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The CCAAT-Binding Factor Dependent Regulation of the Oxidative Stress Response in Candida albicans

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The CCAAT-Binding Factor Dependent Regulation of the Oxidative Stress Response in *Candida albicans*

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Abstract

The success of *Candida albicans* as an opportunistic human pathogen has been attributed to several factors, including the ability to survive in limiting iron environments and the ability to evade the respiratory burst of human macrophages and neutrophils. The goal of this research is to elucidate the role of the CCAAT-binding factor in the oxidative stress response of *Candida albicans*. Prior whole genome microarray studies performed in our lab compared the gene expression of a wild type *Candida albicans* strain versus a *hap5Δ* strain under iron-limiting growth conditions. Among the differentially regulated genes, *CTA1*, encoding catalase, had a four-fold higher level of expression in the *hap5Δ* homozygous mutant under iron limitation. The sensitivity of the *hap5Δ* homozygote to oxidative damage was also assessed by growing the strains in iron replete or iron-limiting conditions and exposing them to various concentrations of hydrogen peroxide. The data obtained from these studies supported our hypothesis that the CCAAT-binding factor was involved in the differential regulation of oxidative stress genes based on iron availability. To further explore the role of the CCAAT-binding factor in the regulation of *CTA1*, wild type and *hap5Δ* homozygote were grown in iron replete and iron-limiting conditions, and the total RNA was isolated and Northern blots performed. Our results indicated a CCAAT-binding factor-dependent regulation of *CTA1* under iron limitation. The oxidative stress response in most organisms, including *Candida albicans*, is due to the concerted actions of many gene products. Therefore, we examined the mRNA levels of genes such as superoxide dismutase, thioredoxin, and glutaredoxin, which are known to confer resistance to oxidative stress. Our data indicates that the CCAAT-binding factor in *Candida albicans* regulates the mRNA expression level of different genes involved in the oxidative stress response. We next performed Northern blots, catalase enzymatic assays and peroxide sensitivity assays to dissect

the role of each of the Hap3 and Hap4 subunits towards the iron-dependent regulation of *CTA1*. Together, our data strongly suggests that the CCAAT-Binding factor not only is essential in the regulation of iron homeostasis, but also regulates the Oxidative Stress Response in *Candida albicans*.

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Dedication

I would like to dedicate this document to my mother Mrs. Kalyani Chakravarti. I couldn't have done this without you Ma.

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1. Literature Overview

The fungal clade harbors more than five million species of which only a small number are capable of causing devastating diseases that are recognized as serious threats to human health and the food security of the world (16,33). The fungal pathogens are characterized by extraordinary genetic flexibility that aids rapid evolution and differing degrees of adaptation to the host or the environment (20). Adaptive evolution is mainly a result of fungal genome plasticity which is not only due to sexual reproduction but also due to parasexuality (113), aneuploidy (127), transposons, telomere instability (136) and horizontal gene transfer (88,116). Besides genome plasticity, fungi also exhibit morphogenetic plasticity thus enabling them to invade and colonize tissues as hyphae (33) and then form spores, fruiting bodies or unicellular yeast cells that can aid rapid dispersal (139). This dissertation focuses on the opportunistic fungal pathogen, *Candida albicans* and this review will specifically outline its pathogenicity as it is currently understood. It briefly describes some of the key virulence factors known with a special emphasis on the molecular mechanisms underlying the regulation of the oxidative stress response, which the pathogen exhibits as a defense mechanism against the host immune response. Free iron is also known to catalyze the formation of reactive oxygen species leading to oxidative stress via the Fenton's reaction (111). Thus, iron poses a dilemma, as it is required as a cofactor for antioxidant enzymes like catalase which helps *Candida albicans* survive oxidative stress, but in toxic concentrations, can in turn generate reactive oxygen species leading to oxidative stress. Hence, owing to the importance of iron towards maintaining the oxidative status of the cell, this dissertation focuses on the iron dependent regulation of oxidative stress response in *Candida albicans*.

***Candida albicans* and pathogenesis**

Candida albicans is an opportunistic pathogen which normally resides in the human gut and other mucosal surfaces as a commensal in more than 70% of healthy individuals, but is capable of causing deep rooted systemic infections in immunocompromised patients (120). Immunocompromised patients includes but is not limited to individuals suffering from AIDS, neutropenia, post transplantation infections, premature babies and cancer patients. In addition, patients that have indwelling catheters, invasive surgery, extensive burns, broad-spectrum antibiotic therapy, and mechanical ventilation are more susceptible to *Candida* infection than normal healthy individuals (9,37,126) . Suppression of the host immune system provides an opportunity for *Candida albicans* to invade and colonize host tissues, and given many of these patients are already ill, these fungal infections increase the overall duration of hospital stay thus increasing the cost of medical care significantly.

In United States hospitals, *Candida* species are the fourth most common cause of hospital acquired bloodstream infection (102). Between 1990 and 1999, *Candida* species accounted for 5-10% of all bloodstream infection (96). A higher mortality rate is associated with *Candida* species bloodstream infections when compared with other nonfungal pathogens (112). The mortality attributed to candidemia is estimated to be greater than 30% (72). This high attributable mortality is most often due to the late detection of the organism during the course of the disease, higher resistance to antifungal drugs like certain azoles, or host toxicity caused by drugs like amphotericin B often necessitating the withdrawal of the drug (26).

Thus, owing to the high morbidity and mortality associated with this fungal pathogen, a lot of research has been exclusively directed to understand the mechanism of its pathogenesis over the last few decades. Its virulence appears to be multifactorial and several factors has been

linked to its disease causing ability in the host. For example, its ability to exist as a dimorphic fungus that responds to specific environmental cues by altering its morphology between the unicellular yeast form (blastospores) and filamentous forms (hyphae and pseudohyphae) during infection has been implicated for its virulence (139). The filamentous forms are not only required for invasion and colonization of the tissues, but also for evasion of host defense mechanism. The unicellular yeast form, however, helps in dissemination of the pathogen through the blood stream during pathogenesis (1,118).

The white-opaque colony switching is another type of morphological plasticity exhibited by this pathogen. Although it is an epigenetic phenomena, environmental cues can significantly alter the frequency of switching (131). Its key role has been implicated in the mating cycle of the pathogen as only the opaque colonies are capable of sexual reproduction (99). It is predicted that the white-opaque switching is tuned to different host micro niches as elevated levels of carbon dioxide and anaerobic conditions trigger the formation of opaque colonies (62,84). Interestingly, the opaque cells are significantly less susceptible to phagocytosis by macrophages than white cells *in vitro* (84). *Candida albicans* can also produce thick walled, asexual resting spores called chlamydospores (115).

Candida albicans can also form dense layers of highly adherent cells on implanted medical devices such as intravenous therapy, catheters, and prosthetics whose properties are distinct from free floating cells. These layers of cells are called biofilms and are inherently resistant to antimicrobial drugs and host defense systems (29,53). In some cases, they can also act as a reservoir for systemic infections. Biofilms consist of yeast cells, pseudohyphae and hyphal cells. Initially the yeast cells adhere to the surface and eventually some of them undergo morphogenetic transition to form hyphae and pseudohyphae. Eventually a layer of carbohydrate

and proteins are secreted around them so as to encase the cells within an extracellular matrix. In order to clear the *Candida albicans* biofilm infections, it is often recommended to remove implanted medical devices (9, 15).

Thus the ability of this fungal pathogen to demonstrate multiple morphological forms is essential for its virulence in the host. Indeed *Candida albicans* strains lacking the *CPH1* and *EFG1* genes produce no hyphae and are significantly less virulent in mice model of infection (85). The hyper filamentous Tup1 mutant also shows less virulence, thus emphasizing the importance of yeast cells for pathogenesis in this organism (83).

Apart from the morphogenetic switch, adhesins have also been implicated for virulence in this pathogen. Adhesins are biomolecules that promote the adherence of *Candida albicans* to host cells or host cell ligands. A number of genes encoding for host recognition proteins have been identified. The agglutinin-like sequence genes, *ALS1* and *ALS5* appear to provide adhesive properties to *Candida albicans*. Highlighting the importance of these factors for *Candida albicans* pathogenesis, Als1 has been found to be essential for virulence in hematogenously disseminated murine model of infection. Similarly, the hyphal wall protein 1 (Hwp1) is essential for the adhesion of *Candida albicans* hyphae to human epithelial cells. More importantly, the *HWP1*-deficient strains caused reduced mortality in mice, germinated less readily in kidneys of mice and caused less endothelial cell damage (134). Integrin like proteins (Int1) also seem to play a role in *Candida albicans* adherence and filamentation. Mntp1 is a mannosyltransferase protein and is used for host-cell recognition. *Candida albicans* strains lacking the gene encoding for this transferase are avirulent (20). Thus, host recognition and adherence to host cells/ligands are indispensable for the pathogenesis of *Candida albicans*.

A number of enzymes that contribute to invasiveness of *Candida albicans* have also been associated with virulence. The Secreted Aspartyl Proteinases (SAPs) and Phospholipases (PL) are two families of such enzymes. Of the four PLs identified, only *PLB1* has been shown to be involved in virulence (79). Plb1 has been found in hyphal tips during tissue invasion (44,103). SAP family comprises of nine proteins and are not limited to *Candida albicans* alone but has been detected in *Candida tropicalis* and *Candida parasilopsis* too (147,159). Sap1-6 appears to be essential for invasive disease in murine model of infection (63,122). *SAP4* and *5* is thought to be involved in early invasion (135), *SAP8* in extensive penetration and *SAP6* for extensive hyphal growth in an *in vitro* mouse epidermis model system (63,152). In vaginitis model, *SAP1* and *SAP2* are essential for disease development although all the *SAPs* have not been evaluated yet (10).

Thus, *Candida albicans* undergo extensive genetic reprogramming to switch its lifestyle from a commensal in healthy individuals to a pathogen in immunocompromised patients. This switch however triggers a host immune response comprising of recruitment of macrophages, neutrophils and other phagocytic cells to the site of infection. The phagocytic cells generate what is called an oxidative burst to counteract the invading pathogen, the mechanism of which is discussed below.

Host Immune Response

Phagocytic cells like the macrophages and neutrophils produce a battery of toxic chemicals like superoxide anions, hydrogen peroxide, hydroxyl radicals, etc., collectively called the Reactive Oxygen Species (ROS), after exposure to a pathogen. ROS such as superoxide anions are also generated by the partial reduction of oxygen during aerobic respiration in the

mitochondrion. Following cytokine activation, phagocytic cells assemble their NADPH oxidase leading to the generation of superoxide anions which can be further processed to hydrogen peroxide by superoxide dismutases, or to hydroxyl radicals and hydroxyl anions via the Haber-Weiss reaction. Enzymes like myeloperoxidases can also convert the hydrogen peroxide to hypochlorous acid (11,15,104).

In addition to NADPH oxidase, phagocytic cells also activate nitric oxide synthase in response to pathogen infection. This nitric oxide synthase can in turn trigger the formation of Reactive Nitrogen Specie (RNS) including nitric oxide and nitrite radicals. Nitric oxide can further react with superoxide to form peroxynitrite, and nitrite can generate nitryl chloride after reacting with hypochlorous acid (15). Therefore, phagocytic cells generate a deadly cocktail of ROS and RNS to neutralize the invading pathogen although the role of RNS is less significant than ROS in eliminating *Candida* infections (60).

To evade these defense mechanisms, the pathogen has to invariably trigger specific stress responses especially Oxidative Stress Response (OSR). Since much knowledge of the stress responses in *Candida albicans* is known from research in other fungal species including benign model yeasts like *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, understanding OSR in general, in these species, is pivotal for understanding the oxidative stress response in *Candida albicans.*

Oxidative Stress Response in *Saccharomyces cerevisiae*

As mentioned above, some of the OSR mechanisms known to us, are conserved among different fungal species including the baker's yeast, *Saccharomyces cerevisiae*. However, as I will highlight later in this review, some transcription factors found in the baker's yeast have

evolved extensively to assume novel roles towards the OSR in *Candida albicans*. Therefore, although understanding the OSR in *Saccharomyces cerevisiae* is an important prerequisite for an in-depth knowledge of the OSR in *Candida albicans*, but, we have to exercise caution while extrapolating the OSR data from other fungal species to OSR in *Candida albicans.*

The OSR in *Saccharomyces cerevisiae*, among other things, comprises of several evolutionary conserved transcription factors. However, many of these proteins perform unique functions in *Candida albicans*, not described previously for *Saccharomyces cerevisiae* as mentioned later in this review.

bZip transcription factors

The Yap1 protein is a bZip transcription factor which when overexpressed in *Saccharomyces cerevisiae* renders yeast resistant to a wide variety of stresses including oxidative stress. The *Saccharomyces cerevisiae* Yap1p orthologue, Cap1, has been discovered in *Candida albicans* and so far research has found considerable overlap between the mechanisms of action of these two transcription factors. Thus, analysis of Yap1 function will provide valuable insights towards understanding the role of Cap1 in *Candida albicans*.

Saccharomyces cerevisiae strains lacking Yap1 have less activity levels of several antioxidant enzymes like superoxide dismutases, glucose-6-phosphate dehydrogenase, glutaredoxins etc (125). Peroxide stress resistance was also severely impaired in a *yap1* mutant strain suggesting that this transcription factor regulates genes involved in peroxide stress (137). The expression of the *YAP1* gene in high copy number has also led to significant increases in total glutathione levels and other antioxidant enzymes (67).

Apart from peroxide stress, Yap1 has also been shown to be involved in diamide stress (74). The Yap1p appears to have a diamide-responsive region at the carboxy terminus of the protein while the response to peroxide stress mainly involves the cysteine rich domain along with

a region near the amino terminal end of the protein (150). A number of promoters of the genes encoding for antioxidant proteins in *Saccharomyces cerevisiae* like *SOD1, GSH1,* and *TRX2* have putative Yap1-binding sites (73,154). However, the Yap1 can not only bind to its own binding sites but also can bind to STress Responsive Elements (STREs) on the promoters of certain genes like *TPS2* which encodes for trehalose phosphate phosphatase in *Saccharomyces cerevisiae* (47). Besides Yap1, Yap2 also plays a role in the hydrogen peroxide induced adaptive stress response since induction of this adaptive stress response is severely diminished in a *yap2* mutant strain (138).

Another bZip transcription factor, Gcn4, although has not been directly implicated for oxidative stress resistance, is important for response to UV-light. Given that UV light can lead to production of ROS in the cell, an indirect role of Gcn4 towards OSR in baker's yeast cannot be ruled out (31).

Copper-binding transcription factors

Metallothionein has been shown to have antioxidant properties. The copper binding transcription factor, Ace1 regulates the expression of *CUP1* which encodes for metallothionein (140,151). *CUP1* is also present in *Candida albicans* (*CaCUP1*) and interestingly, *Candida albicans* strains lacking *CUP1* are killed more readily than the normal wild type cells. Although the direct role of copper binding transcription factors towards OSR is yet to be established in *Candida albicans*, copper stressed *Candida albicans* cells had elevated levels of oxidative stress in their mitochondria and cytoplasm(114).

Besides *CUP1, SOD1* regulation has also been shown to be dependent on this transcription factor (21,49). Another copper-binding protein, Mac1 has been shown to be

involved in the hydrogen peroxide induced activation of the *CTT1* gene which encodes for the cytosolic catalase in *Saccharomyces cerevisiae*. It is speculated that Ace1 and Mac1 can sense the redox status of the cell through their redox active domains (69).

Zinc-finger transcription factors

Hap1 is a zinc finger transcription factor which is involved in the regulation of several oxidative stress response genes like *CTT1* and *CTA1*, the cytosolic and peroxysomal catalase in *Saccharomyces cerevisiae.* The expression of *SOD2* has also been found to be regulated by this transcription factor (48,153). Recently, Hap2, Hap3 and Hap4 have also been shown to be involved in the expression of *SOD2* gene in stationary phase cultures (34). Ironically, this dissertation focuses on the Hap mediated regulation of the *Candida albicans* oxidative stress genes including catalase and Sods. Thus, the Haps appear to be involved in the regulation of oxidative stress in both *Saccharomyces cerevisiae* and *Candida albicans*.

Msn2 and Msn4 are general stress response proteins which are required for STRE-mediated regulation of *CTT1* gene (90,123). These transcription factors initiate a stress response owing to not only oxidative stress but also carbon source deficiency, osmotic stress, heat shock etc. Paradoxically, such a general stress response has not been observed in *Candida albicans* and Msn2 and Msn4-like transcription factors play no role towards the OSR in *Candida albicans* (105), thus highlighting that there has been extensive rewiring of the transcriptional circuitry of OSR in *Saccharomyces cerevisiae*, probably, to fit the ecological niche of *Candida albicans*.

Miscellaneous transcription factors

The Skn7 is involved in hydrogen peroxide induced oxidative stress resistance in *Saccharomyces cerevisiae* and has been shown to bind the promoter of *TRX2 in vitro* (101). The

Imp2 protein although lacks a DNA-binding domain but nevertheless has been shown to impart resistance against peroxide stress. Its actual role in the OSR is still not clear but it might have a role in metal ion homeostasis (91).

Oxidative stress response in *Candida albicans*

As mentioned before, *Candida albicans* has undergone extensive rewiring of the stress regulatory pathways to fit its own ecological niche. A major hindrance in deciphering the mechanism of the oxidative stress response in this opportunistic pathogen is the lack of a powerful genetic system. Nevertheless, with its whole genome sequenced and development of more potent molecular tools like development of conditional mutants and identification of reporter genes that can be used in this organism, manipulation of *Candida albicans* has become comparatively easier. Several proteins have been shown to be regulating genes involved in conferring resistance to oxidative stress either directly or indirectly, many of which have been directly implicated for their role in the virulence of this organism.

Hog1 MAP kinase

The Hog1 MAPK pathway plays an important role in the oxidative stress resistance in *Candida albicans* as mutants lacking this kinase have been found to be susceptible to peroxide stress (6). The oxidative stress signals from the environment are transduced to Hog1 via the two component signaling system including the response regulator protein Ssk1 (22). The histidine kinases that relay the environmental signals and are a component of the two-component signaling system have not yet been discovered in this organism and the downstream signaling pathway underlying the Hog1 mediated oxidative stress response is not very well understood.

Hog1 phosphorylation was found to be almost instantaneous after exposure to hydrogen peroxide in *Candida albicans* over a broad range of concentration from 0.4mM to 100mM, thus emphasizing the specificity of Hog1 phosphorylation by oxidative stress. Recent evidences also suggest that Hog1 inactivation lead to respiratory defects in this pathogen and is also involved in cell wall biosynthesis (5). *Candida albicans* Hog1 shares more than 80% similarity with the *Saccharomyces cerevisiae* homologue, yet it performs a unique function in *Candida albicans* along with the previously described role in osmotic stress for *Saccharomyces cerevisiae* (6).

Cap1

As mentioned earlier, Cap1 is a bZip transcription factor and is the homologue of *Saccharomyces cerevisiae* Yap1. Interestingly, Cap1 has all the cysteines conserved in the c-CRD domain of the *Saccharomyces cerevisiae* Yap1, and like Yap1, is also capable of conferring resistance against both peroxide and diamide stress (2,161). Thus, these data suggest that both Yap1 and Cap1 are regulated similarly by oxidative stress. Indeed, *GLR1* in *Saccharomyces cerevisiae* is known to be regulated by the Yap1 and its homologue, Cap1 has been found to be involved in the regulation of *CaGLR1*. Both, *Candida albicans* Cap1 and *Aspergillus fumigatus* Yap1 are regulated at the level of nuclear localization owing to oxidative stress (80,160).

Paradoxically, Cap1 and Hog1 mediate different oxidative stress responses and Cap1 nuclear localization is not affected in a *hog1* mutant strain. Similarly, Hog1 phosphorylation is not affected in a *cap1* mutant background (6,132).

Ssk1

Ssk1 is a signal protein that adapts cells to peroxide stress. This may be due to the Hog1 MAP kinase pathway. Unlike in *Saccharomyces cerevisiae*, where its major role is to sense environmental cues and generate an osmotic stress response, this protein has little to none role in the osmotic stress resistance in *Candida albicans* (18). Interestingly *ssk1* mutant cells had reduced adherence to human esophageal cell. The *ssk1* mutant strain is also more readily killed by human polymorphonuclear cells (PMNs) like neutrophils in a murine model of hematogenously disseminated candidiasis (19).

Thus, *Candida albicans* stress regulatory pathways share features distinct from the signaling cascades found in other fungal species including the model yeast, *Saccharomyces cerevisiae*. However, the array of antioxidant enzymes activated downstream of these regulators are fairly conserved among different species. *Candida albicans* harbors many distinct antioxidant enzymes including catalase, superoxide dismutases, glutaredoxins and thioredoxin which are described below. A striking feature of this organism is that it harbors multiple isoforms of some enzymes such as superoxide dismutases. One probable explanation is that such a feature may help the pathogen survive disparate niches in the host body.

The antioxidant defense arsenal of *Candida albicans*

Catalase

Catalase is a heme containing antioxidant enzyme which converts harmful hydrogen peroxide to water and oxygen. *Candida albicans* has a single gene, *CTA1*, encoding for catalase, unlike *Saccharomyces cerevisiae* which has two isoforms of this enzyme. Strains lacking catalase are significantly less resistant to human leukocyte killing in vitro. Also, a *cta1* mutant strain had reduced ability to cause progressive murine disseminated candidiasis (155). Catalase

is known to be the downstream target gene of Cap1 although Cap1 independent activation of this enzyme has also been observed (46,163).

Superoxide dismutases

Superoxide dismutases (Sods) are enzymes which convert superoxide anions to hydrogen peroxide, which are in turn processed by catalase. *Candida albicans* has six different isoforms of this enzyme. Sod1, Sod4-6 are CuZnSods while Sod2 and Sod3 are MnSods. These superoxide dismutases are present in either cytoplasm (Sod1 and Sod3), mitochondrion (Sod2) or are cell surface associated proteins (Sod4, Sod5 and Sod6) (23).

Sod1 is a cytoplasmic protein known to impart resistance to menadione, a redox sensing agent. The *sod1* mutant strain also showed delayed hyphal growth on a medium inducing hyphal formation (spider medium). Strains lacking *sod1* have more susceptibility to fungicidal damage caused by macrophages in murine model of disseminated candidiasis. Thus, *SOD1* gene has been implicated as a virulence gene in *Candida albicans* (66).

The mitochondrial Sod2, although is not a virulence factor, is still necessary to detoxify the cells of the ROS produced as a byproduct of respiration. Cells lacking Sod2 also were found to be more susceptible to heat, high concentration of salts and ethanol (65).

Sod3 is an unusual cytoplasmic Mn containing Sod, in contrast to MnSods which are usually mitochondrial. Sod3 is known to confer resistance against oxidants during stationary phase, unlike Sod1 which is repressed under these conditions. Thus, having an unusual MnSod active during stationary phase may help the cells counteract the deleterious effects of copper toxicity during lag phase growth conditions (76,81).

Sod4 is a GPI-anchored cell surface protein which is strongly induced during yeast to hyphal transition. Interestingly, it is also induced during white to opaque colony switching, thus playing a prominent role in detoxifying ROS in the opaque form. This could be advantageous to the pathogen as opaque forms are better colonizers of the skin and also the anaerobic gastrointestinal tract. Therefore, Sod4 may have an important protective role in skin or GI tract infections (77).

Sod5 is one of the most well characterized Sods and is believed to play an indispensable role in protecting the cells from extracellular ROS. However, unlike Sod1, its main function is to detoxify ROS generated by neutrophils. Like Sod4, it is also greatly induced during yeast to hyphal transition. Infact, Sod4 and Sod5 share some redundant functions and in the absence of Sod5, Sod4 can compensate for its loss. The *sod4 sod5* double mutant is severely susceptible to killing by host immune cells in a murine model of infection (42).

The exact function of Sod6 is yet to be known. Although its membrane bound location in the cell would predict a role in the detoxification of extracellular ROS, the presence of *SOD6* could not compensate for the combined loss of *SOD4* and *SOD5* in the cell (42).

Thus, the presence of multiple isoforms of Sods helps *Candida albicans* survive different types of stresses, in either intracellular or extracellular location and at different stages of growth. Different morphological forms like yeast vs hyphae or white vs opaque also trigger the induction of certain unique Sods.

Glutaredoxins

Glutaredoxins are cytosolic proteins that are involved in the reduction of glutathione disulphide. *Candida albicans* possesses four different isoforms of this protein. Grx1 is a

putative uncharacterized glutaredoxin in *Candida albicans*. The most well characterized glutaredoxin is Grx2. Cells lacking Grx2 are more susceptible to neutrophil killing in mice model of infection, thus making it a virulence factor. A strain lacking Grx2 is however very resistant to both menadione and benzamide stresses. Grx2 deficient strains also show defective hyphal formation (24). The roles of Grx3 and Grx5 are yet to be established with respect to oxidative stress response in *Candida albicans*.

Thioredoxins

Thioredoxins are oxidoreductases which catalytically reduce diverse proteins. However, they also have a role in signaling pathways along with their antioxidant function. Unlike *Saccharomyces cerevisiae*, which has three distinct isoforms of this enzyme, *Candida albicans* has Trx1 which is the main thioredoxin of this organism and a putative uncharacterized and enzymatically inactive Trx2. Thioredoxins have been implicated in multiple stress induced roles of which peroxide induced stress response is an important one. It is speculated to be a positive regulator of the Hog1 MAP kinase pathway and a repressor of the Cap1 protein under peroxide stress. Hydrogen peroxide induced oxidation of thioredoxin is thought to be one of the factors responsible for polarized cell growth in the pathogen. This polarized growth may help the pathogen escape from ROS rich environments like host macrophages and neutrophils (128).

Thus, the host immune response attempts to neutralize the invading *Candida albicans* by disturbing their oxidation potential which can have deleterious effects like DNA damage, protein damage and ultimately cell death. The internal homeostasis of redox potential of a pathogen can also be altered by ROS produced as a byproduct of respiration. Besides, toxic levels of several micronutrients including iron have been linked to a disturbed oxidation potential of the cell(111).

Iron possess a dilemma to *Candida albicans* pathogenesis as it is essential for its growth and synthesis of some antioxidant enzymes like catalase, however excess of it can in turn generate ROS via Fenton's reaction. Hence, maintaining an internal iron homeostasis is crucial for maintaining a healthy oxidation potential of the cell.

Since most of the host iron is present in protein-bound form like ferritin, hemoglobin, transferrin etc., *Candida albicans* has evolved exclusive mechanisms to harvest iron from the low-iron host. However, it may also encounter transient toxic doses of this micronutrient in certain niches like human gut. Thus, for successful pathogenesis, its genetic program has to shuttle between an iron limiting environment on one hand and an overdose of this micronutrient in some niches on the other hand.

Iron availability inside the host

There is a constant competition for iron between microorganisms and their host during both, commensalism and pathogenesis. Thus, ability of *Candida albicans* to procure iron from the host has been considered a virulence factor, and successful colonization and infection only occurs if the pathogen gets sufficient amount of this micronutrient. Indeed, the pretreatment of endothelial cells with the iron chelator phenanthroline reduces *Candida albicans* colonization of endothelial tissues (40). Most of the iron present in the human body is associated with specific proteins. This prevents the iron dependent generation of ROS and also limits the availability of this essential nutrient to the invading pathogens.

More than 66% of the total body iron is circulating in the blood as haemoglobin. Haemoglobin is an oxygen binding protein containing haem prosthetic groups. Each

haemoglobin molecule possess four haem groups containing one ferrous ion per prosthetic group (32).

Transferrin is another iron containing protein. It is found in serum and can transport iron from site of absorption and storage to the site of utilization (36). It is synthesized in the liver and about 0.1% of total body iron is present in form of transferrin (32). Lactoferrin is another member of the same family and is present in fluids like milk, saliva, tears, etc. Besides having an iron binding activity it also has iron sequestering properties, thus having antimicrobial activity against *Candida albicans* (145,148,158).

Ferritin is another form of an iron storage protein which is found in almost all human cells. In humans, ferritin comprises about 30% of total body iron (35). Unlike transferrin whose key role is to trap extracellular free iron, ferritin maintains the intracellular iron homeostasis, thus preventing the toxic generation of ROS catalyzed by free iron radicals (142). Deletion of the gene encoding for ferritin has been showed to be lethal in mice. Although majority of the ferritin is intracellular, some ferritin has also been detected in extracellular components like serum even though its function there remains elusive (52).

Other miscellaneous iron containing sources are haem proteins like cytochrome and myoglobin, non-haem proteins like ribonucleotide reductases, and iron sulphur cluster proteins like aconitase and succinate dehydrogenase (43,119). A significantly less amount of iron is also available in the free form called the labile iron pool which consists of both chelatable and redox-active iron and serves as crossroad of cell iron metabolism (71).

As discussed below, *Candida albicans* has evolved three different mechanisms to obtain iron from the various host sources listed above. Mutations in certain genes encoding for proteins

that are required in any of these iron acquisition pathways have resulted in attenuated virulence in mice, thus highlighting the importance of this micronutrient in *Candida albicans* pathogenesis.

Iron acquisition pathways in *Candida albicans*

Siderophore uptake

Siderophores are low molecular weight, high affinity iron chelators that can bind to host iron and make it available to microorganisms. They are secreted by certain bacteria and fungi, although, *Candida albicans* siderophore biosynthetic pathway has not been discovered yet (7). Certain siderophores like desferri-exochelin secreted by *Mycobacterium tuberculosis* have a greater affinity for iron than host protein themselves (45). *Aspergillus fumigatus* are capable of producing two different types of siderophore, each of which can rapidly remove iron from transferrin (55).

Although *Candida albicans* cannot synthesize its own siderophores, it exhibits 'iron parasitism', i.e., it can use iron from siderophores synthesized by other microbes. *Candida albicans* has only one siderophore transporter in the membrane, Sit1/Arn1. Through this membrane bound transporter, siderophores are internalized via an endocytic pathway which is not properly understood. A mutant lacking this membrane transporter was less pathogenic as compared to the strain with the transporter in the reconstituted human epithelium model of infection (54,64).

Haemoglobin uptake

Candida albicans hyphal form has been shown to scavenge iron from haemoglobin by first binding to erythrocytes via the complement-receptor-like molecules. A mannoprotein attached to the cell surface of these pathogens acts like a haemolytic factor by aiding in

adherence of the pathogenic cells to the RBCs, eventually lysing them (100). *Candida albicans* harbors several different haemoglobin receptor proteins in its membrane which can bind to haemoglobin resulting in their uptake. This haemoglobin- receptor gene family comprises the genes *RBT5*, *RBT51*, *WAP1/CSA1*, *CSA2* and *PGA7* (149). Both *RBT5* and *WAP1* have a hyphal specific expression pattern although under low-iron condition the expression of *RBT5* has been found to be morphology independent (13). However, an *rbt5* mutant strain has wild-type virulence in a mouse model of systemic infection. This may be due to the presence of other haemoglobin binding receptor proteins which might compensate for the loss of *RBT5* (13).

After the haemoglobin has been engulfed into the vacuoles via the endocytic pathway distinct from the siderophore uptake pathway, either the vacuolar proteases or the highly acidic pH of the vacuoles help in lysing of the haemoglobin. To harvest iron from haem, *Candida albicans* possess a haem oxygenase encoded by the gene *HMX1*. Haem oxygenase degrades haemoglobin to α-billiverdin and the iron thus obtained is available for microbial use (110).

Reductive uptake

Candida albicans utilizes the reductive pathway to uptake iron from ferritin, transferrin, etc. However, this pathogen cannot uptake iron from lactoferrin, instead it has been shown to inhibit *Candida albicans* growth. *Candida albicans* first binds to these proteins through receptors present on the pathogen's cell surface. Although transferrin receptors have not been yet discovered, Als3 is known to bind to ferritin. Als3 is a member of the ALS family of genes which encodes for the cell-surface GPI anchored glycol-protein, whose original function is to aid adhesion to the host surfaces, and a strain lacking Als3 could not bind to ferritin at all. Als3p is only expressed in hyphae, likewise only the hyphal form can obtain iron from ferritin (59).

However, recent evidence has shed light on the morphology independent expression of Als3 during iron starvation (162).

Once these iron containing proteins are attached to the cell surface, the pathogen uses ferric reductases and multicopper oxidases to assimilate the extracellular iron. Two surface ferric reductases have been identified; Cfl1/Fre1 and Cfl95/Fre10/Rbt2. These reductases reduce the insoluble extracellular ferric ion to soluble ferrous ion (14,157). The multicopper oxidases convert the harmful ferrous ions back to ferric form. *Candida albicans* has five putative multicopper oxidase genes, of which *FET3* and *FET99* have been characterized (86). As copper is essential for oxidase activity, the intracellular copper transporter, Ccc2 is essential for both, reductive pathway and ferritin utilization (3).

Iron permeases form the third component of the reductive pathway. These enzymes form a complex with the oxidases and transport the ferrous ion into the cell. *FTR1* encodes a high affinity iron permease which is essential for sequestering iron from transferrin and ferritin. Cells lacking *FTR1* were completely avirulent in a mice model of systemic infection. Interestingly, *FTR1* has no sequence similarity to any human gene thus making it an attractive drug target (3). Two other genes, *FTH1* and *FTH2* encode for putative high affinity iron transporters (146).

In spite of the presence of multiple pathways to sequester iron from the host, *Candida albicans* endure periods of iron scarcity in certain niches of the host body during pathogenesis. However, as a commensal of the human gut it is also likely to be subjected to comparatively high levels of iron as the majority of dietary iron is not absorbed (93). Thus the transcriptional machinery of this pathogen has greatly evolved in order to adapt itself to such diverse environments.

Transcriptional circuitry regulating iron homeostasis in *Candida albicans*

In the majority of the Ascomycetes, when sufficient iron is present in the environment, a GATA family transcription factor shuts down the expression of the iron acquisition genes as well as the regulatory components of the CCAAT-Binding factor. However, when there is scarcity of iron in the environment, the CCAAT-Binding factor represses the GATA factor and some genes encoding for the iron-requiring proteins (51,70,97). *Candida albicans* has the GATA factor orthologue called Sfu1 which could compensate for the GATA factor in *Schizosaccharomyces pombe* (109). Also, the CCAAT-Binding factor has also been shown to be critical for iron homeostasis under iron deplete conditions and is discussed in great depths in this document.

Intriguingly, *Candida albicans* has incorporated a novel Zn (2) Cys (6) DNA-binding protein, Sef1 in between these two broadly conserved pathways comprised of Sfu1 and the Hap43 member of the CCAAT-Binding complex (106). Sef1 was originally identified as an essential gene required for growth under iron deprivation. Chen e*t al.* showed that this protein acts as an activator of certain iron uptake genes like *SIT1*, *RBT5*, and *CFL1*. They have also shown it to be a positive regulator of Hap2, Hap32 and Hap43 subunits of the CCAAT-Binding factor under iron deprivation. Deletion of *SFU1*, however, resulted in an up regulation of *SEF1* and *HAP43*.

Thus, a simple model was proposed by this group for the maintenance of iron homeostasis in disparate niches by *Candida albicans,* comprising three transcription factors; Sfu1, Sef1 and Hap43. When iron is present in sufficient quantities, Sfu1 directly represses the expression of Sef1 and other iron acquisition genes. However, when iron is limiting in the environment, Sef1 causes the activation of Hap43 and various other iron uptake genes. Hap43 in turn represses the expression of *SFU1* and other iron utilization genes. Interestingly, *SEF1* and *SFU1* are differentially expressed in blood and the human gut respectively. Hence, this might be

one mechanism by which *Candida albicans* is able to alter between high iron and low iron environments in the host body.

Thus the CCAAT-Binding factor plays a major role in maintaining iron equilibrium inside the pathogen, especially during iron limiting conditions. The mechanism by which this transcription factor regulates genes will aid understanding the *Candida albicans* iron homeostasis in general.

CCAAT-Binding Factor

The CCAAT-Binding factor or the Hap complex is an oligomeric transcription factor which comprises multiple subunits namely, Hap2p, Hap3p and Hap5p as the core DNA-binding domain while Hap4p has the activation domain in *Saccharomyces cerevisiae* (89,95). It binds to the consensus CCAAT sequence which occurs in nearly 30% of all eukaryotic promoters (17). It was first discovered in *Saccharomyces cerevisiae* as the transcription factor involved in upregulating the expression of *CYC1* and later several genes encoding proteins of the TCA cycle and respiratory chain enzymes (107). It was shown to be essential for the reprogramming of the yeast metabolism from fermentative to respiratory, a process termed the 'diauxic shift'. The expression of Hap2p, Hap3p and Hap5p has been found constitutive and the expression of Hap4p was induced under diauxic shift. Indeed, there is no visible expression of Hap4p in the presence of glucose in *Saccharomyces cerevisiae*. Hap4p is however expressed when glycerol or galactose is present as the source of carbon in baker's yeast (28,39).

In *Schizosaccharomyces pombe* the CCAAT-binding factor is called the Pap complex and is comprised of the *Saccharomyces cerevisiae* homologues of Hap2p, Hap3p and Hap5p, namely, Php2, Php3 and Php5 respectively. Php4 is the *Schizosaccharomyces pombe* homologue

of Hap4p. The *Schizosaccharomyces pombe* Php2, Php3 and Php5 could complement the respiratory growth deficiency caused by the absence of Hap2p, Hap3p and Hap5p in *Saccharomyces cerevisiae*, but unlike *Saccharomyces cerevisiae*, the php4 mRNA is readily detectable under glucose conditions. Also, *Schizosaccharomyces pombe* can still grow on media containing non-fermentable carbon sources like glycerol in the absence of Php4 (94,108,156). Thus, the CCAAT-Binding factor in the fission yeast regulates the expression of genes involved in different pathways as compared to *Saccharomyces cerevisiae*.

Indeed, the Pap complex has been found to have repressor function in contrast to activator function in baker's yeast. The complex is known to be involved in iron homeostasis and is known to repress genes encoding iron containing proteins via Php4 during iron starvation. The regulation of Php4 is in turn speculated to be dependent on the Fep1. In the absence of iron, Fep1 is inactive and fails to bind to the GATA elements on the promoter of *PHP4*. Thus, Php4 is synthesized and associates with the other subunits of the Pap complex to repress the genes involved in iron utilization. However, when iron is present in the environment, Fep1 represses the expression of *PHP4*, thus activating the genes involved in iron utilization (98).

The CCAAT-Binding factor in the filamentous fungus, *Aspergillus nidulans* is called the AnCF (*Aspergillus nidulans* CCAAT-Binding factor). AnCF comprises the HapB, HapC and HapE as the DNA-binding component (12). It has been previously shown that HapB contains two putative nuclear localization sequences and HapC and HapE are transported to the nucleus only after complex formation with HapB (143).

Apart from the core DNA-binding domain, it also has another protein, HapX which interacts with the complex to repress genes involved in iron utilization during iron starvation. HapX bears no homology with the *Saccharomyces cerevisiae* Hap4p apart from the N-terminal

17 amino acid domain which is essential for its interaction with the core DNA-binding complex. HapX is an iron sensing protein and is thought to sense the redox status of the cell via conserved cysteine residues. Apart from its role in iron homeostasis, AnCF is also involved in penicillin biosynthesis. Recent evidence has also shed light on the auto regulation of this complex via HapB, which is repressed by this complex (57). Thon et *al*. has recently shown that this complex is also involved in sensing the redox status of the cell and the subsequent regulation of the oxidative stress response, including genes encoding the *Aspergillus nidulans* Yap1 orthologue (NapA), catalase, thioredoxin and maintenance of steady state glutathione levels in *Aspergillus nidulans.*

Besides fungi, CCAAT-Binding factor is also present in the model plant *Arabidopsis thaliana* (30), *Xenopus laevis* (82) and mammals. In mammals it is called the NF-Y and binds to the promoter of a number of genes including the ones encoding for collagen, albumin and Major Histocompatibility Complex among others. It is comprised of NF-YA, NF-YB and NF-YC as a part of a trimeric complex (87). In plants this complex is known to regulate photoperiodism, seed germination and abiotic stress tolerance among another things (58,92). Thus, the evolutionary conserved CCAAT-binding factor regulates diverse genes and processes in different organisms.

CCAAT-Binding factor of *Candida albicans*

The CCAAT-Binding factor or the Hap complex of *Candida albicans* is a hetero oligomeric transcription factor that binds to the consensus CCAAT sequence on the promoters of a variety of genes. It comprises Hap2p and Hap5p as a part of the DNA-binding domain. But unlike *Saccharomyces cerevisiae*, this opportunist pathogen is known to harbor two distinct

homologues of Hap3s; Hap31p and Hap32p. *Candida albicans* has been speculated to recruit Hap31 vs Hap32 based on the availability of iron in the environment (129). It also has three distinct homologues of the *Saccharomyces cerevisiae* Hap4, namely, Hap41p, Hap42p and Hap43p. Although not much is known about Hap41 and Hap42, Hap43 has been shown to be the global repressor of genes required for iron utilization in this organism during iron starvation (25,61,129). The Hap complex in *Candida albicans* has been discussed in greater depth in the 'Introduction' of this dissertation.

2. Introduction

The CCAAT-Binding factor or the Hap complex is an evolutionary conserved multi-subunit transcription factor that binds to the consensus CCAAT sequence also called the CAT box, present in the promoters of a variety of genes. In *Candida albicans*, this transcription factor has been shown to be involved in the repression of genes involved in iron utilization, in contrast to the activator function in the model yeast *Saccharomyces cerevisiae* (61,68,107,129).

Previous work in our lab has demonstrated that *CaHAP5* could compensate for the loss of the subunit in *Saccharomyces cerevisiae* by restoring growth on non-fermentable carbon sources, like glycerol or lactate. Further, *Candida albicans hap5Δ* homozygous deletion strain, was defective in yeast to hyphal transition under laboratory conditions, and became hyper filamentous when deprived of glucose in the environment. A total abolishment of DNA-binding activity was observed in the *hap5Δ* strain as seen with the mobility shift assays (68).

Similarly, the strain with homozygous deletion in the gene encoding the Hap2 subunit, *hap2Δ*, was found to be hyper filamentous in the absence of glucose. The loss of either *HAP2* or *HAP5* rendered the colonies defective in hyphal formation on medium inducing hyphal growth. The nitrogen sensing ability in this opportunistic pathogen is thought to be regulated by this protein complex. The *hap2Δ* strain also showed no DNA-binding activity as assessed by mobility shift assays (78).

Interestingly, both *hap5Δ* and *hap2Δ* strains were unable to grow on iron deficient medium. The respiratory genes, *CYC1* and *COX5* were both found to be regulated by this complex in a differential manner depending upon the availability of iron in the medium. When iron was present, this complex acted as an activator of *CYC1* and *COX5*, as the strain lacking the transcription factor showed at least a two-fold down regulation of the expression of these genes in northern blot analysis. In iron deficient conditions, however, this complex acted as a repressor

of the same genes, seen as a significant amount of derepression of *CYC1* and *COX5* in the absence of this complex (78).

Thus, the complex could serve dual yet contrasting roles in the iron responsive gene regulation of *CYC1* and *COX5*. Such regulation of gene expression by the CCAAT-Binding factor under low-iron conditions has also been observed for *Schizosaccharomyces pombe* and *Aspergillus nidulans* (51,57,75). Thus, maintaining the iron homeostasis in several fungal species including *Candida albicans* is a distinct feature of this transcription factor as opposed to regulation of respiratory metabolism in *Saccharomyces cerevisiae*, for which this complex was first described (124).

This differential regulation of genes by the transcription factor is thought to be achieved at least partially by recruiting distinct subunits of the Hap complex. The *Candida albicans* CCAAT-Binding factor has two distinct homologues of the *Saccharomyces cerevisiae* Hap3; Hap31 and Hap32. Both of these homologues are capable of rescuing a *Saccharomyces cerevisiae hap3Δ* respiratory deficient mutant. No growth defect for a strain with homozygous deletion in the gene encoding Hap31, *hap31Δ* was observed in iron-deficient media. However, the strain with homozygous deletion for the gene encoding Hap32, *hap32Δ*, showed a slight growth defect in iron deficient media. Hap31 and Hap32 has been shown to bind to the complex in iron replete and iron deplete conditions, respectively. The strain with homozygous deletion in genes encoding both Hap31 and Hap32, *hap31Δ hap32Δ*, had the same hyper filamentous phenotype when grown in glucose deficient media as that of the *hap2Δ and hap5Δ* mutant strains. The double homozygous knockout strain was unable to grow in iron deficient media and no DNA-binding activity was detected either in the presence or the absence of iron (78).

Therefore, the double *hap31Δ hap32Δ* behaves as a *hap2Δ* or *hap5Δ*, unable to form an active complex.

The Hap31p is a 105 amino acid long protein while Hap32p is 330 amino acid long. Previous mobility shift assays done in our lab showed that in a *hap31Δ* strain, there is no complex formation under iron replete conditions. However, in a *hap32Δ,* there was abundant complex formed which was of the same size as that of the complex in the WT strain under iron sufficient conditions. Likewise, both, in a *hap31Δ* and WT strains under iron deficient conditions, a larger sized complex was formed which was not detected in a *hap32Δ* strain. But, in a *hap32Δ,* a comparatively smaller sized complex was detected when experiments were done under iron limiting conditions. Thus Hap31 and Hap32 form distinct complexes depending upon the presence or absence of iron (78). R.P Singh et *al.* has shown that the expression of *HAP32* mRNA was highly induced and the expression of *HAP31* was almost undetected under iron limiting conditions (129).

Candida albicans also harbors three distinct homologues of the *Saccharomyces cerevisiae* Hap4; Hap41, Hap42, and Hap43, respectively. The expression of *HAP41* and *HAP42* mRNA was unaffected by the presence or absence of iron. However, the expression of *HAP43* was highly induced in the absence of iron and was almost undetected when iron was present in the environment. Thus, similar to Php4 in *Schizosaccharomyces pombe* and HapX in *Aspergillus nidulans*, the Hap43 homologues in these species, Hap43 is involved in maintaining iron homeostasis during low iron conditions (25,130).

Strains with a homozygous deletion in the genes encoding Hap41 and Hap42, *hap41Δ* and *hap42Δ*, had no visible growth defect in iron deficient media. On the contrary, a strain with a homozygous deletion in the gene encoding for Hap43 could not grow in iron deficient media.

The expression levels of the two genes encoding proteins involved in respiration, *CYC1* and *COX5* was unaffected by the presence or absence of Hap41 and Hap42 in both iron sufficient and iron deficient media. However, under iron limiting conditions, there was significant expression of *CYC1* mRNA and *COX5* mRNA in the *hap43Δ,* suggesting that Hap43 is involved in the repression of these genes during iron starvation (78). Interestingly, *HAP2* and *HAP32* expression was also induced by Hap43 under iron limiting conditions. Hap43 has both a Yap-like bZIP domain and a fungal specific Hap4L domain and each of these domains perform an independent function and are essential for Hap43 activity, although their exact roles are yet to be determined. Under low iron conditions, Hap43 has been shown to accumulate in the nucleus, where it facilitates repression of the genes involved in iron utilization. A strain lacking Hap43 has been found to be much less virulent as compared to the wild type strain in mice model of systemic infection (130).

Thus, CCAAT-Binding factor is involved in maintaining iron homeostasis in *Candida albicans* which is similar to its function in *Schizosaccharomyces pombe* and *Aspergillus nidulans*. To understand the global effect of this transcription factor, whole genome microarray was performed in several labs including ours for a WT versus *hap5Δ* strain under iron limiting conditions. Among several genes upregulated in the *hap5Δ* strain under low iron condition, the *CaCTA1* gene encoding the only catalase in this opportunistic pathogen was particularly high. *CTA1* mRNA was over four fold over expressed in the strain lacking the transcription factor as compared to the WT in iron deficient condition. Singh et *al.* found a similar overexpression of *CTA1* in a *hap43Δ* strain under the same environmental conditions (129). Apart from *CTA1*, this group also showed the Hap43 dependent repression of the *Candida albicans* mitochondrial superoxide dismutases, *SOD2* and *SOD3* in iron deficient conditions. Similarly, they also found

a Hap43 mediated induction of the thioredoxin gene, *CaTRX1* under low iron conditions using microarray analysis.

Thus several genes involved in the oxidative stress response in *Candida albicans* appear to be under the control of the CCAAT-Binding factor. Indeed, the CCAAT-Binding factor has been shown to regulate the *SOD2* gene in *Saccharomyces cerevisiae* in stationary phase cultures (34). Besides, it has also been implicated in the oxidative stress response of the filamentous fungi, *Aspergillus nidulans.* In *Aspergillus nidulans*, the CCAAT-Binding factor is regulated by the redox status of the cell and in turn regulates downstream genes which are required for an adequate response to oxidative stress (141).

Given the fact that the oxidative stress response of *Candida albicans* is pivotal in helping the pathogen escape the oxidative burst generated by the host phagocytic cells, like macrophages and neutrophils, the general understanding of the mechanism by which this transcription factor regulates genes involved in OSR will be a considerable step towards understanding *Candida albicans* pathogenesis.

The presence of free iron can also alter the redox potential of the cell, thus generating an OSR in the pathogen. Iron, with its unique chemistry poses a conundrum to *Candida albicans* pathogenesis, as on one hand it is an essential nutrient and is required as a cofactor for several enzymes including catalase, but, on the other hand it can generate Reactive Oxygen Species (ROS) via the Fenton's reaction (106). Moreover, the host body is essentially a low iron environment as most of the iron is bound to host proteins and is unavailable for microbial use (4). Therefore, understanding the regulation of OSR with respect to iron availability will help predict the oxidative stress gene regulation in dynamic micro niches with varying concentrations of this micronutrient inside the host.

This dissertation provides an in-depth analysis of the CCAAT-binding factor mediated regulation of the defense arsenal against oxidative stress in the opportunist pathogen, *Candida albicans*. We first show that the oxidative stress response in *Candida albicans* is differentially regulated based on iron availability in this pathogen. We next corroborate the whole genome microarray data from our lab and prove using immune-blot analysis, Northern blot and luciferase assays that the *CTA1* gene is upregulated in presence of iron and repressed when iron is limiting in the environment by the Hap complex. As many stress genes are regulated at the post transcriptional level as well, we then assessed the Hap complex dependent regulation of the catalase activity in the same condition we had assessed the transcriptome activity.

Since the oxidative stress response is the concerted effort of proteins and enzymes encoded by several genes, we next show the CCAAT-Binding factor dependent regulation of several other genes involved in OSR including superoxide dismutases, glutaredoxins and thioredoxin. We used the standard biomarker of oxidative stress response in *Candida albicans*, *CTA1* as a prototype gene to explore the mechanistic details of the iron based differential regulation. We show that similarly to the regulation of genes involved in respiration like, *CYC1* and *COX5*, the differential regulation of *CTA1* is also partly due to the recruitment of distinct Hap3s and Hap4s depending upon the presence or absence of iron. In summary, we prove that the CCAAT-Binding factor has a critical role in iron homeostasis as well as the oxidative stress response in *Candida albicans*.

3. Materials and Methods

Yeast strain and growth conditions

The yeast strains used in this study are listed in **Table 1**. Lower case letters indicate mutant alleles and upper case letters indicate wild-type (WT) alleles. Strains were routinely cultured in yeast extract-peptone dextrose (YPD) medium (50). For DNA transformations, synthetic complete (SC) medium lacking auxotrophic supplements or synthetic minimal medium (SD) augmented with the auxotrophic requirements were used (50). To generate iron depleted conditions, 200µM bathophenanthroline disulfonate (BPS) (Sigma) was added to the appropriate medium to chelate iron.

Table 1: Yeast Strains and Genotypes

Strain	**Genotype**	**Reference**
BWP17	*ura3Δ::imm434/ura3Δ::imm434his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG*	
DMC 146 (WT)	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ:hisG-ARG4-URA3*	
DMC 117 (*hap5Δ*)	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	
hap31Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG-ARG4 hap31Δ::URA3/hap31Δ::HIS1*	Bates, 2009
hap32Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG-ARG4 hap32Δ::URA3/hap32Δ::HIS1*	Bates, 2009
hap31Δ hap32Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG-ARG4 hap31Δ::hisG/hap31Δ::hisG hap32Δ::URA3/hap32Δ::HIS1*	Bates,2009
hap41Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG-ARG4 hap41Δ::URA3/hap41Δ::HIS1*	McNabb, unpublished
hap42Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-HIS1 arg4Δ::hisG/arg4Δ:hisG-ARG4 hap42Δ::hisG/hap42Δ::hisG*	This study
hap43Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-HIS1 arg4Δ::hisG/arg4Δ:hisG-ARG4 hap43Δ::hisG/hap43Δ::hisG*	This study
hap41Δ hap42Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG-ARG4 hap41Δ::URA3/hap41Δ::HIS1 hap42Δ::hisG/hap42::hisG*	This study
hap41Δ hap43Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG-ARG4 hap41Δ::URA3/hap41Δ::HIS1 hap43Δ::hisG/hap43::hisG*	This study

hap42Δ hap43Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-HIS1 arg4Δ::hisG/arg4Δ:hisG-ARG4 hap42Δ::hisG/hap42Δ::hisG hap43Δ::hisG/hap43Δ::hisG*	This study
hap41Δ hap42Δ hap43Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ:hisG-ARG4 hap41Δ::URA3/hap41Δ::HIS1 hap42Δ::hisG/hap42Δ::hisG hap43Δ::hisG/hap43Δ::hisG*	This study
WT-*CTA1-Rluc*	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-HIS1 arg4Δ::hisG/arg4Δ:hisG-CTA1-Rluc-ARG4*	This study
hap5Δ-CTA1-Rluc	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ: CTA1-Rluc-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	This study
hap31Δ-CTA1-Rluc	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ: CTA1-Rluc-ARG4 hap31Δ::URA3/hap31Δ::HIS1*	This study
hap32Δ-CTA1-Rluc	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ: CTA1-Rluc-ARG4 hap32Δ::URA3/hap32Δ::HIS1*	This study

Oligonucleotides.

The oligonucleotides used in this study are listed in **Table 2**.

Table 2: Oligonucleotides

oDM0341	GGCCGAATTCGGTGCCACTTTATTTAAAACTAGATG
oDM0342	GGCCGGATCCGCTTTCTTCAAATAAGTAACTAAATC
oDM0345	GGCCGGATCCAGTCACGCAAATCATCGATCCAGTATATCTACC
oDM0346	GGCCAAGCTTGGGATTAGACATTTGGTTTGGGATTTTGGTAAAGAATTAACC
oDM0369	GGTGAGGCATGAGTTTCTGCTCTCTCA
oDM0370	CTGTATATCGGCACCACTCAATAAGTTACAGCA
oDM0394	GGCCGGATCCTAAATCTAAACTATTGTAACACTTCTAACCAATCAC
oDM0395	GGCCGTCGACCTACTGGTGCTGGTGCAGGTAATACTATTGCTGGTG
oDM0396	CAGGAATGCGCAGTAGTTTCCGAAATCGGTC
oDM0459	TAGCGGTTCTGACGTGCAAATCGATCGTCG
oDM0460	ACTAACACCTTTTGTGGTGTCTGATGAGCG

oDM0492	CGCAAGAGATATAGAGTTGAGGG
oDM0493	GACTACTTCCTGGCTTCTCGGTATTTGGC
oDM0494	GGGATATGTCTAATATAGAACTG
oDM0495	CCGTTGTTTGTCGTTTTGGCGTTG
oDM0526	CTATCAGATCATCATCTCGTG
oDM0527	GCTTCCTTCCAGTTGATAAC
oDM0528	GTAAACGGGTACAAATGCCGC
oDM0529	GCCTCAGCCCAGTTGATCACG
oDM0530	GAATAATTTCCGTGGC
oDM0531	GGCTGATTGAACCGTCTC
oDM0532	CGAGTCAGAACAACAGTTCAC
oDM0533	CCAATCCACCTTGGAATTCACC
oDM0534	CACGTTGTCACTGAAG
oDM0535	GCTTGTTTAATAGCAGC
oDM0536	CAGTACCAGAATCGAGCTGGCGCCAAGCGC
oDM0588	GGCCAGATCTCCGATAATGTTATCATAAACTGATAG
oDM0589	GGCCAGATCTGGGTTGCTATTTTGGTTTGATTGGG
oDM0590	GGCCAGATCTCCTTTTGCGGGCATGTTGTTCAAATTG
oDM0591	GGCCAGATCTGTTTGACTTACAGTATGAAGTATTTCCG
oDM0617	GACGCAAATACATGATAACCGAGGGT
oDM0620	GGCCACTAGTTCACTCAATTACAATTCCA
oDM0621	CGATATCACTGATATTTGTGC
oDM0622	GGTACCCAATCTGTGTCTGTGAG
oDM0651	GGGTCAAGCCAATGACACC
oDM0652	GTTGCTCTTGCATTAGCAGG

oDM0653	CACCGGCAATACCAGCAGC
oDM0654	GCTTTGGCTGGTGATGCACC
oDM0655	CACCTGCTGCCAAGAGACC
oDM0656	CAATAGCTGCTTCAGACAAG
oDM0657	CCTGCAGCTATTCCCAATAC
oDM0660	GGGGCATGCAAAATCCTGGGTCACAGAAATATGG
oDM0661	GGGGGGATCCAATAAATAATTTATATATAAATAGG

Plasmid construction

The plasmid pDM588 containing *HAP41* was generated by PCR amplification of *Candida albicans* orf19.740 using the oligonucleotide primers oDM0343 and oDM0344. The *HAP41* PCR product was digested with BamHI/PstI cloned into the same sites of pSP65 (Promega Corp.). Plasmid pDM588 was digested with NdeI/ClaI, the ends blunted using T4 polymerase and a BglII linker ligated to generate pDM589 containing a deletion in the *HAP41* coding sequence. To generate pDM592 containing the *hap41::URA3* null allele, *URA3* was amplified from pGEM-URA3 using primers oDM0382/oDM0383 that created BamHI sites on both the 5' and 3' ends of the gene. Plasmid pDM589 and *URA3* was digested with BglII and BamHI, respectively, and the DNA fragments ligated. To generate pDM598 containing the *hap41::HIS1* null allele, *HIS1* was amplified from pGEM-HIS1 with primers oDM0384/oDM0385 that created BclI sites on both the 5' and 3' ends of the gene. Plasmid pDM589 and *HIS1* were digested with BglII and BclI, respectively, and the DNA fragments ligated.

Plasmid pDM571 contains *HAP42* gene that was generated by the PCR amplification of the *Candida albicans* orf19.1481 with the oligonucleotide primers oDM0345 and oDM0346,

which incorporated unique BamHI and HindIII restriction sites into the 5' and 3' ends of the gene, respectively. The plasmid YEplac181 and the *HAP42* PCR product were digested with BamHI and HindIII and ligated to generate pDM571. To create *hap42Δ::hisG-URA3-hisG* knockout allele, pDM571 was digested with BamHI/HindIII and *HAP42* was ligated into pSP65 digested with BamHI/HindIII to generate the pDM800. The plasmid pDM800 was subsequently amplified by PCR using primers oDM0588/oDM0589 that created a PCR amplified plasmid with approximately 500bp of *HAP42* noncoding flanking sequence and a unique BglII restriction site on the 5' and 3' ends. The DNA was digested with BglII and ligated with the BamHI/BglII DNA fragment containing *hisG-URA3-hisG* from the plasmid p5921 (38). The orientation of the knockout cassette was determined by restriction enzyme analysis.

The plasmid pDM602 contains *HAP43* that was generated by the PCR amplification of the *Candida albicans* orf19.681 by PCR with the oligonucleotide primers oDM0394 and oDM0395 that incorporated unique BamHI and SalI restriction sites. The BamHI/SalI digested PCR product was cloned into BamHI/SalI digested pSP65 to generate pDM801. The plasmid pDM801 was subsequently used as a template for PCR with primers oDM0590/oDM0591 that created a PCR amplified plasmid with approximately 1000bp of *HAP43* noncoding flanking sequence and a unique BglII restriction site on the 5' and 3' ends. The DNA was digested with BglII and ligated with the BamHI/BglII DNA fragment containing *hisG-URA3-hisG* from the plasmid p5921. The orientation of the *hisG-URA3-hisG* cassette was determined by restriction enzyme analysis and the plasmid designated pDMAAA.

For construction of pDM802 containing the *CTA1* promoter fused to the *Renilla* luciferase *(Rluc*), a 1kb region of *CTA1*, upstream of the start codon of orf19.13609 was amplified by PCR using the oligonucleotide primers oDM0660/oDM0661, which incorporated

unique SphI and BamHI sites into the 5' and 3' ends of the PCR product, respectively. *CTA1* promoter was digested with SphI/BamHI and cloned into the SphI/BamHI sites of pDM692. The *Rluc* was obtained from pDM659 by digestion with BamHI/PvuII and cloned into the BamHI/PvuII sites of pDM692 to generate the *CTA1-Rluc* fusion.

Construction of *Candida albicans* strain

All DNA transformation procedures were performed using the lithium acetate transformation kit (QBiogene, Inc.) per the manufacturer instructions. The construction of the *hap2Δ/hap2Δ, hap31Δ/hap31Δ, hap32Δ/hap32Δ* and the *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutants was described elsewhere (78). The *hap41Δ* homozygous mutant DMC190 was generated as follows. Two consecutive rounds of transformation of the parent strain BWP17 were performed using the *hap41Δ*::*URA3* and *hap41Δ*::*HIS1* disruption alleles described above. The *hap41Δ*::*URA3* was released from plasmid pDM592 by digestion with BamHI/HindIII and introduced into BWP17 via transformation, and selected on SC-Ura medium. To verify the *HAP4.1/ hap41Δ*::*URA3* heterozygote, genomic DNAs were isolated from multiple transformants as described previously (56), and PCR was used to confirm the appropriate recombination. For PCR, oligonucleotide primers oDM0369 (anneals within the *URA3* gene) and oDM0620 (anneals to *HAP41* loci upstream of the recombination) were used. The *HAP4.1/hap41Δ*::*URA3* heterozygote was subsequently transformed with BamHI/HindIII-digested pDM598 containing the *hap41Δ*::*HIS1* allele as described above, and transformants were selected on SC-His medium. The transformants were subsequently tested on SC-His-Ura medium to verify recombination at the *HAP41* locus versus the *hap41Δ*::*URA3* locus. Genomic DNAs were prepared from His+ Ura+ transformants, and PCR was used to verify the correct

recombination, using oligonucleotide primers oDM0370 (anneals within *HIS1*) and oDM0620. The *hap42Δ* homozygous mutant DMC350 was generated as follows. *Candida albicans* BWP17 was subjected to two consecutive rounds of DNA transformation with the *hap42Δ::hisG-URA3-hisG* cassette after release of the knockout cassette from pDM800 by digestion with BamHI/HindIII. Transformants were selected on SC-Ura medium. To confirm deletion of the first copy of *HAP42*, genomic DNA was isolated from multiple transformants and PCR with oligonucleotides primers oDM0617 (anneals to *HAP42* loci upstream of the recombination) and oDM0369 (anneals within *URA3*) was used to verify the correct recombination. Following confirmation, the *HAP42/hap42Δ::hisG-URA3-hisG* heterozygote was grown on 5-fluoroorotic acid (5-FOA) medium to select from Ura- recombinants. The transformation was repeated for the deletion of the second allele of *HAP42* and the *hap42Δ* homozygous mutant confirmed by PCR with same oligonucleotide primers. The *hap43Δ/hap43Δ* strain DMC351 was generated in a similar manner using the *hap43Δ::hisG-URA3-hisG* cassette after release of the cassette from pDM803 by digestion with BamHI and SalI. The gene disruptions were verified using oligonucleotide primers oDM0396 (anneals to the *HAP43* loci upstream of recombination) and oDM369 (anneals within *URA3*). The *hap42Δ/hap42Δ hap43Δ/hap43Δ* double mutant was constructed by disrupting *HAP43* using the *hap43Δ::hisG-URA3-hisG* in the *hap42Δ* homozygote DMC350 after selection for loss of *URA3* on 5-FOA medium. The *hap41Δ/ hap41Δ hap42Δ/ hap42Δ* and the *hap41Δ/ hap41Δ hap43Δ/ hap43Δ* double mutants were generated from the *hap42Δ* (DMC350) and *hap43Δ* (DMC351) strains, respectively after selection for loss of *URA3* on 5-FOA medium. Both of these strain were transformed with the *hap41Δ::URA3* and the *hap41Δ::HIS1* disruption constructs sequentially and the resulting disruption was confirmed by PCR as outlined above. The *hap41Δ/ hap41Δ hap42Δ/ hap42Δ hap43Δ/ hap43Δ* triple mutant

was generated using the *hap42Δ/ hap42Δ hap43Δ/ hap43Δ* strain DMC352 and disrupting the *HAP41* with *hap41Δ::URA3* and the *hap41Δ::HIS1* disruption constructs sequentially as described above. All of the final strains were confirmed by Southern blot analysis as previously described (68). Following strain constructions, any remaining auxotrophies were rescued by lithium acetate transformation. For *arg4* auxotrophy, the plasmid pDM583 was linearized with HpaI within *ARG4* and transformed into the appropriate strains. For the *ura3* and *his1* auxotrophies, the plasmid pDM605 (containing *URA3* and *HIS1*) was linearized with NruI within *HIS1* and introduced into the appropriate strains. The final prototrophic strains were confirmed by growth on synthetic minimal medium.

Candida albicans strains expressing the *CAT1*-Rluc reporter pDM800 were generated by linearizing pDM800 with HpaI within *ARG4* and introducing the plasmid into the appropriate strains containing only the *arg4* auxotrophy as indicated. The resulting transformants were selected on SC-Arg and multiple transformants of each strain were used for the luciferase assays.

Southern blot analysis

Candida albicans strains were grown to saturation in either YPD medium for iron replete conditions or YPD+ 200μM BPS for iron limiting medium. Genomic DNA from all the mutant strains were extracted using phenol chloroform as previously described (56). To confirm the *HAP42* disruption, genomic DNA from the respective strains were digested with SpeI, resolved by electrophoresis on 0.8% agarose gels, and transferred to GeneScreen Plus membranes (NEN Life Sciences Products). The *HAP42* probe was obtained after digestion of pDM575 with BamHI and SphI, thus releasing the sequence homologous to the 5' end of the non-coding region of the *HAP42* gene.

To confirm the *HAP43* disruption, genomic DNA from the respective strains was digested with EcoRV. The *HAP43* probe was obtained after digestion of pDM606 with BamHI and EcoRI and the fragment thus obtained was homologous to the 5' end of the non-coding sequence of *HAP43*. The digested genomic DNAs of all the samples were separated using a 0.8% agarose gel and transferred to GeneScreen Plus membranes (Dupont-NEN Research products) according to manufacturer's protocol. The membranes were hybridized and washed under standard high-stringency conditions. The probes were purified by agarose gel electrophoresis and GeneClean (Qbiogene, Inc.) and subsequently radiolabeled with [α-^{32}P] dATP (MP Biomedicals, LLC) by use of a random primer labelling kit (U.S. Biochemicals) according to the manufacturer's protocol. The size of the DNA fragments hybridized were visualized on a Molecular Dynamics PhosphoImager.

Northern blot analysis

Candida albicans strains were grown to saturation in YPD for iron replete conditions or YPD+200µM BPS for iron-limiting conditions and subsequently inoculated into the respective medium and grown till OD_{600nm} of 0.5-0.8 at 30^0C. The cells were harvested by centrifugation, and total RNA was prepared by the glass bead-acid phenol method as previously described (8). Approximately 20µg of total RNA was loaded (except for probing with *HAP31*/*HAP32* where 40µg of the total RNA was loaded) , separated by formaldehyde-1% agarose gel electrophoresis, and transferred to GeneScreen Plus membranes (Dupont-NEN Research products) according to manufacturer's protocol. The membranes were hybridized and washed under standard high-stringency conditions (121). The *CTA1*, *CYC1* and *26s rRNA* probes for hybridization were obtained by PCR amplification from *Candida albicans* genomic DNA using the primer pairs

oDM0621/oDM0622, oDM0341/oDM0342, oDM0459/oDM0460, respectively. The *SOD1-6* probes were obtained by PCR amplification using oDM0650/oDM0651, oDM0526/oDM0527, oDM0528/oDM0529, oDM0652/oDM0653, oDM0654/oDM0655 and oDM0656/oDM0657, respectively. The *GRX2*, *GRX3*, *GRX5* and *TRX1* probes were obtained by PCR amplification using oligonucleotide pair oDM0665/oDM0666, oDM0532/oDM0533, oDM0530/oDM0531 and oDM0534/oDM0535, respectively. The *HAP31* and *HAP32* probes were obtained using primer pairs oDM492/oDM0493 and oDM0444/oDM0495, respectively. The probes were purified by agarose gel electrophoresis and GeneClean (Qbiogene, Inc.) and subsequently radiolabeled with [α-^{32}P] dATP (MP Biomedicals, LLC) by use of a random primer labelling kit (U.S. Biochemicals) according to the manufacturer's protocol. The transcript levels were quantified on a Molecular Dynamics PhosphoImager.

Renilla luciferase yeast assays

Renilla Luciferase assays were performed using the *Renilla* Luciferase reporter assay system (Promega Corp., Madison, WI). For the luciferase measurements, all yeast strains were grown overnight to saturation in YPD (Yeast extract-peptone-dextrose) medium, with or without 0.1 mM bathophenanthroline disulfonate, (BPS) (Sigma) for iron replete or iron limiting growth, *respectively*. The cultures were subsequently diluted and grown to mid-log phase on YPD or YPD + 0.15 mM BPS at 30°C. Cultures were standardized by optical density at A_{600}nm. A 1 ml aliquot of each culture was removed and centrifuged at 14,000 rpm for one min. The supernatant was removed and the cells were resuspended on 100µl of 1X lysis buffer (Promega Corp.), and 100µl of sterile glass beads were added. The samples were vortexed for one min; cooled on ice for 30 seconds, and vortexed for another one min. The samples were centrifuged for one minute

at 14,000 rpm and an aliquot of 10µl of the lysate was used for assaying luciferase activity. For the luciferase assay, 10µl was added to a luminometer along with 100µl of Renilla luciferase substrate and luminescence was measured (Turner designs model TD-20/20). The final *Renilla* Luciferase activity was calculated with the following formula: $RLA = RLU/OD*(Va*Vc/Vb)$, where RLA is *Renilla* Luciferase activity, RLU is the initial *Renilla* Luciferase luminescence reading, OD is optical density of the cell culture at A_{600}nm, Va is the volume of sample placed in the luminometer tube (0.01ml), Vb is volume of lysis buffer (0.1ml), Vc is volume taken from original culture (1ml).

Catalase enzymatic assays

Catalase enzymatic activity was determined by monitoring the decomposition of hydrogen peroxide spectrophotometrically at 240nm as previously described (144). The cells were grown to saturation in YPD or YPD + 200 µM BPS medium, the cultures were subsequently diluted in YPD or YPD + 150 µM BPS and grown to an OD_{600nm} of 0.5 to 0.8. The cells were harvested by centrifugation at 14,000 X *g* and washed with water and the cell pellets weighed. The pellet was suspended in 50 mM potassium phosphate buffer (pH 7.2) containing 0.2 mM phenylmethylsulfonylfluoride (PMSF) such that the final cell concentration was 0.25 gm of wet cells/ml. The cells were then disrupted using the Mini-Beadbeater (Biospec products) in the presence of 0.5 mm glass beads and subsequently centrifuged at 14,000 X *g* to obtain cell free lysate for the catalase assay. The total protein concentration of the lysates was determined using the Bradford's protein assay (Biorad). For the catalase activity assay, the Beckman-Coulter DU 800 spectrophotometer was zeroed using 560 µL of 50mM potassium phosphate buffer containing 40 µl of the cell lysate. Following the addition of 400 µl of 30% w/w hydrogen peroxide (Sigma), the decomposition of hydrogen peroxide was determined by

measuring the continuous decrease in absorbance at 240 nm and the activity was calculated from the linear range of the curve. The catalase activity of each strain was proportional to the amount of hydrogen peroxide decomposed as determined by: $\Delta\mu mol\ H_2O_2/min./\mu g$ cell lysate $= \Delta A_{240}$ / (1.5min X 39.7$m^{-1}cm^{-1}$ X 1000 X μg cell lysate), where the $\Delta\mu mol\ H_2O_2$ is the change in micromoles of hydrogen peroxide per min per microgram lysate, ΔA_{240} is the change in absorbance at 240nm, 39.7$m^{-1}cm^{-1}$ is the molar extinction coefficient of hydrogen peroxide at 240 nm.

Hydrogen peroxide sensitivity assays

Each strain evaluated was grown to saturation in YPD or YPD + 200 μM BPS medium, the cultures were subsequently diluted in YPD or YPD + 150 μM BPS, respectively and grown to an OD_{600nm} of 0.5 to 0.8. The cells were harvested by centrifugation for 1 min. at 14,000 X *g*, washed twice with sterile deionized water, and quantified using a hemocytometer. Approximately 1 $X10^7$ cells of each strain were suspended in YPD medium containing 0, 40, and 80 mM hydrogen peroxide and incubated for 2 h at 30ºC. To assess the hydrogen peroxide sensitivity, ten-fold serial dilutions were subsequently plated on YPD medium and incubated at 30ºC.

4. Results

Confirmation of strains using southern blot

The *HAP42* gene in *Candida albicans* genome has two SphI site. After digestion of genomic DNA with SphI, the strain with the wild type gene for *HAP42* would yield among other sized fragments, a 4.5kb band. However the strain disrupted for *HAP42* would yield a 6kb band due to the presence of the *hisG* sequences from *Salmonella typhimurium*.

To confirm *HAP43* disruption, the genomic DNA was digested overnight with EcoRV. For the strains with the WT gene for *HAP43*, a 4.7kb band was expected and for the strains with disrupted *HAP43*, a 1kb band size was predicted as *hisG* sequence from *Salmonella typhymurium* itself has an EcoRV site.

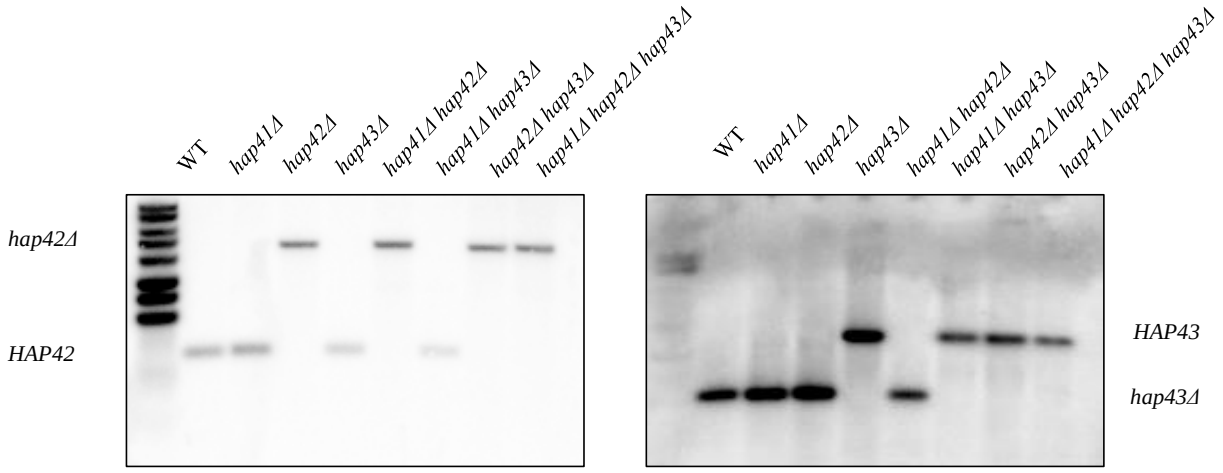

Figure 1. Verification of various *hap4* deletion strains using southern blot.

Southern blot analysis of genomic DNA isolated from the indicated *Candida albicans* strains grown at 30ºC. The membrane was hybridized using radiolabeled probes specific for *HAP42* and *HAP43* as indicated**.**

CCAAT-Binding factor mediated regulation of *CTA1*.

Since the CCAAT-binding factor is known to function as both a transcriptional activator and repressor of genes involved in respiratory metabolism, we predicted that this transcription factor may also be involved in the regulation of oxidative stress response, which is required to protect the cells from ROS generated as a byproduct of respiration or by iron via the Fenton's reaction (111). Indeed, a microarray performed in our lab under low iron conditions showed a fourfold up regulation of *CTA1* in the strain with the homozygous null deletion for the gene encoding the Hap5 subunit (*hap5Δ*), thus lacking the transcription factor as compared to the strain with the transcription factor (WT) (data not shown).

To validate this, we performed northern blots on WT and *hap5Δ* strains in both iron replete (YPD) and iron deficient (BPS) media (Figure 2). The *hap5Δ* strain showed at least a two-fold down regulation of *CTA1* mRNA level as compared to the WT in iron-replete conditions. In contrast, the *hap5Δ* strain showed an up regulation of the *CTA1* mRNA with respect to the WT under iron starvation.

Consistent with the northerns, the *Renilla* luciferase activity of the *hap5Δ* was less than the WT in iron replete medium (Figure 3). Under iron limiting conditions, the luciferase activity was found to be threefold more in the strain lacking the transcription factor as compared to the WT.

Since many genes involved in the OSR are regulated both at the transcriptional and post transcriptional level, we next asked if a similar pattern of *CTA1* regulation exists at the enzyme activity level, as catalase has an iron containing porphyrin cofactor. Catalase activity levels in the WT and *hap5Δ* were measured in both, iron replete and iron deplete media by following the spectrophotometric decomposition of hydrogen peroxide at 240 nm wavelength (Figure 4).

Catalase activity was significantly higher in WT than *hap5Δ* strain when cells were grown in iron sufficient media. In iron limiting conditions, activity was upregulated by two folds in the *hap5Δ* versus the WT.

Next, we wanted to evaluate the overall role of the CCAAT-binding factor towards the oxidative stress response of *Candida albicans* in both YPD and BPS conditions, respectively. To this end, we performed peroxide sensitivity assays with WT and *hap5Δ* under both iron sufficient and iron deficient conditions (Figure 5). As shown in the figure, *hap5Δ* was more susceptible to 40mM and 80mM hydrogen peroxide than WT when iron was present in sufficient quantities. However, under iron deprivation, the *hap5Δ* mutant strain was more resistant to both 40mM and 80mM of hydrogen peroxide as compared to the WT.

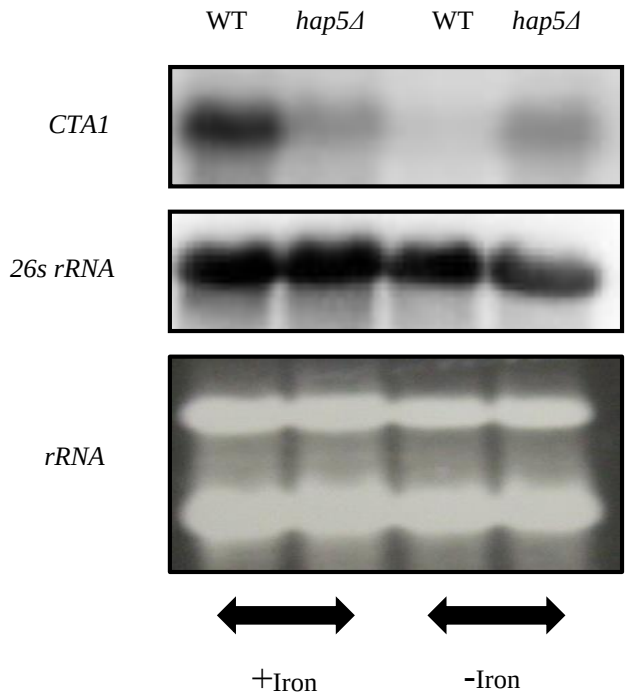

Figure 2. CCAAT-Binding factor regulates *CTA1* differentially based on iron availability.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD+BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *CTA1* as indicated. *26s rRNA* was used to normalize results.

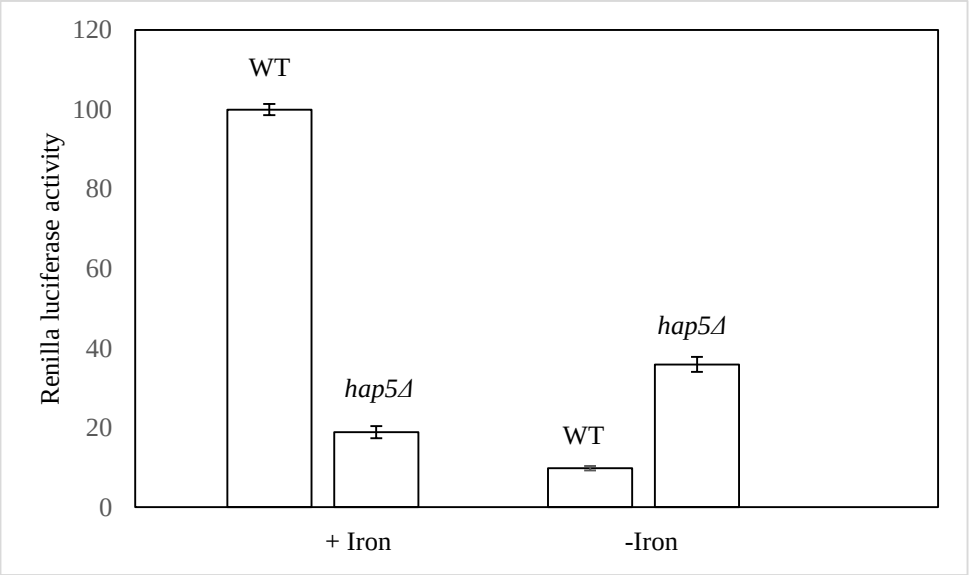

Figure 3. Iron based differential regulation of *CTA1* gene.

Candida albicans strains were transformed with plasmid containing the promoter of *CTA1* gene fused upstream of the Renilla luciferase gene. Strains were either grown in +Iron (YPD) or –Iron (YPD+200µM BPS) media. Error bars represent the standard error of mean from three independent assays.

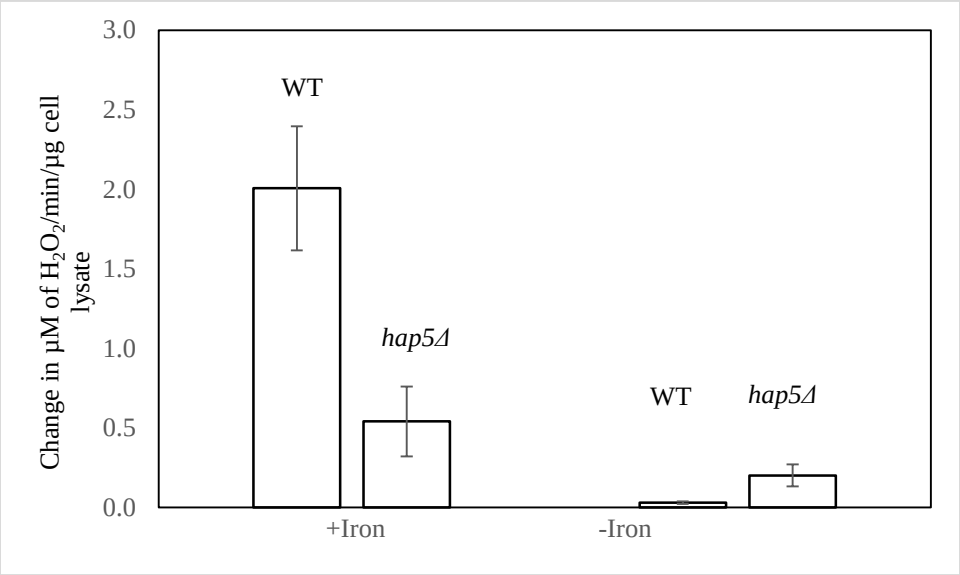

Figure 4. The differential regulation of catalase activity levels by CCAAT-Binding factor.

The catalase activity levels of the *Candida albicans* strains indicated were determined following the spectrophotometric decomposition of hydrogen peroxide at 240nm for five minutes. Strains were either grown in +Iron (YPD) or –Iron (YPD+200µM BPS) media. Error bars represent the standard error of mean from three independent assays.

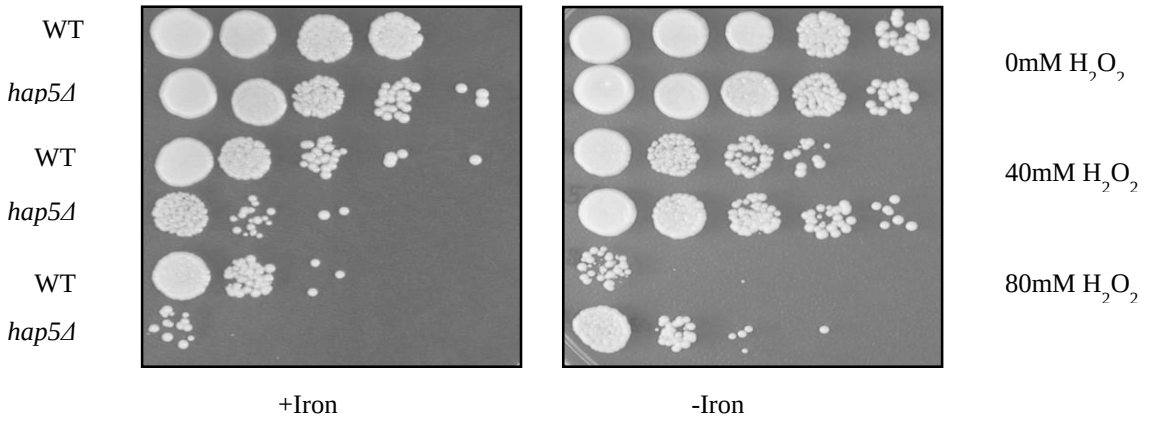

Figure 5. The differential regulation of oxidative stress response by the CCAAT-Binding factor is dependent on iron.

The *Candida albicans* strains indicated above were grown in either +Iron or –Iron media and subsequently exposed to 40mM or 80mM of hydrogen peroxide for two hours at 30ºC. 10-fold dilutions were spot plated to the indicated medium and incubated for 24 hours.

Hap mediated expression patterns of *CaSODs, GRXs* and *TRX1*

We next asked if the CCAAT-binding factor is involved in the transcriptional regulation of other genes involved in the OSR including superoxide dismutases (*SOD*s), glutaredoxins (*GRX*s) and thioredoxin (*TRX1)*. *Candida albicans* has six different isoforms of superoxide dismutases (Sods) of which Sod2 is the only mitochondrial Sod. We observed a slight overexpression of the *SOD2* mRNA in the *hap5Δ* as compared to the WT when the cells were grown in YPD (Figure 6*)*. However, this overexpression was much more pronounced in the *hap5Δ* vs the WT when the cells were exposed to BPS.

The cytoplasmic *SOD3* showed a similar expression pattern as that of *SOD2* with significantly more *SOD3* expression in the *hap5Δ* than the WT for both YPD and BPS. The

cytoplasmic *SOD1* expression was also upregulated in the strain lacking the transcription factor for both iron sufficient and deficient conditions (Figure **7**).

None of the cell surface Sods were expressed in either WT or *hap5Δ* strain when grown in YPD. However, we observed at least a tenfold up regulation of *SOD4* mRNA in *hap5Δ* when the cells were grown in BPS (Figure 8).

We next analyzed the CCAAT-binding factor mediated expression patterns of the *CaGRX*s. In spite of our repeated attempts we did not see any iron dependent or independent expression of *GRX1* in either the WT or *hap5Δ* for both YPD and BPS conditions. *GRX2* was found to be significantly upregulated in the *hap5Δ* with respect to the WT when the cells were grown in iron sufficient medium. Under iron deprivation, however, its expression appeared to be independent of the Hap complex. We found a constitutive Hap and iron independent expression pattern for *GRX3* in both YPD and BPS. Interestingly, the expression pattern of *GRX5* was found to be the same as *CTA1*. *GRX5* expression was significantly down regulated in the strain lacking the transcription factor as compared to the WT strain when the cells were grown in YPD medium. In BPS, the *hap5Δ* showed at least a twofold up regulation of *GRX5* with respect to the WT (Figure 9).

We observed a significant up regulation of *TRX1* in the *hap5Δ* than the WT when the cells were grown in YPD (Figure 10). However, the expression of *TRX1* was at least two folds down regulated in the *hap5Δ* strain vs WT in BPS medium. Thus the expression pattern of *TRX1* was exactly the opposite of *CTA1* and *GRX5*.

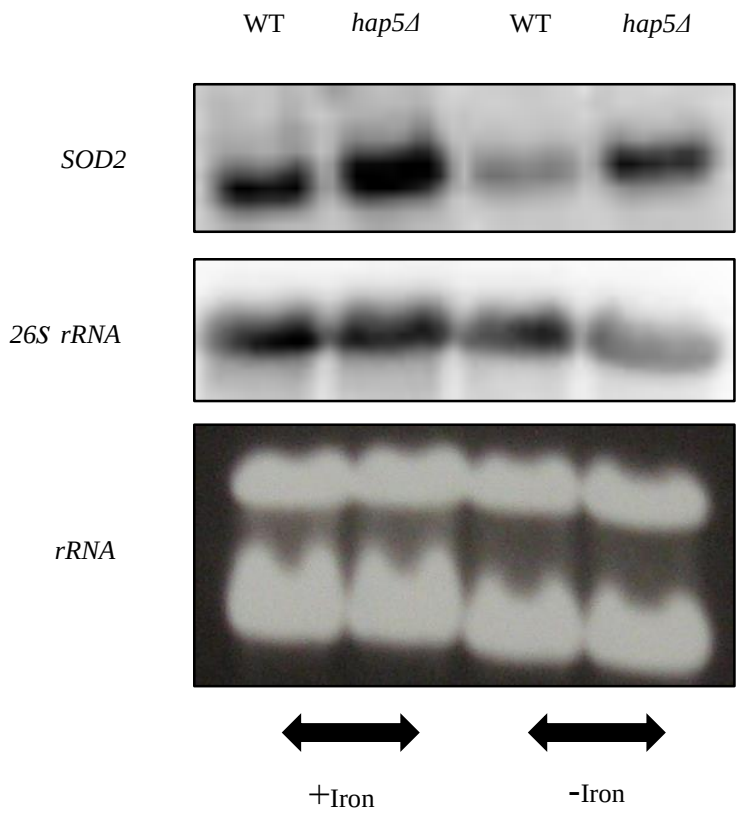

Figure 6. The CCAAT-Binding factor mediated regulation of mitochondrial *SOD2* in *Candida albicans*.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD+200µMBPS) as shown. The membrane was hybridized using radiolabeled probes specific for *SOD2* as indicated. *26s rRNA* was used to normalize results.

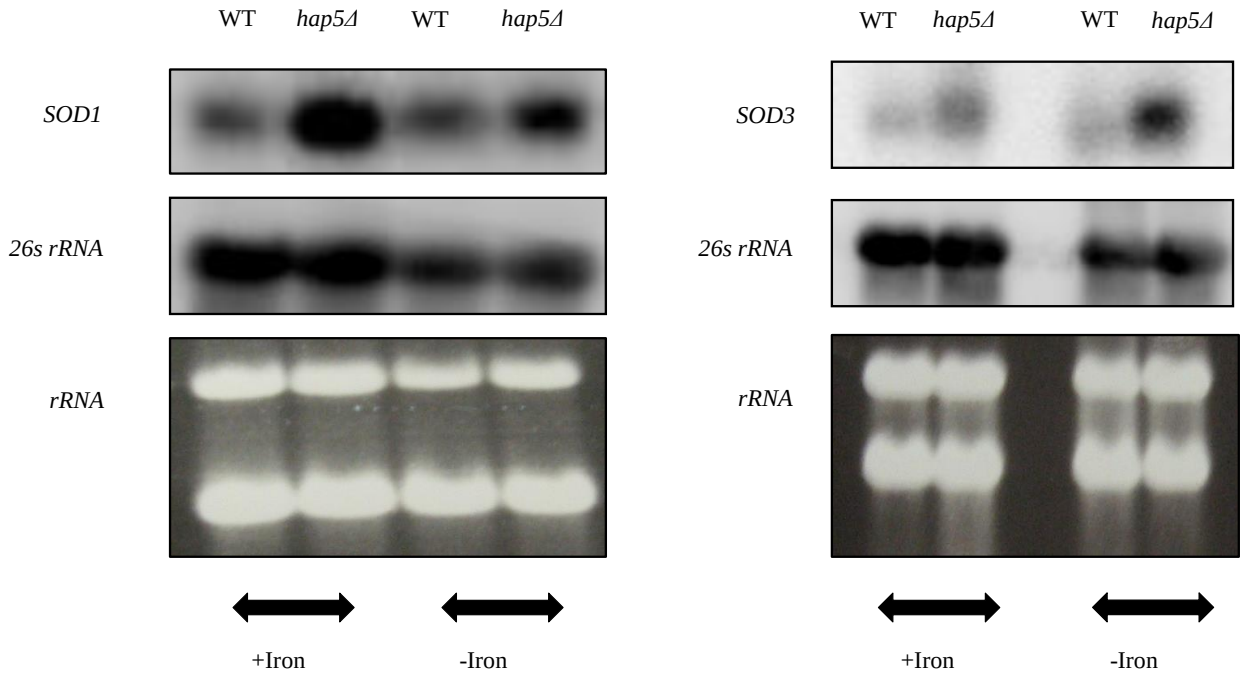

Figure 7. The cytoplasmic *SODs* are repressed by the CCAAT-Binding factor irrespective of the presence or absence of iron.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD +200µM BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *SOD1* and *SOD3* as indicated. 26s rRNA was used to normalize results.

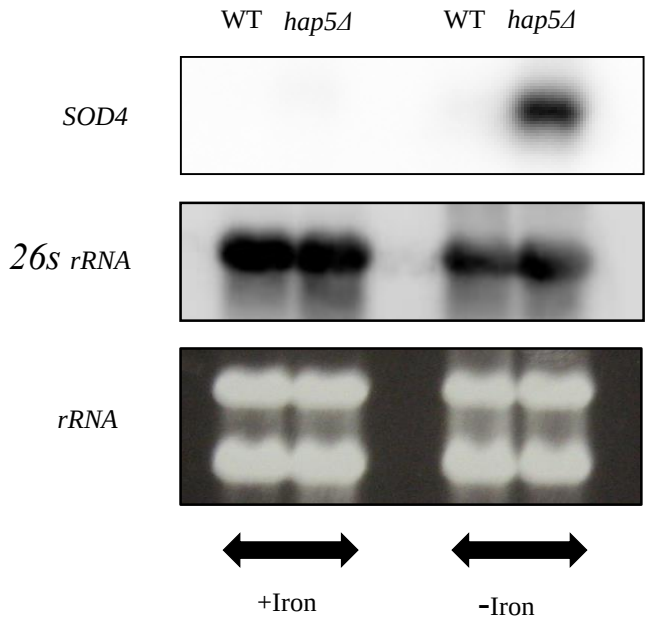

Figure 8. The cell surface associated *SOD4* is repressed by the CCAAT-Binding factor under low iron conditions.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD +200µM BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *SOD4* as indicated. *26s rRNA* was used to normalize results.

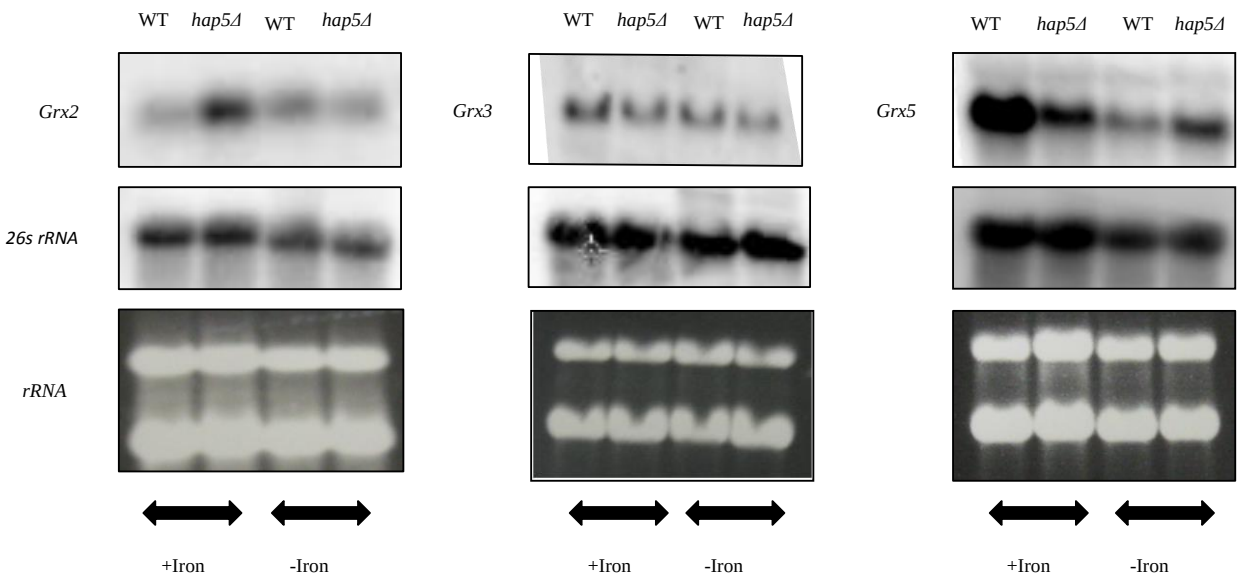

Figure 9. CCAAT-Binding factor dependent regulation of glutaredoxins in *Candida albicans.*

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD +200µM BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *GRX2, GRX3* and *GRX5* as indicated. *26s rRNA* was used to normalize results.

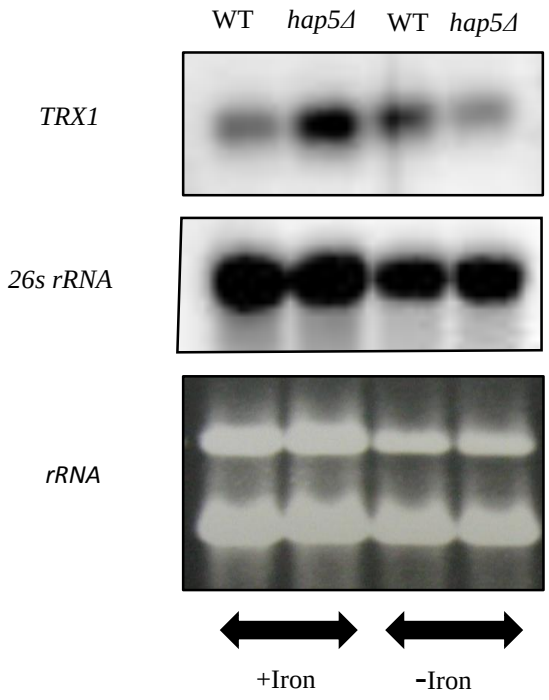

Figure 10. CCAAT-Binding factor dependent regulation of *TRX1* in *Candida albicans*.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD +200µM BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *TRX1* as indicated. *26s rRNA* was used to normalize results.

Role of Hap2p, Hap31p and Hap32p on the iron dependent regulation of the *CTA1*

The CCAAT-binding factor in *Candida albicans* also comprises of the Hap2p as a part of the core DNA binding domain. Previous data from our lab has shown that the homozygous deletion of the gene encoding Hap2p (*hap2Δ*) results in complete abolishment of DNA-binding activity. Hence we predicted the effect of *hap2Δ* on the regulation of *CTA1* to be similar to a *hap5Δ* strain. To validate this, we performed northern blots on WT, *hap2Δ* and *hap5Δ* in both iron sufficient and iron deficient media (Figure 11). The *CTA1* expression was at least two fold down regulated in a *hap2Δ* as compared to the WT in YPD. In BPS, the *hap2Δ* showed a significant over expression of *CTA1* with respect to the WT.

In an effort to understand the role of Hap31 and Hap32 towards regulation of *CTA1*, we performed northern blots on them in both YPD and BPS conditions (Figure 11). In YPD, both *hap31Δ* and *hap32Δ* had *CTA1* mRNA levels more than the WT although the expression level of *hap32Δ* was more than *hap31Δ.* The strain with homozygous deletions in the genes encoding for both Hap31p and Hap32p (*hap31Δ hap32Δ)* had *CTA1* levels comparable to that of *hap2Δ and hap5Δ.* In BPS, the expression level of *CTA1* in *hap32Δ* was same as that of the WT. Interestingly, we observed a subtle yet consistent over expression of *CTA1* in the *hap31Δ* as compared to either WT or *hap32Δ* when the cells were grown in BPS. The *CTA1* expression in *hap31Δ hap32Δ* double deletion strain was significantly over expressed as compared to WT, *hap31Δ* and *hap32Δ* strains.

A similar pattern of *CTA1* expression was obtained with the luciferase assays (Figure 12). In YPD, the *Renilla* luciferase activity of both *hap31Δ* and *hap32Δ* strains was significantly higher than the WT but as observed with the northerns, luciferase activity of *hap32Δ* was at least

two folds more than *hap31Δ* strain. In BPS, both *hap31Δ* and *hap32Δ* had luciferase activity levels comparable to the WT. However *hap31Δ* strain showed slightly more activity levels than both WT and *hap32Δ* which was reproducible in all the three biological replicates. We did not perform luciferase assays on *hap31Δ hap32Δ* as its mRNA expression pattern was found similar to the *hap5Δ* in the northern.

We observed a tenfold down regulation of catalase activity levels in the *hap2Δ* strain as compared to the WT in YPD. In BPS, a tenfold up regulation of the activity levels was detected in *hap2Δ* with respect to the WT. The activity levels of *hap31Δ* and *hap32Δ* was down regulated by more than two folds with respect to the WT strain in YPD (Figure 13). However, both *hap31Δ* and *hap32Δ* had activity levels significantly higher than the *hap5Δ* strain on YPD. In BPS, the activity levels of WT, *hap31Δ* and *hap32Δ* appeared to be less than the *hap5Δ.* Although the *hap31Δ* showed slightly elevated activity levels when compared to the WT, its activity level was nearly two folds higher than a *hap32Δ* in BPS.

In order to understand the global effect of *hap2Δ, hap31Δ* and *hap32Δ* on oxidative stress response in *Candida albicans*, we subjected the exponentially growing cells of the respective strains to 40mM and 80mM of hydrogen peroxide in both YPD and BPS conditions. In YPD, for both the concentrations used, the *hap2Δ* strain displayed enhanced susceptibility to peroxide compared to the wild type strain. In BPS, the mutant displayed enhanced viability compared to the wild-type strain after peroxide exposure, as shown in Figure 14. As shown in Figure 15, no difference in the sensitivities of *hap31Δ* and *hap32Δ* strains were observed with respect to the wild type after exposure to various concentrations of hydrogen peroxide in YPD. Interestingly, in BPS, the viability of *hap32Δ* strain on exposure to peroxide was similar to the wild-type

strain. The *hap31Δ* strain however displayed higher resistance to peroxide stress compared to the wild type when grown in BPS.

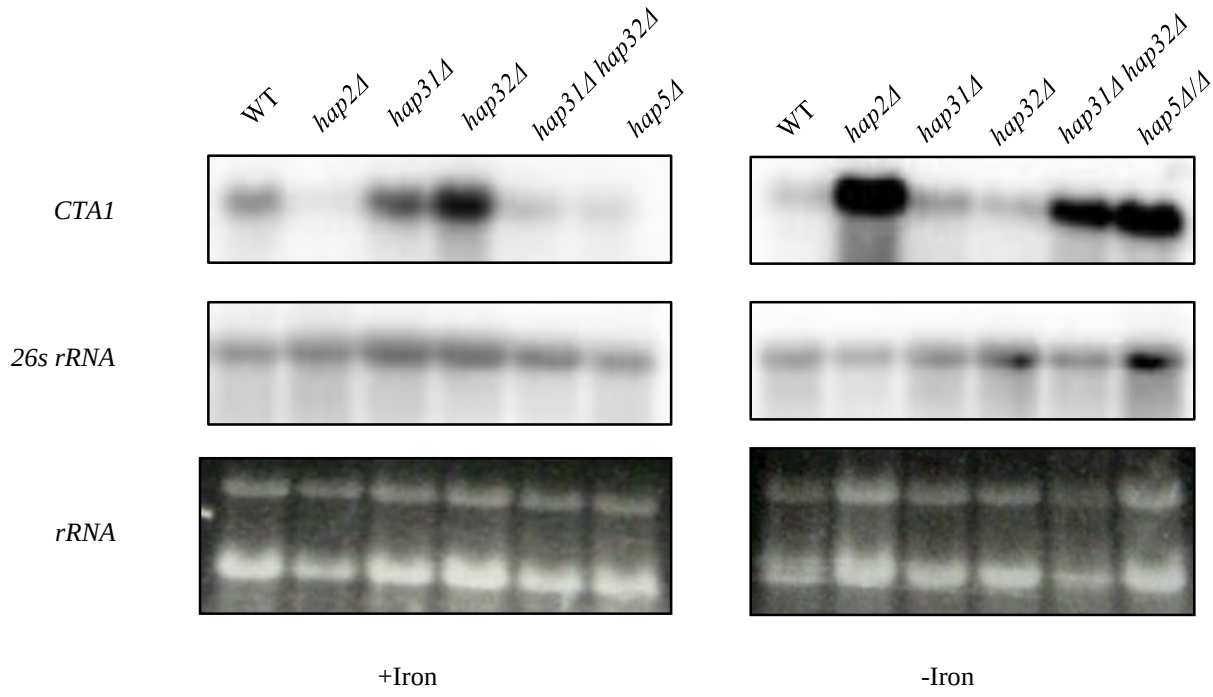

Figure 11. The role of Hap2p, Hap31p and Hap32p in the iron dependent role of *CTA1* in *Candida albicans*.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD +200µM BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *CTA1* as indicated. *26s rRNA* was used to normalize results.

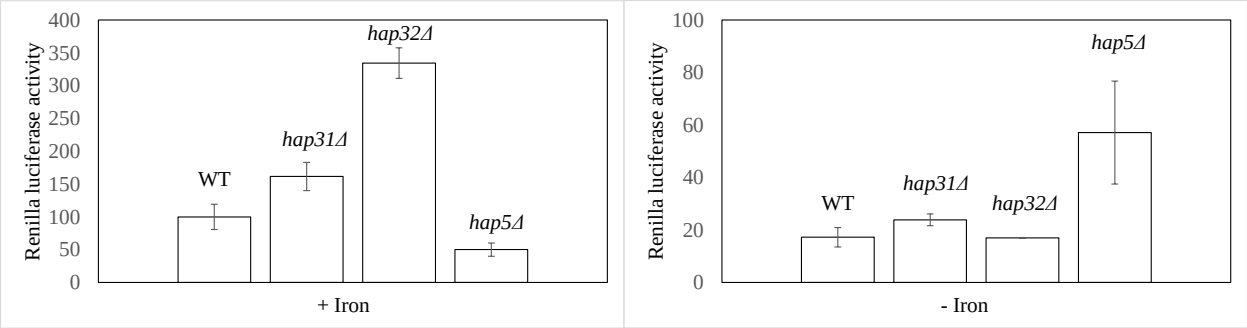

Figure 12. Iron based differential role of Hap31p and Hap32p towards the regulation of *CTA1*gene.

Candida albicans strains were transformed with plasmid containing the promoter of *CTA1* gene fused upstream of the Renilla luciferase gene. Strains were either grown in +Iron (YPD) or –Iron (YPD+200µM BPS) media. Error bars represent the standard error of mean from three independent assays.

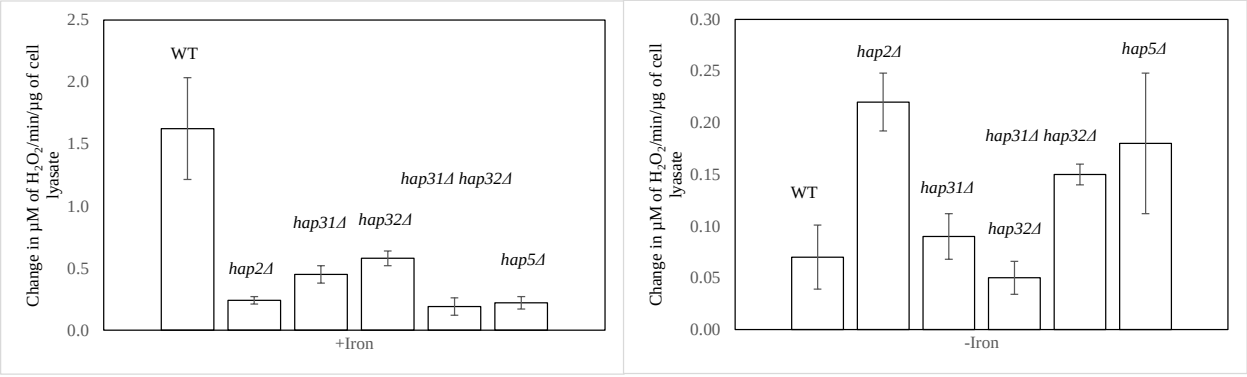

Figure 13. Catalase activity levels in *hap2Δ* and different *hap3* knockout mutants.

The catalase activity levels of the *Candida albicans* strains indicated were determined following the spectrophotometric decomposition of hydrogen peroxide at 240nm for five minutes. Strains were either grown in +Iron (YPD) or –Iron (YPD+200µM BPS) media. Error bars represent the standard error of mean from three independent assays.

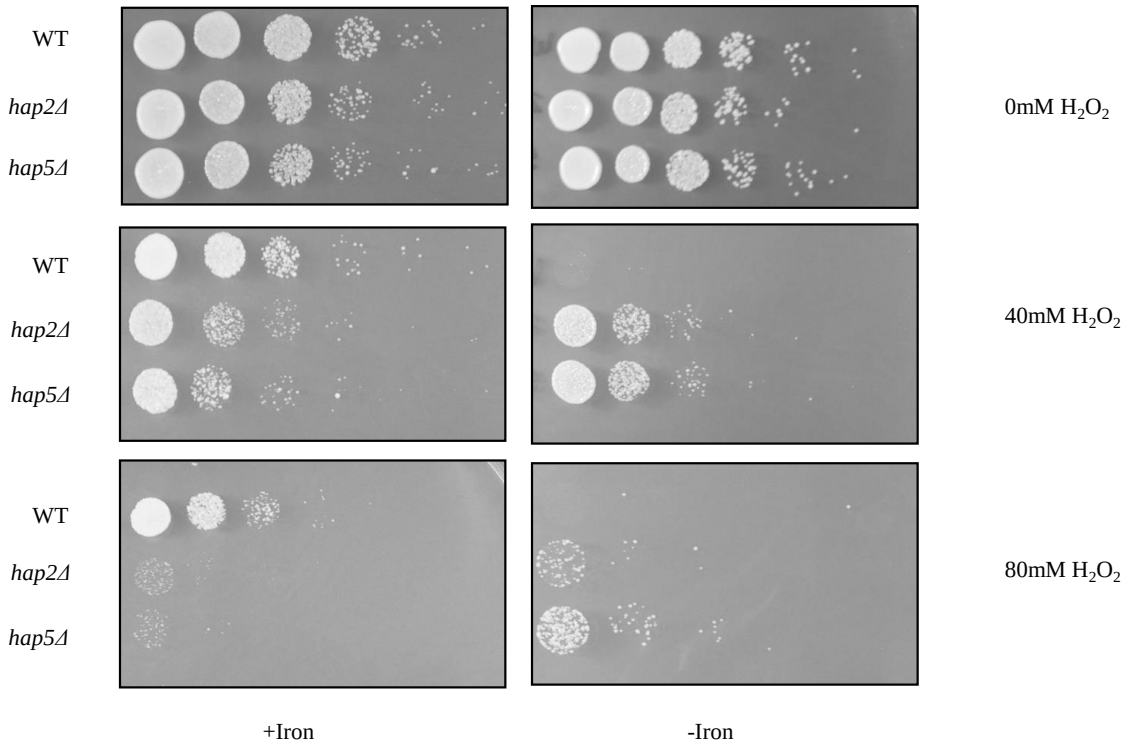

Figure 14. Role of *Candida albicans* Hap2 in the oxidative stress response.

The *Candida albicans* strains indicated above were grown either in +Iron or –Iron media and subsequently exposed to 40mM or 80mM of hydrogen peroxide for two hours at 30ºC. 10-fold serial dilutions were then spot plated on YPD plates and incubated for 24 hours.

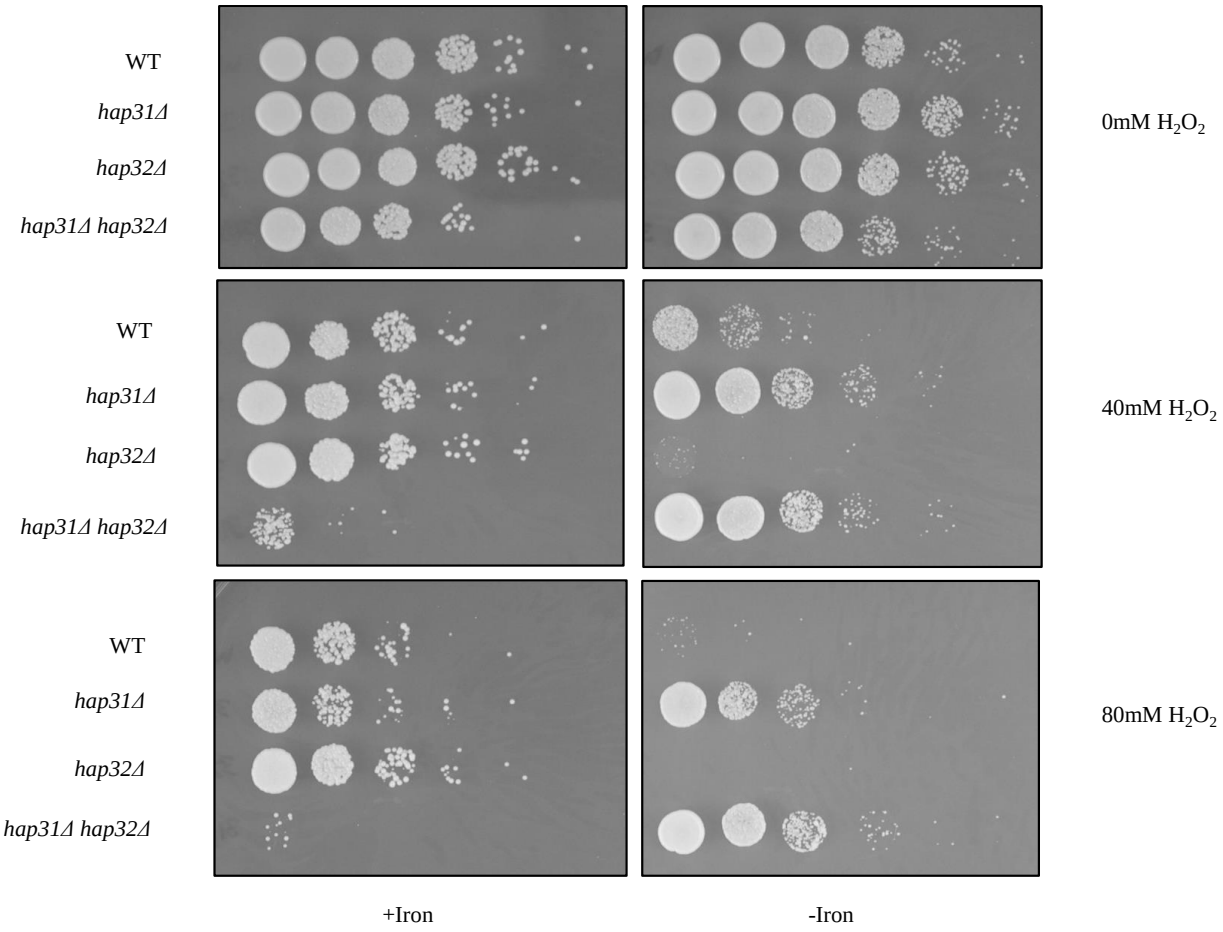

Figure 15. Role of *Candida albicans* Hap31 and Hap32 in the oxidative stress response.

The *Candida albicans* strains indicated above were grown in either +Iron or –Iron media and subsequently exposed to 40mM or 80mM of hydrogen peroxide for two hours at 30ºC. 10-fold serial dilutions were then spot plated on YPD plates and incubated for 24 hours.

Differential expression of *HAP31* and *HAP32* in response to iron availability

We next asked if the expression of *HAP31* and *HAP32* are themselves affected by the presence or absence of iron. As shown in figure 16, *HAP31* is expressed in both YPD and BPS, however, the expression was more in YPD as compared to BPS. *HAP31* expression was upregulated in a *hap32Δ* strain under both YPD and BPS conditions as compared to their WT strains. Strikingly, the expression of *HAP31* was most pronounced in a *hap5Δ* strain as compared to the WT under both, iron replete and iron deplete conditions, respectively. This suggests that the expression of *HAP31* in *Candida albicans* is auto regulated by the CCAAT-binding factor in both iron sufficient and iron limiting conditions.

HAP32 expression was undetected in WT under iron sufficient conditions. However, when iron was limited, it was expressed in the WT strain. *HAP32* was also found to be auto regulated by the CCAAT-binding factor as suggested by the differential expression of *HAP32* in the *hap5Δ* strain, particularly under iron deficient conditions (Figure 16).

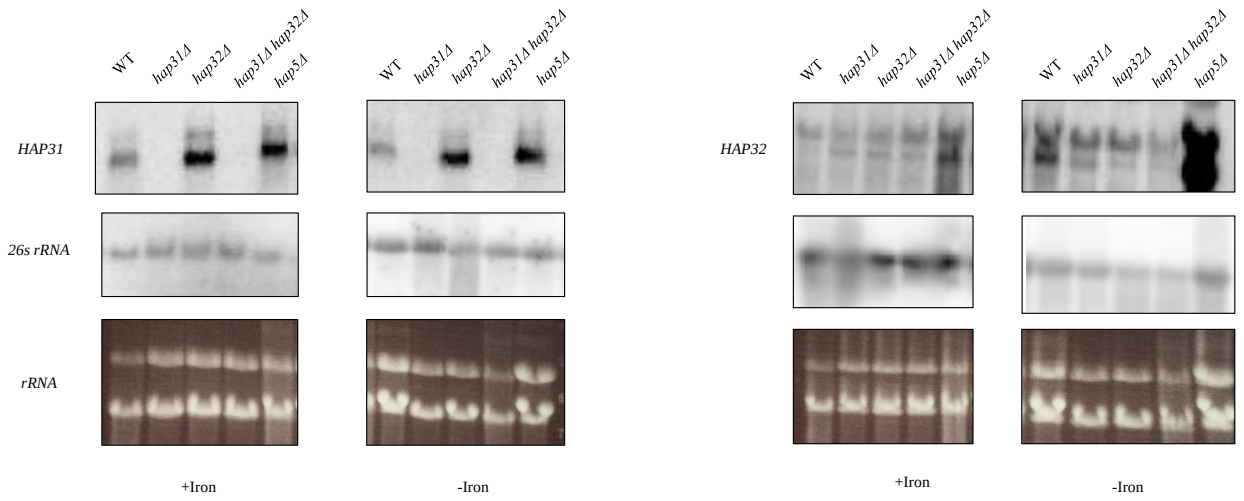

Figure 16. Differential expression of *HAP31* and *HAP32* in *Candida albicans*.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD +200µM BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *HAP31* and *HAP32* as indicated. *26s rRNA* was used to normalize results.

Role of various Hap4 subunits towards regulation of *CTA1* in *Candida albicans*

Candida albicans harbors three distinct homologs of the *Saccharomyces cerevisiae* Hap4p. These three Hap4-like subunits encoded by the *Candida albicans* genome are designated Hap41p, Hap42p and Hap43p respectively. To address the role of each of these subunits towards the iron dependent transcriptional regulation of *CTA1*, we performed northern blots in both YPD and BPS conditions (Figure 17). In YPD, we observed some variability in the expression of *CTA1* mRNA among the different *hap4* single and combination mutants, although, none of the difference in expression levels were significant enough as obtained from three independent northern replicates. Thus, it appeared that none of the Hap4 subunits play an important role towards *CTA1* regulation in iron replete medium. In contrast, the northern blot performed in iron deficient medium clearly suggests that the Hap43 is the only subunit important for repression of *CTA1* in BPS as *hap43Δ*, *hap41Δ hap43Δ*, *hap42Δ hap43Δ* and *hap41Δ hap42Δ hap43Δ* mutant strains had significantly higher expression levels of *CTA1* than the WT.

Similar to the *CTA1* mRNA expression pattern, the catalase activity assay also revealed that the Hap4 subunits do not play a role in the regulation of *CTA1* under iron replete conditions (Figure 18 and Figure 19). Although, the various *hap4* mutant strains displayed less catalase activity levels as compared to the WT, the difference was not found to be statistically significant after analyzing data from three independent biological replicates. The catalase activity levels in iron deficient media was also very consistent with the northern. The expression level of *hap43Δ*, *hap41Δ hap43Δ*, *hap42Δ hap43Δ* and *hap41Δ hap42Δ hap43Δ* mutant strains were around four fold higher than the WT emphasizing that the only relevant Hap4 subunit for *CTA1* repression is Hap43 under iron limiting conditions.

We next examined the sensitivities of various Hap4 mutants towards peroxide stress (Figure 20 and Figure 21). Different homozygous *hap4* mutant strains were exposed to 40 and 80mM hydrogen peroxide for 2 hours at 30 degree Celsius and then spot plated and incubated for 24 hours. As observed in the northerns and catalase enzymatic assays, none of the *hap4* deletion strains impaired the ability of the respective strain to recover from peroxide stress under iron sufficient conditions. These data together suggest that in YPD, none of the Hap4 subunits play a role in the regulation of the catalase gene in *Candida albicans*. On the other hand under iron starvation, only the strains with *hap43Δ* could recover from peroxide stress; most probably due to significantly more amount of catalase than the WT or strains with *hap41Δ* or/and *hap42Δ* as seen previously.

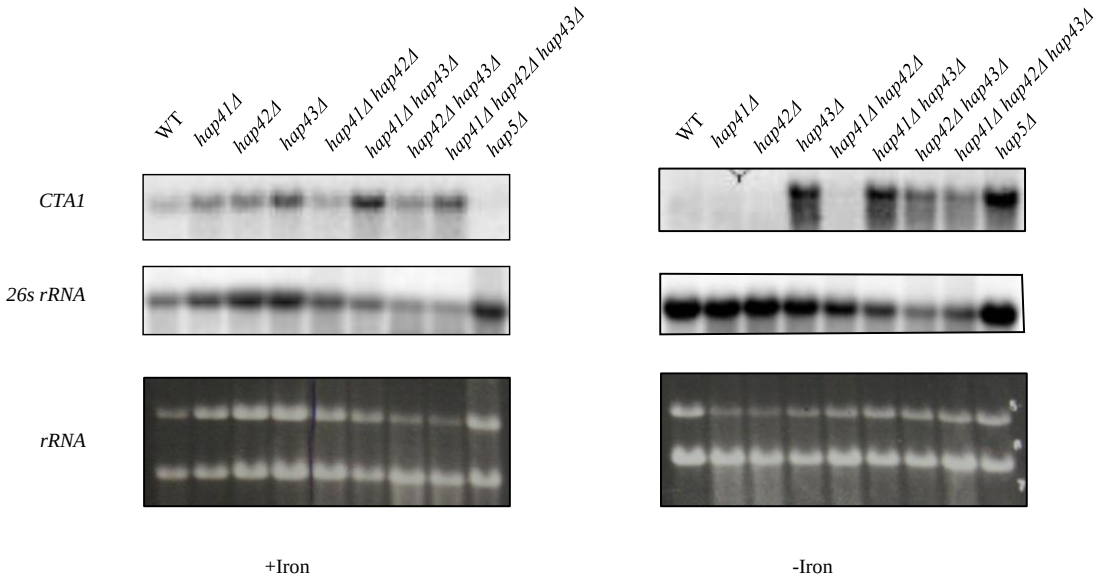

Figure 17. Regulation of *CTA1* mRNA expression by various Hap4 subunits in *Candida albicans*.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD +200µM BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *CTA1* as indicated. *26s rRNA* was used to normalize results.

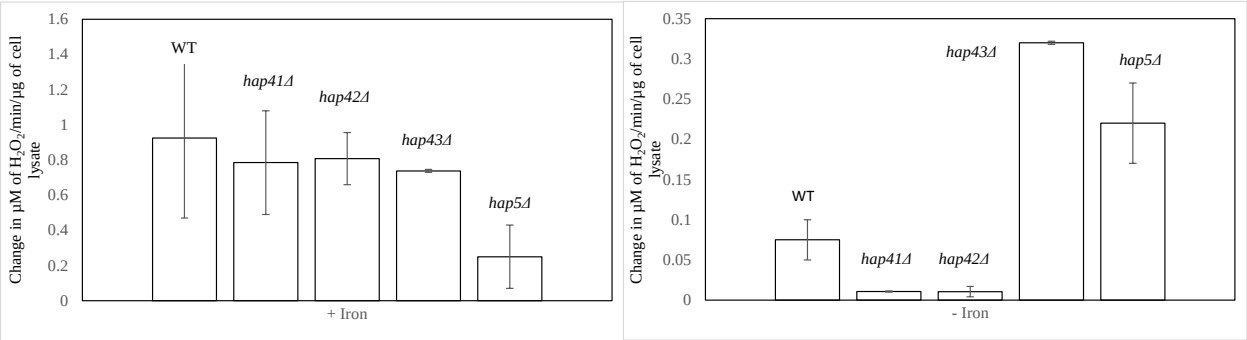

Figure 18. Catalase activity levels of various *hap4* single deletions.

The catalase activity levels of the *Candida albicans* strains indicated were determined following the spectrophotometric decomposition of hydrogen peroxide at 240nm for five minutes. Strains were either grown in +Iron (YPD) or –Iron (YPD+200µM BPS) media. Error bars represent the standard error of means from three independent assays.

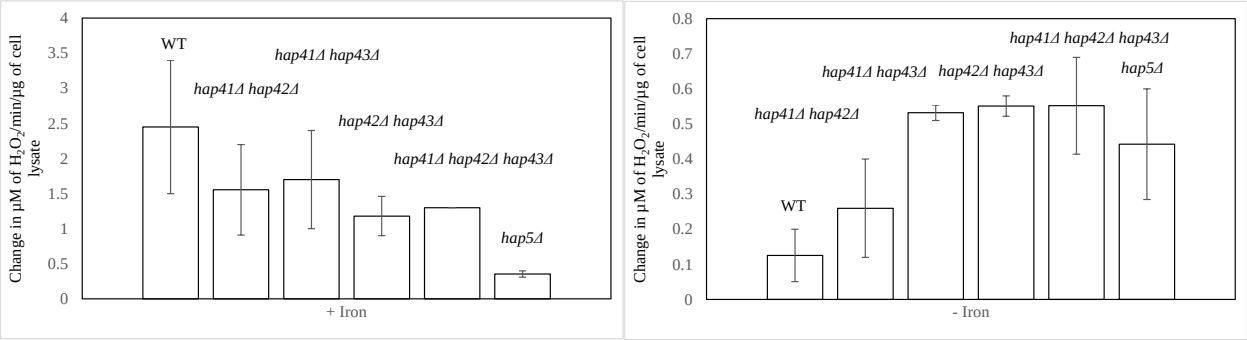

Figure 19. Catalase activity levels of various *hap4* double and triple deletions.

The catalase activity levels of the *Candida albicans* strains indicated were determined following the spectrophotometric decomposition of hydrogen peroxide at 240nm for five minutes. Strains were either grown in +Iron (YPD) or –Iron (YPD+200µM BPS) media. Error bars represent the standard error of means from three independent assays.

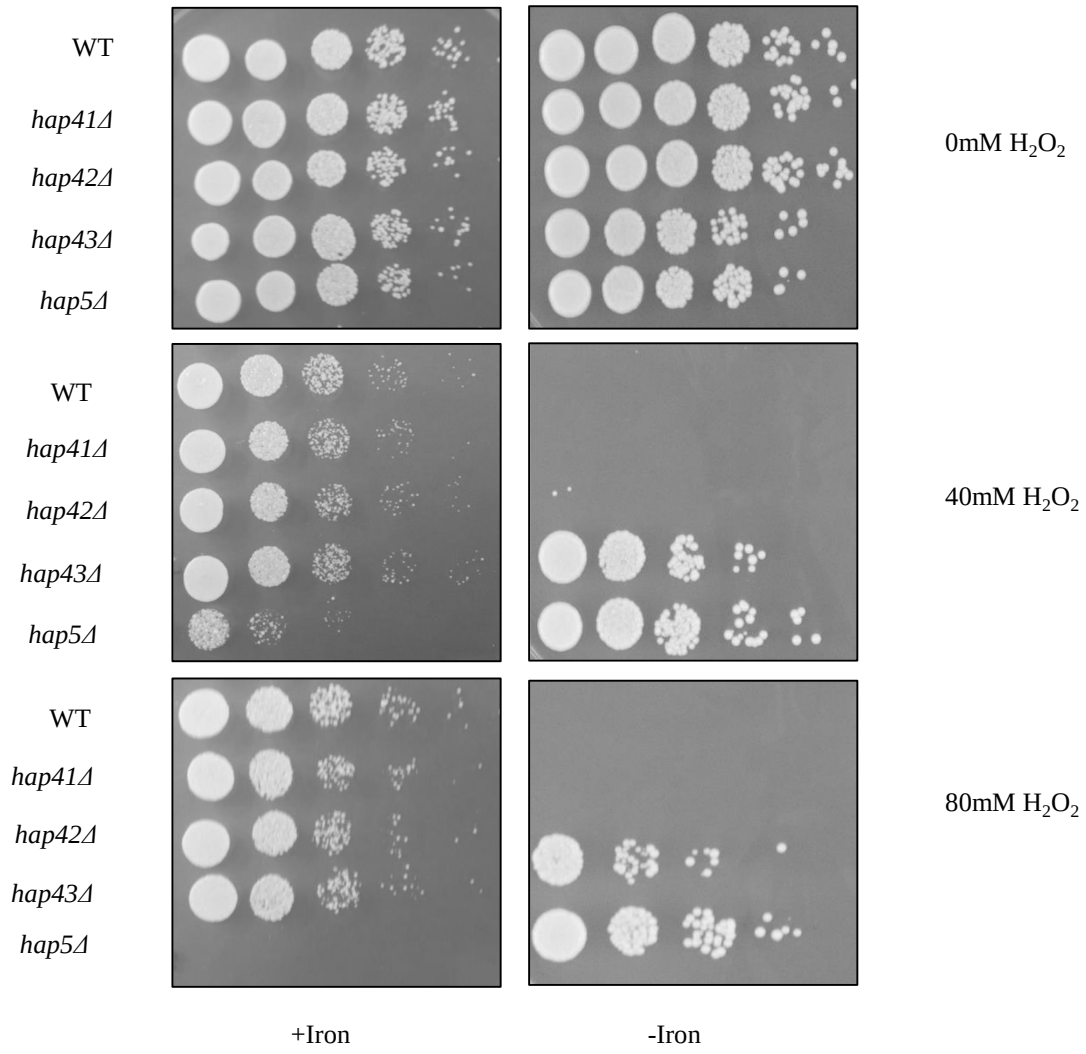

Figure 20. Role of *Candida albicans* Hap4 subunits in the oxidative stress response.

The *Candida albicans* strains indicated above were grown in either +Iron or –Iron media and subsequently exposed to 40mM or 80mM of hydrogen peroxide for two hours at 30ºC. 10-fold serial dilutions were then spot plated on YPD plates and incubated for 24 hours.

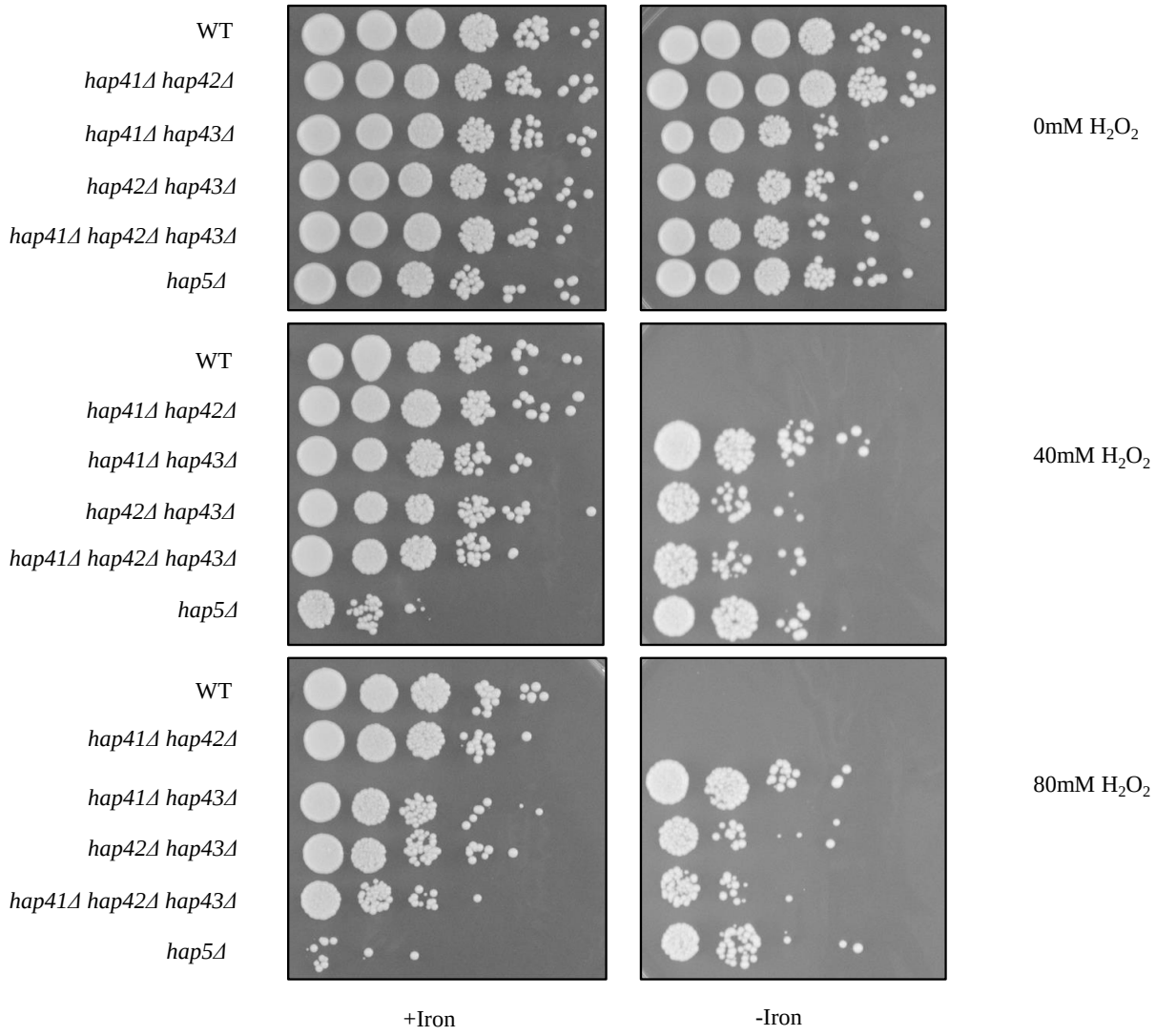

Figure 21. Role of *Candida albicans* Hap4 subunits in the oxidative stress response.

The *Candida albicans* strains indicated above were grown in either +Iron or –Iron media and subsequently exposed to 40mM or 80mM of hydrogen peroxide for two hours at 30ºC. 10-fold serial dilutions were then spot plated on YPD plates and incubated for 24 hours.

Role of various Hap4 subunits on the iron dependent regulation of *CYC1*

CYC1 is the prototype gene studied in our lab to dissect the regulatory function of the CCAAT-binding factor. Previous studies done in our lab has shown that the CCAAT-binding factor is essential for the regulation of *CYC1* in the absence of glucose. It has also been found to be involved in the activation of *CYC1* in iron sufficient medium as a homozygous *hap5Δ* strain and *hap2Δ* strain have at least a twofold downregulated expression level of *CYC1* as compared to the WT in YPD. In contrast, in BPS, the *hap5Δ* and *hap2Δ* strains had significant upregulation of *CYC1* mRNA levels with respect to the WT as seen by northern blots (78).

The expression levels of *CYC1* was downregulated in a homozygous *hap31Δ* and was upregulated in a homozygous *hap32Δ* as compared to the WT in YPD. However, in BPS, the expression levels remained unaffected. A homozygous *hap31Δ hap32Δ* double deletion strain had the same *CYC1* expression pattern as of *hap5Δ* in both YPD and BPS (78).

We next asked the role of the various Hap4 subunits towards the iron dependent regulation of *CYC1*. To this end we performed northern blots in both YPD and BPS conditions. As seen in Figure 22, none of the Hap4 subunits were found to be involved in the regulation of *CYC1* in YPD. In BPS, however, similar to *CTA1*, Hap43 was the only Hap4 subunit involved in repression of *CYC1*. Thus, so far, data from our lab suggest a fairly conserved mechanism by which the CCAAT-binding factor regulate genes depending upon iron availability.

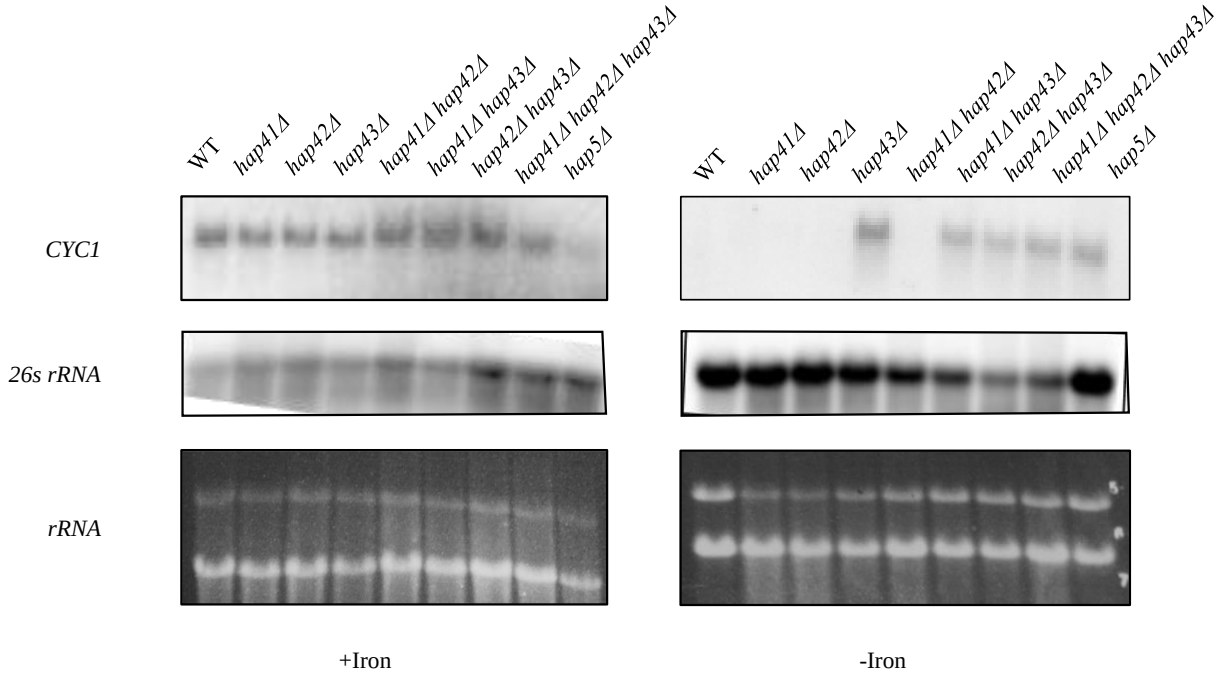

Figure 22. Regulation of *CYC1* mRNA expression by various Hap4 subunits in *Candida albicans*.

Northern blot analysis of total RNA isolated from the indicated *Candida albicans* strains grown at 30ºC in either +Iron (YPD) or –Iron (YPD +200µM BPS) as shown. The membrane was hybridized using radiolabeled probes specific for *CYC1* as indicated. *26s rRNA* was used to normalize results.

5. Discussion

In this study, we have shown that the CCAAT-Binding factor (or Hap complex) in *Candida albicans* is not only an essential regulator of iron homeostasis but also a critical regulator of the oxidative stress response (OSR). First, we show that the CCAAT-Binding factor is involved in the transcriptional regulation of the only catalase encoding gene, *CTA1*, in *Candida albicans*. We have found this regulation to be iron-dependent and although begins at the gene expression level, it is reflected in the enzymatic activity of catalase as well. Second, we show that the CCAAT-Binding factor regulates many genes involved in the OSR, like superoxide dismutases, glutaredoxins and thioredoxin. This regulation is found to be gene-specific and dependent on the iron availability. Third, we exposed the cells to either replete or limited iron conditions, followed by oxidative stress, to demonstrate the iron-dependent *in vivo* regulation of the OSR by the CCAAT-binding factor. Lastly, we investigate the iron-responsive roles of the different subunits of this transcription factor towards the OSR in *Candida albicans.*

CCAAT-binding factor has a dual and contrasting role towards iron-dependent *CTA1* regulation.

Reactive oxygen species (ROS) are primarily generated by the electron transport chain as a byproduct of respiration. Toxic concentration of metal ions like iron also catalyze the production of ROS during cellular metabolism. Since the CCAAT-binding factor is known to be involved in the regulation of respiratory metabolism and iron homeostasis in *Candida albicans*, we hypothesized that it may also play a central role in the regulation of oxidative stress response (OSR).

In our initial gene expression analysis of *CTA1*, under iron replete conditions, the *CTA1* mRNA level was diminished to a low basal level in the absence of the transcription factor. No visible *CTA1* transcript was detected in the WT strain under iron deprivation although the basal level of *CTA1* mRNA level was restored in the strain lacking the transcription factor. Thus the complex seems to act as a transcriptional activator of the *CTA1* gene when iron is available in the environment and as a repressor when iron is limiting in the environment. Importantly, these findings complement previous data demonstrating the role of this transcription factor in the regulation of the respiratory genes in response to carbon source availability (68). Moreover, this work is consistent with previous reports that have shown that the CCAAT-binding factor works as transcriptional repressor/activator of numerous genes encoding proteins that utilize iron as a cofactor in response to iron availability (25, 61,130).

This prompted us to ask if the CCAAT-binding factor also regulates other oxidative stress genes in *Candida albicans.* Among the six genes predicted to encode superoxide dismutase enzymes, three of them, *SOD1*, *SOD2* and *SOD3*, were repressed by the CCAAT-binding factor, but in an iron independent manner, as the transcript levels increased in the *hap5Δ* strain compared to the wild-type under iron replete as well as iron-limiting conditions. The CCAAT-binding factor dependent repression of *SOD2* and *SOD3* has also been reported by microarray studies used to compare the transcriptional profiling of a wild type to a *hap43Δ (cap2Δ)* under iron limitation (130). Since Hap43 is an effector subunit of the CCAAT-binding factor (25, 61,130), one would predict that the null mutations in the genes encoding the other Hap subunits would display a similar profile. Both cytoplasmic Sod1 and Sod3 have been shown to be critical for *Candida albicans* virulence although they are induced under different conditions, consistent with the low expression levels we observed in exponentially grown wild type cells. Sod1 is

known to be induced in hyphal-promoting medium and has been implicated in a protective role against ROS generated by menadione and host macrophages (66). Sod3 is an unusual cytoplasmic MnSod which *Candida albicans* expresses during stationary phase of their growth. Interestingly, Sod1 expression is repressed during the stationary phase as a defense mechanism to evade the copper toxicity (76,81).

The transcript level of mitochondrial Sod2 was most abundant in wild-type cells under iron replete conditions. This observation is consistent with the fact that Sod2 protects the cells against intracellularly produced superoxide anions and under the conditions tested, respiration is the major source of ROS generation. Indeed, the exposure to hydrogen peroxide did not increase the transcript levels of *SOD2* (24), thus emphasizing on an intracellular ROS detoxification role. No visible transcripts were detected for the cell surface associated superoxide dismutases, Sod4, Sod5 and Sod6 on iron replete conditions. This is consistent with the notion that these Sods primarily aid in the detoxification of the extracellular ROS threat, especially those generated by the host macrophages and neutrophils. However, under iron deprivation we observed a strong CCAAT-binding factor dependent repression of *SOD4*, as evidenced by the high mRNA levels present in the *hap5Δ* strain. A connection between superoxide stress and intracellular iron levels has been proposed in *Saccharomyces cerevisiae*, where mutations in superoxide dismutase genes show altered iron homeostasis (41,133). It is possible that a similar mechanism exists in *Candida albicans*, where repression of these *SOD* genes in iron deficient conditions would allow the cell to mobilize iron to its more essential processes.

We found that the two glutaredoxin encoding genes, *GRX2* and *GRX5* are regulated by the CCAAT-binding factor in a contrasting manner. While the CCAAT-binding factor appeared to repress *GRX2*, *GRX5* was activated by the transcription factor in iron-replete medium. In

contrast, during iron limiting growth, *GRX2* expression appeared to be CCAAT-binding factor independent, while *GRX5* was repressed in a manner similar to *CTA1*. Grx2 has been implicated in the resistance to PMN mediated killing by the host (24). On the other hand the Grx5 orthologue in *Saccharomyces cerevisiae* is a mitochondrial matrix protein with a role in incorporation of the Fe-S clusters in proteins such as Complex III of the respiratory chain (117). Thus, on the basis of cellular function, the CCAAT-binding factor seems to regulate *GRX2* and *GRX5* differentially, under the same environmental conditions. *GRX5* repression under iron limitation may help in increasing intracellular iron concentrations, and mobilizing it to essential proteins needed for cell survival. In support of this hypothesis, it has been shown that a *Saccharomyces cerevisiae grx5Δ* strain accumulates iron intracellularly (117).

We also observed the CCAAT-binding factor dependent expression of the thioredoxin gene, *TRX1*, in response to iron; however, it was opposite of that seen with *CTA1* and *GRX5*. The repression and activation observed in iron-replete and iron limiting growth, respectively, may represent an unexplored function of Trx1 related to iron metabolism. CCAAT-binding factor dependent overexpression of *TRX1* in a *hap43Δ* was also reported in a whole genome transcriptional profile that compared a wild type to a *hap43Δ* under iron-limited growth (129). In *Saccharomyces cerevisiae*, it has been shown that thioredoxins and glutaredoxins are responsible for maintaining the cellular thiol-redox system, however, evidence suggests that they operate through distinct non-redundant pathways (128). For *Candida albicans*, additional work is needed to understand the contribution and regulation of these genes in the OSR and iron homeostasis. What is clear is the CCAAT-binding factor can specifically activate or repress some of these genes in response to iron availability during cell growth.

The CCAAT-binding factor regulates the in vivo OSR.

The hydrogen peroxide sensitivity assays allowed us to examine the cellular response of *Candida albicans* confronted to the ROS after being exposed to iron replete or iron deficient environments, the latter mimicking the limited iron environment in the human host upon infection. Overall, the survival to peroxide treatment correlated well with the levels of catalase the cell had in response to the iron availability. Moreover, it showed that the integrity of the CCAAT-binding factor is essential for *Candida albicans* to cope with hydrogen peroxide stress. During iron starvation, the CCAAT-binding factor repressed catalase expression, and also inhibited the survival to the peroxide treatment. These findings are consistent with the need of the fungal pathogen to activate all its iron-scavenger mechanisms upon systemic infection of the human host. Therefore, it appears that *Candida albicans* represses many OSR genes in an iron limited environment, but those genes are expressed as soon as iron is available, making itself ready to fight against the host defense mechanisms. The oxidative stress response has been shown to be regulated by the Hap complex in another fungal pathogen, *Aspergillus nidulans.* In *Aspergillus nidulans* the complex is involved in redox sensing via the evolutionary conserved cysteine residues of HapC which results in the regulation of downstream oxidative stress genes (141). Interestingly, these conserved cysteine residues are found both in *Ca*Hap31 and *Ca*Hap32, suggesting a possible mechanism for their participation in the regulation of OSR. Since either protein can serve as a putative sensor of the redox state of the cell, it is plausible that their function is dependent on environmental cues.

The modular nature of CCAAT-binding complex is essential for the differential regulation of *CTA1* in response to iron.

Since the CCAAT-binding factor serves contrasting roles in environments with and without iron, we hypothesized that this may at least partially be achieved by the differential recruitment of the distinct Hap3 and/or Hap4 subunits. Our data showed that the presence of either Hap31 or Hap32 is essential for activation of *CTA1* in iron replete conditions, since the strain lacking both genes had less catalase and recovered poorly from peroxide stress, mimicking the *hap5Δ* mutant. Thus, it appears that both complexes are capable of compensating each other towards the formation and function of the complex.

In iron limiting conditions, however, the regulation of *CTA1* by the distinct Hap3s is more complex. The mRNA levels indicate that Hap31 or Hap32 can repress *CTA1* expression to similar levels and comparable to the wild type, suggesting a compensatory role between these subunits in the formation of an active CCAAT-binding complex. However, in spite of low levels of catalase the *hap31Δ* strain showed resistance to the hydrogen peroxide treatment. This unexpected result may reflect threshold levels of catalase sufficient for the protective function, or alternatively, a catalase independent mechanism of managing peroxide stress that was only manifested in the *hap31Δ* strain. The latter possibility has been suggested in *Candida glabrata* where a *CTA1Δ* strain remains capable of adaptation to oxidative stress (27).

Our analysis of the CTA1 expression and survival to peroxide stress in the strains containing the single, double and triple combinations of *hap41Δ, hap42Δ* and *hap43Δ* alleles led to the conclusion that none of the Hap4 like subunits were necessary for expression during iron-replete growth. In contrast, during iron limitation, Hap43 was the sole Hap4 like subunit responsible for *CTA1* repression. This is consistent with the fact that *Candida albicans* Hap43

has been reported to be a global repressor of genes encoding proteins that involve utilization of iron in iron-limiting conditions; a mechanism that requires the physical interaction between the Hap43 and Hap5 subunits of the CCAAT-binding factor (129). Evolutionarily, the Hap43 orthologs from other yeast and fungi, including HapX *(Aspergillus nidulans, Aspergillus fumigatus, Cryptococcus neoformans)* and Php4 *(Schizosaccharomyces pombe)*, have been implicated in the regulation of genes involved in iron transport/utilization (57, 75). Hap43 and HapX share three cysteine rich protein domains, which have been proposed to mediate iron sensing through the formation of an iron-binding domain (57). But whether this iron-binding domain coordinates iron or iron sulphur clusters still remains to be established.

Why is it advantageous to co-regulate genes involved in iron homeostasis, respiratory metabolism and oxidative stress response? First, as mentioned before, the human host is essentially a low iron environment as most of the iron available is present in the bound form like transferrin, hemoglobin, ferritin etc. (4). Although *Candida albicans*, like many other pathogens, has evolved multiple sophisticated mechanisms for scavenging iron from the human host (4), the organism must adjust its metabolism to meet this challenge. Also, the availability of iron can vary dramatically in specific micro niches of the host. The co-regulation of respiration, iron metabolism and OSR by the CCAAT-binding factor can help meet this metabolic challenge inside the host. Although our in vitro studies do not aim to mimic the host pathogen interaction, one can envision the iron limited environment of the human host activating all the fungal iron-scavenging mechanisms, while repressing OSR genes and genes that express iron requiring proteins. However, once optimum concentration of iron in the cell has been restored, *Candida albicans* can quickly express the OSR genes and modulate a strong response to the host defense mechanisms such as the oxidative burst following phagocytosis by neutrophils.

6. Literature cited

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