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CCAAT-Binding Factor as a Transcriptional Regulator of CYC1 in Candida albicans

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By

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Abstract

Candida albicans is a normal commensal of the human flora; however, under appropriate circumstances this organism can become pathogenic to the host and cause life threatening conditions. In fact, *Candida* species are the fourth most common nosocomial infection in the Unites States with a mortality rate of over 30%

The CCAAT binding factor is a multi-protein transcription factor highly conserved in eukaryotes. It binds specifically to the consensus sequence 5'-CCAAT-3' in promoters and it is one of the most common *cis*-acting elements in eukaryotes promoters. This transcription factor is composed of three DNA binding subunits; Hap2p, Hap3p and Hap5. In yeast and fungi a fourth subunit, termed Hap4, functions as the effector subunit that regulates the expression of target genes. *C. albicans* has been found to have multiple genes encoding some of the proteins associated with the CCAAT-binding factor, namely Hap31 and Hap32 as well as Hap41, Hap42, and Hap43. In the studies described in this dissertation, the role of the CCAAT-binding factor in the regulation of *CYC1*, encoding cytochrome c, was examined. It was found that the CCAAT-binding factor was the sole transcription factor involved in *CYC1* transcriptional regulation. It was found that Hap31 functions to activate *CYC1* transcription in an iron rich environment, while Hap32 represses *CYC1* transcription when iron is limited. Thus, the differentially utilization of Hap31 and Hap32 serves as a molecular switch to convert a transcriptional activator to a repressor in response to environmental signals. It was also shown that *HAP32* contains a 118-nucleotide intron that is removed during processing; however, the splicing event was not regulated by iron availability. Additional studies were done to construct a *hem1Δ* homozygous mutant of *C. albicans* and this mutant was used to demonstrate that *C. albicans* can acquire iron, in the form of heme, exogenously. We also examined the growth of the *hem1Δ* homozygote under anaerobiosis and discovered that the mutant requires a source of unsaturated fatty acids for anaerobic growth; however, it does not require supplementation with exogenous ergosterol, common in many other fungi. The clinical implications of this finding will be discussed.

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Dedication

I would like to dedicate this document to my loved daughter Carmen Maria Avila Padilla, my husband Carlos Avila and my mother Maria del Carmen Marcia. If it were not for their support and sacrifices I would have never accomplish this dream.

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Chapter 1. Literature Review

Literature review

Candida albicans is the most commonly identified fungal pathogen in humans and it is associated with skin and mucosal infections as well as life threatening systemic infections in persons with a severely compromised immune system (Jones et al., 2004). The importance of invasive fungal infections has increased over the last two decades due to the increase in the population at risk including: persons with human immunodeficiency virus (HIV), patients that have undergone organ transplant, patients with hematologic malignancies, burns and low birth weight infants (Warnock, 2007). In the United States, infections caused by *Candida* species are the fourth most common cause of hospital-acquired blood stream infections with an incidence of 1.5 cases per 10,000 patients per day (Hajjeh et al., 2004) with a mortality rate for candidemia that has been reported to be greater than 30% (Karthaus and Cornely, 2007).

Candida albicans possess the ability to survive and proliferate by adapting to different environments including the extremes of physiological pH, availability of nutrients, and temperature (Tavanti et al., 2006). These characteristics make *Candida albicans* a successful commensal colonizer of healthy individuals as well as an opportunistic pathogen causing symptomatic infections in different regions of the human body (Calderone and Fonzi, 2001).

Morphology

Candida albicans has the ability to grow in a variety of morphological forms. Although the link between virulence and morphology may be circumstantial, it has been shown that these morphological changes increase the efficiency of dissemination in the host (Enjalbert and Whiteway, 2005).
The morphological variations can range from budding yeast to true hyphae, as well as an intermediate form referred to as pseudohyphae (Ernst, 2000; Whiteway and Oberholzer, 2004). During pseudohyphal growth daughter cells elongate and after septum formation the daughter remains attached to the mother cell (Chapa y Lazo et al., 2005). *C. albicans* can also grow in some less common morphological forms such as chlamydospores or opaque cells (Whiteway and Oberholzer, 2004).

The most studied morphological change is the yeast-to-hyphal transition. True hyphae appear as tubes with and without branched forms. In this form, the budding cells grow in a continuous apical

extension followed by septation. This is in contrast to pseudohyphae that are generated by unipolar budding with the daughter cells remaining attached to the mother cell. Both morphological states result in a chain of cells with the difference that true hyphae contain no constrictions at their septa (Sudbery et al., 2004). The yeast to hyphal transition can be induced by a variety of environmental factors such as increased temperature, pH changes, salt concentration, amino acid availability, starvation, and the presence of serum (Maidan et al., 2005). The ability of *Candida albicans* to make the yeast-to-hyphae transition has been considered one of the important factors for virulence. Hyphae are believed to facilitate tissue penetration, adhesion, and invasion to mucosal surface and also to increase the resistance of the yeast to macrophage killing (Adt et al., 2006; Ebanks et al., 2006).

The CCAAT binding factor

The genes encoding the subunits of the CCAAT binding factor were first identified in *Saccharomyces cerevisiae* and a significant amount of information about this transcription factor has been derived from work in this organism. Nevertheless, this transcription factor has been identified in almost every eukaryote that has been analyzed (Mantovani, 1999). Thus, it is considered by many to be one of the most conserved transcription factors in eukaryotes. The CCAAT-binding factor has many different names, depending on the organism. These include the Hap complex in *Saccharomyces cerevisiae, kluyveromyces lactis, Candida albicans* and *Arabidopsis thaliana* (Pinkham and Guarente, 1985; Mulder et al., 1994; Edwards et al., 1998; Johnson et al., 2005), the Php complex in *Schizosaccharomyces pombe* (McNabb et al., 1997), AnCF in *Aspergillu*s species (Li et al., 1998; Brakhage et al., 1999) (Li et al., 1998) and NF-Y in mammals (Hooft van Huijsduijnen et al., 1990; Maity et al., 1990)

The CCAAT-binding factor is a multicomponent transcription activator that specifically binds to promoter sequences containing the consensus sequence 5'-CCAAT-3'. This cis-element is one of the most common present in eukaryotic promoters, with approximately 30% human genes containing a CCAAT site between -50 and -200 base pairs upstream of the transcription start point (Kato, 2005). Despite the structural conservation of the protein subunits that comprise the CCAAT-binding factor, the target genes regulated by this transcription factor vary with the organism.

The CCAAT-binding factor in *Saccharomyces cerevisiae* is composed of four subunits termed Hap2, Hap3, Hap4 and Hap5. The Hap2/Hap3/Hap5 heterotrimer has been shown to be required and sufficient for sequence specific DNA-binding activity (McNabb et al., 1995). In yeast and fungi the fourth subunit Hap4 is the effector subunit required for transcriptional regulation. This subunit does not interact with DNA in a sequence specific manner, rather it interacts with the DNA-bound Hap2/3/5 heterotrimer to regulate transcription (Forsburg and Guarente, 1989).

In *S. cerevisiae*, the CCAAT-binding factor regulates the transcription of target genes in response to carbon source availability (Forsburg and Guarente, 1989; McNabb et al., 1995). The CCAAT-binding factor was first identified in *S. cerevisiae* as a transcriptional regulator of the *CYC1* gene. *CYC1* encodes Iso-1-cytochrome c an electron carrier protein that transfers electrons via a covalently attached heme group from complex III to complex IV in the respiratory chain. Analysis of the *CYC1* promoter revealed two *cis*-acting DNA elements referred to as UAS1 and UAS2 that were important for the transcriptional regulation of *CYC1*. The UAS1 sequence was found to bind the transcription factor Hap1 and regulate *CYC1* transcription in response to heme and oxygen availability (Guarente and Mason, 1983; Pfeifer et al., 1987). Thus, in the presence of oxygen and sufficient iron, in the form of heme, *CYC1* transcription was stimulated. This made perfect sense since respiratory metabolism could not occur in the absence of oxygen (the terminal electron acceptor) or in the absence of heme, a common cofactor for proteins involved in electron transport. In contrast, the UAS2 element was bound by the CCAAT-binding factor and regulated *CYC1* transcription in response to carbon source. *CYC1* transcription was stimulated in the presence of non-fermentable carbon sources, and repressed when fermentable carbon sources were available (Guarente and Mason, 1983; Guarente et al., 1984).

In *S. cerevisiae*, it was found that null mutations in *HAP2, HAP3, HAP4,* or *HAP5* resulted in the failure to activate *CYC1* via UAS2 and phenotypically the yeast were unable to growth in a non-fermentable carbon source, emphasizing the importance of this transcription factor in respiratory metabolism (Hahn et al., 1988; Forsburg and Guarente, 1989; McNabb et al., 1995; McNabb and Pinto, 2005).

As stated earlier, although the CCAAT-binding factor is highly conserved evolutionarily at the sequence level, the transcription factor has functionally evolved to regulate different genetic pathways in

different organisms. For example, in *Aspergillus nidulans*, the CCAAT-binding factor was found to positively regulate the expression of genes involved in penicillin biosynthesis genes (*ipnA* and *aatA*) and the catabolic enzyme acetamidase encoded by *amdS* (Littlejohn and Hynes, 1992; Bergh et al., 1996; Litzka et al., 1996; Kato et al., 2001). In addition, the *A. nidulans* CCAAT-binding factor was found to negatively regulate the homoaconitase *lysA* gene (Weidner et al., 2001) and to repress genes involved in iron-dependent pathways in the absence of iron(Hortschansky et al., 2007), suggesting that the CCAAT-binding factor may function as either a transcriptional activator or repressor.

In mammals, the transcription factor NF-Y consist of three components, named NF-YA (Hap2 ortholog), NF-YB (Hap3 ortholog) and NF-YC (Hap5 ortholog); however, no orthologous proteins related to Hap4 have been found in eukaryotes outside of yeast and fungi (Kato, 2005). Two activation domains have been found in NF-Y, one at N-terminus of NF-YA and one at the C terminus of NF-YC (Coustry et al., 1996; Coustry et al., 1998). Complementation studies have demonstrated that the human NF-YA and NF-YB are functionally interchangeable with Hap2p and Hap3p in *S. cerevisiae*, emphasizing their evolutionary relatedness (Becker et al., 1991; Tuncher et al., 2005). In addition, Sinha *et al* demonstrated that recombinant NF-YC is able to form stable DNA-protein complex with the yeast Hap2 and Hap3 (Sinha et al., 1995). Therefore, CCAAT- binding factor components are highly conserved in eukaryotes with the most relevant domains involved in DNA binding and protein-protein interactions showing the greatest amino acid sequence homology, typically around 70% amino acid identity among different species (Li et al., 1992).

In higher eukaryotes the CCAAT box has been found in a wide variety of promoters. For example, it has been shown to be involved in the regulation of MHC II class genes, globin α, collagen α, osteoporin, albumin, and many others (Berry et al., 1992; Ronchi et al., 1996; Maity and de Crombrugghe, 1998; Mantovani, 1999). However, unlike yeast and fungi, the CCAAT-binding factor in higher eukaryotes is considered a proximal promoter factor, not a gene specific activator. Thus, it serves a more general role in transcriptional regulation, similar to SP1.

The CCAAT-binding factor in *Candida albicans*

The CCAAT-binding factor in *Candida albicans* is similar to *S. cerevisiae*, with four distinct subunits termed Hap2, Hap3, Hap4 and Hap5. Interestingly, some of the CCAAT-binding subunits are expressed from multiple distinct genes. For example, there have been three distinct genetic loci found that encode different variants of Hap4 designated Hap41, Hap42, and Hap43 (Johnson et al., 2005). In addition, it was found that two distinct genetic loci encode Hap3 homologs designated Hap31 and Hap32 (Johnson et al., 2005). The question that arises is why the redundancy of the Hap3 and Hap4 subunits and what is their role in gene regulation.

Johnson *et al.* has been previously demonstrated that the *HAP5* gene from *C. albicans* was able to complement a *S. cerevisiae hap5Δ* mutant to restore normal growth on a non-fermentable carbon source. It was also shown that the deletion of *HAP5* in *Candida albicans* completely abolishes the DNA binding activity of the CCAAT-binding factor (Johnson et al., 2005), analogous to the observation in *S. cerevisiae*. However, in contrast to the activation of transcription by the CCAAT-binding factor in *S. cerevisiae*, the CCAAT-binding factor in *Candida albicans* was suggested to function as a repressor of genes involved in the respiratory metabolism (Johnson et al., 2005). Moreover, it was found that the *hap5Δ* mutant was defective in hyphal development on serum-containing medium and the strain showed defective growth on non-fermentable carbon sources, suggesting that the CCAAT-binding factor may be involved in the regulation of genes involved in respiratory metabolism.

Recently, it was shown that a *hap2Δ* mutant has identical phenotypes to the *hap5Δ* mutant such as abolishment of DNA binding activity, defective growth on non-fermentable carbon sources and a defect in the transition between yeast and hyphal growth forms (Bates, 2009). It was also shown that both the *hap5Δ* and *hap2Δ* mutants were unable to grow in medium that was limited for iron (Bates 2009, Cano and McNabb, unpublished observations). To further explore the defect associated with iron limitation, additional *hap* mutants were examined for phenotypes under iron replete and iron limiting conditions.

As previously mentioned, Johnson *et al.* identified two homologs of Hap3 in *C. albicans* designated Hap31 and Hap32. Complementation studies revealed that either Hap31 or Hap32 could rescue the respiratory defect of a *S. cerevisiae hap3Δ* mutant, suggesting that both were functional orthologs of the CCAAT-binding factor. Interestingly, the *C. albicans hap31Δ* displayed phenotypes

similar to the *hap5Δ* mutant with regard to defects in growth related to carbon source availability; however, the *hap31Δ* mutant did not show a defect in growth under iron limitation as was observed with the *hap5Δ* mutant (Bates 2009). Interestingly, the *hap32Δ* mutant showed a marked reduction in growth on iron limiting medium, but it did not display the carbon source dependent growth defects observed with the *hap2Δ, hap31Δ,* and *hap5Δ* mutants (Bates 2009). These data suggested that Hap31 versus Hap32 may have different functional roles in the CCAAT-binding factor in response to nutrient availability.

To further support the differential roles of Hap31 and Hap32 in CCAAT-binding factor function, it was shown that the two genes are differentially expressed in response to iron availability. In iron-replete growth conditions, *C. albicans* cells express *HAP31* and *HAP32* expression is undetectable. In contrast, when *C. albicans* cells are grown under iron limitation the expression of *HAP32* is present while *HAP31* is repressed (Bates 2009). These data are supported by electrophoretic mobility shift assays that show different CCAAT-binding factor complexes are formed during growth in iron replete versus iron limiting medium. It was shown that in iron replete conditions the CCAAT-binding factor is composed of Hap2/Hap31/Hap5 heterotrimer bound to DNA, while in iron limiting growth conditions the binding complex consist of Hap2/Hap32/Hap5 (Bates, 2009).

Thus, in *C. albicans* the CCAAT- binding factor consist of Hap2p, Hap5p, Hap31p, and Hap32p as components of the DNA-binding protein complex. The complex is composed of Hap2/31/5p under iron replete growth conditions and Hap2/32/5p under iron limiting growth conditions. In addition to the DNA-binding components of the CCAAT-binding factor, *C. albicans* also contains three distinct Hap4-like subunits, Hap41, Hap42, and Hap43 that presumably function as effector subunits that alter gene expression. However, the function of the Hap4-like proteins remains unclear.

Overview of iron regulation and acquisition

Iron is the fourth most abundant element on earth and the transition states of iron make its properties essential for many biological processes. This metal plays an important role in proteins such as ribonucleotide reductase, DNA primase, and DNA helicases that are essential for DNA synthesis; ATPase ABCE1 essential in mRNA translation; ferrochelatase and hemoglobin essential for respiration, electron transport, oxygen transport/storage as well as many other proteins important for metabolic pathways

(Drakesmith and Prentice, 2008). Many of these proteins are highly conserved across prokaryotes and eukaryotes making iron essential in all forms of life. The deficiency of iron in the cellular environment abolishes the function of many iron dependent proteins, disrupting the normal function of the cellular processes (Umbreit, 2005). Despite being abundant, iron has low availability due to insolubility, making it a target of competition between host and microorganism (Schaible and Kaufmann, 2004).

Single-celled organisms and plants have evolved sophisticated strategies to obtain iron from the environment. In humans, iron is poorly absorbed in the diet making iron deficiency the most common nutritional disorder in the world, especially in children and women (Zimmermann and Hurrell, 2007; Philpott and Protchenko, 2008). Anemia is the most common manifestation of iron deficiency, but iron deficiency can also have adverse effect on the immune system and cognitive development. Although the pathogenesis of anemia is well understood, there are many other manifestations of iron deficiency that are not well understood at the cellular and metabolic level. At the same time, accumulation of iron inside the cells is a feature of several human diseases both acquired and inherited that are at this moment far from being understood (Burn and Chinnery, 2006; Hayflick, 2006; Nairz and Weiss, 2006).

Iron homeostasis is a highly regulated process as iron is essential for life, but also toxic at high levels. Excess iron can lead to deleterious oxidative effect on cells due to the Fenton reaction in which free ferrous iron reacts with H_2O_2 or lipid peroxide to generate free radicals. Thus, a precise regulation of this metal inside the cell is required to maintain intracellular levels of iron in a balanced state between the minimal requirement and the cytotoxicity (Hsu et al., 2011).

Previous studies have shown that a metabolic remodeling event occurs in response to iron depletion or repletion during the process of iron homeostasis. In other words, the transcriptional response to iron starvation is to down-regulate the expression of enzymes that require iron to reduce the iron requirement and secondly to activate genes involved in iron uptake pathways (Philpott and Protchenko, 2008; Kornitzer, 2009; Hsu et al., 2011). In this way, the limited amounts of iron can be used more efficiently by the cells for vital functions such as DNA replication and other essential aspects of cell metabolism (Drakesmith and Prentice, 2008; Hsu et al., 2011). By the same token, the transcriptional response to iron repletion is the repression of genes involved in iron acquisition and iron transport to

avoid the detrimental effect of excess intracellular iron (Halliwell and Gutteridge, 1984, 1992; Burn and Chinnery, 2006; Hayflick, 2006).

Many of the studies on iron acquisition mechanisms in yeast and fungi have been performed with the non-pathogenic yeast *Saccharomyces cerevisiae*. This budding yeast can adapt to environments in which the bioavailability of iron is extremely scarce or excessively abundant. *S. cerevisiae* possesses multiple transport iron systems that translocate iron across the cell membrane (Van Ho et al., 2002; Kornitzer, 2009). It possesses two low affinity transporters systems (*FET4* and *SMF1*) and two high affinity transporters systems (reductive and non-reductive) (Lesuisse et al., 1998; Yun et al., 2000; Kaplan et al., 2006). The reductive pathway involves ferric reductases that are located on the cell surface. These ferric reductases (FRE1, FRE2) reduce Fe^{+3} molecules complexed with other molecules (siderophores), and the ferrous iron (Fe^{+2}) can be transported by Fet3 and Ftr1, a multicopper oxidase, and a transmembrane permease, respectively (Dancis et al., 1990; Georgatsou and Alexandraki, 1994). The other iron uptake mechanism in *S. cerevisiae* is the non-reductive system. This mechanism is composed of four different gene products that acquire iron from siderophore-iron complexes followed by the intracellular reduction of the siderophore bound iron (Lesuisse et al., 1998; Heymann et al., 2000; Yun et al., 2000). Siderophores are small organic molecules produced by bacteria and some fungi that bind iron at high affinity. There is no evidence that *S. cerevisiae* can synthesizes its own siderophores, but it has the capability of utilize siderophores from other organism, including bacteria (Lesuisse et al., 1998; Yun et al., 2000).

Pathways for iron acquisition in pathogenic fungi:

Generally, to obtain iron, pathogenic fungi have evolved distinct mechanisms, including a high affinity transporter system (Reductive pathway), a Fe-siderophore uptake system (Non-reductive pathway) and a heme uptake system (Howard, 1999; Ramanan and Wang, 2000; Heymann et al., 2002; Pendrak et al., 2004; Baek et al., 2008).

The reductive iron acquisition pathway or high affinity transporter system relies on the reduction of extracellular ferric iron (Fe^{+3}) to ferrous iron (Fe^{+2}) followed by reoxidation by ferroxidases that are linked to high affinity permeases (Kornitzer, 2009). In *C. albicans* there are two high affinity iron

permeases; Ftr1 and Ftr2. Of these two, only Ftr1 has been found to be important for growth in iron limiting conditions and is expressed only under these conditions (Ramanan and Wang, 2000). It has also been found that Fet3, a multicopper oxidase, and Ccc2, a copper transporter, were also essential for growth under iron limitation and that only an *ftr1*Δ mutant displayed decreased virulence in a model systemic infection of mice (Eck et al., 1999; Weissman et al., 2002). In *Cryptococcus neoformans*, two homologs of the high affinity permease have been identified, Cft1 and Cft2. Only *cft1*Δ shows growth defects under iron limitation and this mutant is also attenuated for virulence in a mouse model, while *cft2*Δ shows only small reduction in virulence (Jung et al., 2008). Homologs of the ferroxidase/permease complex have also been identified in *Aspergillus fumigatus,* FetC and FtrA. *ftra*Δ deletion mutant shows no defect in growth, nor any reduction in virulence; however, this may be due to the compensation by the upregulation of the nonreductive iron acquisition pathway (Schrettl et al., 2004; Jung et al., 2008).

For non-reductive iron acquisition in *C. albicans* only one siderophore transporter has been identified, Sit1/Arn1 (Heymann et al., 2002; Hu et al., 2002). This transporter mediates the uptake of ferrichrome-type siderophores such as ferricrocin and ferrichrysin. The *sit1*Δ did not show any defect in virulence when evaluated in a mouse model, but it exhibited reduce invasion in reconstituted human epithelium, indicating the importance of this iron uptake system in penetration and invasion (Heymann et al., 2002). Early studies suggested the presence of siderophores in *C. albicans* but later studies have not been able to confirm this observation (Ismail et al., 1985). Thus, to date there are no siderophores synthesized by *C. albicans*.

In *C. neoformans,* siderophores have not been identified but the genome sequence indicates the presence of several putative siderophore transporters in which Sit1 was found to be essential for deferoxamine iron utilization (Tangen et al., 2007). On the other hand, *A. fumigatus* is able to synthesize and utilize hydroxamate-type siderophores. It has also been demonstrated that siderophore biosynthesis is essential for pathogenicity of the organism in an animal model of invasive aspergillosis (Schrettl et al., 2004; Hissen et al., 2005). The utilization of siderophores in this organism is not only limited to iron acquisition but it is also necessary for iron storage. These two independent siderophore functions are strictly required for virulence (Schrettl et al., 2007). Lastly, *Histoplasma capsulatum* also synthesizes

siderophores that are necessary for growth in limited iron conditions and also important for virulence in a mouse model infection (Howard et al., 2000; Hwang et al., 2008).

Heme-uptake system

C. albicans utilizes heme differently to the reductive and non-reductive iron uptake pathways (Weissman et al., 2002; Santos et al., 2003). *C. albicans* hemin (oxidized form of heme) utilization depends on cell surface receptors for heme uptake. One of these receptors has been putatively identified as Rbt5. a GPI-anchored receptor (Weissman and Kornitzer, 2004); however, it is clear that other heme receptors must exist. For the utilization of heme as a source of iron cells must also have Hmx1, a heme oxygenase, to catabolizes iron- protoporphyrin IX to alpha biliverdin (Santos et al., 2003; Pendrak et al., 2004). H. *capsulatum* also binds and utilizes hemin as an iron source but the molecular pathway has not been characterized yet (Foster, 2002).

Most of the heme in the animal host is bound to hemoglobin, which constitutes the largest iron pool in the human host. *C. albicans* is able to uptake hemoglobin as an iron source using the same receptor to aquire hemin, Rbt5 (Weissman and Kornitzer, 2004). The hemoglobin iron utilization pathway that emerged from previous studies showed that hemoglobin is bound to a extracellular receptor, such as Rbt5, endocytosis occurs, then in the acidic environment of the endosome a probable dissociation of the heme from the globin, degradation of the protoporphyrin ring by heme oxigenase, and uptake of the free iron into the cytoplasm by a vacuolar iron permease (Santos et al., 2003; Pendrak et al., 2004; Weissman et al., 2008).

Some studies have suggested that *C. albicans* synthesizes a component with hemolytic activity to access the abundant iron reservoir in the bloodstream during a systemic infection (Salvin, 1951; Manns et al., 1994). However, the hemolytic capability of *C. albicans* has not been universally accepted and has not been observed by others (McNabb, personal communication). It has been found that Rbt5 was not required for virulence in a mouse systemic infection model and this could be either because of a limited role for heme in iron acquisition or because *C. albicans* contains Rbt5 homologs, such as Rbt51, that is able of binding heme (Weissman and Kornitzer, 2004).

Candida albicans has also been found to utilize ferritin as a source of iron. Ferritin is an iron storage protein that is found in host cells and in very low concentrations in serum. The utilization of ferritin as an iron source in *Candida albicans* is dependent on the cell surface adhesin protein Als3, and Ftr1 (Almeida et al., 2008). Almeida *et al* showed that Als3, a cell wall protein present only in hyphal cells, was able to bind ferritin and null *als3* mutant was unable to damage epithelial cells *in vitro.* Another host iron storage molecule that *C. albicans* utilizes as iron source is transferrin, which is a serum protein that binds iron with high affinity. Knight *et al* showed the restoration of growth of strains that were iron deprived by iron loaded transferrin and the inability of apotransferrin to promote growth. They also demonstrated that the reductive pathway mediates transferrin iron uptake and the siderophores and heme uptake systems were not involved (Knight et al., 2005).

Despite the evolutionary conservation of the components of the iron acquisition pathways between non-pathogenic *S. cerevisiae* and the pathogenic fungi, the iron response regulators appears to be different. The main transcriptional regulator in response to iron limitation for *S. cerevisiae* is Aft1, which is a protein that is constitutively expressed; however, when intracellular iron levels are abundant, Aft1 is localized in the cytosol and it does not activate transcription (Yamaguchi-Iwai et al., 2002). When intracellular iron levels are low, Aft1 is localized in the nucleus, where bind DNA and activate the transcription of genes involved in iron uptake (Philpott and Protchenko, 2008). In pathogenic fungi an Aft1homolog has not been identified, instead iron regulation seems to depend on GATA-binding (Sfu1) and CCAAT- binding classes of transcription factor. From microarray data in *C. albicans,* around 1000 genes were either up or down-regulated in response to iron starvations conditions, and at least 139 are known to be regulated by Sfu1(Lan et al., 2004). In *C. neoformans,* the main regulator during iron starvation is Cir1, another GATA binding factor (Jung et al., 2006). In some species of *Aspergillus,* iron represses iron acquisition via the transcription factor SreA a GATA-binding factor and induces expression of iron dependent enzymes (Hortschansky et al., 2007; Schrettl et al., 2008).

The CCAAT binding factor has also been found to affect iron regulation in *C. albicans* and *Aspergillus fumigatus*. In *C. albicans*, the Hap43 has been found to stimulate the expression of genes involved in iron acquisition (Baek et al., 2008) and to repress genes encoding iron-dependent proteins

(Hsu et al., 2011). Similarly, *A. fumigatus*, CCAAT- binding factor with HapX is known to repress genes involved in iron utilization pathways during iron limited growth (Hortschansky et al., 2007).

Pathogenic fungi have evolved different but specific mechanisms for iron acquisition in order to take advantage of the animal host as a source of iron. Some of these mechanisms are also essential for virulence and survival of the fungal pathogens in the infected host. In the following chapters I will examine the regulation of one model gene, *CYC1*, in great detail to understand how it is regulated in response to iron availability and the role of the CCAAT-binding factor in the regulation of *CYC1*. As mentioned previously *CYC1* encodes cytochrome c and utilizes heme as a cofactor. Moreover, previous studies have suggested that *CYC1* is regulated in response to iron availability. My studies will probe more deeply the regulation of *CYC1* in response to iron availability and the role of the various CCAAT-binding factor subunits in that regulation.

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Chapter 2. CCAAT-Binding Factor as a Transcriptional Regulator of *CYC1* in *Candida albicans*

Introduction

Candida albicans is the most common opportunistic fungal pathogen found on human mucosal surfaces such as the mouth and gut. This fungal pathogen is able to cause life threatening systemic infections in immunocompromised or debilitated hosts (Knight et al., 2005). Over the last two decades there has been an increase in *Candida* infections due in large part to the increased number of elderly and inmunocompromised individuals, as well as the increased use of antibiotics and more invasive therapeutic procedures (Flisfisch et al., 2008). As a result there is a higher demand for effective antifungal therapies. Since many biological processes are shared between fungi and humans, some antifungal treatments cause harmful side effects and toxicity to the human host (MacCallum and Odds, 2004; Flisfisch et al., 2008). Understanding the metabolic processes associated with the growth of *C. albicans* and related species is a first step toward identifying unique features of the organism that can be exploited for future antifungal therapies.

Iron is an essential nutrient for all microorganisms including *C. albicans*, and it is a common virulence factor for many pathogens. In humans iron is stored intracellularly in ferritin and heme-containing compounds, and extracellularly in iron-binding transport proteins such as transferrin and lactoferrin (Manns et al., 1994; Knight et al., 2005; Almeida et al., 2008). Thus, the human host is an iron-rich environment for a pathogen, but access to that iron is limited because the iron is sequestered. Microbial pathogens have evolved numerous mechanisms to acquire iron from the host environment even when the access to iron is limited (Knight et al., 2005; Weissman et al., 2008). *C. albicans* has developed different iron uptake systems for various iron-containing substrates. These pathways include high affinity iron transporters or permeases, a heme uptake system and an iron siderophore uptake system (Howard, 1999; Heymann et al., 2002; Baek et al., 2008). Some of these pathways are required for virulence such as the high affinity transporters and the siderophore uptake system (Ramanan and Wang, 2000; Heymann et al., 2002) while others are important for survival in the mammalian host, like the heme uptake system (Pendrak et al., 2004). Thus, the ability to acquire iron from a host environment is critical for the survival of *C. albicans* and for its ability to cause disease.

Eukaryotes utilize a variety of mechanisms to regulate gene expression. Eukaryotic genes encoding proteins are transcribed by RNA polymerase II, which in turn requires the prior association of a macromolecular preinitiation complex (PIC) and other regulatory proteins to assemble on the DNA in the promoter region (Cler et al., 2009). This step can be positively or negatively affected by transcription factors that bind at specific sequences in the promoter region (Wray et al., 2003). Transcription factors are proteins or multi-subunit protein complexes that influence transcription either positively or negatively. One such transcription factor is the CCAAT binding factor, a heterooligomeric transcription factor that is highly conserved in eukaryotes and binds to the consensus sequence 5'-CCAAT-3' in the promoters of target genes (Mantovani, 1998). In *Saccharomyces cerevisiae,* the sequence-specific DNA-binding activity of the CCAAT binding factor is mediated by three subunits named Hap2, Hap3, and Hap5; however, this complex lacks the ability of modulate transcriptional activity. In order to activate transcription a fourth subunit, Hap4p is required as the effector subunit. Mutations or deletions that abolish the function of any of these four subunits will result in the inability to activate the expression of genes involved in respiration, and the organism will not grow in a non-fermentable carbon sources (Pinkham et al., 1987; McNabb et al., 1995; McNabb and Pinto, 2005).

The DNA-binding subunits of the *C. albicans* CCAAT-binding factor are highly homologous to those found in *S. cerevisiae* (Johnson et al., 2005). The major difference is the presence of two Hap3-like proteins, termed Hap31 and Hap32, that are differentially expressed in response to iron availability (Bates, 2009). Under iron replete conditions, *HAP31* mRNA is abundantly expressed; whereas, *HAP32* mRNA is not detectable. When iron is limiting for growth, *HAP32* mRNA is expressed and *HAP31* mRNA is not detectable (Bates, 2009). These observations suggest that Hap31 and Hap32 may impart distinct functions to the CCAAT-binding factor in response to iron availability. In support of this premise, electrophoretic mobility shift studies have demonstrated that the CCAAT-binding factor present in cells grown in the presence of iron contains the Hap2/31/5 DNA-binding complex while cells grown under iron limitation contain the Hap2/32/5 DNA-binding complex. In addition, previous studies have demonstrated that *CYC1* mRNA expression is regulated, at least in part, by the CCAAT-binding factor (Johnson et al., 2005; Bates, 2009), and *CYC1* mRNA is highly expressed when cells are grown in iron replete medium,

but repressed in cells grown under iron limitation (Bates, 2009). The goal of the work described in this chapter was to dissect the promoter regulatory elements involved the *CYC1* response to iron and to evaluate whether the Hap2/31/5 versus Hap2/32/5 complex play distinct roles in the regulation of *CYC1* transcription. The working hypothesis was that Hap2/31/5 heterotrimer functions in the activation of *CYC1* during growth under iron replete conditions while the Hap2/32/5 complex functions as a repressor under iron limitation. In addition to the two distinct Hap3-like proteins, *C. albicans* contains three Hap4-like subunits (Johnson et al., 2005). We will also examine the role of two of the Hap4-like subunits in *CYC1* expression in iron replete versus iron limited growth.

Materials and methods:

Plasmid construction: All oligonucleotide primers used for these studies are shown in Table 2-1. The *CYC1* promoter (957bp) was amplified by PCR from *Candida albicans* genomic DNA using primers oDM504 and oDM505. The primer oDM504 contains a unique BamHI site at the 5' end and oDM505 contains a unique 3' HindIII site incorporated into the amplified product to facilitate cloning. The amplification used the following protocol: an initial denaturation of 95°C/2 min., followed by 30 cycles of 95°C/1 min.; 48°C/1 min.; 72°C/3 min., and a final extension of 72°C/8 min. The *C. albicans-E. coli* shuttle vector that contains the *Renilla* luciferase gene was pDM659 (McNabb, unpublished). The PCR product (*CYC1* promoter) and vector pDM659 were digested with BamHI and HindIII, resolved by gel electrophoresis and purified with GeneClean (BIO101). The DNA fragments were ligated with T4 DNA ligase and introduced into $CaCl_2$ competent E. coli DH5α. The correct construction of the plasmid was confirmed by restriction enzyme analysis and the plasmid is named pDM659(CYC1/504-505-Rluc). For the *CYC1* promoter deletions, the pDM659(CYC1/504-505) plasmid was used as the PCR template, and the 3' primer oDM505 was used for all deletions with different 5' primers designated oDM512, oDM513, oDM514, oDM515, oDM520 and oDM521. All of the 5' primers incorporated a unique BamH1 restriction site to each product. PCR reactions were performed as stated above with the following variations in the annealing temperatures: oDM0512, 55°C: oDM0513, 63°C; oDM0514, 58°C; oDM0515, 55°C; oDM0520, 55°C; oDM0521, 62°C. The *CYC1* PCR products were digested with BamHI/HindIII, resolved by gel electrophoresis, purified with GeneClean kit, ligated using T4 ligase and introduced into *E. coli* DH5α. Plasmids were confirmed by restriction enzyme digestion. The plasmids were designated pDM659(CYC1/512-505), pDM 659(CYC1/513-505), pDM659(CYC1/514-505), pDM659(CYC1/515-505), pDM659(CYC1/520-505), pDM 659(CYC1/521-505). Due to the incompatibility of using the auxotrophic marker *HIS1* in pDM659 for selection in *C. albicans*, all of the *CYC1* promoter constructs were digested with BamH1 and PvuII to remove the promoter-luciferase gene fusion and subsequently ligate them into the vector pDM692, which contains an *ARG4* auxotrophic marker. The *CYC1* CCAAT site-directed mutant of the full length promoter was generated using pDM692(CYC1/504-505) as a template to mutate the CCAAT to a TTAAT with the oligonucleotide 0DM241 using the Quik Change Multisite-directed mutagenesis kit (Stratagene, La Jolla CA) as per the manufacturers instructions.

Construction of luciferase reporter yeast strains: All yeast transformation were performed using the lithium acetate transformation method previously described (Gietz et al., 1995). Yeast strains used in this study are listed in Table 2-2. The plasmids were introduced to *C. albicans* BWP17 by homologous recombination. To generate a yeast strain lacking mutations in *his1* and *ura3*, pDM644, a plasmid containing *HIS1* was digested with BamHI and the *URA3* gene was cloned into the BamHI site, generating a plasmid containing both *HIS1* and *URA3*. This newly built plasmid (pDM644 His1+Ura3+) was digested with NruI within the *HIS1* selectable marker to target homologous recombination at the *his1* locus in *C. albicans*. The linearized plasmid was introduced into *C. albicans* BWP17 and transformants were selected on medium lacking histidine and subsequently on medium lacking uridine. After transformation, the only remaining auxotrophy was *arg4*, and this strain, designated *Candida albicans* pDM644+His+Ura, was the recipient for all *Renilla* luciferase reporter plasmids. The gene reporter plasmids containing the *CYC1* promoter fused to *Renilla* luciferase were linearized with HpaI within the *ARG4* auxotrophic marker to direct homologous recombination at the *arg4* locus in *Candida albicans* pDM644+His+Ura and transformants were selected on medium lacking arginine. All strains were confirmed to be prototrophic after the rescue of auxotrophies. After completion of all yeast transformations of the reporter plasmids, several independent transformants of each reporter construct were selected and assayed for luciferase activity.

Construction of *CYC1*-luciferase reporter plasmids in *hap* null mutants: pDM592 carrying the full length *CYC1* promoter (CYC1 504-505) was digested with HpaI to linearize. The linearized plasmid was introduced into various *hap* null mutants of *C. albicans* using the lithium acetate transformation protocol (Geitz et al., 1995). The *CYC1*-luciferase reporter plasmids were integrated at the *arg4* locus of strains containing homozygous null mutations in *hap5Δ/hap5Δ, hap31Δ/hap31Δ, hap32Δ/ hap32Δ,* hap31Δ/hap31Δ *hap32Δ/ hap32Δ*, *hap41Δ/ hap41Δ* and *hap43Δ/ hap43Δ*. The resulting transformants were selected in synthetic medium lacking arginine, and assayed for luciferase activity.

***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 + 0.2 mM BPS at 30°C. Cultures were standardized by optical density at A_{600nm}. 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:

$$RLA = \frac{RLU}{OD \times \frac{Va \times Vc}{Vb}}$$

Where RLA is *Renilla* Luciferase activity, RLU is the initial *Renilla* Luciferase luminescence reading, OD is optical density of the cell culture at A_{600nm}, 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).

Table 2-1: Oligonucleotides used in these studies.

Primer name	Sequence
oDM504	5'-GGCCGGATCCACCCACAATTATTGAACTGTTTACATTGTTGATAGG-3'
oDM505	5'-GGCCAAGCTTTGTAATGATATGTATATAGATTAAATTAAATTAAATTAAATTG-3'
oDM512	5'-GGCCGGATCCCATGGAACAATCCGGAACAAC-3'
oDM513	5'-GGCCGGATCGCTGACCTCTTCTTCTTCTTC-3'
oDM514	5'-GGCCGGATCCGTCCTCTCTTTCTCTTTCTC-3'
oDM515	5'-GGCCGGATGCTTGAATTGAATTGAATTGAGTAAATTTTTCAATTAATAGAAATT TTTCAG-3'
oDM520	5'-GGCCGGATCCTTGAATTGAATTGAATTGAGTAAATTTTTCAACCAATAGAAAT TTTTCAG-3'
oDM521	5'-GGCCGGATGCCGCTTGGACGTATTCCCAAATGTACAC-3'
oDM241	5'-GGGCCAGCTGTTATTGTTCATTTTTGAGAACTCGC-3'

Table 2-2: Yeast strain used for these studies

Strain name	Genotype	Reference
BWP17	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG*	(Romier et al., 2003)
DMC117	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG hap5Δ::URA3/hap5Δ::HIS1*	Johnson et al., 2005
hap31Δ	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG hap31Δ::HIS1/hap31Δ::URA3*	Bates, 2009
hap32Δ	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG hap32Δ::HIS1/hap32Δ::URA3*	Bates, 2009
hap31Δ/hap32Δ	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG hap31Δ::hisG/hap31Δ::hisG hap32Δ::HIS1/hap32Δ::URA3*	Bates, 2009
hap41Δ	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG hap4.1::URA3/hap4.1Δ::HIS1*	McNabb, unpub
hap43Δ	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG hap4.3::URA3/hap4.3Δ::HIS1*	McNabb, unpub
CYC1-Rluc	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1-URA3 arg4Δ::hisG/arg4Δ::hisG-CYC1-RLUC-ARG4*	This study
hap5Δ-CYC1:Rluc	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-RLUC ARG4 hap5Δ::URA3/hap5Δ::HIS1*	This study
hap31Δ-CYC1:Rluc	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-RLUC-ARG4 hap31Δ::HIS1/hap31Δ::URA3*	This study
hap32Δ-CYC1:Rluc	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-RLUC-ARG4 hap32Δ::HIS1/hap32Δ::URA3*	This study
hap31Δ/hap32Δ-CYC1:Rluc	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG- RLUC-ARG4 hap31Δ::hisG/hap31Δ::hisG hap32Δ::HIS1/hap32Δ::URA3*	This study
hap41Δ-CYC1:Rluc	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-RLUC-ARG4 hap4.1::URA3/hap4.1Δ::HIS1*	This study
hap43Δ-CYC1:Rluc	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-RLUC-ARG4 hap4.3::URA3/hap4.3Δ::HIS1*	This study

Results

***CYC1* is transcriptionally regulated by the CCAAT-binding factor during iron replete and iron limited growth.** Previous studies have shown that *CYC1* mRNA expression is altered in a *hap5Δ* homozygous mutant when strains are grown in iron replete (YPD) versus iron limiting medium (YPD + 0.2 mM BPS). *CYC1* mRNA levels were down-regulated in a *hap5Δ* homozygous mutant during growth in iron rich medium; however, under iron limitation (YPD+0.2 mM BPS), the expression of *CYC1* mRNA was up-regulated compared to wild type strain (Bates, 2009). These data suggest that the CCAAT-binding factor may function in both transcriptional activation and repression. Alternatively there could be other cis-acting regulatory elements within *CYC1* promoter that interact with additional transcription factors that act in concert with the CCAAT-binding factor in controlling gene expression in response to iron. To address this question, an analysis of the *CYC1* promoter was carried out to specifically define the *cis*-acting regulatory elements within the promoter that were responsible for the iron-dependent regulation. To dissect the *CYC1* promoter, a series of promoter deletions were fused with the *Renilla* luciferase reporter gene (Figure 2-1) to identify the *cis*-acting elements relevant for iron-dependent expression. Initially the full-length *CYC1* promoter (*CYC1* 504) was fused to *Renilla* luciferase and subsequently assayed in *C. albicans* grown under iron replete and iron limiting conditions. *CYC1* 504 is abundantly expressed in YPD (Figure 2-2); however, it is repressed under iron limiting growth approximately 40-fold consistent with the prior observations (Bates, 2009) To further define the regulatory elements responsible for the expression of *CYC1,* a series of promoter deletions were introduced into *C. albicans* (Figure 2-1). As observed in Figure 2-2, the expression of *CYC1* was not dramatically reduced under iron rich growth conditions whenever the CCAAT consensus site remained intact. The deletion mutant *CYC1* 520 removed all sequences upstream of the CCAAT site, yet *CYC1* expression was maintained (Figure 2-2). When the CCAAT site was removed (*CYC1* 514) or altered by site directed mutagenesis (*CYC1* 515), the expression of *CYC1* was dramatically reduced (8-fold). These data suggests that the CCAAT site is the only cis-acting regulatory element within the *CYC1* promoter that modulates gene expression under iron rich growth conditions. To confirm this observation, the CCAAT site was mutated to TTAAT in the context of the full length *CYC1* promoter (SDM) and the expression of *CYC1* was significantly reduced in iron

replete medium, indicating that the CCAAT site and presumably CCAAT-binding factor were solely responsible for *CYC1* mRNA expression under iron rich growth conditions.

To address whether other promoter elements are present that influence the expression of *CYC1* under iron limited growth conditions, the *C. albicans* strains containing the *CYC1* promoter reporter plasmids were grown in iron limiting medium and assayed for *Renilla* luciferase activity. Importantly, the repression of *CYC1* expression was observed when the CCAAT site was present in the promoter (Figure 2-2). Only with the abolishment of the CCAAT site was derepression observed (compare Figure 2-2 YPD verus BPS for each promoter construct). To confirm this observation, the full length *CYC1* promoter with a CCAAT site mutated to TTAAT (SDM) was evaluated after growth under iron limitation. In this promoter mutant, *CYC1* expression was derepressed three-fold compared to the wild-type suggesting the CCAAT site within the *CYC1* promoter is primarily responsible for transcriptional repression of the gene when cells are grown in iron limiting media.

Expression *of the CYC1*-luciferase reporter in the absence of CCAAT-binding activity. The studies described in the previous section suggested that the loss of the CCAAT-binding activity was responsible for the reduction of *CYC1* expression in iron rich medium and the lack of *CYC1* repression in an iron-limiting environment. From previous studies it was shown that a *hap5Δ* homozygous mutant is defective in growth on iron limiting medium (Bates, 2009), and the deletion of *HAP5* results in the loss of CCAAT-binding activity as determined by DNA mobility shift assays (Johnson et al., 2005). Therefore it is reasonable to hypothesize that if the CCAAT-binding factor is solely responsible for the *CYC1* iron-dependent regulation, a *hap5Δ* homozygous mutant would show a *CYC1* expression pattern similar to that of the SDM reporter containing the CCAAT to TTAAT mutation that abolishes CCAAT-binding activity at the *CYC1* promoter.

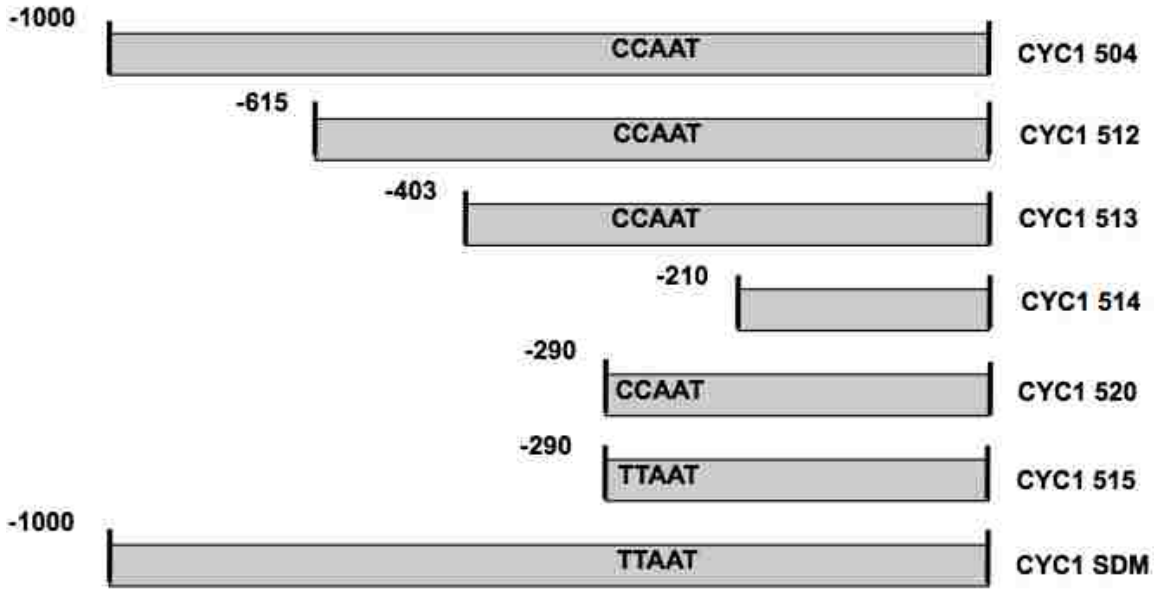

Figure 2-1. **Schematic representation of the *CYC1* promoter deletions used for analysis.** The full length *CYC1* promoter (*CYC1* 504), sequential deletions (*CYC1* 512, *CYC1* 513, *CYC1* 514, and *CYC1* 520), and CCAAT box mutants (*CYC1* 515 and *CYC1* SDM) were fused to *Renilla luciferase* reporter gene as described in the Materials and Methods. The numbers on the left indicate the distance in base pairs from the putative ATG start codon.

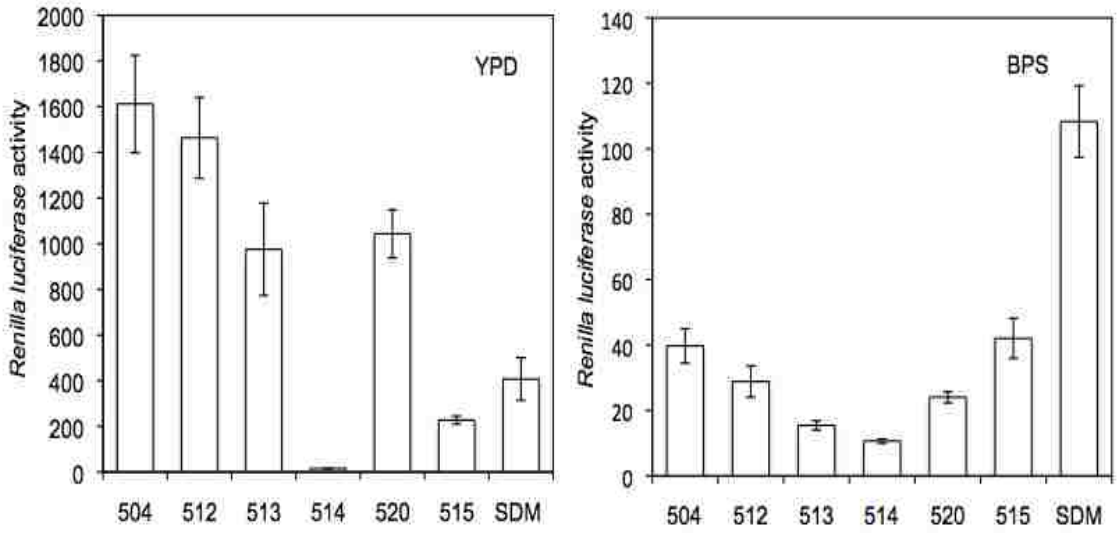

Figure 2-2. The CCAAT site is the only *cis*-element responsible for *CYC1* regulation. *Renilla* luciferase activity was measured in a *C. albicans* wild type strain having the *CYC1*-luciferase reporters integrated at the *ARG4* locus as described in the Materials and Methods. The designations of the promoter constructs are as indicated in Figure 2-1. For the luciferase assays, the strains were grown overnight at 30°C in iron replete (YPD) or iron limited medium (YPD+ 0.1 mM BPS). The following day, the strains were diluted to fresh medium (YPD or YPD+0.2mM BPS), allowed to grown to exponential phase, and subsequently assayed for luciferase activity. The panel of the left and right are strains grown in YPD or YPD + BPS (BPS) as indicated. Error bars represent the mean ±SEM from three independent assays.

To test this hypothesis, the full length *CYC1* promoter-luciferase reporter (*CYC1* 504) was introduced into a *hap5Δ* homozygote and luciferase assays were performed on the strain grown in iron rich (YPD) versus iron limited (YPD + 0.2 mM BPS) medium. In YPD, luciferase expression in the *hap5Δ* homozygous strain was 8-fold decreased compared to the wild type strain (Figure 2-3 YPD), consistent with the data in the previous section showing the loss of the *cis*-acting CCAAT site resulted in decreased *CYC1* expression. When cells were grown under iron limitation the luciferase expression of the wild type strain (*CYC1* 504) was down-regulated 32-fold compared to the wild type in YPD medium (Figure 2-3 BPS). However, when comparing the wild type versus *hap5Δ* homozygous strain, *CYC1* expression was derepressed in the *hap5Δ* homozygote (Figure 2-3, BPS), reinforcing the conclusion that the CCAAT-binding factor is responsible for both activation of *CYC1* in iron replete medium and repression of *CYC1* under iron limitation.

Role of *HAP31* versus *HAP32* in *CYC1* expression. It has been shown previously that *HAP31* is preferentially expressed in iron rich conditions and absent in iron limiting conditions; whereas, *HAP32* is only expressed when cells are grown in iron limiting conditions (Bates, 2009)Further support for the differential expression of *HAP31* versus *HAP32* comes from electrophoretic mobility shift assay studies that revealed the Hap2/31/5 DNA-binding complex is formed when cells are grown in iron replete conditions and Hap2/32/5 is preferred in iron limiting conditions (Bates, 2009)In these same studies, it was also observed that in a *hap32Δ* homozygote, Hap31 can be expressed under iron limitation and form the DNA-bound Hap2/31/5 complex capable of partially substituting for the lack of Hap32 (Bates, 2009). Nevertheless, the previous data strongly suggested that Hap2/31/5 is the DNA-binding complex under iron replete conditions and Hap2/32/5 functions under iron limitation. Given these observations, it was reasonable to hypothesize that Hap31 and Hap32 could serve as the molecular switch for the CCAAT-binding factor. In the presence of Hap31, the CCAAT-binding factor is a transcriptional activator that stimulates *CYC1* transcription and in the presence of Hap32, it functions as a transcriptional repressor.

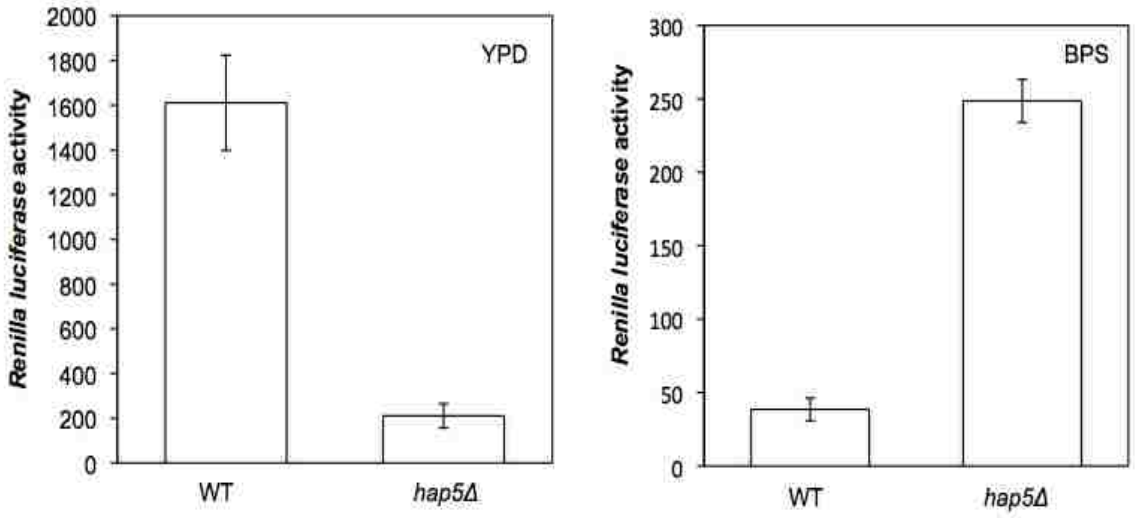

Figure 2-3. The CCAAT-binding factor regulates *CYC1* transcription. The *CYC1* promoter construct (*CYC1* 504) fused to *Renilla* luciferase reporter was integrated into the *arg4* locus of a *C. albicans* wild type and *hap5Δ* homozygous mutant. The strains were grown overnight at 30°C in YPD and YPD containing 0.1mM BPS. The following day cultures were diluted into fresh medium (YPD or YPD+0.2mM BPS), grown to exponential phase and luciferase assays were performed. The panel of the left and right are strains grown in YPD or YPD + BPS (BPS) as indicated. Error bars represent the mean ±SEM from three independent assays.

To further explore this model, the full length *CYC1*-luciferase reporter plasmid (*CYC1* 504) was introduced into *C. albicans* strains including: wild type, *hap31Δ/hap31Δ, hap32Δ/hap32Δ,* and the *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant to determine the role of the Hap3 isoforms in *CYC1* expression. The strains were grown in iron rich medium (YPD) or iron limiting medium (YPD + 0.2 mM BPS) and assayed for luciferase activity. The *hap31Δ* homozygote demonstrated reduced expression of *CYC1* on YPD (Figure 2-4), analogous to the *hap5Δ* homozygous mutant (Figure 2-3 YPD). The *hap32Δ* homozygote did not alter *CYC1* expression (Figure 2-4 YPD); however, this was not surprising since *HAP32* mRNA is not expressed on YPD medium (Bates, 2009), and *HAP31* is expressed to form the Hap2/31/5 complex when cells are grown in YPD medium. When the *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant was examined, the expression of *CYC1* was reduced similar to the *hap31Δ/hap31Δ* (Figure 2-4 YPD). These data support the conclusion that Hap31 is the functional Hap3 isoform when cell are grown in iron rich medium (YPD).

When the same *C. albicans* reporter strains were grown under iron limitation (YPD + 0.2 mM BPS), the wild-type, *hap31Δ* homozygote and the *hap32Δ* homozygote all showed identical repression of *CYC1* (Figure 2-4 BPS). The transcriptional repression observed in the *hap31Δ* homozygote was anticipated since *HAP31* is not expressed under iron limitation (Bates, 2009); therefore, it would not participate in the formation of a CCAAT-binding factor. However, why did the *hap32Δ* homozygote not show derepression since it is the subunit expressed under iron limitation. As mentioned previously, Bates, 2009 demonstrated that in a *hap32Δ* homozygote, *HAP31* is expressed and it can compensate for the loss of *HAP32.* Hence, the CCAAT-binding factor formed under iron limitation in a *hap32Δ* homozygote is Hap2/31/5 (Bates, 2009). However, in the *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant derepression of *CYC1* is observed (Figure 2-4 BPS). Given these observations, it could be argued that Hap32 is the Hap3 isoform responsible for transcriptional repression of *CYC1* when cell are in an iron limited growth environment. The reason the *hap32Δ* homozygote does not show derepression of *CYC1* is because Hap31 is being expressed and compensating for Hap32 loss under conditions it would not normally be present. Therefore to see the effect of Hap32 loss of function, the *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant must be employed and in that circumstance one observes loss of *CYC1* repression. Thus

it appears that Hap31 is involved in the transcriptional activation of *CYC1* as part of the Hap2/31/5 complex when cells are grown under iron rich conditions. In contrast, Hap32 appears to be involved in *CYC1* repression on iron limiting medium as part of the Hap2/32/5 complex bound to the *CYC1* promoter.

Hap43 is essential for the repressor function of the CCAAT-binding factor. It was previously shown that *HAP43* mRNA expression is regulated by Sfu1, a negative regulator in *C. albicans* that is responsible for sensing the levels of iron in the growth environment (Lan et al., 2004) Additional studies have shown that Hap43 is a repressor of iron dependent proteins during iron limited growth (Bates, 2009; Hsu et al., 2011; Singh et al., 2011). Moreover, it has been observed that a *hap43Δ* homozygous mutant has phenotypic growth defects on medium containing limiting iron (Bates, 2009). Taken together, these observations suggest that Hap43 may serve as the effector subunit interacting with Hap2/32/5 at target promoters to facilitate the transcriptional repression of target genes. To further examine the repressor function of Hap43, the *CYC1* promoter fused to *Renilla* luciferase was integrated into the genome of a *C. albicans hap43Δ* homozygous mutant and luciferase expression was measured. Cells containing the *CYC1*-luciferase reporter were grown in iron rich medium (YPD) and iron limiting medium (YPD + 0.2 mM BPS) and luciferase assays performed. When cells were grown in YPD medium, the *hap43Δ* homozygous mutant had no effect on *CYC1* expression (Figure 2-5). This was not surprising since previous studies have showned that *HAP43* is transcriptionally repressed by Sfu1 under iron limitation (Lan et al., 2004). In contrast, when the cells were grown under iron limitation, *CYC1* was derepressed in the *hap43Δ* homozygous mutant implying that Hap43 may be the effector subunit that participates with the DNA-bound Hap2/32/5 complex in the repression of *CYC1* in an iron limiting growth environment.

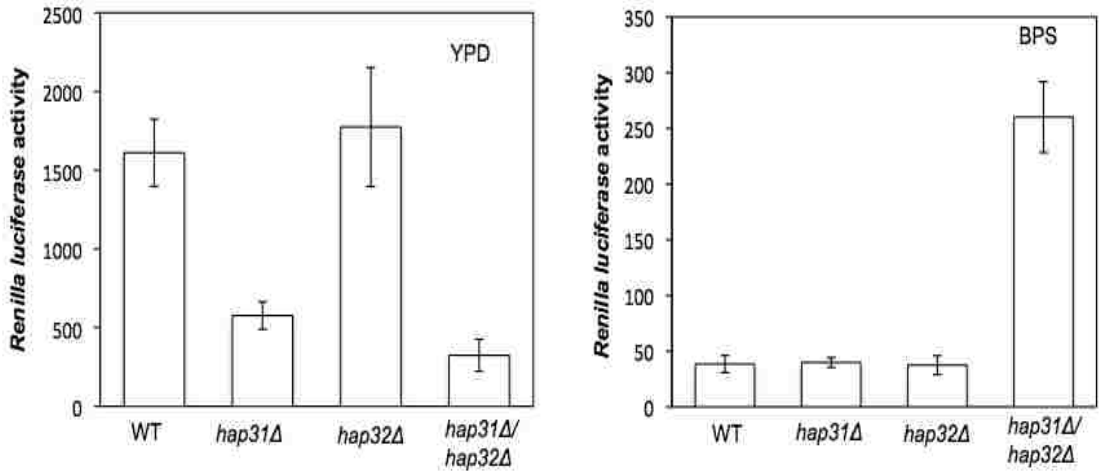

Figure 2-4. Hap31 and Hap32 are necessary for the transcriptional activation versus repression. The *CYC1* promoter construct (*CYC1* 504) fused to *Renilla* luciferase reporter gene was integrated at the *arg4* locus of *C. albicans* wild type, *hap31Δ*, *hap32Δ*, *hap31Δ hap32Δ* homozygous mutants. The strains were grown overnight at 30°C in YPD or YPD+0.1mM BPS and the following day, diluted to fresh YPD and YPD+0.2mM BPS, respectively, and allowed to growth to exponential phase. Luciferase activity was measured for each of the strains. The relevant genotype of each strain is shown and the panel of the left and right are strains grown in YPD or YPD + BPS (BPS) as indicated. Error bars represent the mean ±SEM from three independent assays

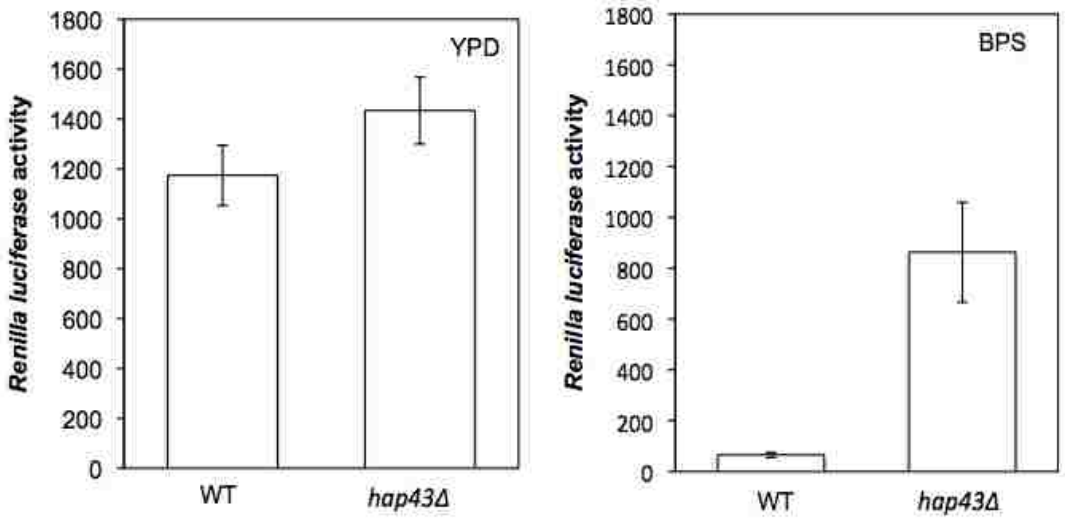

Figure 2-5. Hap43p is essential for the transcriptional repression during iron limitation. The *CYC1* promoter construct (*CYC1* 504) fused to the *Renilla* luciferase reporter gene was integrated at the *arg4* locus of *C. albicans* wild type and *hap43Δ* homozygous mutant. The strains were grown overnight at 30°C in YPD or YPD+0.1mM BPS and the following day diluted in fresh YPD and YPD+0.2mM BPS, respectively, and grown to exponential phase. Luciferase activity was measured for each of the strains. The relevant genotype of each strain is shown and the panel of the left and right are strains grown in YPD or YPD + BPS (BPS) as indicated. Error bars represent the mean ±SEM from three independent assays.

Hap41 does not influence *CYC1* expression. To determine whether the expression of the *CYC1* promoter-luciferase reporter was affected by Hap41p, another Hap4-like protein in *C. albicans*, the full length *CYC1* promoter-luciferase reporter plasmid was introduced into a *hap41Δ* homozygous mutant. The cells were subsequently grown in both iron rich (YPD) and iron limiting medium (YPD +0.2 mM BPS) and luciferase activity determined. As shown in Figure 2-6, the expression of *CYC1* was unaffected by the deletion of *HAP41* suggesting that this subunit is not relevant for the expression of *CYC1* under iron rich or iron poor growth conditions.

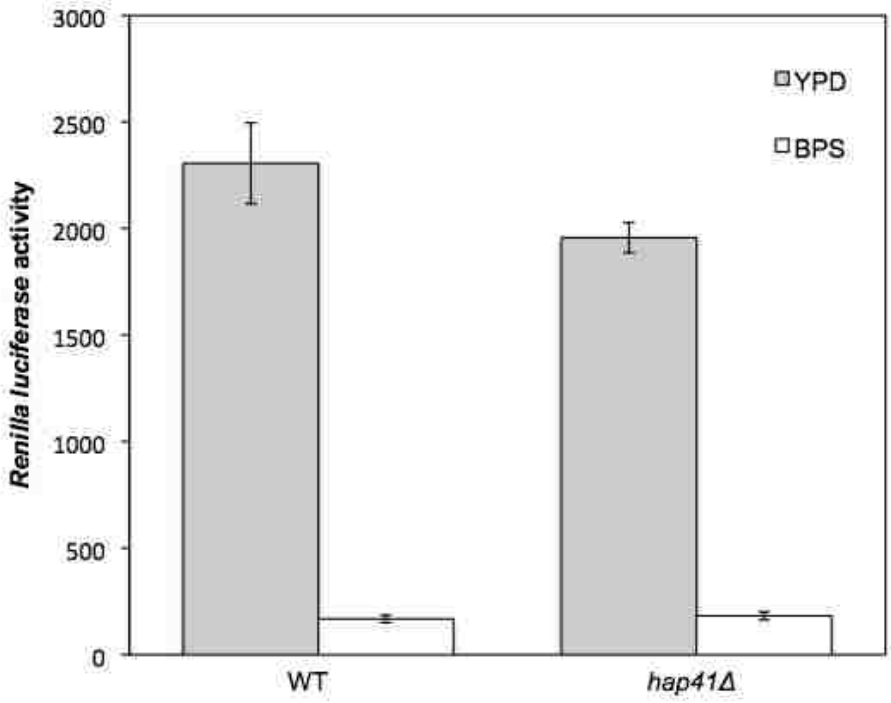

Figure2-6. Hap41 does not play a role in regulation of *CYC1*. The *CYC1* promoter construct (*CYC1* 504) fused to the *Renilla* luciferase reporter gene was integrated at the *ARG4* locus of *C. albicans* wild type and *hap41Δ* homozygous mutant. The strains were grown overnight at 30°C in YPD or YPD+0.1mM BPS and the following day diluted in fresh YPD and YPD+0.2mM BPS, respectively, and grown to exponential phase. Luciferase activity was measured for each of the strains. The relevant genotype of each strain is shown and the strains grown in YPD or YPD + BPS are indicated by the gray and white bars, respectively. Error bars represent the mean ±SEM from three independent assays.

Discussion

The results in this chapter demonstrate that the CCAAT binding site is the only *cis*-acting regulatory element in the *CYC1* promoter that controls the transcriptional activity of this gene in response to iron availability (Figure 2-2). While *CYC1* was used as a model promoter for these studies, it is likely that many other genes containing functional CCAAT sites within their promoter will show a similar regulatory response. We certainly cannot rule out that other promoters may contain additional regulatory elements that influence the overall transcriptional response of a given target gene; nevertheless, the general principles defined by this work lay the groundwork for the evaluation of other genes and how the CCAAT-binding factor influences their function.

The CCAAT-binding factor is highly conserved evolutionarily (Becker et al., 1991; Mantovani, 1999; Thon et al., 2010); however, the *C. albicans* CCAAT-binding factor offers some unique attributes not identified in most eukaryotes, namely the redundancy of the Hap3 and Hap4 subunits. The presence of two isoforms of Hap3 and three isoforms of Hap4 raises questions about why an organism needs multiple isoforms of the same protein to regulate gene regulation. The results presented in this study suggest that Hap31 is important for the activation of *CYC1* under iron replete conditions; whereas, Hap32 is required for repression when cells are grown under iron limitation. These observations are consistent with previous studies from our lab that have shown that *HAP31* mRNA is expressed solely under iron replete conditions and *HAP32* mRNA is expressed exclusively under iron limited growth (Bates, 2009). Moreover, it was also shown that the CCAAT-binding factor subunit composition changes when cells are grown under iron replete versus iron limited conditions. In the presence of sufficient iron, the DNA-binding subunits of the CCAAT binding factor are Hap2, Hap31, and Hap5; however, when iron is limiting the CCAAT-binding factor composition shifts to Hap2, Hap32, and Hap5 (Bates 2009). An important question left unanswered is how Hap32 alters the function of the CCAAT-binding factor in a manner that facilitates transcriptional repression, while Hap31 promotes transcriptional activation. One obvious explanation is that Hap31 versus Hap32 could recruit different Hap4-like proteins that modulate activation versus repression, respectively. This hypothesis remains a viable explanation since Hap43 was shown to facilitate transcriptional repression while Hap41 has no affect (Figure 2-5 versus Figure 2-6). However, it

has also been shown that in a *hap32Δ* homozygous mutant, Hap31 can substitute for Hap32 in facilitating transcriptional repression (Bates, 2009 and Figure 2-4); therefore this could not be the sole explanation.

Host cells are often able to resist microbial infections by a mechanism named natural resistance or nutritional immunity, in which iron is sequestered by host cells to maintain low levels of this metal available for the invading pathogen for growth (Kornitzer, 2009; Hsu et al., 2011). Given the host strategy of limiting iron availability, successful pathogens need to evolve strategies to regulate their genes that require iron to function, but also to develop finely tuned mechanisms to acquire iron and maintain iron homeostasis. These systems must include mechanisms for iron sensing, transport, and storage (Kornitzer, 2009). Thus, it is not surprising that metabolic remodeling must occur within the host in response to available iron. From this perspective, the function of the CCAAT-binding factor as the single regulator of genes that require iron as a cofactor would seem reasonable. Consider the situation in which there is sufficient iron, the Hap2/31/5 complex would bind to the CCAAT site in target promoters and activate those genes which would result in an increased growth response when iron is not limiting. In the opposing scenario, when iron is limiting, the Hap2/32/5 complex would bind to the same CCAAT site in the same promoters to repress those genes, thereby limiting the iron requirement and the growth rate. Under such conditions, the organism may have a reduced growth rate, but it would be able to survive. Therefore, one could consider the CCAAT-binding factor as a molecular switch that can reprogram metabolism when faced with either an iron rich or iron poor environment.

In *S. cerevisiae*, Hap4 is the effector subunit required for activation of transcription, but it is not required for DNA binding (Forsburg and Guarente, 1989). *HAP4* is itself transcriptionally repressed by the presence of glucose and induced in the presence of a non-fermentable carbon source (McNabb and Pinto, 2005). In other words, the activation function of the CCAAT-binding factor is regulated by controlling the expression of Hap4 and this control represents the switch between fermentation and respiration. Hap4 homologs were not found until recently in other organism because the homology was restricted to a small region of the proteins in the N terminus, the domain that interact with Hap2/Hap3/Hap5 (Sybirna et al., 2005). Since that time, Hap4-like proteins have been identified in

several fungi, but they appear to have different functions. For example, Hap43 in *C.albicans,* Php4 in *S. pombe and* HapX in *A. nidulans* are putative Hap4 orthologs that have been shown to have a role in the transcriptional repression of iron-dependent genes during iron limited growth (Lan et al., 2004; Johnson et al., 2005; Hortschansky et al., 2007; Mercier et al., 2008). When iron is abundant another regulatory protein Sfu1 represses Hap43, HapX is downregulated by SreA and Php4 by Fep1 (Lan et al., 2004; Mercier et al., 2006; Hortschansky et al., 2007; Mercier et al., 2008). These DNA-binding proteins, Sfu1, SreA and Fep1, also repress the transcription of iron uptake genes under iron rich conditions. Thus, Sfu1, SreA, and Fep1 appear to be the master regulators of iron metabolism in fungi.

The studies in this chapter demonstrate that Hap43 is necessary for the transcriptional repression of *CYC1*. It has been previously shown that a *C. albicans hap43Δ* homozygous mutant is defective for growth under iron limiting conditions, suggesting its importance in iron homeostasis. As mentioned previously, *C. albicans* has been shown to have three distinct genetic loci that encode Hap4-like homologs, named *HAP41, HAP42, HAP43* (Johnson et al., 2005). In this chapter was also examined the role of Hap41 in the expression of *CYC1*. The results indicated that *CYC1* expression is unaffected in the *hap41Δ* homozygous mutant. Moreover, a *hap41Δ* homozygote shows no defective growth in iron rich or iron deficient medium, further supporting our data (Bates, 2009). However, there is one caveat to this conclusion. It is possible that Hap42 could functionally substitute for Hap41 during growth on iron rich or iron deficient conditions and that may masked the phenotype and reporter expression studies. Studies are currently underway in the McNabb lab to generate double mutant combinations and a triple mutant for *HAP41, HAP42,* and *HAP43* to identify the relevant components for transcriptional activation and repression under iron rich and iron limiting growth conditions.

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Chapter 3. Intron Identification and Splicing in *HAP32* in *Candida albicans*

Introduction

Candida albicans is an opportunistic fungal pathogen found as part of the normal microflora in healthy individuals; however, it can cause life-threatening disease in inmunocompromised individuals. *C. albicans* is considered the major causative agent of fungal infections acquired in hospitals in the United States and worldwide. (Beck-Sague and Jarvis, 1993; Viudes et al., 2002; Jones et al., 2004). This versatile commensal can sense environmental changes and alter its morphology between yeast and hyphal forms during infections to allow invasion of various organs of the host (Lo et al., 1997; Saville et al., 2006). By the same token, successful pathogens must also sense the growth environment within the host and regulate their metabolism to survive the varying nutrient availability.

Iron is the fourth most common element on earth. It is an essential metal for almost all living organisms due to chemical properties that are essential for many biological processes (De Domenico et al., 2008; Drakesmith and Prentice, 2008; Hsu et al., 2011). The same chemical properties that make iron essential for biological systems, namely the ability to easily lose or gain electrons, can also generate problems in cells since the donation of electrons to oxygen results in the formation of oxide anions and hydroxyl radicals that cause oxidative damage to the cells. In light of this problem, organisms have developed exquisite mechanisms to tightly regulate the levels of iron within cells to avoid cellular toxicity and damage. (Wessling-Resnick, 1999; De Domenico et al., 2008). Pathogens must also develop mechanisms to acquire iron from the host under circumstances in which iron may be limiting due to the regulatory mechanisms of the host designed to control iron uptake and utilization.

Candida albicans can acquire iron using several distinct mechanisms involving either a reductive or non-reductive uptake pathway (Kornitzer, 2009). The reductive mechanism relies on the reduction of extracellular ferric to ferrous iron followed by uptake and re-oxidation of the iron within the cell (Eck et al., 1999; Ramanan and Wang, 2000; Weissman et al., 2002). The non-reductive mechanism of uptake involves the use of hydroxamate-type siderophores synthesized by other microorganisms (Heymann et al., 2002; Hu et al., 2002). Although *C. albicans* is unable to synthesize and secrete siderophores, it does possess the ability to bind and internalize the siderophores produced by other microorganisms. *C.*

albicans can also acquire iron in the form of heme from host proteins such as hemoglobin. It is currently thought that GPI-anchored receptors Rbt5 and Rbt51 mediate the uptake of exogenous heme (Weissman and Kornitzer, 2004); however, this has not been definitively proven. Finally, *C. albicans* can scavenge iron from other host cell proteins such as ferritin or transferrin via binding to receptors on the fungal cell surface and subsequent internalization by receptor-mediated endocytosis (Weissman and Kornitzer, 2004; Knight et al., 2005; Almeida et al., 2008; Weissman et al., 2008).

C. albicans possess two functionally distinct CCAAT binding factors. As shown in the previous chapter, the Hap2/31/5 DNA-binding complex is involved in the activation of *CYC1* transcription under nutrient rich growth conditions. In contrast, when cells are grown under iron limitation, the Hap2/32/5 DNA-binding complex, with the participation of Hap43, represses *CYC1* expression. Thus, the organism encodes distinct Hap3-like homologs (Johnson et al., 2005) that differentially influence target gene expression. These proteins are preferentially expressed under differing environmental conditions, with *HAP31* expressed in iron rich conditions and *HAP32* expressed during iron limitation. Hence, the differential expression of *HAP31* versus *HAP32* appears to be a molecular switch that converts the CCAAT-binding factor from a transcriptional activator to repressor, presumably though the recruitment of different Hap4-like homologs as the effector subunit for gene expression.

Previous bioinformatics work has suggested the presence of a putative intron in *HAP32* (Mitrovich et al., 2007). In addition, Northern blotting studies have suggested the presence of two discreet *HAP32* mRNA transcripts that differed in size when cells were grown in iron rich versus iron limited medium (Bates, 2009). When cells were grown in iron rich medium, a poorly expressed mRNA transcript was observed that was slightly larger than the mRNA transcript generated when cells were grown in iron limiting medium (Bates, 2009). Given the bioinformatics studies that suggested the presence of an intron in *HAP32* mRNA, it was conceivable that the splicing of *HAP32* mRNA could be regulated by the presence or absence of iron. In this chapter, I focused on addressing whether the 118 nucleotide intron in *HAP32* was differentially spliced in response to iron availability. In addition, I wanted to examine whether there are properties unique to Hap31 versus Hap32 that could correlate with the differential expression of these

proteins on iron rich versus iron limited medium, respectively. To address this question, I constructed hybrid genes in which *HAP31* transcription was under the control of the *HAP32* promoter and *HAP32* was under the control of the *HAP31* promoter. Hence, expression of Hap32 protein would occur on iron rich medium while expression of Hap31 would occur only under iron limitation with the goal of determining whether the two proteins could functionally replace each other as a subunit of the CCAAT-binding factor under differing environmental conditions.

Materials and methods

Strains and culture conditions: The *Candida albicans* strains used in this study are listed in Table 1. For isolation of total RNA from cells, overnight cultures were grown in 5ml of yeast extract/peptone/ dextrose (YPD) medium or YPD + 0.1 mM bathophenanthroline disulfonate (BPS) (Sigma), an iron chelator. The following day 40 ml of YPD or YPD +0.2mM BPS was inoculated from the overnight cultures and allowed to grow until exponential phase ($OD600_{nm}$ of 0.6 to 1).

Total RNA isolation: Exponentially growing cultures were harvested by centrifugation 3000 X g for 5 min. The cells were resuspended in 400μl of TES and RNA was isolated by the hot phenol method as previously described (Wise, 1991). Briefly, 400μl of cold acid phenol was added to the samples, vortexed for 10 seconds and incubation at 65°C for 45 minutes. The samples were then incubated on ice for 5 minutes and the cell debris removed by centrifugation at 12,000 X g for 5 min at 4°C. The aqueous phase was retrieved; the sample was re-extracted with acid phenol, transferred to a new tube and extracted with chloroform. The aqueous phase was again recovered and 0.3M sodium acetate was added, followed by 2.5 vol. of cold 100% ethanol to precipitate total RNA. Following centrifugation at 12,000 X g for 5 min, the supernatant was removed and the RNA pellet washed with 70% ethanol, and centrifuged again. The supernatant was removed and the samples were allowed to dry. The total RNA was resuspended in 50μl of dH_2O plus 1 μl RNasin Plus (Promega Corp.) and the samples were incubated at 65°C water bath for 30 min to dissolve the RNA. The total RNA samples were stored at -20°C until use. Total RNA was quantified at 260_{nm} absorbance and a concentration of RNA was calculated using an extinction coefficient of 40.

PolyA mRNA isolation: PolyA mRNA was isolated using the PolyA Tract mRNA system (Promega Corp.) following the manufacturer instructions. Briefly, total RNA was diluted with RNase-free dH_2O in a total volume of 500μl. The RNA was denatured at 65C° for 10 min, and 3μl of Biotinylated- oligo(dT) plus 13μl of 20X SSC was added to the samples, mixed and incubated a room temperature until cooled. Streptavidin paramagnetic particles (SA-PMPs) were added to allow binding of the streptavidin to the biotinylated-oligo(dT). The PolyA mRNA bound to the magnetic particles was recovered using a

magnetic rack (Promega Corp) to attract the magnetic particles to the walls of the tube. The supernatant was removed from each sample, and the samples were washed three times with 0.5X SSC, followed by a magnetic capture after each wash. The samples were washed four times with 0.1X SSC. To elute the mRNA, the pellet was resuspended in 100μl of RNase-free dH_2O and the unattached SA-PMPs were removed by magnetic capture and the eluted mRNA was transferred to a fresh tube. The polyA RNA samples were tested for DNA contamination using PCR with oligonucleotide primers oDM0612 and oDM0613 without a reverse transcription step. No amplification product was found in the polyA mRNA samples in the absence of reverse transcription, indicating the lack of DNA contamination.

RNA gel electrophoresis: To confirm the quality of the total RNA and the polyA mRNA, a formaldehyde gel was prepared as described previously (Johnson et al., 2005). Briefly, 0.48 g of agarose was mixed in 51ml of dH_2O following heating to dissolve agarose. After cooling of the agarose, 6ml of 10X MOPS and 3 ml of 37% formaldehyde was added to the agarose and the gel was poured. The gel was placed in the electrophoresis chamber along with 1X MOPS running buffer and the gel was run without sample for 10 min. The RNA samples were prepared for gel electrophoresis with following procedure: 5 μl of RNA was combined with 20μl of loading buffer. The loading buffer contained 750μl formamide, 75μl 37% formaldehyde, 150μl 10X MOPS, 7.5μl of 5mg/ml ethidium bromide, and 17.5μl of dH_2O. The RNA samples in loading buffer were incubated for 5 min. at 65°C, placed on ice, and 2μl of 6X loading dye was added to each sample. The gel was loaded and electrophoresis was performed for 1-1.5h at 65V, and the gel was viewed and photographed under UV light at 304_{nm}.

Reverse transcription: All oligonucleotide primers used for these studies are listed in Table 3-1. For reverse transcription, the Access RT-PCR system (Promega Corp.) was used as described by the manufacturer. Nuclease free water, AMV/*Tfl* 5X reaction buffer, dNTPs, upstream primers (oDM0612 or oDM0615), downstream primer (oDM0613), 25mM $MgSO_4$, mRNA (from cell grown in YPD or YPD +BPS), AMV Reverse transcriptase, and *Tfl* DNA polymerase were combined. The reaction was performed using the following protocol. First-strand cDNA synthesis was performed with one cycle at 45°C/45 min for reverse transcription, followed by one cycle of 94°C/2 min to inactivate the reverse

transcriptase. For subsequent reactions the following program was used: 40 cycles at 94°C/30 sec, 60°C/1min, 68°C/2 min, and a final extension at 68°C for 7 min. The resulting PCR products were analyzed by electrophoresis using a 2% agarose gel and visualized by ethidium bromide staining.

Plasmid constructions: All oligonucleotide primers used for the plasmid constructions are listed in Table 3-1. The DNA fragments containing the promoters, coding regions and 3' non-coding regions of *HAP31* and *HAP32* were generated independently by PCR. The *HAP31* and *HAP32* promoters were amplified by PCR using the oligonucleotide primers oDM0599/oDM0600 and oDM0603/oDM0604, respectively. NotI and BamHI restriction sites were incorporated into the 5' and 3' primers of the *HAP31* and *HAP32* promoters, respectively. The promoters were cloned into the vector pDM583 by digestion of the PCR products and the vector with NotI and BamHI. The appropriate cloning of the promoter sequences was confirmed by restriction enzyme analysis and agarose gel electrophoresis. The *HAP31* and *HAP32* coding sequence and 3' non-coding regions were amplified by PCR using oligonucleotide primers oDM0601/oDM0602 and oDM0616/oDM0611. For these PCR products, BamHI and SalI restriction sites were introduced into the 5' and 3' ends of the PCR product by the oligonucleotide primers during PCR. The *HAP31* and *HAP32* coding sequence PCR products were digested with BamHI and SalI and ligated into the pDM583 vectors containing either the *HAP31* or *HAP32* promoter. Four plasmids were generated in which either the *HAP31* or *HAP32* promoter was fused to the *HAP31* or *HAP32* coding and 3' noncoding regions. All plasmid ligations were introduced into $CaCl_2$ competent *E. coli* DH5 alpha. All plasmid constructions were confirmed by restriction enzyme digestion and agarose gel electrophoresis.

Integration of plasmids into the *C. albicans* genome: For integration of the *HAP3* hybrid plasmids into *C. albicans*, the plasmids were digested with HpaI within the *ARG4* auxotrophic marker to linearize the plasmid and to direct homologous recombination at the *arg4::hisG* allele in *C. albicans*. The recipient strain for the plasmids was *C. albicans hap31Δ hap32Δ* homozygous double mutant. *C. albicans* was transformed by the lithium acetate transformation method (Geitz et al., 1995), and the transformants were selected on synthetic medium lacking arginine.

Table 3-1: *C. albicans* strains used in this study.

Name	Genotype	Reference
SC5314	Wild type clinical isolate	(Gillum et al., 1984)
hap31Δ/hap32Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG hap31Δ::hisG/hap31Δ::hisG hap32Δ::HIS1/hap32Δ::URA3 arg4Δ::hisG/arg4Δ::hisG-pDM583*	Bates, 2009
HAP3131	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG hap31Δ::hisG/hap31Δ::hisG hap32Δ::HIS1/hap32Δ::URA3 arg4Δ::hisG/arg4Δ::hisG-pDM583-HAP3131*	This study
HAP3132	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG hap31Δ::hisG/hap31Δ::hisG hap32Δ::HIS1/hap32Δ::URA3 arg4Δ::hisG/arg4Δ::hisG-pDM583-HAP3132*	This study
HAP3231	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG hap31Δ::hisG/hap31Δ::hisG hap32Δ::HIS1/hap32Δ::URA3 arg4Δ::hisG/arg4Δ::hisG-pDM583-HAP3231*	This study
HAP3232	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG hap31Δ::hisG/hap31Δ::hisG hap32Δ::HIS1/hap32Δ::URA3 arg4Δ::hisG/arg4Δ::hisG-pDM583-HAP3232*	This study

Table 3-2: Oligonucleotide primers used in these studies.

Primer name	Sequence
oDM0599	5'-GGCCGCGGCCGCTGAATTGAGCCAAGGCAAAGG-3'
oDM0600	5'-GGCCGGATCCAATATTCTTATTTTAACCTGGATTATG-3'
oDM0601	5'-GGCCGGATCCATGAATCAACAAAACGCAAGAGATATAG-3'
oDM0602	5'-GGCCGTCGACTACTAGTGAAGTTGTCCTCTC-3'
oDM0603	5-'GGCCGCGGCCGCTTAATGGAATATTGGAAATTAGAAATTAAAG-3'
oDM0604	5'-GGCCGGATCCGTTTTTTAATTGAATGGCTATATTAGGG-3'
oDM0611	5'-GGCCGTCGACCAATTCAATTTTAACTTCTTGGTGTAGTAGT TGTAGTTCC-3'
oDM0612	5'-CAAATGAAACCATACAACTAACCCTAATATAGCC-3'
oDM0613	5'-GTTCGATTTGTTGAAAGTCTTGGTCTTGGTC-3'
oDM0615	5'-GTTCTCCTTTACATGTGAGGAGAAGTATCG-3'
oDM0616	5'-GGCCACTACTTCACTCAATTACAATGCCA-3'

Results

Identification of the intron within *HAP32.*

A computational approach had previously identified a putative 118 nucleotide intron within *C. albicans HAP32* (Mitrovich et al., 2007). Our lab had observed by Northern blot analysis that *HAP32* appeared to generate two transcripts, one larger and less abundant transcript found exclusively when cells were grown in iron rich medium. In contrast, cells grown under iron limitation showed a smaller, more abundant *HAP32* mRNA species. It was conceivable that the larger *HAP32* transcript could correspond to the size of the mRNA with a 118 nucleotide intron present, raising the questions of whether *HAP32* could be regulated both transcriptionally and post-transcriptionally (RNA splicing). It the absence of mRNA splicing, the functional Hap32 would not be synthesized due to a stop codon within the intron; hence, appropriate splicing was required for Hap32 expression.

To address this possibility, *C. albicans* SC5314 was grown in iron rich (YPD) or iron limiting medium (YPD +BPS) and total RNA was isolated from the cells. To determine whether the isolated RNA was contaminated with DNA, PCR was performed using oligonucleotide primers oDM0612 and oDM0613 with Taq DNA polymerase. If the RNA were pure no PCR product would be anticipated; however, if DNA contamination was present, a PCR product of 634bp would be observed. Analysis of the total RNA from *C. albicans* grown in YPD and YPD + BPS showed a PCR product of 634bp indicating DNA contamination (data not shown). To alleviate the problem, polyA RNA was isolated as described in the Materials and Methods, tested for DNA contamination, and no PCR product was observed. These highly purified RNA samples were then subjected to reverse transcription followed by PCR using primer pair oDM0612/oDM613 and oDM0615/oDM0613 and the PCR strategy depicted in Figure 3-1. Following PCR, the samples were evaluated by agarose gel electrophoresis (Figure 3-2). As controls. *C. albicans* genomic DNA was examined with each primer pair. As seen in Figure 3-2, the intron appears to be spliced regardless of whether the growth medium was iron rich or limiting, suggesting that the splicing of *HAP32* is not regulated by iron availability. It should be noted that the intron was observed when using the primer pair oDM0615/0DM613; however, that could result from trace amounts of unspliced transcript present in the polyA mRNA sample we isolated for the PCR. Nevertheless, it was clear that *HAP32*

mRNA splicing occurred during growth in iron rich and iron limiting medium suggesting that *HAP32* mRNA splicing is not regulated by iron availability.

As noted above, computational studies suggested the presence of a 118 nucleotide intron in *HAP32* (Mitrovich et al., 2007). To confirm the presence size and sequence on the intron sequence, the PCR products obtained with primers oDM0612/oDM0613 were subjected to DNA sequencing. The size and location of the intron was identified and it was found to be consistent with the bioinformatics prediction for *HAP32* (data not shown).

Hap31 cannot fully complement Hap32 during iron limited growth.

Previous studies have shown that a *C. albicans hap32Δ* homozygous mutant had a slight defect when grown under iron limitation (Bates, 2009). It was also shown that Hap32p is preferentially expressed to form the Hap2/32/5 DNA binding complex during iron limited growth; however, it was also shown that Hap31p could substitute, at least partially, for Hap32p in the *hap32Δ* homozygote. In the studies shown in Chapter 2, *HAP32* did not play a role in the expression of the *CYC1* reporter gene in iron rich conditions. However, *HAP32* was needed for repression of the *CYC1* reporter gene during iron limitation, but in a *hap32Δ* homozygote, Hap31 was able to substitute for the *hap32Δ* null mutations to facilitate transcriptional repression. This raises the question of why there are two distinct Hap3-like proteins and why they are seemingly necessary under different growth conditions. Does Hap31 impart different properties on the CCAAT-binding factor than Hap32? If so, then why can Hap31 partially replace Hap32, but Hap32 does not appear to effectively replace Hap31.

To address these questions, it seemed reasonable to evaluate whether changing the expression program of *HAP31* and *HAP32* would have any phenotypic effect on *C. albicans* growth in iron rich versus iron limitation. To accomplish this, the promoter for *HAP31* was fused with the coding sequence of *HAP32* (designated *HAP3132*), and the *HAP32* promoter was fused to the coding sequence of *HAP31* (designated *HAP3231*). The logic of this experiment was to determine whether the expression of these

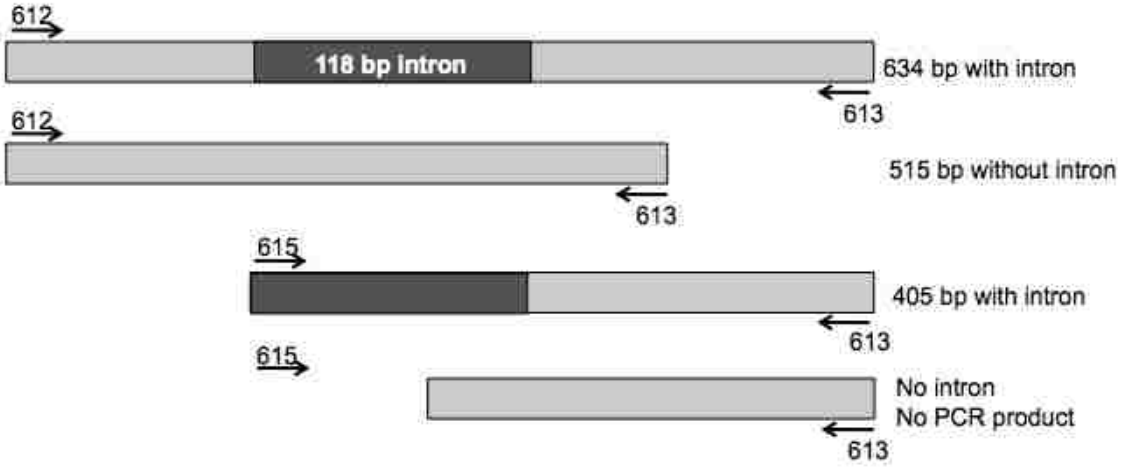

Figure 3-1. Schematic of the region of *HAP32* containing the putative intron. The strategy for the RT-PCR reactions is shown with the primer pairs indicated by arrows. The predicted size of each PCR product is shown on the right.

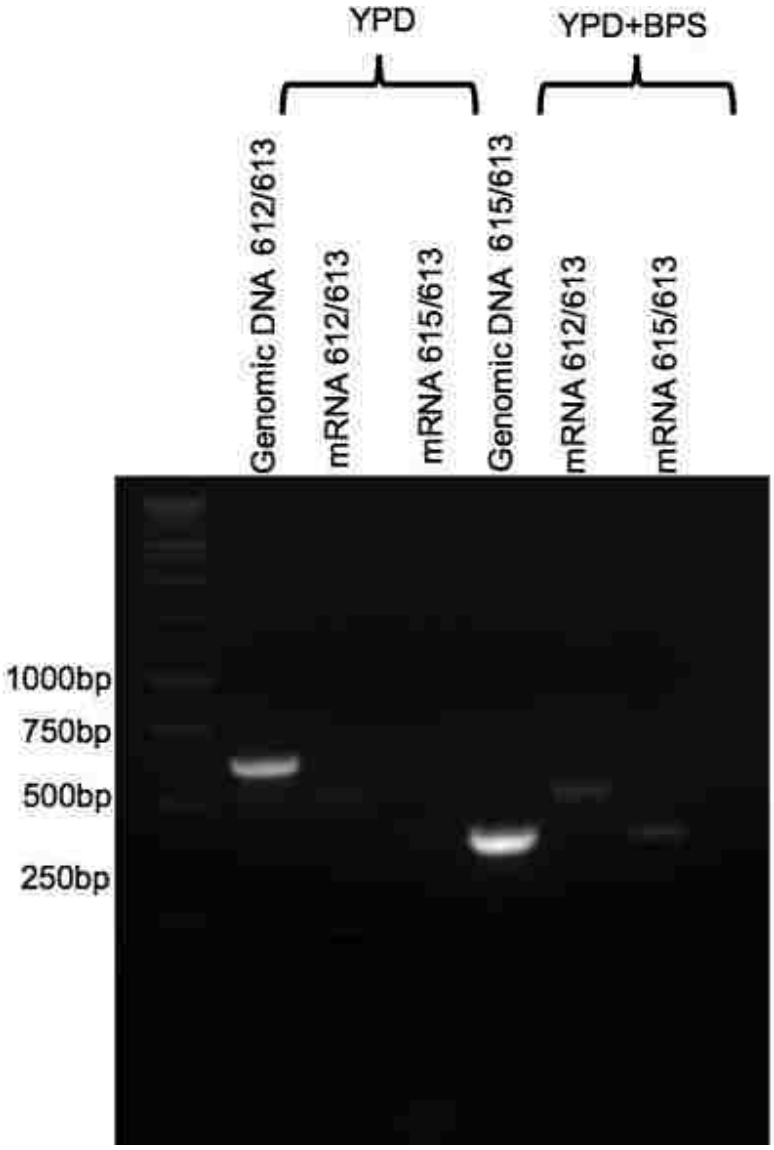

Figure 3-2. Analysis of *HAP32* mRNA splicing. PolyA RNA was isolated from *C. albicans* SC5314 grown in iron rich (YPD) or iron limited (YPD + 0.2 mM BPS) medium. The RNA was subjected to RT-PCR followed by agarose gel electrophoresis as described in Materials and Methods. Lane 1 is the 1 kb DNA ladder (Promega Corp) with the relevant sizes indicated on the left. The RT-PCR samples are indicated in each lane by the growth conditions and the primer pairs used for amplification.

proteins under opposing conditions, namely *HAP32* expression during iron rich growth and *HAP31* expression under iron limitation, would affect the growth the *C. albicans*. Since restriction sites were artificially introduced into the promoter and coding sequences of both *HAP31* and *HAP32* to generate these hybrid genes (Figure 3-3), two positive controls were also constructed in which *HAP31* promoter was fused with the *HAP31* coding sequence (designated *HAP3131*) and the *HAP32* promoter was fused with its corresponding coding region (designated *HAP3232*). All four constructs were made in an identical manner as described in the Materials and Methods section. A schematic representation of the hybrid genes is shown in Figure 3-3.

The plasmids containing the *HAP3131, HAP3232, HAP3132*, and *HAP3231* genes were integrated at the *arg4::hisG* locus of a *hap31Δ hap32Δ* homozygous double mutant strain of *C. albicans*. Thus, the only source of *HAP3* would be the allele expressed from the hybrid constructs. As a negative control, the *hap31Δ hap32Δ* homozygous mutant contained the *ARG4* vector integrated at the *arg4::hisG* locus. To evaluate the phenotype, each strain was grown overnight in YPD or YPD + 0.1 mM BPS and serial dilutions were prepared and spotted on YPD or YPD + 0.15 mM BPS agar plates, respectively, as shown in Figure 3-4. In iron replete medium (YPD), the strains all grew normally as compared to wild type with the exception of the *hap31Δ hap32Δ* homozygous mutant that displayed a slightly slower growth phenotype consistent with previous observations (Bates, 2009). When the strains were spotted on YPD+BPS, the *hap31Δ hap32Δ* homozygous mutant did not grow, consistent with previous studies (Bates, 2009). The *HAP3131* expressing strain grew weakly on YPD + BPS (Figure 3-4) consistent with previous observations with a *hap32Δ* homozygote (Bates, 2009). In addition, the growth of the *HAP3232* strain was comparable to the wild-type on YPD + BPS (Figure 3-4). These observations suggest that the introduction of restriction sites to construct the hybrid genes was not detrimental to gene expression. The strain containing the *HAP3132* hybrid allele grew slightly poorer on YPD + BPS; however the difference was not dramatic. This is not surprising given the previous observation that the *HAP31* promoter is transcriptionally induced in a *hap32Δ* homozygous mutant, and *HAP31* can partially replace *HAP32* for function. The most interesting observation from this experiment was the fact that *HAP31*, expressed from a *HAP32* promoter grows poorly on YPD + BPS. In fact, the growth rate was very similar to the *HAP3131*

hybrid allele. These data strongly suggests that there is some unique properties associated with Hap32p that are absent from Hap31p and these properties are relevant for regulating gene expression during iron limitation.

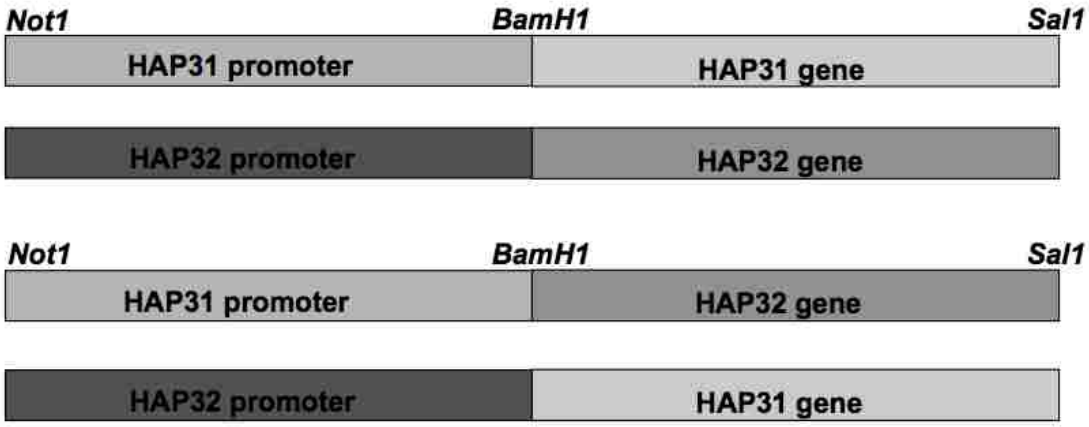

Figure 3-3. Schematic of the *HAP3* gene fusions. The figure indicates the restriction enzyme sites that were artificially introduced into each gene. The locations of the restrictions sites are the same for each fusion.

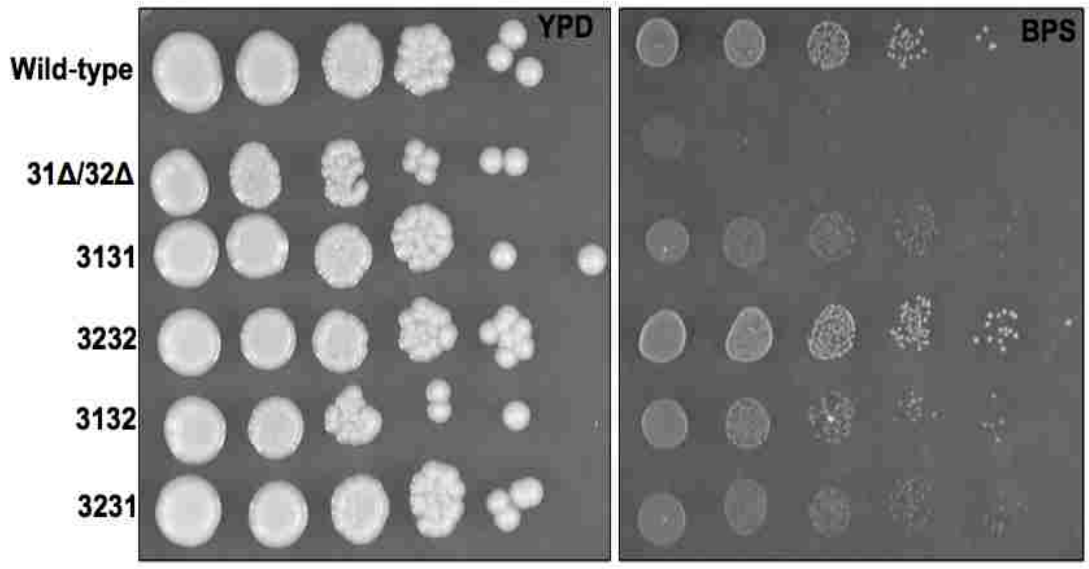

Figure 3-4. Growth of strains containing hybrid *HAP3* genes on YPD and YPD+BPS. The indicated strains were grown overnight in YPD or YPD + 0.1 mM BPS. The cells were quantified by hemocytometer and 10-fold serial dilutions were prepared for each strain. The cells were spotted to YPD or YPD + 0.15 mM BPS and grown for 3 days at 30^{o}C. The relevant hybrid gene is shown on the left of the panels.

Discussion

In most eukaryotes, including mammals, each of the subunits of the CCAAT-binding factor is encoded by a single genetic locus (Maity and de Crombrugghe, 1998); while in plants, such as *Arabidopsis thaliana*, the different subunits of the CCAAT-binding factor are encoded by multiple distinct genetic loci (Riechmann and Ratcliffe, 2000; Gusmaroli et al., 2001; Siefers et al., 2009). Interestingly, the different genes encoding the *HAP2, HAP3*, and *HAP5* orthologs in *A. thaliana* are expressed during different developmental stages in the plant (Edwards et al., 1998; Kwong et al., 2003; Wenkel et al., 2006), suggesting that they impart different functions to the CCAAT-binding protein complex at different times during plant development (Gusmaroli et al., 2001; Kato, 2005; Chen et al., 2007; Siefers et al., 2009). By analogy, *C. albicans* contains two genetic loci encoding *HAP3*: *HAP31* and *HAP32*. These proteins are expressed differentially in response to the availability of nutritional iron with *HAP31* expressed when iron is abundant, and *HAP32* expression occurs only under iron limitation. This suggests that *C. albicans*, like *A. thaliana*, has evolved to use a different CCAAT-binding factor subunits to alter the function of the transcription factor while maintaining its ability to recognize the same *cis*-acting sequence element, 5'-CCAAT-3'. Our goal in this chapter was to understand if there were some characteristics of the Hap31 versus Hap32 proteins that provided an advantage to the cell under different environmental growth conditions.

From bioinformatics studies, it was observed that *HAP32* contained a 118nucleotide intron at the 5' end of the coding region of the gene (Mitrovich et al., 2007). We hypothesized that the presence of an intron in *HAP32* could provide a mechanism for posttranscriptional control by differentially controlling splicing in response to iron availability. To our knowledge, there are no examples of differential splicing in response to iron, so if this were true it would offer the opportunity to study a unique mechanism of iron sensing to control gene expression in response to iron. While our studies did not indicate that *HAP32* mRNA splicing was iron-dependent, we did confirm the *in silico* observation that *HAP32* contains a 118 nucleotide intron.

The second aspect of this work was to examine whether there were unique properties associated with Hap31p versus Hap32p that were important for growth under iron rich versus iron limiting conditions, respectively. As a preliminary approach to address this question, the promoters for *HAP31* and *HAP32* were exchanged so that *HAP32* would be expressed when iron is abundant and *HAP31* would be expressed when iron is limiting. We could then observe whether the aberrant expression of the Hap3 isoforms yielded a visible phenotype when cells were grown in iron rich versus iron limited medium. The major observations from this work was that Hap31p does not restore normal growth to a *C. albicans hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant grown under iron limitation whether it is expressed from the *HAP31* or *HAP32* promoter. This simple observation suggests that Hap32p does have unique properties important for normal cellular growth under iron limiting conditions. What is that property? That question remains unanswered; however, one could speculate that it is some structural feature of Hap32 that is absent from Hap31. Additional hybrid alleles that generate chimeras within the coding regions of *HAP31* and *HAP32* may allow us to uncover these differences.

Due to sequestration of iron, mammalian hosts are iron limiting environments for microbial pathogens to survive (Ratledge and Dover, 2000). As a human opportunistic pathogen, *Candida albicans* has evolved to survive in both iron rich and iron limited environments. Presumably the presence of two Hap3-like proteins allows it to better reprogram gene expression to the different habitats that *Candida albicans* encounters in the human host. Understanding the unique properties associated with Hap31p versus Hap32p that impart the ability of the CCAAT-binding factor to function as both an activator and repressor will help elucidate how this transcription factor can differentially control the expression of genes within *C. albicans* in response to nutrient availability.

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Chapter 4: Phenotypic Characterization of a *hem1Δ* Homozygote of *Candida albicans*

Introduction

Candida albicans is a commensal in the gastrointestinal, genitourinary tract and to a less extent on the skin (Sudbery et al., 2004; Ebanks et al., 2006). This opportunistic pathogen is capable of causing disease once the host immune system is weakened or suppressed due to cancer chemotherapy, HIV infection, or even when the normal flora has been eliminated due to antibiotic therapy (Flisfisch et al., 2008). This human pathogen has been associated with 78% of fungal nosocomial infections and 10% of all nosocomial infection (Dumitru et al., 2004). *C. albicans* is a dimorphic fungus with the ability to switch between hyphal and budding yeast forms, and this dimorphic transition is a required characteristic for virulence (Noverr and Huffnagle, 2004). Another virulence factor, typical of most microbial pathogens, is the ability to acquire iron from the human host. As reviewed in previous chapters of this dissertation, the host, although rich in iron, has the iron sequestered such that it is not readily available for invading pathogens. For pathogens to gain access to these iron stores, they have evolved sophisticated mechanisms of iron uptake. *C. albicans*, encodes genes that facilitate both reductive and non-reductive iron acquisition (Eck et al., 1999; Hammacott et al., 2000; Lesuisse et al., 2002; Lan et al., 2004). In addition, *C. albicans* has cell surface receptors for ferritin and transferrin that allows uptake to extract the iron (Knight et al., 2005; Almeida et al., 2008). Finally, *C. albicans* can utilize the heme found in hemoglobin as a source of iron for growth (Weissman and Kornitzer, 2004).

Heme is an essential molecule for both eukaryotic and prokaryotic cells (Phillips and Kushner, 2001). It is required for ergosterol, methionine and unsaturated fatty acid biosynthesis (Bard et al., 2005). *HEM1* encodes for δ-aminolevulinate synthase, an enzyme that catalyses the first step of the heme biosynthetic pathway. In *Saccharomyces cerevisiae,* the deletion of *HEM1* has been shown to prevent the transcription of genes involved in the high affinity iron uptake system (Crisp et al., 2003). In *C. albicans*, whole genome microarray studies have suggested that *HEM1* is down-regulated in response to growth in low iron medium (Lan et al., 2004). During anaerobiosis, it has been shown that *S. cerevisiae* must acquire exogenous sterols since the organism is unable to synthesize ergosterol in the absence of oxygen. This phenomenon has been called aerobic sterol exclusion.(Lorenz and Parks, 1991; Crowley et al., 1998). A *S. cerevisiae hem1Δ* mutant cannot grow in an anaerobic environment without

supplementation of the growth medium with ergosterol and a source of unsaturated fatty acids, typically Tween-80 is used (McNabb, unpublished). The importance of heme in the regulation of sterol biosynthesis is well known; however, any mutation that affects heme synthesis will result in defects in several heme-dependent processes such as respiration, unsaturated fatty acid synthesis, sterol synthesis and aerobic sterol exclusion.(Trocha and Sprinson, 1976; Gollub et al., 1977).

Candida albicans has been often referred as a facultative anaerobe, but there are very few studies that describe the characteristics or metabolism during the anaerobic growth of this human pathogen. Dumitru *et al.* published a defined anaerobic medium that allows *Candida albicans* to grow anaerobically *in vitro* and strains grown under these conditions were more resistant to antifungal compounds (Dumitru et al., 2004). This was a particularly relevant observation because *C. albicans* forms biofilms on medical implant devices (Kojic and Darouiche, 2004), and many of the cells within a biofilm are surviving in a virtually anaerobic environment (Davey and O'Toole G, 2000). Hence, understanding the anaerobic metabolism of *C. albicans* is critical to understand how this organism responds to antifungal compounds in the absence of oxygen. *C. albicans* has been found to grow anaerobically in medium supplemented with oleic acid and nicotinic acid (Dumitru et al., 2004). Dumitru *et al* showed that *C. albicans* may not need ergosterol as a supplement in the media for growth, which was somewhat surprising since ergosterol is only produced in presence of oxygen. No additional studies were performed to identify how *C. albicans* can meet the sterol requirement under anaerobic conditions; however, it is interesting to note that the antifungal compounds tested by Dumitru et al. (2004) targeted the ergosterol pathway. Thus, the lack of sensitivity to antifungals may result from the lack of ergosterol in the membrane when cells are grown in anaerobic conditions.

This chapter describe the construction of a *C. albicans hem1Δ* homozygous mutant and the studies on the media supplementation needed for the growth of the *hem1Δ* mutant both aerobically and anaerobically. These studies were initiated because it has been shown that a *S. cerevisiae hem1Δ* mutant, when supplemented with ergosterol and Tween-80 for growth, showed a gene expression pattern characteristic of cells grown anaerobically. If *C. albicans* showed a similar response, it would allow us to

conveniently investigate gene expression under anaerobic conditions without the need for sophisticated anaerobic growth chambers. Hence, our initial goal was to generate a *C. albicans hem1Δ* homozygote and determine the growth requirements for the strain under both aerobic and anaerobic conditions. This would then allow us to simulate anaerobiosis in the laboratory to study drug sensitivity.

Materials and Methods

Yeast strains and growth medium: *C. albicans* strains used in this study are listed in Table 4-1. Yeast strains were grown, aerobically or anaerobically, on Yeast extract-Peptone-Dextrose (YPD) medium. The medium was supplemented with 0.03 mg/ml ergosterol, 0.02 mM oleic acid, 10% Tween-80, 0.08 mM nicotinic acid, 1 mg/ml 5-aminolevulonic acid (ALA) or 50 μM hemin as needed. For the dilution spot plating, *C. albicans* strains were grown overnight in YPD supplemented with 1 mg/ml ALA. For the growth assay, 10 μl of the overnight culture was added to 90ul of phosphate-buffered saline (PBS) and 10-fold serial dilutions were prepared with 2μl of each dilution spotted to the appropriate medium. The *hem1Δ* homozygote was maintained by growth on YPD supplemented with ALA as described above. For anaerobic growth, *C. albicans* strains were incubated in a 100% nitrogen atmosphere (facility generously provided by Dr. Dan Lessner, Department of Biological Sciences, University of Arkansas).

Construction of the *hem1Δ* deletion and rescue plasmids: The oligonucleotide primers used in these studies are shown in Table 4-2. The *HEM1* gene was obtained by PCR amplification of the gene from *C. albicans* genomic DNA. The PCR reaction was performed with KOD polymerase and oligonucleotide primers oDM0545/oDM0546 using the following program: an initial denaturation at 94C°/1 min, followed by 30 cycles of 94C° for 30 sec, 55C°/10 sec, and 74C°/5 min followed by a final extension at 72C°/10 min. The primer oDM0545 incorporated a BamHI restriction site on the 5' end and oDM0546 added a HindIII site to the 3' end of the PCR product for cloning. The *HEM1* PCR product and the vector pSP65 (Promega Corp.) were digested with BamHI and HindIII. The digested DNA fragments were resolved by electrophoresis on a 1% agarose gel and the DNA fragments were excised from the agarose gel, purified by GeneClean (BIO101) and ligated using T4 DNA ligase. The ligation reaction was introduced into $CaCl_2$ competent *E. coli* DH5α using standard DNA transformation procedure. The correct construct was verified by restriction enzyme analysis of the plasmid DNA purified from *E. coli*. The *HEM1* rescue plasmid was generated in an identical manner except the starting vector was pDM644 that contains a *HIS1* auxotrophic marker for selection in *C. albicans*. For construction of the hem1Δ::hisG-URA3-hisG knockout plasmid, the pSP65-HEM1 was digested with BglII to remove 954bp of coding sequence. The plasmid was alkaline phosphatase treated and the hisG-URA3-hisG cassette (Sharkey et al., 2005)

digested with BamHI and BglII was ligated into the plasmid. The correct construct was verified by restriction enzyme digestion.

Construction of the *C. albicans hem1Δ* homozygous mutant: All *C. albicans* transformation were performed using the lithium acetate method as previously described (Gietz et al., 1995). The strategy for constructing the *hem1Δ/hem1Δ* mutant of *C. albicans* is depicted in Figure 4-1. To delete the first copy of *HEM1*, the plasmid pSP65-HEM1-hisG was digested with BamHI and HindIII and introduced into C. albicans BWP17 by lithium acetate transformation. The transformants were selected on synthetic medium lacking uridine. To confirm the deletion, genomic DNA was isolated from several independent transformants as previously described.(Hoffman and Winston, 1987) and used as the template PCR with the primers oDM0555/oDM0369. The correct transformants were again evaluated by PCR using primers oDM0555 and oDM0536 (anneals within the hisG repeat). Strains that were positive for *hem1Δ* gene disruption were grown on medium containing 5-fluoroorotic acid (5-FOA) to select for strains that lost the *URA3* marker via homologous recombination between the flanking hisG repeats. The second allele of *HEM1* was deleted using the same strategy, except the starting strain for the transformation was the *HEM1/hem1Δ* heterozygote. Following transformation of the knockout allele, the correct transformants were selected on synthetic medium lacking uridine and supplemented with 1 mg/ml of ALA to support cell growth in the absence of heme biosynthesis. Verification of the gene disruption was performed as outlined above. Following construction of the *HEM1/hem1Δ* and *hem1Δ/ hem1Δ* strains, the remaining auxotrophies were rescued to prototrophy. For the *HEM1/hem1Δ and hem1Δ/ hem1Δ* mutants, the *his1::hisG* was rescued by digestion of the *HIS1*-containing plasmid pDM644 with NruI to target integration at the *his1* locus following transformation. The *arg4::hisG* allele was rescued to prototrophy by digestion of the *ARG4* plasmid pDM583 with HpaI to target integration at the *arg4* locus. To construct the *hem1Δ/ hem1Δ* mutant strain with the knockout mutation restored, the plasmid pDM644-HEM1 was digested with NruI and transformed into the *hem1Δ/ hem1Δ* mutant to target integration of *HEM1* to the *his1::hisG* locus.

Table 4-1: Strains used in this study

Strain name	Genotype	Reference
DMC146	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4-URA3*	Johnson et al., 2005
BWP17	*ura3Δ::imm434/ura3 Δ::imm434his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG*	Romier et al., 2003
HEM1/hem1Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HEM1/hem1::hisG-URA3-hisG*	This study
hem1Δ/hem1Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 hem1Δ::hisG /hem1Δ::hisG-URA3-hisG*	This study
hem1Δ/hem1Δ HIS1-HEM1	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-HEM1-HIS1 argΔ::hisG/arg1Δ::hisG-ARG4 hem1Δ::hisG/hem1::hisG-URA3-hisG*	This study

Table 4-2: Oligonucleotide primers used in these studies

Name	Oligonucleotide primers
oDM0545	5’-GGCCGGATCCACTATAACGTATGGACTGGGCGAACATCAGCC-3’
oDM0546	5’-GGCCAAGCTTTATCGAATCCCAGAAAGATTTGTATTTGAAGATGTG-3’
oDM0555	5’-GCTTTGGGCTTACTGGTATCAAACACC-3’
oDM0536	5’CAGTACCAGAATCGAGCTGGCGCCAAGCGC-3
oDM0369	5'-GGTGAGGCATGAGTTTCTGCTCTCTCA-3'

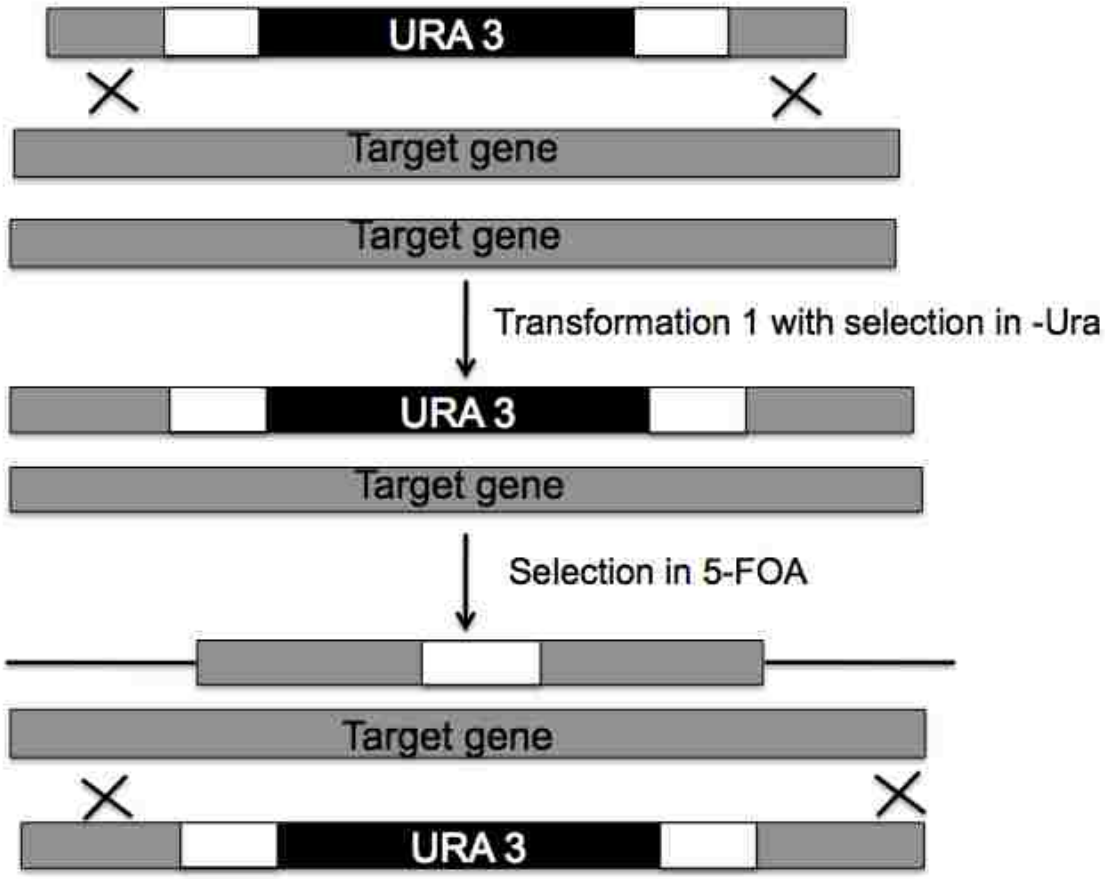

Figure 4-1: Strategy for generating the *hem1Δ* homozygous mutant of *C. albicans*. The first allele of *HEM1* was disrupted using the hisG-URA3-hisG cassette via homologous recombination directed by 5' and 3' HEM1 flanking sequences (shown in gray). Following verification of the heterozygote, the strain was grown on 5-FOA medium to select for strains that loss the *URA3* through recombination between the *hisG* repeats flanking *URA3* (depicted in white). After verification of the marker loss, the *HEM1/hem1Δ* strain was again transformed with the same knockout construct and the homozygous *hem1Δ* was selected on medium lacking uridine and supplemented with ALA. The correct gene knockout was confirmed by PCR.

Results

The *hem1Δ/hem1Δ* mutant was generated by two sequential rounds of gene disruption using the hisG-*URA3*-hisG cassette flanked on the 5' and 3' ends by *HEM1* DNA sequence to target homologous recombination at the *HEM1* locus. To propagate the *hem1Δ/hem1Δ* mutant, the strain was grown on medium supplemented with 5-aminolevulonic acid (ALA). To determine whether any other single nutrient could support the growth of the *hem1Δ* homozygote in the absence of ALA, YPD medium was prepared and supplemented with either Tween-80, oleic acid, nicotinic acid or ergosterol (Figure 4-2). The wild-type, *HEM1/hem1Δ, hem1Δ/hem1Δ,* and the *hem1Δ/hem1Δ/HEM1* strains were spot plated on medium containing each of the individual supplements and grown either aerobically or anaerobically for 2 d or 15 d, respectively. The results of these studies are shown in Figure 4-2. These studies demonstrated that under aerobic conditions, no single supplement supported the growth of the *hem1Δ* homozygote; however, the *hem1Δ/hem1Δ* mutant with a reintegrated *HEM1* at the *HIS1* locus restored normal growth to the strain indicating that the knockout of *HEM1* was solely responsible for the lack of growth. When the same strains were grown under anaerobic conditions it was observed that Tween80 and oleic acid stimulated growth; whereas, the nicotinic acid and ergosterol did not support growth of the *hem1Δ* homozygote. We also evaluated growth of the *hem1Δ* homozygote on YPD medium supplemented with hemin, ALA, and hemoglobin under anaerobiosis; however, no significant growth response was observed (data not shown).

The next series of experiments were performed using a combination of supplements to identify conditions in which the *hem1Δ* homozygote would grow better in the absence of oxygen. Since Dumitru et al. (2004) suggested that nicotinic acid was important for anaerobic growth of *C. albicans*, nicotinic acid was combined with either ergosterol, Tween-80 or oleic acid to determine whether growth would be enhanced under anaerobic conditions (Figure 4-3). It was found that the combination of nicotinic acid and ergosterol would not promote growth. Moreover, the growth of strains in nicotinic acid and Tween-80 or oleic acid did not show significantly improved growth over that observed with either Tween-80 or oleic acid alone (Figure 4-2 compared to Figure 4-3). The growth of individual colonies were visibly larger when

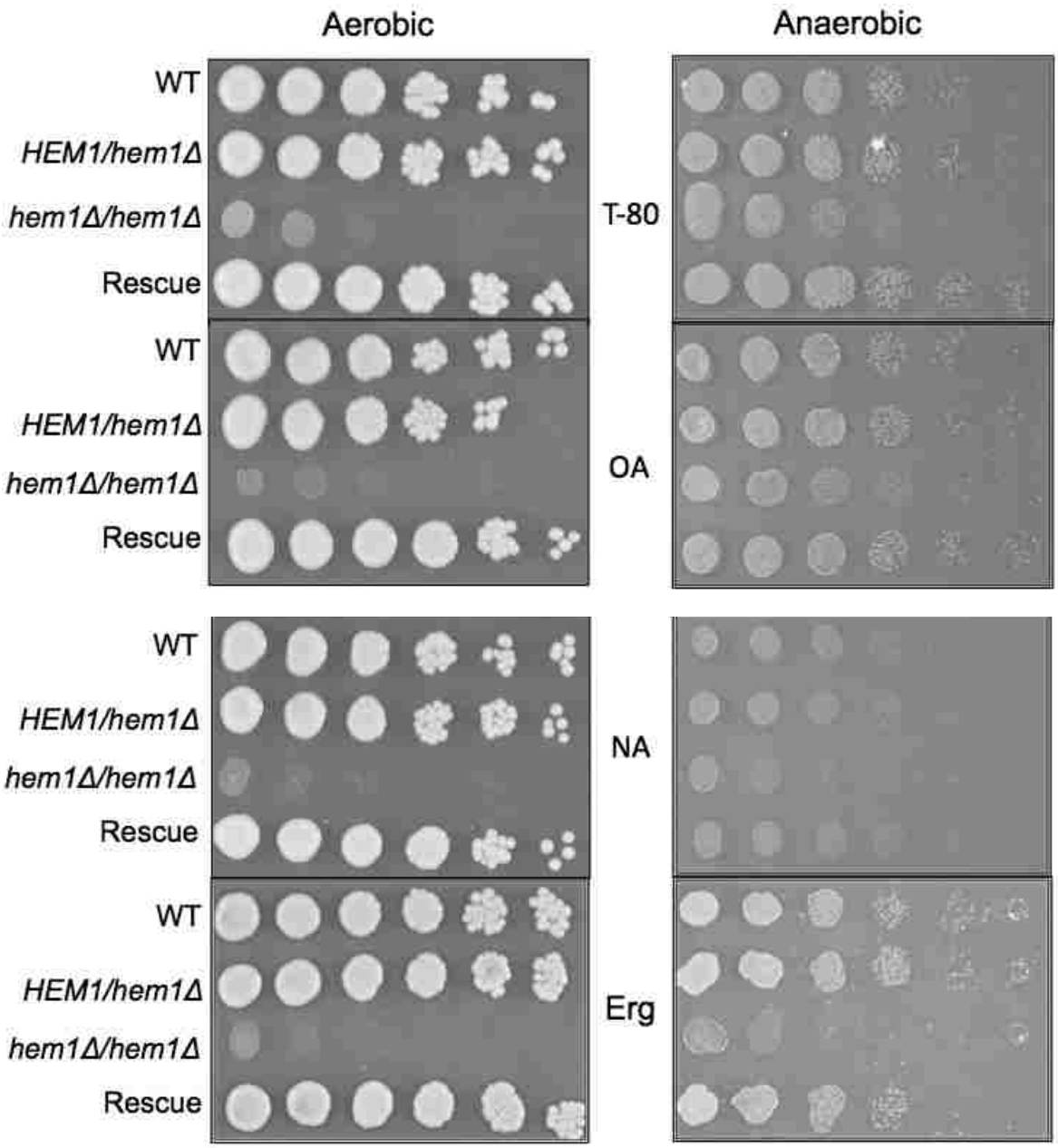

Figure 4-2. Growth of *hem1Δ* homozygous mutant under aerobic and anaerobic conditions. *C. albicans* strains with the indicated genotypes (left of the panels) were grown either aerobically or anaerobically in YPD medium supplemented with Tween-80 (T-80), oleic acid (OA), nicotinic acid (NA) or ergosterol (Erg) as described in the Materials and Methods. For aerobic growth the strains were incubated for 2 days and for anaerobic growth for 15 days. The rescue genotype indicates the *hem1Δ* homozygous mutant with *HEM1* reintegrated into the genome at the *arg4* locus.

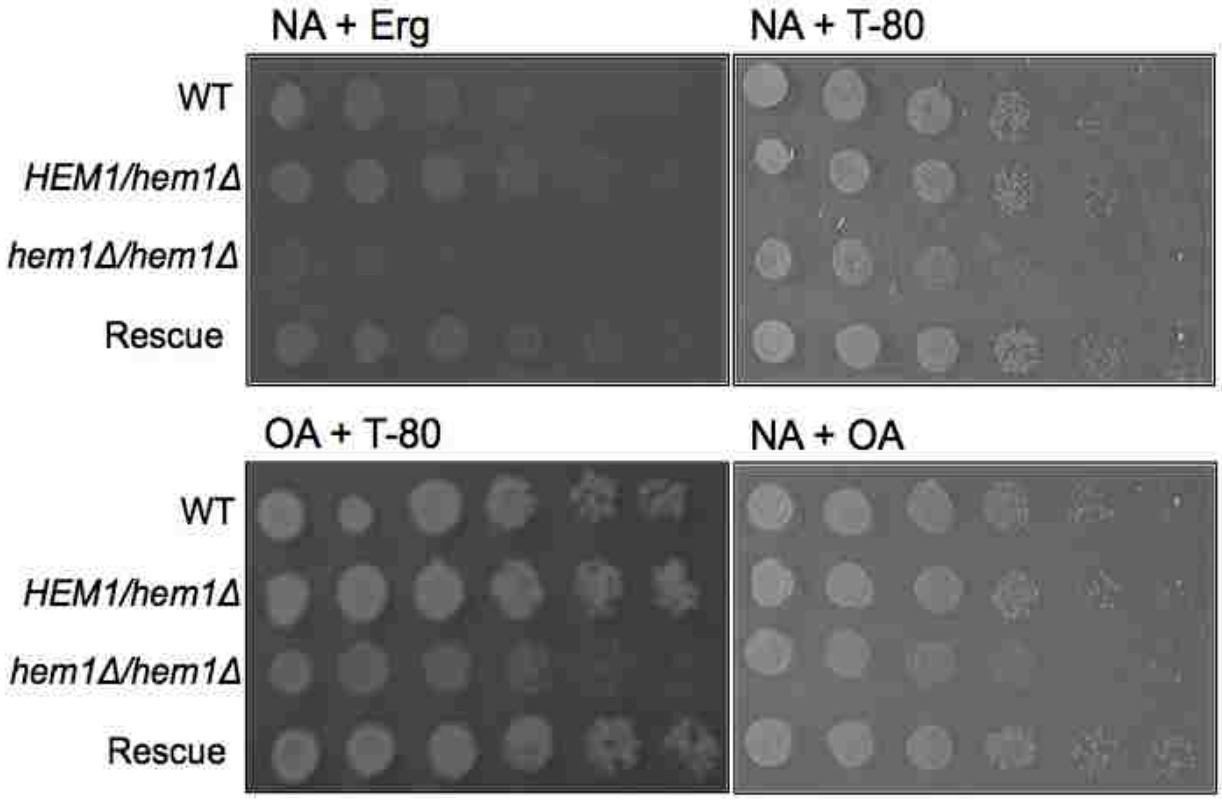

Figure 4-3. Growth of *hem1Δ* homozygous mutant under anaerobic conditions with multiple nutritional supplements. *C. albicans* strains with the indicated genotypes (left of the panels) were grown anaerobically in YPD medium supplemented with Tween 80 (T-80), oleic acid (OA), nicotinic acid (NA) or ergosterol (Erg) as described in the Materials and Methods. The strains were incubated for 15 days. The rescue genotype indicates the *hem1Δ* homozygous mutant with *HEM1* reintegrated into the genome at the *arg4* locus.

Tween-80 and oleic acid were both added to the medium suggesting that increasing the concentration of either Tween-80 or oleic acid in the YPD medium would likely provide the same response, namely increasing the availability of unsaturated fatty acids.

Previous studies have suggested that *C. albicans* could internalize exogenous heme as the sole source of iron (Santos et al., 2003; Weissman and Kornitzer, 2004); however, these studies were performed using an iron chelator in the growth medium to limit ferrous or ferric iron uptake and supplementing the medium with hemin. The *hem1Δ* homozygous mutant offered a unique opportunity to examine definitively whether *C. albicans* could acquire heme exogenously. In the absence of ALA, the *hem1Δ* mutant is unable to grow on rich medium (YPD) (Figure 4-4); however, if *C. albicans* could acquire exogenous heme, the growth of the mutant should be restored. To test this hypothesis, the wild-type, *HEM1/hem1Δ, hem1Δ/hem1Δ,* and the *hem1Δ/hem1Δ/HEM1* strains were spot plated on YPD medium or YPD containing ALA, heme, and on sheep blood agar plates to evaluate their growth (Figure 4-4). As controls, it was found that the *hem1Δ/hem1Δ* mutant could not grow on YPD without supplements, and the addition of ALA to the medium restored normal growth, bypassing the requirement for Hem1 enzyme. It was also observed that the addition of heme to the YPD medium restored growth of the *hem1Δ/hem1Δ* mutant, demonstrating definitively that *C. albicans* can acquire exogenous heme to sustain near normal growth. Interestingly, it was also found that the *hem1Δ/hem1Δ* mutant could acquire sufficient heme from blood agar plates to grow, albeit at a somewhat slower rate. These results provide the first direct evidence that *C. albicans* can take up exogenous heme from the medium to sustain growth in the absence of heme biosynthesis.

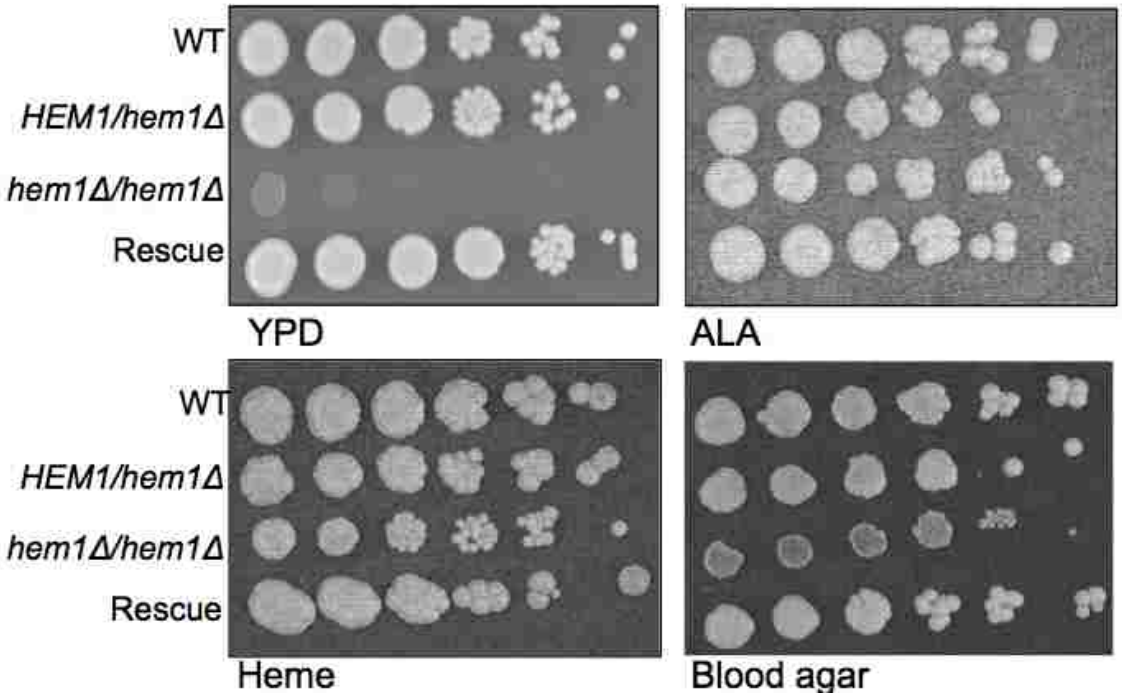

Figure 4-4. Growth of *hem1Δ* homozygous mutant in the presence of exogenous heme. *C. albicans* strains with the indicated genotypes (left of the panels) were grown aerobically in YPD medium, YPD supplemented with ALA or heme or sheep blood agar plates . The strains were incubated for 3 days. The rescue genotype indicates the *hem1Δ* homozygous mutant with *HEM1* reintegrated into the genome at the *arg4* locus.

Discussion

Candida albicans has been referred to as a facultative anaerobe, but surprisingly very few studies have examined the requirements for anaerobic growth and metabolism of this human pathogen. *C. albicans* is commonly found in the gastrointestinal tract (Biswas and Chaffin, 2005) and can be associated with biofilms either alone or in combination with other intestinal microflora. The cells that constitute a biofilm are frequently surviving in an anaerobic or microaerophilic environment (Davey and O'Toole G, 2000). In 2004, Dumitru *et al* showed that *C. albicans* can grow anaerobically in a defined media supplemented with oleic acid and nicotinic acid. They also found that anaerobically grown *C. albicans* was at least four-fold more resistant to antifungal compounds, such as the polyenes and azoles, as compared to strains grown aerobically (Dumitru et al., 2004). Given the inherent difficult in combating *C. albicans* biofilms, coupled with the increased resistance of this organism to antifungals in an anaerobic environment commonly associated with biofilms, an understanding of anaerobic growth has clinical significance to understanding infection.

Our results have shown that the *C. albicans hem1Δ/ hem1Δ* can grow anaerobically in rich medium (YPD) supplemented with oleic acid or Tween-80, either of which would meet the requirements for unsaturated fatty acids necessary for anaerobic growth when the heme biosynthetic pathway has been interrupted. The addition of ergosterol to the medium did not support the growth of the *hem1Δ/ hem1Δ* mutant. The nutritional requirement for anaerobic growth for some fungi is well documented. For example, *S cerevisiae* requires both ergosterol (Andreasen and Stier, 1953) and a source of unsaturated fatty acid (Andreasen and Stier, 1954; Bartnicki-Garcia and Nickerson, 1961). *Mucor rouxii* requires thiamine and nicotinic acid (Bartnicki-Garcia and Nickerson, 1961) and as mentioned above *C. albicans* requires oleic acid and nicotinic acid (Dumitru et al., 2004). We did not observe that nicotinic acid improved growth when added to the growth medium with either Tween 80 or oleic acid (Figure 4-2 and Figure 4-3). It was surprising that exogenous ergosterol is not required for growth in anaerobic conditions because molecular oxygen is required for the biosynthesis of both heme and ergosterol. Given that ergosterol is a major component of fungal membranes, analogous to cholesterol in higher eukaryotes, it was anticipated that it

would be required. It will be interesting to determine the membrane ergosterol content of C. albicans cells grown aerobically and anaerobically to determine whether the ergosterol content is significantly different.

Heme is an indispensable cofactor for numerous proteins in the respiratory chain of most organisms. For example, heme functions as a cofactor for proteins such as catalases, peroxidases, cytochromes of the P450 class, and a cofactor for enzymes involved in numerous biosynthetic pathways (Heinemann et al., 2008). Studies in this chapter have shown that aerobically grown *C. albicans hem1Δ/ hem1Δ* can be sustained by growth on medium supplemented with 5-aminolevulonic acid to bypass the requirement for the enzyme 5-aminolevulinate synthase encoded by the *HEM1* gene. This provides a unique platform in which to study how cells acquire heme exogenously. We have demonstrated that a *hem1Δ* homozygous mutant can take up exogenous heme from the growth medium. This observations offers an excellent model system to uncover the mechanism of heme uptake by eukaryotic cells. Previous studies have suggested that Rbt5 and possibly its homolog Rbt51 are putative heme receptors on the surface of *C. albicans* (Weissman and Kornitzer, 2004); however, this has not been definitively proven. Studies identifying authentic heme-binding receptors have been notoriously difficult to perform because heme has a tendency to bind nonspecifically to many proteins. The McNabb lab is now in the position to use genetics to uncover the heme-binding receptor. For example, it is now possible to generate gene disruptions of *RBT5* and/or *RBT51* in the *C. albicans hem1Δ/ hem1Δ* mutant background to address whether these proteins are true receptors receptors. It is anticipated that if either Rbt5 or Rbt51 is the heme receptor, then a *hem1Δ/ hem1Δ* mutant containing a knockout mutation of either gene alone or in combination would result in a mutant incapable of grow on YPD medium containing heme; however, it would grow on YPD medium supplemented with ALA.

The *hem1Δ/ hem1Δ* mutant was also found to grow on medium containing sheep red blood cells, implying that *C. albicans* can acquire heme from red blood cells, presumably from the hemoglobin present in the cells. Interestingly, one report has suggested that *C. albicans* secretes a hemolytic factor when grown on blood agar (Manns et al., 1994), and this hemolytic factor lyses red blood cells to release the hemoglobin. It has also been shown that *C. albicans* can use either heme or hemoglobin as a source of iron for growth

(Weissman and Komitzer, 2004). The availability of the *hem1Δ/ hem1Δ* mutant offers a unique tool to investigate the mechanism of heme acquisition during infection and how this relates to the overall virulence and pathogenesis of *C. albicans*.

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Chapter 5: Summary and Conclusions

The CCAAT-binding factor is a highly conserved heterooligomeric transcription factor (Mantovani, 1999). Despite the high degree of evolutionary conservation at the structural level, the target genes regulated by this transcription factor vary depending widely on the organism (Mantovani, 1998, 1999; Gusmaroli et al., 2001). In the yeast *Saccharomyces cerevisiae,* this transcription factor is composed of four subunits, each encoded by a single gene. The protein subunits of the *S. cerevisiae* CCAAT-binding factor are designated Hap2, Hap3, Hap4 and Hap5. The Hap2/3/5 heterotrimer forms the DNA-binding complex that recognizes the consensus DNA sequence 5'-CCAAT-3' within the promoters of target genes. The Hap4 subunit is the effector protein that stimulates target gene expression is response to glucose availability (McNabb and Pinto, 2005). In humans, the CCAAT-binding factor is composed of three subunits termed NF-YA, NF-YB, and NFY-C that form the DNA-binding complex orthologous to Hap2/3/5 (Mantovani, 1999). The human CCAAT-binding factor is a proximal promoter factor present in approximately 30% of all genes in the genome (Mantovani, 1999) and it functions in cooperation with other gene specific activators to regulate gene expression.

In *Candida albicans,* orthologs to the S. cerevisiae Hap2, Hap3, Hap4, and Hap5 have been identified a (Johnson et al., 2005). Interestingly, there are two genetic loci that encode distinct isoforms of the Hap3-like protein that were designated *HAP31* and *HAP32* (Johnson et al., 2005). These genes were found to be differentially expressed in response to iron availability with *HAP31* expression occurring when cells are grown in an iron rich environment while *HAP32* is expressed in an iron limited environment (Bates, 2009). As a consequence of differential expression, there are two distinct DNA-binding complexes formed in *Candida albicans,* Hap2/Hap31/Hap5 and Hap2/Hap32/Hap5 (Bates, 2009). Both complexes bind the identical consensus sequence on DNA (Bates, 2009); however, the Hap2/31/5 complex is seen during growth on iron rich medium and the Hap2/32/5 complex is observed when iron is limiting (Bates, 2009). Given this observation, it was presumed that Hap2/31/5 and Hap2/32/5 protein complexes function to alter gene expression differently in response to nutritional signaling. The studies presented in Chapter 2 of this dissertation have shown that Hap31 is required for transcriptional activation of *CYC1* in iron rich media and Hap32 is used for the repression of *CYC1* when iron is limiting. It has also been shown that in the absence of Hap32, Hap31 can assemble with Hap2 and Hap5 to form a heterotrimeric complex during

iron deficient growth (Bates, 2009) and the Hap2/31/5 complex can, at least partially, substitute for the absence of Hap32 in the repression of *CYC1*. These observations are consistent with the data shown in Chapter 3 of this dissertation in which a hybrid *HAP3231* gene containing the *HAP31* coding region expressed from the *HAP32* promoter could partially compensate for the lack of growth of a *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant on iron deficient medium. However, it is important to note that the complementation of the growth phenotype is only partial, suggesting that Hap32 possesses some unique properties not found in Hap31. Additional studies will be required to identify the regions of the Hap32 protein that are important for complete complementation of the growth phenotype.

As mentioned above, *S. cerevisiae* also encodes a fourth subunit, named Hap4, which is the effector subunit and does not form part of the DNA binding complex (McNabb and Pinto, 2005). The transcription of this subunit is induced in the absence of glucose and repressed in presence of glucose, representing the switch between respiration and fermentation (McNabb and Pinto, 2005). It was not until recently that Hap4-like homologs were identified in other fungi; HapX in *Aspergillus nidulans,* Hap43 in *Candida albicans* and Php4 in *S. pombe*; however, these proteins appear to have a repressor function as opposed to the activator function of Hap4 in *S. cerevisiae* (Lan et al., 2004; Johnson et al., 2005; Mercier et al., 2006). Interestingly, as with the Hap3-like proteins, there is redundancy in the Hap4-like proteins in *C. albicans*. There are three distinct genetic locus that encode isoforms of the Hap4 that have been designated *HAP41, HAP42*, and *HAP43 (Johnson et al., 2005).* In Chapter 2 of this work, it was shown that Hap43 is essential for the repression of *CYC1* and Hap41 is not necessary for *CYC1* activation or repression; however, this does not rule out the possibility that Hap41 is relevant as a subunit of the CCAAT-binding factor. It is conceivable that *HAP41* may be expressed under a different environmental condition that was not examined in this dissertation.

As mentioned above, *C. albicans* expresses *HAP31* when iron is abundant and *HAP32* in iron depleted medium (Bates, 2009). Previous bioinformatics studies suggested that *HAP32* may contain a 118 nucleotide intron (Mitrovich et al., 2007). Moreover, Northern blot analysis suggested that there were two distinct *HAP32* mRNA transcripts of differing sizes when cells are grown in iron rich medium versus iron

depleted medium (Bates, 2009). Studies presented in Chapter 3 have confirmed the presence of the intron; however the splicing of the intron was not found to be dependent on the iron availability.

Microorganisms have evolved exquisite iron acquisition mechanisms to be successful colonizers in the iron limited mammalian host. *C. albicans* is an opportunistic pathogen that has adapted to survive in iron rich and limited conditions. At this point stage it could be hypothesized that the presence of two Hap3 isoforms serve to fine tune the genetic program of this human pathogen to better adapt to changing nutritional environments. Our studies in Chapter 3 demonstrated that Hap31 is unable to fully compensate for the absence of Hap32. This suggests that there must be something unique about Hap32 that is important for gene regulation in iron limiting conditions. That unique feature remains to be determined.

C. albicans is capable of obtaining iron from the host using multiple mechanism including; a reductive and non-reductive pathway (Kornitzer, 2009), as well as directly from ferritin, transferrin or heme-containing proteins such as hemoglobin (Weissman and Kornitzer, 2004). Heme is an essential molecule for every organism. Heme biosynthesis is important in the regulation of sterol biosynthesis, respiration, unsaturated fatty acid synthesis and aerobic sterol exclusion (Trocha and Sprinson, 1976; Gollub et al., 1977). *HEM1* encodes for 5-aminolevulinate synthase, the enzyme that catalyzes the first step in heme biosynthesis. *S. cerevisiae hem1* mutants have been show to be unable to grown anaerobically if not supplemented with ergosterol and a source of unsaturated fatty acids (McNabb, unpublished). *C. albicans* has been showed to grow anaerobically in media supplemented with nicotinic acid and oleic acid (Dumitru et al., 2004). *Candida albicans* have been referred as a facultative anaerobe that is typically found in the gastrointestinal tract of humans (Biswas and Chaffin, 2005). Moreover, it is also able to form biofilms by itself or in combinations with other microorganisms (Kojic and Darouiche, 2004). Biofilms are often virtually anaerobic environments and the microorganism that form the biofilm are usually more resistant to antimicrobial treatment (Davey and O'Toole G, 2000). Our studies in Chapter 4 have shown that *Candida albicans hem1Δ* homozygous mutant is able to grow anaerobically in rich medium (YPD) supplemented with oleic acid and Tween-80. Surprisingly, ergosterol was not required for the growth of the *hem1Δ* homozygote anaerobically. This observation deserves further investigation since ergosterol biosynthesis

requires molecular oxygen. In fact, in fungi it is commonly thought that the biosynthesis of heme and ergosterol are the molecular sensors for oxygen in the microbial eukaryotes (Kwast et al., 1998; Davies and Rine, 2006). Thus, the question arises as to how C. albicans can survive anaerobically without ergosterol. Is there an alternative, oxygen-independent mechanism of ergosterol synthesis? Does the organism grow anaerobically without ergosterol? From a clinical perspective these are important questions to address. The two most common classes of antifungal drugs used to treat *C. albicans* infections are the azoles and polyenes, both of which target ergosterol biosynthesis (azoles) and ergosterol in the membrane (polyenes). If *C. albicans* grows in the absence of ergosterol anaerobically that scenario would present a major barrier to antifungal treatment, particularly in cases where *C. albicans* is associated with biofilms. It was also shown that a *hem1Δ* homozygote can grow aerobically in medium supplemented with hemin or in blood agar plates corroborating previous results that this opportunistic pathogen is able to uptake exogenous heme (Weissman and Kornitzer, 2004; Weissman et al., 2008). More importantly, the *hem1Δ/hem1Δ* mutant will provide a useful genetic tool to further investigate the use of exogenous heme by *C. albicans*. Previous studies have suggested that Rbt5 and its homolog Rbt51 may be the heme receptors responsible for the acquisition of heme by *C. albicans*. Using reverse genetics, along with the *hem1Δ* homozygote, studies can be performed to definitively demonstrate whether Rbt5 and Rbt51 are authentic heme receptors as outlined in Chapter 4. This would be a major discovery because true cell surface heme receptors have not been identified in eukaryotes, although they have been found in prokaryotic pathogens (Anzaldi and Skaar, 2010).

It should be noted that the CCAAT-binding factor in *S. cerevisiae* is involved in the regulation of heme biosynthesis (Keng and Guarente, 1987); however, this have not been investigated in *C. albicans*. Nevertheless, it would not be surprising that the *C. albicans* CCAAT-binding factor may be involved in the regulation of heme biosynthesis since it regulates genes that use heme as a cofactor, namely cytochrome c encoded by *CYC1*.

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