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MECHANISM (S) OF METAL-INDUCED APOPTOSIS IN *SACCHAROMYCES CEREVISIAE*.

by

AMRITA M. NARGUND

Under the direction of Dr. John E. Houghton

ABSTRACT

Heavy metals, such as copper and cadmium have been linked to a number of cellular dysfunctions in single and multicellular organisms that are associated with apoptosis. The yeast, *Saccharomyces cerevisiae*, provides a valuable model for elucidating apoptosis mechanisms, and this study extends that capability to Cu and Cd-induced apoptosis. We demonstrate that *S. cerevisiae* undergoes a glucose-dependent, programmed cell death in response to low cadmium concentrations, which is initiated within the first hour of Cd exposure. The response was associated with induction of the yeast caspase, Yca1p, and was abolished in *YCA1*Δ mutant. Other apoptotic markers, including sub-G₁ DNA fragmentation and hyper-polarization of mitochondrial membranes, were also evident among Cd-exposed cells. We also show that low levels of copper can induce a similar apoptotic response in yeast within the first hour of

exposure. Such cellular responses were verified by analyzing mitochondrial perturbation, generation of superoxide ions, activation of the yeast caspase1, and the eventual fragmentation of nuclear DNA (through TUNEL). In analyzing the response of yeast to the different metals, we also demonstrated that the metal-induced PCD is instigated through the sequential activity of at least two caspase-like proteins (i.e., Yca1 and Atg4), both of which appear to be involved in the process of inducing mitochondrial stress. The additional caspase-like activity is shown to be derived from an enzyme involved in the latter stages of autophagy (Atg4), and provides an intriguing association of apoptosis with autophagy. Here we also demonstrate that metals such as copper and cadmium causes oxidative damage to mitochondrial proteins. Such oxidative attack is targeted and we show that oxidation of certain crucial proteins is required for apoptosis upon metal exposure. By showing that such targeted protein oxidation is dependent on *YCA1* and *ATG*, we also confirm the finding that in yeast that have been exposed to a heavy metal, *YCA1* and *ATG* are essential for damaging mitochondria and to initiate apoptosis. These novel findings highlight several new perspectives about the mechanism of metal-dependent apoptosis, while opening up future analyses to the power of the yeast model system.

INDEX WORDS: Heavy metals, Apoptosis, Yeast, Oxidative stress

MECHANISM (S) OF METAL-INDUCED APOPTOSIS IN *SACCHAROMYCES CEREVISIAE*.

by

AMRITA M. NARGUND

A Dissertation Submitted in Partial Fulfillment of the Requirements for the

Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2010

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2010

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May 2010

DEDICATION

This is to my husband Mandar Mangalvedhekar and to my daughter Gandhali for their unconditional love and support, which made this accomplishment possible.

To my parents, brothers, sister-in-laws and nieces for their continued support and encouragement.

To my parents-in-law for their support.

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GENERAL INTRODUCTION

Why study metal induced oxidative stress?

Metals generate reactive oxygen species (ROS) and hence cause oxidative stress in the cell (Fig 1) ^{1,51}. Metal induced oxidative stress has been implicated in the pathogenesis of Alzheimer's disease, Parkinson's disease, spongiform encephalopathy, and familial amyotrophic lateral sclerosis ²⁻³. The imbalance in copper homeostasis is observed in hereditary neurodegenerative disorders (ND) such as Menke's and Wilson's disease. The impairment of copper transporting gene leads to fatal neurodegeneration in Menke's syndrome ⁴. Moreover, the excessive accumulation of copper in liver, kidney and brain leads to hepatic, renal and neurological abnormalities in case of Wilson's disease ⁵. Cadmium (Cd) is known to be carcinogenic and known to be associated with renal damage.

In the above mentioned disorders, metal alters mitochondria and decreases cytochrome c oxidase activity giving rise to burst of ROS. These ROS that can cause oxidative stress are also neurotoxic, because they trigger neuronal death due to apoptosis (programmed cell death) ^{3,6,7}. Loss of neurons because of apoptosis in ND is of serious health concern.

Why study metal toxicity, apoptosis and mitochondria dysfunction in *Saccharomyces cerevisiae*?

Saccharomyces cerevisiae is an excellent model system for the molecular study of metal toxicity, because its genome and proteome are well characterized. Yeast is easier to manipulate experimentally than higher eukaryotes. The effects of metal toxicity and the response to oxidative stress, which can cause apoptosis, are similar to those seen in higher eukaryotes. Cellular mechanisms are conserved between higher eukaryotes and yeast (Fig 2)³².

Recently, unicellular yeast have been seen to undergo apoptosis under different stress conditions. As seen in case of higher eukaryotes, ROS are found to be the major cause of apoptosis in yeast⁸. Orthologs of genes such as *caspase*, *AIF1* and *BI-1* that are important for apoptosis, have been characterized in yeast^{8-9,10}. Yeast has been found to be useful in elucidating some of the problems related to aging and neurodegeneration. A mutation in *CDC48*, a homologue of VCP in mammalian cells, was first shown to result in apoptosis in yeast and then later was found to be involved in the formation of inclusion bodies in Paget's disease and in neurodegeneration caused by polyglutamine¹¹⁻¹⁴. Likewise yeast has been proven to be a useful system in the studies related to Alzheimer's, Parkinson's and Huntington's disease¹⁴⁻¹⁵. Hence, yeast is a good tool for studying apoptosis related to ageing and neurodegeneration.

In the past, yeast have been studied to elucidate mitochondrial function in cells. Recently, mitochondria have gained importance even in the pathway for cell death

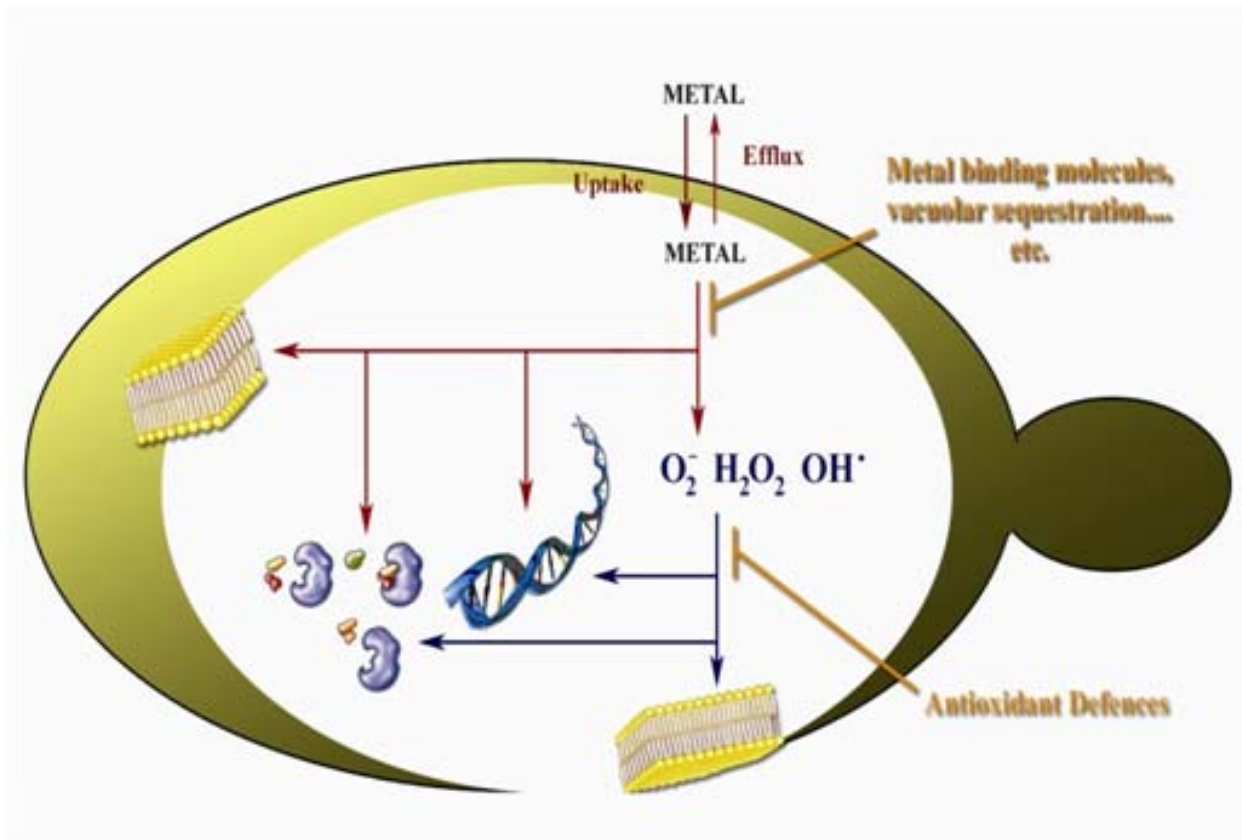


Figure 1: Metal generate ROS and cause oxidative stress.

Metal can generate ROS and cause oxidative stress by damaging cellular macromolecules.

pathway, as yeast have been shown to undergo mitochondrial-mediated apoptosis under various stress conditions ¹⁶. Although mitochondrial dysfunction has also been associated with oxidative stress and apoptosis in cases of neurodegenerative diseases (ND), it is difficult to elucidate even this form of cell death in higher eukaryotes. In yeast, it is simple to perform mitochondrial manipulations because it is easier to create specific mutations in mitochondrial DNA. In addition, cells can be grown on a variety of different carbon sources (fermentative and non-fermentative) to control the mode of respiration, i.e., aerobic and anaerobic, and thus the different involvement of mitochondria in cellular activities. As a result, yeast is an excellent model organism to elucidate the events of mitochondrial-mediated cell death. Even so, the exact path of mitochondria-mediated apoptosis in yeast is still unclear. The greater the understanding of yeast mitochondria mediated apoptosis, the easier it will be to solve some important issues regarding these above mentioned ND ¹⁴.

Metal generates Reactive oxygen species (ROS) and cause oxidative stress

In the cell, ROS originates in mitochondria. Mitochondria, a powerhouse of a cell, can generate energy through the electron transport chain by producing ATP. In doing so, mitochondria also generate ROS, such as peroxide and superoxide anions but to a lesser extent. Mitochondria are equipped with antioxidant defenses such as superoxide dismutase (SOD) and glutathione (GSH imported from cytosol); these convert superoxide and peroxide to other harmless products before these species can become a substrate for Haber-Weiss or Fenton's reaction, which generates more potent ROS ^{3,17}.

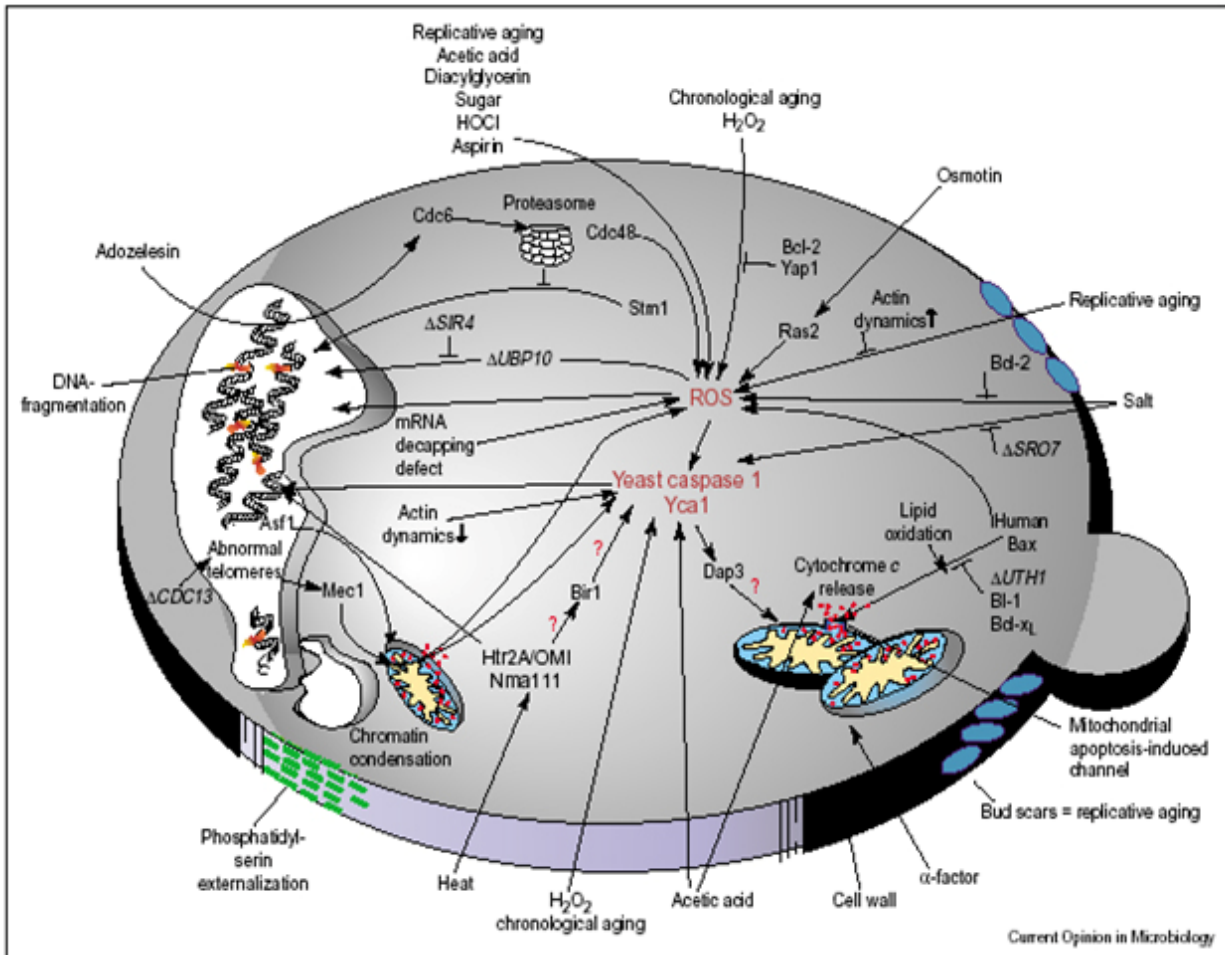


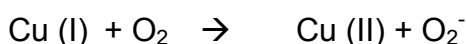
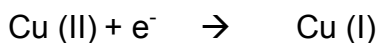
Figure 2: Apoptosis pathways in Yeast.

All the components and known yeast proteins involved in apoptosis are depicted

Copper is a transition metal, because it is able to cycle between Cu (II) and Cu (I) as shown in the reaction below. In doing so, copper can generate superoxides, a reactive oxygen species (ROS). Copper also qualifies as a redox active metal because by using Fenton's reaction it is able to generate more potent ROS such as hydroxyl radical (OH°) from the less reactive species such as peroxide (H_2O_2) and superoxide (O_2^-) that are generated due to cellular respiration in the mitochondria ³.

Copper is also able to catalyze Haber-Weiss reaction to give OH° ¹⁸. Hence, in the presence of excess of copper, cells will be overloaded with potent ROS. Such humungous amounts of ROS are difficult to be disarmed by antioxidants. Excess ROS damages all of the cellular macromolecules, such as proteins, lipids and DNA, and hence a condition called oxidative stress develops inside the cell ¹⁹ (Fig 2) ³².

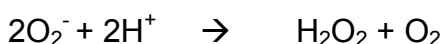
Transition metal Cu:



Fenton's reaction:



Haber-Weiss cycle:



Cadmium by nature is a redox-inactive metal. Cadmium hence cannot catalyze Fenton Haber-Weiss reaction, but it can generate ROS indirectly by depleting the cell's

anti-oxidant defenses or by displacing redox-active metals from proteins²⁰. Therefore, cadmium is able to still cause oxidative stress in the cells.

Even though there is a clear difference in ROS species formed by copper and cadmium, there is no overt difference in cellular oxidation levels between both metals⁴⁷ (Shanmuganathan, A., personal communication).

Apoptosis and the role of oxidative stress or ROS in the process

Apoptosis is a programmed cell death i.e. a cell commits suicide. Apoptosis is not a random process; rather it happens in a very controlled and coordinated manner. Apoptosis involves a series of events that ultimately lead to the removal of cells from a population with no harmful substances released in the environment. An apoptotic cell shows a typical morphology (e.g., shrinkage of a cell, condensed chromatin, fragmented DNA), and finally a cell is broken into numerous small apoptotic bodies that are phagocytosed rapidly. Apoptosis involves a complex signaling network and is an active process that requires energy. The two pathways by which apoptosis can occur are the extrinsic and intrinsic pathways (Fig 3,4)^{52,53}. In the extrinsic pathway, ligands serve as stimuli when they bind to the receptors on a target cell and trigger activation of caspases. Caspases are able to fragment DNA and, hence, cause apoptosis. The other apoptotic path is the intrinsic pathway or mitochondria mediated pathway, in which the signals for the cell death are internal (e.g., DNA damage, oxidative stress, mutations in mitochondrial DNA). ROS induced oxidative stress precedes apoptosis in many

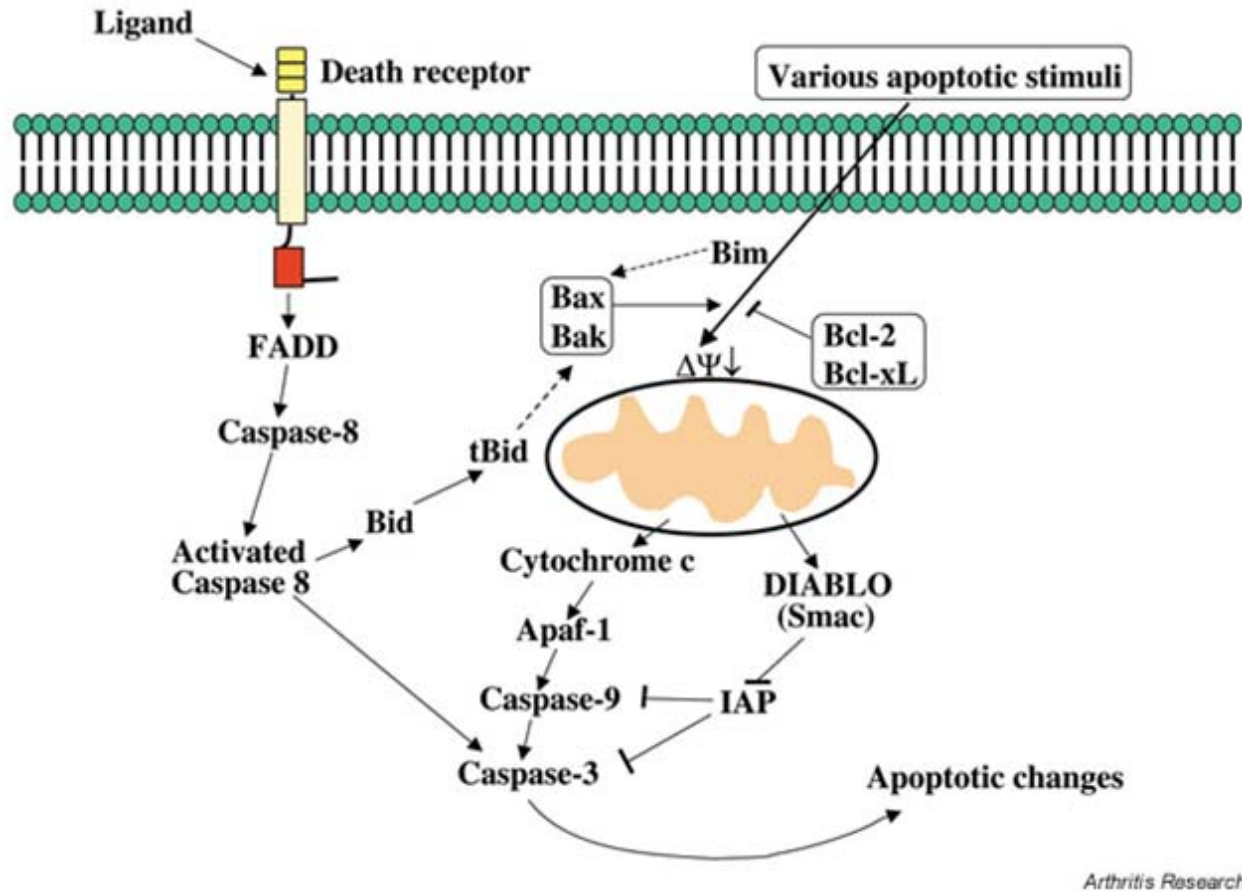


Figure 3: Intrinsic and extrinsic pathway of apoptosis.

There are two different pathways of apoptosis in higher eukaryotes- intrinsic or mitochondria mediated and extrinsic pathway or death receptor mediated apoptosis.

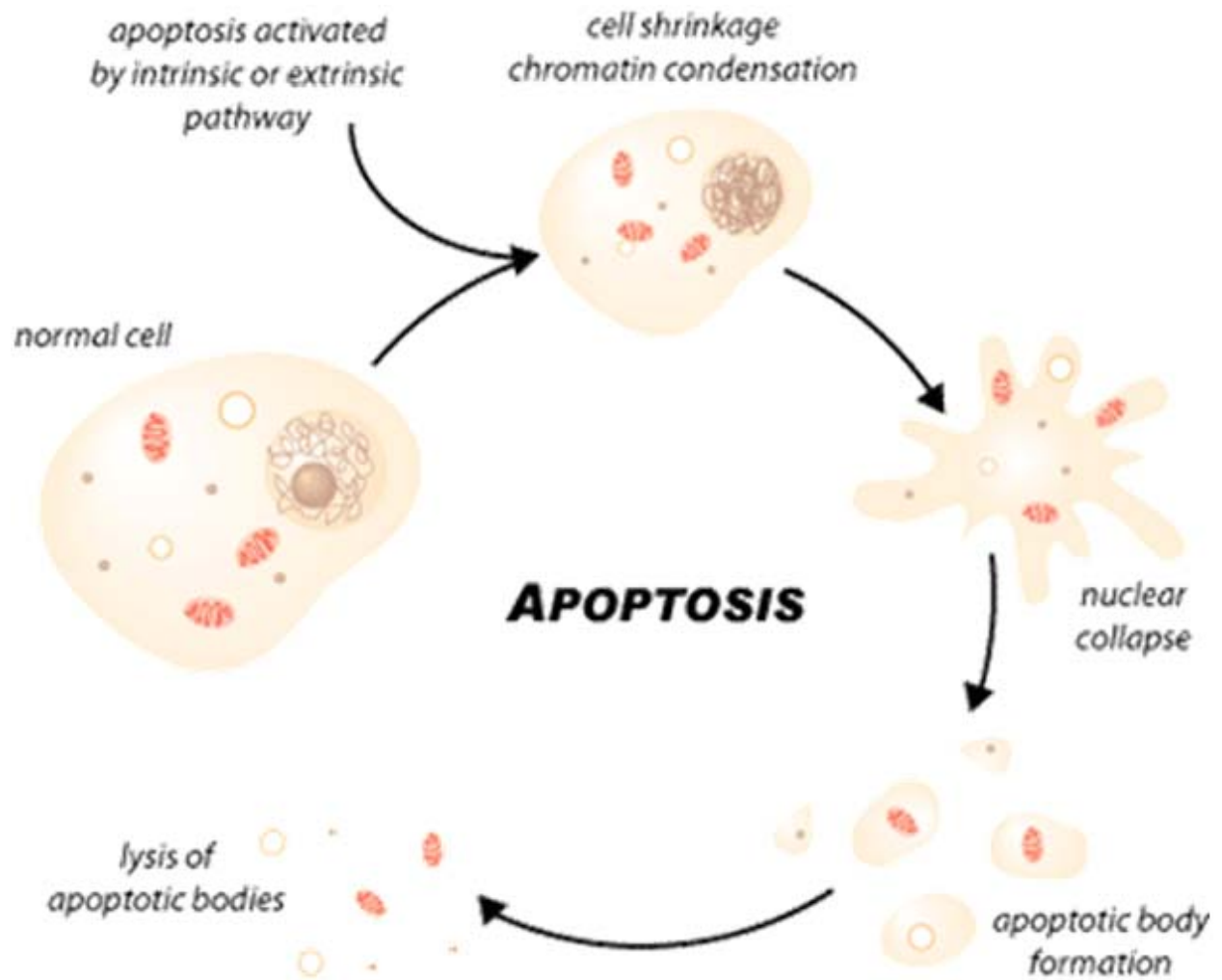


Figure 4: Apoptotic cell death.

Cells destined for apoptosis triggered by either mitochondria or death receptor shows certain features before the cell death.

cell death scenarios indicating that ROS is an important stimulus for the intrinsic pathway of apoptosis. Numerous studies with antioxidants have shown that oxidative stress when prevented by scavenging ROS, there is no apoptosis ²¹. This indicates that ROS can trigger apoptosis. However, the steps involved in ROS triggered mitochondria mediated apoptosis are unclear.

ROS is generated and released from mitochondria. Studies have also shown that once released from mitochondria, ROS can oxidize mitochondrial pore proteins. Such an oxidation can lead to mitochondrial fission or dysfunction. Mitochondria fission is associated with apoptosis. Mitochondria are, therefore, not only a source but also the target of ROS during apoptosis ²².

Mitochondria in apoptosis

In ND, mitochondria dysfunction and apoptosis are commonly observed toxic effects. Mitochondria are key players in the intrinsic pathway of apoptosis (Fig 5) ¹⁴. Mitochondria dysfunction is a cause of apoptosis in intrinsic pathway. In some cell types, ROS released from mitochondria causes transient hyperpolarization of the mitochondrial inner membrane leading to mitochondrial inner membrane permeabilization (MMP). Because of MMP, solutes less than 1.5KDa can enter the mitochondrial matrix along with an influx of water leading to mitochondrial swelling and rupture of an outer mitochondrial membrane. This phenomenon causes a drop in the mitochondrial membrane potential or depolarization.

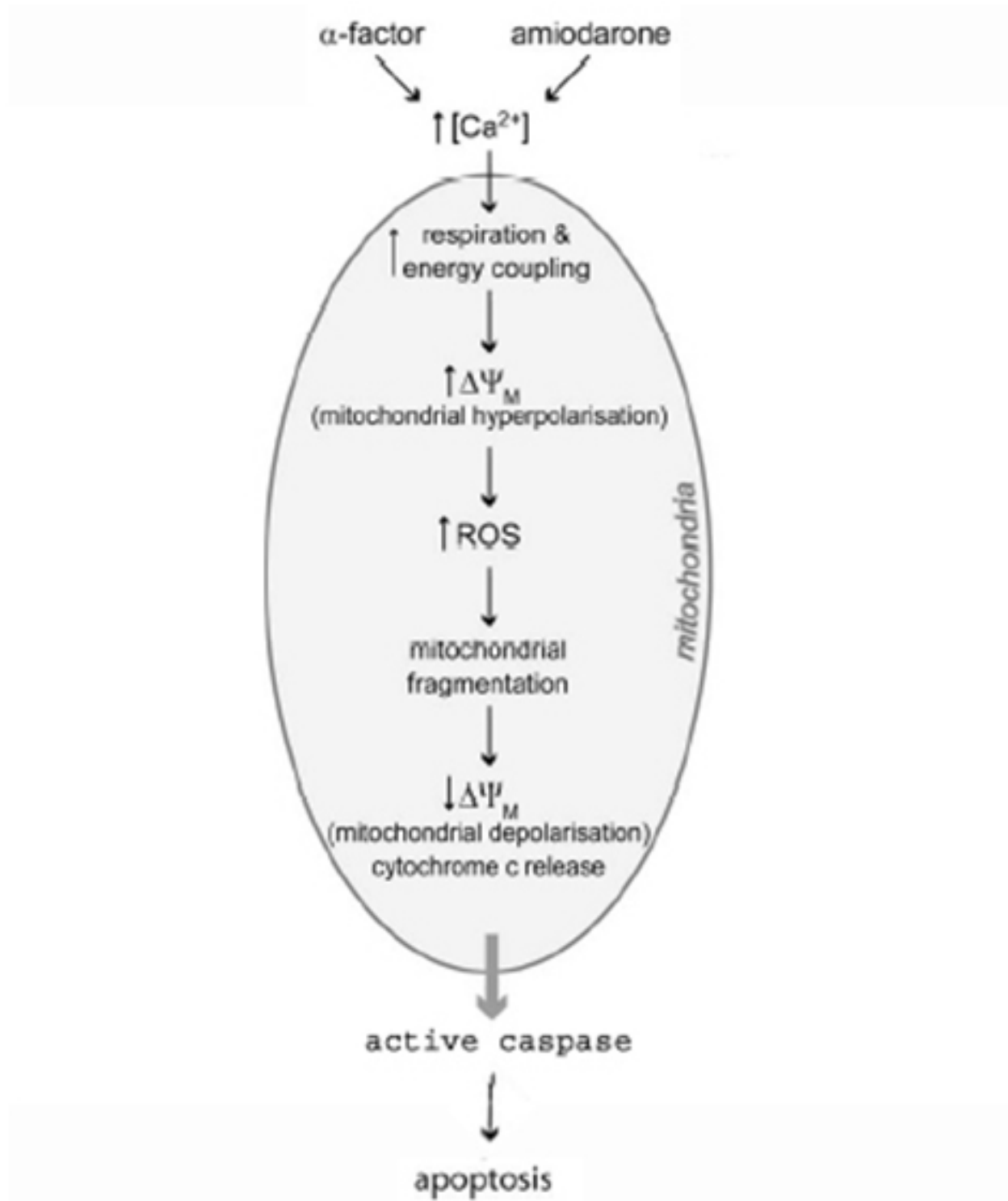


Figure 5: Mitochondria in yeast apoptosis.

The events leading to mitochondrial dysfunction in yeast apoptosis are shown.

The pro-apoptotic factors(e.g., Apoptosis inducing factor(AIF) and cytochrome c) are then released from the inner-mitochondrial space into the cytosol²³⁻²⁵. Both AIF and cytochrome c are able to trigger apoptosis. Not only AIF and cytochrome c, but several other mitochondrial proteins can participate in apoptosis.

Mitochondrial proteins and apoptosis

Mitochondria are not only the source but also the target of ROS (Fig 6)⁵⁴. Mitochondrial proteins are oxidized upon exposure to certain oxidants¹⁸. In some mammalian cell types, oxidation of some of the mitochondrial proteins leads to mitochondrial fission²⁹⁻³¹.

Isolated yeast mitochondria lacking Voltage dependent anionic channel (a mitochondrial protein), are not sensitive to human Bax mediated apoptosis as suggested in Fig 3.³² Yeast strains lacking mitochondrial pore genes(e.g., *POR1* and *ANT1*) do not undergo HIV-1 viral protein R (VPR) induced apoptosis³³. On exposure to oxidizing agent such as paraquat, mitochondrial proteins are carbonylated (oxidized) in yeast¹⁸. Both copper and cadmium cause oxidative stress by oxidizing the cellular proteins in yeast (Shanmuganathan A, personal communication)⁴⁷. High concentration of cadmium causes mitochondria mediated pathway in higher eukaryotes²⁷. To understand the toxicity associated with relatively low concentrations of copper and cadmium, we will investigate their role in oxidation of mitochondrial proteins

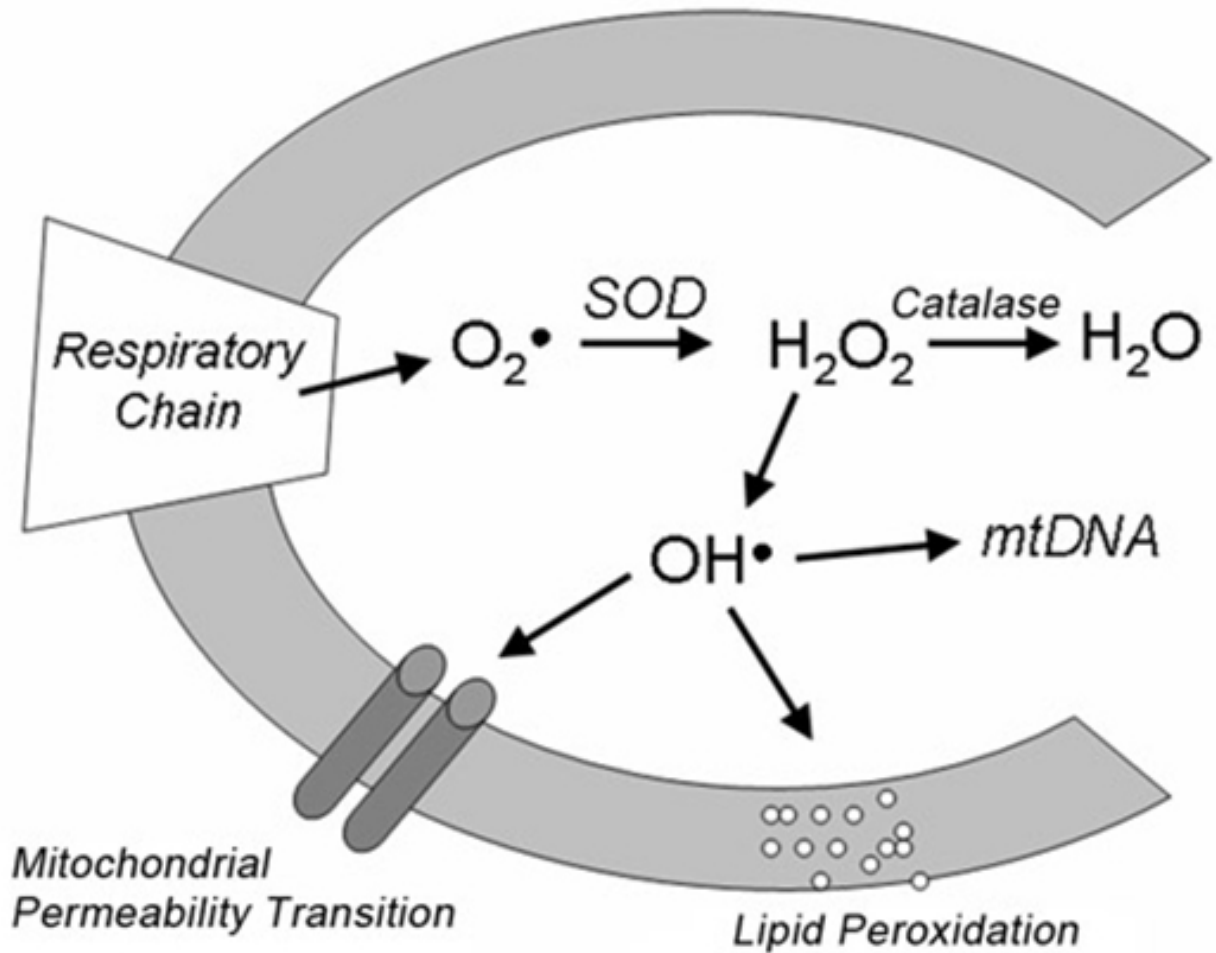


Figure 6: Mitochondria permeability transition during apoptosis.

Mitochondrial damage during apoptosis could be due to lipid peroxidation or oxidation of mitochondrial proteins.

(e.g., POR1 and ANT1) and mitochondrial collapse in apoptosis.

Mitochondrial flavoprotein, AIF, is present in the inter-mitochondrial space and has oxidoreductase activity³⁴. It is an important regulator of cell death in mammals, because it is involved in mitochondria mediated apoptosis³⁴. In higher eukaryotes, on induction of apoptosis, AIF translocates from mitochondria to the nucleus^{10,35}. In the nucleus, it fragments DNA itself or with the help of endonuclease G^{26,36}. AIF has a DNA binding domain, which is essential for its apoptogenic activity³⁷. AIF can also contribute to the release cytochrome c from inter-mitochondrial space with the mechanism still unclear. Cytochrome c can lead to activation of caspases. Hence, AIF and caspase can cooperate and cause apoptosis^{26,36}. The structure and function of AIF are conserved in *Saccharomyces cerevisiae*¹⁰. In yeast, AIF essentially performs the same role as in higher eukaryotes, upon peroxide exposure¹⁰.

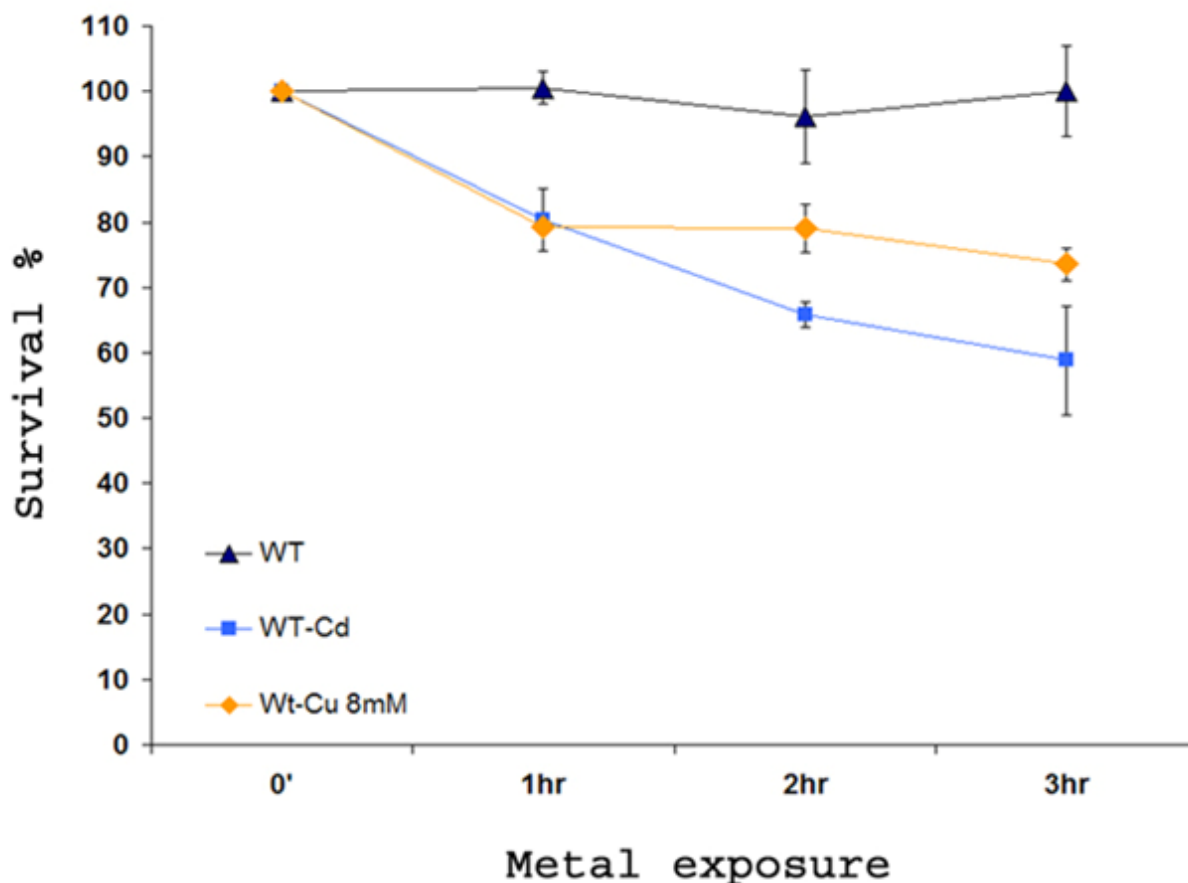


Figure 7: Viability of yeast upon copper and cadmium exposure.

S. cerevisiae YCA1Δ was incubated in the presence of 8mM $\text{Cu}(\text{NO}_3)_2$ and $30\mu\text{M}$ $\text{Cd}(\text{NO}_3)_2$ for up to 3 h. Viability was determined at intervals after the addition of metals by spreading aliquots from dilutions ($\text{OD}_{600} = 1.0$) cultures onto YEPD agar and incubated for 3 d at 30°C . Colonies were enumerated after incubation for 3 days at 30°C . Percentage viability was calculated with reference to the number of colonies formed by untreated cells at zero time.

Experimental Question

Copper (Cu) and cadmium (Cd) are implicated in many neurodegenerative disorders.^{5,40} Cadmium is a redox-inactive metal, whereas copper is a redox-active metal and therefore is able to generate potent reactive oxygen species (ROS) directly as opposed to cadmium, which is able to do so indirectly. Both the metals cause oxidative stress in the cell⁴¹. Metal induced oxidative stress is associated with mitochondria dysfunction and apoptosis (programmed cell death) in cases of neuronal death^{39,42-44}. In neurodegenerative disorders, neuronal death due to apoptosis is the major concern¹. To prevent apoptotic cell death, it is crucial to know the cascade of events leading to apoptosis and involvement of mitochondria in the process.

In *Saccharomyces cerevisiae* (yeast), metals such as copper and cadmium generate ROS and cause cell death with the induction of cell death genes. Yeast, being a simple unicellular eukaryote, serves as an excellent model to elucidate the mechanism of metal toxicity. **The hypothesis of this dissertation study is that heavy metals induce apoptosis in yeast in a mitochondria-dependent manner that involves the role of solitary yeast caspase, Yca1.**

SA1: In yeast, what is the cellular response on exposure to copper and cadmium?

Copper has been associated with apoptosis in human hepatoma cells and breast cancer cell line⁴⁰⁻⁴¹. Cadmium also has been associated with apoptosis in human hepatoma cells and cultured renal tissues^{3,26}. However, cadmium has not been shown to cause apoptosis in yeast. In addition, low doses of copper have not yet been associated with apoptosis in yeast. When treated with very low doses of metals, such as copper and cadmium, yeast cells do show, however, a decrease in viability (Fig 7). Moreover, upon exposure to either copper or cadmium, proteins are damaged through metal-induced oxidative stress (Shanmuganathan, personal communication)⁴⁷, and oxidative stress (when induced by hydrogen peroxide) is a known trigger for apoptosis in yeast (32, 33). To examine the relationship between cadmium or copper and reduced yeast viability, the following questions were asked:

Specific Aim 1.1: Does exposure to very low doses of cadmium (~30 μ M) cause apoptosis (programmed cell death)?

Specific Aim 1.2: Does exposure to very low dose of copper (8mM) cause apoptosis?

SA2: What is the role of mitochondria in copper and cadmium induced apoptosis, and how is Yeast caspase-1 involved in the process?

Copper and cadmium toxicity at low concentration has not been studied extensively in yeast or higher eukaryotes. In higher eukaryotes, Cd-induced apoptosis appears to involve mitochondria although not much is known about the precise mechanism. In mitochondria-mediated pathways, mitochondrial proteins such as VDAC and ANT1 are oxidized in higher eukaryotes ²⁸. Thereafter, this oxidation of mitochondrial proteins is thought to lead to mitochondrial dysfunction and collapse, which is believed to trigger apoptosis by releasing pro-apoptotic factors such as AIF and cytochrome c ^{32,18,29-30}. In yeast, some of the events associated with mitochondrial perturbation have been observed in case of pheromone induced apoptosis ¹⁴. However, the cascade of such mitochondrial events related to oxidative stress is poorly understood ¹⁴. Exposure of yeast to copper and cadmium does generate oxidative stress (Shanmuganathan A, personal communication) ⁴⁷. Therefore, it is important to study mitochondrial events, such as mitochondrial protein oxidation and involvement of AIF, in response to the presence of cadmium.

Importantly, cadmium induced apoptosis in higher eukaryotes is thought to be independent of caspase ⁴⁹⁻⁵⁰. However, preliminary data in our laboratory does suggest that in yeast, cadmium-induced apoptosis involves both mitochondria and Yeast caspase-1. Intriguingly, *YCA1*Δ mutant do not show any mitochondrial perturbation in response to cadmium suggesting that Yca1 plays a significant role in mitochondrial perturbations. It is considered important, therefore, in any determination of metal induced apoptosis whether the singular caspase in yeast is involved in triggering each

of the mitochondrial events and/or subsequent stages of the apoptotic process. We have designed experiments to address the following questions:

Specific Aim 2.1: Does metal-induced oxidative stress include increase oxidation of mitochondrial proteins? Are proteins such as VDAC and ANT1 targeted?

Specific Aim 2.2: Are proteins such as AIF (Apoptosis Inducing Factor) involved in apoptosis? If so, do they translocate to the nucleus.

Specific Aim 2.3: Does mitochondrial protein oxidation depend upon YCA1?

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CHAPTER 1

CADMIUM INDUCES A HETEROGENEOUS AND CASPASE-DEPENDENT APOPTOTIC RESPONSE IN *SACCHAROMYCES CEREVISIAE*

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The toxic metal cadmium is linked to a series of degenerative disorders in humans, in which Cd-induced programmed cell death (apoptosis) may play a role. The yeast, *Saccharomyces cerevisiae*, provides a valuable model for elucidating apoptosis mechanisms, and this study extends that capability to Cd-induced apoptosis. We demonstrate that *S. cerevisiae* undergoes a glucose-dependent, programmed cell death in response to low cadmium concentrations, which is initiated within the first hour of Cd exposure. The response was associated with induction of the yeast caspase, Yca1p, and was abolished in *YCA1Δ* mutant. Cadmium-dependent apoptosis was also suppressed in a *GSH1Δ* mutant, indicating a requirement for glutathione. Other apoptotic markers, including sub-G₁ DNA fragmentation and hyper-polarization of mitochondrial membranes, were also evident among Cd-exposed cells. These responses were not distributed uniformly throughout the cell population, but were restricted to a subset of cells. This apoptotic subpopulation also exhibited markedly elevated levels of intracellular reactive oxygen species (ROS) and unusually, permeabilized plasma membranes. The heightened ROS levels alone were not sufficient to induce apoptosis. These findings highlight several new perspectives to the mechanism of Cd-dependent apoptosis and its phenotypic heterogeneity, while opening up future analyses to the power of the yeast model system.

Introduction

As a form of programmed cell death (PCD), apoptosis has been shown to provide a critical contribution to metazoan development, homeostasis and cellular differentiation ¹⁻². It has also been implicated as a major element in the response network of cells to external and internal stressors ³, aging ⁴⁻⁵ and disease control; being involved (for example) in tumor suppression ⁶⁻⁷. Unicellular organisms, such as *Saccharomyces cerevisiae* also undergo programmed cell death ⁸. While this finding was initially surprising, some of the potential benefits of selective apoptosis, of a few for the sake of the population as a whole, have now been rationalized ⁹⁻¹⁰. Moreover, the discovery of apoptosis in yeast has proven to be invaluable for the elucidation of several key apoptotic regulators in a variety of other organisms ¹¹⁻¹⁵. Despite the conservation of only a single caspase-like protein, Yca1p, which regulates caspase-dependent apoptosis in yeast ¹⁶, the retention of specific cell death pathways in common with humans ¹¹ has made *S. cerevisiae* a key model system in which to study the mechanisms that may underlie these shared apoptotic pathways.

The toxic effects of heavy metals, such as cadmium, have been known for a long time. Cadmium itself is a carcinogen, and elicits a number of toxic effects within cells. These range from the more chronic genotoxic effects, such as altering the capacity of cells to mismatch repair DNA ¹⁷, to more acute responses involving protein damage and/or lipid peroxidation ¹⁸. The cellular responses to Cd overlap markedly with those attributed to other forms of oxidative stress ¹⁹⁻²². While cadmium is unable to catalyze the production

of ROS directly, it has been shown to promote oxidative stress by depleting the cell's anti-oxidant defenses ²³⁻²⁴, or by displacing redox-active metals from proteins ²⁵. Similarly, the sensitivities of individual yeast cells to oxidative stressors such as peroxides, superoxide and cadmium are heterogeneous ²⁶, and have been shown to fluctuate (under varying concentrations of oxidative stressors) during yeast metabolic oscillations (ultradian rhythms; period <24 h) ²⁷⁻²⁹, which are associated with concerted transcriptomic and metabolomic changes ^{15,30-31}.

A major consequence of ROS induced oxidative stress in yeast is apoptosis ¹⁶, although this has not previously been tested in the case of cadmium-induced ROS. Cadmium, however, has been associated with apoptosis in rat testes ³², human hepatoma cells ³³ and cultured renal tissue ³⁴⁻³⁵. Even so, the precise role(s) that cadmium plays in these processes is only now starting to become apparent ^{34,36-37}. In particular, the caspase-dependency of Cd-induced apoptosis remains uncertain ³⁸⁻³⁹. Here, we demonstrate that low levels of cadmium induce apoptotic cell death in yeast, and that this induction is heterogeneous among individual cells within a population. Furthermore, the programmed cellular response within the affected cells requires the resident caspase, Yca1p, and, perhaps more intriguingly, glutathione; a critical component in the cellular response of yeast to oxidative- and cadmium- induced stresses ⁴⁰⁻⁴¹.

Materials and methods

Strains and growth conditions

Saccharomyces cerevisiae BY4741 (MAT α *his3* Δ 1 *leu2* Δ 0 *met15* Δ 0 *ura3* Δ 0) and the isogenic mutants *YCA1* Δ and *GSH1* Δ were obtained from Euroscarf (Frankfurt, Germany). Experimental cultures were inoculated from 24 h starter cultures derived from single colonies and grown overnight to exponential phase (OD₆₀₀~2.0) at 30°C with orbital shaking (120 rev min⁻¹) in YEPD broth ⁴². When necessary for experiments, cadmium nitrate [Cd(NO₃)₂] was added to growing cultures to a final concentration of 30 μ M. Also, when necessary, to test for the requirement for glucose, additional glucose was not added to the medium.

RNA isolation, microarray analysis and realtime RT-PCR

Total RNA was isolated using Qiagen RNeasy kit. RNA samples were prepared from BY4741 untreated and samples treated with Cd(NO₃)₂ for 5, 15, 30 and 60 mins. Relative quantification RT-PCR was used to confirm the expression levels of *YCA1* with a One Step RT-PCR kit (Qiagen) and the 7500 SDS system (PE Biosystems). Gene specific probes and primers were as follows: *YCA1* (forward primer 5'GGATGCGCAACCCAATGA^{3'}; reverse primer 5'AAATCTTCAGTTTGGC -CACCAT^{3'} ; probe FAM-TCTTTGTTTCCTTCATTATT-CTGGA-TAMRA), *ACT1* -endogenous control: (forward primer 5'ATGCAAACCGCTGCT-CAA^{3'}; reverse primer 5'AGTTTGGTCAATACCGG-CAGA^{3'} and probe 6 FAM-TGGTAACGAA -

AGATTCAGAGCCC–TAMRA). Fold changes in gene expression were calculated using the comparative $C_t\Delta\Delta C_t$ method⁴³⁻⁴⁴.

Cell viability and tests for apoptotic markers

The viability of Cd-treated and untreated cells was determined according to colony forming ability. Cultures were diluted and spread plated onto YEPD plates. Colonies were enumerated after incubation for 3 d at 30°C.

For determination of apoptosis, cell cultures were grown to an $OD_{600} \sim 2.0$ and induced by exposure to 30 μM $\text{Cd}(\text{NO}_3)_2$ for 1 h (unless otherwise specified). Cells were harvested by centrifugation. To test for DNA fragmentation, the cell pellet was resuspended in 300 μl of ddH₂O, and 700 μl of 95% ethanol was added before incubation for 3 d at 4°C. Cells were harvested by centrifugation and the pellet resuspended in 1 ml of 50 mM citrate buffer, pH 7.4. Cells were then re-pelleted and resuspended in citrate buffer with 0.25 mg ml⁻¹ RNase, followed by incubation for 2 h at 50°C. Finally, cells were pelleted and resuspended in 1 ml of 16 μg ml⁻¹ propidium iodide (PI) in 50 mM citrate buffer. After incubation for 30 min at room temperature, the PI-stained cells were analyzed (613 nm) by flow cytometry with a FACSCanto (BD Biosciences)²⁰.

The presence of yeast caspase was detected using an SR_FLICA activated-caspase detection kit (Immunochemistry Technologies, LLC)⁴⁵. Cell pellets, obtained as described above, were resuspended to $OD_{600} \sim 1.0$ in YEPD broth and incubated with SR_FLICA reagent (SR-VAD-FMK/ SR-DEVD-FMK) at 30°C for 3 h. The relative intensity of FAM/sulphorhodamine fluorescence in the cells was determined at

530nm/590nm emission using a FACSCanto (BD Biosciences). Images were captured using a Zeiss Axioimager fluorescent microscope equipped with Zeiss CP-ACHROMAT 100X/1.25 oil objective, a rhodamine filter and a Zeiss AxioCam MRc5.

Intracellular ROS were detected using the oxidant-sensitive dye dihydro-rhodamine-123 (DHR123, Sigma Aldrich) ⁴⁶. DHR123 was added at 5 μg per ml of cell culture, from a 2.5 mg ml⁻¹ stock solution in ethanol, and cells were incubated at 30°C for 3 h. Cells were observed by fluorescence microscopy as described above. Fluorescence was also determined with the FACSCanto at 525 nm to measure the ROS content. To determine whether the same cells that have high intracellular ROS are also caspase-positive, cells were incubated with both DHR123 and SR_FLICA reagent for 3 h. Spectral overlap of the dye emissions at 535 nm and 590 nm was compensated using FACS Diva software (v 5.0.1), with reference to control cell samples.

To evaluate mitochondrial membrane potential, cells (obtained as described above) were incubated with a final concentration of 2 μM rhodamine-123 and analyzed with the FACSCanto at an emission wavelength of 535 nm

PI assay of plasma membrane permeabilization

Cells were grown to an OD₆₀₀ ~ 2.0 and treated with Cd as described above. Cells were harvested by centrifugation and the pellet was resuspended in YEPD and diluted to OD₆₀₀ ~1.0. PI was added to 1 ml of the Cd treated and control (untreated) cells to a final concentration of 25 μg ml⁻¹, and cells were incubated for an additional 1 to 3 h at

30°C. Cells were examined by fluorescence microscopy, and by flow cytometry (emission 613 nm) using a FACSCanto. To determine whether the same cells had high intracellular ROS and high PI, cells were incubated with both DHR123 and PI for 3 h. Compensation for spectral overlap between the two fluorochromes at emission wavelengths of 535 nm and 613 nm was calculated using FACS Diva software v (5.0.1).

Results and discussion

Cadmium induces the yeast caspase, and a glucose-dependent apoptosis

Previously it was established that 30-50 μM $\text{Cd}(\text{NO}_3)_2$ was sufficient to initiate discernible oxidative stress in yeast, but insufficient to cause acute cellular damage and extensive loss of viability^{22,47}. In preliminary microarray analyses of the transcriptional response of *S. cerevisiae* to 30 μM Cd (Fig 16), we noted that a principal upregulated gene in Cd-treated cultures was *YCA1*. *YCA1* encodes the pivotal apoptotic marker protein, yeast caspase^{16,48}. Consequently, RT-PCR measurements were used to investigate this upregulation of *YCA1* more rigorously (Fig. 8A). These analyses revealed a 2-log increase in the *YCA1* mRNA levels of Cd-treated versus control cells, an increase that was detectable within 5 min and sustained over a 1 h time course of cadmium exposure.

YCA1 induction has previously been observed in yeast cells that have been shown to undergo apoptosis^{16,46}. We hypothesized, therefore, that 30 μM $\text{Cd}(\text{NO}_3)_2$ provokes a similar caspase-mediated apoptotic response in *S. cerevisiae*. In support of this hypothesis, we initially made observations of DNA fragmentation in Cd-exposed cells using the fluorescent DNA stain propidium iodide (PI)^{20,49}. Preliminary experiments indicated that after 3 h Cd exposure, ~10% of cells had a well-defined, weakly staining “sub-G1” DNA content, indicative of apoptosis. Moreover, given that significant *YCA1* induction occurred between 5 and 60 min of Cd exposure (Fig. 8A), the possibility that 1 h exposure was sufficient to initiate an apoptotic response was tested.

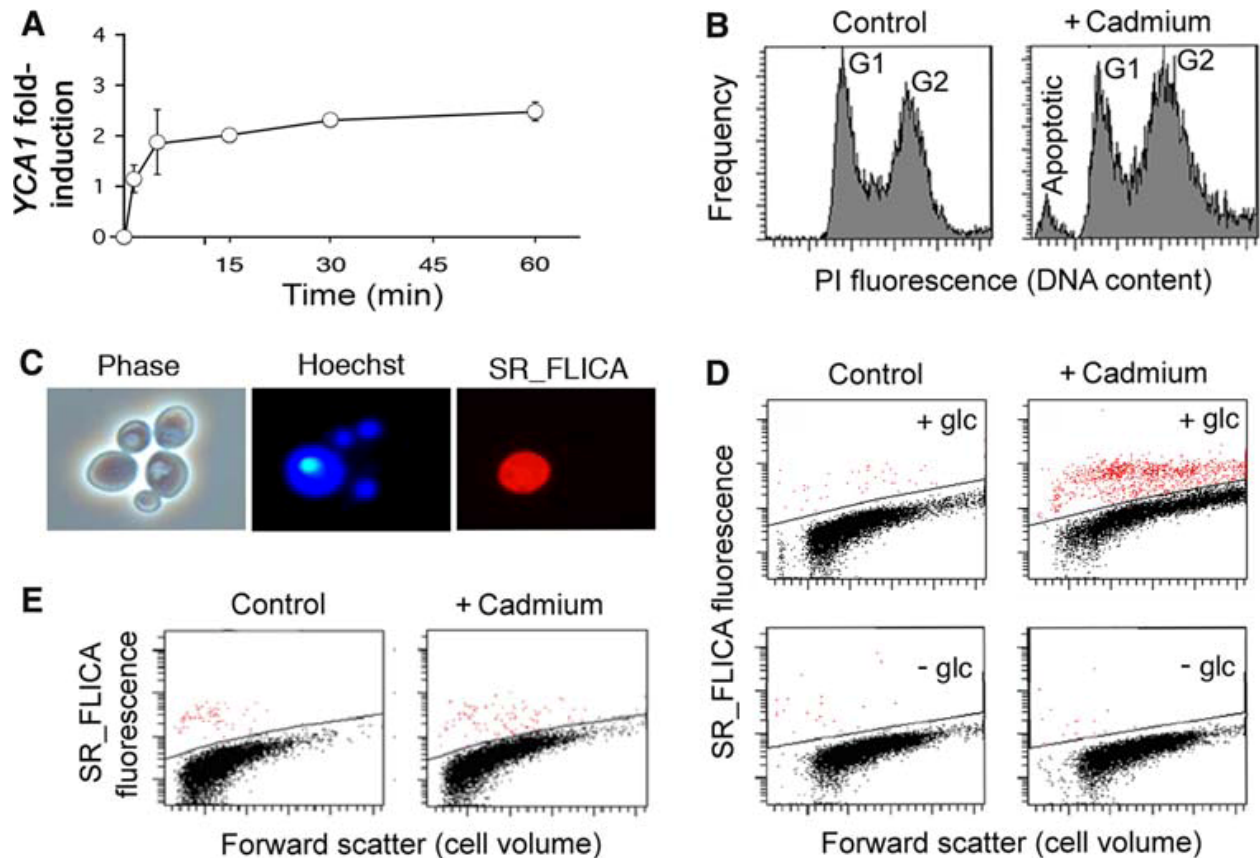


Figure 8: Cadmium induces the yeast caspase, and a glucose-dependent apoptosis

(A) Transcriptional induction of the yeast caspase gene (*YCA1*) was determined using realtime RT-PCR at various intervals after the exposure of *S. cerevisiae* (BY4741) to 30 μM $\text{Cd}(\text{NO}_3)_2$ in YEPD medium. Fold changes in *YCA1* mRNA were calculated using SDS software through the $\Delta\Delta C_\tau$ method, with *ACT1* mRNA as an internal control. The data represent fold-changes (\log_2) in *YCA1* mRNA levels. (B) Cells were exposed to 30 μM $\text{Cd}(\text{NO}_3)_2$ for 1 h then 'recovered' in fresh YEPD medium for 3 h. Cellular DNA was stained with propidium iodide, before the relative DNA contents of individual cells were determined with flow cytometry. (C) Cells treated as in (B) were tested for caspase activation with SR_FLICA detection chemistry (Immunochemistry, LLC) and analyzed by fluorescence microscopy: the top panel is a representative phase contrast image, the lower two panels are fluorescent micrographs of the same cells stained with Hoechst (middle) and SR_FLICA (bottom). No brightly fluorescent cells were observed within control populations that were not exposed to Cd. (D) Cells prepared as described in (C) were analyzed by flow cytometry. The lower panels show the corresponding analyses for cells that were incubated in the absence of glucose during the 3 h recovery period.

Accordingly, cells were incubated with 30 μM $\text{Cd}(\text{NO}_3)_2$ for 1 h, followed by a 3 h ‘recovery’ period in fresh YEPD growth medium to allow common apoptotic indicators, such as DNA fragmentation, to become apparent. The presence of a tell-tale, distinctive sub-G1 peak (Fig. 8C), representative of a subpopulation of cells within the cultures, indicated that 1 h exposure to Cd was indeed sufficient to promote an apoptosis-like response (Fig. 8C). This was despite the fact that overt cellular indicators of apoptosis (e.g., enhanced cellular complexity associated with cellular “blebbing”) did not become apparent until much later (Fig 32B).

As increased expression of *YCA1* was observed by both microarray and real time RT-PCR analyses (Fig. 8A), we ascertained whether activated caspase could be used to monitor apoptotic responses of individual cells to cadmium. We employed a fluorescent dye with the caspase inhibitor SR_VAD_FMK (SR_FLICA), which is a strong and selective indicator of the presence of “activated” caspase^{45,50}. Staining of cells that had been exposed to Cd revealed that some cells had markedly increased levels of activated caspase (Fig 8C, D). Flow cytometric analysis further indicated that this caspase-positive subpopulation comprised approximately 10-15% of the total cell population. Specificity of the fluorescent marker for caspase was confirmed by showing that the SR-FLICA-positive subpopulation was completely abolished in a (Cd-exposed) *YCA1* Δ mutant (Fig 8E).

Unlike necrotic cell death scenarios, programmed apoptotic responses to cellular stress are known to require ongoing protein synthesis and cellular metabolism^{46,51}. We

applied SR_FLICA to determine the requirement for glucose in the response of *S. cerevisiae* to 30 μ M Cd. Cells that were exposed to cadmium (as above) failed to exhibit any signs of caspase-specific apoptosis in the absence of glucose (Fig 8D). Similarly, cells that were exposed to cadmium in the presence of glucose, but which were subsequently allowed to recover from Cd exposure in its absence, also failed to exhibit any caspase-specific apoptotic response (Fig. 8D). These results indicated that the apoptotic response to cadmium is dependent on the continued metabolism of glucose, consistent with other types of stress-induced apoptotic responses observed in yeast^{46,49}.

Cadmium induces mitochondrial membrane hyper-polarization and enhanced ROS levels in a cell subpopulation

To substantiate an apoptotic response to 30 μ M Cd and to examine a potential role for mitochondria in the apoptotic process, we tested additional markers of programmed cell death. Mitochondrial dysfunction in yeast has been discovered in a number of apoptotic scenarios, the effects including mitochondrial membrane hyper-polarization, oxidative bursts and, ultimately, breakdown of membrane potential and mitochondrial fragmentation⁵²⁻⁵⁴. Alterations in the mitochondrial membrane potential ($\Delta\psi_m$; an early indicator of apoptosis) were assessed in Cd-treated cells, according to uptake of the cationic, lipophilic dye

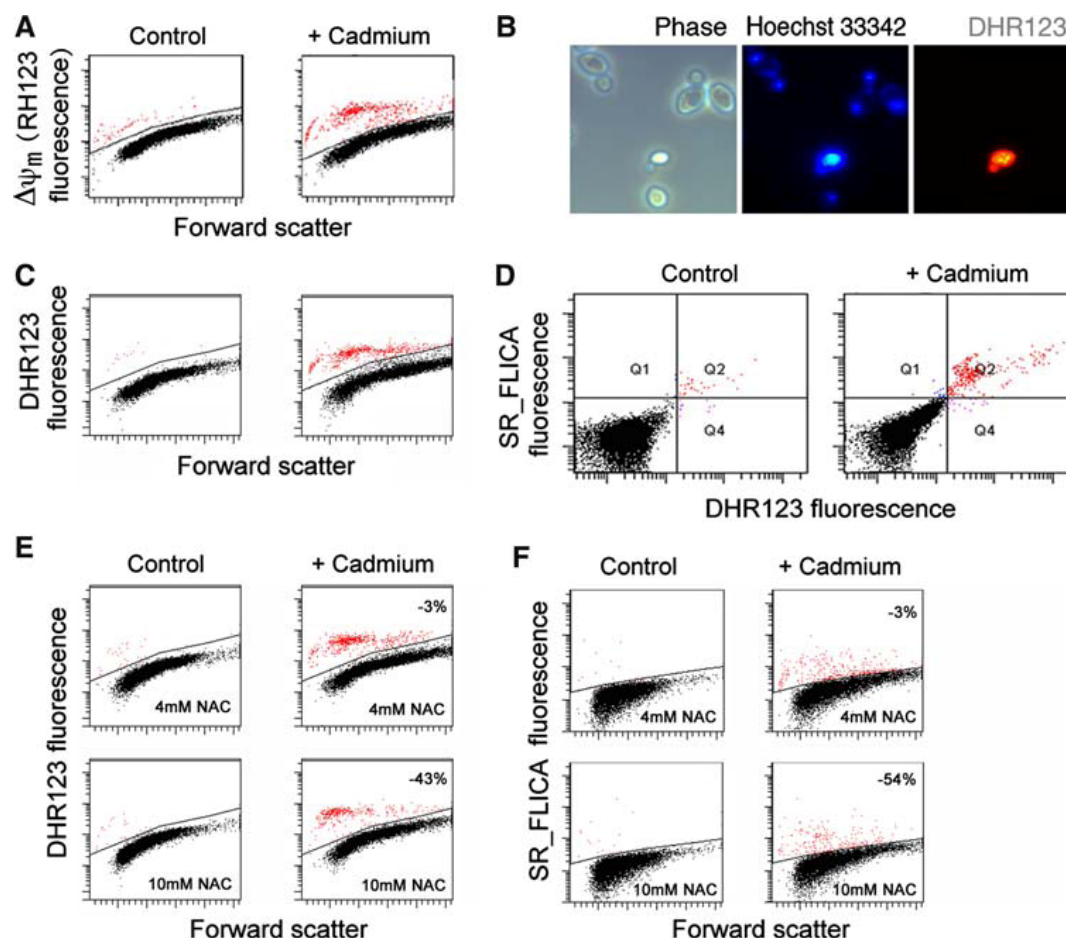


Figure 9: Cadmium induces mitochondrial membrane hyperpolarization and enhanced ROS levels in a cell subpopulation

Cells were incubated in the absence or presence of 30 μM $\text{Cd}(\text{NO}_3)_2$ for 1 h then recovered in fresh YEPD medium for 3 h. (A) Changes in the mitochondrial membrane potential as a consequence of Cd treatment were determined following staining with RH123. Fluorescence from RH123 was analyzed with flow cytometry. Control cells were treated in exactly the same way as the +Cd cells (except for the exclusion of Cd from incubations). (B) Heterogeneous production of intracellular ROS was detected using the oxidant-sensitive fluorescent dye DHR123. Cells were stained with DHR123 after incubation in the absence or presence of Cd as above. The left-most panel is a representative phase contrast image, and the images to its right are fluorescent micrographs of the same cells stained with Hoechst (middle) and DHR123 (right). (C) The dot plots show cells treated in the same way as in (B) and analyzed with flow cytometry. (D) Cells were dual-stained with DHR123 (for ROS levels) and SR_FLICA (for caspase activation), before flow cytometric analysis. The sector labeled 'Q2' contains cells that are both ROS- and activated caspase- positive. rhodamine123 (RH123) [Gross, 2000].

In contrast to control (untreated) cells, a sub-population (comprising ~5-10% of the total population of Cd-exposed cells) exhibited a distinct hyper-polarization of the mitochondrial membrane (Fig. 9A). This fraction of cells corresponded well with that exhibiting high levels of bound SR_FLICA (Fig. 8D), suggesting that these same cells induce a caspase-related, hyper-polarization of their mitochondrial membranes.

Elevated cellular ROS levels are also a characteristic of apoptotic yeast cells ^{16,46}. Furthermore, even though cadmium itself cannot induce ROS directly, it is a potent pro-oxidant and can promote oxidative stress even at the relatively low concentrations used here ²². Considering also that mitochondria are principal sources of cellular ROS and that Cd-treatment is associated with mitochondrial membrane hyper-polarization (Fig. 9A), we tested for ROS over-production in cells exposed to 30 μ M Cd. Dihydrorhodamine123 (DHR123) was used to monitor the presence of ROS in cells, as this dye readily permeates cells and is quantitatively oxidized to its fluorescent product in the presence of ROS ¹⁰. Analysis of ROS levels in this way showed that cadmium treatment did indeed elicit a marked increase in ROS production, and in a similar manner to that observed for caspase and mitochondrial membrane hyper-polarization (Fig. 9B,C). It should be noted that there is a potential for dead cells to bind fluorescent dyes, such as fcDHR123 ⁵⁵⁻⁵⁶, nonspecifically, giving rise to the artifactual labeling of dead cells and cellular debris. Such concerns were alleviated by confirming that the stained cells were still living, by their location in forward scatter plots, and by the fact that the cells remained responsive to antioxidants, such as N-Acetylcysteine (NAC: Fig. 9E).

To determine whether the cells generating increased ROS, following Cd treatment (Fig. 9B, C), constitute the same apoptotic sub-population of cells as those showing increased activated caspase (Fig. 8D), cells were simultaneously labeled with caspase inhibitor SR_VAD_FMK and DHR123. These analyses required compensation for the spectral overlap of the fluorochromes (see Materials and Methods section). The resultant dot-plots showed that the cells, which had accumulated high ROS were the same cells that exhibited high levels of caspase-induced apoptosis (Fig. 9D).

As ROS have been shown to be key regulators of apoptosis in yeast ⁴⁶, and knowing that there are marked overlaps in the responses of cells to cadmium and hydrogen peroxide ^{21,57}, it could be argued that it is the heightened production of ROS that is responsible for the caspase-dependent apoptotic response of yeast to cadmium.

To test this idea, various concentrations of the antioxidant, N-Acetylcysteine (NAC) were added to the cells to determine whether the removal of ROS was involved in the apoptotic response. N-Acetylcysteine is a thiol containing antioxidant that is commonly used as an ROS scavenger. At low concentrations (4mM) addition of NAC had little, if any affect, upon either the levels of ROS in the cells, or their apoptotic response. At higher levels (10 mM, 20 mM and 40mM), however, NAC was seen to reduce the proportion of cells that exhibited exaggerated levels of ROS (above threshold) by ~36%, 96% and >98%, respectively. This effect was also reflected in a reduced apoptotic response, which was completely abrogated at the higher concentrations (Fig. 8E). Unfortunately, especially at the higher concentrations, it is feasible that NAC may also be chelating Cd, and thereby influencing the apoptotic response in this way.

Consequently, to test the role of heightened ROS in the Cd-induced apoptosis, apoptotic indicators of cells treated with cadmium (namely caspase activation or mitochondrial membrane hyper-polarization, described above [Fig. 9]), were compared with those of cells exposed to a range of H₂O₂ concentrations, under otherwise similar conditions. Even at the highest concentration of H₂O₂ tested (5 mM), the presence of H₂O₂ in the growth medium for 1 h failed to cause any perceptible apoptotic response within the exposed cells, according to an absence of caspase activation or mitochondrial membrane hyper-polarization (Fig. 10A). H₂O₂-induced caspase activation and apoptosis evidently requires prolonged exposures such as those used previously¹⁶. Nonetheless, relatively high concentrations of H₂O₂ (1 - 5 mM) did cause elevated levels of ROS in cells after a 1 h exposure (Fig. 10B). These levels were at least as great as those observed with 30 µM Cd(NO₃)₂ (Fig. 9B) without, however, eliciting any concomitant apoptotic cell death. Consequently, the levels of ROS in Cd-exposed cells, alone, are not sufficient to explain the apoptotic response to Cd seen after 1 h exposure.

An additional observation made during the above series of experiments was that cellular ROS levels increased gradually with increasing H₂O₂ concentration (Fig. 10B), and in a much more homogeneous fashion than those seen with Cd (Figs. 9B). Thus, Cd exposed cell populations, stained with DHR123, exhibited a clear binary phenotype (i.e.,

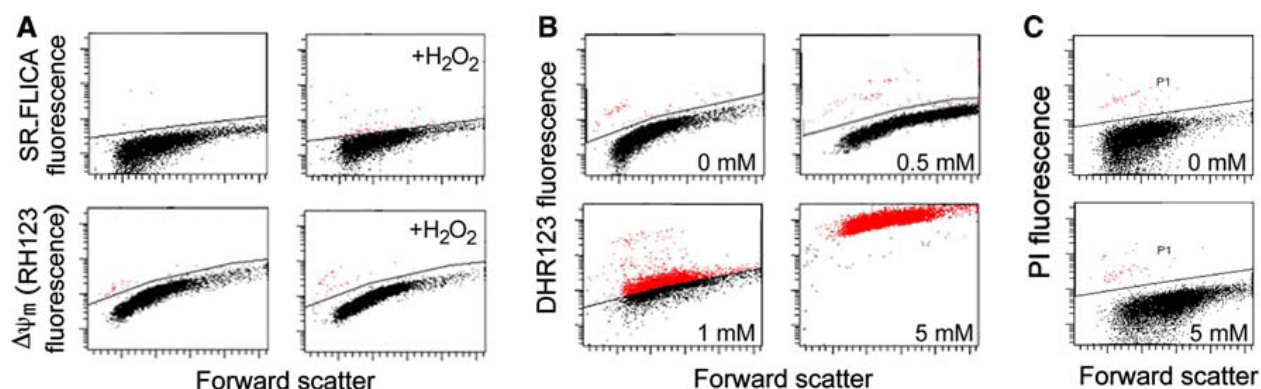


Figure 10: Hydrogen peroxide exposure causes a homogeneous increase in ROS levels in the absence of apoptosis

Cells were incubated in the absence or presence of H₂O₂ for 1 h then allowed to recover in fresh YEPD medium for 3 h. (A) The cells were stained for caspase activation with SR_FLICA (upper panels) or for mitochondrial membrane hyperpolarization with RH123 (lower panels) and analyzed by flow cytometry. Control cells (left panels) were not incubated with H₂O₂, whereas the test cells (right panels) were treated with 5 mM H₂O₂. Similar data as for 5 mM H₂O₂ were obtained for cells treated with 0.5 mM or 1 mM H₂O₂ (not shown). (B) Cells treated with the indicated concentrations of H₂O₂ were analyzed for ROS levels (DHR123 fluorescence) using flow cytometry.

individual cells had either low or high ROS), whereas ROS levels were relatively uniform across the population after 1 h exposure to any concentration of H₂O₂. Binary phenotypes commonly stem from bistable gene expression patterns upstream. In turn, these can arise from positive auto-regulatory feedback and similar regulatory mechanisms, increased input of noise in regulatory cascades, or changes in the rates of promoter state transitions ²⁶. The binary nature of ROS generation among Cd-exposed cells sets this apoptotic response apart from that of cells exposed to H₂O₂, and reinforces the value of Cd-induced stress as a model heterogeneous phenotype ²⁷.

Cadmium causes membrane damage

In an attempt to obtain additional labels for differentiating apoptotic and non-apoptotic cells within the Cd-exposed population, propidium iodide (PI) staining was employed. The application of PI as a DNA stain (Fig. 8B) relies on a membrane-permeabilization step to allow entry of the dye. The PI-impermeability of live (or apoptotic) yeast cells is commonly exploited to distinguish these from necrotic cells, in which the cellular entry of PI indicates that the membranes have been severely compromised ⁵⁸. As even low Cd concentrations cause peroxidation of membrane lipids in yeast ²⁰, we speculated that cadmium could be causing some cellular necrosis, in addition to the observed apoptosis; a possibility that we sought to test with PI (Fig. 11). Unexpectedly, the proportion of Cd-treated cells that we found to be PI-positive (~10%) approximated the proportion which we had shown earlier to be undergoing apoptotic,

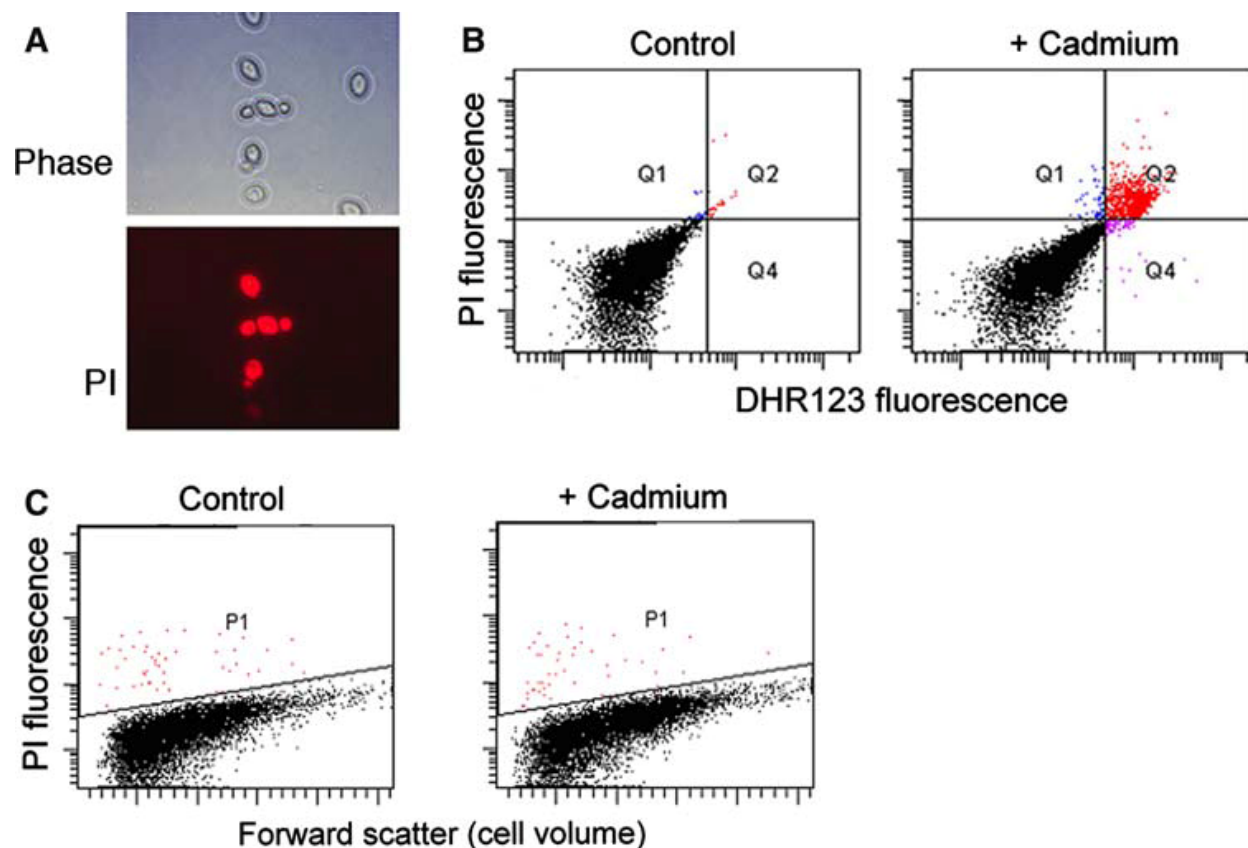


Figure 11: Cadmium-induced apoptosis in yeast is characterized by plasma membrane damage

Cells were incubated in the absence or presence of 30 μM $\text{Cd}(\text{NO}_3)_2$ for 1 h then recovered in fresh YEPD medium for 3 h, before staining for loss of plasma membrane integrity with propidium iodide (PI). (A) The upper panel is a representative phase contrast image, and the lower panel a fluorescence micrograph of the same PI-stained cells. (B) Cells were dual-stained with DHR123 (for ROS levels) and PI, before flow cytometric analysis. The sector labeled 'Q2' contains cells that are both PI- and ROS-positive.

programmed cell death. To ascertain whether the cells that were undergoing apoptosis were the same cells that had become PI-permeant, Cd-exposed cells were dual-labeled. Owing to a significant overlap in the fluorescence properties of PI and most of the other dyes used in this study, only DHR123 could be used in combination with PI here (with appropriate compensation). Dual-labeling with PI and DHR123 demonstrated that the cells which accumulated high ROS (which were also those with hyper-polarized mitochondrial membranes; Fig. 9C) were the same cells that had become sufficiently permeabilized for PI to gain entry (Fig. 11B). While permeabilization of plasma membranes is normally associated with late apoptosis, it is unusual for it to be evident so early after the onset of apoptosis.. Moreover, permeabilization of the plasma membrane does not occur in the *YCA1Δ* mutant (Fig. 11C), indicating that this, seemingly Cd-specific, membrane damage requires an active caspase. In this regard it is interesting to note that, even after prolonged exposure to H_2O_2 , which does initiate a caspase dependent apoptosis ¹⁶, there is no associated permeability to PI (data not shown). One common cause of membrane permeabilization in cells is lipid peroxidation, which is known to be a principal mechanism of Cd toxicity ^{18,20}. Lipid peroxidation has previously been implicated in the apoptotic cell death of yeast, mediated by the heterologous action of human Bax ⁵⁹. Furthermore, fatty acid induced lipoapoptosis in the fission yeast, *Schizosaccharomyces pombe*, has also been shown to have an essential ROS component ⁶⁰. Thus, it is possible that Cd-induced lipid peroxidation (and associated plasma membrane permeabilization) provides additional cellular cues that enhance the ability of this metal to elicit an apoptotic response in yeast.

The apoptotic response to cadmium is abrogated in caspase- and glutathione-deficient mutants

Having demonstrated the apparent involvement of caspase in Cd-induced membrane damage and apoptosis (Fig. 8, 2D), *YCA1*Δ mutant was further used to investigate whether the apoptotic response, itself, was dependent on this activated caspase. Cell viability was determined according to colony forming ability on YEPD agar after incubation in broth with 30 μM Cd(NO₃)₂. These analyses showed that there was a progressive loss in viability (from ~20–40% cell death) of wild type cells with time of incubation in the presence of Cd (Fig. 12A). This progression was mirrored by the proportions of cells undergoing caspase-induced apoptosis under similar conditions of prolonged exposure to cadmium (Fig. 12A). In corroboration of the suggestion that the viability loss was due to a caspase-mediated response, ~100% of *YCA1*Δ cells retained their viability under identical conditions of Cd exposure (Fig. 12A). Not unexpectedly, the *YCA1*Δ mutant did not exhibit any upregulation of activated caspase within the first hour of Cd exposure (Fig 8E). Moreover, these cells also failed to show any overproduction of intracellular ROS (Fig. 12B left panels) or mitochondrial membrane hyper-polarization (Fig. 12B, right panels), which all occur in wild type cells (Fig. 9). This indicates not only that caspase production is required for Cd-induced apoptosis, but also that the activation of caspase precedes (and is potentially a prerequisite for) the stages of the programmed cascade in this cell death response that involve mitochondrial hyper-polarization and increased ROS production⁶¹. The latter

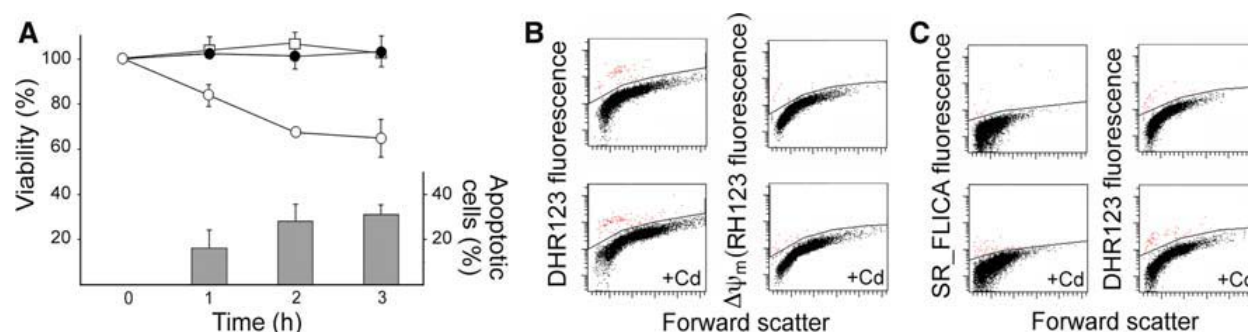


Figure 12: Cadmium-induced apoptosis is caspase- and glutathione-dependent

S. cerevisiae BY4741 (wild type) or the isogenic mutants *YCA1Δ* and *GSH1Δ* were incubated in the presence of 30 μM $\text{Cd}(\text{NO}_3)_2$ for up to 3 h. (A) Viability was determined at intervals after the addition of Cd by spread plating aliquots from dilutions ($\text{OD}_{600} = 1.0$) of the wild type (\circ), *YCA1Δ* (\bullet) and *GSH1Δ* (\square) cultures onto YEPD agar. Colonies were enumerated after incubation for 3 d at 30°C. Percentage viability was calculated with reference to the number of colonies formed by untreated cells (zero time). The histogram shows the proportion of apoptotic cells within wild type cultures at intervals after Cd addition, determined with SR_FLICA staining. No SR_FLICA-positive cells were detected in *YCA1Δ* cultures. (B) Cells of the *YCA1Δ* mutant were tested for ROS levels (DHR123 fluorescence) and mitochondrial membrane hyper-polarization (RH123 fluorescence), after incubation for 1 h in the absence (top panels) or presence of 30 μM $\text{Cd}(\text{NO}_3)_2$ (lower panels), followed by 3 h recovery in fresh YEPD. The corresponding analyses with wild type cells are shown in Fig. 9B. (C) Cells of the *GSH1Δ* mutant were tested for caspase activation (SR_FLICA fluorescence) and ROS levels (DHR123 fluorescence) after incubation, as described in (B). The corresponding analyses with wild type cells are shown in Figs. 8D (top panels) and 9B.

conclusion is consistent with the observation (Figs. 9B, 10B) that elevated levels of ROS are not sufficient, in themselves, to initiate apoptosis within 1 h.

Glutathione (GSH) plays a major role in the cellular regulation of redox homeostasis, being directly (and indirectly) involved in the sequestration of oxidatively damaged proteins^{40,62}. Glutathione is also a key component in the cellular response to cadmium-induced stress as, in addition to its role in relieving oxidative stress, it is associated with the direct chelation and vacuolar detoxification of cadmium^{41,63-64}. Consequently, cells lacking the ability to synthesize glutathione were tested to see if the absence of glutathione might precipitate an exaggerated response to cadmium. Intriguingly, *GSH1*Δ cells lacking γ-glutamyl synthetase, which catalyzes the rate-limiting step of glutathione (GSH) synthesis, retained ~100% viability following incubation in the presence of 30 μM Cd (Fig. 12A). In addition, this glutathione-deficient mutant failed to show any discernible induction of activated caspase, or heightened intracellular ROS (Fig. 12C).

Given the detoxification role of glutathione against cadmium toxicity (see above), the finding that a *GSH1*Δ mutant effectively resists Cd-induced apoptotic cell death is seemingly contradictory to the known glutathione function. Furthermore, glutathione is reported to suppress apoptosis in a number of other systems^{10,46,65-67}, although there are also examples of glutathione-stimulated apoptosis similar to our findings³⁶⁻³⁷. Evidently, in yeast there is a specific requirement for glutathione in the process of apoptotic cell death that is triggered by low concentrations of Cd. Glutathione is known

to play a critical role in a number of other cellular processes, including FeS-cluster biosynthesis and metabolic oscillations (ultradian rhythms; period <24 h) ²⁷⁻²⁹. These oscillations are associated with concerted transcriptomic and metabolomic changes ^{15,30-31}, and are susceptible to particular perturbations, such as altered levels of glutathione ⁶⁸⁻⁷⁰. Indeed, rhythm-dependent oscillations in cellular levels of reduced-glutathione (GSH) have recently been shown to underpin the variable Cd resistances of individual yeast cells ²⁷. It is possible; therefore, that such variation in GSH could also play a role in the marked cell-to-cell heterogeneity in Cd-induced apoptosis which we describe. That apoptosis only occurs within some cells of a population (i.e., is heterogeneous) is a key feature of the anticipated benefits to unicellular organisms of apoptotic cell death ⁹⁻¹⁰.

Conclusions

This paper provides the first demonstration that yeast cells mount an apoptotic response to cadmium. Moreover, there are a number of features of this response that distinguish it from other apoptotic scenarios. In particular, the plasma membranes of apoptotic cells were permeabilized to an extent that allowed entry of PI. This raises questions about the general applicability of PI as a common marker of necrosis, at least with regard to toxins that are known to induce membrane damage directly. Furthermore, the apoptotic response to Cd was highly heterogeneous, even when examined with markers such as DHR123 that, under similar growth conditions, produced a homogeneous response to another apoptotic agent, H₂O₂. These differences reflect a more complex and rapid cellular response to the presence of Cd than that described for H₂O₂, potentially mediated by a caspase-dependent mitochondrial pathway. The caspase- and glutathione-dependency of Cd-induced apoptosis provide further key insights into the nature of this response. Common apoptotic mechanisms are now known to exist in yeast and mammalian cells, and new tools for apoptosis research are being developed with yeast. Consequently, the discovery of this novel apoptotic phenotype should prove a timely stimulus to progress in our understanding of Cd-induced apoptosis.

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CHAPTER 2

METAL TOXICITY RESULTS IN CHANGE IN GENE EXPRESSION PATTERN

Introduction

Heavy metal such as copper and cadmium cause oxidative damage to the proteins leading to oxidative stress in the cells ¹. Such oxidative stress is known to be involved in pathogenicity of Alzheimer's and Parkinson's disorder ²⁻³. Oxidative stress also seems to alter protein homeostasis by causing very high rates of protein unfolding ⁴. Such unfolded proteins can aggregate and become a cause of malfunction and cell death in neurodegenerative disorder ⁵. Oxidative stress also influences the transcriptional expression in the cells. Oxidative stress causes transcriptional induction of antioxidant genes and proinflammatory genes ⁶. Certain transcription factors like *ATF4* are known to be induced during glutathione depletion during oxidative stress ⁷. In yeast, on copper exposure, Copper Responsive Transcription factor (*CRF1*) is activated, and it offers copper and cadmium resistance ⁸. Metal responsive transcription factor 1 is also induced due to oxidative stress caused by heavy metal and peroxide toxicity. In all, metals can induce stress responsive transcription factors. These transcription factors can induce several different stress related genes.

When cells are treated with copper, metabolic enzymes are targeted. Most of the enzymes of glycolysis are damaged. Such damage has been suggested to be a targeted response, even in peroxide treated yeast cells, to route the carbon flow through pentose phosphate pathway, to generate NADPH/reducing power. NADPH is important to maintain cellular redox and overcome stress ¹. Oxidation of glycolytic enzymes leads

to reduced activity of these enzymes (Shanmuganathan A, personal communication). Because metals in fact can regulate transcription factors, metals possibly repress glycolysis pathway genes and induce pentose phosphate pathway genes. Such regulation would ensure the flow of carbon through pentose phosphate pathway to deal with long term oxidative stress.

Metals can also cause an oxidative damage to the important machinery of chromatin such as DNA, histones, and protamines ⁹ Exposure to metals can lead to genetic and epigenetic changes. Gene expression can also be influenced by these changes therefore; it would be therefore interesting to study whether metals influence the transcription levels of proteins responsible for histone modification ¹⁶.

Copper and cadmium both have been shown to cause apoptosis in yeast ¹⁰ (Nargund et. al 2010). Apoptosis is programmed cell death with a cascade of events leading to cell demise. There are several genes and pathways involved in apoptosis. Transcription factors such as NF- κ B, p53, Mad, Max and c-Myc, are induced by different kinds of apoptotic stimulus. These transcription factors are involved in expression of pro-apoptotic genes in higher eukaryotes ¹¹⁻¹⁴. Different transcription factors could be induced or repressed in metal induced yeast cells- metal specific transcription factors (e.g., *SKN1* and *YAP1*) and apoptosis specific transcription factor such as AP-1. Due to the effect of metals on transcription factors, it is expected that on addition of metal, there are changes in the gene expression patterns. It is important to study the differential gene expression patterns to understand the metal toxicity and its role in apoptosis and other metabolic changes in the cells.

Here we report that the genes that belong to Apoptosis, Autophagy, cell death, and cell cycle pathway show significant changes in transcriptional expression upon metal exposure. Cell cycle pathway genes are induced more than ten-fold upon copper and cadmium insult. On analyzing changes in metabolic pathway genes e.g., glycolysis, pentose phosphate pathway and TCA, we found that glycolysis and TCA pathway genes are barely induced but some genes from Pentose phosphate pathway (e.g., *ZWF1*, *GND1*, and *TKL1* are highly induced on metal exposure.

Material and methods

Microarray

7 µg of total RNA was used to synthesize cDNA. A custom cDNA kit from Life Technologies was used with a T7-(dT)₂₄ primer for this reaction. Biotinylated cRNA was then generated from the cDNA reaction using the BioArray High Yield RNA Transcript Kit. The cRNA was then fragmented in fragmentation buffer (5X fragmentation buffer: 200mM Tris-acetate, pH8.1, 500mM KOAc, 150mM MgOAc) at 94°C for 35 minutes before the chip hybridization. 15 µg of fragmented cRNA was then added to a hybridization cocktail (0.05 µg/µl fragmented cRNA, 50 pM control oligonucleotide B2, *BioB*, *BioC*, *BioD*, and *cre* hybridization controls, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 100mM MES, 1M [Na⁺], 20mM EDTA, 0.01% Tween 20). 10 µg of cRNA was used for hybridization. Arrays were hybridized for 16 hours at 45°C in the GeneChip Hybridization Oven 640. The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 400. After this, the arrays were scanned with the Hewlett Packard GeneArray Scanner. Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning, and basic analysis. Sample quality was assessed by examination of 3' to 5' intensity ratios of certain genes. Arrays were scaled to the same target value '500'. Data was normalized against housekeeping gene actin. Data was analyzed and represented using GenSpring GX.

Realtime RT-PCR

Real-time qualitative RT-PCR was used to measure the mRNA expression levels of the one reference gene actin-a house keeping control and 3 candidate genes *ZWF1*, *GND1*

and *GSH1*. The One step RT-PCR kit, and 7500 SDS system (PE Biosystems) were utilized in these studies. Gene specific primers and FAM-TAMRA dual labeled probes for these genes were designed using the Primer express software. Two hundred nanograms of total RNA with 1 μ M primers and 0.2 μ M probes were used for the RT-PCR under the following conditions: reverse transcription (30 min at 48°C), one cycle of denaturation at 95°C for 10 mins, and PCR reaction of 45 cycles with denaturation (15s at 95°C), annealing and elongation (1min at 55°C). Data are presented as a fold change in gene expression for the candidate genes *ZWF1*, *GND1* and *GSH1* as compared to the actin (house keeping control) using the SDS software $\Delta\Delta$ Ct method.

Results

Highly differential expression of genes on copper and cadmium exposure.

On copper exposure, when the genes are clustered according to the fold change in expression, there are approximately 75 genes that are either more than 10 fold upregulated or down regulated during copper treatment time course in yeast cells (Fig 13). Cell cycle is the most significant pathway found in the result set of highly differential expression of genes on copper and cadmium exposure. The other interesting genes that are highly expressed in copper exposed yeast cells are genes of heat shock proteins. Some genes from glutathione metabolism and pentose phosphate pathway such as *GSH1*, *STR3* and *SOL4* respectively are also upregulated more than 4 fold. Gene that produced protein important for the cell to mount a response to oxidative stress (*MXR1*) is also upregulated 4.3 fold. Even gene such as *ERO1* that produces a protein involved in oxidative protein folding is upregulated more than 4 fold. *VPS51*, a gene that produces a product protein that recycles the proteins from endosomes to GOLGI, is upregulated 4.6 fold.

Similar to copper treated WT yeast cells, cadmium treated WT yeast cells also show a lot of genes that are highly expressed or repressed as compared to the untreated WT cells (14). There are about ~120 genes that are different more than 10 fold at time point during the 1 hour exposure. Like copper, the most represented pathway in these highly expressed or repressed genes is cell cycle pathway. Also, heat shock proteins and glutathione metabolism genes are higher induced on cadmium exposure. *FMO1*, a gene for a protein that is required to balance the redox condition so

Affymetrix ID	Gene Symbol	Fold change	Affymetrix ID	Gene Symbol	Fold change
4284_at	SDL1	7.284472	5773_at	CIN8	4.470686
9657_i_at	SPT5	7.143987	7565_at	MET16	4.444236
9162_at	MET2	6.952915	4361_at	LIN1	4.434847
7295_at	HSP26	6.945589	6317_at	HSP42	4.425799
6704_at	RTN2	6.651601	7322_at	YRO2	4.405058
5589_at	RTT105	5.971642	7296_at	RDH54	4.396254
7944_at	OYE3	5.949958	5697_at	MXR1	4.374726
6180_i_at	HNT2	5.781418	6905_at	FUS1	4.361638
8167_at	RAD17	5.714473	9549_at	SPG4	4.347899
9481_at	ALD3	5.599236	8982_i_at	THO2	4.333972
5588_at	SSA4	5.452825	7554_at	ARR3	4.324071
10705_g_at	SSH4	5.446249	7422_at	SSA3	4.314199
10178_at	PEP3	5.342266	7599_i_at	TPO3	4.296729
4540_at	SPO11	5.257585	4432_at	HXT5	4.289351
6580_at	PHO2	5.196295	10681_at	LAP4	4.282139
6223_at	HSP78	5.163519	9802_at	ERO1	4.23699
9996_at	TMA10	5.144405	10375_at	YCT1	4.21482
4388_at	SPS100	5.063557	9991_at	VPS65	4.207464
10416_at	GTT2	5.028066	8193_at	CIN1	4.191351
3793_f_at	MAG1	5.01718	8575_at	OPI10	4.166413
10338_at	SPO75	5.014542	9757_at	MSC1	4.159949
4423_at	RTC3	5.006745	4924_at	CTT1	4.156556
11177_at	FMP33	4.974575	4969_at	NQM1	4.153585
4768_at	SOL4	4.931758	10360_at	HSP104	4.127781
11032_at	MET3	4.872504	10501_at	ECM4	4.122262
5196_at	STR3	4.749688	5582_at	MAG1	4.105054
6394_at	FMP16	4.71277	10653_at	SRX1	4.069553
6579_at	MSS2	4.693594	5700_at	MEI4	4.061202
4842_at	BTN2	4.660311	4578_at	ECM29	4.039833
10537_at	VPS51	4.651886	5424_at	AAD6	4.036646
5751_at	CYC7	4.55653	9739_at	NUP188	4.010874

Figure 13: Highly differential expression of genes, upon 1hour exposure of wild type yeast, to copper.

GeneSpring analysis of microarray data of cells exposed to copper versus untreated cells was performed to determine the fold-change of the highly differentially expressed genes. Up-regulated and down-regulated genes referred by a positive or negative sign, respectively.

Affymetrix ID	Gene Symbol	Fold change	Affymetrix ID	Gene Symbol	Fold change
2125_s_at	TRA1	-5.7389307	2532_g_at	SEC16	-4.343775
2464_s_at	UTP20	-5.595478	AFFX-YEL021w/URA3_at		-4.3411617
2706_s_at	FKS3	-5.513602	2349_s_at	TOM1	-4.287122
2463_s_at	UTP20	-5.452354	2446_at	NUP170	-4.279956
8183_at	UBC11	-5.450941	5566_at	NSA2	-4.2772975
2362_s_at	TRM3	-5.369112	9710_at	PRM6	-4.252561
2884_g_at	KIN2	-5.363969	2108_at	MYO1	-4.2403107
2467_s_at	UTP20	-5.3351164	8642_at	IZH4	-4.2331486
2707_s_at	FKS3	-5.3047013	2967_s_at	FAS1	-4.2296343
8652_at	SPO21	-5.297813	7859_at	MEI5	-4.2075214
2215_g_at	UTP22	-5.2708817	AFFX-MurL4_ell4		-4.1851873
2964_at	FAS1	-5.2599373	2410_g_at	UGA4	-4.1822286
2128_s_at	TRA1	-5.065705	2447_g_at	NUP170	-4.175209
9920_at	SWC7	-5.044712	11414_s_at	VTH1 /// VTH2	-4.1703873
2497_s_at	CSR2	-4.892049	2264_s_at	GCN1	-4.16467
5365_at	PES4	-4.8035936	3069_s_at	CYR1	-4.1626234
2589_g_at	RPA190	-4.8026934	2181_s_at	GCN1	-4.1405277
2917_s_at	FLO10	-4.8007355	2161_at	YHB1	-4.119506
2618_s_at	IRA2	-4.777817	9694_at	OGG1	-4.103435
7308_at	FIG1	-4.7049637	5676_at	FCY22	-4.0993056
2119_g_at	TRA1	-4.6255083	2704_s_at	FKS3	-4.0962324
2965_g_at	FAS1	-4.557335	2620_s_at	IRA2	-4.0840225
2725_s_at	POL2	-4.55617	2265_s_at	GCN1	-4.0408144
2216_s_at	UTP22	-4.5450983	2564_s_at	SWI1	-4.036532
7958_at	CSM4	-4.525732	6394_at	FMP16	4.0776796
2280_s_at	GEA2	-4.480426	6579_at	MSS2	4.0981627
2378_g_at	DNF2	-4.465152	11146_at	GSH1	4.1150384
2966_s_at	FAS1	-4.429349	3793_f_at	MAG1	4.190215

Figure 14: Highly differential expression of certain genes, upon 1hour exposure of wild type yeast, to cadmium.

GeneSpring analysis of microarray data of cells exposed to cadmium versus untreated cells was performed to determine the fold-change of the highly differentially expressed genes. Up-regulated and down-regulated genes referred by a positive or negative sign, respectively.

that protein with disulfide bond will be accurately folded, is induced 5 fold at the end of 60mins. *SRX1* (gene product: Sulfiredoxin) is 4.6 fold upregulated in cadmium treated cells and is responsible for resistance to oxidative stress.

Fold change analysis of genes involved Autophagy, apoptosis and cell death in response to metals.

Metal such as copper and cadmium induce apoptosis in yeast. As expected therefore, apoptotic genes such as *AIF1*, *YCA1*, *RNY1*, *CDC48* and *POR1* are induced in both copper and cadmium treated cells (15). Although the above genes are induced there is a difference in their level of induction. Apoptosis inducing factor (*AIF1*) is induced 6 fold in copper as compared to 2.5 fold in cadmium exposed yeast cells. However, Yeast caspase 1 (*YCA1*) is induced more in cadmium 4 fold than in copper treated cells. Cell division cycle (*CDC48*) that has been shown to be important for apoptosis goes up 3 fold in copper and 2.7 fold in cadmium (16). Ribonuclease from yeast (*RNY1*) that can promote apoptosis in yeast is expressed 6 fold higher as compared to the control in both copper and cadmium treated cells. There is no change in the expression of *PET9*, an ADP/ATP carrier is required for apoptosis when compared to untreated samples.

On metal exposure proteins are oxidized and damaged. Damaged proteins and organelles are usually removed by a process called Autophagy (Fig 17,18) ⁶⁵. Hence on metal treatment it is possible that autophagic genes are upregulated. Autophagic genes such as *ATG1*, *ATG2*, *ATG3*, *ATG4*, *ATG5*, *ATG7*, *ATG8*, *ATG9*, *ATG10*, *ATG11*, *ATG13*, *ATG14*, *ATG16*, *ATG17*, *ATG20*, *ATG21*, *ATG22*, *ATG23*, *ATG26*,

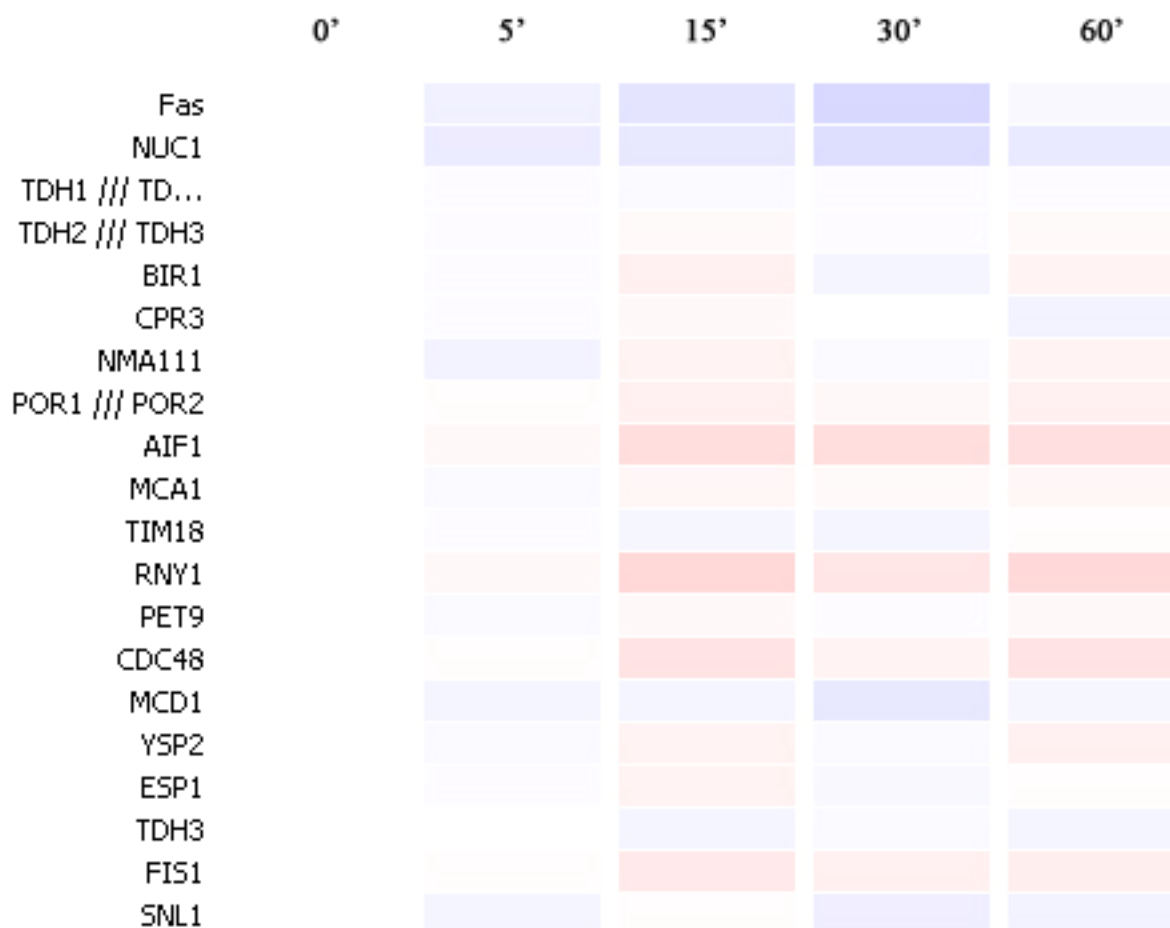


Figure 15: Fold change in expression of apoptotic genes, upon exposure of wild type yeast to copper.

GeneSpring analysis of microarray data of cells exposed to copper for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to apoptosis. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

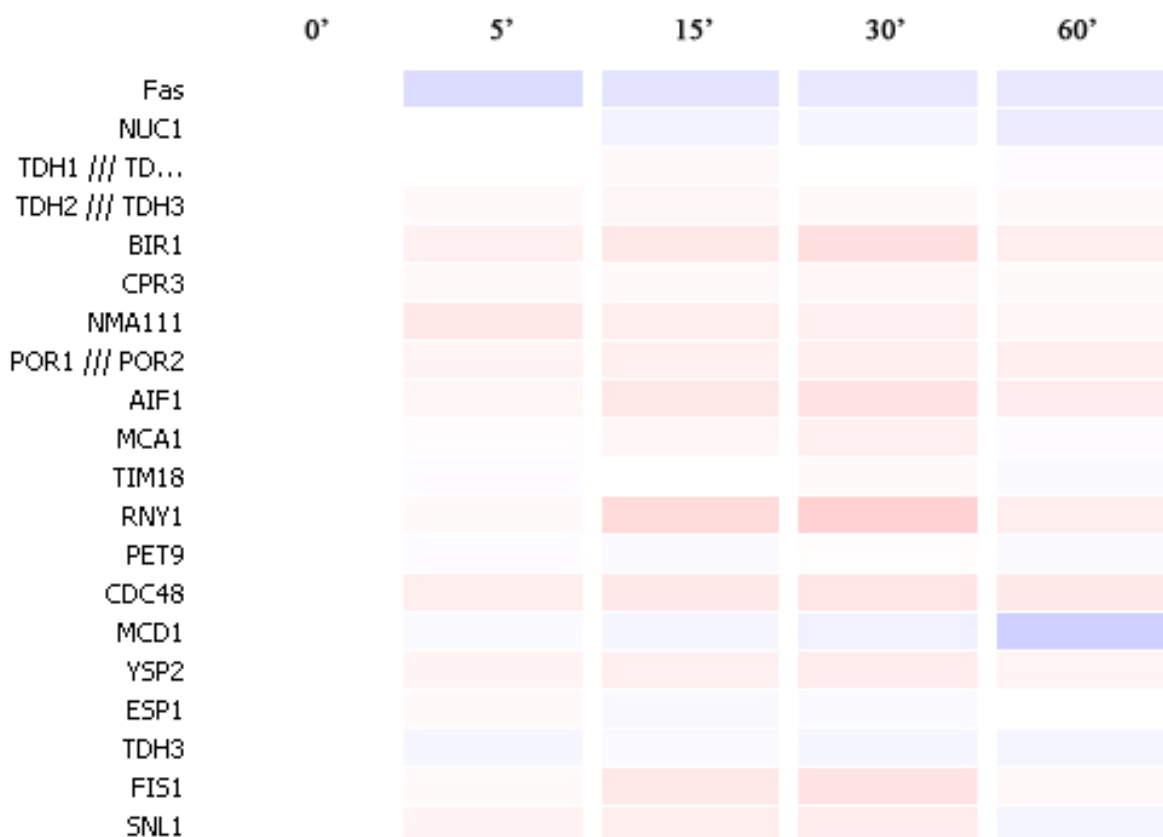


Figure 16: Fold change in expression of apoptotic genes, upon exposure of wild type yeast to cadmium.

GeneSpring analysis of microarray data of cells exposed to cadmium for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to apoptosis. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

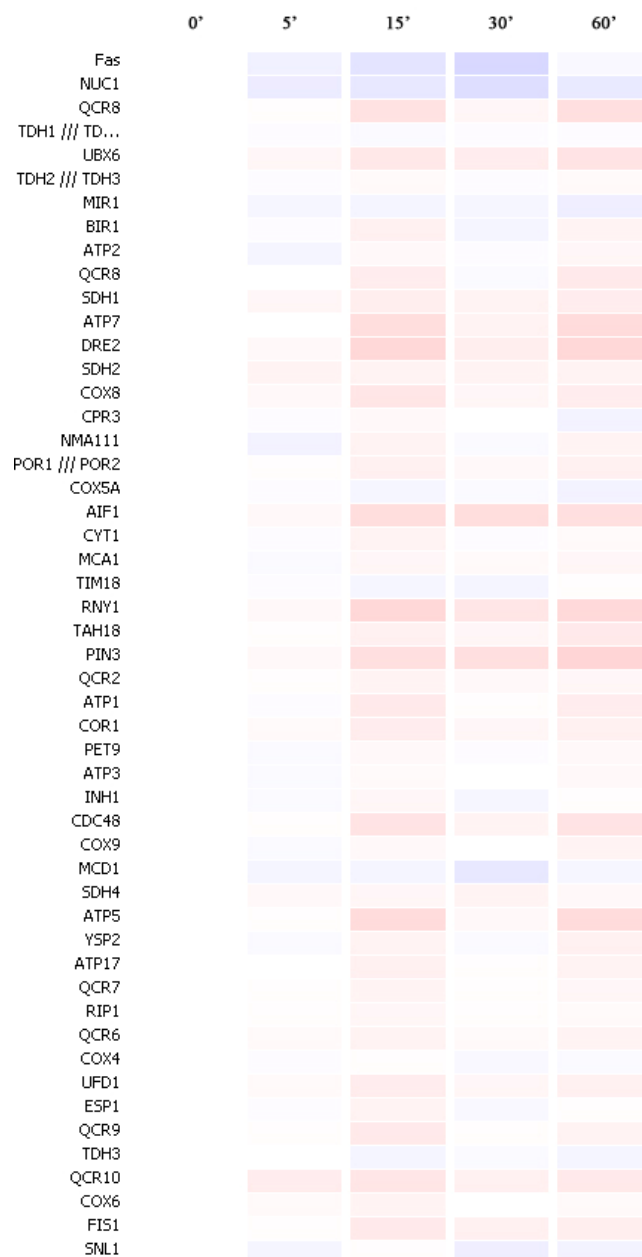


Figure 17: Fold change in expression of cell death genes, upon exposure to copper.

GeneSpring analysis of microarray data of cells exposed to copper for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to cell death. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

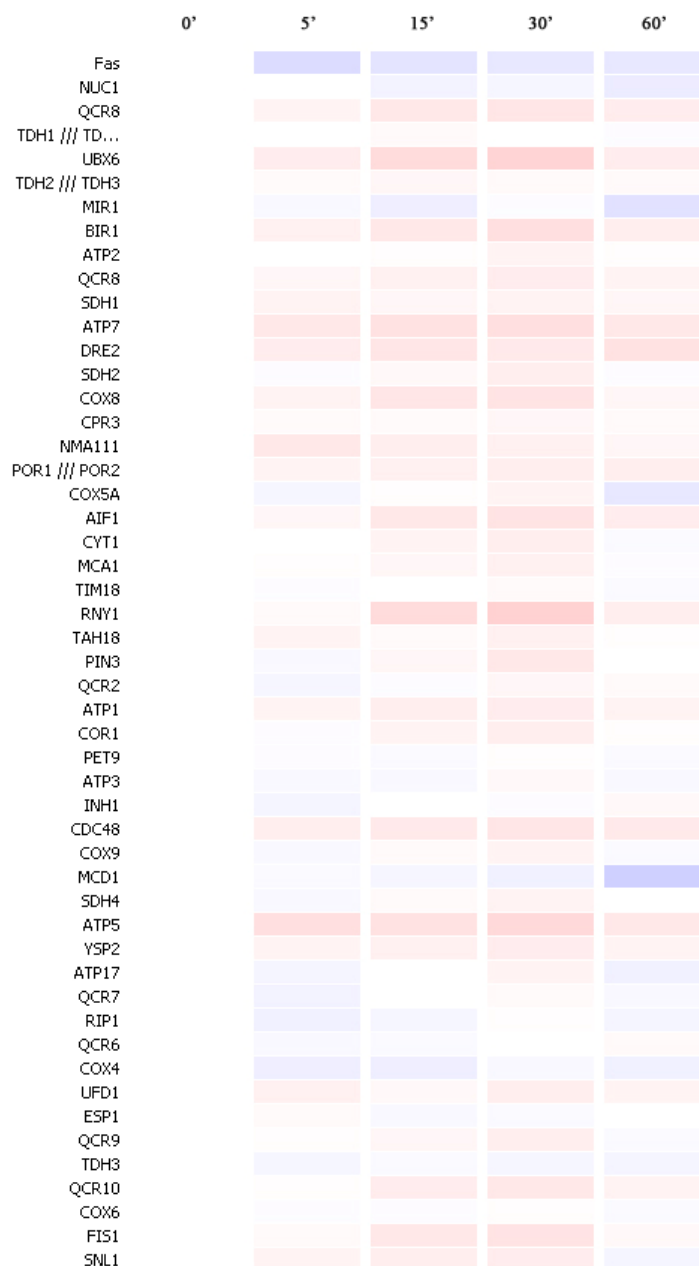


Figure 18: Fold change in expression of cell death genes, upon exposure to Cd.

GeneSpring analysis of microarray data of cells exposed to cadmium for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to cell death. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.



Figure 19: Fold change in expression of autophagy genes, upon exposure to Cu

GeneSpring analysis of microarray data of cells exposed to copper for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to autophagy. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.



Figure 20: Fold change in expression of autophagy genes, upon exposure to Cd

GeneSpring analysis of microarray data of cells exposed to Cd for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the trend in the expression of genes related to autophagy. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

ATG29 are induced on metal exposure except for genes like *ATG12*, *ATG15*, *ATG18*, *ATG19* and *ATG27* that are either repressed or not changed on metal exposure.

Several other genes that are involved in cell death such as *UBX* (Ubiquitin regulatory X), *COX3*, *CPR3*, *COX8*, *QCR2*, *COR1*, *ATP1* etc are at least somewhat induced on metal treatment. Whereas some genes such as *RIP1*, *COX4* and *NUC1* though involved in cell death in certain scenarios, remain unchanged under metal exposure.

Fold change analysis of genes involved metabolic pathways in response to metals.

We analyzed Glycolysis, Pentose phosphate pathway and TCA amongst several different metabolic pathways available (Fig 18,19,20). In glycolysis, expression of the genes except for *GPM2* and *TDH1* do not change on copper exposure. Even expression of *GPM2* and *TDH1* is barely 2 fold higher than the untreated sample. In general, there is no significant difference in the transcriptional induction of glycolytic genes in copper treated and untreated cells. In cadmium treated cells, genes such as *GPM2*, *GLK1* and *TDH1* are induced about more than 1.5 fold when compared to untreated cells.

On analysis of pentose phosphate pathway, we found that genes such as *SOL4*, *NQM1* and *GND2* are induced more than 3 fold on both copper and cadmium treatment as compared to untreated. And genes such as *RPE1* and *RKI1* are down regulated in WT treated with copper and cadmium when compared to the untreated WT.

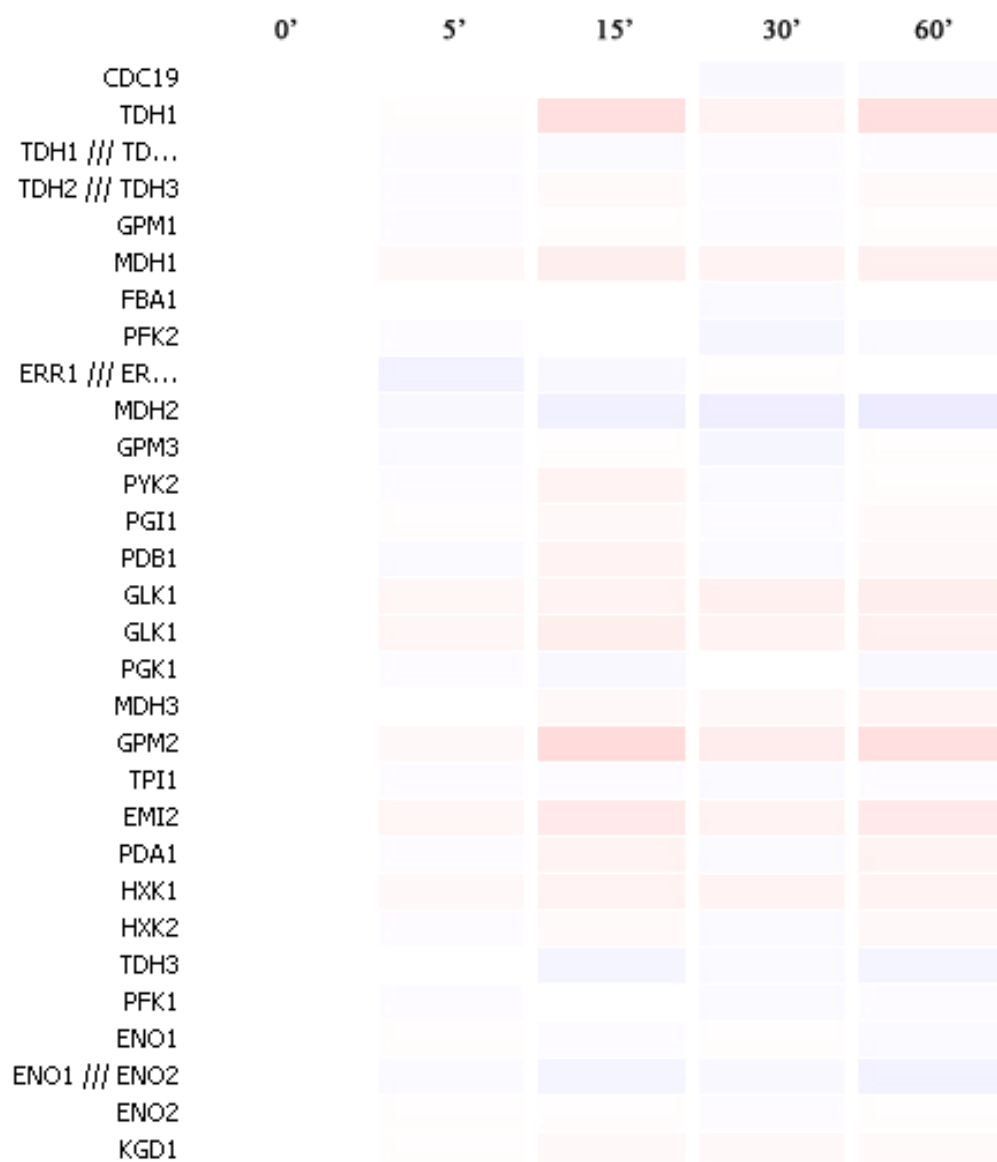


Figure 21: Fold change in expression of glycolytic genes, upon exposure to copper.

GeneSpring analysis of microarray data of cells exposed to copper for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to metabolic pathway of glycolysis. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

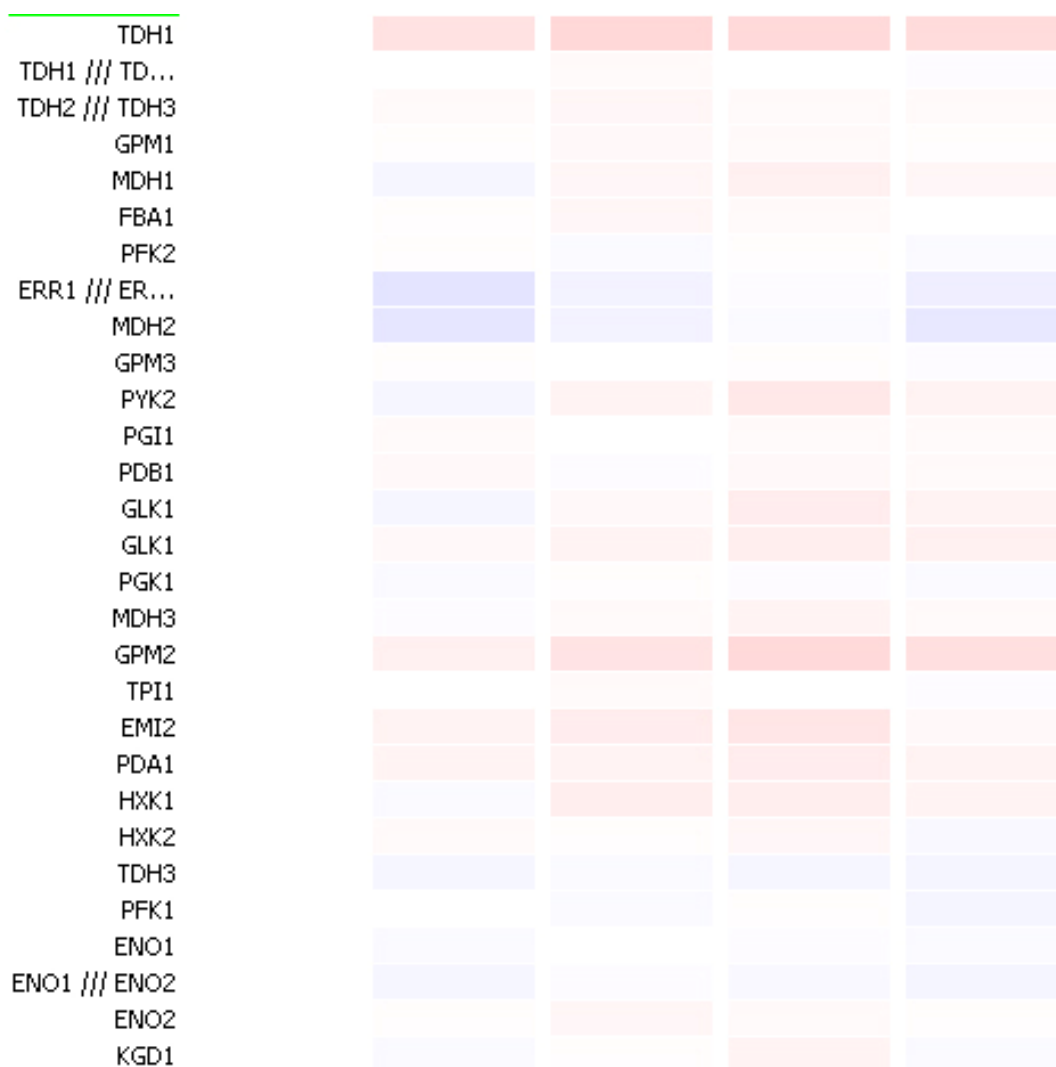


Figure 22: Fold change in expression of glycolytic genes, upon exposure to cadmium.

GeneSpring analysis of microarray data of cells exposed to cadmium for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to metabolic pathway of glycolysis. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

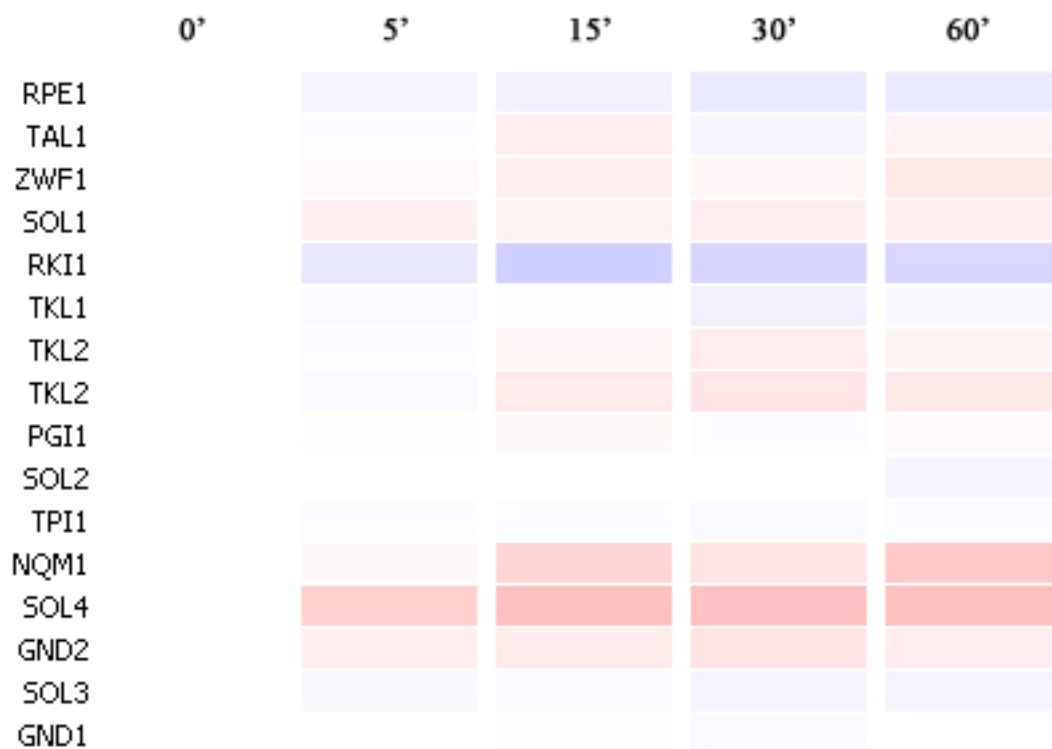


Figure 23: Fold change in expression of pentose phosphate pathway genes, upon exposure to copper.

GeneSpring analysis of microarray data of cells exposed to copper for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to metabolic pathway of glycolysis. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

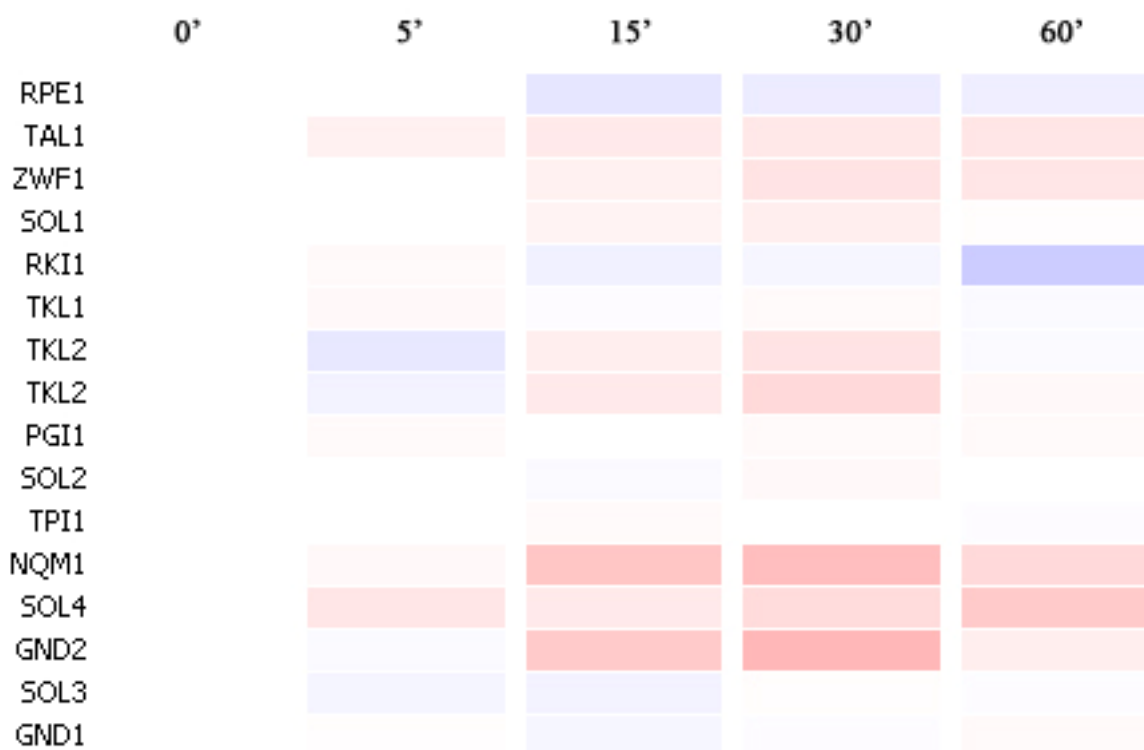


Figure 24: Fold change in expression of pentose phosphate pathway genes upon cadmium exposure.

GeneSpring analysis of microarray data of cells exposed to cadmium for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to metabolic pathway of glycolysis. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

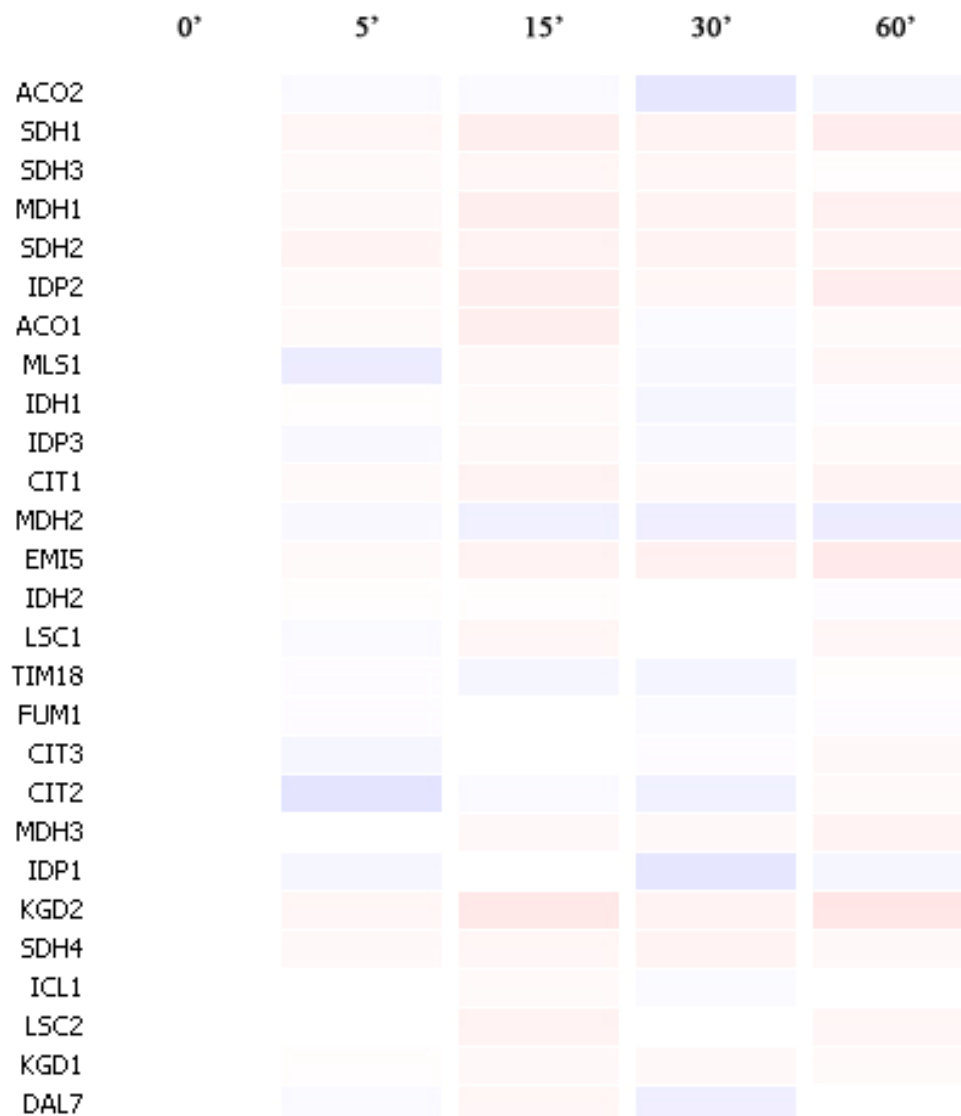


Figure 25: Fold change in expression of TCA pathway genes, upon copper exposure.

GeneSpring analysis of microarray data of cells exposed to copper for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the fold change in the expression of genes related to metabolic pathway of TCA. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

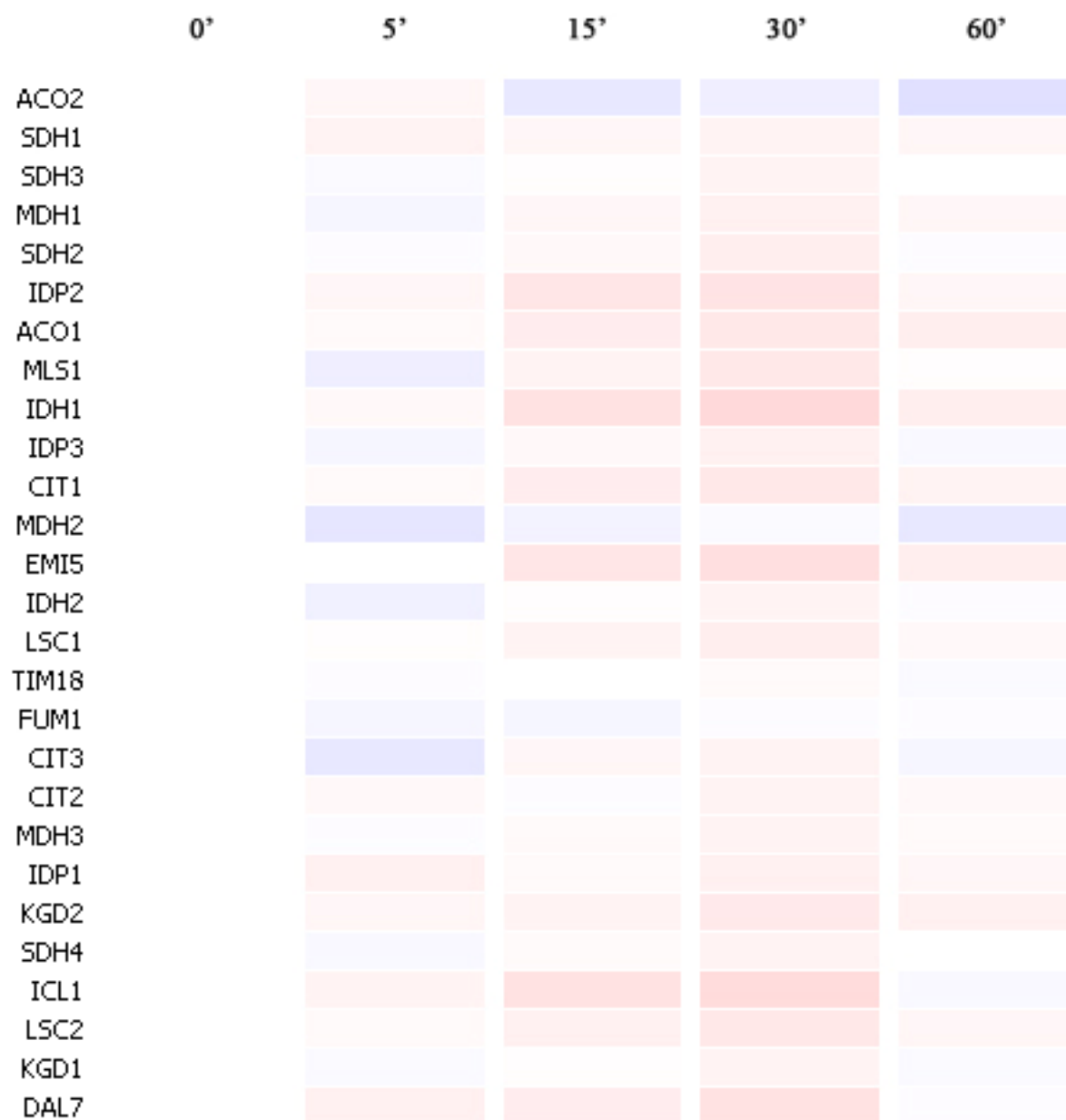


Figure 26: Fold change in expression of TCA pathway genes, upon cadmium exposure.

GeneSpring analysis of microarray data of cells exposed to Cd for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the trend in the expression of genes related to metabolic pathway of TCA. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

In WT cells treated with copper and cadmium, there is an increase in transcript levels of *IDP2* and *EMI5*; there is a decrease in the transcript level of *ACO1*. In cadmium treated cells, there is an increase in transcript levels of *IDH1*.

Fold change analysis of genes involved histone modification in response to metals.

On cadmium treatment some genes such as *SPT8*, *SWD1*, *ACS1* and *FPR4* that are involved in histone modification are upregulated transcription (21). Only some genes such as *TRA1* and *MEC1* are down regulated on cadmium exposure. There are more histone modification genes that are down regulated in copper treatment as compared to cadmium treatment.

Fold change by realtime RTPCR

Microarray being a high through put method; we verified our data by using real time RT-PCR (Taqman probe based assay). We used the sensitive FAM-TAMRA chemistry to detect the changes in the gene expression over the time period studied upon copper or cadmium exposure. Because glycolytic proteins are oxidized upon metal exposure, it is expected that flow of carbon will be routed through pentose phosphate pathway. We studied transcription of glycolysis and pentose phosphate pathway genes to better understand or speculate the carbon flow. On testing a few genes by real time RT-PCR, as seen in case of microarray we also found that transcription of *adh1* and *eno2* are not impacted as much on metal exposure. But as expected and Similar to microarray data, genes from pentose phosphate pathway such as *ZWF1* is upregulated and *TKL1* is

down regulated in real time RT-PCR. Our data from microarray corroborated very well with the real time RT-PCR data.

Hyp2 is a translation elongation factor, which we suppose will be impacted upon metal exposure, to control the level of protein translation in the cell during stress. We analyzed this factor by both microarray and real time RT-PCR and we found that *HYP2* is down regulated both in copper and cadmium treated cells. To understand the implication of such result, *HYP2* has to be further studied.

Discussion

When the WT yeast cells are treated with metals such as copper and cadmium, cells generate potentially toxic oxygen radicals (ROS). These ROS can cause oxidative stress in the cells – one potential cause of metal toxicity. Oxidative stress is also implicated in apoptosis. Previously we have demonstrated that copper and cadmium can cause apoptosis in yeast. In general, oxidative stress may compromise protein homeostasis, and is likely to induce a change in transcription pattern. In the current study we demonstrate that in the subset of genes that we analyzed that oxidative stress caused by both copper and cadmium mostly lead to similar changes in the transcription induction pattern. There are very few metal specific transcriptional differences. The reason could be that these metals though they belong to different categories such as redox active and redox inactive and though they preferentially produce different types of ROS, the induction pattern is a result of generic ROS toxicity and not as a result of direct effects of metals.

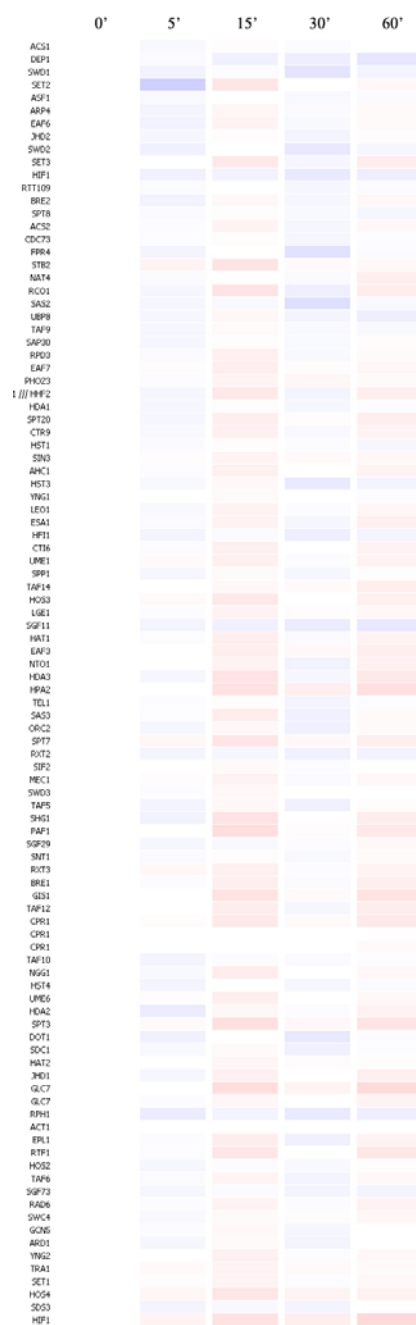


Figure 27: Fold change in expression of histone modifiers, upon Cu exposure.

GeneSpring analysis of microarray data of cells exposed to copper for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the trend in the expression of genes related to histone modifiers. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

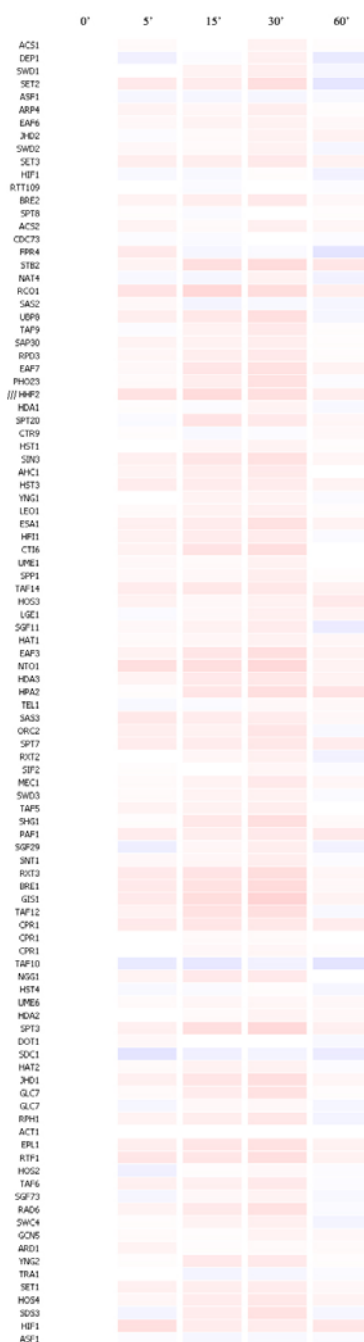


Figure 28: Fold change in expression of histone modifiers, upon Cd exposure.

GeneSpring analysis of microarray data of cells exposed to Cd for 5min, 15min, 30min and 60mins versus untreated cells was performed to determine the trend in the expression of genes related to histone modifiers. Data is represented using heat maps. Up-regulated and down-regulated genes are indicated by a blue or red color, respectively.

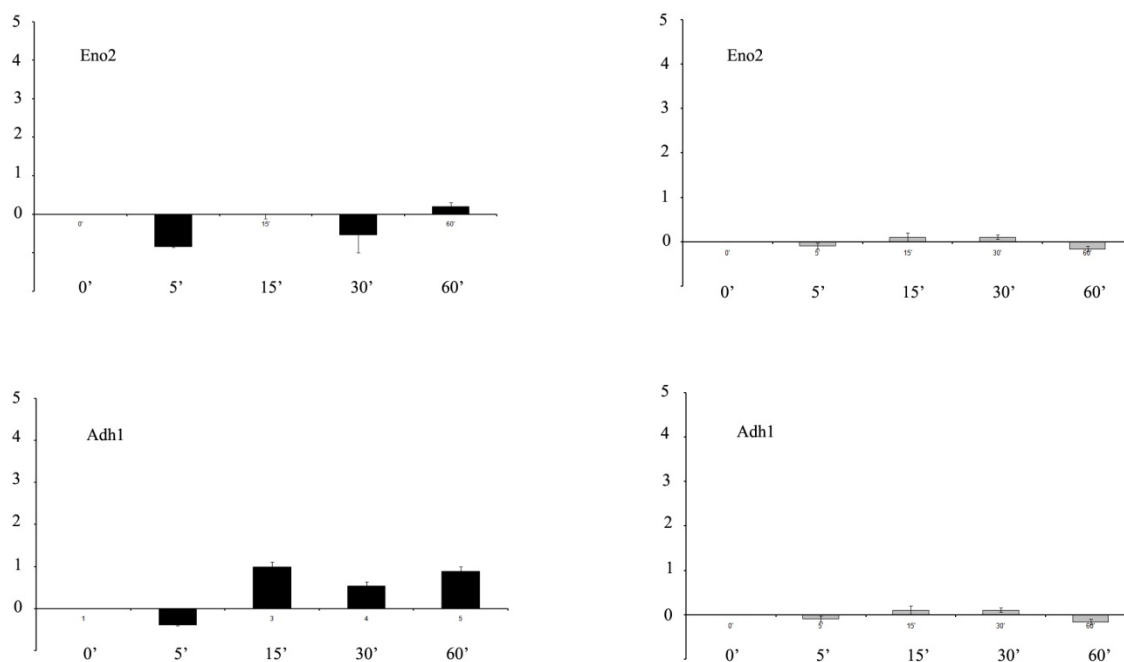


Figure 29: Fold change in expression of glycolytic genes, upon metal exposure as measured by real time RT-PCR.

Real time RT-PCR of cells treated with Cu and Cd for various time points are represented as black bars on left panel and grey bars (right panel) respectively. The fold-change was determined between untreated and treated using $\Delta\Delta C_t$ method, for the genes *Adh1* and *Eno2*.

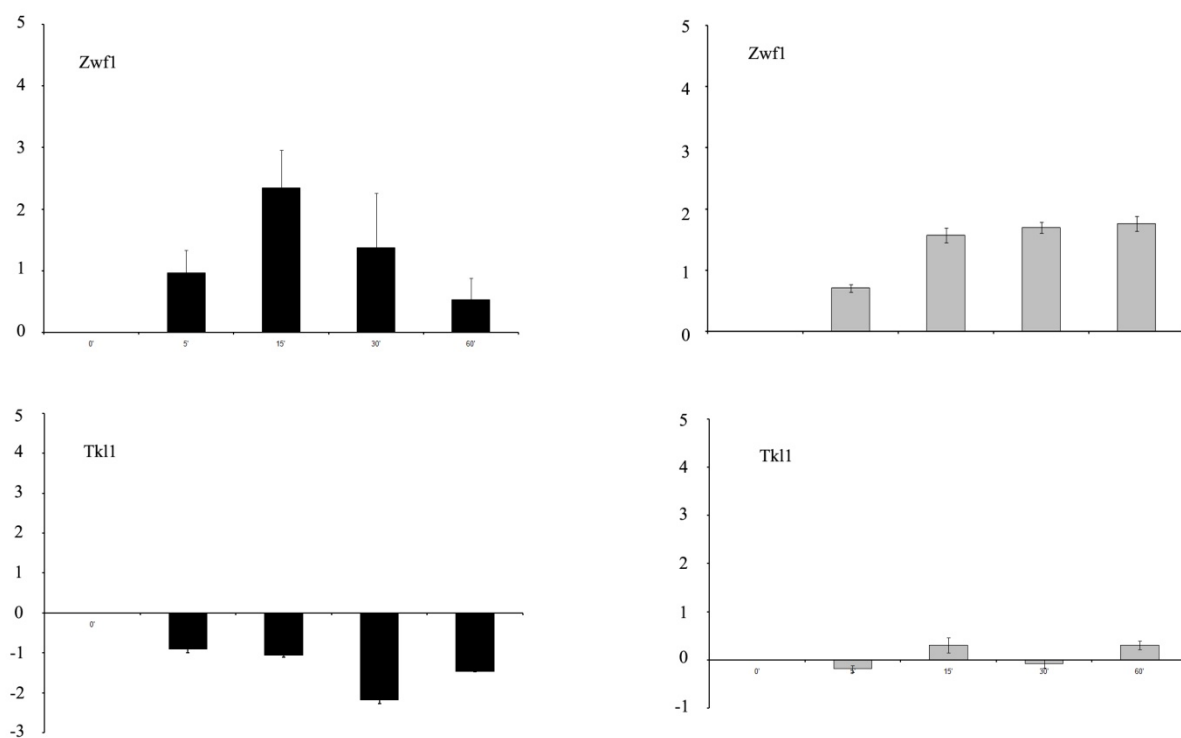


Figure 30: Fold change in expression of pentose phosphate pathway genes, upon metal exposure as measured by real time RT-PCR.

Real time RT-PCR of cells treated with Cu and Cd for various time points are represented as black bars on left panel and grey bars (right panel) respectively. The fold-change was determined between untreated and treated using $\Delta\Delta C_t$ method, for the genes *Zwfl* and *Tkl1*.

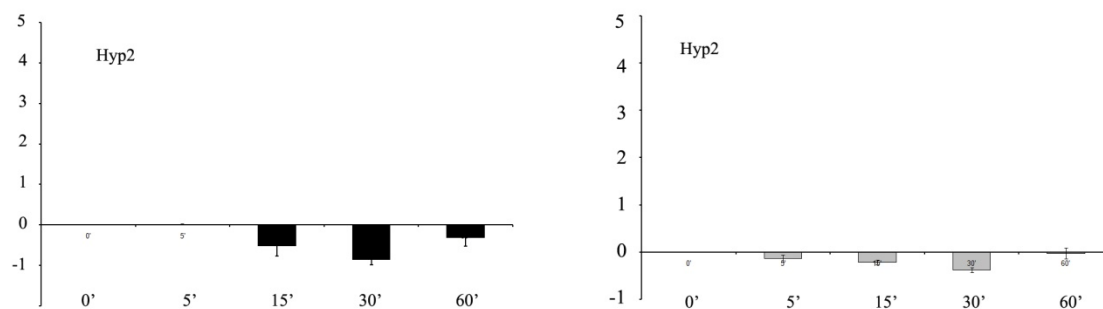


Figure 31: Fold change in expression of translation elongation factor, upon metal exposure as measured by real time RT-PCR.

Real time RT-PCR of cells treated with Cu and Cd for various time points are represented as black bars on left panel and grey bars (right panel) respectively. The fold-change was determined between untreated and treated using $\Delta\Delta C_t$ method.

In this study, we also show that Autophagy genes are induced along with certain key apoptotic and cell death pathway genes. Both the metals cause oxidative damage and such damage has been reported to trigger apoptosis. Because proteins are damaged, autophagy may be triggered to get rid of damaged proteins. Autophagy can also remove damaged organelles. In copper and cadmium treatment, we know organelles such as mitochondria are also damaged ¹⁵. Therefore, it may be possible that induction of Autophagy is a mechanism to deal with stress. If the cells are still not able to cope with stress, then may be apoptosis will be triggered. So induction of both apoptosis and autophagy genes could be a potential mechanism by which cells keep the machinery ready. It has become more and more evident that autophagy and apoptosis are connected but the connection is not known. Therefore, to guarantee a failsafe mechanism, it may be possible that one process may lead to another. To ensure such system, it is required to induce both set of genes are induced as seen in case of the current study.

Metal can not only damage protein by causing protein oxidation but also damage DNA and histones to cause epigenetic changes. Such changes can lead to a complete change in transcription pattern in the cell. Here we show that metals can induce or repress transcription of some crucial chromatin modifiers/histone modifiers. We suspect that such induction or repression may lead to the change in the histone modification and hence lead to epigenetic changes. Such DNA and/or chromatin modification is shown to be the major cause of cancers ⁹.

In this study, we show that there is an induction of pentose phosphate pathway. But the TCA and glycolytic pathway genes do not show much difference in induction

between a control and treated samples. It has been previously reported that metals such as copper and cadmium causes oxidation of protein. Such oxidation seems to preferentially damage glycolytic proteins leading to reduced rate of glycolysis. It is predicted that such reduction of the rate of glycolysis is a targeted response in the cell to increase the flow of carbon from glycolysis onto pentose phosphate pathway to produce more NADPH. NADPH, a reducing power of the cell is produced to equip Gsh, an antioxidant moiety to combat oxidative stress¹. Although, the current study does not completely prove the flux but it does indicate the flux through pentose phosphate pathway.

Finally in this study, we not only show that copper and cadmium toxicity is not only at the level of proteins by causing post translational modification but is also at the level of genetics and epigenetics.

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16. **Adriana Arita and Max Costa 2009 metallomics**

CHAPTER 3

HEAVY METAL –INDUCED APOPTOSIS IN *SACCHAROMYCES CEREVISIAE* REQUIRES THE INTERCESSION OF AN AUTOPHAGIC PROTEASE, ATG4

Amrita M. Nargund and John E. Houghton*

Heavy metals, such as copper and cadmium have been linked to a number of cellular dysfunctions associated with autophagy and apoptosis in both uni- and multicellular organisms. Having recently demonstrated that low levels of cadmium elicit a caspase-specific programmed cell death (PCD) in *Saccharomyces cerevisiae*¹, we here confirm that low levels of copper can also provoke a similar apoptotic response in yeast -as evidenced by mitochondrial hyperpolarization, superoxide generation, activation of the Yeast caspase-1 (Yca1) and resultant DNA fragmentation. In attempting to delineate the mechanism of apoptosis, induced by such inherently different redox-reactive metals, we determined a number of similarities and differences in the way that each involves an additional caspase-like protease, which (along with Yca1) precedes hyperpolarization of the mitochondrial membrane in the apoptotic process. We further demonstrated the intriguing dependence of this caspase-like proteolytic activity on Atg4, a cysteine protease that is intimately involved in the latter stages of autophagy. This study also shows that Atg4 participates in the cleavage activation of Yca1 in cells exposed to either metal, and as such, provides a critically important link between metal-induced autophagy and apoptosis in *Saccharomyces cerevisiae*.

Introduction

(Macro)autophagy and apoptosis specify two similar, but distinct programs for organellar and cellular fragmentation, the one -effectively to recycle damaged or toxic cellular components (sequestered within double membrane autophagosomes), the other -to dismantle the cell from within, and thus minimize the spread of cell debris resulting from cell death ^{2, 3}. Both cellular programs provide integral components of cellular homeostasis, cell development and differentiation ^{4, 5}, with each also providing a potential counterpoint to unfettered cell proliferation and, in higher eukaryotes, aberrant cancerous growth (at least in the early stages of tumorigenesis) ^{4, 6-8}. Both apoptosis and autophagy have been shown to occur in the budding yeast, *Saccharomyces cerevisiae*, which not only demonstrates a remarkable mechanistic conservation of these cellular programs among eukaryotes, but also that yeast can provide a useful cellular repository for the heterologous expression and evaluation of autophagic and apoptotically related proteins ^{9, 10}.

While the potential for *Saccharomyces cerevisiae* to undertake autophagy has been well documented, and the autophagic pathway is well characterized ^{11, 12}, the role of an apoptotic program in its cellular response to stress, are less well established. Indeed, from the first determination that yeast encoded a solitary caspase (Yca1p) ^{13, 14}, the potential for programmed cell death (PCD) within a unicellular organism raised more than a few concerns as to why such a process should be retained in a unicellular organism. Moreover, given the paucity, in yeast, of additional analogues of many of the

other apoptotic-related intermediates, significant questions have been raised as to whether a singular yeast caspase (Yca1p) is even capable of initiating a fully-fledged response^{15, 16}. Even so, modeling of the yeast caspase has indicated that Yca1p retains sufficient structural homology with other caspases, which, together with its caspase-like processing from an ~45 KDa inactive form to a smaller 35KDa active cysteine protease with a substrate preference for an aspartate residue within its target sequence^{14, 17, 18}, provide sufficient justification for Yca1p to be considered as a *bone fide* caspase. Moreover, a number of benefits for the retention of Yca1p activity in mediating oxidative stress, osmotic shock and viral toxins have been proposed^{16, 19, 20}. To this list of environmental stressors that induce some form of Yca1p-dependent cellular response, we have recently been able to add the heavy metal, cadmium, which elicits a caspase-dependent apoptosis within a subset of exposed cells with markers of apoptosis such as ROS generation, Yca1p activation, mitochondrial hyperpolarization and DNA fragmentation¹. Moreover, as cadmium is unable to generate ROS by itself^{21, 22}, we were also able to conclude that Yca1p was necessary for the induction of any and all discernable markers of apoptosis (including heightened levels of ROS), indicating that at least some of the required Yca1p activity preceded any mitochondrial membrane perturbation. This was in contrast to similar caspase-dependent cellular responses of yeast to oxidative stress induced by peroxide or menadione^{14, 17, 23}, in which the activation of the yeast-caspase was found to be subsequent to any mitochondrial damage. These findings prompted a series of questions as to the potential mechanism(s) that underpin the response of yeast to a low level exposure of the heavy metal, cadmium, and whether such a response was limited to cadmium, or

whether other heavy metals -even those able to generate ROS directly- initiate a similar apoptotic response.

Here, we show that copper (a *redox-active* heavy metal, fully capable of directly inducing high levels of ROS) appears to induce a pattern of apoptotic response in yeast that is similar, but distinct to that induced by the *redox-inactive* heavy metal, cadmium. We further demonstrate that the caspase-dependent apoptotic response to either metal, in *Saccharomyces cerevisiae*, is more complex than had been anticipated, and that this metal-induced programmed cell death requires the intercession of an autophagic protease, Atg4p.

Materials and methods

Strains and treatments

S. cerevisiae strains wild type BY4741 (MAT α his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0), Δ ATG4 (YNL223W) and Δ YCA1 (YOR197W) were obtained from Euroscarf (Frankfurt, Germany). Δ ATG4/ Δ YCA1 double knockout strain was made using PCR based gene deletion strategy as described in Janke et. al. To achieve a double knockout Δ ATG4/ Δ YCA1 strain, ATG4 gene deletion was carried out in Δ YCA1 mutant background. Strains were inoculated from 24 h starter cultures into YEP broth with 2% glucose as a carbon source. For induction, at the beginning of the experiments, Cu(NO₃)₂ or Cd(NO₃)₂ was added to culture flasks (OD₆₀₀ ~2.0) to a final concentration of 8mM and 30 μ M respectively. Cells were then washed and incubated in YEPD for 3 hrs for apoptosis markers to develop. Again when necessary N-acetyl cysteine was added to the final concentration of 10mM as described in Nargund *et al.* '08¹

Cell viability

For Viability, both untreated and BY4741 cells treated Cu(NO₃)₂ for 1, 2, and 3 hours were analyzed by serial 10-fold dilutions spotted onto YEPD plates and incubated for 3 d at 30°C. Cell death was quantified by plating 1:1000 dilution onto YEPD plates and counting colonies. The percentage of colony-forming units (c.f.u.) of cells treated with cadmium nitrate was obtained by relating the c.f.u. counts of treated cells to those of untreated cells, which were considered 100 %.

Test for Apoptotic markers.

To assess apoptosis, presence of the caspase was detected by FLICA Apoptosis detection kit (Immunochemistry Technologies, LLC). Cells were induced as mentioned in strains and treatments and stained with SR_FLICA reagent as per manufacturer's protocol. The relative intensity of Sulphorhodamine fluorescence in the cells was determined at 590 nm emission using a FACSCanto (BD Biosciences). Images were captured using a Zeiss Axioimager fluorescent microscope equipped with Zeiss CP-ACHROMAT 100X/1.25 oil objective, a red filter and a Zeiss AxioCam MRc5.

Intracellular ROS were detected by using the oxidant-sensitive Dihydroethidium (DHE, Sigma aldrich) as described in Madeo *et al.* '99³³. Cells were then imaged under fluorescent microscope as described above using a red filter and quantified on FACSCanto (BD Biosciences) at 590nm emission filter.

To assess mitochondrial membrane potential, cells were incubated with final concentration of 2 μ M Rhodamine 123 and analyzed on FACSCanto (BD biosciences) at 525nm as emission wavelength as described in Gross *et al.* '00²⁶ and Nargund *et al.* '08¹. To determine live cells from dead cells, fluorescein diacetate (FDA) was added to the cells at a final concentration of 10 μ g/ml and incubated for 15mins. Cells were then observed under microscope equipped with Zeiss CP-ACHROMAT 100X/1.25 oil objective, a green filter and a Zeiss AxioCam MRc5 and were also quantified using FACSCanto at 525nm emission wavelength to make sure FDA gets into the cells and is metabolized to Fluorescein by cellular esterases.

Fluorimetric analysis of caspase activity

Caspase activity assay was carried out as described in Saraiva *et al.* '06³². Cells were grown and induced as mentioned in the strains and treatment section above. Cells were harvested and mechanically disrupted in lysis buffer (20mM Hepes pH7.4, 84mM KCl, 10mM MgCl₂, 0.2mM EDTA, 0.2mM EGTA, 1mM DTT and 10% glycerol). Caspase activity was determined for per µg of protein by incubation of protein lysate with 50µM fluorogenic caspase substrates N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) and N-acetyl-Ile-Glu-Thr-Asp (Ac-IETD-AMC) in 200µl assay buffer (50mM Hepes pH7.4, 100mM NaCl, 0.1% CHAPS, 10mM DTT, 1mM EDTA and 10% glycerol). Untreated cells were used as a negative control. AMC release was monitored using Victor³ microtitre plate reader (excitation 380nm and emission 460nm) for 90mins at 30°C. Caspase activity was measured by calculating slope of linear regressions and expressed as arbitrary fluorescence unit/min/µg of protein.

Western blot analysis of caspase activity

Proteins were harvested from WT, $\Delta ATG4$ before and after metal treatment for 1hr and then 3hr of incubation in YEPD. Proteins from $\Delta YCA1$ were also prepared to serve as negative control. Cadmium treated WT cells were also sorted using FACS Aria to obtain non-apoptotic cell population (98% purity). Proteins prepared from these non-apoptotic cells were also used to serve as a negative control. Proteins were then run on 8% SDS-PAGE and blotted overnight onto nitrocellulose membrane. Yca1 was then detected by western blotting using rabbit anti-yca1 antibody (manufactured by Genscript Inc, using custom peptide 'CGGQTEDLDGDEEDG' as an antigen).

Results and Discussion

Copper induces apoptosis in the presence of an activated yeast caspase

We have previously demonstrated that relatively low concentrations of copper and cadmium are able to induce oxidative-stress in yeast, and that, for cadmium at least, this stress results in the rapid onset (within 4 h) of apoptotic, programmed cell death in some of the exposed yeast cells ^{1, 24}. These studies have further shown that metal-induced oxidative stress peaked within 1h of exposure, and that such a limited exposure to cadmium was sufficient to elicit an apoptotic response in some of the cells, which accounted for any and all loss of cell viability as a result of exposure to cadmium. A similar series of apoptotic cellular responses as indicated by some of the more diagnostic events that are known to characterize the apoptotic process; such as activation of caspase, DNA fragmentation and apoptotic bodies formation (Fig 32A, B, C & D); ^{1, 20, 25}, are also apparent for cells exposed to low levels of copper (8mM) for 1hr and then incubated in YEPD (devoid for copper) for 3 hrs for the apoptosis to develop. That the yeast caspase, Yca1p, was involved in this apoptotic response was confirmed by the absence of any apoptotic response in the deletion mutant to an extended exposure (up to 3 h; Fig 32A). Data also confirm that apoptosis accounts for any and all loss of cell viability in the wild-type cells exposed to copper, and that such a short term exposure is sufficient to induce a caspase-specific apoptotic response in a sub set of the population of exposed cells (15-20%; Fig 32A).

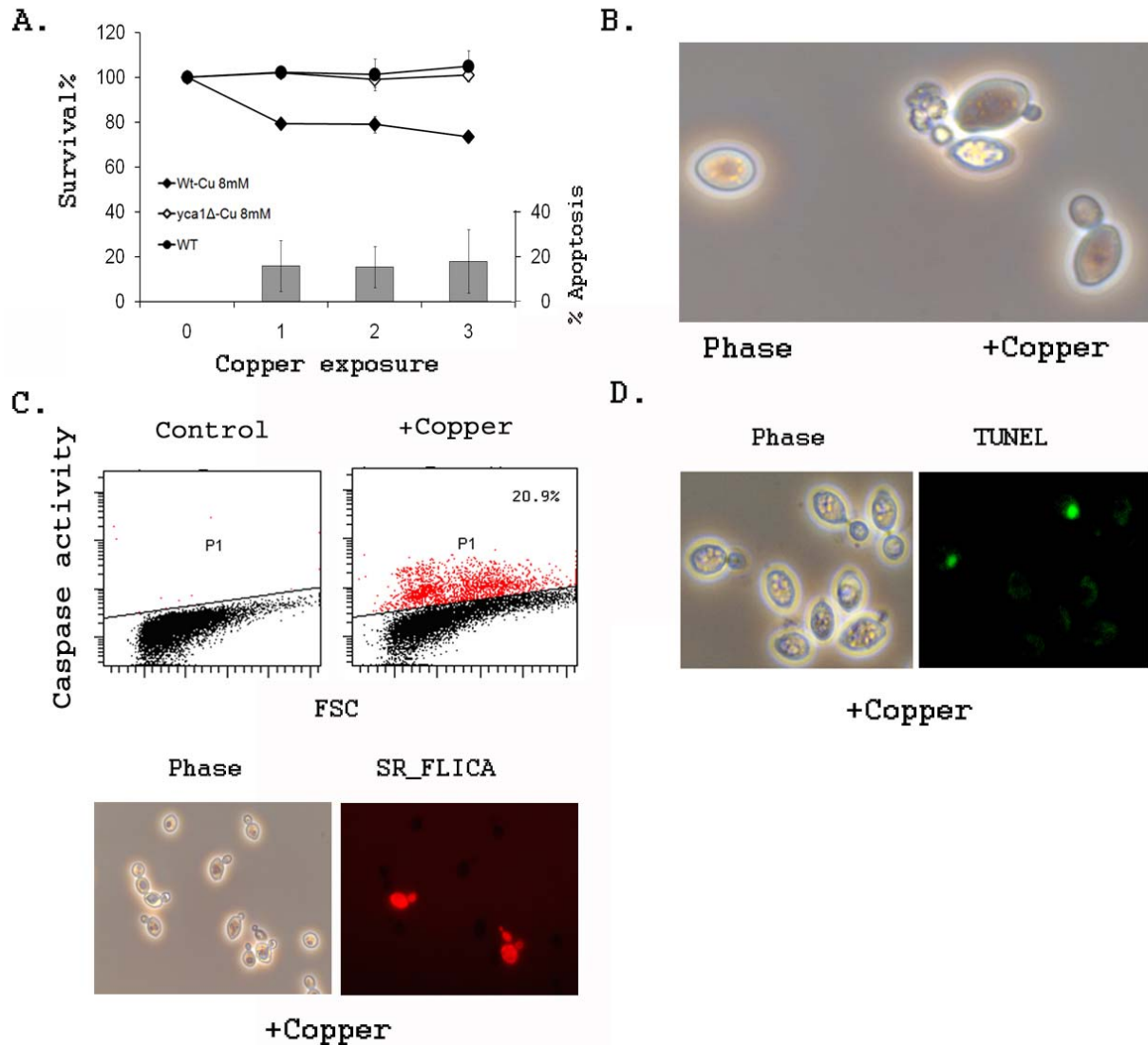


Figure 32: Copper induces caspase-dependent apoptosis in *Saccharomyces cerevisiae*

(A) *S. cerevisiae* strains, BY4741 (wild type) and YCA1Δ, were incubated in the presence of 8 mM Cu(NO₃)₂ for up to 3 h. Viability was determined at hourly intervals after the addition of Cu by the spotting of aliquots from dilutions (OD₆₀₀ = 1.0) cultures onto YEPD agar, and incubation for 3 d at 30°C. (B) Formation of apoptotic bodies after treatment of wild-type cells, using phase contrast microscopy. Cells were exposed to 8mM Cu(NO₃)₂ for 1 h then allowed to 'recover' in fresh YEPD medium for 3-4 h. (C) WT Cells were treated as in (B), after which time the cells were stained for active caspase (red) with SR_FLICA detection chemistry (Immunochemistry, LLC), and detected by FACS Canto (top panel) and fluorescent microscope (bottom panel). (D) Cells were prepared as in (B), and subsequently stained (green) with TUNEL reagents, as described in Madeo *et al.* '99³³ and observed under fluorescent microscope.

Copper induces mitochondrial membrane hyper-polarization and enhanced superoxide levels in a sub population cells.

Copper, as one of the more potent *redox-active* metal, is capable of generating ROS, even after only 1h of exposure, as opposed to cadmium, which is considered to be redox-inactive and cannot. The extent to which ROS are produced in cells treated with copper is seen to be far in excess of any produced by cells exposed to cadmium (Fig 33A). Yet the presence of either heavy metal seems to modulate an apoptotic response after just an hour of metal exposure, suggesting that the two metals could take potentially different routes for inducing apoptosis. This rationale prompted further evaluation of the action and route of copper mediated apoptotic process. Mitochondrial membrane hyperpolarization ($\Delta\psi_m$) is an early indicator of apoptosis, and was assessed in Cu-treated cells by analyzing the uptake of the cationic, lipophilic dye rhodamine123 (RH123) ²⁶. In contrast to untreated cells, ~15-25% of the total population of cells that had been exposed to copper exhibited hyper-polarization of their mitochondrial membrane (Fig 33C). Elevated levels of ROS, due to stress, is also a characteristic of apoptotic yeast cells ^{14, 17}. Consequently, dihydroethidium (DHE) was used to demonstrate the presence of increase superoxide within ~19% of the cells that had been exposed to the copper (Fig 33C). Copper is a “*redox-active*” metal, and is therefore capable of inducing reactive oxygen species (ROS) within the cell directly, though Fenton or Harber-Weiss reactions ^{27, 28}. Thus ROS in copper-treated cells can result from either direct formation of free radicals through these reactions, or through hyperpolarized mitochondrial membranes, which can result in oxidative bursts and, ultimately the breakdown of membrane potential and mitochondrial fragmentation ^{1, 29-31}.

This would not be the case for cells exposed to the “*redox-inactive*”, cadmium, which requires the intercession of other metals or cellular functions to produce ROS ^{21, 22}. What is more, previous comparisons of wild-type and *YCA1* Δ mutant responses to cadmium strongly implicated a requirement for an activated Yca1p to provide both mitochondrial hyperpolarization and ROS production ¹.

Yeast Caspase-1 is also involved in copper mediated mitochondrial membrane hyperpolarization.

In order to take advantage of this potential difference between the two types of heavy metals, and to ascertain whether an activated caspase is also a minimal requirement for mitochondrial perturbations in copper-induced apoptosis, ROS and mitochondrial membrane hyperpolarization ($\Delta\psi_m$) were monitored in different yeast cell types exposed to either cadmium or copper, and then in the presence or absence of a pan-caspase inhibitor Z-VAD-FMK (Figs 33B & C).

The results demonstrate that Z-VAD-FMK is able to mitigate Yca1p activity, as it effectively abrogates any Yeast caspase-1-mediated mitochondrial membrane hyperpolarization and ROS production in cells that were exposed to cadmium (Fig 33B) in a manner that is equivalent to a *YCA1* deletion mutant (*YCA1* Δ). The effects of Z-VAD-FMK on cells that had been exposed to copper were less dramatic, however (Fig 33C), as it reduced, but did not completely abolish mitochondrial hyperpolarization in these cells. The incomplete removal of mitochondrial membrane hyperpolarization and ROS in similar experiments using *YCA1* Δ cells that were exposed to copper, further indicates that Yca1p is not the sole mechanism through which

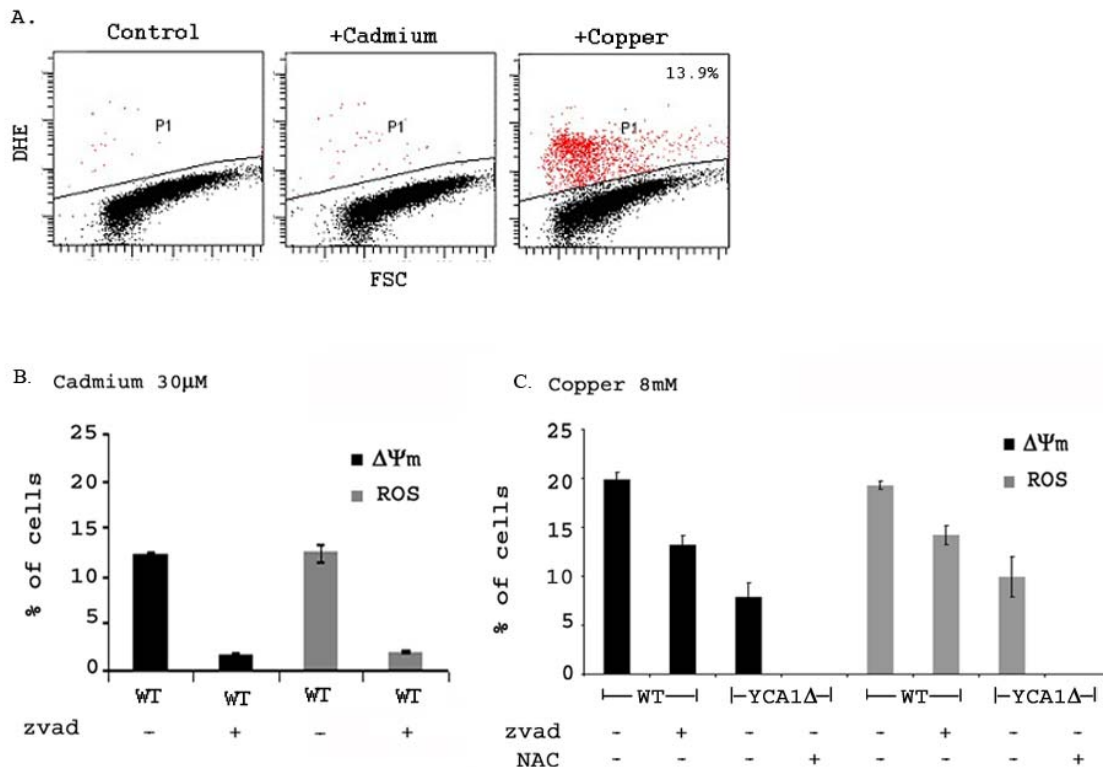


Figure 33: Yca1 is not the only route for copper mediated mitochondrial membrane hyperpolarization

(A) Differential production of ROS in cells within 1h of exposure to either cadmium, using oxidant sensitive dye Dihydroethidium (DHE) and flow cytometry analysis on FACS Canto. Untreated control cells labeled with DHE are on the left, with cells exposed to cadmium and copper for 1h in the middle and right panels respectively. (B) Differing roles of cadmium and copper in mitochondrial membrane hyperpolarization and WT, z-vad-fmk treated WT cells were incubated in the presence of Cd for 1 h then allowed to recover in fresh YEPD medium for 3 h. Cells were then stained for mitochondrial membrane hyperpolarization using RH123 or stained with DHE to determine ROS and then analyzed by flow cytometry. The percentages of positive cells were then represented as bar graphs (black for $\Delta\Psi_m$ and grey for ROS). Control cells that were not incubated with Cd did not show any positively labeled cells. (C) WT, z-vad-fmk treated WT, YCA1Δ, and N-Acetyl L-Cysteine (NAC) treated YCA1Δ cells were incubated in the presence of Cu for 1 h then allowed to recover in fresh YEPD medium for 3 h. Cells were then stained for mitochondrial membrane hyperpolarization with RH123 or stained with DHR123/DHE to determine ROS and then analyzed by flow cytometry. The percentages of positive cells were then represented as bar graphs (black for $\Delta\Psi_m$ and grey for ROS). Control cells that were not incubated with Cu did not show any labeling. Data are representative of at least three independent experiments.

integrity of the mitochondrial membrane can be compromised, as it is in Cd exposed cells. This would be consistent with the expectation that the various ROS, which are generated directly by copper, also impinge upon the mitochondrial membrane integrity. To address this question directly, the antioxidant N-acetyl-L-cysteine (NAC) was used to assess mitochondrial membrane hyperpolarization in copper-exposed cells in which ROS levels had been depleted by NAC. The results, shown in Fig 33C, demonstrate that no mitochondrial membrane hyperpolarization was apparent in cells that are mutant for Yca1p or that had been pre-treated with NAC. These data are consistent with the previous finding that ROS -generated directly through exposure to copper- are able to cause mitochondrial membrane perturbation, and hence Yca1p- mediated mitochondrial hyperpolarization is not the only route through which the mitochondria can be perturbed upon Cu exposure. While the involvement of Yca1p in mitochondrial perturbation is not a traditional activity associated with a caspase-like protease, it is not without precedence. Both caspase 3 and caspase 7 have been shown to mediate mitochondrial damage directly in mice ⁵, perhaps through interactions with electron transport complexes within the membrane ³⁰.

In this regard, it is important to note that, even though ROS in Cu-treated YCA1Δ cells may be able to cause mitochondrial hyperpolarization, by itself, Cu is unable to cause DNA fragmentation or apoptosis (Fig 32A). Copper-induced apoptosis, therefore, remains dependent upon the activation of the yeast caspase, suggesting that -while Yca1p presumably plays role in the perturbation of mitochondria upon exposure to heavy metals- it also plays a critical role in apoptotic events that occur subsequent to mitochondrial damage, as has been proposed by Eisenberg *et al.* 2007 ²³.

An additional caspase-like protease is involved in Cu-induced Yca1p-dependent mitochondrial perturbation

To confirm the requirement for an activated Yca1p in apoptotic response upon copper exposure, *YCA1Δ* cells were exposed to copper and stained for the presence of activated yeast caspase-1, using SR-FLICA. Curiously, a substantial portion of these cells (~12.6%) still demonstrated significant levels of activated caspase (Fig 34A), although (as mentioned previously) none of these *YCA1Δ* cells were found to subsequently undergo apoptosis. Even so, given our expectation that *Saccharomyces cerevisiae* encodes for only one caspase, these results proved to be quite puzzling. A number of research groups have expressed caution in using FITC-based fluorochromes to interpret any apoptotic events in yeast, which (they suggest) are possibly due to anomalous measurements in differentially stressed cells, and the added concern that dead yeast cells naturally present a high background fluorescence^{15, 16}. However, we have previously demonstrated the specificity of the SR-FLICA assay in detecting activated caspase activity in yeast¹. To address viability concerns, the metabolizable fluorescein diacetate (FDA) and appropriate control groups were employed to distinguish between living and dead cells (Fig 34B). In so doing, we were able to reaffirm that the SR-FLICA stained *YCA1Δ* cells, which had been exposed to copper were viable and not, as yet, dead (Fig 34B). To determine further that these *YCA1Δ* cells truly exhibited caspase activity, and that the residual fluorescence is not an artifact, caspase activity was independently confirmed using commercially available caspase peptide substrates conjugated to the cleavable fluorescent dye, 7-Amino-4-methylcoumarin (AMC) by performing enzyme assays³². In this way, proteins prepared

from wild-type cells that had been exposed to either copper or cadmium exhibited both IETD and DEVD-like caspase activity (3E and 3D), consistent with previously published data for yeast caspase activity in cells exposed to peroxide³³.

These results also confirmed that a proportion of *YCA1Δ* cells still exhibit a specific activity for an IETD-AMC linked substrate, more so when exposed to copper than cadmium. Furthermore this proteolytic activity disappears upon pretreatment of the cells with the pan caspase inhibitor, Z-VAD-FMK, prior to extraction of proteins (Fig 34E). Both lines of evidence strongly suggest that the residual SR-FLICA-based activity that was previously obtained in the *YCA1Δ* cells was not an artifact, and that an additional caspase-like enzyme activity, other than Yca1p, is present in cells exposed to both copper and cadmium (Figs 34D and 34E).

The additional caspase-like activity is through ATG4

A second caspase-like activity has been previously reported in apoptotic analyses in yeast under other stress yeast³⁴. Also, the intensity and specificity of the reactions in metal exposed *YCA1Δ* cells (as defined by both SR-FLICA and/or IETD-AMC cleaving capability) warranted further investigation as to the source of this additional caspase-like activity. Database searches and primary sequence comparisons yielded little primary sequence similarities among the evolutionarily distant metacaspases and true caspases found in metazoans³⁵. The positional conservation of histidine and cysteine residues in the active sites of known caspases was, however, used to identify similar residue conservations in 5 - 6 cysteine proteases of yeast. One of these candidate proteases, ATG4, is a requisite protease involved in the final stages of autophagy^{36, 37}. The 3-

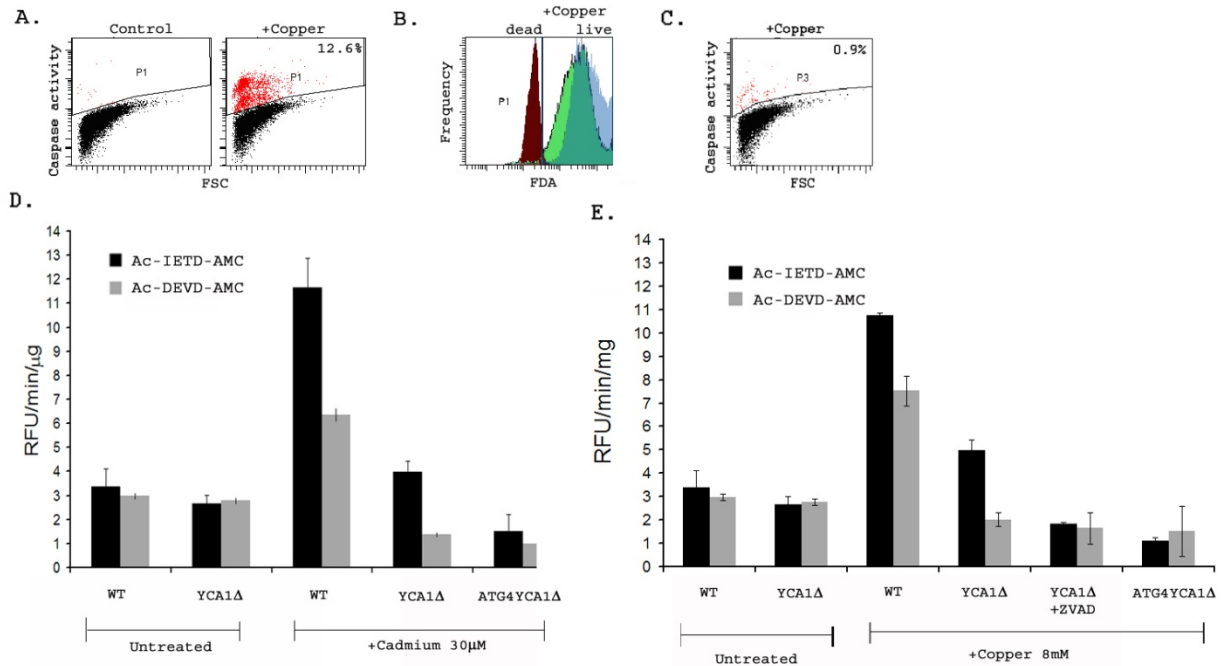


Figure 34: Caspase like protein may be responsible for Cu induced apoptosis

(A) *YCA1Δ* mutant cells were exposed to 8mM $\text{Cu}(\text{NO}_3)_2$ for 1 h then 'recovered' in fresh YEPD medium for 3 h. Cells were stained for active caspases (red) with SR_FLICA detection chemistry (Immunochemistry, LLC) and cells demonstrating elevated levels of activated caspase were detected using flow cytometry. Control *YCA1Δ* cells were treated in exactly the same way as the treated Cu cells (except for the exclusion of Cu from incubations). (B) Copper treated *YCA1Δ* cells (blue histogram) were incubated with FDA and analyzed with flow cytometry. Heat killed and live cells were used as controls (red and green histogram respectively), and their fluorescence levels overlayed with cells that had been exposed to copper as in (A) for comparison. (C) *ATG4YCA1Δ* double mutant cells were treated and stained for active caspases (red) as explained in (A). (D) To determine caspase activity using IETD-AMC and DEVD-AMC substrate, protein extracts from cadmium treated Wild Type, *YCA1Δ* mutant, *ATG4YCA1Δ* double mutant under indicated conditions. Protein extracts were tested for the cleavage of fluorescence labeled substrate such as IETD-AMC and DEVD-AMC as described in ³². The resultant fluorescence was read using a plate reader. (E) Protein extracts from copper treated wild-type cells, *YCA1Δ* mutant, *ATG4YCA1Δ* double mutant under indicated conditions were tested as described in (D). The resultant fluorescence was read using a plate reader.

dimensional structure of the human Atg4 protease analog (HsAtg4B) has recently been determined ^{36, 37}, and while HsATG4B has certainly not been classified as having a caspase-like structure, it does bear significant resemblance to papain-like cysteine proteases, which have previously been shown to be inhibited by caspase-specific substrates, including DEVD ^{38, 39}. Moreover, HsATG4B shares some functional and distinctive regional primary sequence homology with Atg4p from yeast, especially around a conserved cysteine residue (cys159 in Atg4p) deemed essential for proteolytic activity in HsAtg4B ⁴⁰. Interest in this protease was further heightened by the potential interplay and possible cross talk between autophagy and apoptosis, which has recently received some recognition ^{41, 42}; especially since it was shown that activity of Atg4p is specifically regulated by increased ROS ⁴³.

The secondary nature of any additional caspase-like protease activity would, presumably, be masked by the presence of Yca1p activity in wild-type cells. Consequently, in an effort to implicate ATG4 as having such capase-like substrate specificity an *ATG4/YCA1* double deletion mutant was constructed (in addition to obtaining *ATG4Δ*, courtesy of Euroscarf). The *ATG4Δ/YCA1Δ* double mutant failed to exhibit any caspase-like proteolytic activity in cells that had been exposed to copper (Fig 34C), which suggested that Atg4 indeed plays some role in providing background SR-FLICA fluorescence. In an effort to corroborate this finding, and to identify substrate specificity, fluorescently labeled peptide substrates IETD-AMC and DEVD-AMC were used to assay for caspase activity. When tested, two similar (but quite distinct) caspase-like activities in the wild-type and Yca1Δ strains were observed, following the

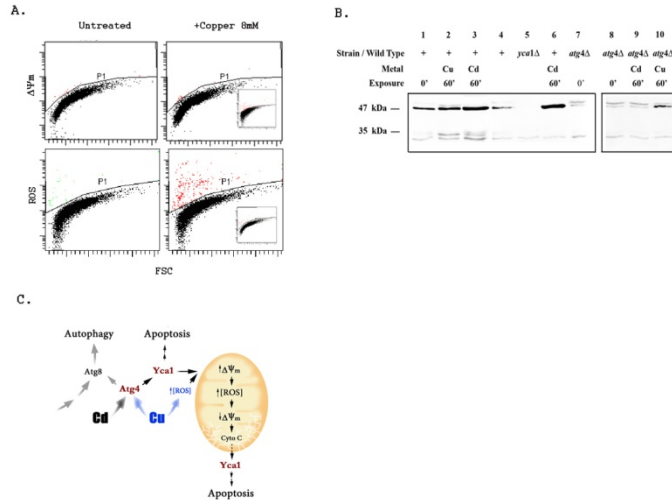


Figure 35: Atg4 is involved in processing of Yca1p

(A) To detect mitochondrial perturbations in *ATG4Δ* mutant, cells incubated in the presence or absence of 8mM $\text{Cu}(\text{NO}_3)_2$ for 1 h then allowed to 'recover' in fresh YEPD medium for 3 h. Cells were subsequently either stained for mitochondrial membrane hyperpolarization with RH123 and detected by flow cytometry (upper panels), treated with oxidant sensitive DHE (red) to detect the presence of ROS using flow cytometry (lower panels). For comparison, *ATG4Δ* were independently exposed to 30μM $\text{Cd}(\text{NO}_3)_2$ and tested for mitochondrial hyperpolarization and the presence of ROS in the same way (upper and lower panel inserts, respectively). (B) *ATG4Δ* and wild-type cells were harvested at 0 min and after exposure to either copper and cadmium exposure for 1h. before being allowed to 'recover' in fresh YEPD medium for 3 h. Proteins were then extracted from these cells and electrophoresed though SDS-PAGE and blotted onto nitrocellulose membrane. Western blot analysis was then performed to detect Yca1p using an anti-Yca1p antibody (raised against an antigenic site 'CGGQTEDLDGDEEDG' within the yeast caspase, itself). Additional protein extracts were taken from similarly exposed *YCA1Δ* and "non-apoptotic" wild-type cells, which had been sorted away from their "apoptotic" counterparts, using a FACS-Aria cell sorter. Both the *YCA1Δ* and sorted "non apoptotic" cells were used as controls in order to validate the presence of and changes to- *YCA1* specific bands within the gel. The absence of any 35 KDa band expressed in the sorted wild-type cells demonstrates that the specifically-cleaved 35KDa band of "activated" caspase in lanes 2 & 3 is only present in the fraction of harvested cells, ranging from 5-15% of the treated cells that is defined by the apoptotic sub-population. (C) The cascade of events predicted or, in part, elucidated for metal induced apoptosis in yeast. Atg4, an autophagic protease that is known to activate Atg8 in the later stages of autophagy, is also directly (or indirectly) involved in activation of the Yeast caspase 1 (Yca1). As such, Atg4 potentially provides a "sensory" role in metal-induced stress, wherein it can either activate Atg8 or activate Yca1, and thus initiate apoptotic programmed cell death. The intercession of both Atg4 and Yca1 is required for mitochondrial hyperpolarization in cells exposed to cadmium, but is not required for ROS-mediated mitochondrial damage in cells exposed to copper.

exposure of each strain to either of the two heavy metals, cadmium and copper (Figs 34D & E). The absence of any IETD-like activity in *ATG4Δ/YCA1Δ* double mutant suggested that Atg4 is minimally required for the “secondary” caspase-like activity. The apparent specificity of yeast caspase-1 for DEVD is seen with the loss of DEVD-like activity in *YCA1Δ*, which contrasts slightly with the results of a similar series of experiments undertaken by the Madeo group ^{14, 17}, who reported that Yca1p demonstrated a preference for the substrate IETD over DEVD. Not surprisingly, this conclusion assumed the presence of only a single caspase-like proteolytic enzyme activity, and was derived from data acquired from a yeast strain that overexpressed *YCA1* ^{14, 17}, not from a *YCA1Δ* strain, lacking its function. It is entirely possible, therefore, that -given the presence of an additional caspase-like protein in yeast- the elevated levels of IETD-specific activity that were previously attributed to Yca1p resulted from the relative over expression of more than one caspase-like protease, exhibiting a mixed preference for the two peptide substrates ⁴⁴, with the over expression of the one, potentially increasing the specific activity of the other.

***ATG4* is required for metal-stressed activation of Yca1**

Having implicated the cysteine protease, Atg4p, as playing an essential role in the secondary “caspase-like” activity, the contribution of this protease in the metal-induced apoptosis was addressed further by analyzing the activation of Yca1p and the production of ROS in a metal stressed *ATG4Δ* strain. Interestingly, both mitochondrial membrane hyperpolarization and ROS were significantly compromised in this *ATG4Δ* strain in cells exposed to copper (Fig 35A), and completely abolished in cells similarly

exposed to cadmium (Fig 35A inserts), These results, along with the result showing the presence of secondary caspase-like activity in a *YCA1Δ* upon metal exposure would indicate that *ATG4* is required for any ROS-independent metal-induced activation of the Yca1 (3D, 3E, 4A). While this would not be the first time that autophagy and apoptosis have exhibited significant levels of cross-talk, it is the first instance in which the autophagic *ATG4*^{4, 43, 45} has been shown to influence activation of a caspase in yeast or in any other organism. To determine further whether *ATG4* is involved in the actual cleavage-activation of yeast caspase^{14, 17}, a series of Western blot analyses were undertaken, using an anti-Yca1p antibody to monitor the potential cleavage of Yca1p in a variety of wild-type and mutant strains exposed to either copper or cadmium (Fig 35B). These data reinforce the argument that *ATG4* is required for (and thus anticipates) activation of Yca1p and, indeed, that *ATG4* is absolutely required for any metal-induced proteolytic “activation” of Yca1 from a 47kDa procaspase to an active 35 KDa form, as defined previously by Madeo *et al.*^{14, 17}. Intriguingly, wild-type cells that had been sorted through a FACS Aria (BD Biosciences) –based upon their inability to demonstrate any apoptotic fluorescent marker- also lacked any caspase activity, suggesting that any and all activated caspase activity is confined to the 12-25% sub population of cells that have been shown to undergo apoptosis. Furthermore, the presence of an additional caspase-specific band that is slightly larger than the 47KDa procaspase band, potentially indicates additional levels of protein modification of the Yca1 procaspase in yeast. While such modifications have not been addressed in this report, it is worthy of note that this additional band >47KDa is more prevalent in the

ATG4Δ, which only adds to the potential importance of *ATG4* in regulating activity of Yca1p.

Even though the precise role of Atg4p in heavy metal-induced activation of yeast caspase has not yet been identified, the results of these experiments clearly suggest a working model for heavy metal-induced apoptosis in yeast (Fig 35C). In this model Atg4p would play a pivotal regulatory role in defining the ultimate fate of yeast cells that have been exposed to low concentrations of either cadmium or copper. This model does not argue against the ability of Yca1 to be activated through the more standard mechanism of cytochrome C release from damaged mitochondria. Given that activation of Yca1p appears to be critical for any mitochondrial perturbation -at least for cadmium exposed cells and (in part) for cells exposed to copper (Fig 33) ¹ - a more directed activation of Yca1 through Atg4p would effectively bypass the need for mitochondrial damage to be an essential mechanistic component of metal-induced apoptosis. Such a model would be entirely consistent with the more rapid apoptotic response that yeast cells present upon heavy metal exposure, as opposed to hydrogen peroxide ¹. The direct involvement of Atg4 in the cleavage of Yca1 remains to be confirmed, although the paucity of cysteine proteases in yeast and the requirement for Atg4p for any secondary “caspase-like” activity argues in favor of just such a direct role. Regardless, the evidence presented in this paper clearly demonstrates that metal-induced, caspase-specific apoptosis in *Saccharomyces cerevisiae* is intimately associated with the autophagic protease, Atg4, which makes it potentially far more varied and complex than the involvement of a singular yeast caspase, Yca1, would have indicated.

Acknowledgements

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CHAPTER 4

YEAST CASPASE-1 MEDIATED OXIDATIVE DAMAGE OF MITOCHONDRIAL PROTEINS IN YEAST APOPTOSIS

Amrita M. Nargund and John E. Houghton

Heavy metals, such as copper and cadmium, have been linked to several cellular dysfunctions, neurodegenerative disorders and associated apoptosis. *Saccharomyces cerevisiae* has been shown to elicit a metal-induced stress response that ultimately results in a caspase-dependent apoptosis involving typical, key response markers such as high reactive oxygen species generation, activation of the singular yeast caspase (Yca1p), and DNA fragmentation. Given the simplicity of the yeast genetic system, and its well characterized genome, this unicellular budding yeast proved to be an excellent model organism to study the potential effects that are associated with initial exposure to such heavy metals. Previously we have also demonstrated the involvement of a key, autophagic gene, *ATG4*, which together with *YCA1* is required for mitochondrial perturbation leading to apoptotic programmed cell death. Here we indicate further the importance of *ATG4* in the activation of Yca1p and the overall apoptotic programme. We demonstrate that metals such as copper and cadmium cause oxidative damage to mitochondrial proteins, and that such damage appears to be targeted to the oxidative damage of certain, crucial proteins that appear to be required for metal-induced apoptosis. By showing that such targeted protein oxidation is dependent on *YCA1* and *ATG4*, we also confirm our initial finding that, in yeast, both *YCA1* and *ATG4* activities are essential for any significant mitochondrial damage. In so doing, this study attempts

to identify and examine the mechanism(s) by which mitochondria can be damaged as a result of cellular exposure to heavy metals.

Introduction

Metal-induced oxidative stress has been implicated in the pathogenesis of Alzheimer's disease, Parkinson's disease, spongiform encephalopathies, and familial amyotrophic lateral sclerosis¹⁻². While the precise role of heavy metals in these pathologies is still unclear, the ability of heavy metals to generate reactive oxygen species (ROS) –invariably leading to mitochondrial stress and dysfunction- is thought to play a significant part in the mechanisms that underpin damage to cells, potentially leading to apoptosis³⁻⁴. Besides playing a role in bioenergetics, mitochondria are intricately involved in a variety of cell death programmes, including apoptosis^{3,5}. Mitochondria dysfunction is known to be an integral component of apoptosis in intrinsic pathway⁶⁻⁷. In higher eukaryotes, mitochondria-mediated apoptosis (intrinsic pathway results from a transient hyperpolarization of the mitochondrial membrane, the generation of increased amounts of ROS leading to mitochondrial inner membrane permeabilization (MMP) and finally depolarization⁸⁻¹⁰, promoting the release of pro-apoptotic factors, such as AIF and cytochrome c, from the inner-mitochondrial space into the cytosol¹¹⁻¹². Both AIF and cytochrome c are able to trigger apoptosis. AIF, itself can translocate to the nucleus and initiate DNA fragmentation¹³⁻¹⁴, while cytochrome c is able to activate downstream “executioner” caspases and trigger further targeted protease activity, ultimately leading to programmed apoptotic cell death¹⁵⁻¹⁶.

Recently, unicellular yeast has been seen to undergo apoptosis under different stress conditions such as peroxide, copper, cadmium and acetic acid¹⁷⁻¹⁹. As with higher eukaryotes, elevated levels of ROS are found to be among some of the major

causes of apoptosis in yeast^{17,20-22}. In yeast, apoptosis that has been shown until now, is with mitochondrial involvement²¹. Consequently, yeast appears to be an excellent model organism to elucidate some of the mechanisms by which mitochondrial-mediated cell death occurs. Even so, many of the components of mitochondria-mediated apoptosis in yeast are still unclear, as they are in higher eukaryotes²³.

During apoptosis there are several death signals that can affect mitochondria leading to their dysfunction. Of these the most heavily characterized mechanism is through tBID that can cause homodimerization of *BAK* or *BAX* form channels in the mitochondrial membrane that facilitate the release of key signaling molecules, including cytochrome c²⁴. However, BH3 domain -like proteins such as Bcl, Bak or Bax have yet to be found in *Saccharomyces cerevisiae* (although at least one potential BH3 domain analogue has been found in the related yeast, *S. pombe*²⁵). Consequently, the precise cause of mitochondrial dysfunction, allowing the release of apoptotic agonists, such as cytochrome c, has yet to be identified. We have previously proposed another potential mechanism for mitochondrial perturbation, wherein Yeast Caspase1, itself, can cause mitochondrial membrane hyperpolarization and increased ROS generation in *Saccharomyces cerevisiae* upon exposure to heavy metal such as copper and cadmium²⁶. While such a role for any caspase has yet to be reported *in vivo*, some *in vitro* studies involving caspase 3, have shown that caspase 3 is able to cause mitochondrial damage and collapse²⁷. Once mitochondria are hyperpolarized by Yca1p intervention, ROS will be produced. However, what causes depolarization or mitochondrial dysfunction thereafter is not clear in yeast. Mitochondrial ROS can cause damage to

mitochondrial DNA, lipid peroxidation and oxidation of crucial mitochondrial membrane proteins leading to mitochondrial membrane permeability transition²⁸⁻²⁹.

Previous research in our laboratory has shown that specific oxidation of proteins is among the most immediate cellular responses to metal-induced stress³⁰. Moreover, oxidation of certain mitochondrial proteins in some mammalian cell types, has been shown to lead to mitochondrial fission [England, 2001]³¹⁻³². Indeed, oxidation of mitochondrial proteins such as Bcl-2 and ANT has been shown to precede Nitric oxide induced apoptosis³¹. Although not studied specifically for the purpose of mitochondrial dysfunction and apoptosis, oxidizing agent such as paraquat have also been shown to carbonylate (oxidize) mitochondrial proteins in yeast³³.

The possibility, therefore, that oxidative damage of mitochondrial proteins might be part of the mechanism leading to mitochondrial membrane dysfunction in yeast, warranted further investigation. We therefore set out to determine if specific mitochondrial proteins are targeted for oxidation during metal-induced apoptosis. In yeast, mitochondrial proteins such as VDAC have to present for mitochondria to be sensitive to human Bax mediated apoptosis³⁴. Yeast strains lacking mitochondrial pore genes such as *POR1* and *ANT1* fail undergo viral proteinR (VPR) induced apoptosis³⁵. It is possible, therefore that oxidation of mitochondrial proteins, such as these, may provide part of the mechanism by which mitochondrial fission and/or dysfunction is initiated during the metal-induced apoptotic response

Here we show for the very first time that specific mitochondrial proteins are oxidized during copper and cadmium induced apoptosis in yeast. Oxidation of some key proteins is required to cause apoptosis upon metal exposure and such oxidation is dependent on *YCA1* and *ATG4* – caspase like protease, confirming the role of these proteases in causing mitochondrial perturbation.

Materials and methods

Strains and growth conditions

Saccharomyces cerevisiae BY4741 (MAT α *his3* Δ 1 *leu2* Δ 0 *met15* Δ 0 *ura3* Δ 0) and the isogenic mutant strains. *YCA1* Δ and *ATG4* Δ , were obtained from Euroscarf (Frankfurt, Germany). Experimental cultures were inoculated from 24 h starter cultures derived from single colonies and grown overnight to exponential phase (OD₆₀₀~2.0) at 30°C with orbital shaking (120 rev min⁻¹) in YEPD broth³⁰. When necessary, either copper nitrate [Cu(NO₃)₂] or cadmium nitrate [Cd(NO₃)₂] were added to growing cultures to a final concentration of 8 mM and 30 μ M, respectively. After 1 h of incubation, cells were washed with YEPD and incubated in fresh YEPD for 3 hrs to allow cells to refract and any apoptotic program to develop.

Preparation of protein extracts

Mitochondrial protein extracts were prepared, as described in O'Brien *et. al.* [O'Brien, 2004], with all steps being performed on ice, unless otherwise noted. Briefly, yeast cells were treated with metals as described previously in strains and growth conditions. The cells were harvested and washed twice with ice-cold distilled water before being resuspended in 0.5 g/ml spheroblast buffer (10 mM NaPO₄ 1.35 M sorbitol, 1 mM EDTA, 2.5 mM dithiothreitol, pH 7.5) along with zymolyase 20T (final conc. of 3 mg /g of cells), and allowed to incubate at 30°C with gentle shaking. The resulting spheroplasts were then lysed using an homogenizer in lysis buffer (0.6 M mannitol, 2

mM EDTA, pH 7.0 along with protease inhibitors such as; pepstatin, aprotinin and leupeptin). Cell debris was removed by centrifugation at 1,900 x g for 5 min. Cells were centrifuged at 12, 100 x g for 10 min to pellet the mitochondria. To remove the majority of the debris from the mitochondrial pellet, the pellet was again washed with lysis buffer and centrifuged at 1,900 x g for 5 min. Supernatant obtained from the above step was then further centrifuged at 23,000 x g and then resuspended in 10 mM NaPO₄ to obtain relatively pure mitochondria.

Two-dimensional gel electrophoresis and Western blot analysis

The general methods used for 2D protein analysis and Western blotting were as defined by Shanmuganathan *et al.*, 2004³⁰. For all 2D analyses, either 100 µg (for protein detection) or 100 µg (for Western blotting) of protein was used.

For the analysis of carbonylated proteins by Western blotting, rabbit anti-DNP was used as the primary antibody (Molecular Probes Inc; 1:2500 dilution) and peroxidase-linked goat-anti rabbit IgG was used as the secondary antibody (Sigma; 1:2500 dilution). Carbonylated proteins were detected using a chemiluminescent peroxidase substrate (Pierce), the presence of which was visualized using a LAS3000 image analyzer (Fuji). Chemiluminescence was quantified using Multigauge software v2.3

To identify specific proteins spots in various gels, the spot profile from the different sources were compared to each other and compared with the YMP database. Also, to confirm the specific identity of particular protein spots of interest these proteins were analyzed further and their sequence verified using an ABI 4800 Plus MALDI-TOFF/TOFF MS Analyzer.

Results

Mitochondrial proteins are oxidized on metal treatment.

ROS is generated and released from mitochondria³⁶⁻³⁷. Studies have also shown that once released from mitochondria, ROS can oxidize mitochondrial proteins. Such oxidation can lead to mitochondrial fission or dysfunction³¹. Such mitochondria dysfunction is associated with apoptosis¹². Mitochondria are therefore, not only a source of ROS, but also the potential target of ROS that are generated during apoptosis. Since yeast undergo apoptosis in response to metal treatment and also demonstrate significant mitochondrial perturbation, we wanted to determine whether yeast cells show any targeted oxidation of mitochondrial protein as a consequence of exposure to heavy metals such as Cd and Cu. To address these questions directly, yeast mitochondrial proteins were obtained from mitochondrial preparations that had been harvested from a series of yeast strains, following transient exposure to either cadmium or copper. As previously explained, the transient exposure to either metal defined as being for 1 hr with an additional 3 hours of growth in metal free media, to provide an adequate period for the apoptotic program to progress. The mitochondrial proteins were then analyzed for oxidative damage, using iwestern blot technique with anti-DNPH antibodies, and displayed n a two-dimensional array through 2D gel electrophoresis. The total mitochondrial protein oxidation data show 5.5 times more oxidation in copper treated yeast mitochondrial extracts than untreated Wild type samples. Also, cadmium induced mitochondrial protein oxidation is 4 times higher than untreated Wild type samples (Fig 36).

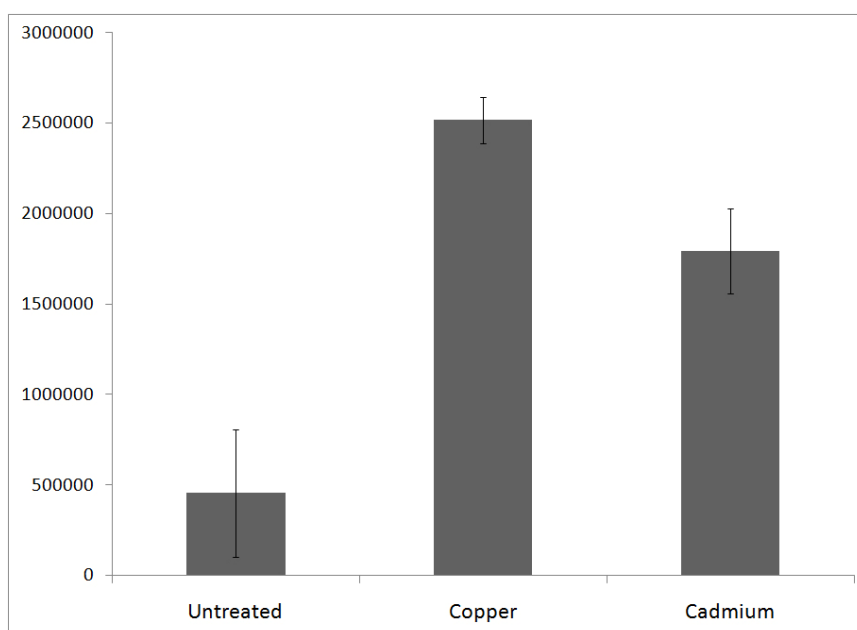


Figure 36: Total protein oxidation during apoptosis in Wild type.

Cells were harvested as described in experimental procedures and mitochondrial fractions were prepared as described in O'Brien et. al ¹⁸. Carbonylation/Oxidation was determined by probing 2D western blots using anti-DNP antibodies. Oxidation was quantified using Multigauge software. Total oxidation was normalized to actin (loading control). Data consist of results from 2 independent experiments.

When looking at the individual proteins targeted and oxidized upon metal exposure, data shows that there is some background level oxidation even in the absence of metal treatments- heat shock proteins such as Hsp60, Ssa1 and Ssc1. Also, labile metabolic enzyme such as Aco1, Kgd2 and Pdh1 are somewhat oxidized even in the untreated samples. But on copper exposure about 8 proteins and upon cadmium exposure 7 proteins are oxidized that are untouched in untreated cells. These proteins are therefore, oxidized as a result of metal toxicity. Some proteins like Atp1, Cor1, Tdh3 and Por1 are oxidized in both copper and cadmium treatment (Fig 37). Since both these metals induce apoptosis and mitochondrial damage, the proteins that are oxidized in both could be important for apoptosis. Two isoforms of Atp1 are oxidized on metal treatment. Atp1 is an alpha subunit of mitochondrial F1F0 ATP synthase. Data shows that isoform1 of Atp1 is oxidized 200 units in copper treated and 75 units in cadmium treated cells as opposed to not oxidized at all in untreated samples. Similarly isoform2 of Atp1 is oxidized more in copper treated samples than cadmium (Fig 38A). Cor1 is a Core subunit of the ubiquinol-cytochrome c reductase complex (bc1 complex), which is a component of the mitochondrial inner membrane electron transport chain. Proteins from ETC are targeted during apoptosis ²⁷. Cor1p is about more than 110 units oxidized in copper and 70 units in cadmium treated cells (Fig 38B). Tdh3 is glycolytic enzyme Glyceraldehyde 3 phosphate dehydrogenase and it has been linked to apoptosis ³⁸.

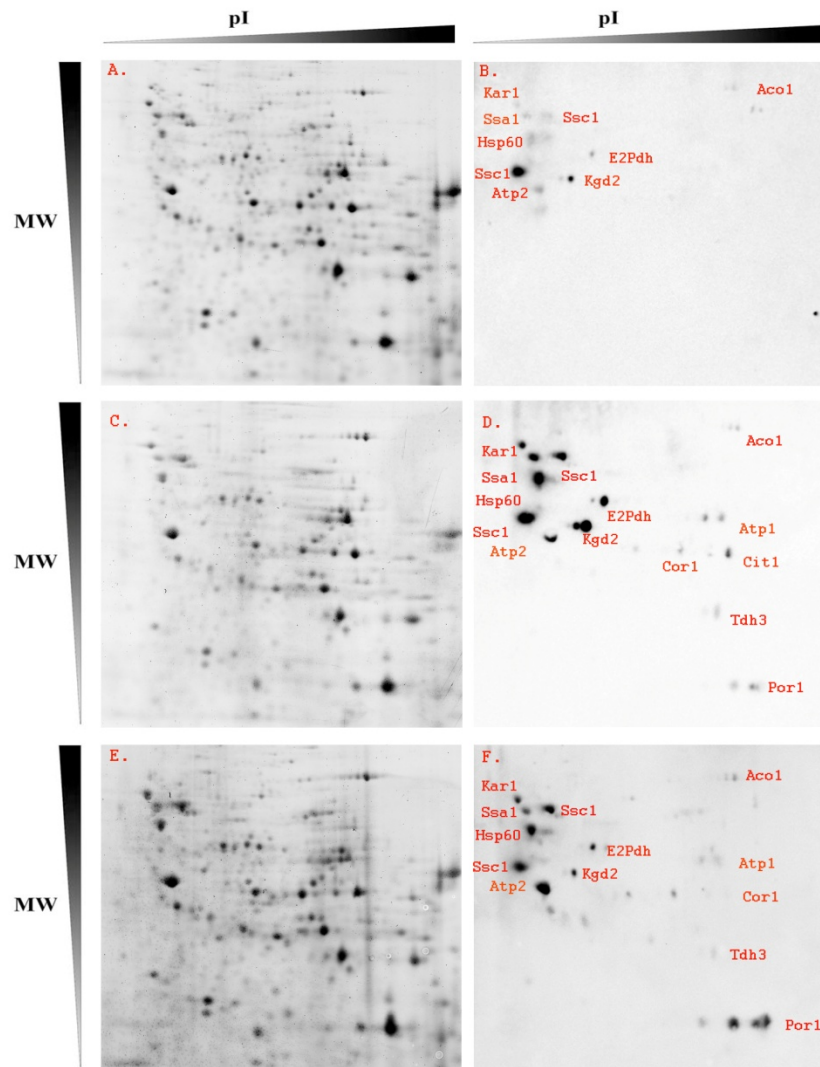


Figure 37: Wild type mitochondrial proteins are oxidized on metal treatment.

Cells were harvested and mitochondrial proteins were prepared as described in experimental procedures. Panels A,C,E are 2D-profile of protein abundance and panels B,D,F are corresponding western blots probed with anti-DNP antibodies to detect oxidation. (A) Mitochondrial proteins from untreated WT and (B) shows corresponding oxidized mitochondrial proteins. (C) Mitochondrial proteins from copper treated WT and corresponding oxidized proteins are shown in (D). (E) Mitochondrial proteins from cadmium treated WT and corresponding oxidized proteins on the right in panel (F).

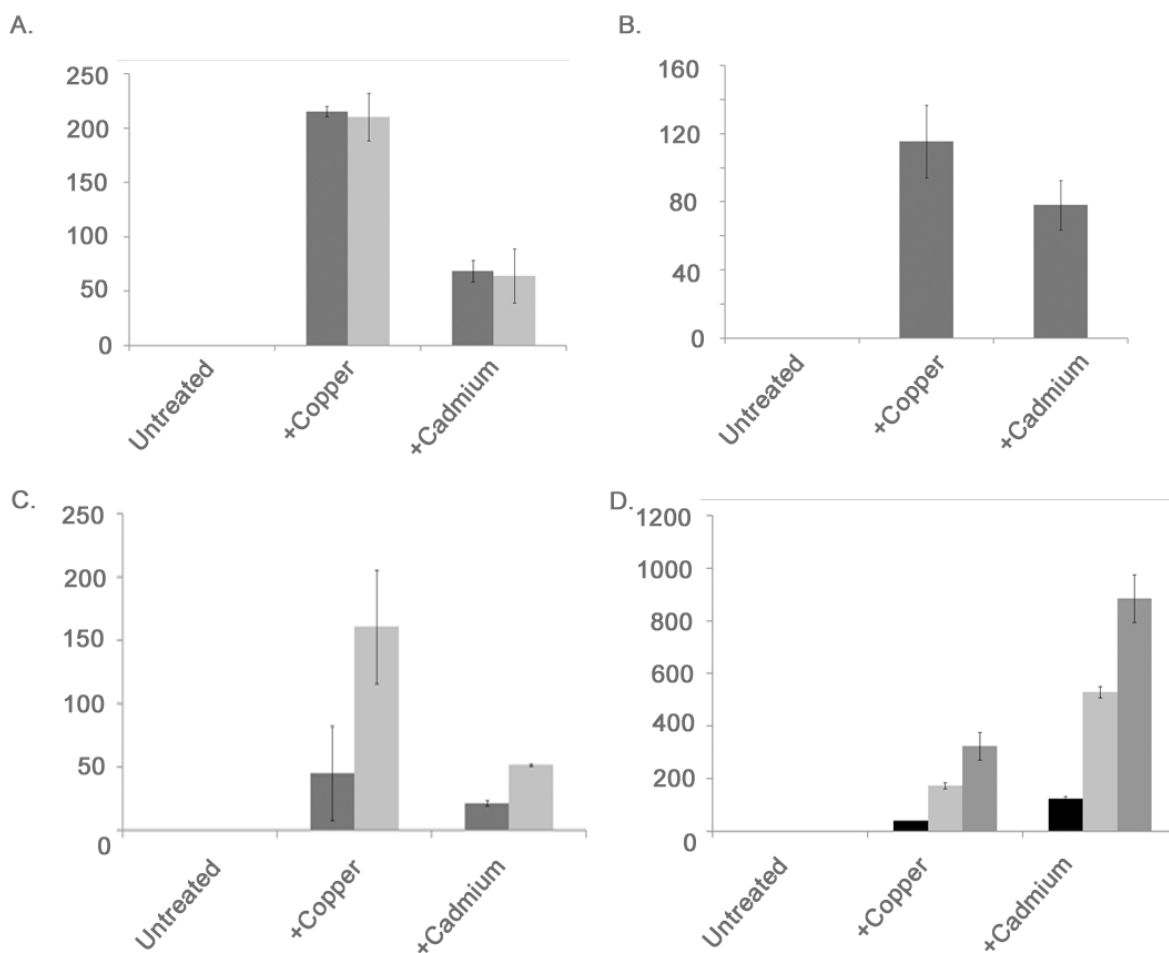


Figure 38: Oxidation levels of individual proteins that are affected during the apoptosis upon metal exposure.

Oxidation levels of the targeted individual proteins from copper and cadmium treated Wild type samples are quantified from 2D-western blot using Multigauge software. The oxidation values were then normalized to relative abundance of actin. Data consist of results from 2 independent experiments.

Two isoforms of Tdh3 are oxidized on Cu and Cd exposure. Isoform1 of Tdh3 is less oxidized about 40 units in copper and 20 units in cadmium as compared to Isoform2 of Tdh3 which is heavily oxidized approximately 175 units in copper and 50 units in cadmium (Fig 38C). Por1 is a Porin (Voltage dependent anionic channel) and is known to be important for apoptosis in yeast ³⁴. All the three isoforms of Por1 are oxidized with isoform 3 being the most oxidized and isoform1 is the least oxidized (Fig 38D). From the above data we can say that these mitochondrial proteins could be important for apoptosis. And that oxidation of these proteins could potentially act as a signal for mitochondrial collapse and apoptosis.

Yeast caspase 1 is required for mitochondrial protein oxidation during cadmium induced apoptosis.

In yeast and mammalian apoptosis, it is predicted that stress inducer will somehow cause mitochondrial dysfunction during mitochondria mediated apoptosis. Such dysfunction has been shown to release proapoptotic factors, followed by activation of caspase and apoptosis. However, in cadmium treated *YCA1Δ* (caspase knock out), there is no mitochondrial membrane hyperpolarization and ROS suggesting that Yca1 is required for mitochondria collapse in cadmium induced apoptosis ¹⁸. Such caspase-mediated ROS generation is also seen in a study of the effect of caspase 3 on ETC complex I and II ²⁷. The reason for mitochondrial dysfunction, once ROS is released by mitochondria, is still unclear. It is known that oxidation of mitochondrial proteins in neuronal cells can lead to cellular dysfunction ^{2,28}. Consequently, we wanted to

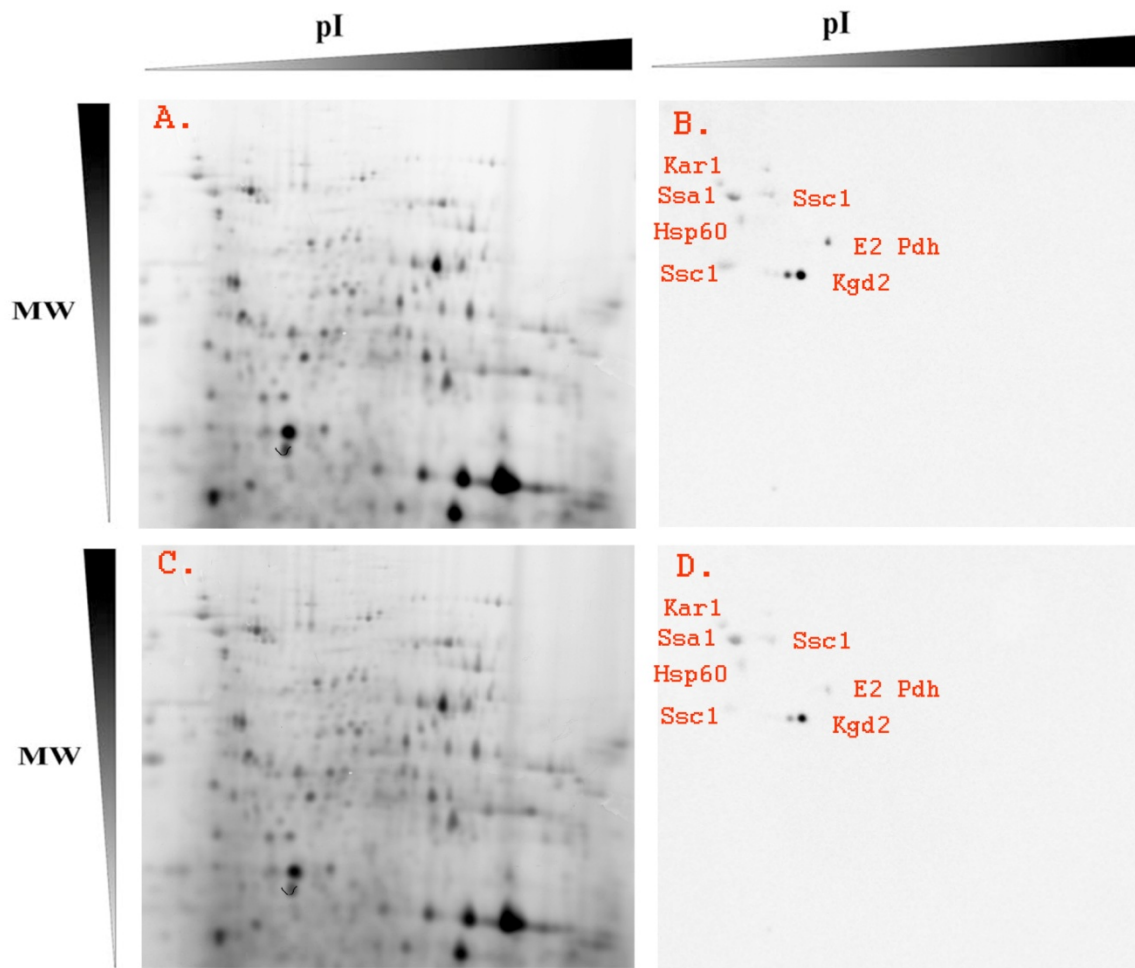


Figure 39: YCA1 is required for mitochondrial protein oxidation during apoptosis upon exposure to Cd.

Cells were harvested and mitochondrial proteins were prepared as described in experimental procedures. Panels A,C are 2D-profile of protein abundance and panels B,D are corresponding western blots probed with anti-DNP antibodies to detect oxidation. (A) Mitochondrial proteins from untreated YCA1Δ and (B) shows corresponding oxidized mitochondrial proteins. (C) Mitochondrial proteins from cadmium treated YCA1Δ and corresponding oxidized proteins are shown in (D).

determine whether ROS were produced because of *YCA1*, and that such activity plays a role in mitochondrial protein oxidation. Mitochondrial proteins were harvested from *YCA1Δ* following metal treatment and detected mitochondrial protein oxidation after subjecting mitochondrial proteins to 2D gel electrophoresis. We found that in the absence of yeast caspase 1, there is background level oxidation as seen in wild type but the proteins predicted to be important apoptosis are not oxidized (Fig 39). This data confirms firstly that *YCA1* is required for mitochondrial protein damage and secondly, that proteins such as Atp1, Cor1, Cor2, Tdh3 and Por1 are potentially important and linked to apoptosis.

ATG4 is required for mitochondrial protein oxidation during cadmium induced apoptosis.

Having recently suggested that Atg4, an autophagic protease is required for activation of Yca1 in metal-induced apoptosis, which (in turn) is involved in the hyperpolarization of the mitochondrial membrane ^{18,26}. To confirm these previous findings we also wanted to determine oxidation in *ATG4Δ*. We harvested mitochondrial proteins from *ATG4Δ* after metal treatment and detected mitochondrial protein oxidation after subjecting mitochondrial proteins to 2D gel electrophoresis. We found that in the absence of *ATG4*, for reasons yet unknown, oxidation of chaperone protein Ssc1 is 2.5 times more than seen in wild-type and *YCA1Δ* (Appendix A). Significantly, the important apoptotic are not oxidized in *ATG4Δ* (Fig 40). This data confirms again that *ATG4* is required for mitochondrial protein damage and that proteins such as Atp1, Cor1, Cor2, Tdh3 and Por1 are important and linked to apoptosis.

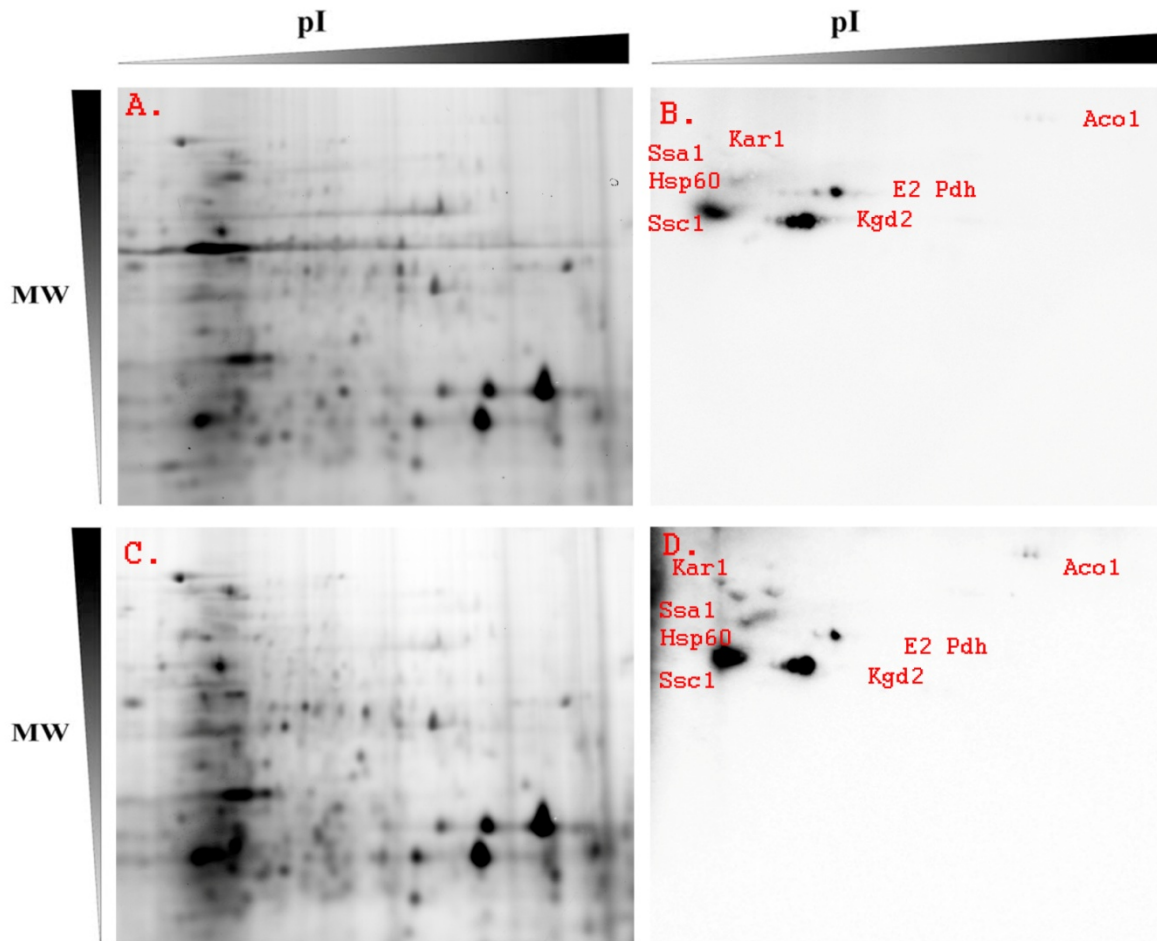


Figure 40: *ATG4* is required for mitochondrial protein oxidation during apoptosis upon exposure to Cd.

Cells were harvested and mitochondrial proteins were prepared as described in experimental procedures. Panels A,C are 2D-profile of protein abundance and panels B,D are corresponding western blots probed with anti-DNP antibodies to detect oxidation. (A) Mitochondrial proteins from untreated *ATG4Δ* and (B) shows corresponding oxidized mitochondrial proteins. (C) Mitochondrial proteins from cadmium treated *ATG4Δ* and corresponding oxidized proteins are shown in (D).

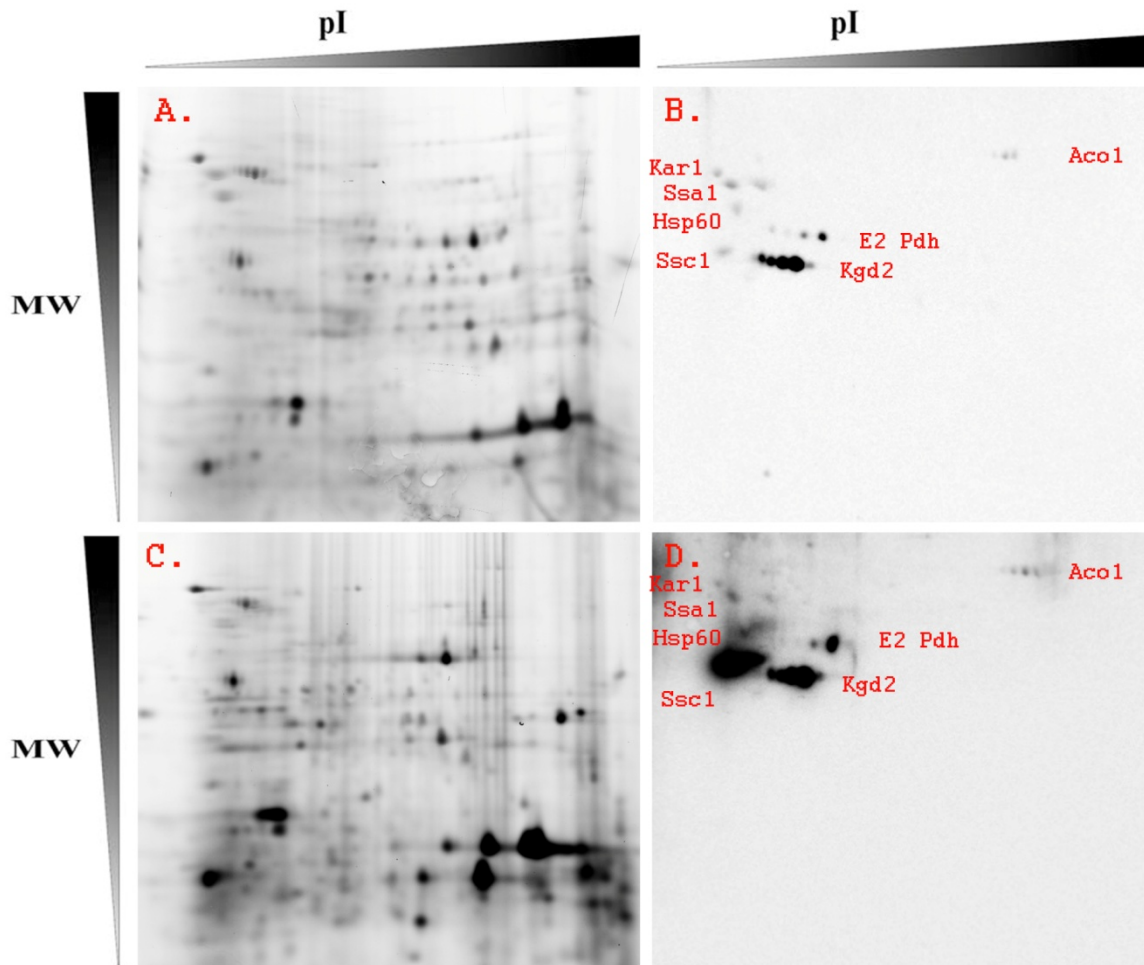


Figure 41: Unlike Cd, Cu can oxidize some mitochondrial proteins even in the absence of *YCA1* and *ATG4*.

Cells were harvested and mitochondrial proteins were prepared as described in experimental procedures. Panels A,C are 2D-profile of protein abundance and panels B,D are corresponding western blots probed with anti-DNP antibodies to detect oxidation. (A) Mitochondrial proteins from copper treated *YCA1Δ* and (B) shows corresponding oxidized mitochondrial proteins. (C) Mitochondrial proteins from copper treated *ATG4Δ* and corresponding oxidized proteins are shown in (D).

Unlike cadmium, copper can oxidize some proteins even in the absence of *YCA1* and *ATG4*

Unlike cadmium, copper has 2 potential routes to induce mitochondrial ROS generation - one through the insidious ROS that it can produce and another via Yca1²⁶. Even though the presence of ROS in Cu-treated *YCA1Δ* cells may be able to cause mitochondrial membrane hyperpolarization but these ROS levels are insufficient to cause DNA fragmentation, or apoptosis²⁶. Since there is no apoptosis in copper treated *YCA1Δ* or *ATG4Δ* cells, we wanted to find if the oxidation profile of these copper treated mutants that still show some amount of ROS, are any different from Wild type. To determine the proteins oxidized on copper treatment in the absence *YCA1* and *ATG4*, we performed 2D gel electrophoresis followed by detection of oxidation on the mitochondrial proteins prepared from *YCA1Δ* and *ATG4Δ*. When we looked at the oxidation levels in copper treated wildtype, *YCA1Δ* and *ATG4Δ*, we found proteins relevant to apoptosis are not oxidized. This data suggests that oxidative damage of these proteins is a prerequisite to apoptosis. Although, copper itself can oxidize some mitochondrial proteins independent of *YCA1* and *ATG4*, ROS induced by copper is not sufficient to cause damage to the crucial proteins leading to non-apoptotic phenotype. Metabolic enzymes such as Aco1, Kgd2, 4 isoforms of E2-PDH1 and Cit1 are heavily oxidized in copper treated *YCA1Δ* and *ATG4Δ* (Fig 41).

Discussion

Protein carbonylation is known to play a significant role in several human diseases such as Alzheimer's disease, chronic renal failures and diabetes, and is considered to be a major contributor to resulting oxidative stress and apoptosis³⁹. Similar mechanisms have been shown to be involved in drug induced apoptosis, where ROS and protein modifications, such as oxidation, occur at an early stage in the apoptotic program of cells undergoing apoptosis⁴⁰. Oxidative stress that results in protein modification could be a cause for apoptosis for a couple of reasons; either a specific protein could be targeted for modification and thus initiate a signaling cascade (leading to apoptosis), or it could be a more generic effect such as loss of cellular function due to protein damage⁴¹. Targeted protein carbonylation is shown to be associated with oxidative stress and apoptosis induced by photodynamic therapy. Such targeted response was defined as a signaling mechanism for apoptosis induced by photodynamic therapy⁴².

In this study we show that metals, such as copper and cadmium, are able to generate ROS, and mount an oxidative "attack" on mitochondria by causing carbonylation of mitochondrial proteins. In this study we also demonstrate that there is a targeted carbonylation of mitochondrial proteins such as ATP1, POR1, COR1, COR2, TOM40 and TDH3 along with some other heat shock chaperone proteins. Carbonylated TDH3 (GAPDH) in CNS-derived cells is thought to be involved in transporting apoptotic proteins from the cytoplasm to the nucleus⁴³. It is, therefore, possible that the observed carbonylation of TDH3 –resulting from metal exposure in yeast- could also be an important signal for apoptosis. While it is possible that the oxidized TDH3 could be

a result of cytosolic contamination of the mitochondrial preparation, the distribution pattern of TDH3 isoforms in the mitochondrial preps appear to be slightly different from those previously determined for the cytosol ³⁰. This would suggest that the TDH3 present in the mitochondrial fractions is indeed from the mitochondria. Damage of Mitochondrial ATP synthase caused by peroxynitrite causes permeability transition pore opening and cytochrome c release ⁴⁴. Oxidation of ATP1(ATP synthase) upon metal exposure can also cause a damage and decline in activity which could lead to apoptosis. Similarly, oxidation of components of permeability transition pore- ANT and VDAC is required for apoptosis ²⁸. These proteins are involved in the apoptotic process under different stress conditions. Targeted oxidation of the above mentioned proteins upon metal exposure could potentially cause mitochondrial fragmentation or dysfunction in yeast apoptosis.

So oxidation of these proteins and the involvement of caspase in the process give new insight regarding a potential mechanism. The fact that five different proteins are targeted also explains the notion of apoptosis process in yeast being even more complex. And that mitochondria damage during apoptosis may be regulated at different levels. It could be also that there is a redundancy to ensure a failsafe mechanism.

During intrinsic pathway of apoptosis, cytochrome c is released after mitochondrial permeability transition in ratbrain ⁴⁵. Cytochrome c can then activate caspases and apoptosome formation takes place. We have reported previously and also demonstrated in the current study that upon metal exposure Yeast caspase 1 is required for of mitochondrial perturbation during apoptosis in yeast. Similar observations have been reported recently, wherein; Caspase 3 has been shown to impact complex I

and complex II of ETC in the mitochondria ²⁷. In this study the authors report that the release of ROS is due to the impact of caspase 3 on complex I and II, caspase is therefore, said to be required for ROS generation ²⁷. Even so, the potential mechanism by which Yca1 leads to some form of mitochondrial permeability remains unknown. In this study we clearly show that mitochondrial protein oxidation takes place as a result of cellular exposure to metals, and that these oxidation patterns are quite different depending upon whether or not the cells are exposed to copper or cadmium (Figure 37) and that such oxidation is dependent on the presence and activation of Yeast caspase1 (Figure 39). Oxidation of mitochondrial proteins has been shown to be the cause of mitochondrial permeability transition in insulin-secreting RINm5F cells ³¹. Such a mechanism, if initiated directly by Yeast Caspase1, would be unusual. In previous investigations the focus has been upon the Bcl2 family proteins as the only mechanism known to cause mitochondria mediated apoptosis -through tBid, Bax and Bcl2 ⁴⁶⁻⁴⁷. With the evidence of presence of alternate route such as Yeast caspase1 mediated mitochondrial dysfunction, it is revealed that there could be several mechanisms to initiate mitochondrial perturbation that are still largely unexplored.

Oxidation of metabolic enzymes such as aconitase and α -Ketoglutarate dehydrogenase has been demonstrated upon peroxide stress. Aconitase is a labile enzyme and known to be oxidized even at low concentrations of ROS. α -Ketoglutarate dehydrogenase is known to be oxidized and inhibited only at high concentrations of ROS. Such inhibition limits NADH/NADPH production leading to low reducing power environment ⁴⁸. It is known that low reducing environment will slow down the ETC and hence avoid generation of more ROS. Although oxidation of metabolic mitochondrial

enzymes occurs in cells that have been exposed to copper, even in the absence of *YCA1* and *ATG4*, such damage is unable to only cause cell death (Fig 32A).

In the light of recent evidence, Apoptosis and Autophagy seem to be interconnected processes ²⁶. Proteins of either pathway are sometimes shown to be involved in the regulation of the other ⁴⁹. Our previous study indicates the role Atg4, an autophagic cysteine protease, plays in mitochondria perturbation by activating Yeast caspase1 during apoptosis in yeast. By showing the absence of targeted mitochondrial protein oxidation in *ATG4Δ* upon cadmium exposure, we have been able to confirm that like *YCA1*, *ATG4* is involved in oxidation of key mitochondrial proteins. Intriguingly, when exposed to the presence of either Cd or Cu, *ATG4Δ* cells show heavy carbonylation/oxidation of chaperone protein such as Ssc1, above and beyond the levels experienced by *Yca1 Δ* cells (Fig 40-41, Appendix A). It is possible that these elevated levels could be attributed to the absence of an active autophagic pathway that would have been capable of removing these damaged proteins.

Whether the mitochondrial protein oxidation is a targeted mechanism, as argued in this study, or the consequence of the ROS, if such specific patterns of carbonylation are proven to be specific to each of the metal-induced stress response, such patterns of oxidation can potentially be used as biomarker for metal exposure (and the consequences thereof). Moreover, the relatively “novel” mechanism of oxidation of mitochondrial proteins via the activities of *YCA1* and perhaps *ATG4* cysteine proteases (previously suggested by the in vitro experiments of Ricci et al, 2003 ²⁷) provides an

additional level of regulation/control in the apoptotic response of cells to the presence of heavy metals.

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GENERAL DISCUSSION

Heavy metals have been associated with degenerative disorders and have also been identified as carcinogen¹⁻². Both in degenerative disorder and cancer, abnormal apoptosis or programmed cell death is the major concern. Apoptosis involves a series of events such as mitochondrial fall apart, caspase activation, and nuclear fragmentation that leads to the cellular demise. Apoptosis is essential in developmental process. Abnormal apoptosis, however, leads to various diseases. Abnormal up-regulation of apoptosis, in the cells, may lead to degenerative disorders. Similarly down-regulation of apoptosis may lead to cancer³⁻⁴. Heavy metal such as copper is a redox-active metal and hence is capable of generating toxic reactive oxygen species directly by catalyzing Fenton Haber weiss reactions. Cadmium, on the other hand, is a redox-inactive metal and, therefore, unable to generate reactive oxygen species on its own. We chose to study these two metals copper and cadmium to understand the toxicity associated with different category of metals.

Yeast, has been considered to be a very good model system to study cellular responses to heavy metal exposure. We found that on exposure to metals, genes involved in the pathway of apoptosis, autophagy and cell death were up regulated in yeast (Fig 16-18). Such transcriptional responses lead us to investigate possibility of induction of cell death upon metal exposure. Surprisingly, both copper and cadmium were able to cause cell death in spite of the inherent difference in the nature of these metals. Here we discovered for the first time that very low concentration of copper and cadmium induced apoptosis in a sub-population of the exposed yeast cells, with

canonical markers such as ROS, mitochondrial damage, caspase activation and DNA fragmentation⁵.

Yeast, a unicellular organism, undergoing apoptosis was unanticipated. It was hard to rationalize such altruistic behavior of yeast. More and more studies have now speculated several reasons behind such “altruism”. The presence of genes that are required for apoptosis ensures the benefit of the population as a whole in aging cells⁶. But the ability of dead yeast cells to accumulate fluorochrome nonspecifically also raised concerns over the possibility of yeast to commit apoptosis⁷. We confirmed, however, that the labeled cells in our experiments were alive at the time of analysis, by using an additional dye Fluorescein diacetate (FDA). FDA can be metabolized to Fluorescein only in living cells, and as such can discriminate between and among live and dead cells. By staining live non-apoptotic cells, apoptotic cells, and dead cells with FDA, we here able to clearly demonstrate that yeast cells that demonstrated all apoptotic signals were alive (Fig 34).

In this report, we also discovered the concentration of the metal that is toxic to yeast cells. When we treated Wild type and *YCA1Δ* with different concentration of copper, we could differentiate between a toxic metal concentration and a metal concentration that allows certain cells to undergo apoptosis for the benefit of the population (Fig 42). At the higher concentration of copper (9mM), we see that there is less cell death in WT cells that are able to offset certain population by apoptosis than *YCA1Δ*. That at higher concentration of copper (toxic concentration) having apoptosis gives advantage to WT cells. We can hence say that there is difference in toxic dose Vs the dose that allows cell to respond (Fig 42). Using *YCA1Δ* we could demonstrate that

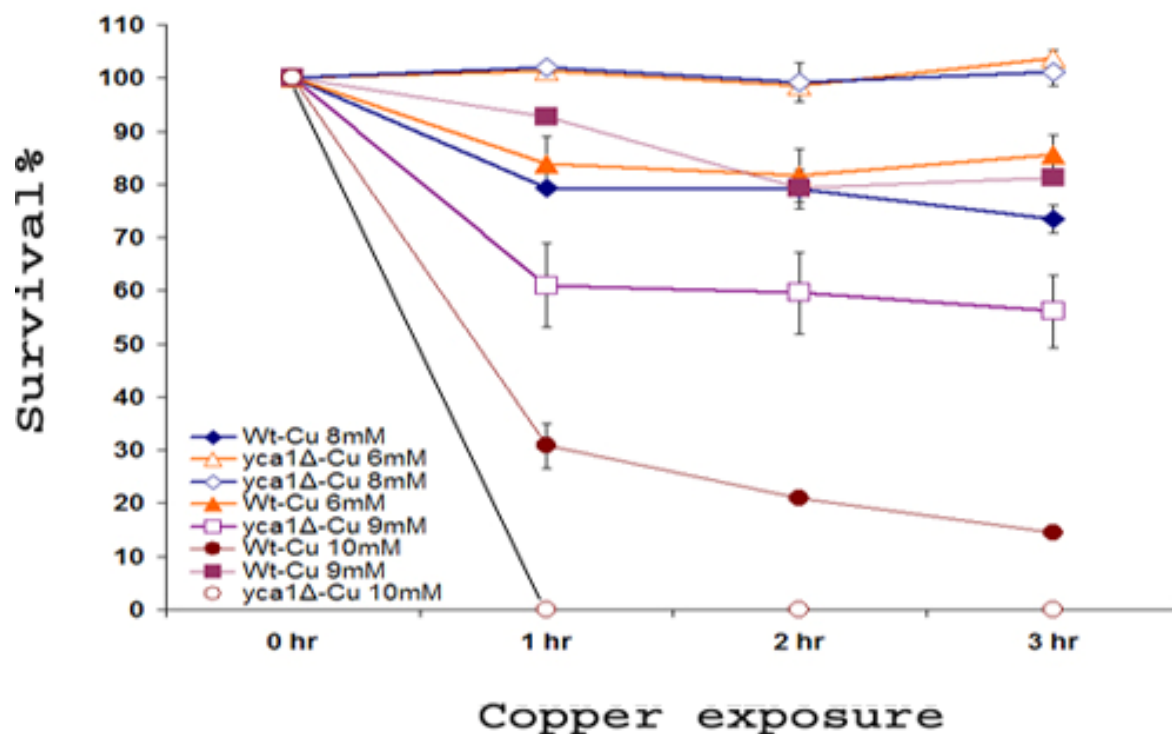


Figure 42: Viability curve for Wild type and YCA1Δ yeast cells exposed to different concentrations of copper

S. cerevisiae Wild type and *YCA1Δ* were incubated in the presence of 6, 8, 9, and 10mM $\text{Cu}(\text{NO}_3)_2$ for up to 3 h. Viability was determined at intervals after the addition of metals by spreading aliquots from dilutions ($\text{OD}_{600} = 1.0$) cultures onto YEPD agar and incubated for 3 d at 30°C. Colonies were enumerated after incubation for 3 days at 30°C. Percentage viability was calculated with reference to the number of colonies formed by untreated cells at zero time.

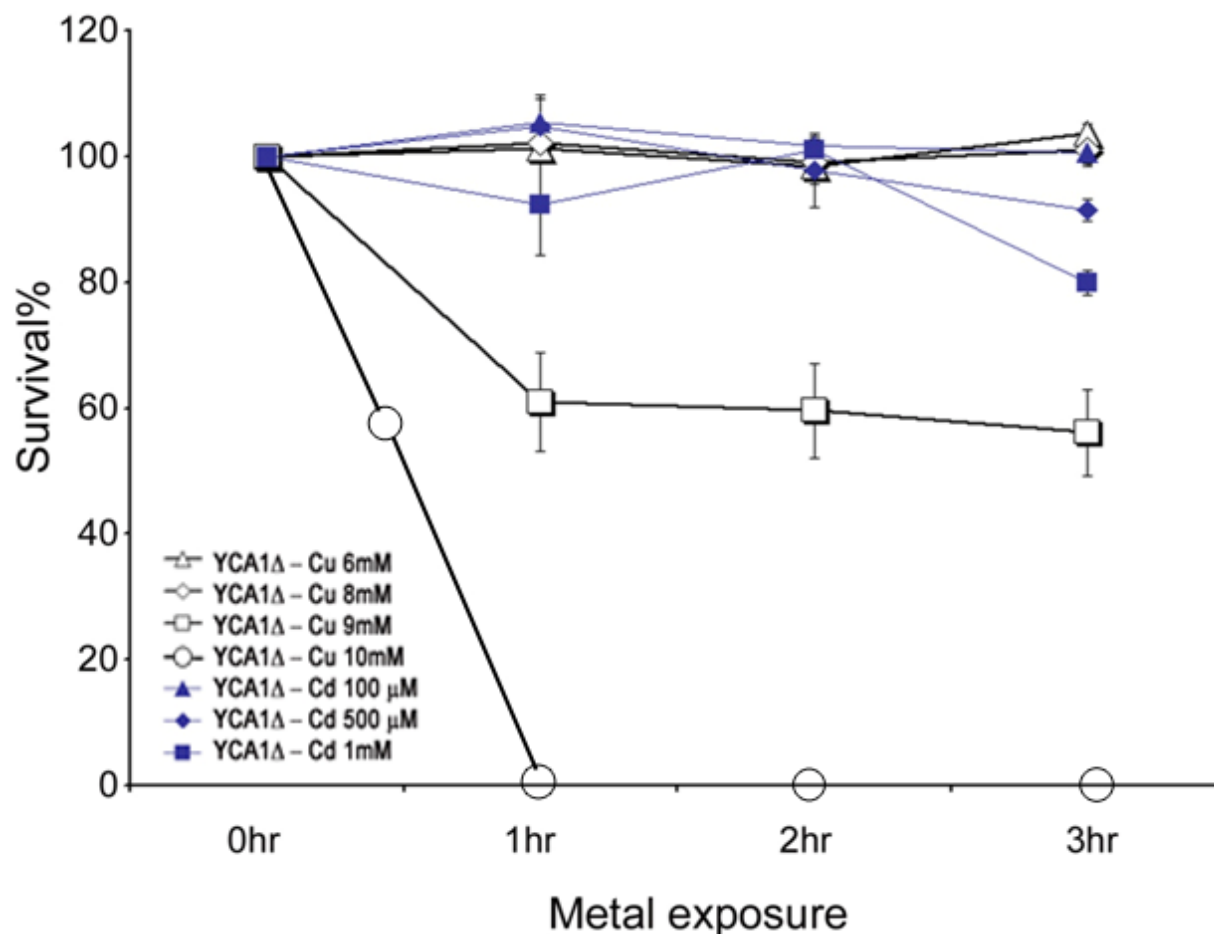


Figure 43: Viability curve for yeast cells exposed to different concentrations of copper and cadmium

S. cerevisiae YCA1Δ was incubated in the presence of 6, 8, 9 mM $\text{Cu}(\text{NO}_3)_2$ and 100 μM, 500 μM and 1mM $\text{Cd}(\text{NO}_3)_2$ for up to 3 h. Viability was determined at intervals after the addition of metals by spreading aliquots from dilutions ($\text{OD}_{600} = 1.0$) cultures onto YEPD agar and incubated for 3 d at 30°C. Colonies were enumerated after incubation for 3 days at 30°C. Percentage viability was calculated with reference to the number of colonies formed by untreated cells at zero time.

concentration greater than or equal to 9mM Cu and 500 μ M Cd was toxic to the cells. The concentration of metals we used in this report therefore is not toxic to cells. Cells are able to respond and survive as a population at this concentration (Fig 43).

When yeast cells undergo apoptosis under different kinds of stress, mitochondria play a significant role in the process^{5,8}. Given that the inductive signals are from within the cell, it is, therefore, considered to be equivalent to an intrinsic pathway of apoptosis in higher eukaryotes⁹, in which mitochondria provide the major trigger mechanism for apoptosis. In such a response, mitochondria are depolarized with the release of cytochrome c that activate caspases and form apoptosomes¹⁰⁻¹¹. In higher eukaryotes also, Bcl2 family proteins play major roles in the permeabilization of the mitochondria¹². In *Saccharomyces cerevisiae*, however, even though mitochondria still appear to play a significant part in the apoptotic program, no Bcl2 family proteins have yet been found¹³. As a consequence there is still a considerable lack of knowledge as to precisely what is involved in the break-apart of the mitochondria. Some of the more novel findings in this report demonstrate address this particular issue, and suggest that –at least in metal-induced apoptosis in yeast- the yeast caspase (Yca1), itself, is actually required for any mitochondrial membrane perturbations. While such a role for a caspase would be somewhat unusual, it is not without precedence, In this report we show that yeast caspase1 is not only required for generation of toxic oxygen radicals, but also for the directed oxidation of mitochondrial proteins.

In the past mitochondrial protein oxidation has been reported during apoptosis in some studies, as has the carbonylation of Bcl2 and ANT1 apoptosis¹⁴⁻¹⁵. Such oxidation may lead to mitochondrial damage leading to depolarization of mitochondria. We have

further demonstrated that proteins involved in electron transport chain are oxidized upon exposure to copper and cadmium, and that such oxidation minimally involves the activation of Yca1 (in cadmium treated cells it is required for the process to occur). Any such damage to electron transport chain proteins can lead to depolarization of mitochondria. We, therefore propose that the yeast caspase 1 is minimally responsible for initiating the disruption of mitochondrial membranes. While this is not the most well characterized attribute of caspases, it has been shown, by *in vitro* studies in higher eukaryotes, that caspase can interact directly with ETC protein to cause mitochondrial perturbation and depolarization¹⁶. As a result, it will be interesting to study further the interaction(s) between Yeast caspase 1 and mitochondrial proteins such as ETC proteins that may lead to the breakup of mitochondria in yeast. Certain other mitochondrial proteins such as Por1 (Voltage dependent anion channel) are suspected to be involved in apoptosis in yeast¹⁷. The main function of VDAC is to allow metabolite diffusion and to maintain mitochondrial potential¹⁸. It is interesting to note that, in this study, we clearly show that Por1 is oxidized specifically, and in a Yeast caspase-1 dependent manner, during metal-induced apoptosis. Oxidation of VDAC could, therefore, provide the all important link between Yca1 activity and mitochondrial stress - leading to mitochondrial damage and eventual collapse.

Another interesting protein that is specifically oxidized upon metal exposure is Tdh3, glucose 3 phosphate dehydrogenase. Tdh3 is a cytosolic enzymes that catalyzes the conversion of glyceraldehyde 3 phosphate to 1,3 Biphosphoglycerate. Tdh3/Gapdh, however, is considered to be a pleiotropic enzyme, in that It has been shown to be involved in many different cellular activities in addition to its usual role in glycolysis¹⁹.

One of the functions it has been shown to perform is that of a signaling molecule during apoptosis²⁰. It has been shown to translocate to nucleus prior to apoptosis²¹. Also, in some studies, Tdh3 has been demonstrated to translocate into mitochondria and interact with VDAC/Porins. Such interactions, it is believed, can cause mitochondrial disruptions and, perhaps, apoptosis²². In this study, and in previous studies within our laboratory, we have shown that Tdh3 is specifically oxidized under metal-induced stress and we speculate that such oxidized protein may have a function unusually thereby causing damage to mitochondria that then causes depolarization.

Following depolarization, it has been shown (in higher eukaryotes) that the apoptosis inducing factor (AIF) can translocate from mitochondria to the nucleus and cause DNA fragmentation²³⁻²⁴. AIF1, an orthologue of AIF in yeast can perform similar function upon peroxide stress⁸. Even though not analyzed thoroughly, we have seen that AIF1 is also involved in metal induced apoptosis. Using microarray analysis, we have been able to show that AIF1 is induced upon metal exposure during apoptosis. We also confirm the involvement of AIF1 in apoptosis by showing that an AIF1Δ strain is unable to undergo apoptosis (Appendix B). GFP tagged AIF1 was created and studied for the nuclear translocation in copper and cadmium treated cells by determining the presence/absence of AIF1-GFP in sub cellular fractions. AIF1-GFP from mitochondria and nucleus was immuno-precipitated with anti-*AIF1-GFP* antibody and then analyzed for the presence/absence in respective sub-cellular fractions. We found that AIF1-GFP translocates to the nucleus upon metal treatment- confirming the involvement of AIF1 in metal induced apoptosis (Fig 44). In higher eukaryotes, there are 2 categories of apoptosis- caspase dependent and caspase independent apoptosis. AIF

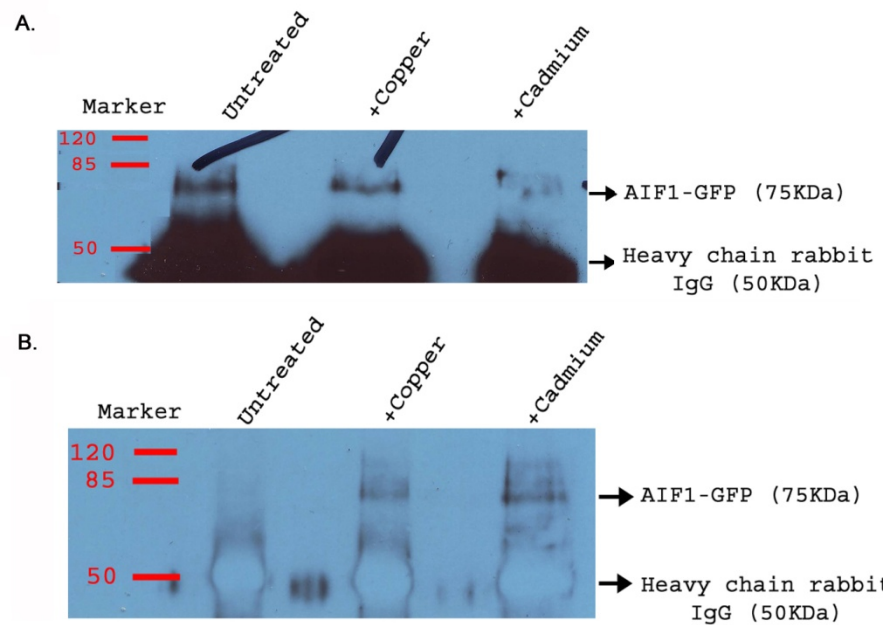


Figure 44: AIF1 is involved in metal induced apoptosis

Untreated and metals treated *AIF1-GFP* cells were fractionated to obtain mitochondrial and nuclear fraction. *AIF1-GFP* nuclear and mitochondrial extracts were confirmed using anti-cox3p as mitochondrial marker and anti-homocitrate-synthase as nuclear marker. (A) The presence of AIF1-GFP in the mitochondria was assessed using anti-GFP antibody in the mitochondria extracts. (B) The presence of AIF1-GFP in the nucleus upon metal treatment was assessed using anti-GFP antibody in the nuclear extracts.

has been shown to be involved in caspase independent apoptosis²⁵. It is called caspase independent because only executioner caspases are involved in the process. In yeast however, there is only one caspase-yeast caspase1. And AIF1 and Yca1 both seem to participate in apoptosis under peroxide stress^{8,26}. We also observe the same phenomenon under metal induced apoptosis.

Although yeast has only one known caspase, there have been many cases where *YCA1Δ* showed caspase like activity under different kinds of stress. We also noted such extra caspase-like activity on treatment of yeast cells with copper. In this study, we show for the first time that Atg4, a cysteine protease is responsible for extra caspase-like activity in yeast upon metal exposure. We confirmed the role of Atg4 by showing that *ATG4Δ* is unable to undergo apoptosis upon metal exposure. By showing the removal of extra caspase-like activity in a double knockout of *ATG4YCA1Δ*, we were also able to confirm that *ATG4* can directly (or indirectly) contribute to “background” caspase-like activity.

Atg4 is an autophagic protein that is required to cleave-activate any Atg8 that is outside the vesicle, and in so doing allow completion of the “phagosome”²⁷. While initially it was surprising to find any involvement of an autophagic gene in the apoptotic process, more and more evidence now suggests that autophagy and apoptosis are interconnected processes²⁸⁻²⁹. Although autophagy is considered to be a cell survival mechanism through the sequestration and removal of damaged proteins/organelles, it has also been suggested that, when the damage to the cell is overwhelming, autophagy may promote cell death, and perhaps trigger apoptosis. Recently ATG6, an autophagic protein has been shown to be cleaved by caspase suggesting that these two processes

are interconnected³⁰. In another recent study, it was suggested with evidence that Beclin-1/ATG6 is a molecular switch that decides the fate of the cell³¹. Autophagy and apoptosis are both very important cellular processes that have been associated to cancer and degenerative disorders. Therefore it is of great interest to understand any interconnections between these two discrete cellular programs. In this study, we confirm that such interconnection do exist in yeast, independently from any Beclin or BCL-2-like interactions. Even so, the exact signaling mechanism between autophagy and apoptosis are not yet clear. We have, however, confirmed that Atg4 can either instigate directly the cleavage activation of Yca1 in metal-induced apoptosis, or that it is minimally required for the process.

The purpose of choosing 2 different types of metal in this study was to understand the mechanisms that underlie metal toxicity, in general, and also to determine whether these metals that are inherently different in nature, possess similar or different pathways of cellular response. We found that copper and cadmium, not only generate different types of ROS, but also that copper can generate ROS more rapidly than cadmium (Fig. 33). This finding was not entirely unexpected, in that copper, unlike cadmium, is a redox-active metal, and therefore able to generate potentially toxic oxygen radicals directly. Even so, we have also shown that cadmium also “perturb” the redox balance, but in a manner that is directly dependent upon the activation of the yeast caspase. In our studies, we further show that copper and cadmium exhibit similar but distinct patterns of transcriptional induction. Genes involved in apoptosis, autophagy and certain other pathways are induced both in copper and cadmium treated yeast cells (Fig 16-29). As discussed previously, both metals can induce an apoptotic response in

yeast. Even so, their unique metallic characteristics invoke subtle differences in the potential mechanisms through which they initiate their distinct cellular response. Overall in this study we have shown that, even after a relatively low level exposure to either copper and cadmium, yeast demonstrates a full-blown apoptotic response, involving mitochondrial membrane hyperpolarization, increased levels of ROS, mitochondrial protein oxidation and DNA fragmentation, and that this response requires the presence of the Yeast caspase 1. Moreover, for first time, we have also shown that the autophagic, cysteine protease, ATG4, is directly involved in this cellular response, providing additional levels of regulatory constraints upon this process.

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APPENDICES

Appendix A

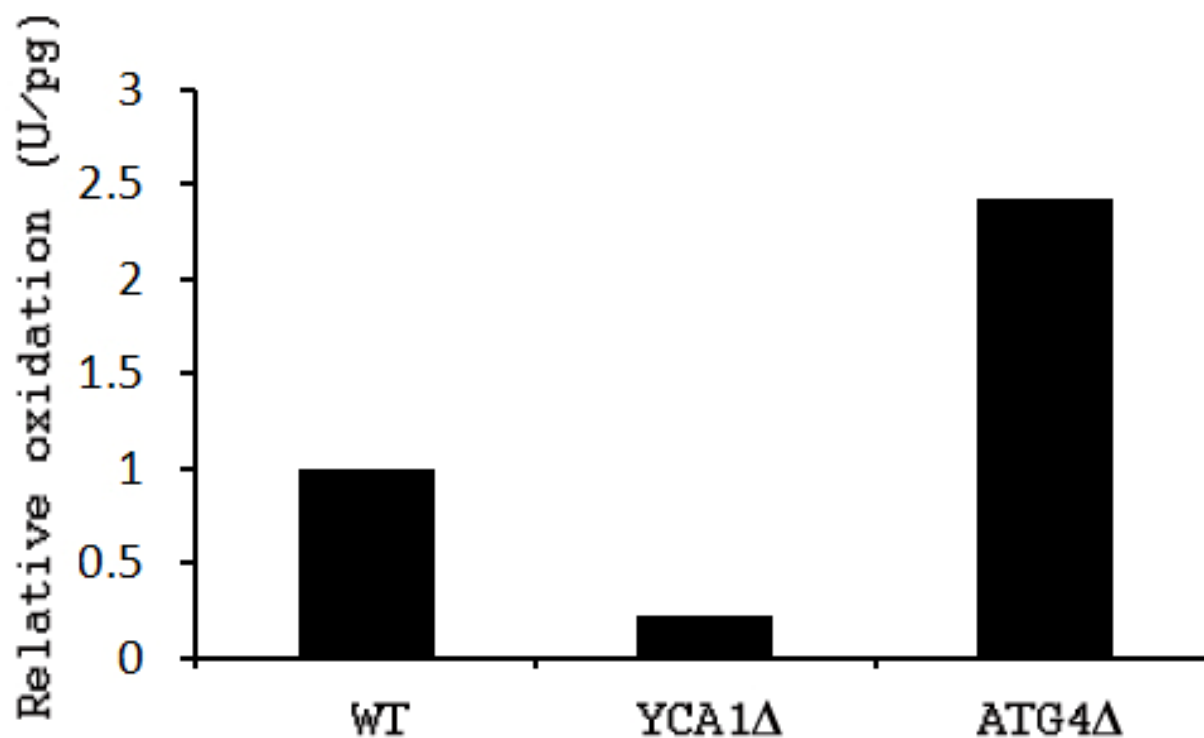


Figure 45: Relative oxidation of Ssc1

Oxidation levels of Ssc1(Hsp70 family protein) in Wild type, YCA1Δ, and ATG4Δ samples are quantified from 2D-western blot using Multigauge software. The oxidation values were then normalized to relative abundance of actin. Data consist of results from 2 independent experiments.

Appendix B

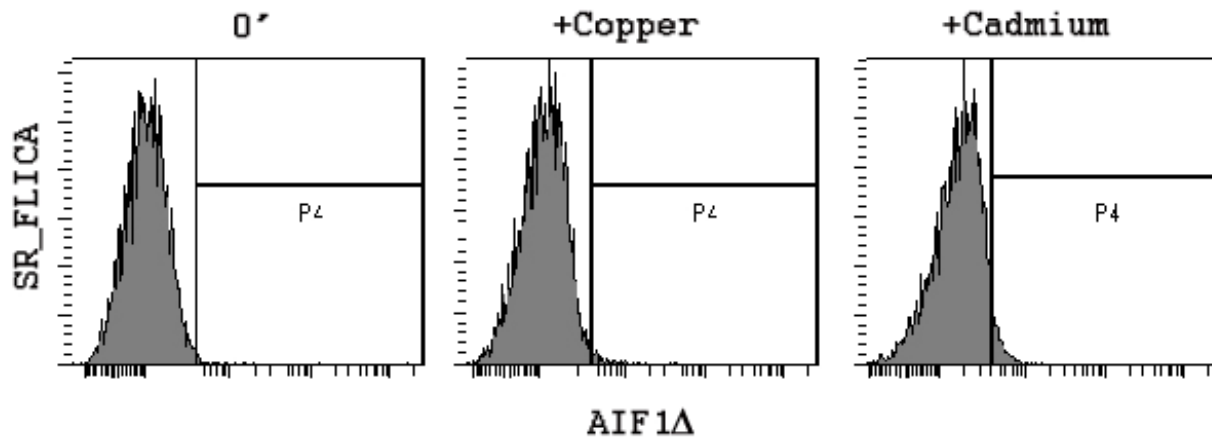


Figure 46: *AIF1Δ* is non apoptotic upon copper and cadmium exposure

AIF1Δ mutant cells were exposed to 8mM $\text{Cu}(\text{NO}_3)_2$ and 30 μM cadmium for 1 h then 'recovered' in fresh YEPD medium for 3 h. Cells were stained for active caspases (red) with SR_FLICA detection chemistry (Immunochemistry, LLC) and then activated caspase were detected using flow cytometry. Control *AIF1Δ* cells were treated in exactly the same way as the treated cells (except for the exclusion of metals from incubations).

Appendix C

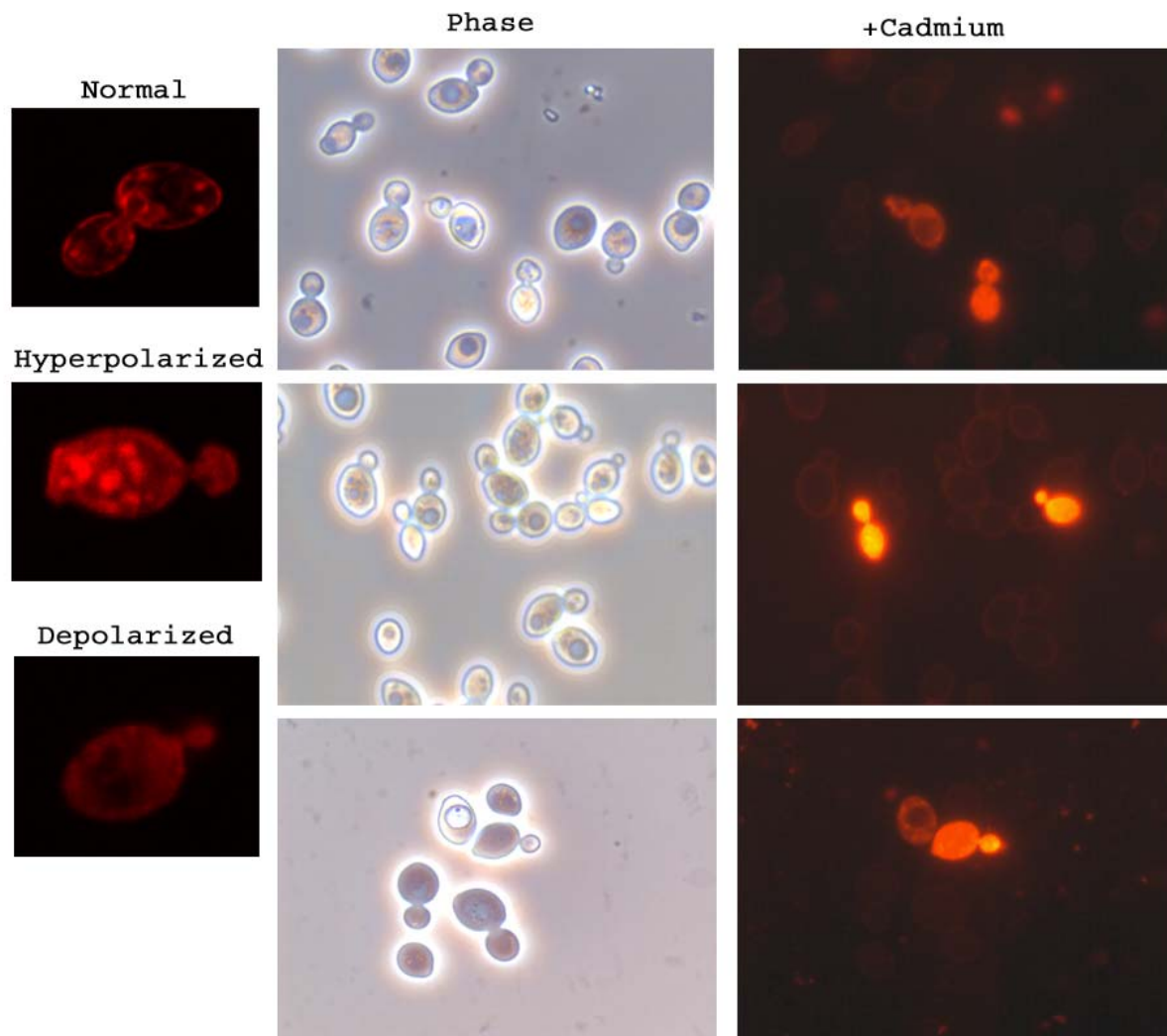


Figure 47: Mitochondrial membrane depolarization study upon cadmium exposure.

Cells were exposed to 30 μM $\text{Cd}(\text{NO}_3)_2$ for 1 h then 'recovered' in fresh YEPD medium for 3 h. Mitochondrial membrane depolarization was assessed using rhodamine 123 and analyzed by confocal microscopy: No brightly fluorescent cells were observed within control populations that were not exposed to Cd (left top panel). Cell exposed to cadmium showing hyperpolarized mitochondria is shown in middle left panel and left bottom panel show treated cell with depolarized mitochondria.

Appendix D

Table 1: Proteins that are differentially expressed within 60 minutes of copper and cadmium exposure in YCA1Δ

Sr. No	Category	Gene	Protein Name	~MW (KD)	~pI	Fold change after 60' Cu exposure	Fold change after 60' Cd exposure
1	Metabolic enzymes	Aco1	Aconitase isoform1	85,368	8.15	3.54	2.41
		Aco1	Aconitase isoform2	85,368	8.15	2.49	2.18
		Tdh3	Glyceraldehyde 3 phosphate	35,746	6.96	Similar	Similar
			dehydrogenase			Similar	Similar
		Cit1	Citrate synthase	53,360	8.86	Similar	2.23
		Cit1	Citrate synthase isoform	53,360	8.86	Similar	1.83
2	Heat shock proteins	Ssa1	Atpase	69,657	4.82	Similar	1.89
		Ssc1	Atpase Hsp70 family	70,627	5.34	Similar	2.21
		Hsp60	Heat shock protein	60,751	5.06	Similar	2.41
						Similar	Similar
3	Karyogamy	Kar1	Karyogamy protein	50,653	10.09	Similar	2.83
4	Electron transport chain	Atp1	Mitochondrial F1F0 ATP synthase	58,617	9.85	2.28	Similar
		Qcr1	Ubiquinol-cytochrome c reductase	50,227	7.34	-2.11	-2.06
			Ubiquinol-cytochrome c reductase			-2.44	-2.39
		Qcr1	Isoform	50,227	7.34	-2.44	-2.39

Appendix E

Table 2: Proteins that are differentially expressed within 60 minutes of copper and cadmium exposure in *ATG4Δ*

Sr. No	Category	Gene	Protein Name	~MW (KD)	~pI	Fold change after 60' Cu exposure	Fold change after 60' Cd exposure
1	Metabolic enzymes	Aco1	Aconitase isoform1	85,368	8.15	Similar	Similar
		Aco1	Aconitase isoform2	85,368	8.15	Similar	Similar
		Tdh3	Glyceraldehyde 3 phosphate	35,746	6.96	Similar	Similar
			dehydrogenase			Similar	Similar
		Cit1	Citrate synthase	53,360	8.86	Similar	-2.35
		Cit1	Citrate synthase isoform	53,360	8.86	Similar	Similar
2	Heat shock proteins	Ssa1	Atpase	69,657	4.82	Similar	Similar
		Ssc1	Atpase Hsp70 family	70,627	5.34	Similar	Similar
		Hsp60	Heat shock protein	60,751	5.06	Similar	1.95
						Similar	Similar
3	Karyogamy	Kar1	Karyogamy protein	50,653	10.09	Similar	Similar
4	Electron transport chain	Atp1	Mitochondrial F1F0 ATP synthase	58,617	9.85	Similar	Similar
			Ubiquinol-cytochrome c reductase				
		Qcr1	Ubiquinol-cytochrome c reductase	50,227	7.34	Similar	-3.55
		Qcr1	Isoform	50,227	7.34	-2.43	-2.09

Appendix F

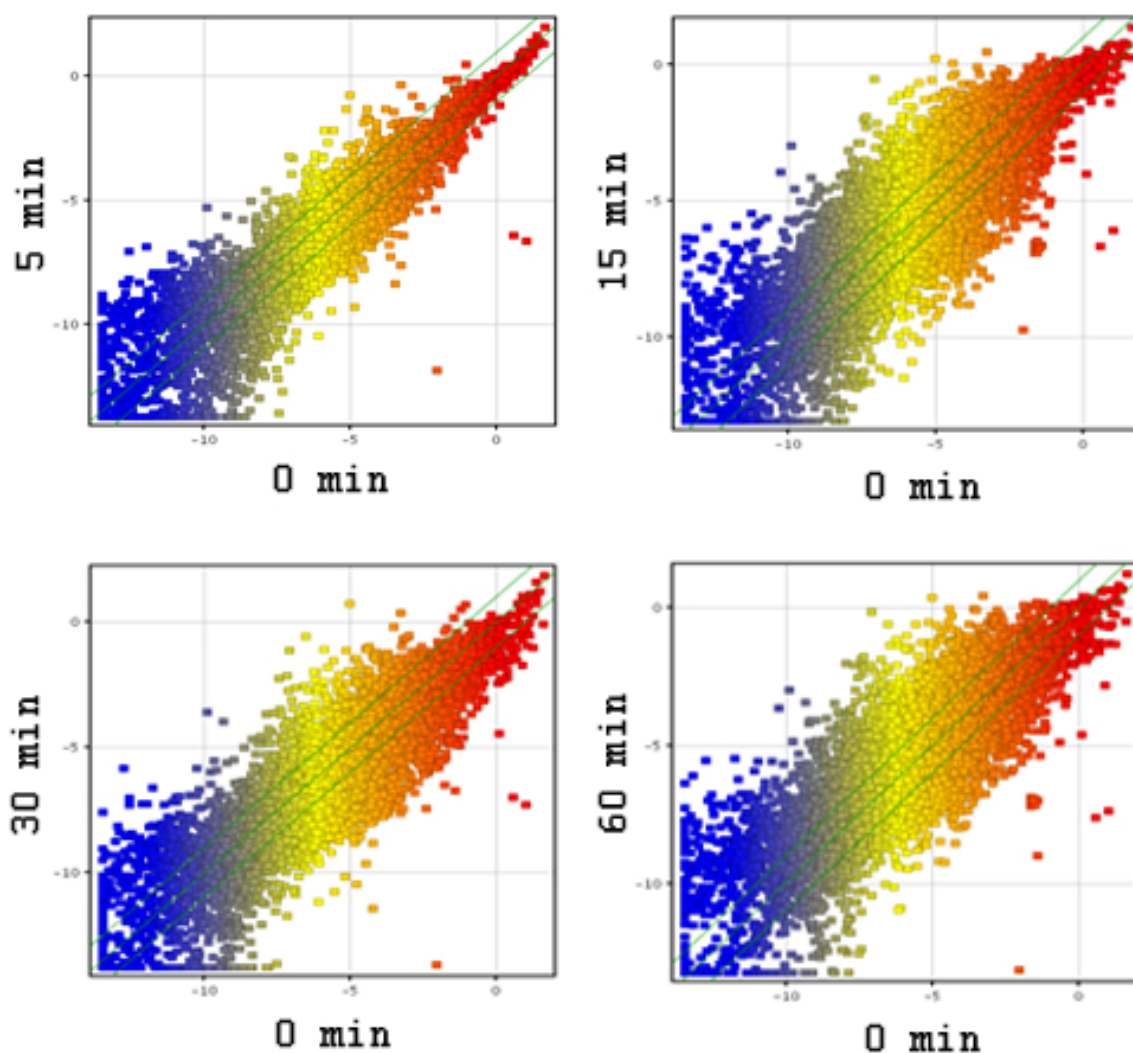


Figure 48: Gene expression at various time points upon copper exposure.

Total mRNA isolated from copper (8mM) treated wild type *Yeast* BY4741 and from, untreated control cells was hybridized to Affymetrix SG-98 microarray chips. The four scatter plots represent gene expression profiles comparing the treated to the untreated yeast cells at different time points after exposure: (Left top) 5 minutes, (Right top) 15 minutes, (Left bottom) 30 minutes, and (Right bottom) 60 minutes. Yellow dots represent unchanged gene expression, blue dots indicate down-regulated genes, and orange/red dots symbolize up-regulated genes compared with the control. Green bars represent two fold-change ranges for each chip hybridized. Each point depicts an individual element of the microarray.

Appendix G

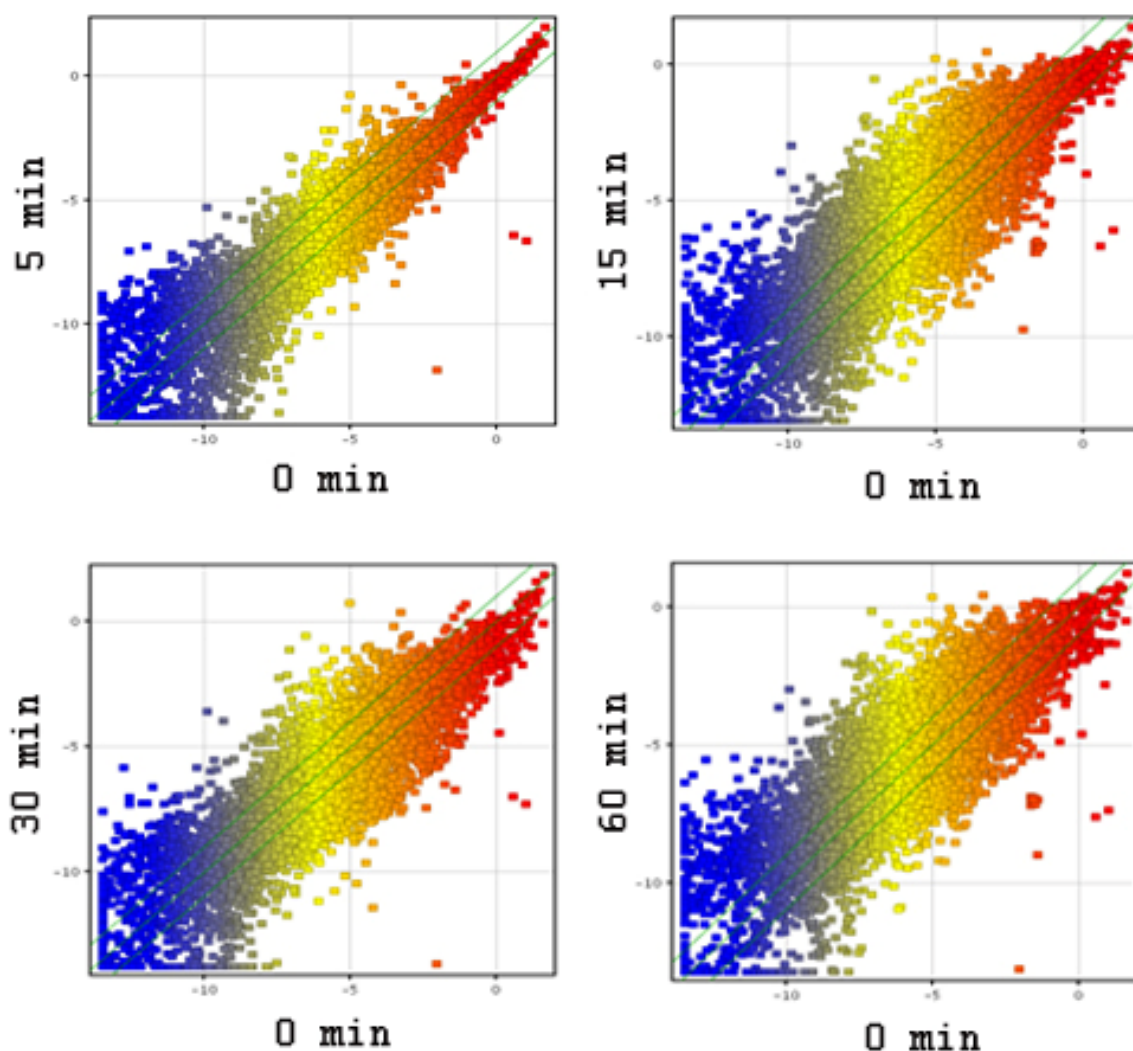


Figure 49: Gene expression at various time points upon cadmium exposure.

Total mRNA isolated from cadmium (30 μ M) treated wild type Yeast BY4741 and from, untreated control cells was hybridized to Affymetrix SG-98 microarray chips. The four scatter plots represent gene expression profiles comparing the treated to the untreated yeast cells at different time points after exposure: (Left top) 5 minutes, (Right top) 15 minutes, (Left bottom) 30 minutes, and (Right bottom) 60 minutes. Yellow dots represent unchanged gene expression, blue dots indicate down-regulated genes, and orange/red dots symbolize up-regulated genes compared with the control. Green bars represent two fold-change ranges for each chip hybridized. Each point depicts an individual element of the microarray.