SOD ENZYMES IN A HUMAN FUNGAL PATHOGEN: OXIDATIVE STRESS PROTECTION VERSUS CELLULAR SIGNALING

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Abstract

Candida albicans is a human opportunistic pathogen of important public health relevance. The capacity of this organism to adapt to drastic changes in nutrient availability in the host allows *C. albicans* to be a successful pathogen. More specifically *C. albicans* adapts to fluctuations in bioavailability of the metal nutrient copper by substituting a copper requiring superoxide dismutase enzyme (Cu/Zn SOD1) with a non-copper alternative (Mn SOD3). Both are antioxidant enzymes that remove superoxide free radicals. However, the exact function of these two SOD enzymes inside the cell and whether they perform redundant or diverging roles remained largely unknown, and is the focus of investigation in this thesis.

We found that Cu/Zn SOD1 but not Mn SOD3 enters the mitochondria intermembrane space to protect against mitochondrial oxidative stress (Chapter 2). During copper starvation when cells repress Cu/Zn SOD1, *C. albicans* induces an iron requiring alternative oxidase (AOX) to maintain mitochondrial superoxide at low levels. We find that this replacement of Cu/Zn SOD1 with non-copper alternatives (Mn SOD3 and Fe AOX) helps spare copper for cytochrome c oxidase respiration, which is essential for pathogenesis.

Both Cu/Zn SOD1 and Mn SOD3 localize to the cytosol and in Chapter 3 we investigate their possible role in cell signaling involving reactive oxygen species (ROS). In the related yeast *Saccharomyces cerevisiae,* Cu/Zn SOD1 participates in a cell signaling process involving ROS that promotes glucose uptake and represses respiration. Whether *C. albicans* Cu/Zn SOD1 and/or Mn SOD3 were capable of similar cytosolic cellular signaling was unknown. We established that both SODs of

C. albicans participate in glucose signaling, however as a fungal pathogen, *C. albicans* has rewired the SODs such that they signal repression of glucose uptake, rather than activation of glucose uptake as is seen in the fermentative yeast *S. cerevisiae*. *C. albicans* heavily relies on respiration, not fermentation for pathogenesis, consistent with the repurposing of its SODs in glucose signaling.

Altogether, this work illustrates unique mechanisms in *C. albicans* for responding to changes in nutrients, specifically copper and glucose. These traits likely reflect the need to adapt rapidly to changes in environment within the human host.

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Abbreviations and Nomenclature

° C	degrees Celsius
µg	micrograms
µM	micromolar
AAS	atomic absorption spectroscopy
ATP	adenosine triphosphate
AOX	alternative oxidase
BCS	bathocuproine sulphonate
CCS	copper chaperone for superoxide dismutase I
cDNA	complementary deoxyribonucleic acid
COX	cytochrome c oxidase
Cu	copper
Cu(I)	monovalent copper
DHE	dihydroethidium
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
ETC	electron transport chain
Fe	Iron
Fig.	figure
g	gram

GFP	green fluorescent protein
H_2O_2	hydrogen peroxide
HIS	histidine
HGT	high affinity glucose transporter
ICP-MS	inductively coupled plasma mass spectrometry
IMS	intermembrane space
KCN	potassium cyanide
L	liter
Leu	leucine
M	molar
Mito	mitochondria
mg	milligram
mL	milliliter
Mn	manganese
M^{ox}	oxidized metal
M^{red}	reduced metal
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
nm	nanometer
NOX	NADPH oxidase
OD_{600}	optical density at 600nm
O_2	molecular oxygen

$O_2^{\bullet-}$	superoxide
P144	proline at residue 144 of *C. albicans* SOD1 protein
PMS	post mitochondrial supernatant
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qRT-PCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SHAM	salicylichydroxamic acid
SC	synthetic complete yeast medium
SOD	superoxide dismutase
SRR	sensor/receptor-repressor
URA	uracil
WT	wild type
X g	times gravity
YPD	yeast extract, peptone, dextrose enriched yeast medium
Zn	zinc

Yeast Nomenclature

CEN	centromere, single copy plasmid
2 micron	high copy number yeast vector
All caps, italics	wild type gene (e.g. *SOD1*)

No caps, italics	mutant gene (e.g. *sod1*)
All caps, no italics	protein (e.g. SOD1)
Δ	gene deletion in haploid yeast (i.e. *S. cerevisiae*)
Δ/Δ	homozygous gene deletions in diploid yeast (i.e. *C. albicans*)

Chapter 1

Introduction and Background

Superoxide Dismutase Enzymes

Superoxide dismutases (SODs) are a family of abundant antioxidant enzymes that scavenge the free radical superoxide anion. SODs are highly conserved from bacteria to humans and catalyze the disproportion of superoxide anion into molecular oxygen and hydrogen peroxide via a two step metal catalyzed reaction where M= the catalytic metal ion [1-3]:

$$M^{ox} + O_2^{\bullet -} \rightarrow M^{red} + O_2$$

$$M^{red} + O_2^{\bullet -} + 2H^+ \rightarrow M^{ox} + H_2O_2$$

The disproportionation reaction occurs at reaction rates of $\sim 10^9\ M^{-1}\ s^{-1}$, approaching diffusion limits, which makes SODs some of the fastest enzymes in biology [1].

The free radical, superoxide, is produced through a one-electron donation to molecular oxygen, often as a consequence of aerobic respiration. Once formed the highly reactive superoxide anion can act as either an oxidant or reductant reacting directly with macromolecules in the cell. Additionally, superoxide can be subsequently reduced or will spontaneously disproportionate to form hydrogen peroxide. Superoxide has been shown to oxidize proteins containing Fe-S clusters (4Fe-4S or 2Fe-2S) [2], which leads to the release of Fe(II). Free Fe(II) in the cell can react with hydrogen peroxide through Fenton chemistry yielding the formation of the highly reactive and dangerous hydroxyl radical ($OH^{\bullet}$) for which there is no means for detoxification [4]. The resulting hydroxyl radical can act as a powerful oxidant of DNA, proteins, carbohydrates and fatty acids [5, 6]. Therefore SODs are

the first line of defense in the protection of cells against oxidative stress by scavenging toxic superoxide.

SOD enzymes have evolved on three separate occasions resulting in three classes of SODs that diverge in sequence and structure but all utilize a redox active metal cofactor to catalyze the same disproportionation reaction [1]. These include a Cu/Zn SOD family where copper is the catalytic metal ion and zinc serves a structural role; a large family of Mn and Fe SODs [1], which utilize either manganese or iron for catalysis; and a rare family of nickel containing SODs [7]. The variety in SOD enzymes likely reflects the bioavailability of metal ions. In gram-negative bacteria, copper is restricted to the periplasmic/extracellular space, while manganese and iron can accumulate inside the cell. As a consequence of the localization of bioavailable metal ions, Cu/Zn SODs are predominately found in the periplasm, while Mn and Fe SODs are localized in the cytosol (Fig. 1-1). This partitioning of SOD enzymes has been conserved in the mitochondria of eukaryotes, as Cu/Zn SOD is localized to the intermembrane (IMS) space of the mitochondria, equivalent to the bacterial periplasm, and Mn SODs are found in the mitochondrial matrix resembling the bacterial cytoplasm [8, 9] (Fig. 1-1).

Sources of Superoxide

The superoxide anion is negatively charged and cannot cross a biological membrane, therefore SODs must be localized in different compartments in the cell where superoxide scavenging is required. There are mitochondrial and cytosolic sources of superoxide and for the purpose of this work great emphasis will be

placed on mitochondrial-derived superoxide produced as a byproduct of the electron transport chain [10].

The electron transport chain (ETC) consists of four multi-subunit complexes embedded in the mitochondrial inner membrane, which allow the transfer of electrons from NADH to molecular oxygen (Fig. 1-2A). The transfer of electrons from one complex to another is coupled to proton pumping across the inner mitochondrial membrane producing an electrochemical gradient that is utilized to generate ATP. With the exception of the final transfer of electrons to molecular oxygen, all other steps catalyze single-electron transfers [11]. Single electrons can leak during these transfers reducing molecular oxygen and forming the free radical, superoxide.

Experimental evidence has shown that the main sources of superoxide production in the ETC occur at complex I and complex III [12-14]. Complex I transfers electrons from NADH to ubiquinone which can leak into the mitochondrial matrix, producing superoxide in this compartment [12, 15] (Fig. 1-2, top). Complex III which transfers electrons to cytochrome c has been shown to release electrons to both sides of the inner membrane, producing superoxide in both the mitochondrial matrix and IMS [16, 17] (Fig. 1-2, top). Superoxide produced in the IMS is not restricted to this compartment as superoxide can leak to the cytosol through voltage dependent anion channels [18]. Approximately 1-5% of oxygen consumed through respiration is reduced to superoxide, which underscores the importance of having superoxide dismutase enzymes in mitochondria. The partitioning of SOD enzymes to the matrix and IMS of the mitochondria reflects corresponding locations where

superoxide is produced. Cu/Zn SOD1 serves to scavenge superoxide in the IMS whereas Mn SOD2 is localized to the matrix to eliminate superoxide in this compartment.

Superoxide can be produced outside of the mitochondria by proteins localized in the cytosol and other organelles such as the endoplasmic reticulum. NADPH cytochrome P450 reductase and xanthine oxidase are two classes of enzymes involved in cellular metabolism that reduce molecular oxygen to produce superoxide as a byproduct of catalysis [19, 20]. The superoxide from these enzymes is released into the cytosol and is the substrate for cytosolic Cu/Zn SODs. NADPH oxidases (NOX) enzymes are often located in the plasma membrane, but also exist in intracellular sites such as the endoplasmic reticulum. NOX enzymes produce either intracellular superoxide in the cytosol as is seen in the bakers' yeast *Saccharomyces cerevisiae*, or extracellular superoxide as is observed in mammalian immune cells [21-23]. NOX derived superoxide has been shown to play a role in cellular signaling and a key strategy in host defense against microbes [24, 25].

Cellular roles of Cu/Zn SOD1

Amongst all of the SOD enzymes, the intracellular Cu/Zn SOD1 of eukaryotes is the best understood. Cu/Zn SOD1 exists as a homodimer, where each monomeric unit binds one catalytic copper ion and one structural zinc ion in the active site [26]. Metallation and activity of SOD1 depends on the copper chaperone for SOD1 (CCS) [27]. CCS delivers copper to the active site of SOD1 and is required for the formation of a disulfide bond essential for enzyme catalysis [28, 29]. In a typical eukaryote

SOD1 is largely localized to the cytosol, but also exists in the nucleus [30] and secretory pathway [31]. Additionally a small fraction of SOD1 has been localized to the mitochondrial IMS [32, 33]. As SOD1 resides in a number of cellular compartments, the role of SOD1 is not limited exclusively to cytosolic oxidative stress protection. Cu/Zn SOD1 is a key player in cytosolic cellular signaling as well as protecting the mitochondria from oxidative stress. These dual roles of SOD1 in cytosolic cell signaling and mitochondrial oxidative stress protection are the focus of this thesis.

SOD1 in cytosolic signaling

The role of eukaryotic SOD1 in cell signaling has been documented in both mammalian cells and the bakers' yeast *Saccharomyces cerevisiae.* Of particular relevance to this thesis is the role of yeast SOD1 in casein kinase signaling. Members of the casein kinase-1 gamma family including YCK1 and YCK2 play important roles in cellular signaling by regulating glucose sensing, repression of respiration, and amino acid sensing [34] . Recent studies from the Culotta lab show a role for SOD1 in this process whereby SOD1 enzyme catalysis works upstream of YCK1 and YCK2 to help promote glucose repression. In the presence of oxygen and glucose, the NADPH oxidase, YNO1, produces the superoxide substrate for Cu/Zn SOD1 needed for regulating YCK1 and YCK2. SOD1 was shown to physically bind to the C-terminus of the YCK1 protein, and when SOD1 catalyzes superoxide disproportionation at this site, the local production of hydrogen peroxide somehow protects a degron located in the C-terminus of YCK1, preventing degradation of the kinase [35] (Fig. 1-4). This

regulation is indeed dependent on hydrogen peroxide, as YCK1 can also be stabilized when yeast cells are exposed to a continuous source of hydrogen peroxide [35]. While the exact mechanism of how hydrogen peroxide protects the C-terminus from degradation is not fully understood, it may rely on the modification of three lysine residues localized in the YCK1 C-terminus. When these lysines are mutated to arginine residues, YCK1 is no longer regulated by SOD1 [35].

The ability of Cu/Zn SOD1 to prevent degradation of members of the casein kinase-1 gamma is not exclusive to *S. cerevisiae*. Cu/Zn SOD1 from humans and *C. elegans* can stabilize YCK1 when expressed in *S. cerevisiae*, and human Cu/Zn SOD1 can stabilize the mammalian homolog CK1γ in a human cell line [35]. It is curious that only Cu/Zn SODs seem capable of participating in this signaling paradigm as the mitochondrial Mn SOD2 from *S. cerevisiae* was unable to prevent YCK1 degradation [35]. This will further addressed in this thesis (Chapter 3).

In mammalian cells, Cu/Zn SOD1 is also involved in the regulation of receptor tyrosine kinase (RTK) signaling [36]. A NADPH oxidase (NOX, gp91 Phox) is coupled to tyrosine kinase receptors and upon stimulation by growth factors such as epidermal growth factor (EGF) this NOX produces superoxide, the substrate for cytosolic SOD1 [37]. Superoxide dismutation produces hydrogen peroxide and the SOD1-derived hydrogen peroxide inactivates the protein tyrosine phosphatases (PTPs) for the RTKs through oxidation of a catalytic cysteine in the PTP susceptible to ROS. Thus RTKs prevent their own deactivation through ROS and SOD1 and the signal is amplified [36].

Cu/Zn SOD1 not only participates in cellular signaling through the production of hydrogen peroxide, but can also act as a transcription factor. Tsang and colleagues showed the rapid localization of SOD1 to the nucleus of *S. cerevisiae* and humans cells in response to endogenous and exogenous oxidative stress [30]. The ROS is sensed by the ATM kinase, MEC1, promoting the phosphorylation of SOD1 crucial for translocation of SOD1 in to the nucleus. Once inside the nucleus SOD1 binds to promoter regions regulating the transcription of genes involved in ROS-induced DNA replication stress, DNA damage responses, and the maintenance of cellular redox state. Therefore, nuclear SOD1 acts as transcription factor regulating gene expression necessary to maintain the integrity of genomic DNA during oxidative stress [38].

SOD1 in mitochondrial oxidative stress protection

While Cu/Zn SOD1 is largely localized to the cytosol, a small fraction of Cu/Zn SOD1 is localized to the intermembrane space (IMS) of the mitochondria [32, 39-42]. A typical protein destined for the mitochondria is targeted to the organelle by a mitochondrial targeting presequence that is cleaved upon entry into the mitochondria. Cu/Zn SOD1, however contains no such presequence and is imported through a disulfide relay system in a manner that is dependent on the copper chaperone, CCS1 [32, 43-45]. The majority of SOD1 molecules in the cytosol exists in a form where the disulfide bonds are oxidized, disabling SOD1 to pass through the pores of the outer mitochondrial membrane. A small fraction of SOD1 that does not react with CCS1 in the cytosol is reduced, and this reduced form can freely pass

through the TOM channels of the outer membrane into the mitochondrial IMS. CCS1 is imported into the mitochondrial IMS through a disulfide relay system involving MIA40, trapping CCS1 in the IMS [46, 47] (Fig. 1-4, left). Once IMS SOD1 interacts with IMS CCS1, the SOD1 disulfide is oxidized, trapping SOD1 in the IMS [48-50] (Fig.1-4, right).

Inside the mitochondrial IMS, Cu/Zn SOD1 serves to scavenge superoxide that is released into this compartment by the electron transport chain (ETC) during aerobic respiration. Work from the Culotta lab in *S. cerevisiae* demonstrates that *sod1Δ* mutants exhibit an elevation in carbonylation of mitochondrial proteins and rapid death during stationary phase, both markers of mitochondrial oxidative stress [32]. SOD1 has also been found in the IMS of human cells and mutant forms of human SOD1 lead to oxidation of mitochondrial proteins and lipids [33], defects in mitochondrial respiration and ATP synthesis as well as changes in mitochondrial morphology [33, 51, 52]. These data emphasize the importance of Cu/Zn SOD1 in protecting mitochondria against respiratory derived oxidative stress.

From bakers yeast to mammalian cells, the Cu/Zn containing SOD1 is the major SOD, representing as much as 1% of the total cellular protein and functioning in both cell signaling and oxidative stress protection. However there are rare eukaryotic organisms that do not express Cu/Zn containing SODs and substitute this with a cytosolic manganese containing SOD (described in more detail below). Mn SODs are a distinct family of metalloenzymes and to date, there has been no report of Mn SODs participating in cytosolic signaling or localizing to the mitochondrial IMS for oxidative stress protection as described above for Cu/Zn SOD1. Research in

this thesis will address how a Mn containing SOD may substitute for Cu/Zn SODs in an organism that evolved with a complex family of multiple SOD enzymes, namely the fungal pathogen *Candida albicans.*

The opportunistic pathogen *Candida albicans*

Candida albicans is a Saccharomycete yeast that is found as a commensal of the human flora, primarily inhabiting mammalian mucus membranes such as the gastrointestional tract, oral cavity and the vagina. When left unchecked by the host immune system or surrounding microflora, *C. albicans* can become an opportunistic pathogen. This yeast can be found in approximately 30-70% of the human population [53], and is responsible for mild infections such as thrush or vaginal yeast infections. When the epithelial integrity is compromised or when the immune system is suppressed life threatening systemic infections can occur with a mortality rate of ~ 30% [54].

Systemic candidiasis can occur when the surrounding bacterial community is disrupted through the administration of antibiotics and in immunocompromised populations, including individuals with HIV/AIDS, patients undergoing cancer therapeutics and premature infants [54, 55]. While current antifungals such as fluconazole are widely used, the prevalence of life-threatening systemic infections remains high, especially in hospital settings [56]. Additionally, with the emergence of drug resistant strains, *C. albicans* continues to be an important public health concern, and the development of new antifungal strategies is necessary.

C. albicans is a polymorphic fungus, existing as a budding yeast and as filamentous forms including pseudohyphae and true hyphae [57, 58]. It is this ability to switch between different morphologies that allow *C. albicans* to penetrate tissues, invade the blood stream and infect peripheral organs such as the kidney, liver and spleen [59]. Bioavailability of oxygen and nutrients such as glucose and metal ions has been shown to vary in different niches of the body. The ability to adapt to drastic changes in nutrient availability allows *C. albicans* to be a successful pathogen.

Unusual collection of SOD enzymes in *Candida albicans*

A typical eukaryote contains a Cu/Zn SOD1 that resides largely in the cytosol and a manganese containing SOD, SOD2 that is localized to the mitochondrial matrix. *C. albicans* displays an unusually large collection of SOD enzymes. In addition to cytosolic Cu/Zn SOD1[60] and mitochondrial Mn SOD2, *C. albicans* possesses three extracellular copper only SODs, (SODs 4,5 and 6) [61, 62], and of particular interest to the thesis research described here, a second manganese containing SOD, SOD3 [63] (Fig. 1-1). Mn SOD3 is highly homologous to the mitochondrial Mn SOD2, however it lacks the presequence required for import into the mitochondria and has a predicted cytosolic localization [64]. Throughout evolution copper and manganese SODs have been separated to different cellular compartments due to the bioavailability of their respective metal-cofactors [8, 9]. The vast majority of eukaryotes solely express Cu/Zn SOD1 in the cytosol. Crustaceans and certain photosynthetic microbes lack a Cu/Zn SOD1 and express only a Mn SOD in the

cytosol. In the blue crab, copper is largely used for oxygen transport, through the protein hemocyanin, instead of antioxidant protection by Cu/Zn SOD1 [65]. The localization of both a copper containing SOD and a manganese containing SOD to the same cytosolic compartment is extremely rare and is exclusive to *C. albicans* and closely related fungi.

Recent work in the Culotta lab has shown that *C. albicans* expression of two cytosolic SODs serves as an adaptation to copper starvation [66]. When copper is abundant Cu/Zn SOD1 is the predominat cytosolic SOD expressed; however, when copper levels become scarce, Cu/Zn SOD1 is repressed and Mn SOD3 is expressed as the sole cytosolic SOD. The reciprocal regulation of Cu/Zn SOD1 and Mn SOD3 is regulated at the level of transcription by the copper sensing transcription factor, MAC1. In copper depleted conditions, MAC1 binds to a consensus sequence in the intron of SOD1 to block transcription of *SOD1* mRNA and conversely activates transcription of *SOD3* by binding to a consensus sequence in the *SOD3* promoter region [66]. By switching to a non-copper SOD alternative in the cytosol, *C. albicans* ensures SOD activity regardless of copper status.

The switch from SOD1 to SOD3 is not only seen in laboratory cultures but has been observed in clinical isolates of *C. albicans* and during a mouse model of disseminated candidiasis [66]. In this model, *C. albicans* is introduced into the mouse through a lateral tail vein injection, where the kidney is the main target of infection. Work in our lab has demonstrated that during early infection there is abundant copper in the kidney, however as infection progresses kidney copper levels decline [66]. It was additionally observed that *C. albicans* adapted to this

decrease in kidney copper during the course of infection by swapping SOD enzymes. When kidney copper levels are high in early stages of infection, *C. albicans* express Cu/Zn SOD1. During late stages of infection, where kidney copper levels are low, *C. albicans* repress *SOD1* mRNA and induce the *SOD3* transcript [66]. While much is known about the regulation of SOD1 and SOD3 in *C. albicans*, the role these two SOD enzymes play in ROS homeostasis remained largely unknown and will be extensively investigated in this thesis.

Copper sparing

The aforementioned switch of a copper containing enzyme for a non-copper alternative, i.e., Mn SOD3 represents a means for sparing copper micronutrients. Copper is an essential metal nutrient for most organisms and is utilized as a cofactor for proteins involved in numerous processes such as respiration, metal transport, and oxidative stress resistance. While essential for growth, high levels of copper can be toxic to the cell and thus intracellular levels of bioavailable copper are extremely low (10^{-19}M) [67]. With such low levels of bioavailable intracellular copper, the metal must be strategically distributed among cuproenzymes, hence the concept of "copper sparing". During times of copper limitation a dispensable copper containing protein is down-regulated sparing copper for use in a more essential cuproprotein. An instance of copper sparing has been reported in the photosynthetic algae, *Chlamydomonas reinharti* [68]. In *C. reinharti* copper is partitioned between three copper containing enzymes, plastocyanin, utilized for photosynthesis; ferroxidase, essential for iron assimilation; and cytochrome c

oxidase, required for respiration. During moments of copper starvation plastocyanin is degraded and replaced by a non-copper alternative, cytochrome $c_{6,}$ which utilizes heme [69]. The copper spared by down-regulation of plastocynanin is utilized by cytochrome c oxidase for respiration, since a copper independent alternative does not exist for this protein [68]. Through the copper sparing mechanism, *C. reinharti* maintains functionality of the respiratory chain during times of copper limitation.

As described, *C. albicans* is susceptible to copper deprivation in the kidney during disseminated candidiasis and the limited amount of copper must be partitioned to all of the copper containing proteins in the cell. In addition to Cu/Zn SOD1, *C. albicans* needs copper for respiration through cytochrome c oxidase and for a family of multicopper ferroxidases, such as FET3 [70-72], that are required for iron acquisition and assimilation. Copper is also utilized by an amine oxidase responsible for polyamine degradation. The replacement of Cu/Zn SOD1 by a non-copper alternative, Mn SOD3 during copper starvation is reminiscent of what has been observed in *C. reinharti*. How copper is prioritized during moments of copper limitation in *C. albicans* remained largely unknown and in Chapter 2 of this thesis, we will describe how the metal is spared for respiration in *C. albicans* during copper starvation.

Conventional and Alternative Modes of Respiration

In a majority of eukaryotic organisms, oxygen consumption through mitochondrial respiration occurs through the conventional electron transport chain. This mode of respiration involves all four respiratory complexes including

cytochrome c oxidase (COX) as the terminal oxidase (Fig. 1-2, top). Cytochrome c oxidase is a member of the heme-copper oxidase family and is responsible for the reduction of molecular oxygen to water, the final step in the ETC [11]. Cytochrome c oxidase is copper dependent and is inhibited by cyanide. Though COX-driven respiration, a total of 34 molecules of ATP are generated per mole of oxidized glucose as a product of electron transport.

In addition to cytochrome c oxidase, an alternative oxidase (AOX) has been found in certain fungi, plants and protists [73-78]. The alternative oxidase is a di-iron containing protein located on the matrix side of the inner mitochondrial membrane that can also catalyze the reduction of molecular oxygen to water [79, 80]. Unlike COX respiration, electrons are donated to AOX directly from co-enzyme Q bypassing complex II and complex III of the ETC. Since alternative respiration is uncoupled from complexes II and III, utilization of this pathway does not result in the formation of an electrochemical gradient and generates little ATP [81]. Alternative oxidase respiration has been described as cyanide-insensitive, but can be inhibited by hydroxamic acids. Expression of AOX is induced by a number of factors, including oxidative stress, inhibitors of the conventional respiratory pathway, and viral infections [74, 82-85].

Since AOX respiration is not coupled to ATP production much research has been done to investigate why certain organisms utilize this form of respiration. Interestingly, the conventional COX respiratory pathway and alternative AOX pathway can be utilized simultaneously [84] suggesting that AOX may serve a function in the cell that cannot be performed by COX. Work in plant mitochondria

suggest that the main function of AOX respiration is to maintain the flux of electrons through the ubiquinone pool when the conventional COX respiratory chain is disabled [82, 86]. An increase in oxidative stress when the conventional COX respiratory chain is blocked has been reported in a number of eukaryotic cells, and it has been proposed that induction of AOX respiration allows the flow of electrons to continue preventing the formation of ROS, such as superoxide [73, 87-90]. Indeed it has been shown that when AOX respiration is inhibited in certain plant cells, there is an increase in reactive oxygen species [87, 88]. *C. albicans* is amongst the fungi that expresses an alternative oxidase [91]. The role of AOX respiration in *C. albicans* will be addressed in Chapter 2.

Respiration versus fermentation: Glucose control of metabolism in yeast

In most organisms, glucose is completely oxidized through glycolysis and aerobic respiration, leading to a total of 38 ATP molecules produced per mole of glucose oxidized. Respiration through the electron transport chain of course requires oxygen as the terminal electron acceptor. When oxygen is deplete, cells can switch from aerobic respiration to fermentation, and the decision to ferment versus respire as a function of oxygen is known as the Pasteur effect [92]. However, in rapidly dividing cells, such a cancer cells, fermentation is preferred over respiration regardless of the level of oxygen through a process known as the Warburg effect [93]. A similar phenomenon, the Crabtree effect has been described for the bakers' yeast *S. cerevisiae*, where fermentation is also utilized in the presence of oxygen instead of respiration [93, 94]. The main difference between the Crabtree

and Warburg effect is glucose, whereby glucose signals to repress respiration and promote fermentation in the case of the Crabtree effect.

Glucose metabolism and its control of respiration and fermentation has been well studied in bakers' yeast [95]. Glucose regulation is mediated by two pathways; one involves glucose sensing and the other dependent on glucose uptake (Fig. 1-5). Glucose sensing is mediated through a sensor/receptor-repressor (SRR) pathway that is activated when glucose binds to two sensors (SNF3/RGT2) located in the plasma membrane. These sensors transmit a signal to a member of the casein kinase-1 gamma family YCK1 that as mentioned above, is stabilized by SOD1. The stabilization and activation of YCK1 initiates a signaling cascade resulting in the inhibition of the transcriptional repressor, RGT1 from entering the nucleus. This pathway leads to the induction of genes required for glucose uptake, fermentation, and repression of respiration (Fig. 1-5, left) [96]. The second pathway for glucose repression is mediated by the uptake of glucose through glucose transporters [97]. As more glucose enters the cell the increase in glycolysis causes the degradation of a signaling kinase SNF1[97-99], allowing the transcriptional repressor MIG1 to enter the nucleus to block the transcription of genes required for respiration, the utilization of alternative carbon sources, and high affinity glucose transporters that are used only when glucose is scarce [100, 101] (Fig 1-5, right).

In contrast to *S. cerevisiae*, *C. albicans* is not a fermentative yeast, it is Crabtree negative, relying heavily on respiration in the presence of oxygen. Glucose does not repress respiration in *C. albicans.* Even so, *C. albicans* possesses many of the same key components of the glucose sensing and glucose uptake pathways (Fig.

1-6) [102]. The glucose sensing pathway (Fig. 1-6, left) contains a single glucose sensor, HGT4 that has been shown to act upstream of the transcriptional repressor, RGT1 [103]. Experiments confirm that RGT1 in *C. albians* represses genes required for glucose uptake, fermentation, alternative respiration and utilization of alternative carbon sources in the absence of glucose [104]. Two casein kinase homologs to bakers' yeast YCK1 have been identified in *C. albicans*, YCK2 and YCK22, however only YCK2 is predicated to act downstream of the glucose sensor HGT4 [105]. In the glucose uptake pathway (Fig. 1-6, right), *C. albicans* possess a large family of glucose transporters, as well as the kinase SNF1 and transcriptional repressor MIG1. Deletion of SNF1 is lethal to *C. albicans*, therefore most of the research on this pathway has focused on the repressor, MIG1. MIG1 is responsible for repressing glucose transporters in high glucose [104]. It is unknown whether SOD enzymes are involved in either pathway for glucose signaling in *C. albicans* and a potential role will be investigated in Chapter 3.

Overview of Thesis Research

The work described in this thesis is aimed at understanding the role of Cu/Zn SOD1 and Mn SOD3 in mitochondrial oxidative stress protection and cytosolic cellular signaling in the pathogenic fungus *C. albicans*. Chapter 2 focuses on the cytosolic SODs in mitochondrial oxidative stress protection. We found that *C. albicans* SOD1 is localized to the intermembrane space of the mitochondria where it protects against mitochondrial derived superoxide. During copper starvation Cu/Zn SOD1 is replaced by two non-copper alternatives: Mn SOD3 in the cytosol and the

expression of an alternative form of respiration, AOX. We show that AOX respiration serves to maintain mitochondrial superoxide levels low in the absence of IMS SOD1. We also found that the repression of SOD1 during copper starvation serves to spare copper for cytochrome c oxidase, to maintain mitochondrial respiration required for pathogenesis of *C. albicans*. Chapter 3 investigates the role of Cu/Zn SOD1 and Mn SOD3 in glucose signaling and regulation. Previous work from the Culotta lab described a role for Cu/Zn SOD1 in a glucose-signaling paradigm in *S. cerevisiae*. We found that both Cu/Zn SOD1 and Mn SOD3 participate in cytosolic cellular signaling by repressing glucose transporters in *C. albicans*. Compared to *S. cerevisiae* SOD1 which acts in the YCK/RGT1 glucose-signaling pathway to promote glucose uptake, *C. albicans* Cu/Zn SOD1 and Mn SOD3 are involved in the MIG1 glucose repression pathway, that inhibits glucose uptake. Additionally we find that regulation of glucose transporters by the cytosolic SODs of *C. albicans* does not involve either of the casein kinase homologs YCK2 and YCK22, contrary to what is observed in *S. cerevisiae*. Taken altogether, these studies highlight the clever adaptations of *C. albicans* in mitochondrial oxidative stress and glucose signaling that likely reflect the lifestyle of this yeast in the mammalian host.

Figure 1-1: Cellular distribution of SOD enzymes in gram-negative bacteria and eukaryotes.

In gram-negative bacteria (left) Cu is restricted to the periplasm while Mn and Fe metal ions are found in the cytosol. Distribution of SOD enzymes is dictated by this metal availability; Cu/Zn SODs are found in the periplasm and Mn and Fe SODs are generally found in the cytosol. In a typical eukaryote, (middle) the mitochondria thought to evolve from a gram-negative bacteria, contains a Mn-SOD2 in the mitochondrial matrix and Cu/Zn SOD1 that is found in the intermembrane space. Cu/Zn SOD1 largely resides in the cytosol. *C. albicans* (right) expresses Mn-SOD2 in the mitochondrial matrix, and Cu/Zn SOD1 in cytosol. The pathogenic yeast also contains a second cytosolic SOD, Mn SOD3 and three extracellular Cu-only SODs 4, 5 and 6.

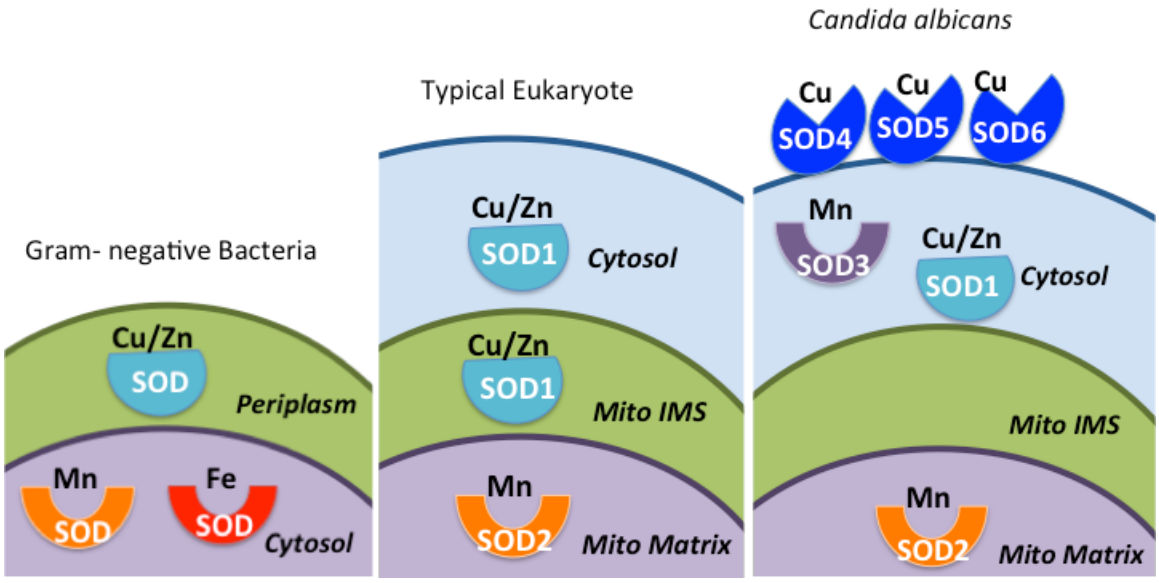


Figure 1-2: Modes of respiration and sources of mitochondrial superoxide. Conventional respiration (top) involves the full electron transport chain (complexes I-IV) and utilizes cytochrome c oxidase (COX) as the terminal oxidase. Mitochondrial superoxide is produced by the electron transport chain by complex I that releases superoxide to the mitochondrial matrix and complex III that releases superoxide to the matrix and intermembrane space. Alternative respiration (bottom) bypasses complexes II and III of the electron transport chain by donating electrons from coenzyme Q to an alternative oxidase (AOX).

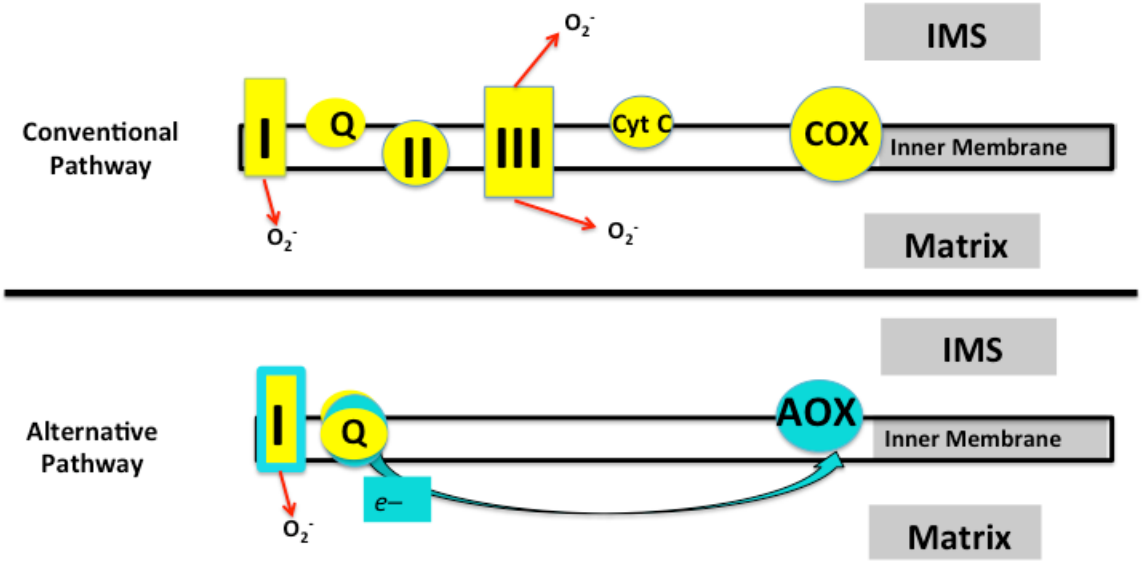


Figure 1-3: Role of Cu/Zn SOD1 in cytosolic glucose signaling.

Oxygen and glucose provide the superoxide substrate for Cu/Zn SOD1. The local production of hydrogen peroxide by SOD1 protects a degron located in the C-terminus of YCK1, preventing degradation of the kinase. Stabilization of YCK1 by SOD1 results in a downstream signaling cascade that induces genes required for glucose uptake, fermentation and amino acid sensing, while repressing genes required for respiration.

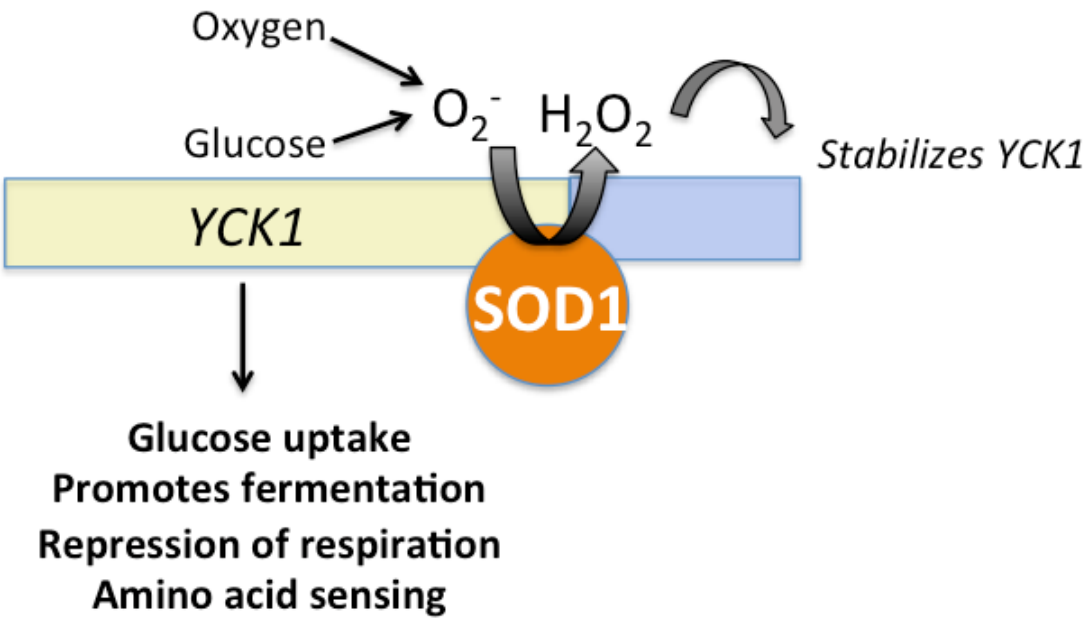


Figure 1-4: Import of Cu/Zn SOD1 into the mitochondrial IMS by the disulfide relay system.

The copper chaperone, CCS1 is imported into the IMS by disulfide relay using MIA40 (left). Active SOD1 enzyme in the cytosol with an oxidized disulfide bond (right) cannot enter the mitochondria. Reduced SOD1 can enter the mitochondrial outer membrane pores and becomes oxidized through interactions with CCS, trapping active SOD1 in the IMS.

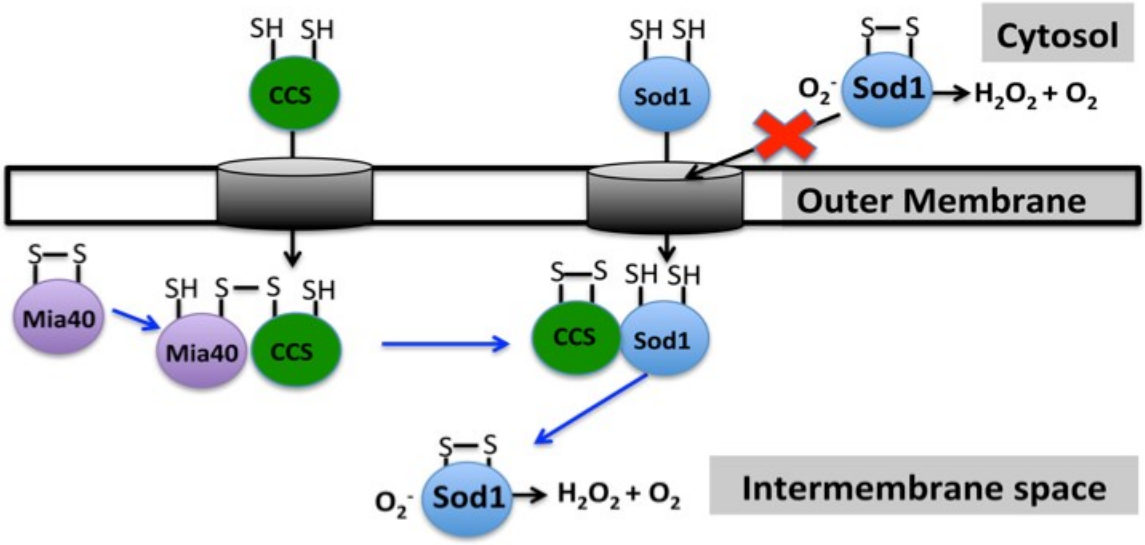


Figure 1-5: Glucose metabolism in *S. cerevisiae*.

Glucose metabolism is mediated through pathways involving glucose sensing and glucose uptake. A) Glucose sensing (left) is mediated through the binding of glucose to two glucose sensors SNF3 and RGT2 that transmit a signal to the casein kinase, YCK1. Activation of YCK1 leads to a downstream signaling cascade that prevents the transcriptional repressor RGT1 from entering the nucleus (red cross), which allows the transcription of genes required for fermentation, glucose uptake, and the repression of respiration. In the glucose uptake pathways (right), the influx of glucose through glucose transporters leads to an increase in glycolysis that causes the degradation of a signaling protein kinase, SNF1 (red cross). The degradation of SNF1 allows the transcriptional repressor MIG1 to enter the nucleus and repress genes required for respiration.

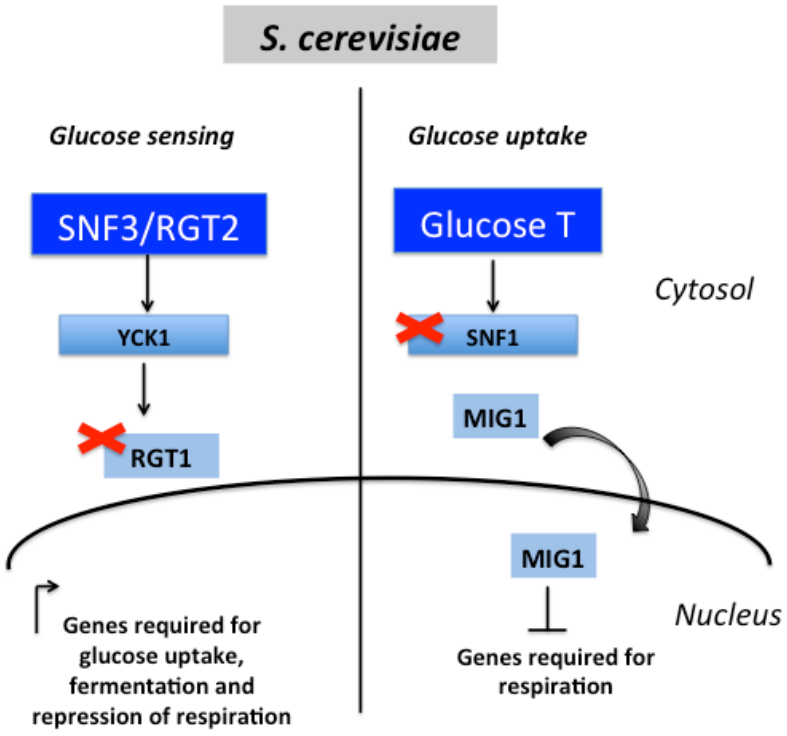
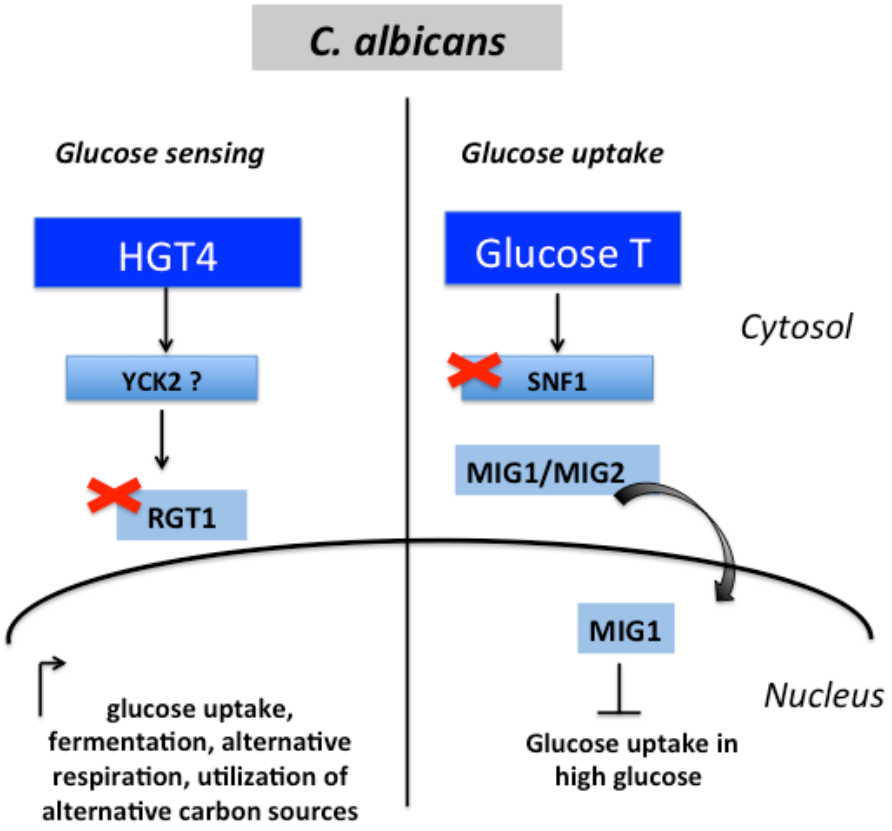


Figure 1-6: Glucose metabolism in *C. albicans*.

Glucose sensing and glucose uptake pathways are conserved in the pathogenic yeast *C. albicans*. (Left) Glucose sensing is mediated through a single glucose sensor HGT4 and the casein kinase, YCK2 is predicated to function upstream of the transcriptional repressor, RGT1. (Right) A large family of glucose transporters exists in *C. albicans*, as well as the kinase SNF1. Transcriptional repressors MIG1/MIG2 repress the induction of glucose transporters in high glucose instead of repressing genes required for respiration as observed in *S. cerevisiae*.



CHAPTER 2

An Adaptation to Low Copper in *Candida albicans* Involving SOD Enzymes and the Alternative Oxidase

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INTRODUCTION

Superoxide dismutases (SOD) play vital roles in the biology of reactive oxygen species (ROS) by disproportionating superoxide anion free radicals into hydrogen peroxide and molecular oxygen [2, 3]. There are three major SOD families including copper and zinc (Cu/Zn) SODs that use copper as the catalytic co-factor, a separate SOD family with either manganese or iron [1], and a rare family of nickel containing SODs [7]. Gram-negative bacteria restrict copper containing SODs to the periplasmic/extracellular space while manganese and iron SODs are intracellular/cytosolic [8, 9]. An analogous partitioning occurs in eukaryotic mitochondria where a Cu/Zn SOD (known as SOD1) lies in the intermembrane space (IMS) and a manganese SOD (known as SOD2) resides in the mitochondrial matrix [33, 39-42, 52, 106-108]. SOD1 is also cytosolic in eukaryotes and its distribution between the cytosol and mitochondrial IMS involves a disulfide relay system and the copper chaperone for SOD1, CCS [47-49].

In mitochondria, superoxide anion is formed as a byproduct of respiration and is released into both the matrix and IMS where it reacts with SOD2 and SOD1 respectively [16, 109]. With conventional respiration involving the full electron transport chain and cytochrome c oxidase (COX), superoxide is released to the matrix by complex I [110], while complex III releases superoxide to both the IMS and matrix [16, 111]. COX is not the only form of respiration and certain fungi, plants, and protists express an alternative oxidase (AOX) that accepts electrons directly from coenzyme Q [78, 79, 82, 83, 85, 112, 113]. Unlike COX, AOX respiration is not coupled to ATP production [85]. One example of an organism that utilizes

both COX and AOX respiration is the polymorphic fungus *Candida albicans* [81, 84, 91, 114]. *C. albicans* is an opportunistic human pathogen that exists as a commensal of the human flora, but can become pathogenic in immune compromised individuals. Unlike fermenting yeasts such as *Saccharomyces cerevisiae, C. albicans* relies heavily on COX respiration for ATP [115, 116]. Thus, the rationale for retaining AOX respiration is not clear, particularly since AOX could potentially compete with COX for coenzyme Q electrons.

Aside from dual modes of respiration, *C. albicans* has an unusually large collection of six SOD enzymes including the Cu/Zn containing SOD1 [117, 118], the manganese containing SOD2 in the mitochondrial matrix [119], three extracellular copper-only SODs (SOD4,5,6) [61, 120-122] and a second manganese containing SOD3 predicted to be cytosolic, as is the case with SOD1 [63]. We have recently shown that SOD1 and SOD3 are reciprocally expressed according to copper status: copper replete yeast only express SOD1 while copper starved cells repress SOD1 and induce SOD3 [66]. This switch in SOD enzymes is mediated by the copper sensing regulator MAC1 and is set into motion during fungal invasion of the kidney [66]. While both SOD1 and SOD3 are predicted to be cytosolic, it is unknown whether either can enter the mitochondrial IMS to deal with superoxide release in this compartment.

Herein we investigate the role of *C. albicans* SOD1 versus SOD3 in mitochondrial oxidative stress protection. We demonstrate that under copper replete conditions, the Cu/Zn containing SOD1 partitions between the cytosol and mitochondrial IMS and protects against mitochondrial superoxide. However, during

copper starvation, the mitochondrial IMS becomes devoid of a SOD enzyme since the manganese containing SOD3 localizes exclusively to the cytosol. In spite of no IMS SOD, mitochondrial superoxide is not elevated. We find that during copper limitation, *C. albicans* induces AOX respiration, which suppresses mitochondrial superoxide and bypasses the need for an IMS SOD. In spite of extreme copper starvation, copper dependent COX respiration remains high and we provide evidence for a copper sparing mechanism whereby repression of SOD1 helps maintain high COX respiration in the face of copper starvation.

MATERIALS AND METHODS

Yeast strains and culture conditions

C. albicans strains used in this study were derived from SC5314 including CA-IF100 (*arg4Δ/arg4Δ, leu2Δ/leu2Δ::cmLEU2, his1Δ/his1Δ::cdHIS1, URA3/ura3Δ*) and isogenic strains CA-IF001 (*sod1Δ::cmLEU2/sod1Δ::cdHIS1*) and CA-IF011 (*sod3Δ::cmLEU2/sod3 Δ::cdHIS1*) [121]. The SN152 strain (*his1Δ/his1Δ, leu2Δ/leu2Δ, arg4Δ/arg4Δ, URA3/ura3Δ::imm434, IRO1/iro1Δ::imm434*) and isogenic *mac1Δ::LEU2/mac1Δ::HIS1* strain were obtained from the Fungal Genetics Stock Center [123, 124]. The *ccs1Δ/Δ* strain *(ccs1Δ::cmLEU2/ccs1::cdHIS1)* was derived from SN78 (*ura3Δ::imm434/ura3Δ::imm434, leu2Δ/leu2Δ, his1Δ/his1Δ*) as previously described [125]. The strain constitutively expressing SOD1 was derived from CA-IF001 (*sod1Δ::cmLEU2/SOD1*) as previously described in [66]. In the so-called SOD1^{con} strain, the single allele of *SOD1* was engineered with a mutation in the MAC1 binding site at position +148 by gene replacement. A similar gene

replacement was used to engineer the control SOD^{rep} with an intact MAC1 binding site [66].

C. albicans cells were cultured at 30°C in either enriched media (YPD; BD Difco) containing 1% yeast extract, 2% peptone, and 2% dextrose (w/v) or in a synthetic complete (SC) media with 0.67% yeast nitrogen base (US Biologicals) and 2% dextrose (w/v). Where indicated, cells were starved for copper by supplementing the growth media with 800 μM of the extracellular Cu(I) chelator BCS.

Cell fractionation and analysis of SOD proteins and enzymatic activity

Mitochondria and post-mitochondrial supernatant (PMS)/largely cytosolic fractions were obtained from *C. albicans* essentially as described [84]. Briefly, cells were grown in YPD to an OD_{600} between 1.0 and 4.0, were harvested and successively washed in MilliQ deionized water and 0.1 M Tris-SO_4 pH 9.4, 10 mM DTT, followed by incubation in the same buffer for 30 minutes at 30°C shaking at 100 RPM. Cells were harvested, and spheroplasts formed by incubating cells in 1.2 M sorbitol, 20 mM KH_2PO_4/K_2HPO_4 pH 7.4 with 0.3 mg/L Lyticase (Sigma). Spheroplasts were isolated by centrifugation at 1000 x g for 5 minutes and resuspended in 0.6 M sorbitol 20 mM K^+HEPES pH 7.4 and lysed by dounce homogenization to create whole cell lysates. The lysate was spun at 12,000 x g for 10 minutes at 4°C to resolve PMS and crude mitochondria fractions. Mitochondria were washed twice in 1.2 M sorbitol 20 mM KH_2PO_4/K_2HPO_4 pH 7.4 buffer and where indicated, were resolved further into IMS and matrix fractions by

resuspending in 20 mM K^+ HEPES pH 7.4 and briefly vortexing for 5 seconds to rupture the outer membrane. The mitochondrial fraction was centrifuged at 12,000 x g for 10 minutes at 4°C to separate the matrix (pellet) from the soluble IMS. Prior to analysis by gel electrophoresis, crude whole mitochondria or matrix fractions were resuspended in a 10 mM sodium phosphate buffer 7.4 containing 0.1% Triton X-100 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, 10% glycerol, a procedure that solubilizes the matrix and allows for visualization of matrix SOD2 [126].

SOD protein expression and localization was analyzed by immunoblot as previously described [66]. 70 μg of whole cell spheroplast lysates and the same cell equivalents of PMS and mitochondrial fractions were loaded onto 4-12% Bis-Tris gels (Thermo Fisher). Where indicated whole cell lysates of *C. albicans* were prepared by glass bead homogenization [66] and 30 μg of lysate protein was used. Blots were probed with anti-SOD1 and SOD3 primary antibodies as previously described [66]. Anti-SOD1 is a polyclonal antibody directed against *C. elegans* Sod-1 (JH766) that reacts well with copper and zinc containing SOD molecules across eukaryotes [126]. The anti-SOD3 polyclonal antibody is directed against a peptide unique to *C. albicans* SOD3 that is absent in mitochondrial SOD2 [125]. Anti-PGK1 at a 1:5000 dilution (Acris Antibodies, #AP21371AF-N) was used as a cytosolic marker. The polyclonal anti-SOD2 antibody is directed against *S. cerevisiae* SOD2 and was prepared as described [127]. This anti-SOD2 cross-reacts well with *C. albicans* SOD2 and was used at a 1:5000 dilution as a mitochondrial marker. On immunoblots, all four antibodies are highly specific. SOD enzymatic activity was

analyzed by native gel electrophoresis and nitroblue tetrazolium staining as previously described [66] using 30 μg total cell lysate.

RNA analysis

C. albicans cells were grown in SC medium in the absence or presence of 800 μM BCS to an OD_{600} between 1.0 and 2.0. RNA was isolated from 20 OD_{600} cell units using an RNeasy mini kit (Qiagen), and then subsequently converted to cDNA using Superscript IV Reverse Transcriptase kit (Thermo Fisher Scientific). Real time PCR was performed as previously described [66]. cDNA was diluted 20-fold before PCR amplification with iQ SYBR Green Supermix (Bio-Rad) and values were normalized to *TUB2* transcripts in each sample. The *SOD3* and *TUB2* primers were used as described [66]; *AOX2* primers are GTTGGTCAAGGGGTTTTCACTAATG and ACTGCCACTTCAGGGATTTTCATGG.

Measurements of mitochondrial superoxide, oxygen consumption and cellular copper

Mitochondrial superoxide was measured in *C. albicans* cells initially cultured in YPD media to early stationary phase (OD_{600} 6.0 - 10.0), then diluted back to OD_{600} ≈0.1 in SC medium and grown to OD_{600} of 1.0-2.0. Background fluorescence is minimized in cells from SC medium. 5 μM of MitoSOX Red (Life Technologies) in DMSO or the equivalent volume of DMSO control was added to 500 μl of culture and incubated for 45 minutes in round bottom tubes at 250 rpm in the dark at 30°C. Where indicated, cells were co-incubated with MitoSOX red and 500 nM

MitoTracker Green FM (Thermo Scientific). To monitor effects of AOX inhibition, cultures were incubated for 1 hour with 5 mM SHAM prior to MitoSOX addition. Cells were washed three times in phosphate buffered saline (PBS), resuspended in PBS and subjected to either microscopic visualization of MitoSOX Red fluorescence using a Zeiss Observer Z1 fluorescence microscope with a Zeiss Plan-Apochromat 63x objective, or to fluorescence quantification on a Biotek Synergy HT microplate reader with excitation and emission at 510 nm and 580 nm respectively. Fluorescence quantification used 2 x 10^7 of cells in 200 µl PBS.

Oxygen consumption measurements were conducted on whole cells essentially as described [116] using cells grown in YPD to early log (OD_{600} of 1.0 – 2.0). Ten OD_{600} units of cells were harvested, washed once in PBS and resuspended in 2 ml of YPD. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode in a magnetically stirred, thermostatically controlled 1.5-ml chamber at 25°C (Oxytherm; Hansatech). Following 1 min, 5mM salicylhydroxamic acid (SHAM) was added to the oxygen chamber to inhibit AOX respiration followed by addition of 1mM potassium cyanide (KCN) to inhibit COX respiration. The change in oxygen saturation over time was used to calculate rates of oxygen consumption. The degree to which the oxygen consumption rate is inhibited by SHAM or KCN was used to derive the percent oxygen consumption by AOX or COX, respectively.

Copper content of whole cells was measured on the same cultures used for oxygen consumption. 20 OD_{600} units of cells were washed twice in 10 mM Tris pH 8.0 1 mM EDTA, and twice in deionized water. Cells were resuspended in 500 µl

MilliQ deionized water before analysis of copper by atomic absorption spectroscopy (AAS) on a PerkinElmer Life Sciences AAnalyst 600 graphite furnace instrument as described [66].

RESULTS

A fraction of *Candida albicans* SOD1, but not SOD3, localizes to the mitochondrial intermembrane space.

In standard enriched medium, *C. albicans* cells are copper replete and only express SOD1, not SOD3 (Fig. 2-1A). Supplementing cultures with the extracellular Cu(I) chelator BCS (bathocuproinedisulfonic acid) results in a ten-fold reduction in intracellular copper (Fig. 2-1B), and cells respond to this copper starvation stress by repressing SOD1 and inducing the manganese containing SOD3 (Fig. 2-1A) [66]. Using these differential copper conditions we examined the localization of SOD1 versus SOD3.

We first addressed whether the Cu/Zn containing SOD1 partitions between the cytosol and mitochondria as was shown for *S. cerevisiae* and mammalian cells [33, 106, 128]. Whole cell lysates were subjected to differential centrifugation to resolve crude mitochondria from the largely cytosolic or post mitochondrial supernatant (PMS) fraction. Identical cell equivalents of each fraction were analyzed by western blot. As seen in Fig. 2-1C, the majority of SOD1 fractionates with the cytosolic maker PGK1, consistent with a largely cytosolic localization of the protein. We also observed a small fraction of SOD1 partitioning with the mitochondria together with the mitochondrial marker, SOD2. By quantification of

the results from five experimental trials, we find that mitochondrial SOD1 represents 5% of total cellular SOD1 (standard deviation= 0.0247). This value is quite comparable to the ≈1-5% reported for human and *S. cerevisiae* mitochondrial SOD1 [52, 106]. As was shown for human and *S. cerevisiae* SOD1 [48, 129], *C. albicans* SOD1 appears to require its copper chaperone CCS for mitochondrial uptake, as SOD1 was not detected in mitochondria from *ccs1Δ/Δ* cell (Fig. 2-2A, compare lanes 4 and 7). Additionally, upon fractionation of IMS and matrix components, we observed that SOD1 localizes to the IMS while the manganese containing SOD2 partitions to the mitochondrial matrix (Fig. 2-2B), precisely as was shown for *S. cerevisiae* and mammalian SODs [33, 40-42, 52, 106, 107]. Altogether, the studies of Figs. 2-1C and 2-2 demonstrate that under copper replete conditions, *C. albicans* parallels other eukaryotes by importing a small fraction of SOD1 into the mitochondrial IMS.

We next addressed the status of *C. albicans* mitochondria when cells are starved for copper and switch from Cu/Zn containing SOD1 to the manganese containing SOD3. In the experiment of Fig. 2-1D, the mitochondrial and PMS fractions of copper starved cells were probed for SOD3. We observed that unlike SOD1, SOD3 was only visible in the PMS, and over three experimental trials there was no detectable SOD3 in the mitochondrial fraction (representative result shown in Fig. 2-1D).

Mitochondrial superoxide under copper replete versus copper starvation conditions

We sought to understand the consequences of no IMS SOD during copper starvation. To monitor mitochondrial superoxide we utilized the superoxide specific fluorescent probe MitoSOX Red, a derivative of dihydroethidium designed to target mitochondria. As seen in Fig. 2-3A, MitoSOX yields a punctate pattern of fluorescence in *C. albicans* wild type cells mirroring that of MitoTracker Green FM, validating its use as a specific marker of mitochondrial ROS in *C. albicans.* Under copper replete conditions, *sod1Δ/Δ* mutants also display a punctate pattern of fluorescence (Fig. 2-3C) that appeared enhanced compared to the signal obtained with wild type cells analyzed in parallel (Fig. 2-3B). To quantitate any changes between wild type and *sod1Δ/Δ* cells, MitoSOX fluorescence was measured spectrophotometrically at 580 nm. Over four experimental trials with eight independent samples, *sod1Δ/Δ* cells consistently exhibited a ≈2- fold increase in MitoSOX detectable fluorescence (Fig. 2-4A). The MitoSOX signal was also increased in *sod2Δ/Δ* cells lacking the mitochondrial matrix SOD2 (Fig. 2-4B). Thus, MitoSOX Red is an effective indicator of mitochondrial ROS in *C. albicans,* sensitive to both IMS (SOD1-relevant) and mitochondrial matrix (SOD2-relevant) superoxide.

Using MitoSOX Red, we probed the mitochondrial ROS status of copper deficient cells. We anticipated an elevation in mitochondrial superoxide, since the mitochondrial IMS appears devoid of a SOD enzyme under these conditions. However, MitoSOX Red fluorescence was not elevated in either wild type cells or *sod3Δ/Δ* strains treated with BCS (Fig. 2-4C) consistent with the notion that SOD3 does not enter the mitochondria. It was therefore possible that copper starvation stress induces a non-SOD antioxidant to minimize mitochondrial ROS.

Modes of mitochondrial respiration under copper replete versus copper starvation conditions

As mentioned above, *C. albicans* uses two forms of respiration: COX respiration that employs the full electron transport chain and is a major source of cellular ATP, and AOX respiration that accepts electrons directly from coenzyme Q and is not coupled to ATP production [81, 82, 84]. *C. albicans* expresses dual AOX isoforms, *AOX1* and *AOX2* [91, 114], and *AOX2* was previously shown to be a target of the same MAC1 regulator that controls *SOD1* and *SOD3* expression [130]. Copper regulation of *AOX2* had not been previously reported and we find using quantitative real-time PCR a very strong induction of *AOX2* mRNA in cells starved for copper (Fig. 2-4D). This pronounced induction of *AOX2* mimics that of *SOD3* mRNA and is abolished by *mac1Δ/Δ* mutations (Fig. 2-4D).

We tested whether the induction of *AOX2* mRNA correlates with utilization of the AOX pathway for respiration. Fig. 2-5A,B shows representative oxygen consumption experiments where cells were successively treated with salicylhydroxamic acid (SHAM) to inhibit AOX respiration followed by treatment with potassium cyanide KCN to inhibit COX [84]. By calculating the rates of oxygen consumption in the presence and absence of these inhibitors, the fraction of respiration due to AOX versus COX can be ascertained. We find that during copper replete conditions, COX respiration accounts for essentially all of the oxygen consumption while AOX respiration is undetectable (Fig. 2-5C,D). However, during copper starvation, there was a pronounced induction of AOX respiration, accounting

for nearly 25% of total oxygen consumption; COX respiration proportionately declined (Fig. 2-5C,D). AOX respiration is clearly induced during copper starvation.

AOX respiration bypasses complex III of the electron transport chain, and since complex III is a source of IMS superoxide [16], AOX induction could conceivably minimize mitochondrial ROS. To test the impact of AOX on mitochondrial superoxide, MitoSOX Red fluorescence was measured in cells in which AOX was inhibited by SHAM. As seen in Fig. 2-6, SHAM had no impact on mitochondrial ROS in copper replete wild type cells, consistent with the observation of no AOX respiration under these conditions (Fig. 2-5B). However, SHAM treatment had a marked effect on mitochondrial ROS under copper starvation conditions. In wild type cells, MitoSOX Red fluorescence was enhanced 3-4 fold when AOX was inhibited with SHAM (Fig. 2-6). The same is true of *sod1Δ/Δ* and *sod3Δ/Δ* strains starved for copper, demonstrating that the change in superoxide occurs independent of these SOD enzymes (Fig. 2-6). Together, the studies of Fig. 2-5 and 2-6 demonstrate that during copper starvation, *C. albicans* induces AOX respiration to reduce mitochondrial superoxide.

Evidence for copper sparing with SOD1

Since cytochrome c oxidase is a copper dependent enzyme, it was curious that COX respiration falls no more than 30% (Fig. 2-5D) when intracellular copper levels drop by ≥90% (Fig. 2-1B). Under these same copper starvation conditions, activity of copper dependent SOD1 is undetectable, largely due to *SOD1* gene

repression. Does this repression of *SOD1* serve to spare copper for COX? To address this, we used a *C. albicans* strain engineered to constitutively express *SOD1*.

C. albicans SOD1 contains a single intronic MAC1 site that mediates *SOD1* repression during copper starvation [66] (illustrated in Fig. 2-7A). As seen in Fig. 7B top, mutating this MAC1 site in chromosomal *SOD1* results in constitutive *SOD1* expression and cells express both SOD1 and SOD3 with copper starvation, consistent with previous findings [66]. We observe that constitutively expressed SOD1 is not fully active with copper starvation due to limitation of its copper co-factor; nevertheless it can secure sufficient copper for 30-40% of the activity seen with copper replete conditions (Fig. 2-7B bottom and legend). We tested whether this limited copper activation of SOD1 can impact COX respiration. As seen in Fig. 2-7C, the control strain expressing repressible *SOD1* with intact MAC1 site exhibited the anticipated ≈30% decrease in COX respiration with copper starvation. By comparison, COX respiration drops ≈60% with copper starvation in the strain that constitutively expresses SOD1. Thus, even a small retention in SOD1 activity during copper starvation conditions is sufficient to inhibit COX respiration. We conclude that the repression of SOD1 during copper starvation helps maximize COX respiration.

DISCUSSION

Copper is an essential nutrient of restricted availability and when cellular copper declines, priorities must be established to spare the nutrient. In the case of *C. albicans,* the response to low copper involves down-regulating the cuproprotein

SOD1. Like other eukaryotes, SOD1 resides in both the *C. albicans* cytosol and mitochondrial IMS, and when copper declines, *C. albicans* maintains ROS homeostasis by replacing SOD1 with two copper-independent enzymes including SOD3 in the cytosol and AOX in the mitochondria (see model, Fig. 2-8). We show here that AOX serves to minimize mitochondrial ROS in the absence of an IMS SOD enzyme. The role of SOD3 in the cytosol is less clear, but may act to substitute for SOD1 in cell signaling. Previously, we have shown that in *S. cerevisiae,* cytosolic SOD1 functions in glucose sensing and signaling [35], and the laboratories of Thiele and Zheng have shown a role for nuclear SOD1 in gene regulation and the response to DNA damage [38, 131]. *C. albicans* SOD3 could very well replace SOD1 in one or more of these extra-mitochondrial roles in signaling through ROS.

We propose that the down-regulation of *C. albicans* SOD1 during copper starvation serves to free up substantial metal for other processes. We find that copper-dependent COX respiration remains high in *C. albicans* during copper starvation; however this activity can be compromised when even a small level of copper is distributed to SOD1, as was found using a constitutively expressed allele of *SOD1*. By down-regulating SOD1 during copper starvation, *C. albicans* can spare this metal for COX respiration, crucial for this opportunistic fungal pathogen that relies heavily on COX respiration for ATP. The notion of sparing copper for respiration is reminiscent of what has been reported by Merchant and colleagues for the photosynthetic algae, *Chlamydomonas reinharti* [68]. *C. reinhardtii* does not express a copper requiring SOD1 [132], rather a major source of cellular copper is plastocyanin used for photosynthesis. In this organism when copper levels are low,

plastocyanin is degraded and allows for copper to be allocated for the synthesis of COX [68]. To compensate for loss of plastocyanin, *C. reinhardtii* induces iron requiring Cytochrome c_6 [69, 133], analogous to the induction of the non-copper alternatives SOD3 and AOX that substitute for SOD1 in *C. albicans*.

AOX respiration is not coupled to ATP synthesis, and the rationale for retaining this apparently futile mode of oxygen consumption has been the subject of much investigation. Many previous studies support a role for AOX in offsetting certain defects associated with inhibition of COX respiration. By directly accepting electrons from coenzyme Q, AOX can help maintain the NAD/NADH balance through complex I activity [134] or prevent superoxide formation when downstream portions of the electron transport chain are interrupted [73, 87, 88]. Consistent with its role in correcting COX deficiencies, AOX gene expression is induced by inhibitors of respiration in several organisms [77, 90, 135]. Here we provide a new layer to AOX functionality, specifically under copper limiting conditions. In *C. albicans,* AOX not only offsets deficiencies in COX respiration but compensates for loss of IMS SOD1 by minimizing mitochondrial ROS. Inside the animal host, *C. albicans* is subject to great fluctuations in copper availability and experiences copper starvation stress during invasion of the kidney [66]. The adaptation described here involving SOD1, SOD3, AOX and COX is expected to minimize mitochondrial oxidative damage while maximizing COX respiration required for pathogenesis [115, 116].

Lastly, why has *C. albicans* retained the copper containing SOD1 when the fungus is susceptible to copper starvation stress *in vivo* and the requirement for

SOD1 appears to be bypassed by cytosolic Mn SOD3 and mitochondrial AOX? Our previous work in *S. cerevisiae* has shown that in addition to scavenging superoxide, SOD1 serves as a copper buffer and can minimize copper toxicity in cases of copper excess [136]. *C. albicans* SOD1 may have a similar role in copper detoxification. In the case of *Chlamydomonas*, it has been suggested that Cu-plastocyanin acts as a storage depot for copper that can be rapidly allocated to COX when copper becomes limiting [68]. By the same token, SOD1 may temporarily hold copper that is re-distributed to COX or other sites as needed.

Figure 2-1: A fraction of *C. albicans* SOD1 but not SOD3 localizes to the mitochondrial IMS.

C. albicans strain CAIF-100 was grown in enriched medium in either the presence or absence of 800 μM of the extracellular Cu(I) chelator BCS as indicated. (A) Lysates were prepared from spheroplasts and were analyzed for SOD1 and SOD3 by immunoblot. (B) Total cellular copper was measured by atomic absorption spectroscopy. (C-D) Spheroplast cell lysates (Total) were fractionated into the largely cytosolic post-mitochondrial supernatant (PMS) and crude mitochondria (mito) by differential centrifugation. 70 μg of total lysate protein and the same cell equivalents of PMS and mitochondria were analyzed by immunoblot for SOD2 (marker of mitochondrial matrix), PGK1 (marker of cytosol) and either SOD1 (C) or SOD3 (D). MW = molecular weight markers. SOD1 often appears as two bands, both of which are down-regulated by copper starvation.

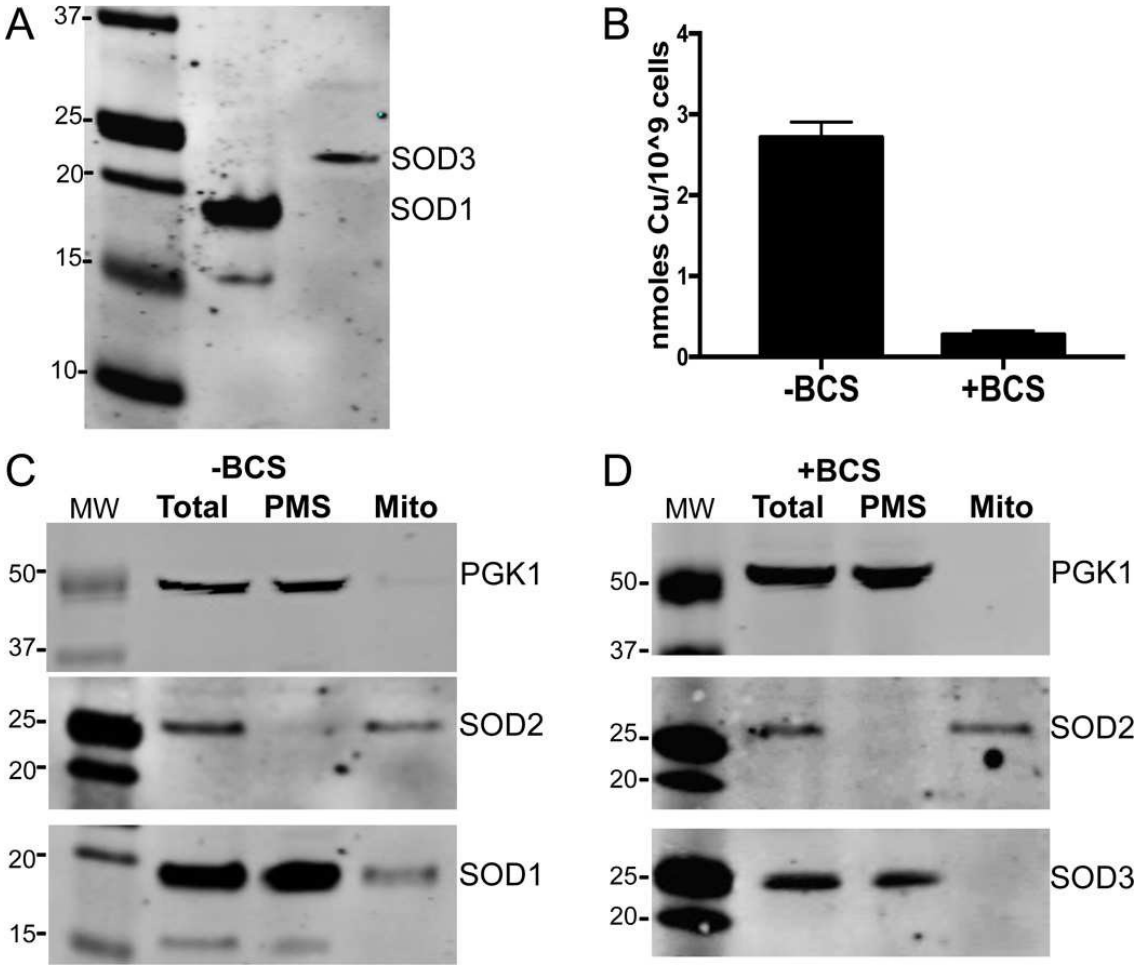

A
37
25
20
15
10
SOD3
SOD1
B
nmoles Cu/10^9 cells
4
3
2
1
0
-BCS
+BCS
C
-BCS
MW
Total
PMS
Mito
50
37
PGK1
25
20
SOD2
20
15
SOD1
D
+BCS
MW
Total
PMS
Mito
50
37
PGK1
25
20
SOD2
25
20
SOD3

Figure 2-2: *C. albicans* SOD1 localizes to the mitochondrial IMS and requires its copper chaperone CCS1 for mitochondrial localization

(A) Cell lysates from wild type (CAIF-100) and the isogenic *ccs1Δ/Δ* strain were fractionated into PMS and mitochondria and analyzed by immunoblot for SOD1 and SOD2. (B) The mitochondrial fraction derived from 70 μg total lysate protein was further fractionated into IMS and matrix as described in *Materials and Methods* and analyzed for SOD1 and SOD2 by immunoblot. Numbers on left indicate molecular weight markers.

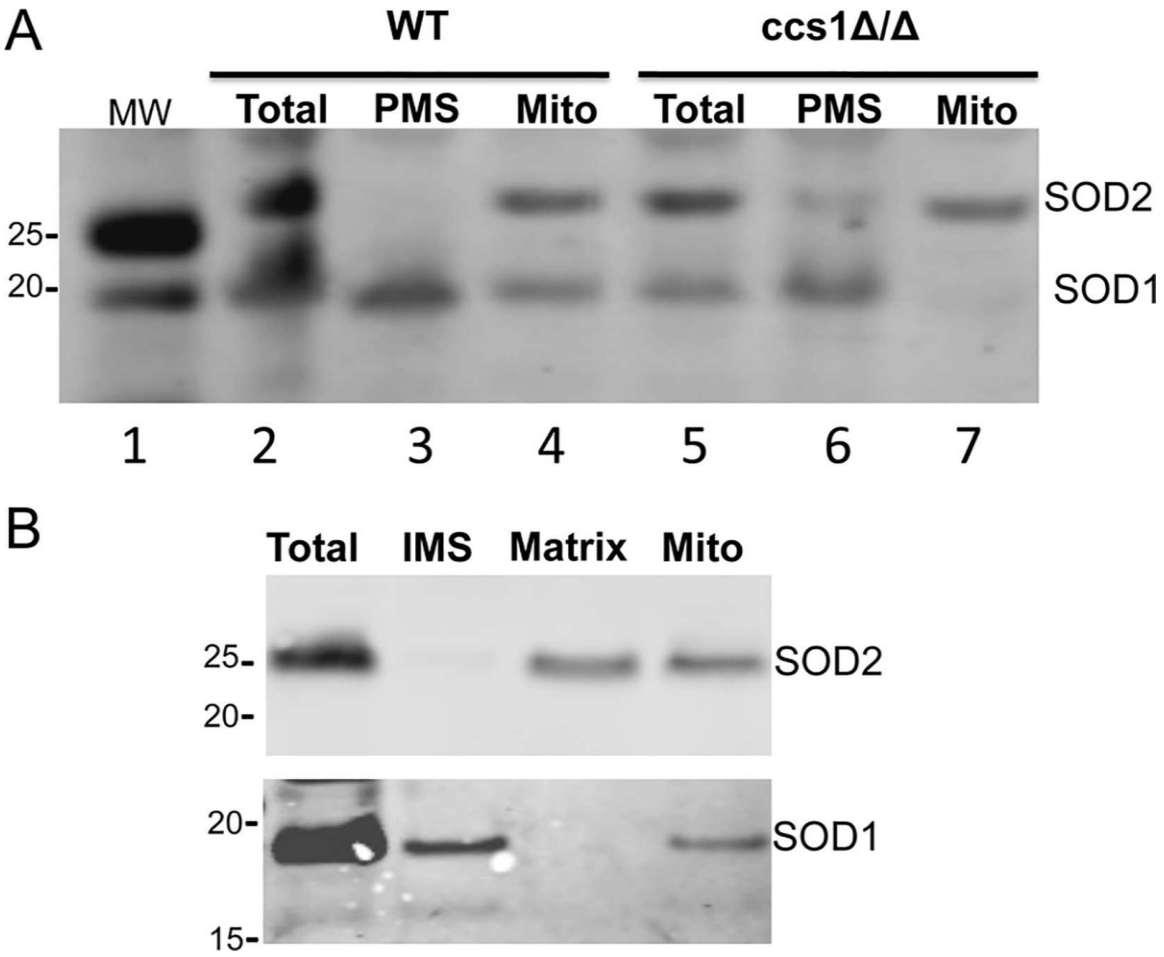


Figure 2-3: MitoSOX Red as a probe for mitochondrial superoxide in *C. albicans*.

Log phase CA-1F100 *C. albicans* wild type cells (A, B) or the isogenic *sod1Δ/Δ* strain (C) grown under non-stress conditions were incubated with 5 mM MitoSOX Red as a marker for mitochondrial superoxide and imaged by fluorescence microscopy at 63X. (A) "DIC" = light microscopy images and "MitoTracker" = cells treated with both MitoSox Red and 500 μM MitoTracker Green as a marker for mitochondria. (B,C) Wild type and *sod1Δ/Δ* cells were examined in parallel where the top and bottom rows show representative MitoSox Red fluorescence and DIC images, respectively.

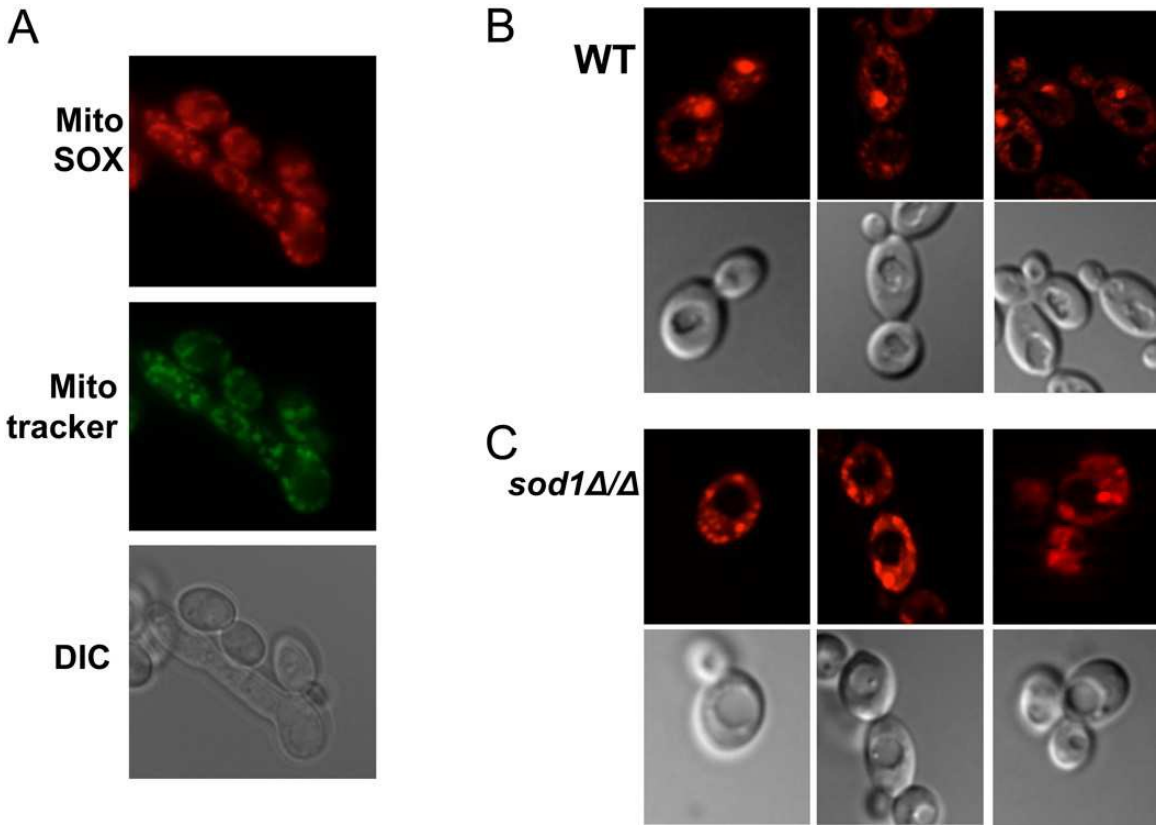


Figure 2- 4: Effects of copper starvation and *sod* gene mutations on mitochondrial superoxide and *AOX2* expression.

(A-C) MitoSOX Red fluorescence was measured spectrophotometrically at 580 nm where fluorescence intensity is the signal obtained with $2x10^7$ cells as described in *Materials and Methods*. (A-B) A comparison of the effects of *sod1Δ/Δ* versus *sod2Δ/Δ* mutations on MitoSOX Red fluorescence where results represent the averages of eight and four independent samples respectively, and statistical significance was determined using a paired two-tailed Student's t-test. Asterisks indicate statistical significance with P values < 0.05. Error bars represent standard deviation. The baseline MitoSOX Red fluorescence of non-stressed wild type cells typically varies between 20-40 fluorescent units, but regardless of this baseline, *sod1Δ/Δ* and *sod2Δ/Δ* mutants exhibited enhanced fluorescence. (C) The indicated strains were grown in the presence or absence of 800 μM BCS as designated. Results represent the averages of three independent cultures where error bars are standard deviation. The difference between minus and plus BCS with the wild type strain is not statistically significant (p = 0.2063). (D) *AOX2* and *SOD3* mRNA were quantified by qRT-PCR from wild type (SN152) and the isogenic *mac1Δ/Δ* strain as described in *Materials and Methods.* Values are normalized to that obtained with wild type SN152 *C. albicans* cells without BCS. Shown are averages of two biological replicates where error bars are standard deviation.

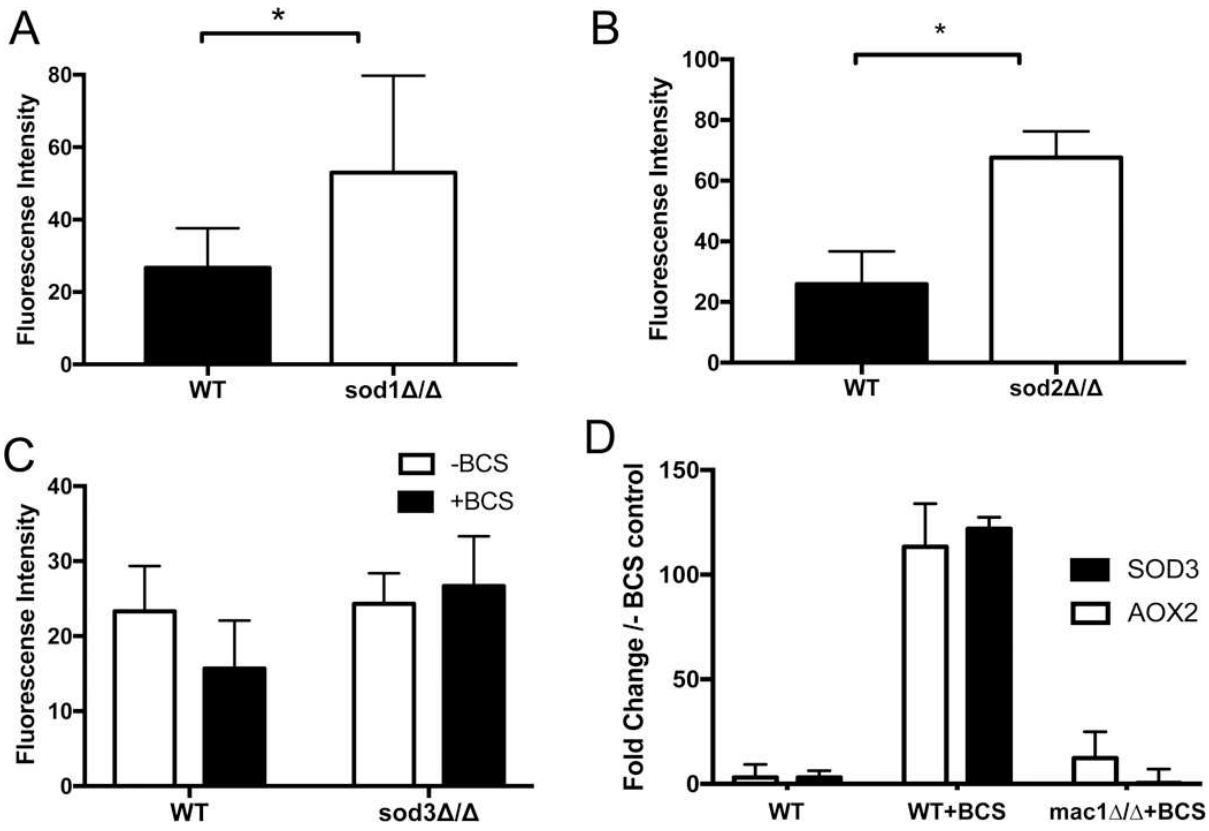

A
*
Fluorescense Intensity
80
60
40
20
0
WT
sod1Δ/Δ
B
*
Fluorescense Intensity
100
80
60
40
20
0
WT
sod2Δ/Δ
C
-BCS
+BCS
Fluorescense Intensity
40
30
20
10
0
WT
sod3Δ/Δ
D
Fold Change /- BCS control
150
100
50
0
SOD3
AOX2
WT
WT+BCS
mac1Δ/Δ+BCS

Figure 2-5: AOX respiration contributes to oxygen consumption during copper starvation.

C. albicans strain CA-IF100 grown in the presence or absence of BCS was subjected to measurements of oxygen consumption using a Clark electrode. (A,B) Shown are representative profiles of oxygen consumption as monitored by percent oxygen saturation in solution. At the indicated time points, cells were treated with 5 mM SHAM to inhibit AOX respiration followed by 1 mM KCN to inhibit COX respiration. (C,D) The rates of oxygen consumption were derived from oxygen saturation curves. Plotted are the percentages of oxygen consumption inhibited by SHAM and attributed to AOX (C) and the oxygen consumption inhibited by KCN and attributed to COX (D). Results represent the averages of three independent samples with error bars representing standard deviation. Statistical significance was determined using a paired two-tailed Student's t-test. Asterisks indicate statistical significance with P values < 0.05.

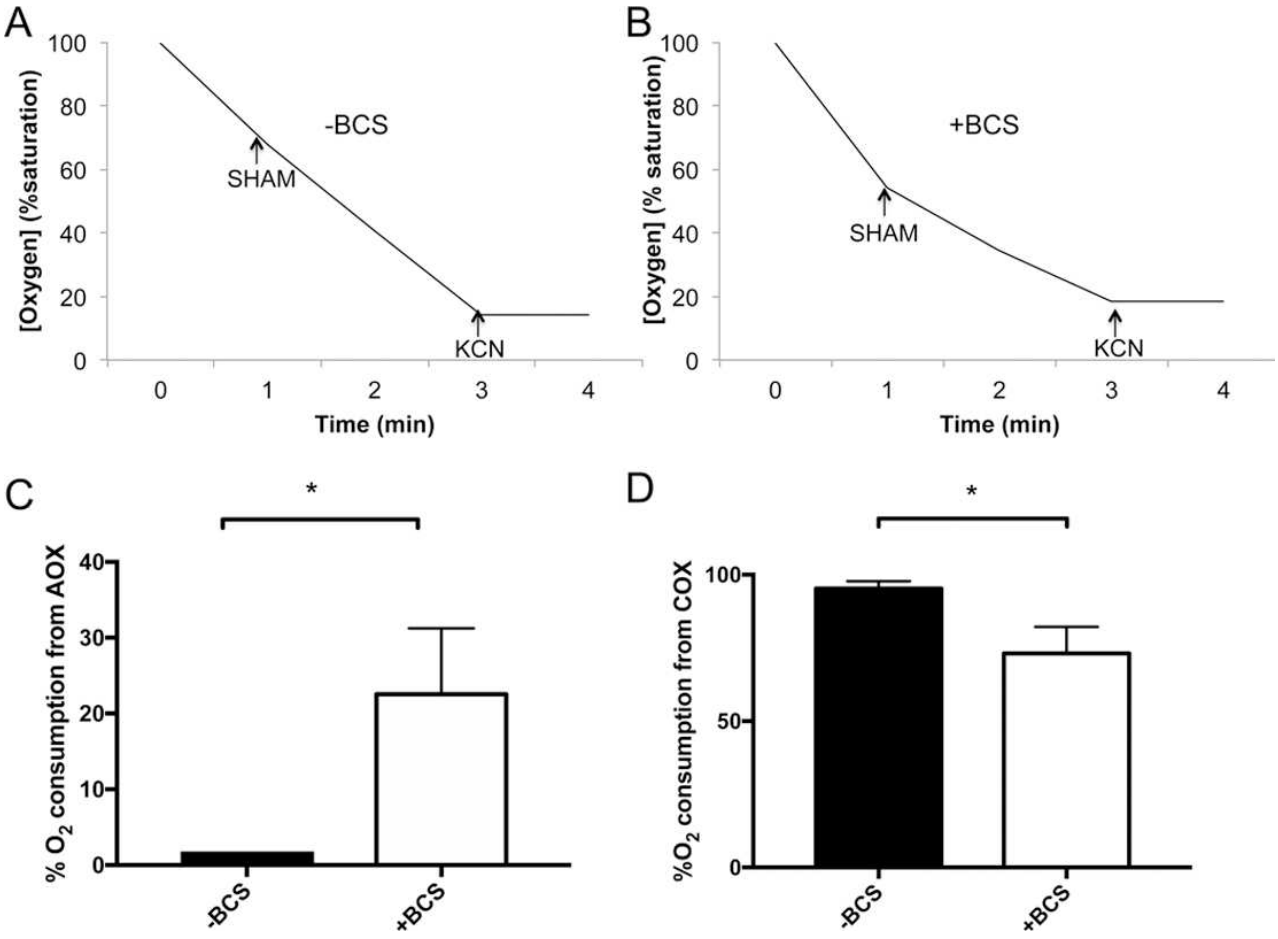

A
[Oxygen] (%saturation)
100
80
60
40
20
0
-BCS
SHAM
KCN
0
1
2
3
4
Time (min)
B
[Oxygen] (% saturation)
+BCS
SHAM
KCN
Time (min)
C
*
% O2 consumption from AOX
40
30
20
10
0
-BCS
+BCS
D
*
%O2 consumption from COX
100
50
0
-BCS
+BCS

Figure 2-6: Effect of inhibiting AOX respiration on mitochondrial superoxide under copper starvation.

MitoSOX Red fluorescence was measured in the designated strains. Where indicated, cells were treated with 5 mM SHAM for 1 hour to inhibit AOX respiration prior to MitoSOX Red incubation. Results represent the averages of two independent cultures with error bars representing standard deviation.

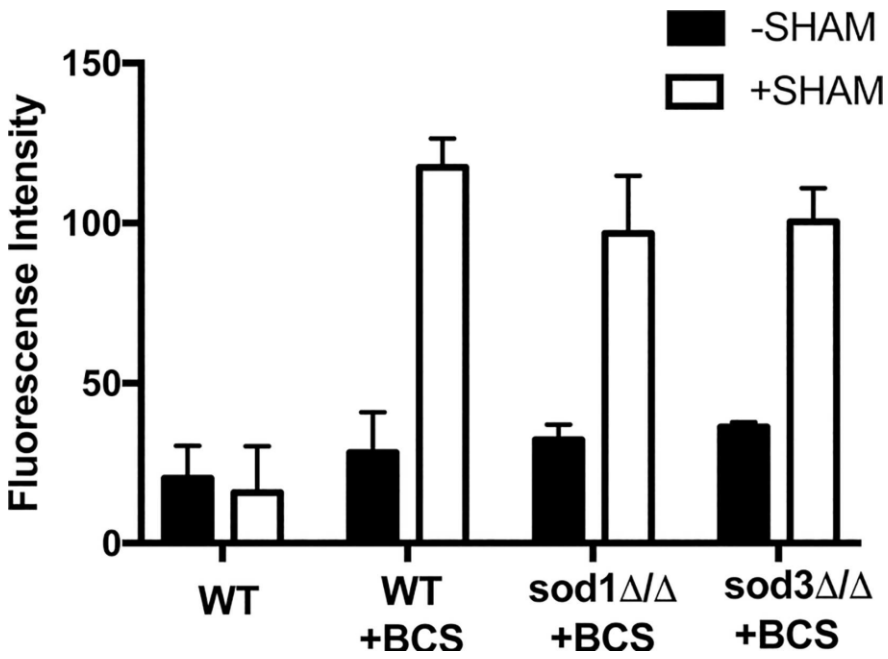


Figure 2- 7: Constitutive expression of SOD1 inhibits COX repression during copper starvation.

(A) Schematic showing the coding region (black) and intron (white) of the *SOD1* gene with sequence of the wild type and mutated MAC1 sites in $SOD1^{rep}$ (*SOD1* repressed by copper) and $SOD1^{con}$ (*SOD1* constitutively expressed). (B) Strains expressing a single copy of either $SOD1^{rep}$ or $SOD1^{con}$ were grown in the presence or absence of BCS and whole cell lysates were (top) immunoblotted for SOD1 and SOD3 as in Fig. 2-1A and (bottom) subjected to SOD enzymatic activity analysis by the native gel assay. Numbers on left of immunoblot represent molecular weight markers and the positions of SOD1 and SOD2 migration on the native gel are indicated on the right. Densitometric tracings show that the intensity of SOD1 activity in $SOD1^{con}$ +BCS is 38% that of the corresponding –BCS sample. (C) Cytochrome c oxidase respiration was measured as in Fig. 2-5D as a function of KCN inhibitable oxygen consumption. Results represent the averages of two independent experiments with a total of four biological replicates with error bars representing standard deviation. Statistical significance was determined using a paired two-tailed Student's t-test. Asterisks indicate statistical significance with P values < 0.005.

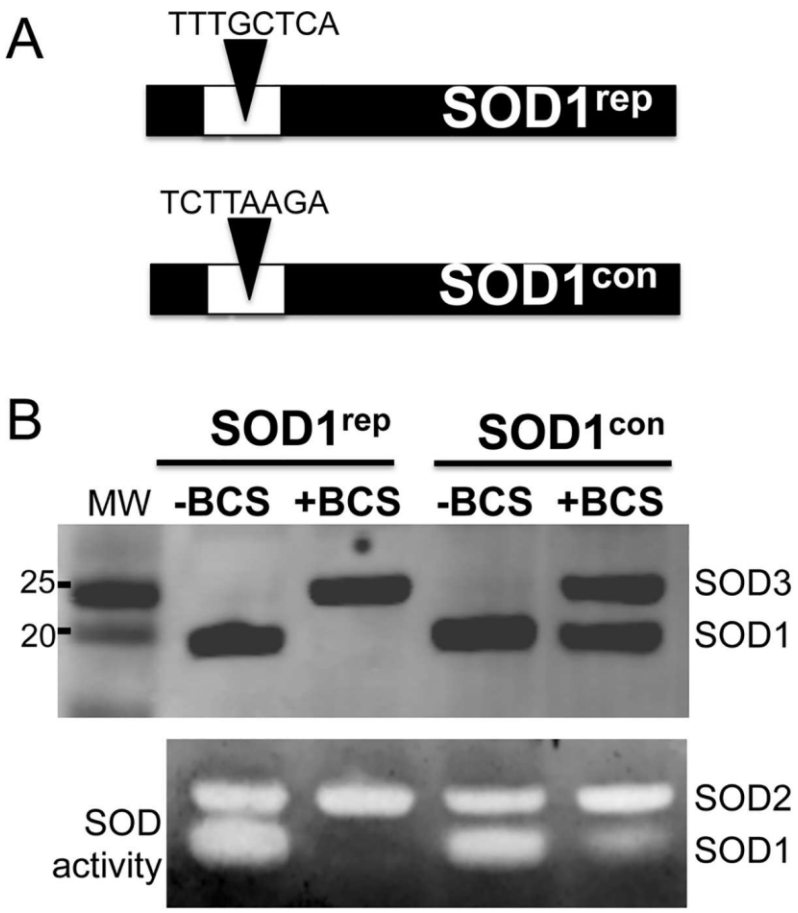
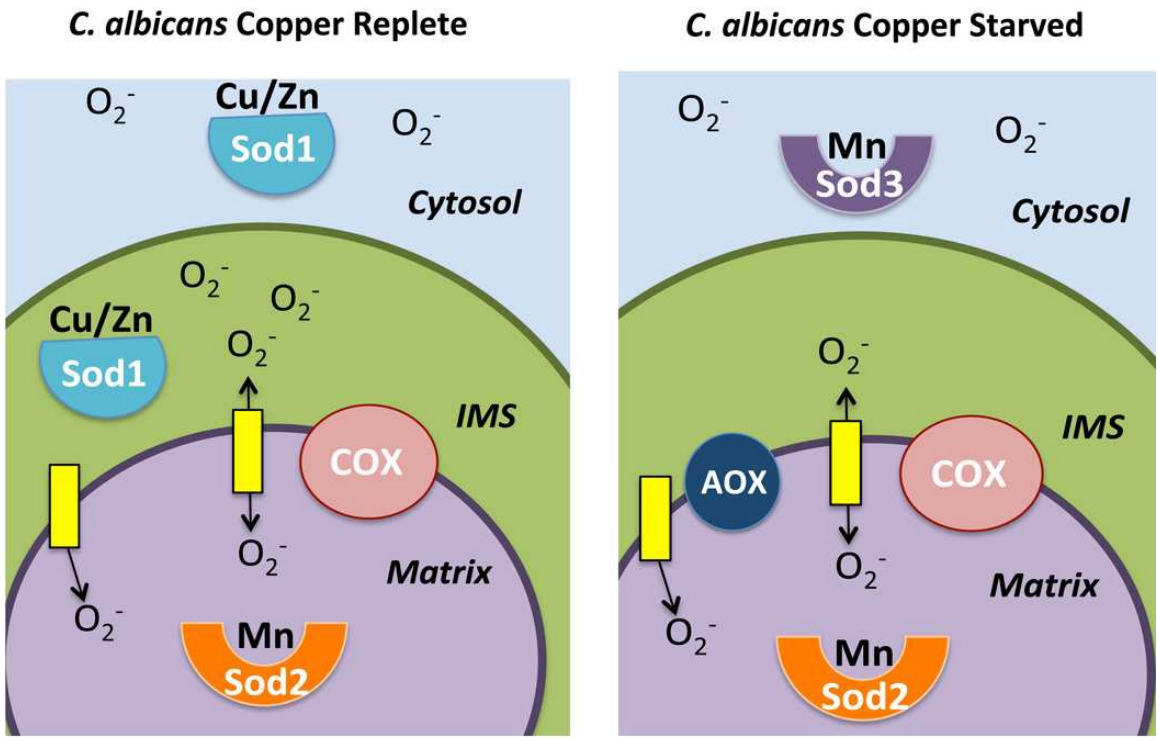

A
TTTGCTCA
SOD1rep
TCTTAAGA
SOD1con
B
SOD1rep
SOD1con
MW
-BCS
+BCS
-BCS
+BCS
25
20
SOD3
SOD1
SOD
activity
SOD2
SOD1
C
**
%O2 consumption from COX
100
50
0
-BCS
+BCS
-BCS
+BCS
SOD1rep
SOD1con

Figure 2-8: Adaption to copper in *Candida albicans* involving SOD enzymes and the mode of mitochondrial respiration.

Cartoon to illustrate *C. albicans* adaptation to low copper by alternating SOD enzymes and inducing AOX respiration. When copper is abundant (left), *C. albicans* utilizes COX respiration and SOD1 is active in both the IMS and the cytosol. During copper starvation (right), cells switch to expressing SOD3 in the cytosol, but the IMS becomes devoid of a SOD enzyme. AOX respiration is induced which helps to lower IMS superoxide. Regardless of copper conditions, COX respiration remains active and mitochondrial matrix superoxide is managed by SOD2.



CHAPTER 3

The role of *C. albicans* cytosolic SOD enzymes in glucose signaling

INTRODUCTION

The nutrient glucose is utilized to generate metabolic energy through the processes of glycolysis and oxidative phosphorylation. In a number of eukaryotic cells, the pathway by which glucose is metabolized is often dictated by the concentration of oxygen, a phenomenon known as the Pasteur effect [92]. As oxygen concentration increases, cells will utilize respiration to generate the maximal amount of ATP and when oxygen levels become scarce cells will switch their metabolism and utilize fermentation for energy production. An exception to this phenomenon exists in rapidly dividing cells such as cancer cells that favor aerobic fermentation through a process known as the Warburg effect[93] . Another example occurs in the baker's yeast *Saccharomyces cerevisiae*.

In *S. cerevisiae*, glucose is preferentially metabolized through fermentation and genes required for mitochondrial respiration are repressed by glucose even in the presence of high concentrations of oxygen, a process referred to as the Crabtree effect [93, 94, 97, 137]. At least three signaling pathways have been shown to mediate glucose uptake and repression of respiration in *S. cerevisiae*. One involves the glucose sensors (SNF3 and RGT2) located in the plasma membrane. When stimulated by glucose, these sensors act together with a casein kinase, namely YCK1, to induce genes for glucose transport and repress genes for mitochondrial respiration. This re-programming of gene expression involves the transcriptional repressor, RGT1 [95, 138-141]. Recent studies by the Culotta lab has shown that the YCK1/RGT1 glucose signaling pathway in *S. cerevisiae* involves the antioxidant enzyme Cu/Zn superoxide dismutase (SOD1) [35]. SOD1 is located largely in the

cytosol, but also in the mitochondrial intermembrane space, and scavenges the free radical superoxide to produce molecular oxygen and hydrogen peroxide [1, 3, 142]. Hydrogen peroxide is a known signaling molecule [36, 143], and the hydrogen peroxide produced by the cytosolic form of SOD1 has been shown to stabilize YCK1 by protecting a degron located in the C-terminus of the protein [35]. This stabilization of YCK1 appeared specific for Cu/Zn containing SODs, as an unrelated Mn containing SOD2 expressed in the cytosol was ineffective [35]. Stabilization of YCK1 by SOD1 requires both oxygen and glucose that help generate the superoxide substrate for SOD1. By integrating signals from glucose and oxygen, SOD1 allows *S. cerevisiae* to rapidly adapt to changes in oxygen tension and carbon sources in its environment [35].

This well characterized glucose signaling pathway in *S. cerevisiae* may not be common among all yeast species. One example includes the opportunistic pathogen *Candida albicans,* a Saccharomycete yeast cousin of *S. cerevisiae* that often exists as a harmless commensal of the human flora, but can become pathogenic in immunocompromised individuals. *C. albicans* can inhabit a variety of niches inside the host and experience both high and low concentrations of glucose [144]. Unlike its Crabtree positive cousin, *S. cerevisiae, C. albicans* is Crabtree negative [145]. The effects of glucose are largely opposite to that seen with *S. cerevisiae.* In the presence of glucose, glucose uptake is repressed and genes for respiration are expressed in *C. albicans* such that oxidative phosphorylation is utilized as the primary means for energy production [146]. *C. albicans* relies heavily on respiration for growth, and respiration is required for pathogenesis of the organism [115, 116].

Aside from being Crabtree negative, *C. albicans* also differs from *S. cerevisiae* in that *C. albicans* has an unusual collection of SOD enzymes. *Candida albicans* uniquely expresses two cytosolic SODs, a Cu/Zn containing SOD1 and Mn containing SOD3, which are differentially expressed in response to copper bioavailability. As shown in Chapter 2, both of these SODs have a cytosolic localization, but only SOD1 resides in the mitochondrial intermembrane space where it protects against respiratory superoxide. But the role of SOD1 and SOD3 in the cytosol has never been examined. Do these enzymes also participate in cell signaling processes?

Herein, we investigate the role of *C. albicans* SOD1 and SOD3 in glucose sensing and regulation. Using *S. cerevisiae* as a heterologous expression system [147], we demonstrate that *C. albicans* Cu/Zn containing SOD1 can substitute for *S. cerevisiae* SOD1 in participating in the glucose regulatory pathway of bakers' yeast involving stabilization of YCK1. Surprisingly, despite being a Mn SOD, we show that *C. albicans* SOD3 can also stabilize *S. cerevisiae* YCK1 through a mechanism that requires oxygen and glucose similar to Cu/Zn SODs. We additionally examined whether SOD1 and SOD3 could in participate glucose signaling and regulation in the native *C. albicans* host. We find that in *C. albicans* SOD1 and SOD3 are involved in the repression of glucose transporters genes, in stark contrast to the activation of glucose transporter genes seen with *S. cerevisiae* SOD1. Also in *C. albicans,* SOD mediated repression of glucose transporters does not involve RGT1 as is the case with *S. cerevisiae,* but rather a separate glucose regulatory pathway involving the MIG1 transcription factor. The exact target of *C. albicans* SOD1 and SOD3 is not

known, but does not involve either of the *C. albicans* casein kinases YCK2 and YCK22, homologs to *S. cerevisiae* YCK1. Collectively, our studies demonstrate that unlike *S. cerevisiae* where SOD1 promotes glucose uptake, *C. albicans* has repurposed the cytosolic SODs to repress glucose uptake using a distinct glucose-signaling pathway.

MATERIALS AND METHODS

Yeast strains and plasmids

S. cerevisiae strains were all derived from the *sod1Δ* strain AR203 (MATa*, met15Δ0, ura3Δ0, his3Δ1 sod1Δ::LEU2*), kind gift of Amit Reddi. This *sod1Δ* strain was transformed with various SOD expressing plasmids including pLJ175 (*CEN URA3*) expressing *S. cerevisiae SOD1* sequences –674 to +584, the pJG102 plasmid (*CEN LEU2*) for expressing *C. albicans* P144L SOD1 under the *S. cerevisiae SOD1* promoter[125], the pLJ486(*URA3* 2 micron) for expressing *S. cerevisiae SOD1* under the *ADH1* promoter [148], plasmid pVTSOD3 (*URA3* 2 micron) for expressing *C. albicans* SOD3 under *S. cerevisiae ADH1* , plasmid pCB002 for expressing *S. cerevisiae* SOD2 under *ADH1* (see below), or empty vectors pRS426 [149] and pVT102u [150]. All strains co-expressed either *TEF1* driven GFP fused to full length *YCK1* (pAR113) [35], or *MET25* driven GFP fused to the C-terminus of *YCK1* residues 368-538 (pAR119) [35]. The plasmid for expressing *ADH1* driven *S. cerevisiae* cytosolic SOD2 (pCB002) was constructed through amplification of *SOD2* nucleotide sequences +265 to +889 lacking the mitochondrial localization signal from *S. cerevisiae* genomic DNA with primers containing *Bam*HI restriction sites. The resulting PCR

fragment was digested with *Bam*HI and ligated into the *Bam*H1 digested pVTSOD3 plasmid.

C. albicans strains used in this study were derived from SC5314 including CA-IF100 (*arg4Δ/arg4Δ, leu2Δ/leu2Δ::cmLEU2, his1 Δ/his1 Δ::cdHIS1, URA3/ura3Δ*), and isogenic strains CA-IF001 (*sod1Δ::cmLEU2/sod1Δ::cdHIS1*) and CA-IF011 (*sod3Δ::cmLEU2/sod3 Δ::cdHIS1*) provided by K. Kuchler, Medical University of Austria, Vienna [121]. The SN250 strain (*ura3□-iro1□::imm^{434}/URA3-IRO1, his1□/his1□, arg4□/arg4□., leu2□□□C.m.LEU2/leu2□□□C.d.HIS1*) and isogenic strains *rgt1Δ/Δ* and *mig1Δ/Δ* as well as DAY286 (*ura3Δ:: λimm^{434}/ura3Δ:: λimm^{434}pARG4::URA3::arg4::hisG/arg4::hisG*) and the isogenic *yck22Δ/Δ* strain were obtained from the fungal genetics stock center. Strain BWP17 *(ura3Δ::λimm^{434}/ura3Δ:: λimm^{434}his1::hisG/his1::hisG arg4::hisG/arg4::hisG)* and isogenic *yck2Δ/Δ* (*yck2-Tn7::UAU1/yck2-Tn7::URA3::pHIS1*) were provided by Hyunsook Park [151].

Culture conditions

S. cerevisiae and *C. albicans* cells were cultured at 30°C in enriched yeast extract and peptone (YP) media containing 1% yeast extract and 2% peptone with either 4% glucose, 2% glucose (H), 0.2% glucose (L) or 5% glycerol (G) (w/v). For anaerobic growth of *S. cerevisiae* cells were grown in enriched media supplemented with 15 mg/L ergosterol and 0.5% Tween-80 and were cultured in a COY chamber under 95% nitrogen and 5% hydrogen gas (COY Laboratory Products, Inc., Grass Lake, Michigan, USA) as described [35]. Where indicated *C. albicans* cells were

starved for copper by supplementing the growth media with 800 μM of the extracellular Cu(I) chelator bathocuproine sulfonate (BCS).

Whole cell lysis, immunoblot, and SOD enzymatic assay

Whole cell lysates of *S. cerevisiae* and *C. albicans* cells was prepared by glass bead homogenization [35, 66]. *S. cerevisiae* strains were grown from freezer stocks originally plated on synthetic completed media (SC) lacking histidine and uracil, or lacking histidine and leucine to select for yeast containing the GFP-YCK2 plasmid and the respective SOD enzymes. Cultures were inoculated at an OD of 0.005 in YPD media as described above and shaken for ~ 12-16 hours at 30°C. In *S. cerevisiae*, stability of casein kinase YCK1 was monitored by immunoblot. 100 μg of whole cell lysate protein was loaded onto 12% Tris-Glycine gels (Thermo Fisher) and blots were probed with anti-GFP and anti-PGK1 antibodies as previously described [35]. To monitor the effects of *mig1Δ/Δ* mutations on SOD1 protein in *C. albicans*, 30 μg of *C. albicans* whole cell lysate protein was loaded onto 4-12% Bis-Tris gels and SOD1 protein was detected by immunoblot using anti-SOD1 antibody as described in Chapter 2. *S. cerevisiae* and *C. albicans* SOD enzymatic activity was analyzed by native gel electrophoresis and nitroblue tetrazolium staining as previously described ([35, 66] and Chapter 2).

RNA analysis

For RNA-seq analysis, *C. albicans* cells were grown in YP+2% glucose medium in the presence or absence of 800 μM BCS to an optical density at 600 nm

(OD_{600}) between 1.0 and 2.0. RNA was isolated from 20 OD_{600} units of cells using RNAeasy (Qiagen) and subjected to RNA-seq analysis at the Institute for Genome Science, University of Maryland School of Medicine. Cells for quantitative real time PCR (qRT-PCR) or RT-PCR were grown either as above for RNA-seq, or were tested for glucose repression by first growing in YP+.2% glucose to OD_{600} 1.0-2.0, followed by diluting to $OD_{600} \approx 0.04$ in YP + 2% glucose or 5% glycerol and growing to a final $OD_{600} \approx 2.0$. RNA was isolated as above and converted to cDNA using Superscript IV reverse transcriptase (Thermo Fisher Scientific). The cDNA was diluted 20-fold prior to analysis by qRT-PCR and RT-PCR. For qRT-PCR, PCR amplification used iQ SYBR Green Supermix using parameters as previously described [66]. Values were normalized to *TUB2*. *TUB2* primers were used as described in [66]. *HGT17* primers used are TGGACTATGGGTTTCAATTC and CATGACTCCGACAAAGAAAT; *HGT12* primers, CTGTCACTGCTTCATACCAA and CTTGGAAACCCAGTATCTTG; and HGT2 primers, ACTTGACAACAAATCTGCCT and ATACATGGCAATAGCGAAAT.

RT-PCR was accomplished using Taq DNA Polymerase (NEB). Protocol consisted of initial denaturation at 95°C for 30 seconds, followed by 30 cycles of amplification (denaturing at 95°C for 30 second, annealing at 55°C for 30 second, and extension at 72°C for 1 minute) (HGT17, HGT2 and HGT12 genes) or 25 cycles of amplification (HGT7) with a final extension at 72°C for 10 minutes. Primer sequences used for amplification were previously described [152].

Analysis of glucose consumption

C. albicans cells were cultured in YP+2% glucose to early stationary phase, then diluted back to $OD_{600} \approx 0.200$ in 10 mL of fresh YP+2% glucose media. At the indicated OD, 200 µL of culture was removed and centrifuged at 12,000 x g for 2 minutes to remove whole cells and cell debris. The spent media was removed as supernatant and glucose consumption was determined by measuring extracellular glucose using the Glucose Colorimetric Assay Kit (Cayman Chemical Company product #: 10009582). Spent media was diluted 1:5 with sodium phosphate sample buffer and 2 uL of diluted media was added to 98 uL of sample buffer in a 96 well plate. Addition of 100 uL of the Glucose colorimetric enzyme mixture results in a pink color when glucose is present and samples were read on a Biotek Synergy HT microplate reader at 520nm.

RESULTS

C. albicans SOD1 and SOD3 can stabilize the *S. cerevisiae* casein kinase, YCK1.

In the bakers' yeast *Saccharomyces cerevisiae*, cytosolic SOD1 participates in glucose signaling by preventing the degradation of a signaling casein kinase, YCK1 [35]. The pathogenic fungus *Candida albicans* uniquely expresses two cytosolic SOD enzymes, Cu/Zn containing SOD1 and Mn containing SOD3 [60, 63, 66] but a possible role for these SODs in cytosolic cellular signaling had yet to be explored. Using *S. cerevisiae* as a heterologous expression system it has been shown that SOD1 from both *C. elegans* and humans can substitute for *S. cerevisiae* SOD1 in stabilizing YCK1 [35]. We used the same heterologous expression system to examine whether *C. albican*s SOD1 and SOD3 could participate in cytosolic cellular signaling.

We first addressed whether *C. albicans* SOD1 could stabilize *S. cerevisiae* YCK1. For these studies we used *C. albicans* P144L *SOD1* under the *S. cerevisiae SOD1* promoter. WT *C. albicans* SOD1 is normally inactive in *S. cerevisiae* due to poor interaction with the *S. cerevisiae* copper chaperone CCS1. But P144L *C. albicans SOD1* is activated independent of CCS and exhibits abundant activity in *S. cerevisiae* [153]. *S. cerevisiae sod1Δ* cells expressing a N-terminal GFP-YCK1 fusion were transformed with vectors for expressing *S. cerevisiae SOD1*, *C. albicans* P144L *SOD1* or empty vector and YCK1 protein levels were monitored by immunoblot with anti-GFP. As previously reported, *S. cerevisiae* SOD1 could stabilize YCK1 (Fig. 3-1A, lane 1), and this stabilization is lost when there is no SOD1 expressed, as seen when *sod1Δ/Δ* mutants are transformed with empty vector (Fig. 3-1A, lane 2). Stabilization of YCK1 is recovered when *C. albicans* SOD1 is expressed (Fig. 3-1A, lane 3). This result is consistent with previous findings that SOD1 from various organisms including humans and *C. elegans* can substitute for *S. cerevisiae* SOD1 in stabilizing YCK1 [35].

Next we asked if *C. albicans* SOD3 could participate in stabilization of YCK1. Mn containing SODs are not predicted to stabilize YCK1, as has been demonstrated when a mitochondrial Mn SOD2 from *S. cerevisiae* is targeted to the cytosol [35]. Using the same heterologous expression system mentioned above, *S. cerevisiae sod1Δ/Δ* mutants were transformed with *C. albicans SOD3* expressed under the *ADH1* promoter or a cytosolic form of mitochondrial *SOD2* from *S. cerevisiae* also expressed under the *ADH1* promoter. Controls included *S. cerevisiae SOD1* under *ADH1* and empty vector. Using a native gel assay for SOD enzymatic activity, we

ensured that SOD activity from all three SODs were comparable (Fig. 3-1B). Surprisingly, we observed that *C. albicans* Mn SOD3 could stabilize YCK1 (Fig. 3-1B, lane 3) similar to the Cu/Zn SOD1 control (Fig. 3-1B, lane 1). By comparison, *S. cerevisiae* Mn SOD2 targeted to the cytosol failed to stabilize YCK1 in spite of abundant enzymatic activity (Fig. 3-1B, lane 4). Despite the strong homology between the two Mn SODs, cytosolic SOD3 must have evolved with a unique capacity for operating in this signaling pathway (see Discussion).

We sought to determine whether SOD3 stabilization of YCK1 shared the same requirements as had been shown for SOD1. The protection of YCK1 by SOD1 requires oxygen which is needed to generate the superoxide substrate necessary for hydrogen peroxide production [35]. To test whether SOD3 also requires oxygen to stabilize YCK1, cells expressing *C. albicans SOD3*, *S. cerevisiae SOD1* or empty vector were grown in 20% oxygen or anaerobically (0% oxygen) and GFP-YCK1 protein levels were monitored. As previously reported [35], stabilization of YCK1 by SOD1 seen in 20% oxygen (Fig. 3-2A, lane 1) is lost when cells are grown in 0% oxygen (Fig. 3-2A, lane 4). *C. albicans* SOD3 displays a similar trend of regulation as YCK1 was no longer stabilized under anaerobic conditions (Fig. 3-2A, lane 6). This result demonstrates that SOD3 is dependent on oxygen or more likely the superoxide substrate to regulate the YCK1 protein.

In addition to the requirement for oxygen, YCK1 regulation by SOD1 requires glucose which favors superoxide production [35]. To address whether *C. albicans* SOD3 also depends on glucose to regulate YCK1, cells expressing either *S. cerevisiae SOD1*, *C. albicans SOD3*, or empty vector were grown in the presence of media

containing 4% glucose (high glucose) or 0.2% glucose (glucose starvation conditions) and YCK1 protein levels were monitored. As previously described [35], SOD1 is able to stabilize YCK1 in the presence of high glucose (Fig. 3-2B, lane 2), but not low glucose (Fig. 3-2B, lane 5). Identical to these effects of SOD1, we observed that in cells expressing SOD3, YCK1 was abundant in 4% glucose (Fig. 3-2B, lane 3), but absent when glucose was low (Fig. 3-2B, lane 6).

Work in *S. cerevisiae* has shown that SOD1 protects a degron found in the C-terminus of the YCK1 protein, and that the C-terminus is sufficient for protein stabilization [35]. To investigate whether *C. albicans* SOD3 regulates YCK1 by stabilizing the C-terminal domain, *S. cerevisiae* SOD1, *C. albicans* SOD3, or empty vector were co-expressed with a fusion protein consisting of N-terminal GFP fused to the C-terminus of YCK1 amino acid residues 367-538 and this short GFP fusion was monitored by immunoblot. As shown in Fig. 3-2C bottom, SOD3 can indeed stabilize GFP containing this minimal C-terminal domain of YCK1 (lane 3), similar to what is observed with *S. cerevisiae* SOD1 (lane 1). Altogether these findings suggest that despite being a Mn SOD, SOD3 from *C. albicans* can stabilize YCK1 through a mechanism similar to what has been shown for Cu/Zn SODs. *C. albicans* SOD3 has appeared to have evolved with the potential for stabilizing casein kinases, at least in this heterologous *S. cerevisiae* expression system.

A role for SOD1 in control of glucose transporter expression in *Candida albicans*

Although both cytosolic SODs from *C. albicans* seem capable of participating in glucose regulation when heterologously expressed in *S. cerevisiae,* it was not clear whether these SODs play a role in glucose signaling in their native host *C. albicans.* Glucose metabolism varies drastically between *S. cerevisiae* and *C. albicans*. *S. cerevisiae* is a Crabtree positive yeast, where glucose promotes glucose uptake and fermentation and results in repression of respiration [93, 97] (illustrated in Fig. 3-3). *C. albicans*, however, is a Crabtree negative yeast. In the presence of glucose and oxygen, respiration is promoted and genes for glucose uptake and fermentation are repressed (Fig. 3-3). Due to these differences in glucose metabolism, we were curious as to whether SOD1 and/or SOD3 played a role in glucose sensing in *C. albicans*.

We first used an RNA-seq approach to investigate any connection between the cytosolic SODs and glucose control in the native *C. albicans* host. In copper-replete media Cu/Zn containing SOD1 is the only cytosolic SOD expressed in *C. albicans*, however when copper levels decrease, SOD1 is repressed and Mn containing SOD3 is expressed as the only cytosolic SOD [66]. To identify genes in *C. albicans* whose expression may be affected by the cytosolic SODs we performed RNA-seq analysis on copper-replete WT (solely expressing *SOD1*), copper-replete *sod1Δ/Δ* mutants (expressing neither *SOD1* or *SOD3*), and copper-starved WT yeast treated with 800μM of the copper chelator, BCS (solely expressing *SOD3*). These samples were grown in media containing high (2%) glucose to log phase and gene expression profiles were compared. We observed that deletion of *SOD1* resulted in a prominent increase in the RNA from eleven members of the glucose transporter

gene family (Table 3-1). To confirm these findings, quantitative real-time PCR (qRT-PCR) was carried out on the top three glucose transporters induced by deletion of *sod1Δ/Δ*, namely *HGT17*, *HGT12*, and *HGT2*. All three genes showed a strong induction of expression in *sod1Δ/Δ* cells compared to WT cells (Fig. 3-4A). Thus it appears that *SOD1* participates in repression of glucose transporter gene expression in the native *C. albicans* host. This effect is opposite to the induction of glucose transport genes seen with *S. cerevisiae* SOD1 [35].

Evidence for *C. albicans* SOD3 in control of glucose transporter gene expression.

As mentioned above, *C. albicans* starved for copper will switch from expressing Cu/Zn containing SOD1 to Mn containing SOD3 [66]. We surmised that if SOD3 can fully substitute for SOD1 in glucose control, there would be no de-repression of glucose transporters during copper starvation. However, copper starved cells did exhibit some level of induction in eight of the eleven glucose transporters genes that are induced in *sod1Δ/Δ* mutants (Table 3-1). Even so, the level of induction observed with copper starvation is much reduced compared to that seen in *sod1Δ/Δ* cells expressing neither SOD. These results alone suggested that SOD3 may *partially* substitute for SOD1 in repression of glucose transporters.

To address this further, expression levels of the glucose transporter *HGT17* were examined using qRT-PCR. Consistent with RNA-seq, copper starvation through BCS treatment did yield a small induction of *HGT17* (no more than 2 fold) compared to the ≈20 fold induction seen with *sod1Δ/Δ* mutations (Fig. 3-4B). To

determine if the presence of SOD3 was helping to repress *HGT17* in copper starved cells, we examined the effects of *sod3Δ/Δ* mutations. As seen in Fig. 3-4B, expression of *HGT17* in BCS treated cells was significantly increased with *sod3Δ/Δ* mutations, indicating that SOD3 can contribute to repression of glucose transporters. We also tested whether the de-repression of *HGT17* in *sod1Δ/Δ* cells could be reversed by inducing *SOD3.* In the experiment of Fig. 3-4B, *sod1Δ/Δ* mutants were treated with BCS to induce *SOD3*. We observed that induction of *HGT17* mRNA in *sod1Δ/Δ* mutants was greatly attenuated with BCS (Fig. 3-4B), consistent with a role for SOD3 in substituting for SOD1 in glucose repression. Even so, *HGT17* was not fully repressed upon expression of SOD3 (compare *sod1Δ/Δ* + BCS to WT, Fig. 3-4B). Thus, while both SOD1 and SOD3 appear to contribute to repression of glucose transporters genes, SOD1 seems more effective in this regard (see Discussion). For the remainder of the studies presented here, we will focus on SOD1 repression of glucose transporters.

C. albicans SOD1 does not affect glucose consumption

There are two possible explanations for why glucose transporters are induced in *C. albicans* lacking a cytosolic SOD. The yeast could have a defect in glucose uptake and as a result glucose transporters are induced to bring more glucose into the cell. It is also possible that *sod1Δ/Δ* mutants have a defect in glucose sensing, and induce glucose transporters even though there is sufficient glucose uptake. To assess glucose uptake, WT and *sod1Δ/Δ* mutants were grown in medium containing 2% glucose and extracellular glucose was measured over 8 hours of

growth. We observed that *sod1 Δ/Δ* mutants consume glucose at the same rate as WT cells (Fig. 3-5), indicating no apparent defect in glucose consumption. This data suggests that *sod1Δ/Δ* mutants may have a defect in glucose sensing.

SOD repression of glucose transporter genes

As mentioned above, *C. albicans* is a Crabtree negative yeast and rather than inducing glucose uptake in response to high glucose (as is the case with *S. cerevisiae*), *C. albicans* will repress glucose uptake when glucose is abundant [104, 105]. Expression of many glucose transporters becomes maximal under conditions of low glucose and when glucose is totally absent, certain glucose transporters are repressed (illustrated in Fig. 3-6A). This complex regulation of glucose sensing is mediated by two transcriptional repressors, namely *C. albicans* MIG1 and RGT1. MIG1 is responsible for the repression of glucose transporters when glucose is abundant, and RGT1 is responsible for repressing certain glucose transporters when glucose is absent (see cartoon of Fig. 3-6A) [104, 139]. It is possible that SOD1 may be involved in one or more of these glucose-sensing pathways.

To begin to address whether SOD1 is involved in the MIG1 or RGT1 or both pathways, we surveyed our list of SOD1-regulated genes. *HGT17*, *HGT2* and *HGT12* are strongly induced by *sod1Δ/Δ* mutations (Table 1 and Fig. 3-4A). Of these, glucose transporters *HGT2* and *HGT12* are known targets of MIG1 [104], but *HGT17* had yet to be identified as a MIG1 or RGT1 target. In the experiment of Fig. 3-6B, WT, *mig1Δ/Δ*, and *rgt1Δ/Δ* yeast were grown in high glucose (H), low glucose (L) or glycerol (G) and *HGT17*, *HGT2*, and *HGT12* mRNA expression was analyzed by RT-

PCR. Consistent with previous findings [104], *mig1Δ/Δ* mutants show a de-repression of *HGT2* and *HGT12* in high glucose but there is no effect of *rgt1Δ/Δ* mutations (Fig. 3-6B); these genes are clearly MIG1 targets. We observed that *HGT17* is also de-repressed by *mig1Δ/Δ* mutations in high glucose, but is not affected by *rgt1Δ/Δ* mutations, indicating that this gene is also a target of MIG1 (Fig. 3-6B). This data shows that all three SOD1 regulated HGT genes are targets of MIG1, but not RGT1.

To rule out the possibility that *C. albicans* SODs are involved in the RGT1 signaling pathway, we asked whether deletion of SOD1 had an effect on the regulation of a known RGT1 target, *HGT7* [104]. Based on RNA-seq, there was no significant change in *HGT7* expression with *sod1Δ/Δ* cells or copper starvation under high glucose (Table 3-1). We also examined glucose regulation of *HGT7*. Copper replete WT and *sod1Δ/Δ* null cells and copper-starved WT cells (express SOD3 but not SOD1) were grown in high glucose or glycerol and *HGT7* mRNA detected by RT-PCR. In WT cells, *HGT7* is expressed in high glucose but is repressed when glucose is absent (glycerol), consistent with RGT1 control (Fig. 3-7, lane 2). We observed that the same repression of *HGT7* in the absence of glucose occurs in copper starved cells (Fig. 3-7, lane 4) and in *sod1Δ/Δ* mutants (Fig. 3-7, lane 6) consistent with the notion that *C. albicans* cytosolic SODs are not involved in the RGT1 glucose sensing pathway.

The aforementioned studies demonstrate that *C. albicans* SOD1 participates in regulation of the same glucose transporter genes as MIG1. To address whether SOD1 and MIG1 are acting in the same pathway or in separate parallel pathways, we

tested whether the effects of low glucose and *sod1Δ/Δ* mutations were additive with regard to regulating glucose transporters. WT and *sod1Δ/Δ* mutants were grown in high versus low glucose and *HGT17* mRNA expression was monitored by qRT-PCR. As seen in Fig. 3-8A, *sod1Δ/Δ* mutations did not further increase *HGT17* expression over that obtained with WT cells grown in low glucose (Fig. 3-8A). Since the effects of *sod1Δ/Δ* mutations and low glucose are not additive, it appears that SOD1 is acting in the same pathway as MIG1. To test whether SOD1 works downstream of MIG1, we addressed whether SOD1 protein levels or enzymatic activity were altered in *mig1Δ/Δ* mutants grown in high glucose. By immunoblot, there was no difference in SOD1 protein levels in WT versus *mig1Δ/Δ* mutant yeast (Fig. 3-8B, Top). Additionally using a native gel assay, SOD1 activity was comparable between *mig1Δ/Δ* mutants and the WT control (Fig. 3-8B, Bottom). Therefore, SOD1 does not appear to be a downstream target of MIG1 repression. Instead, SOD1 may be upstream of transcriptional control by MIG1.

Casein kinases and glucose control in *Candida albicans*

In *S. cerevisiae*, SOD1 participates in glucose sensing by acting upstream of the RGT1 transcription factor through stabilization of YCK1, and we were able to demonstrate that the cytosolic SODs from *C. albicans* can also function in this RGT1 pathway and stabilize YCK1 when expressed heterologously in *S. cerevisiae* (Fig. 3-1 and 3-2). It is very curious therefore that in *C. albicans*, SOD1 has evolved to function in the MIG1, not the RGT1 pathway. Even so, *C. albicans* SOD1 may help stabilize a casein kinase in the MIG1 pathway. There are three predicted casein

kinases in *C. albicans*, HHR25, YCK2, and YCK22. HHR25 belongs to the casein kinase delta family, and lacks the critical C-terminal domain that serves as the SOD1-regulated degron of *S. cerevisiae* YCK1. *C. albicans* YCK2 and YCK22 share 67% and 53% sequence similarity to *S. cerevisiae* YCK1 respectively. Both proteins have a similar C-terminal domain and belong to the same casein kinase gamma family as *S. cerevisiae* YCK1. Of these two, YCK2 has been predicted to act in the RGT1 pathway of *C. albicans,* based solely on the analogy to *S. cerevisiae* [102, 151], but this has never been tested. Furthermore, nothing is known about the possible role of YCK22 in glucose sensing in *C. albicans.*

We asked whether deletion of *YCK2* or *YCK22* from *C. albicans* affected MIG1 and/or RGT1 target genes. RNA was isolated from WT, *yck2Δ/Δ* and *yck22Δ/Δ* mutants grown in high glucose or glycerol, and expression of *HGT17*, a MIG1 target, and *HGT7* a RGT1 target, were analyzed by RT-PCR. As previously shown in WT yeast (Fig. 3-6B and [152]), *HGT17* is repressed in high glucose and expressed in glycerol (Fig. 3-9A, top panel). Deletion of *YCK2* did not affect repression of *HGT17* in high glucose, indicating that YCK2 is not involved in the same pathway as MIG1 and SOD1 (Fig. 3-9A, top panel). We did, however, observe that the RGT1 target *HGT7* is de-repressed in glycerol by *yck2Δ/Δ* mutations (Fig. 3-9A, bottom panel, lane 4) compared to what is seen in the WT control (Fig. 3-9A, bottom panel, lane 2). Thus, YCK2 appears to act in the RGT1 glucose-sensing pathway of *C. albicans* as previously proposed [102], and parallels the role of *S. cerevsiae* YCK1 in the RGT1 pathway of bakers yeast [96, 141]. However, unlike effects of *yck2Δ/Δ* mutations there was no change in *HGT17* or *HGT7* regulation observed in *yck22Δ/Δ* mutants

compared to the WT control (Fig. 3-9B), suggesting that this casein kinase may not participate in either of the glucose signaling pathways. We conclude that *C. albicans* cytosolic SODs play a role in regulating genes in the MIG1 glucose-signaling pathway through a mechanism that does not involve the casein kinases YCK2 or YCK22, therefore diverging from the role of *S. cerevisiae* SOD1 in glucose signaling.

DISCUSSION

The largely cytosolic SOD1 enzyme has been shown to participate in signaling pathways involving ROS in mammalian cells [30, 36] and in *S. cerevisiae* [35], and here we report a role for the dual cytosolic SODs of *Candida albicans* in signaling. As with *S. cerevisiae,* the cytosolic SODs of *C. albicans* participate in glucose sensing and signaling, although in a fashion that is apparently opposite to that of *S. cerevisiae*. *C. albicans* SOD1 and SOD3 are involved in the repression of glucose transporters, acting in the same pathway as the transcriptional repressor, MIG1. By contrast, *S. cerevisiae* SOD1 participates in the induction of glucose transporters mediated through the transcription factor, RGT1 [35, 141]. Although the SODs act divergently in these two yeasts, it seems completely in line with the disparate effects of glucose in these organisms: glucose uptake is repressed by glucose in *C. albicans* [104, 105] whereas glucose uptake is induced by glucose in *S. cerevisiae* [141].

Previous work from *S. cerevisiae* suggested that glucose signaling involving the casein kinase YCK1 was restricted to Cu/Zn containing SODs, not Mn containing SODs [35]. Therefore we found it surprising that *C. albicans* Mn SOD3 could stabilize

YCK1 when expressed heterologously in *S. cerevisiae*, while a cytosolic version of Mn SOD2 from *S. cerevisiae* could not. *C. albicans* SOD3 and *S. cerevisiae* SOD2 are over 60% identical in amino acid sequence and the recombinant proteins show comparable catalytic activity *in vitro* [64]. In spite of these similarities, these two Mn containing SODs behave differently when expressed in the cytosol of *S. cerevisiae*. SOD2 has difficulty acquiring its manganese co-factor when expressed in the cytosol and is inactive unless millimolar quantities of manganese are added to the growth media [35]. By comparison, *C. albicans* SOD3 is active in the cytosol of both *S. cerevisiae* and *C. albicans* without manganese supplements [66](Fig. 1A). While SOD2 has evolved to function in the mitochondrial matrix, C*. albicans* SOD3 has evolved to function in the cytosol, including a capacity for cellular signaling involving glucose.

Although *C. albicans* SOD3 can contribute to glucose signaling, this SOD could not fully compensate for the loss of SOD1 in the repression of glucose transporter genes. SOD1 and SOD3 catalyze the same superoxide disproportionation reaction, but the two enzymes completely diverge in primary sequence and structure [1] which may account for their differential capacities for glucose signaling. Is this apparent inadequacy of SOD3 fortuitous or intentional? It is possible that the lower capacity for glucose repression seen with SOD3 is by design. SOD1 is expressed when there is abundant copper, the rate of respiration is high and cells are rapidly dividing. SOD3 is expressed when copper levels decline and also in stationary phase cells that are no longer rapidly dividing [63]. These conditions may not require

repression of glucose uptake to the same extent as when cells are rapidly dividing and respiring.

What is the target of SOD1 and SOD3 that allows for the repression of glucose transporters in *C. albicans*? As both cytosolic SODs can stabilize YCK1 from *S. cerevisiae*, the target in *C. albicans* likely shares similarities to this casein kinase. The C-terminal domain of *S. cerevisiae* YCK1 contains a degron crucial for stabilization by SOD enzymes and a search for *C. albican*s proteins that shared sequence similarity to the C-terminal domain revealed the casein kinases YCK2 and YCK22. Despite high sequence similarities neither of these proteins repress glucose transporters in the same pathway as SOD1 or SOD3. The target of *C. albicans* cytosolic SODs may not share primary sequence similarity to *S. cerevisiae* YCK1, but instead may have a degron that is similar in secondary or tertiary structure. There are two kinases involved in the glucose repression pathway, the hexokinase HXK2 and the SNF1 kinase that act upstream of the MIG1 transcriptional repressor [105]. As HXK2 and SNF1 kinases do not share primary sequence similarities to YCK1, they may possess similarities in secondary and tertiary structure that allows for their regulation by the cytosolic SODs. It is also possible that MIG1 itself may be a target of regulation by the SODs or an unidentified molecule in the MIG1 pathway.

Separate from the signaling functions described here, does either SOD1 or SOD3 act as anti-oxidants to guard against oxidative stress in the cytosol? In Chapter 2 we described a role for SOD1, but not SOD3 in mitochondrial oxidative stress protection, but a similar anti-oxidant function for either SOD in the cytosol was not clear. Using a cytosolic probe for superoxide, dihydroethidium (DHE), *C.*

albicans sod1Δ/Δ and *sod3Δ/Δ* mutants do not show an increase in cytosolic superoxide compared to wild type cells (data not shown). Additionally *C. albicans sod1Δ/Δ* cells do not exhibit a defect in methionine biosynthesis [154] a hallmark of cytosolic superoxide stress found in *S. cerevisiae sod1Δ* mutants [155] . This evidence suggests that the main role of *C. albicans* SOD1 and SOD3 in the cytosol is not oxidative stress protection but rather in signaling involving ROS, specifically glucose signaling.

The utilization of cytosolic SOD enzymes in glucose repression rather than glucose activation, is another example of how pathways can be rewired in *C. albicans* versus *S. cerevisiae*. Regulatory circuits in *S. cerevisiae* have also been repurposed in *C. albicans* to promote morphological changes, such as the formation of hyphae [147, 156], adherence to endothelial cells required for virulence [157], and the utilization of alternative carbon sources such as lactate, important for host colonization [145, 158]. Together these multiple tiers of rewiring including that involving SOD enzymes help promote *C. albicans* existence within the mammalian host as opposed to the environmental habitats of *S. cerevisiae*.

Figure 3-1: *C. albicans* Cu/Zn SOD1 and Mn SOD3 can stabilize *S. cerevisiae* casein kinase, YCK1. *S. cerevisiae* strains expressing GFP-YCK1 together with the indicated SODs or empty vector (EV) were grown in enriched YP media +4% glucose and whole cell lysates were prepared by glass bead lysis. (A) 100 µg of whole cell lysate protein from cells expressing *S. cerevisiae* SOD1 (Sc SOD1) or *C. albicans* P144L SOD1 under control of the *S. cerevisiae SOD1* promoter (Ca SOD1) were analyzed by immunoblot for GFP and PGK1, a loading control for cytosolic proteins. (B) 100 µg of whole cell lysate protein from strains expressing *S. cerevisiae SOD1* (Sc SOD1), *C. albicans SOD3* (Ca SOD3), *S. cerevisiae* cytosolic *SOD2* (Sc SOD2) all under the *ADH1* promoter were analyzed for GFP and PGK1 by immunoblot (top) and 30 µg of lysate protein was subjected to SOD enzymatic analysis by native gel assay (bottom). *S. cerevisiae* SOD2 is inactive when expressed in the cytosol unless manganese is added to the media; 0.75mM manganese was supplemented to *S. cerevisiae* strains expressing the cytosolic form of SOD2.

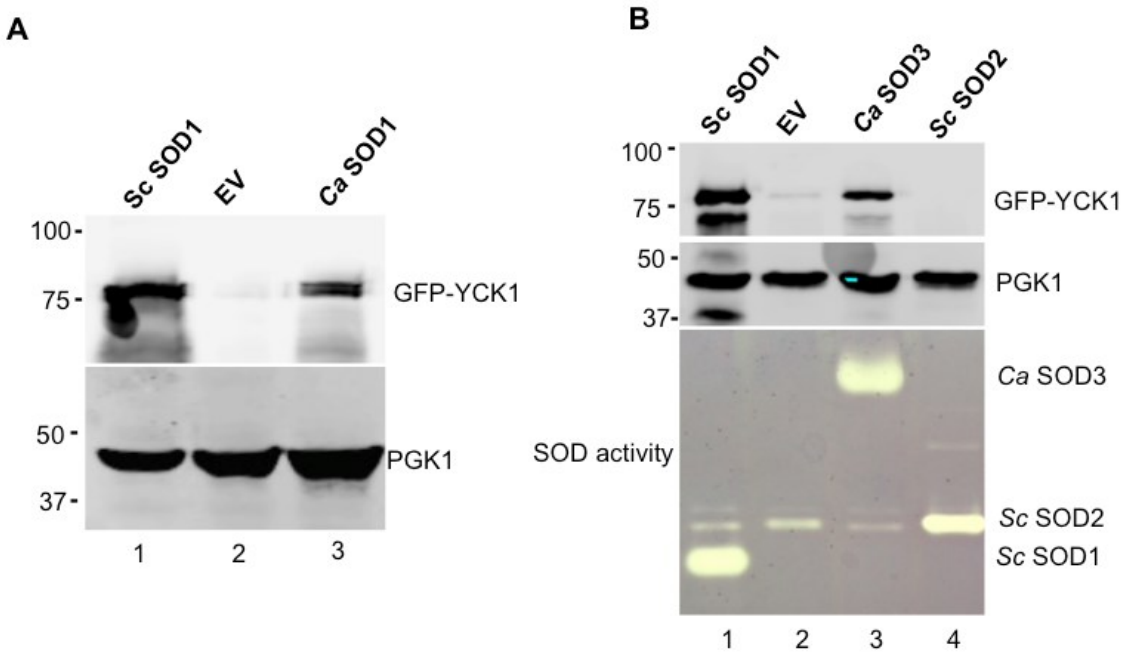

A
Sc SOD1
EV
Ca SOD1
100
75
50
37
GFP-YCK1
PGK1
1
2
3
B
Sc SOD1
EV
Ca SOD3
Sc SOD2
100
75
50
37
GFP-YCK1
PGK1
Ca SOD3
SOD activity
Sc SOD2
Sc SOD1
1
2
3
4

Figure 3-2: *C. albicans* Mn containing SOD3 shares the same requirements as *S. cerevisiae* SOD1 for the stabilization of YCK1.

100 μg of whole cell lysate protein from the indicated strains as described in Fig. 3-1B were probed for GFP and PGK1 by immunoblot. (A) Strains were grown in enriched YP media+4% glucose in atmospheric conditions (20% oxygen) or under nitrogen (0% oxygen). (B) Strains were grown where indicated in enriched YP media supplemented with high (4% glucose) or low (2%) glucose. (C) *S. cerevisiae* strains expressing either full length GFP-YCK1 (top) or GFP fused to the YCK1 C-terminal amino acids 367-538 (bottom) were grown in YP media + 4% glucose.

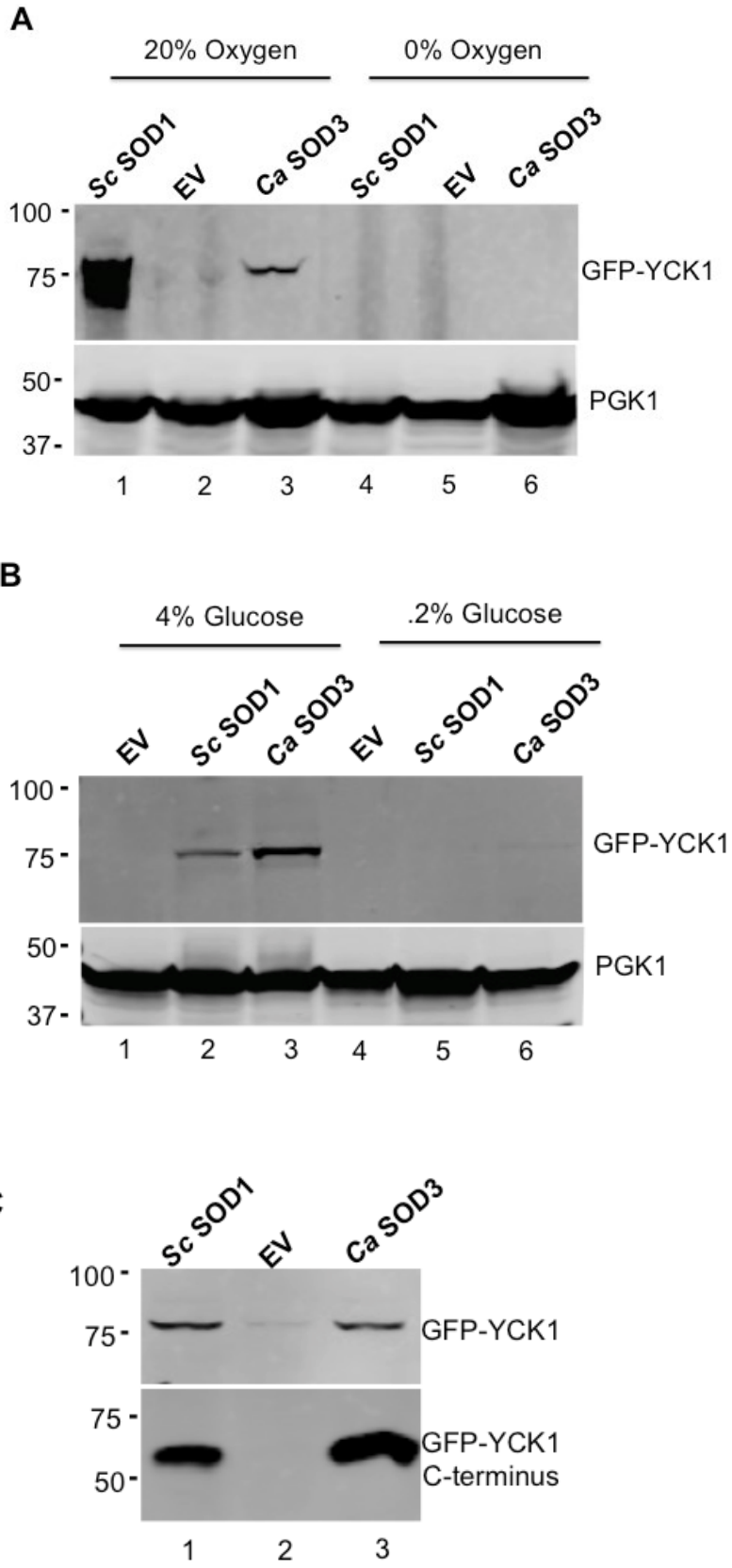

A
20% Oxygen
0% Oxygen
Sc SOD1
EV
Ca SOD3
Sc SOD1
EV
Ca SOD3
100
75
GFP-YCK1
50
37
PGK1
1 2 3 4 5 6
B
4% Glucose
.2% Glucose
EV
Sc SOD1
Ca SOD3
EV
Sc SOD1
Ca SOD3
100
75
GFP-YCK1
50
37
PGK1
1 2 3 4 5 6
C
Sc SOD1
EV
Ca SOD3
100
75
GFP-YCK1
75
50
GFP-YCK1
C-terminus
1 2 3

Figure 3-3: Glucose metabolism in *S. cerevisiae* and *C. albicans*.

Shown is a schematic to illustrate the differences in glucose metabolism between *S. cerevisiae* and *C. albicans*. *S. cerevisiae* (left) is Crabtree- positive where the presence of oxygen and glucose promotes fermentation and genes required for respiration are repressed. Cu/Zn containing SOD1 plays a role in integrating signals from oxygen and glucose to repress respiration and induce glucose utilization through fermentation. *C. albicans* (right) is Crabtree negative, and the presence of oxygen and glucose promote respiration and genes involved in the utilization of glucose by fermentation are repressed. Prior to these studies, a role for cytosolic SOD1 or SOD3 in *C. albicans* glucose metabolism was unknown.

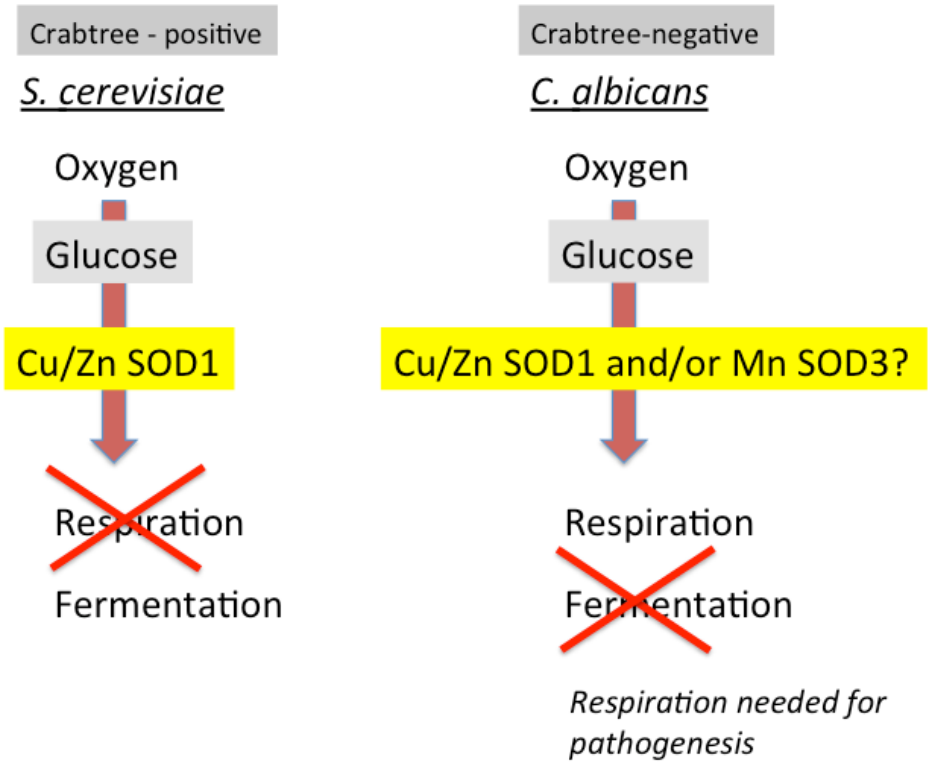


Table 3-1: Expression of glucose transporter genes in *sod1Δ/Δ* and copper starved yeast

Fold change increases in mRNA as measured by RNA-seq in *sod1Δ/Δ* versus WT strains and Cu-starved WT versus Cu-replete WT *C. albicans*

Gene	Cu-replete *sod1Δ/Δ* versus Cu-Replete WT	Cu- Starved WT versus Cu-Replete WT
HGT17	247	28
HGT12	92	45
HGT2	22.3	5.1
HXT5	13.9	15.5
HGT19	12.3	4.6
HGT13	9	2
HGT16	6	1.5
HGT6	4.4	1.6
HGT1	3.9	4.1
HGT4	2.9	2.2
HGT9	2	0
HGT7	1.4	1

Figure 3-4: Effect of *sod1Δ/Δ* and *sod3Δ/Δ* gene mutations on the expression of glucose transporters in *C. albicans*.

The expression of glucose transporter genes *HGT17* (A and B), *HGT2* and *HGT12* (B) was quantified by qRT-PCR in cells grown as described in *Materials and Methods*. Shown are averages of two biological replicates where error bars are standard deviation. (A) Wild-type (CA-IF100) and the isogenic *sod1Δ/Δ* strain were grown in enriched YP+ 2% glucose media. mRNA values are normalized to that obtained with wild type CA-IF100 *C. albicans* cells. (B) Wild-type (CA-IF100) and isogenic *sod1Δ/Δ* and *sod3Δ/Δ* strains were grown in YP+ 2% glucose media in the presence or absence of 800 μM BCS where indicated. mRNA values are normalized to that obtained with wild type CA-IF100 *C. albicans* cells in the absence of BCS.

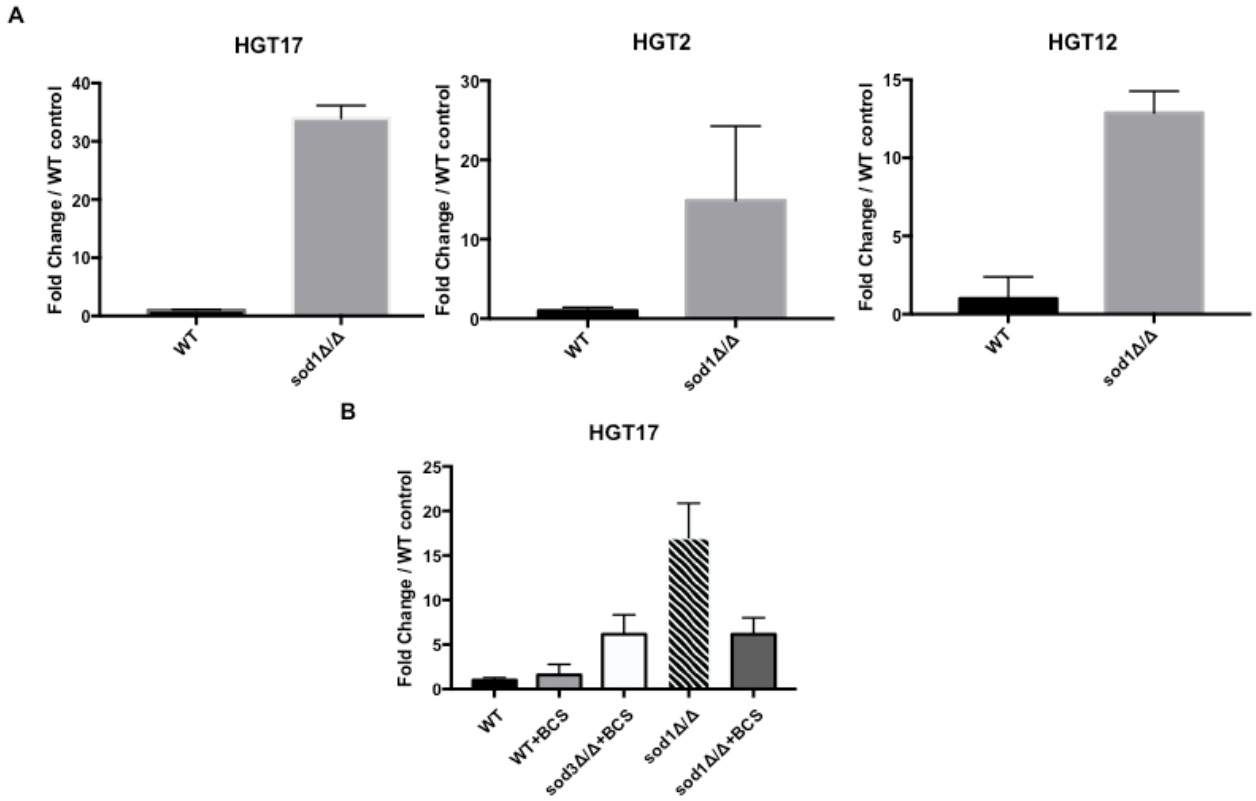


Figure 3-5: SOD1 does not affect glucose consumption in *C. albicans*.

Extracellular glucose was measured as described in *Materials and Methods*. Wild type (CA-IF100) and *sod1Δ/Δ* mutants were grown in YP+ 2% glucose media and fungal consumption of glucose was determined as the level of extracellular glucose remaining during yeast growth measured at an optical density of 600 nm. Data shown represents an average of two biological replicates where error bars are standard deviation.

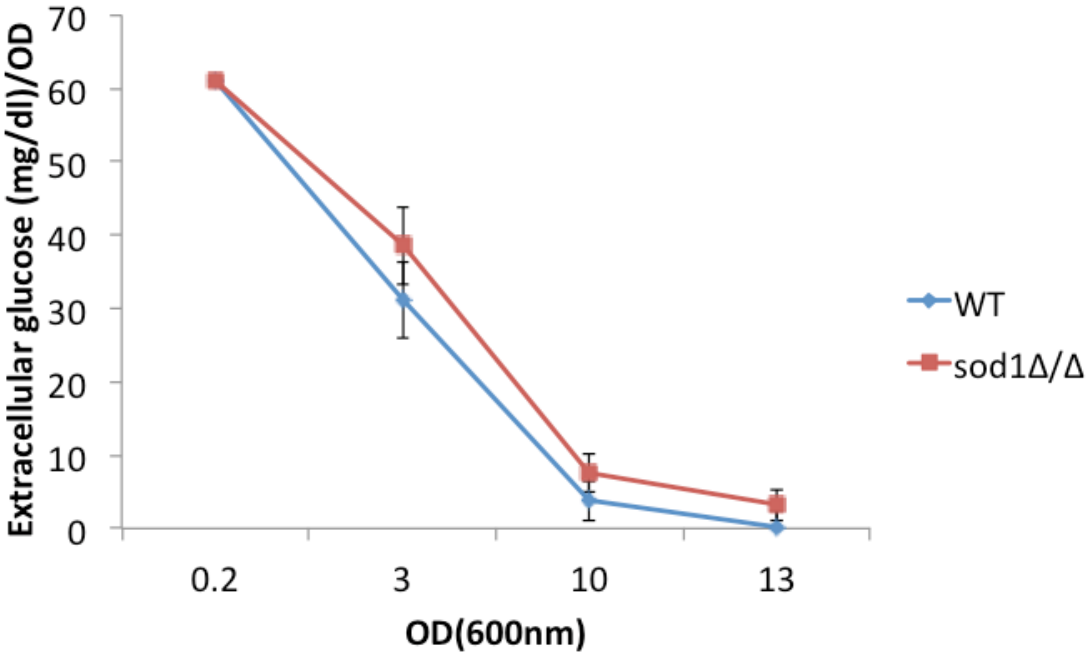


Figure 3-6: Regulation of glucose transporters by the transcriptional repressors RGT1 and MIG1 in *C. albicans*.

(A) Schematic illustrating glucose transporter expression as a function of glucose concentration. When extracellular glucose is virtually absent, glucose transporters are repressed by RGT1. At the opposite extreme, the transcriptional repressor MIG1 represses glucose transporters when glucose is high. At intermediate/low glucose, neither repressor is active and glucose transporter expression is maximal. (B) Effect of *MIG1* and *RGT1* gene deletions on the expression of glucose transporter genes. Wild type (SN250) and isogenic *mig1Δ/Δ* and *rgt1Δ/Δ* mutant strains were grown in either YP+ 2% glucose (H), YP+ .2% glucose (L), or YP + 5% glycerol (G). Expression of *HGT17, HGT2* and *HGT12* mRNA was measured by RT-PCR as described in *Materials and Methods*. PCR products were run on a 2% agarose gel and detected by staining with ethidium bromide. cDNA reactions with reverse transcriptase are indicated as +RT and cDNA reaction without reverse transcriptase are indicated as –RT.

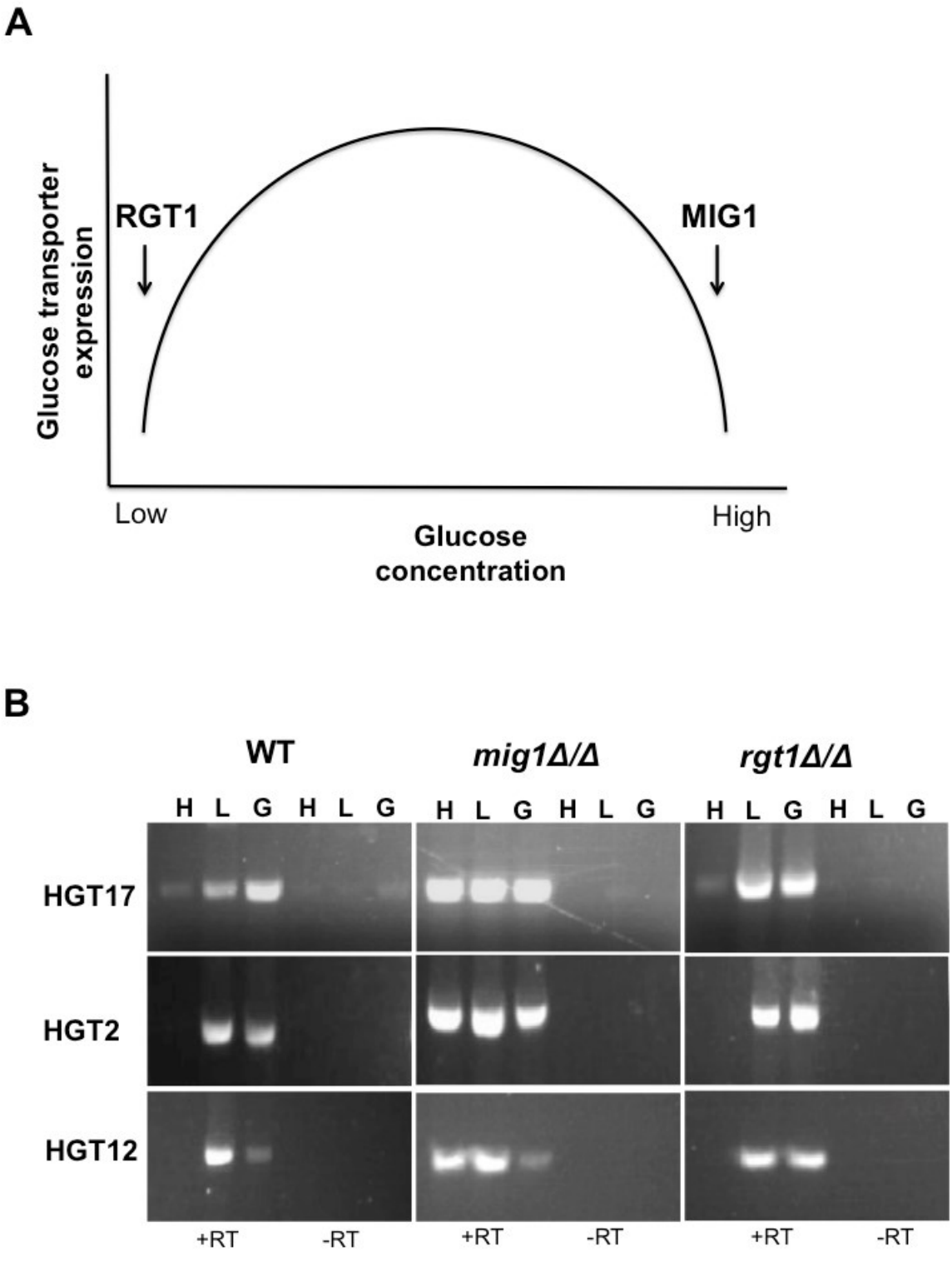

A
Glucose transporter expression
RGT1
MIG1
Low
High
Glucose concentration
B
WT
mig1Δ/Δ
rgt1Δ/Δ
H L G H L G
HGT17
HGT2
HGT12
+RT
-RT

Figure 3-7: Deletion of SOD1 does not affect expression of the RGT1 target, *HGT7*. Wild type (CA-IF100), the isogenic *sod1Δ/Δ* mutant and wild type cells treated with 800 μM BCS were grown in YP+ 2% glucose (H) or YP + 5% glycerol (G). *HGT7* mRNA was measured by RT-time PCR as described in *Materials and Methods*. The PCR product was run on a 2% agarose gel and detected by staining with ethidium bromide.

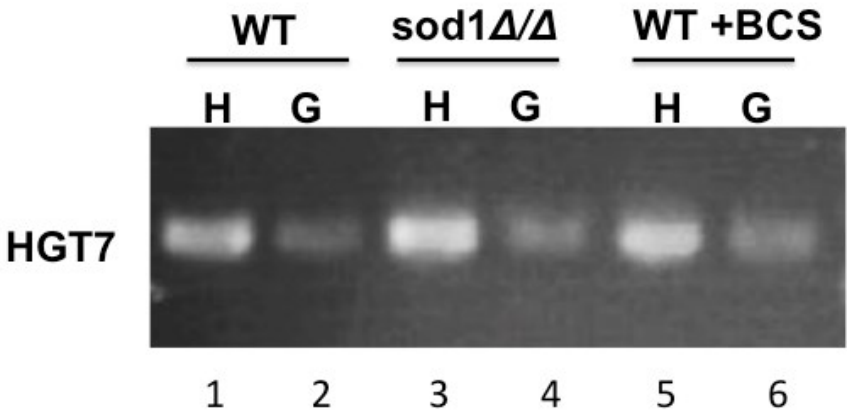
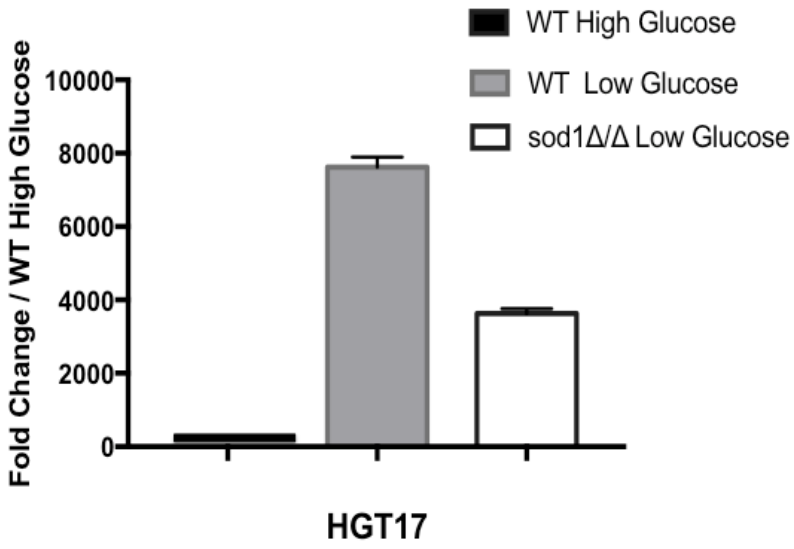


Figure 3-8: SOD1 represses glucose transporters in the same pathways as the transcriptional repressor, MIG1.

(A) Wild type (CA-IF100) and isogenic *sod1Δ/Δ* mutants were grown in YP+ 2% glucose (High) or YP + .2% glucose (Low) and *HGT17* gene expression was monitored by qRT-PCR as described in *Materials and Methods*. Values are normalized to that obtained by wild type CA- IF100 grown in high glucose. Data represents the average of two biological replicates where error bars represent standard deviation. (B) Wild type (SN250) and isogenic *mig1Δ/Δ* mutant strains were grown in YP + 2% glucose and whole cell lysates were prepared by glass bead homogenization. 30μg of lysate protein were immunoblotted for SOD1 (top) and subjected to SOD enzymatic assay analysis by native gel assay (bottom).

A


WT High Glucose
WT Low Glucose
sod1Δ/Δ Low Glucose
Fold Change / WT High Glucose
10000
8000
6000
4000
2000
0
HGT17

B

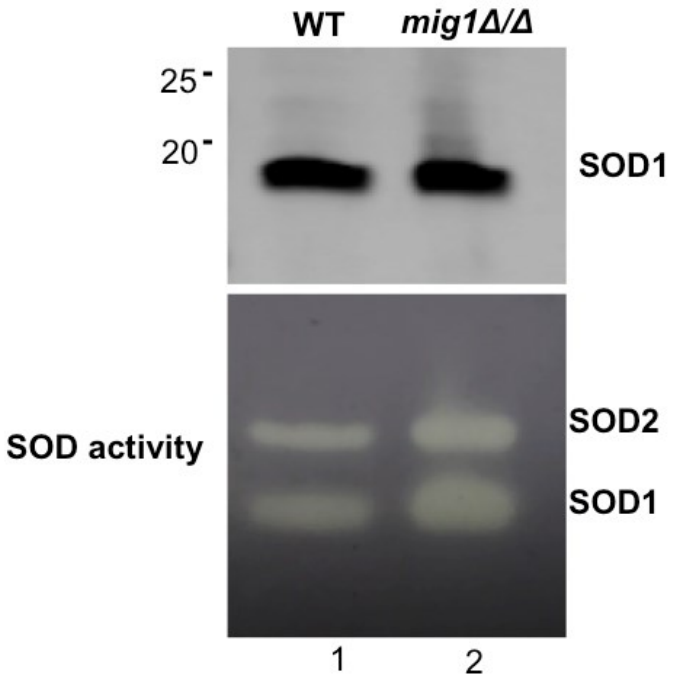
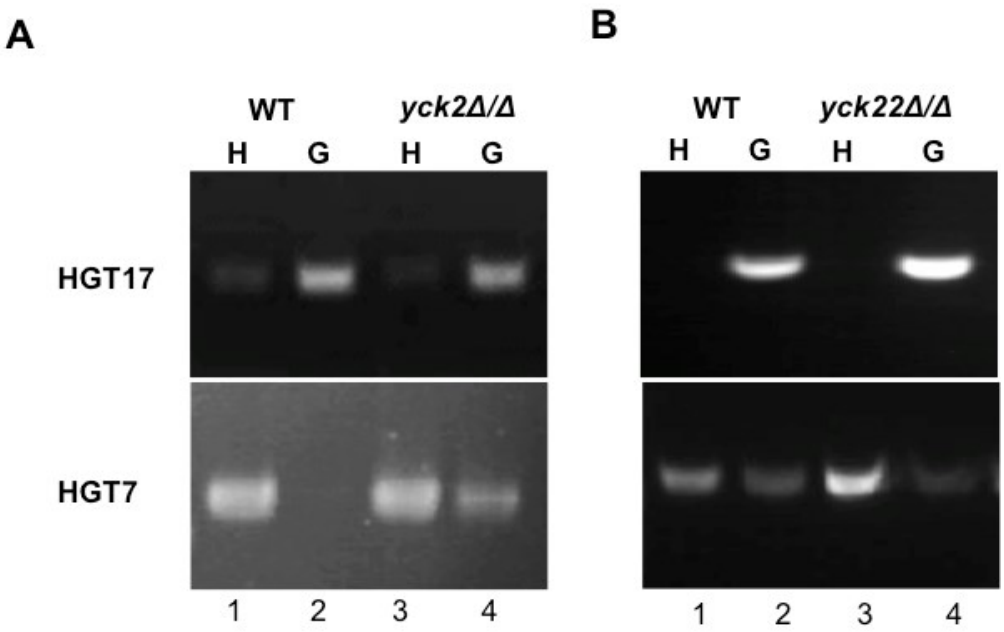

WT
mig1Δ/Δ
25
20
SOD1
SOD activity
SOD2
SOD1
1
2

Figure 3-9: *C. albicans* casein kinases, YCK2 and YCK22 do not repress the same glucose transporters as *C. albicans* cytosolic SODs.

C. albicans strains were grown in YP+ 2% glucose (H) or YP+ 5% glycerol (G) and *HGT17* and *HGT7* mRNA expression was monitored by RT-PCR as described in *Materials and Methods*. (A) *HGT17* and *HGT7* mRNA expression of wild type (BWP17) and isogenic strain *yck2Δ/Δ*. B) *HGT17* and *HGT7* mRNA expression in wild type (DAY286) and *yck2Δ/Δ* isogenic strain.



Future Directions

The research in this thesis focused on Cu/Zn SOD1 and Mn SOD3 in mitochondrial oxidative stress and cellular signaling in the opportunistic fungal pathogen, *C. albicans*. While these studies have elucidated important information on the role of these SODs, many questions remain unanswered, which provides an opportunity for new investigations in the future.

Exploring the role of the alternative oxidase in fungal pathogenesis

In Chapter 2, we describe a role for the alternative oxidase in protecting the mitochondria against respiratory superoxide during copper deprivation. Recent work from the Culotta lab shows that *C. albicans* experiences copper starvation during the course of disseminated candidiasis [66], and it's possible that the utilization of alternative oxidase respiration is required for survival inside the mammalian host. There are two genes in *C. albicans* that encode for the alternative oxidase, *AOX1* and *AOX2,* however only *AOX2* is induced during copper starvation. To identify whether the alternative oxidase is required for pathogenesis *aox1Δ/Δ* and *aox2Δ/Δ* mutants can be generated using the SAT-Flipper protocol currently used by the Culotta lab to generate *C. albicans* mutants. Using a model of disseminated candidiasis, mice will be injected with wild type, *aox1Δ/Δ* and *aox2Δ/Δ* yeast through tail-vein injection and survival of mice will be monitored over time.

Understanding copper sparing and prioritization in C. albicans

Work in this thesis provided evidence that the down regulation of Cu/Zn SOD1 spares copper to maintain cytochrome c oxidase respiration. *C. albicans* expresses a number of copper containing proteins in addition to Cu/Zn SOD1 and cytochrome c oxidase, including a family of multicopper ferroxidases required for iron acquisition. Does repression of SOD1 spare copper for ferroxidase activity and is respiration through cytochrome c oxidase always the first priority? This question can be addressed using the *C. albicans* strain engineered to constitutively express SOD1, described in Chapter 2. To determine whether SOD1 spares copper for iron acquisition, ferroxidase activity can be measured using a native gel activity assay [159] during copper starvation when SOD1 is repressible and when it is constitutively expressed as in Fig. 2-7 of Chapter 2. To identify the order in which copper proteins are prioritized during copper starvation *C. albicans* wild type cells will be starved for copper and cytochrome c oxidase respiration and ferroxidase activity will be monitored over time. If cytochrome c oxidase is prioritized over ferroxidase activity we would expect to see a decrease in ferroxidase activity while respiration through cytochrome c oxidase is maintained.

Possible roles of C. albicans SOD in metal homeostasis and phosphate transport as revealed by RNA-seq

RNA-seq can be a very powerful tool for identifying new pathways and new gene function. Using RNA-seq, we obtained the first clues that in the native *C. albicans* host SOD1 and SOD3 are involved in a glucose- signaling pathway that leads to the repression of glucose transporter expression (Chapter 3). But are there other

genes and pathways that are regulated by SOD1 and SOD3 in *C. albicans?* Using the same transcriptome data set described in Chapter 3 for SODs and glucose control we sought to identify additional genes and pathways that are regulated by the cytosolic SODs in *C. albicans*.

HAP43 target genes: Through further analysis of this RNA-seq data set we observed a clear repression of HAP43 regulated genes in *sod1Δ/Δ* mutants compared to wild type (FD-Table 1). HAP43 is involved in the cellular response to low iron, acting as a transcriptional repressor to repress genes encoding iron-dependent proteins and genes involved in iron-sulfur cluster biogenesis [160, 161]. The repression of HAP43 regulated genes in *sod1Δ/Δ* suggests that these cells may be experiencing iron starvation and that SOD1 could be involved in iron homeostasis. We also analyzed these HAP43 target genes in copper starved cells, conditions where SOD1 is repressed and SOD3 is induced. As seen in FD- Table 1, we observed no repression of HAP43 regulated genes in copper starved yeast; therefore it appears that Mn SOD3 is substituting for Cu/Zn SOD1 in this role for regulating HAP43. Both forms of cytosolic SODs appear to contribute to iron homeostasis involving HAP43 repression and the mechanism can be probed in future directions (see below).

Ferric reductases: a response to copper starvation? C. albicans cells lacking SOD1 exhibit an induction of genes encoding ferric reductases FRE7 and FRE30 (FD-Table 1). *FRE7* is a known target of the copper sensing transcription factor MAC1 and is highly expressed during copper starvation [130]. Consistent with this known regulation of *FRE7* the gene is strongly induced by copper starvation in our RNA-seq

analyses (FD-Table 1). We also observed a drastic increase in *FRE30* expression in copper starved wild-type cells indicating that this ferric reductase is regulated by copper deprivation and may also be a target of MAC1. The *sod1Δ/Δ* mutants appear to mimic copper starvation by inducing these ferric reductase genes and this can be explored in future directions (see below).

PHO84 – a response to phosphate starvation or metal homeostasis? PHO84 is a high affinity phosphate transporter and through RNA-seq analysis we observe a repression of this transporter in both *sod1Δ/Δ* mutants and copper starved yeast compared to the copper replete wild type control (FD-Table 1). Work in *S. cerevisiae* shows a connection between phosphate accumulation by PHO84 and metal ion homeostasis [162]. When intracellular phosphate levels are high by uncontrolled expression of PHO84, yeast are more susceptible to toxicity from metals such as manganese, copper and zinc and perturbations in phosphate control leads to an iron starvation response [163]. Additionally, PHO84 plays a role in metal homeostasis by acting as a low affinity transporter of metal phosphate complexes [164]. It is not clear whether the repression of PHO84 is linked in any way to the *sod1Δ/Δ* effects on iron and copper starvation as mentioned above, but this can be investigated in future studies. Since P*HO84* expression is similar in both *sod1Δ/Δ* and copper starved WT cells it does not appear that Mn SOD3 can substitute for Cu/Zn SOD1 in the regulation of this gene.

Future directions on the RNA-seq dataset

To further investigate the findings from the RNA-seq analysis, the expression of candidate genes will be confirmed using qRT-PCR in wild type, *sod1Δ/Δ* mutants

and copper starved wild type yeast. As it appears that metal homeostasis is disrupted, experiments can be performed to determine whether *sod1Δ/Δ* mutants exhibit symptoms of defects in iron and copper homeostasis. Changes in intracellular metal content can be detected by ICP-MS or atomic absorption spectroscopy (AAS). The accumulation of inorganic and polyphosphate can be measured by the molybdate and acid treatment assays commonly used in the Culotta lab for phosphate [162]. Mutant yeast can additionally be tested for metal toxicity (Mn, Zn, Cu) as well as the activity of Fe and Cu containing proteins.

Elucidating the target of SOD1 and SOD3 in the MIG1 glucose signaling pathway

In *C. albicans* we show that cytosolic SOD1 and SOD3 repress glucose transporters in the MIG1 glucose-signaling pathway. In contrast to work in *S. cerevisiae*, the casein kinase gamma homologs in *C. albicans* YCK2 and YCK22 are not involved in the same glucose-signaling pathways as the cytosolic SODs. Therefore the question remains, what is the target of SOD1 and SOD3 that mediates the regulation of glucose transporters? As mentioned in Chapter 3 (discussion), there are two kinases, HXK1 and SNF1, in the glucose repression pathway that may be targets of SOD regulation[105]. It is also possible that the transcription factor MIG1, responsible for repressing glucose transporters may be a target of SOD1. To test whether HXK1, SNF1 or MIG1 require SOD1 for stabilization, GFP could be fused to HXK1, SNF1 and MIG1 and these fusion proteins can be expressed in wild type versus *sod1Δ/Δ* cells under varying glucose conditions. The expression and stabilization of these proteins can subsequently be monitored by immunoblot using

an anti-GFP antibody as described in Chapter 3. Once the target protein is identified, truncations of this protein can be fused to GFP to determine which portion of the protein is required for SOD mediated stabilization.

Future Directions Table 1: Representative metal metabolism genes induced and repressed in *sod1Δ/Δ* and copper starved yeast.

RPKM values as measured by RNA-seq in Cu-Replete WT, *sod1Δ/Δ*, and Cu-Starved WT *C. albicans*.

Gene	Description	Copper Replete WT	*sod1Δ/Δ*	Copper starved WT
MAK16	Uncharacterized; Hap43 induced	10.06	1.00	10.15
DIM1	Putative 18S, rRNA dimethylase; Hap43 induced	2.53	0.00	3.33
NOC4	Putative nucleolar protein; Hap43 induced	9.71	0.57	9.19
NAN1	Putative snoRNP; Hap43 induced	12.00	1.00	6.56
SMM1	Putative dihydrouridine synthase; Hap43 induced	1.54	0.13	0.83
UTP18	Putative U3 snoRNA-associated protein; Hap43 induced	15.29	1.38	13.30
PHO84	Predicted phosphate transporter	830.05	233.57	115.02
FRE7	Ferric reductase	8.80	40.56	676.78
FRE30	Predicted Ferric reductase	4.04	22.62	401.22

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EDUCATION

Johns Hopkins Bloomberg School of Public Health — Baltimore, MD
PhD candidate — 2011 – Present
Department of Biochemistry and Molecular Biology

The College of Saint Elizabeth — Morristown, NJ
Bachelor of Science — 2006 – 2010
Major: Biochemistry Minor: Mathematics

LABORATORY EXPERIENCE

Johns Hopkins Bloomberg School of Public Health — Baltimore, MD
PhD Candidate, Department of Biochemistry and Molecular Biology — August 2011 – Present
Advisor: Valeria C. Culotta, PhD
Project: Studying the role of the two cytosolic superoxide dismutases in cellular signaling and mitochondrial oxidative stress protection during copper starvation.

DPT Laboratories — Lakewood, NJ
Laboratory Technician — May 2010 – June 2011
Supervisor: Susan Tierney, MS
Tasks: Quality control testing of finished pharmaceutical products for release and stability

Syracuse University — Syracuse, NY
Undergraduate Researcher, Department of Chemistry — June 2009 – August 2009
Advisor: Philip N Borer, PhD
Project: Progress towards a nucleic acid switch for the protein Human Alpha Thrombin.

RESEARCH GRANTS

Ruth L. Kirschstein National Service Research Award (NRSA) — National Institutes of Health
Predoctoral fellowship award to promote Diversity in Health Related — July 2015 – Present
Research 1F31GM113637-01- $62,794.00

FELLOWSHIPS AND AWARDS

Excellence in Diversity Symposium graduate student travel award recipient — September 2016
Johns Hopkins Diversity Postdoctoral Alliance Committee
Baltimore, MD

J. Howard Brown Award for Outstanding Graduate Student — June 2016
The Maryland Branch of the American Society for Microbiology

Baltimore, MD

Sharon Krag Award for Outstanding Leadership — January 2016
Johns Hopkins Bloomberg School of Public Health
Baltimore, MD

Elsa Orent Keiles Fellowship in Biochemistry — March 2015
Johns Hopkins Bloomberg School of Public Health
Baltimore, MD

Jorge Crosa Poster Award for Graduate Students — July 2014
9th International Biometals Symposium
Durham, NC

2nd Place Poster Award — May 2010
NJ-ACS Research Conference
Morristown, NJ

NSF- Research Experience for Undergraduates Fellowship — June 2009
Syracuse University
Syracuse, NY

CRC Chemistry Award — May 2007
The College of Saint Elizabeth
Morristown, NJ

PUBLICATIONS

Broxton CN, Culotta VC (2016) An Adaptation to Low Copper in *Candida albicans* Involving SOD Enzymes and the Alternative Oxidase. PLoS ONE 11(12): e0168400. doi:10.1371/journal.pone.0168400

Broxton CN, Culotta VC (2016) SOD Enzymes and Microbial Pathogens: Surviving the Oxidative Storm of Infection. PLoS Pathog 12(1): e1005295. doi:10.1371/journal.ppat.1005295

ORAL PRESENTATIONS

Chynna N. Broxton, Valeria C. Culotta. Divergent Roles of the Dual Cytosolic SODs in Candida albicans. Presented at the 1st Annual Excellence in Diversity Symposium, Baltimore, MD. 2016

Chynna N. Broxton, Valeria C. Culotta. Divergent Roles of the Dual Cytosolic SODs of Candida albicans. Presented at the Maryland Branch of the American Society for Microbiology, Baltimore, MD. 2016.

Chynna N. Broxton, Valeria C. Culotta. The Two Cytosolic SODs of the pathogenic yeast, Candida albicans. Presented at the JHSPH Biochemistry and Molecular Biology Retreat. Sheppard Pratt Conference Center, Towson, MD. 2015

Chynna N. Broxton, James E. Crill II, Philip N. Borer. Progress Towards a Three- way Nucleic Acid Switch for the Protein Human Alpha Thrombin. NJ-ACS Research Conference, Drew University, Madison NJ, 2010.

POSTER PRESENTATIONS

Chynna N. Broxton, Valeria C. Culotta. Divergent Roles of the Dual Cytosolic SODs of *Candida albicans*. Presented at the JHSPH Biochemistry and Molecular Biology Retreat. Sheppard Pratt Conference Center, Towson, MD. 2016

Chynna N. Broxton, Valeria C. Culotta. Divergent Roles of the Dual Cytosolic SODs of *Candida albicans*. Presented at the American Society for Microbiology conference on Candida and Candidiasis, Seattle, Washington. 2016

Chynna N. Broxton, Vincent M. Bruno, Valeria C. Culotta. The Dual Metal Containing Cytosolic SODs of the Pathogenic Yeast *Candida albicans.* Presented at Gordon Research Conference: Cell Biology of Metals, Mount Snow, VT. 2015.

Chynna N. Broxton, Vincent M. Bruno, Valeria C. Culotta. The Dual Metal Containing Cytosolic SODS of the Pathogenic Yeast *Candida albicans*. Presented at the JHSPH Biochemistry and Molecular Biology Retreat. Sheppard Pratt Conference Center, Towson, MD. 2014

Chynna N. Broxton, Vincent M. Bruno, Valeria C. Culotta. The Dual Metal Containing Cytosolic SODS of the Pathogenic Yeast *Candida albicans*. Presented at the 9th International Biometals Symposium, Duke University, Durham, NC. 2014

PROFESSIONAL AND STUDENT ASSOCIATIONS

American Society for Microbiology
American Association for the Advancement of Science

TEACHING AND MENTORING

Rotation Student Mentor, JHSPH — November 2015
Johns Hopkins Bloomberg School of Public Health
Department of Biochemistry and Molecular Biology

Teaching Assistant, JHSPH — Winter term 2016
Department of Biochemistry and Molecular Biology
Class: Genomics for Public Health

LABORATORY TECHNIQUES

Immunoblotting, quantitative - real time PCR, molecular cloning, subcellular fractionation, Atomic Absorption Spectroscopy, fluorescence spectroscopy, fluorescence microscopy, SDS-PAGE, RNA/DNA isolation, oxygen consumption analysis, RNA-Seq, mouse dissection, UV-Vis spectroscopy, organic enzyme extraction, solid phase DNA synthesis, and HPLC chromatography.

SCIENTIFIC AND DEPARTMENTAL SERVICE

Co-Coordinator, Biochemistry and Cancer Journal Club — 2015 – 2016
Johns Hopkins Bloomberg School of Public Health
Department of Biochemistry and Molecular Biology

PhD Professional Development Group — Summer Term 2015
Inaugural Member
Johns Hopkins Bloomberg School of Public Health
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PhD education reform focus group
Student Representative — 2016 – Present
Johns Hopkins Bloomberg School of Public Health

Diversity and Inclusion Committee — May 2016 – Present
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COMMUNITY SERVICE AND OUTREACH

Lead Mentor -Thread Mentoring Program — 2014 – Present
Baltimore, MD

Counselor, Jim Holland Summer Science Research Program — July 2016
Indiana University, Bloomington IN

Secretary, Black Graduate Student Association — 2015 – 2016
Johns Hopkins Bloomberg School of Public Health, Baltimore MD