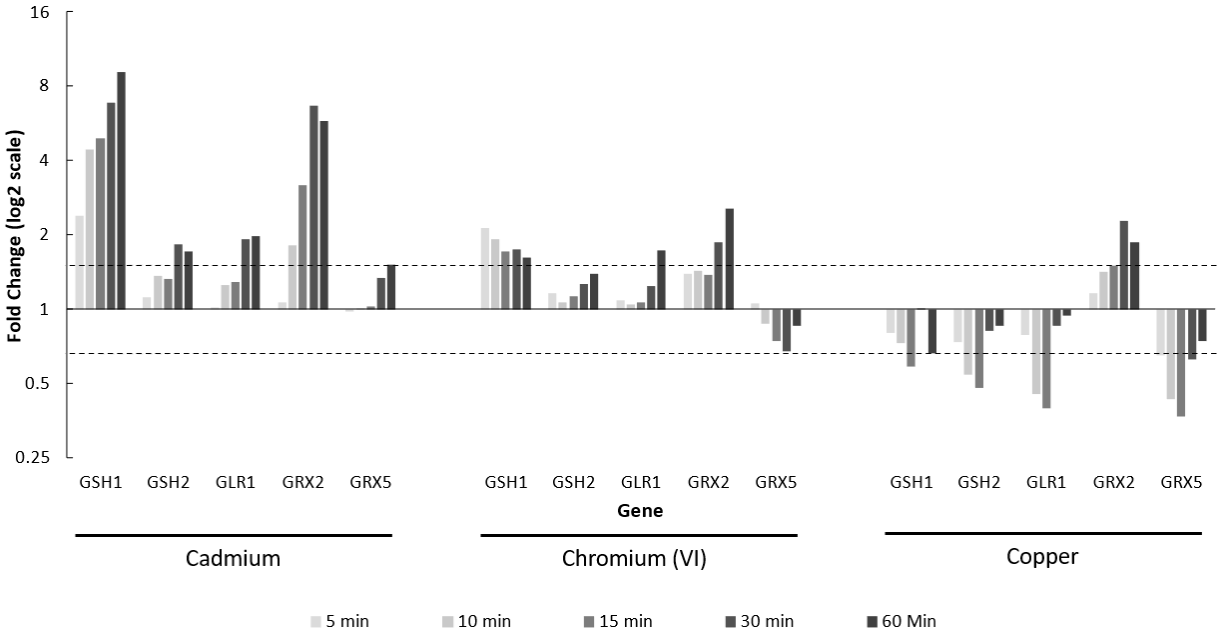


Figure 12. Genes involved in glutathione synthesis.
A temporal profile of glutathione related genes in yeast during one hour treatment with 80µM cadmium, 8mM copper, and 2.4mM chromium (VI) respectively. Increasing density of the bars references increasing time for after initial metal. Each bar indicates the average fold change calculated from expression rates as normalized to untreated expression rates in a log_2 scale. Dotted lines denote 1.5-fold increase or decrease in expression relative to untreated cells.

The second set of gene expression profiles of a select series of genes, Cys3 and Cys4 involved in biosynthesis of cysteine within the cell, were analyzed. Under cadmium exposure, both CYS3 (cystathionine gamma-lyase) and CYS4 (cystathionine beta-synthase) are significantly upregulated within the first hour of treatment (Fig. 13). Conversely, treatment of cells with copper for one hour leads to a downregulation of both genes, primarily in the first 30 minutes of metal exposure. Treatment with chromium (VI) does give rise to a significant (>1.5 fold) increase in expression of both genes, with a discernable delay in onset of gene expression.

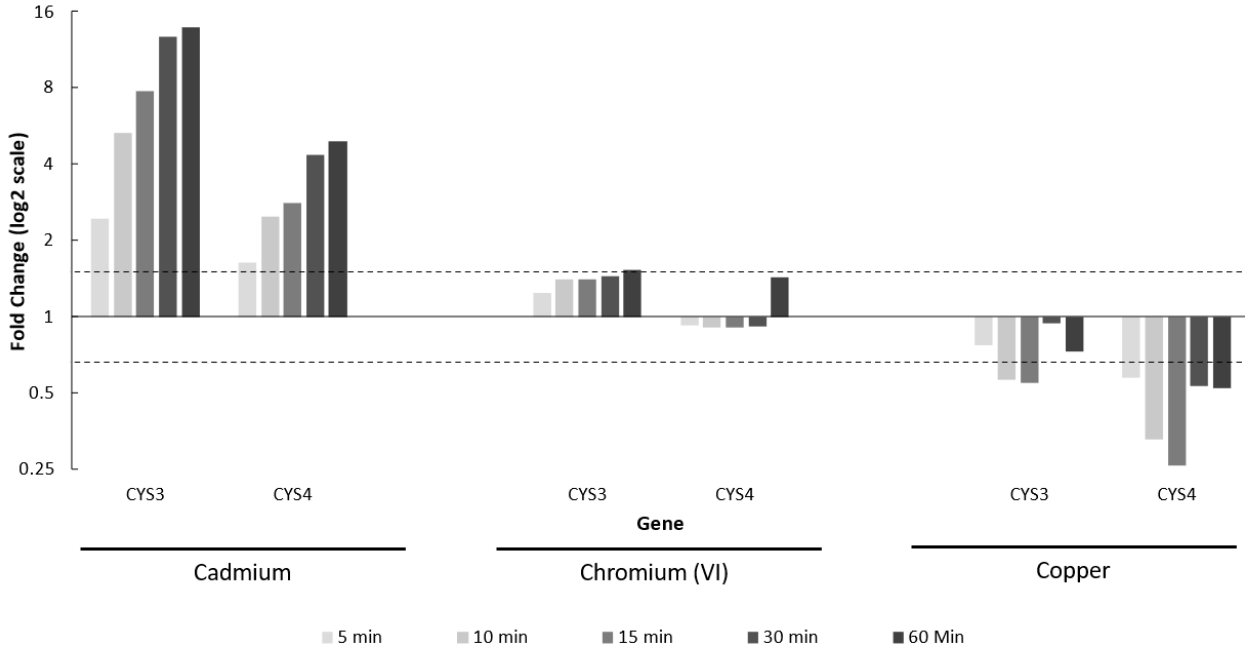


Figure 13. Genes involved in cysteine biosynthesis.
A temporal profile of cysteine biosynthetic related genes Cys3 and Cys4 in yeast during one hour treatment with 80µM cadmium, 8mM copper, and 2.4mM chromium (VI) respectively. Increasing density of the bars references increasing time for after initial metal. Each bar indicates the average fold change calculated from expression rates as normalized to untreated expression rates in a log_2 scale. Dotted lines denote 1.5-fold increase or decrease in expression relative to untreated cells.

The genes involved in the HSP70 family of protein misfolding and translocation were also profiled (Fig. 14). Three of the four HSP70 related genes SSA1 (ATPase/ NLS-directed nuclear transport), SSC1 (Hsp70 family ATPase), and SSE1 (ATPase component of heat shock protein Hsp90 chaperone complex) were all dramatically upregulated in cells exposed to cadmium, significantly downregulated (>1.5 fold) in cells exposed to copper and were relatively unaffected by the presence of chromium (VI). SSA4 (HSP70 protein -induced via cellular stress) showed significant increase in response to the presence of all metals and was dramatically increased (128-fold) in the presence of cadmium.

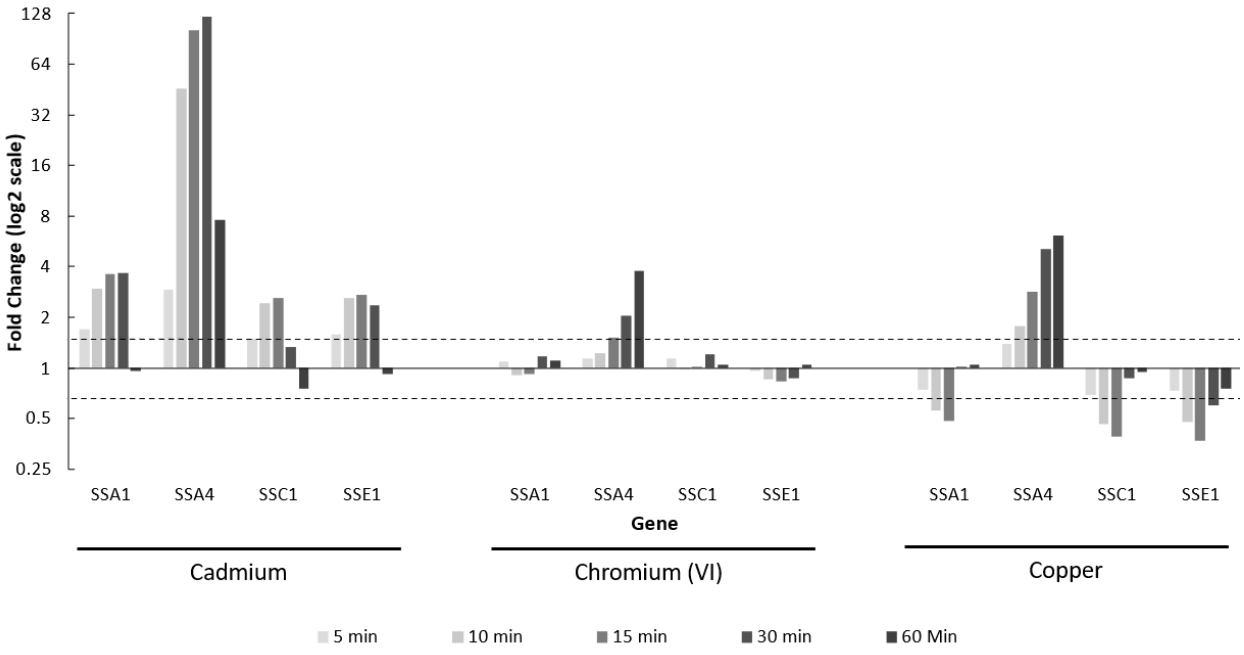


Figure 14. Genes involved in protein misfolding and translocation.
A temporal profile of chaperone and protein folding genes related genes in yeast during one hour treatment with 80µM cadmium, 8mM copper, and 2.4mM chromium (VI) respectively. Increasing density of the bars references increasing time for after initial metal. Each bar indicates the average fold change calculated from expression rates as normalized to untreated expression rates in a log_2 scale. Dotted lines denote 1.5-fold increase or decrease in expression relative to untreated cells.

An additional subset of genes involved in cellular stress responses were also analyzed. As shown with most of the other mRNA transcriptional profiles, each of the stress response genes analyzed were significantly upregulated under cadmium treatment within the first hour. These genes include AHP1 (thiol-specific peroxiredoxin), HSP30 (negative regulation of ATPase), MCA1 (Ca^{2+} -dependent cysteine protease; Yeast metacaspase), and MXR1 (methionine-S-sulfoxide reductase) (Fig. 15). Under acute chromium (VI) exposure, AHP1, MCA1, and MXR1 demonstrated significant inducible levels, with the marked exception of HSP30. Copper had a mixed effect on all genes, with a significant diminution in MCA1 (yeast metacaspase) and MXR1 expression in the first 30 minutes, before normalizing expression levels by the end of the 60 time. Expression of AHP1 initially was decreased and recovered to a slight significant increase in the second half hour of treatment while HSP30 did the inverse, increasing initially and then decreasing significantly. The other two stress response genes analyzed, MCA1 and MXR1, both significantly decreased within the hour of acute copper treatment.

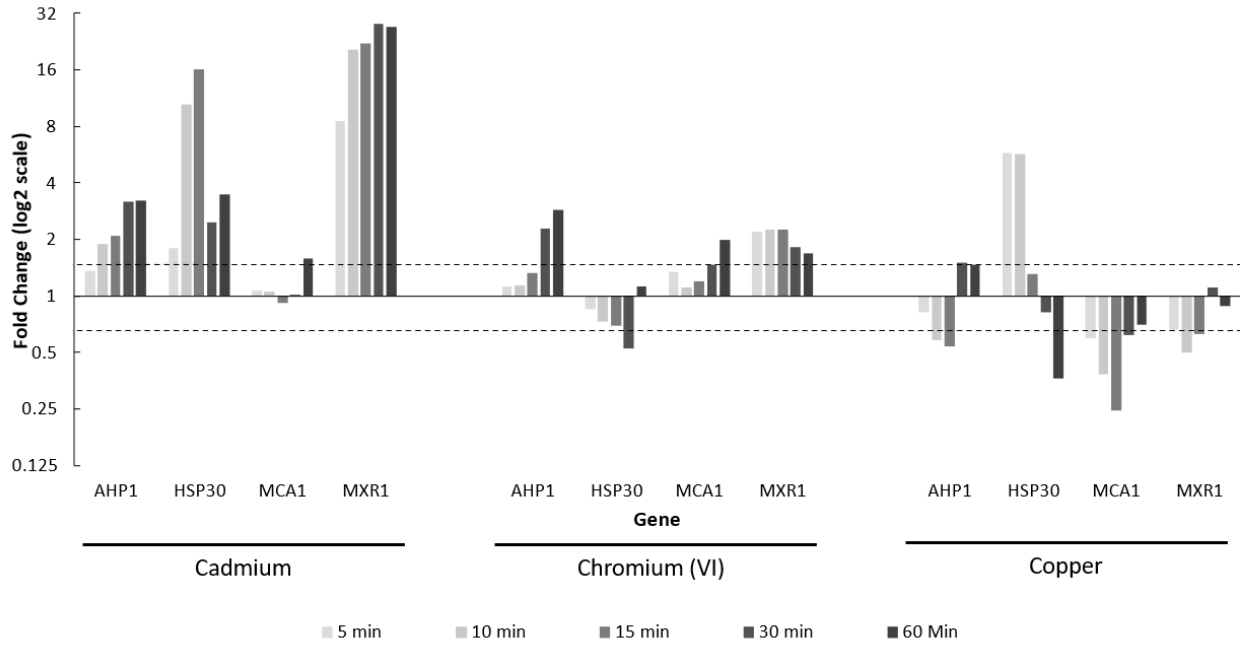


Figure 15. Stress response gene transcripts.
A temporal profile of "stress response" genes related genes in yeast during one hour treatment with 80µM cadmium, 8mM copper, and 2.4mM chromium (VI) respectively. Increasing density of the bars references increasing time for after initial metal. Each bar indicates the average fold change calculated from expression rates as normalized to untreated expression rates in a log_2 scale. Dotted lines denote 1.5-fold increase or decrease in expression relative to untreated cells.

The final gene set that was analyzed was a miscellany of genes that would potentially have responded to cellular exposure to one or the other heavy metals, but which do not fit into any generic category (Fig. 16). The first gene, ATG6 (subunit of phosphatidylinositol 3-kinase complexes I and II), is involved in vacuolization and protein folding due to autophagy and curiously does not respond to any of the metals, except perhaps a rapid diminution in the presence of copper before it reverts to normal expression numbers. COX2 (subunit II of cytochrome c oxidase) acts similarly to the HSP70 family of genes, and while it is induced by cadmium, like most genes that were analyzed it shows a significant decrease in expression in the presence of copper before normalizing by the end of the hour-long exposure. The copper binding protein CUP1 (Metallothionein) not too surprisingly shows a dramatic increase in

expression in the presence of copper, but is also significantly increased, albeit not as much in response, to cadmium. RRN5 (regulator of RNA polymerase I) is slightly upregulated by cadmium exposure while its expression is suppressed in both chromium (VI) and copper exposure.

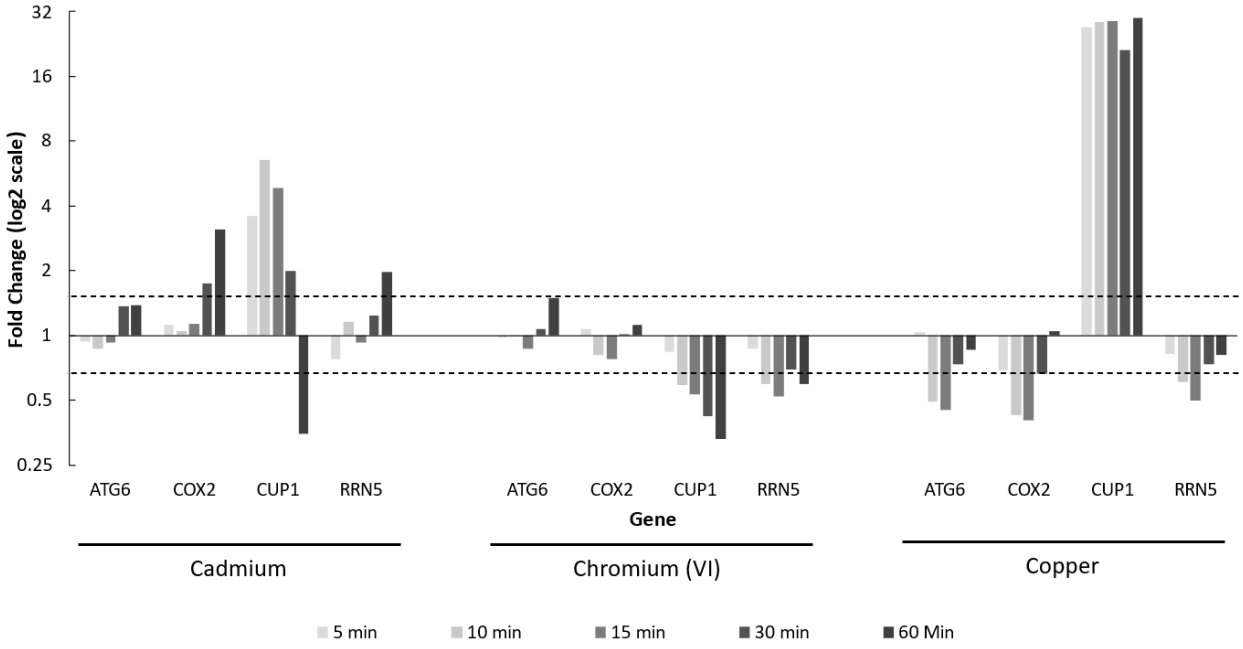


Figure 16. Miscellaneous gene transcripts.

A temporal profile of miscellaneous genes related to specific activities in yeast cells that might be affected by one hour treatment of cells with 80µM cadmium, 8mM copper, and 2.4mM chromium (VI) respectively. Increasing density of the bars references increasing time for after initial metal. Each bar indicates the average fold change calculated from expression rates as normalized to untreated expression rates in a log_2 scale. Dotted lines denote 1.5-fold increase or decrease in expression relative to untreated cells.

4 Discussion

4.1 Concentrations of heavy metals

Studying the impact of heavy metal toxicity in eukaryotes has become increasingly important due to increased sources of contact in the environment. Understanding the impact that these heavy metals have on the cellular processes in various model organisms helps for a better appreciation of their effects upon the cells and a better understanding of their toxicity overall. In this study, the use of *S. cerevisiae* as a model organism allows for a general, basic understanding of the potentially different mechanisms by which such a model eukaryotic organism may be impacted due to this metal assault. At this point, it is important to distinguish between acute and chronic effects of metal assault cellular metabolism, as in these studies, yeast cultures were exposed to the heavy metals over a relatively short period of time (1 hour) which aimed to assess the more acute levels of heavy metal poisoning. Previous research on heavy metal toxicity by Gardner and Shanmuganathan have indicated that there are "subtoxic" levels of treatment for each metal. This subtoxic level of acute treatment has been determined to be the maximum concentration of heavy metal that does not influence growth rate while simultaneously leading to a subpopulation of apoptotic cells.

Previous studies in our laboratory have established the optimal range of metal concentrations for acute cadmium treatment to be 30μM - 80μM for the one-hour treatment as being "subtoxic", but not necessarily "sublethal" -as a result of apoptotic attrition within a sub population of the growing cells over subsequent generations [*Shanmuganathan, 2008; Nargund, 2008*], and more recently, (*Gardner, 2019*). Similar growth curves had been conducted to determine the effects of concentrations of copper and chromium (IV) exposure to yield similar acute responses in yeast [*Shanmuganathan, 2008; Nargund, 2008*]. These optimal

concentrations were confirmed in this work through similar growth curve analyses, of various *S. cerevisiae* cell cultures treated with different concentrations of copper and chromium (IV), which showed no changes in the effective concentrations of heavy metals that were used (Fig. 7). The optimal, experimental subtoxic concentration of copper was determined to be 8mM while the optimal level of chromium (VI) was determined to be 2.4mM. These concentrations of each metal are shown to have equivalent doubling times in relation to untreated cell cultures approximating 90 minutes, which has been determined to be the average growth rate of *S. cerevisiae* as demonstrated by previous studies in this lab [*Gardner, 2019; Shanmuganathan, 2008; Nargund, 2008*]. Higher concentrations led to a significant increase in doubling time, reflecting a more direct, toxicity. It is important for this study that these levels be currently established so that the consequences of acute treatment of heavy metal exposure can be referenced.

4.2 Apoptotic impact of acute heavy metal assault

Understanding how yeast cells react to acute exposure to each of the heavy metals being tested is important as it leads to a better overall understanding of how higher eukaryotes might react to similar environmental insults. The subtoxic amounts of exposure for each heavy metal treatment has been determined, as stated, by observing the growth rate of the cell cultures, but also acknowledging the demonstrable impact of an apoptotic response of a sub population of cells to the presence of each heavy metal. In this experiment, *S. cerevisiae* cell cultures were introduced to acute treatments (1 hour exposure) of 80µM cadmium, 8mM copper, 2.4mM chromium (III), and 2.4mM chromium (VI) before being allowed to grow in the absence of metal for 3 hours to allow for metacaspase activity and ensuing cellular responses to present

themselves. The addition of heavy metal assault to each of the cell populations caused a significant increase in apoptosis in all treated cells (Fig. 8). This is determined by observing the number of cells that are shown by excitation of the FAM-FLICA dye at 488 nm (referenced as FITC-A on the cytometric tracings), which exposes the presence of activated caspase within the cell (*Nargund, 2014*). Of note, the type of heavy metal introduced to each cell culture had a varying effect on the levels of caspase activation.

Redox-active heavy metals, such as chromium (VI) and copper, consistently led to a significant and relatively pronounced increase in overall apoptosis within the cells approximating to 10% and 9% respectively. This indicates that the generation of ROS within the cell due to either the cytoplasmic breakdown of chromium (VI) to chromium (III) or the Fenton-like reactions of copper. These inherent redox potential of each metal leads to an increase in ROS, which in turn leads to a rapid and substantial response in a subpopulation of cells, triggering cell death (Fig. 8B/E). As expected, activation of caspase is more pronounced in these subpopulations of cells, consistent with the onset of apoptotic related cell vacuolization and cell shrinkage (Fig. 8B/E and *Falcone and Mazzoni, 2016*).

The redox-inactive metal, cadmium, led to an apoptotic response that was significantly lower when compared to the redox-active metals. Acute cadmium assault led to a 4.3% apoptotic population that is loosely spread among the overall population of cells (Fig. 8C). Previous research on intercellular ROS production by Nargund et al. and later confirmed by Gardner has shown that, in marked contrast to redox reactive metals such as copper and chromium (VI), exposure to cadmium fails to elicit any increase in superoxide ROS within the first hour or so of metal treatment [Nargund, 2008; *Gardner, 2019*]. This would be entirely consistent with the finding of Nargund et al. who also demonstrated that activation of yeast

caspase by the redox-inactive metal, cadmium, preceded any mitochondrial membrane perturbation and release/production of ROS [*Nargund, 2008; Nargund, 2014*]. Unfortunately, I was unable to confirm these findings for chromium (III) or chromium (VI), as the necessary technology to undertake such analysis was unavailable for the last year or more. Even so, absent such confirmation, the differing inducible levels of activated caspase by redox-inactive (cadmium) and redox-active (copper and chromium) metal exposure would indicate potentially significant differences in the ability of each metal-type to influence the onset of apoptosis. Chromium (III) has been shown to have a direct interaction with DNA causing nucleic acid damage once it is present in the cell (*Beyersmann, 2008*). This would be theorized to cause significant damage to the cell leading to ROS generation and apoptosis. However, it has been shown that the chromium (III) ion is not membrane permeable. Previous studies have shown that the chromium (III) metal ion is unable to cross the cellular membrane in other organisms and, moreover, can only cause DNA damage when bound to other complexes [*Warren et al., 1981; Stearns et al., 2002*]. The inability for chromium (III) to cross cell membranes could go a long way to explain why treatment of yeast with this form of chromium fails to induce higher levels of an apoptotic response. It would further demonstrate that there is a clear mechanistic difference as to how redox-active and redox-inactive heavy metals can potentiate activation of yeast caspase, there are also several different metal effects in cells that might modify the cellular response to each of these metals.

4.3 Impact of glutathione biosynthesis and regulation due to acute heavy metal treatment

Regulation of antioxidant defense is important to ensure an organism's survival when exposed to environmental factors such as heavy metal toxicity. One of the major, influential

antioxidant responses of the cell to combat increased ROS is glutathione, which can be recycled between oxidized (GSSG) and reduced (GSH) forms. For the formation of each oxidized glutathione two reduced glutathione molecules donate their free electrons to reduce and thereby provide sufficient reducing power to neutralize ROS molecules, among other functions. Oxidized glutathione (GSSG) is then recycled, using $NADPH_2$ as an electron donor, to produce reduced glutathione (GSH) that can then be used, potentially, to bind to more ROS molecules. An understanding as to how this powerful resource of reducing power is maintained and employed in cells undergoing metal-induced oxidative stress is important for any appreciation of cellular response to the presence of heavy metals and elevated levels of ROS in the cell. In the cycling process, GSH molecules are needed to donate their free electron to minimize the effect of ROS accumulation in the cell which can be directly produced via the electron transport chain of the mitochondria (*Branca, et al., 2020*). This is eventually rectified by glutathione reductase converting the GSSG molecules that accumulate back to two GSH molecules. This recycling pathway, along with a significant feedback inhibition of glutathione biosynthesis itself, keeps a tight regulation on the overall amounts of glutathione present within the cell at any time.

S. cerevisiae cells, when exposed to acute heavy metal assault, leads to significant impact on the overall glutathione antioxidant response after one hour of treatment (Fig. 9). Acute assault with copper or chromium (VI) does not lead to a significant change in the total levels of glutathione, however each metal does play a role on the ratios of GSH/GSSG within the cell. The lack of a significant increase in the total levels of glutathione in the cell is critical as these redox-active metals are not directly bound to glutathione. This indicates that the stress induced by these metals is more closely linked to the ROS production in the cell rather than direct metal interactions. Previous studies have been conducted in the lab which have shown that this ratio is

critically important to linking oxidative stress to the levels of glutathione present in the cell (*Gardner, 2019*). In this case, the basal level of GSH/GSSG ratio has been determined as 14, which demonstrates the amount of oxidative stress that the cell is under without any extracellular treatment. It was determined that any change greater than 25% was a significant impact on the stress levels of the cell. This more in depth look at glutathione regulation shows that both 8mM copper and 2.4mM chromium (VI) treatments caused a significant shift from GSH to GSSG within the cell, with ratios of 5 and 9 respectively (Fig. 9). This shift in ratios is caused by the significant increase in ROS production within the hour of treatment of these redox-active heavy metals. Previous data conducted in the lab has also demonstrated that copper assault causes immediate ROS production, however data for chromium (VI) was not obtained due to issues with the flow cytometry machinery (*Gardner, 2019*).

The next metal that was tested was chromium (III), however this study was conducted using an older, now discontinued Enzo Life Sciences kit, whose availability precluded the completion of all glutathione related analyses to be undertaken with the same kit. While each glutathione assay had individual enzymes and substances, the overarching trends in glutathione levels remain the same between the two kits once normalized. As such, the acute exposure of cells to 2.4mM chromium (III) presented little effect to the overall levels of glutathione, and again failed to induce any significant changes to the GSH/GSSG ratios (Fig. 9). Chromium (III) seems to be acting more similarly to the redox-inactive metal cadmium rather than acting similarly to the chromium (VI) form. This is important to note as further studies must be conducted to understand the oxidative stress that may be caused due to acute chromium (III) exposure. This demonstrates that the oxidative state of chromium is important for the overall

toxicity that the metal causes and has significant implications as to the mechanism by which even the same metal can initiate apoptotic programmed cell death.

Treatment with 80μM cadmium leads to a significant 83% increase in total glutathione within the cell after one hour of treatment ($p<0.05$). This remarkable response is thus far unique to cadmium exposure, as similar, subtoxic treatments with either copper, chromium (III) or chromium (VI) failed to give rise to such a marked increase in the total levels of glutathione. Cadmium subtoxic exposure leads to a novel increase in total glutathione levels compared to all other metals assayed, which could be due to its unique ability to be bound by glutathione in the cell and exported via vesicles (*Li et al., 1997*). Two molecules of glutathione bind to the cadmium in the cell forming a complex (Gs-Cd-Gs) which can be exported. This sequestration of metal causes excess GSH molecules to be used up, which in turn are unable to be present to negatively inhibit the gamma-ECS protein of glutathione biosynthesis. Therefore, the increase in overall glutathione is most likely due to the cell being able to manufacture more antioxidant in response to it being used to export the toxic metal. Cadmium treatment, when compared to the other redox-active metals, does not cause an impact with a GSH/GSSG ratio of 14 (Fig. 9). This further demonstrates that the glutathione is being utilized as a mechanism for removing the metal as a whole rather than any ROS within the cell. This is further demonstrated in other organisms in which exposure to cadmium leads to eventual depletion of GSH within the cell and causing ROS buildup as a result over time (*Ross et al., 2019*). This is potentially also the case in *S. cerevisiae* cells, showing that cadmium has a further metal-specific effect on the metabolism of the cell preceding any ROS effect that might happen at a chronic level.

4.4 The effect of FFAAP supplementation on metacaspase activation

FFAAP supplementation has been hypothesized to help diminish the apoptotic response of heavy metal assault due to potentially being able to induce higher levels of glutathione within the cell. This result was not found to be true, as the relative apoptotic levels of each metal treatment remained similar to those without supplementation (Fig. 10). Copper exposure led to a similar value of 8.9% apoptotic activity while chromium (VI) treatments resulted in an increase in metacaspase activity relative to non-supplemented cultures of 11.8% (Figs. 10B/E). The trend of slightly increased caspase activity due to FFAAP supplementation was further demonstrated under cadmium and chromium (III) treatments of 6.3% and 4.9% respectively (Figs. 10C/D). These data show that FFAAP supplementation was indeed not able to rescue metacaspase activity within *S. cerevisiae* cultures treated with acute heavy metal assault.

4.5 The effect of FFAAP supplementation on glutathione biosynthesis and regulation

FFAAP supplementation was also studied in relation of glutathione biosynthesis and regulation. FFAAP contains the building blocks of amino acids required for glutathione production, so it was hypothesized that supplementation would lead to a change in glutathione activity along with apoptotic stress within the cell. Under no metal assault, FFAAP supplementation did not have a significant effect on the overall levels of glutathione within the cell compared to previous untreated samples (Fig. 11). Under all subtoxic heavy metal treatments, however, supplementation of FFAAP led to a significant decrease in total glutathione levels relative to the untreated supplemented treatment (as denoted by red asterisks) (Fig. 11). Cadmium treated cells followed this same trend, even though under non supplemented conditions the total value of glutathione was significantly increased (Fig. 11). Chromium (III)

was the outlier in this group as FFAAP supplementation did not decrease the overall levels of glutathione within the cell post exposure, which could be caused by the metal not easily being taken up by the cell as previously stated. These data show that supplementation seems to be uniform across all metal assault experiments in that the added amino acid pool caused each culture to decrease the total amounts of glutathione within the cell. This result leaves some questions unanswered as to why the addition of amino acids to the media may cause a decrease in overall biosynthesis, which needs to be studied further.

FFAAP supplementation also caused a relative change in the GSH/GSSG ratios of samples tested. Initially, the untreaded sample had higher ratios relative to non-supplemented cultures changing from 15 to 23 (Fig. 11). This is interesting, as the addition of FFAAP seems to decrease the relative oxidative stress of the cell by shifting to more GSH being available to bind to free oxygen radicals in the cell. This trend was mirrored in both copper and chromium (VI) treatments where the initial non supplemented samples had lower ratios compared to FFAAP supplemented ones. Chromium (VI) treated cultures had a specific interaction where pre supplementation the oxidative stress levels were significantly increased, however post supplementation, the GSH/GSSG ratio was significantly increased relative to untreated cells demonstrating a decrease in overall oxidative stress. Cadmium treatment was again the outlier in this data set showing that the supplementation of FFAAP resulted in an unchanged oxidative stress level of 15 (Fig. 11). Cadmium has been shown over and over to be an outlier in how the cell responds to acute subtoxic assault. The specific interactions of the metal with biological machinery results in a novel interaction when looking at cadmium specifically.

These data show that supplementation of FFAAP before heavy metal exposure leads to a rescue in response to the assault in the biosynthesis and regulation of glutathione. The cell is

either able to both reduce total levels of glutathione and decrease oxidative stress ratios as seen with the redox active metals or is simply able to reduce the total levels of glutathione as seen in the redox inactive metal, cadmium. The supplementation of FFAAP to the cell cultures pre metal assault leads to a direct reduction on the glutathione pools under heavy metal stress, which could indicate that the supplement itself is being used as a chelating agent to either the ROS produced by redox active metals or cadmium itself. These direct interactions would lead to the cell needing less glutathione overall as the supplement would allow for direct reduction of stress independent of the antioxidant. This shows further proof that redox activity of the metal exposure at the acute subtoxic treatment is important to how the cell responds metabolically.

4.6 Acute heavy metal assault causes a significant impact on transcriptional profiles in *S. cerevisiae*

S. cerevisiae reacts to acute heavy metal assault differently depending on the different type of metal treatment. This is key to understanding how the cell can react to different exogenous environmental factors and how these cause a change to the genetic expression profiles during one hour of treatment. As stated above, the heavy metals used in these experiments are categorized as either redox active or redox inactive. Redox activity is important in this case as it leads to different, highly specific impacts on the overall transcriptional machinery of yeast.

Redox-active heavy metals directly produce ROS within the cell, which leads to immediate, significant levels of expression changes compared to untreated cell cultures. Copper and chromium (VI) treatments are both redox-active metals, however the transcriptional effect of each is significantly different within one hour of treatment. Interestingly, the glutathione biosynthesis and regulation machinery utilized by the cell to combat free radicals within the cell

are affected differently by these two metals. The biosynthesis genes (GSH1 and GSH2) are both significantly down regulated under copper poisoning however GSH1 expression is increased while GSH2 remains unchanged due to chromium (VI) treatment (Fig. 12). This is further represented by the GLR1 gene, which is increased due to chromium (VI) exposure but is decreased by copper. This is rather interesting, as the glutathione assay data presented above shows that both treatments have total glutathione values that remain unchanged while the GSH/GSSG ratio of oxidative stress is decreased due to both (Fig. 12). This shows that even though both metals produce ROS within the cell and create apoptotic populations, each have a different effect on the biosynthesis and regulation machinery of glutathione. The slight variation in metabolic glutathione ratios, with copper inducing higher oxidative stress (Fig. 12), is coupled with the decrease in production and regulation of the glutathione molecules within the cell at a transcriptional level.

The redox-inactive metal, cadmium, leads to a different story of transcription compared to the redox-active metals studied. In this case, every gene involved in glutathione biosynthesis and regulation that was analyzed was increased significantly within the hour at a much higher fold change compared to either of the redox active metals (Fig. 12). This is interesting to note, as the only metal to change the total values of glutathione within the cell within one hour of treatment was cadmium (Fig. 12). These data show that cadmium treatments lead to a highly specific, unique impact on both metabolic and transcriptional profiles within the cell. This could be explained because cadmium has been shown to interact directly with glutathione and other cellular machinery which can cause change within the cell.

The impact of redox active versus redox inactive metals is further shown to follow a similar trend when other genes are analyzed more specifically. CYS3 and CYS4, genes involved

in cysteine biosynthesis, are impacted similarly to the glutathione genes observed above. Under cadmium treatment, transcription of these genes is highly increased while they are decreased due to copper exposure (Fig. 13). This coupled with the glutathione metabolism (Fig. 13) and the glutathione transcriptional regulation (Fig. 13) shows that cadmium has an even more specific role on this pathway. The up regulation of cysteine biosynthesis genes would make sense under cadmium exposure as cysteine is needed to produce more glutathione molecules which have been shown to be increased significantly.

This trend of cadmium-specific impacts on cellular machinery is continued when observing the protein misfolding and stress genes analyzed. In these cases, transcription of all genes involved in stress response and protein management was significantly increased within the hour of treatment due to cadmium exposure (Figs. 14 and 15). This is important to note, as acute cadmium exposure leads to a highly increased stress response even though it has been shown to be redox-inactive by previous experiments (*Gardner, 2019*). Stress response was similar due to chromium (VI) exposure, however at a much lower fold change when compared to cadmium assault. The one exception to this rule was HSP30, which was significantly decreased due to chromium (VI) (Fig. 15). Interestingly, copper exposure led to the completely opposite result in decreasing almost all stress response activity within one hour while simultaneously increasing HSP30 expression initially. This HSP30 response was quickly down regulated towards the end of the hour mirroring the other stress response genes (Fig. 15). It is important to note that MCA1, which is the yeast metacaspase involved in the signal cascade of apoptosis, was increased transcriptionally at the end of the hour under cadmium and chromium (VI) assault, however it was lowered initially under copper exposure and began to return to normal levels at the hour (Fig. 15). This finding is interesting, as previous studies have demonstrated that copper

assault does not lead to caspase-specific apoptosis at a chronic level, however this study has shown that copper activates caspase at an acute level (Fig. 8). The transcriptional decrease in MCA1 expression could be linked to the decreased response of chronic exposure to caspase activity (*Liang and Zhou, 2007*). This shows that the different metals all have a specific role on stress response and metacaspase expression, however they all show induced apoptotic populations due to a one-hour acute treatment (Fig. 8), which further illustrates that each metal has specific routes of apoptosis induction due to the different pathways described above.

4.7 Future experimentation

Having begun to address the complexity of the role(s) that glutathione plays in confounding heavy metal-induced oxidative stress in yeast cells and the varied ability of FFAAP to potentiate this function, the first line of analyses that would be informative would be to broaden the spectrum of genes that were analyzed for transcriptional expression and to expand the conditions under which their expression was responsive, using Nanostring, or related, technology. Furthermore, this study was only able to observe targeted gene expression in cell cultures exposed to each of the three heavy metals. These analyses, however, did not extend to the effects of FFAAP which, as previously shown, leads to a rapid decrease in total glutathione levels under stressed conditions that could be mirrored at the transcriptional level. I would hypothesize that the supplementation of FFAAP would directly influence the glutathione biosynthesis and regulatory pathways, as indicated in the metabolic data from this study. Having a better understanding of the affects that such targeted amino acid supplementation might have on genetic expression of *S. cerevisiae* could help determine if FFAAP has merely a metabolic and/or transcriptional impact metal induced stress under varied conditions.

In addition, given that all the work defined herein was undertaken using wild-type yeast strain one additional approach would be to begin to dissect the precise nature of glutathione's role(s) in metal-induced oxidative stress by addressing the different effects of the different metals using specific mutations in genes that maintain the glutathione cycle. This would be further studied by observing some of the related genes that are so dramatically influenced by the presence of the metals and/or metabolic response of the cells to these resulting cellular stressed conditions. In this way the individual stressors that initiate and modulate the metabolic response would potentially be determined.

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APPENDICES

Appendix A

Nanostring Panel Genes

All gene descriptions were taken from the *Saccharomyces* Genome Database

1. ADE5/7 - Purine biosynthesis enzyme
2. ADE6 - FGAM synthetase
3. ADO1 - Adenosine kinase
4. AHP1 - Thiol-specific peroxiredoxin
5. ATG1 - Serine/threonine kinase
6. ATG2 - Protein for autophagic vesicle formation
7. ATG4 - Cysteine protease
8. ATG6 - Subunit of phosphatidylinositol 3-kinase complexes I and II
9. ATG7 - Autophagy related protein
10. ATG8 - Protein involved in autophagosome formation
11. BUD21 - Component of ribosomal subunit processome
12. CDC19 - Pyruvate kinase
13. COX2 - Subunit II of cytochrome c oxidase
14. CUP1 - Metallothionein
15. CYS3 - Cystathionine gamma-lyase
16. CYS4 - Cystathionine beta-synthase
17. DDR48 - DNA damage-response protein
18. EFB1 - Translation elongation factor 1 beta
19. EFT2 - Elongation factor 2
20. ENO1 - Enolase 1
21. FBA1 - Fructose 1,6-bisphosphate aldolase
22. FKH1 - Forkhead family transcription factor
23. FKH2 - Forkhead family transcription factor
24. GAD1 - Glutamate decarboxylase
25. GLR1 - Cytosolic and mitochondrial glutathione oxidoreductase
26. GPP1 - DL-glycerol-3-phoshate phosphatase
27. GRX2 - Glutathione-dependent disulfide oxidoreductase
28. GRX5 - Glutathione-dependent oxidoreductase
29. GSH1 - Gamma glutamylcysteine synthetase
30. GSH2 - Glutathione synthetase
31. HCM1 - Forkhead transcription factor
32. HSP26 - Small heat shock protein
33. HSP30 - Negative regulation of ATPase
34. HSP31 - Methylglyoxalase
35. HYP2 - Translation elongation factor eIF-5A

36. IPP1 - Cytoplasmic inorganic pyrophosphatase
37. KAR2 - ATPase
38. KTR7 - Putative mannosyltransferase
39. MCA1 - Ca^{2+} -dependent cysteine protease
40. MCR1 - Mitochondrial NADH-cytochrome b5 reductase
41. MSN2 - Stress-responsive transcriptional activator
42. MSN4 - Stress-responsive transcriptional activator
43. MXR1 - Methionine-S-sulfoxide reductase
44. OYE3 - NADPH oxidoreductase
45. PDB1 - E1 beta subunit of the pyruvate dehydrogenase complex
46. PDI1 - Protein disulfide isomerase
47. PET9 - ADP/ATP carrier
48. PFK1 - Alpha subunit of heterooctameric phosphofructokinase
49. PGI1 - Phosphoglucose isomerase
50. PGK1 - 3-phosphoglycerate kinase
51. POR1 - Mitochondrial porin
52. RPP0 - Ribosomal protein
53. RRN5 - Regulator of RNA polymerase I
54. SAM1 - S-adenosylmethionine synthetase
55. SAM4 - S-adenosylmethionine-homocysteine methyltransferase
56. SEC4 - Rab family GTPase
57. SEC53 - Phosphomannomutase
58. SIR2 - NAD+ dependent histone deacetylase
59. SSA1 - ATPase/ NLS-directed nuclear transport
60. SSA4 - HSP70 protein induced via stress
61. SSB1/2 - Cytoplasmic ATPase
62. SSC1 - Hsp70 family ATPase
63. SSE1 - ATPase component of heat shock protein Hsp90 chaperone complex
64. TDH1/2/3 - Glyceraldehyde-3-phosphate dehydrogenase
65. TPI1 - Triose phosphate isomerase
66. TSA1 - Thioredoxin peroxidase
67. UBI4 - Ubiquitin
68. VMA2 - Subunit B of V1 peripheral membrane domain of vacuolar H+-ATPase
69. YPT32 - Rab family GTPase
70. ALG9 - Mannosyltransferase
71. TAF10 - Subunit of TFIID and SAGA complexes
72. TFC1 - Subunit of RNA polymerase III transcription initiation factor complex