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The Role of Multiple CCAAT-binding Factors in *Candida albicans* Gene Expression

THE ROLE OF MULTIPLE CCAAT-BINDING FACTORS IN *CANDIDA ALBICANS* GENE EXPRESSION

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

By

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December 2009
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Abstract

The CCAAT-binding factor is a heterooligomeric transcription factor that is evolutionarily conserved in eukaryotes. In yeast, the DNA-binding component that interacts with the CCAAT consensus sequence in promoters consists of the subunits termed Hap2p, Hap3p and Hap5p. In yeast and fungi, a fourth subunit, Hap4p, is required for regulating gene expression. The goal of this research is to understand the function of the *Candida albicans* CCAAT-binding factor and how it relates to virulence and pathogenicity. *C. albicans* is a human opportunistic pathogen responsible for a variety of mucosal and systemic infections that result in significant morbidity and mortality, particularly in immunosuppressed individuals. *C. albicans* responds to environmental changes by altering its morphology between the yeast and hyphal forms during infection, and the ability to transition between the two forms is required for virulence. We have previously demonstrated that *C. albicans hap5Δ/hap5Δ* mutants are defective in the yeast-to-hyphal transition *in vitro* under several conditions and become hyperfilamentous when deprived of glucose as a sole carbon source (Eukaryotic Cell 4:1662-1676, 2005). Moreover, the *hap5Δ/hap5Δ* mutant shows no CCAAT-binding activity, suggesting the lost of the CCAAT-binding factor alters the ability of cells to undergo the normal yeast-to-hyphal transition. This research will show that the *hap2Δ/hap2Δ* mutant also abolishes DNA-binding activity and exhibits the same phenotypic deficiencies as *hap5Δ/hap5Δ* mutants. Two distinct functional homologs of *S. cerevisiae* Hap3p have been identified in *C. albicans,* designated Hap31p and Hap32p. These subunits form separate CCAAT-binding complexes with Hap2p/Hap5p. Hap31p is a member of the complex under iron replete conditions and Hap32 under low iron conditions. The Hap complex effects the regulation of *CYC1* and *COX5.* Also three distinct homologs of *S. cerevisiae* Hap4p have been identified in *C. albicans,* designated Hap41p, Hap42p, and Hap43p.

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Acknowledgements

I would like to thank Dr. McNabb for giving me a chance. If it was not for your willingness to take me on, I would not be where I am now, accomplishing more than I thought possible, achieving my dream and making a difference! To Dr. Pinto for being such a good co-mentor. I appreciate the way you two work together and the support you offer your students. It is nice to work for two people who you can look up to.

Thanks to Dr. Kirby, Dr. Deaton, Dr. Koski, and Dr. Korth, for support when I needed it. Also, thanks to Dr. Rhoads, for filling in the gap when I needed a new committee member.

Thanks to Marsha Rhoads, for answering my unending questions! For support when I thought I was crazy. You deserve way more thanks than I can give you.

Last, but not least, to Gary for his support, faith, and love through it all.

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Chapter 1. Introduction: The importance of the CCAAT-binding factor in *Candida albicans*.

Introduction

Candida albicans is an opportunistic fungal pathogen that is normally a part of the mammalian microflora and is found in and on more than 70% of the healthy human population (Ruhnke and Maschmeyer 2002). It is located in the gastrointestinal and urogenital tracts, and can cause a variety of skin and mucosal infections in otherwise healthy individuals. Increases in *C. albicans* infections have followed advances in medical and surgical treatments which have expanded the size of the population at risk. This population consists of individuals suffering from AIDS, neutropenia, post-transplantation infections, neonates, and cancer patients. In addition, patients that have indwelling catheters, invasive surgery, extensive burns, broad-spectrum antibiotic therapy, and mechanical ventilation are at risk for *Candida* infection. Suppression of the host immune system provides an opportunity for *C. albicans* to invade and colonize host tissues. Since many of these patients are already ill, fungal infections increase the overall expense of health care by prolonging hospital stays.

Most of the antifungal drugs commonly available have deleterious side effects and are fungistatic. Superficial infections, such as skin, mouth, or throat infections, are not life threatening and can be treated, but recurrent superficial infections can lead to drug resistance. Advanced colonization can cause severe localized or disseminated infections resulting in pyelonephritis, peritonitis, abdominal abscesses, arthritis, pneumonitis, myositis, macronodular skin lesions, osteomyelitis, endophthalmitis, meningitis, and/or multiorgan involvement (Odds 1988, Alexopoulos *et al.* 1996, Fridkin and Jarvis 1996).

In United States hospitals, *Candida* species are the fourth most common nosocomial bloodstream infection (NNIS 1999). *C. albicans* was responsible for 45-55% of *Candida* species bloodstream infections in North America during the 1990's (Edmond *et al.* 1999, Kao *et al.* 1999, Rangel-Frausto *et al.* 1999, Diekema *et al.* 2002, Hajjeh *et al.* 2004, Laupland *et al.* 2005). A higher mortality rate is associated with *Candida* species bloodstream infections when compared with nonfungal pathogens (Pittet *et al.* 1997). The mortality attributed to blood-born infections with *C. albicans* is estimated to be greater than 30% (Odds 1988, Gudlaugsson *et al.* 2003, Benjamin *et al.* 2006, Acar *et al.* 2008).

Understanding how *C. albicans* persists in healthy individuals, yet causes such a wide variety of disease states in immunocompromised individuals is an area of significant biological interest as well as of major medical and economical importance. The research described in this dissertation focuses on the CCAAT-binding factor, a transcriptional regulator, in *Candida albicans*.

Antifungals and Resistance

Due to the evolutionarily conserved basic functions of both *Candida* species and the human host, the modes of action of several antifungals affect both the target fungi and the host. Antifungal drugs carry either a high risk of host toxicity, high rate of resistance, or low efficacy. Accurate diagnosis of *C. albicans* can be difficult and therefore, treatment of candidaemia may be delayed. A delay in treatment is correlated with an increase in mortality (MacCallum and Odds 2004).

Antifungal agents currently available for the treatment of candidaemia include the polyenes, the azoles, and the allylamines which all target ergosterol biosynthesis. The fluoropyrimidines inhibit nucleic acid synthesis and the echinocandins inhibit synthesis of fungal β-1,3 glucan (Odds *et al.* 2003).

The ergosterol pathway is divided into an early pathway in which squalene is produced from acetate and the late pathway which proceeds from squalene to ergosterol. Polyenes target ergosterol directly whereas the azoles decrease ergosterol synthesis by targeting inhibition of fungal cytochrome P450 enzymes and allylamines inhibit fungal squalene epoxidase causing the accumulation of squalene and the loss of ergosterol production (Georgopapadakou and Bertasso 1992, Ryder and Mieth 1992, Ghannoum and Rice 1999).

Polyenes include Amphotericin B and nystatin. Polyenes bind to ergosterol, compromising membrane integrity and ultimately causing cell death (Gallis *et al.* 1990, Lyman and Walsh 1992). Amphotericin B interacts with membrane sterols to form a transmembrane channel compromising the cell membrane and allowing small molecules to diffuse across the membrane. Due to the mode of action, polyenes are only fungicidal at physiologic

concentrations, therefore there is a risk of relapse in the infection. Amphotericin B is the gold standard for the treatment of most fungal infections. However, there are problems of solubility, stability, and toxicity (Gallis *et al.* 1990, Kelly *et al.* 1996). Oral preparations of amphotericin are used to treat thrush and may cause harsh side effects including gastrointestinal upset, anorexia, shortness of breath and hyperventilation. Intravenous doses are used to treat systemic fungal infections and are associated with multiple organ damage, electrolyte imbalances, and anemia.

The azoles include the imidazoles (miconazole, ketoconazole), which are normally used for localized and surface infections, and triazoles (fluconazole, itraconazole, voriconazole), commonly use for systemic infections (White and Goetz 1994, Kelly *et al.* 1996, Ghannoum and Rice 1999). These drugs inhibit the product of *ERG11,* lanosterol demethylase. They bind to heme in the active site of lanosterol demethylase which inhibits the biosynthesis of ergosterol, a major membrane sterol of fungi (Kelly *et al.* 1993, Sanglard 2002). This leads to the incorporation of methylated sterols into the plasmid membrane. Azole inhibits cell growth, but is not fungicidal. Allylamines (naftifine and terbinafine) inhibit squaline epoxidase, a membrane-bound enzyme, thereby inhibiting ergosterol production (Georgopapadakou and Bertasso 1992) .

The echinocandins inhibit the synthesis of β-1,3-glucan. This polysaccharide is a major component in the fungal cell wall (Groll and Walsh 2001), that is absent from mammalian cells. The fungal cell wall stabilizes osmotic potential and is important in cell growth and division. Available echinocandins are Caspofungin, Micafungin and Anidulafungin. They have low oral bioavailability and must be administered intravenously. The rate of antifungal activity is related to the concentration of the drug (Lyman and Walsh 1992, Groll and Walsh 2001).

Flucytosine is a fluoropyrimidine that inhibits RNA, DNA and protein synthesis by interfering with nucleotide metabolism (Ghannoum and Rice 1999). It is transported by cytosine permease into the cell and is then converted into 5-fluorouracil, a pyrimidine analog which is then incorporated into RNA and interrupts protein synthesis. Flucytosine is easily absorbed in the gastrointestinal tract, but must be administered under close medical supervision because there is a rapid development of resistance. Flucytosine can damage bone marrow and cause

gastrointestinal problems. It cannot be used in life threatening candiaemia due to the fast development of resistance.

Drug resistance is bound to occur in all pathogenic microorganisms, including fungi, when exposed to antimicrobial agents because the environmental stress initiates a selective pressure causing the population to shift due to natural selection. Mutations provide genetic viability that allows fit individuals to reproduce. Resistance occurs when antimicrobial agents are not used in a way that totally destroys the pathogenic population. Patients that get these fungal infections tend to have recurrent infections due to the nature of their immunosuppression. Many of the drugs used to treat candidaemia have drug usage limited by toxicity and resistance. Also the treatment differs among patients, for example, AIDs patients usually receive low doses for long periods of time while bone marrow transplants receive high doses for short periods of time.

C. albicans has developed resistance to the echinocandin caspofungin by a mutation in the catalytic subunit of β-1,3-glucan synthase (Balashov *et al.* 2006). Azole resistance has occurred by target enzyme alteration and expression of transporter proteins (Banerjee *et al.* 1991). Resistance is high to azole derivatives and has been found for itraconazole, fluconazole, and ketoconazole (Sanglard 2002). Amphotericin B resistance has been correlated with a decrease in ergosterol in the fungal cell wall, although the mechanism is unknown (Kelly *et al.* 1996, Ghannoum and Rice 1999). A mutation in cytosine deaminase allows *C. albicans* to become resistant to flucytosine. This mutation stops the conversion of 5-flucytosine to 5-fluorouracil (Defever *et al.* 1982, Whelan *et al.* 1986, Whelan 1987). This resistance is quite common and therefore flucytosine is usually administered concurrently with other treatments. One study found that resistance of *C. albicans* strains isolated from cancer patients was 17.5% to fluconazole, 3.4% to flucytosine and 4% to amphotericin B (Maksymiuk *et al.* 1984) . Another study found that 50% of *C. albicans* strains isolated from AIDS patients were resistant to fluconazole (Barchiesi *et al.* 1994).

The increase in resistant strains of *C. albicans* and other *Candida* species has lead to the need for further research to understand metabolism, genetics, and pathogenicity of these organisms so that better resources can be developed to fight infections.

Taxonomy

Kingdom: Fungi, Phylum: Ascomycota, Subphylum: Ascomycotina, Class: Ascomycetes, Order: Saccharomycetales, Family: *Saccharomycetaceae*, Genus: *Candida*

Candida species are members of the family *Saccharomycetaceae*. This family of fungi is composed of typical yeasts which develop in a predominantly unicellular manner, reproduce by budding, and ferment carbohydrates. *Saccharomyces cerevisiae*, a model eukaryote organism, is also a member of this family. Much of what is known about *Candida* species was originally based on homology with *S. cerevisiae.*

The genus *Candida* is comprised of more than 150 species. These species were originally grouped into the genus based on the main common feature of lack of an observed sexual form. The most commonly isolated pathogenic species causing invasive candidiasis are *C. albicans*, *C. tropicalis*, *C. glabrata*, C. *parapsilosis*, *C. krusei*, and *C.lusitaniae*, with *C. albicans* being the most commonly isolated species (Odds 1987, Odds 1988).

C. albicans is the only species in this genus that forms true hyphae and chlamydospores (Odds 1988). It is a diploid, with 16 chromosomes, that until recently was thought to lack a sexual cycle (Hull *et al.* 2000), therefore, conventional strategies to study its genetics have not been possible. However with the completion of the *C. albicans* genome sequence (Jones *et al.* 2004, Braun *et al.* 2005) molecular techniques are now applicable.

In the 1970's and 1980's, *C. albicans* was discovered to be a diploid, but no sexual cycle was identified (Olaiya and Sogin 1979, Whelan *et al.* 1980, Riggsby *et al.* 1982). In the late 1990's, genes for mating were identified by homology to *Saccharomyces cerevisiae* genes known to be involved in mating. Many of these orthologs from *C. albicans* could substitute in *S. cerevisiae* (Sadhu *et al.* 1992, Clark *et al.* 1995, Diener and Fink 1996, Leberer *et al.* 1996, Raymond *et al.* 1998, Hull and Johnson 1999, Tzung *et al.* 2001), but it was not until recently that mating was verified (Hull *et al.* 2000, Magee and Magee 2000, Tzung *et al.* 2001, Lockhart *et al.* 2002, Magee *et al.* 2002, Miller and Johnson 2002).

The mechanism of the *MAT* locus in *S. cerevisiae* and the existence of two mating types *MATa* and *MATα* have been found in *C. albicans* (Magee and Magee 2000). The *C. albicans*

mating-type-like locus *(MTL)* and white-opaque switching is involved in a parasexual cycle (Bennett and Johnson 2003, Forche *et al.* 2008), where genetic exchange can occur in the absence of meiosis. So far the mechanism of meiosis has not been identified, but homologs to some *S. cerevisiae* meiosis genes have been found (Forche *et al.* 2008). Mating products in *C. albicans* are mononuclear and tetraploids (Bennett and Johnson 2003, Legrand *et al.* 2004). The tetraploids undergo chromosome loss to become diploids (Bennett and Johnson 2003). Population studies find that there is largely clonal reproduction with genetic recombination occurring rarely (Graser *et al.* 1996).

Morphology

C. albicans is a dimorphic fungus that responds to environmental changes by altering its morphology between the yeast and hyphal forms during infection. This dimorphic switch has been implicated in the ability of *C. albicans* to become pathogenic, as a mutation that blocks the transition from yeast to hyphae is avirulent (Lo *et al.* 1997). Host cell invasion and infection appears to be dependent on the morphological switch. Yeast form alone can exit blood vessels and disseminate to target organs, but the ability to switch to hyphal form is required for host organ invasion and death (Saville *et al.* 2006). For example, a *tup1Δ* homozygous mutant is constitutively filamentous, yet it is unable to colonize the kidney or liver (Bendel *et al.* 2003).

The morphological switch from yeast-to-hyphae is a rapid response to external signals. These signals include temperature, pH, presence of serum, nitrogen or carbon starvation, low oxygen, and nonfermentable carbon sources (Odds 1988). The relationship between pathogenicity and morphology is a complex interaction that is not entirely understood.

C. albicans has at least three morphological forms, round budding yeast (blastospores, blastoconidia), pseudohyphae (elongated cells that appear filamentous), and true hyphae (Alexopoulos *et al.* 1996, Calderone and Fonzi 2001, Sudbery *et al.* 2004). *C. albicans* can also form chlamydospores, which are thick walled asexual resting spores (Odds 1988). Yeast cells are unicellular forms of the fungi that divide by budding. This process involves the growth of a new cell, the bud, which begins at the pole distal to the birth scar. Nuclear division takes place

and the septum is created to divide mother and daughter cells. The two cells will separate and become blastospores (Odds 1988, Alexopoulos *et al.* 1996).

Hyphae are elongated branching structures that consist of multiple fungal cells divided by septae. Blastospores give rise to germ tubes which are the cylindrical beginnings of hyphae. These germ tubes grow continuously at the distal pole. Mitotic division occurs within the extending structure and septa are formed to divide cells. This occurs without any decrease in the extension rate. Branching can occur on already existing hyphae or by germination of blastospores. Branching is important in forming mycelium which is the aggregation of fungal cells including hyphae and their branches.

White-opaque colony switching is also a change in morphology. This is an epigenetic phenomenon that has become increasingly important since the discovery that opaque cells alone are able to undergo mating. The opaque cells are twice the size of white cells, oblong in shape and have pimples on the cell wall (Whiteway and Bachewich 2007, Ramirez-Zavala *et al.* 2008).

C. albicans can also form dense layers of cells on implanted medical devices such as intravenous therapy, catheters, and prosthetics. These layers of cells are called biofilms and are inherently resistant to antimicrobial drugs and host defense systems. Biofilms consist of yeast cells, pseudohyphae and hyphal cells. They are created by attachment and colonization of a surface, growth of anchoring cells, and growth of hyphae and pseudohyphae to create a matrix (Hawser and Douglas 1994, Baillie and Douglas 1999, Chandra *et al.* 2001, Ramage *et al.* 2001, Douglas 2003). In order to clear the *C. albicans* biofilm infections, it is often recommended to remove implanted medical devices.

Hyphal morphogenesis is important for biofilm formation. Creation of a double mutant of *CPH1* and *EFG1*, both positive regulators of hyphal morphogenesis, lead to a strain that was unable to form hyphae in most situations (Lo *et al.* 1997). This strain was also unable to form a biofilm (Lewis *et al.* 2002, Ramage *et al.* 2002). Adherence is an important factor in biofilm production, but also in systemic infections. Initiation of a biofilm depends on adherence, both for colonization of a surface, but also for anchoring of cells to one another (Douglas 2003, Verstrepen and Klis 2006).

Biofilms appear to possess multiple mechanisms involved in resistance that occurs rapidly after surface adhesion (Chandra *et al.* 2001, Mateus *et al.* 2004). Some of the possible mechanisms involve poor drug penetration, reduced ergosterol levels (Mukherjee *et al.* 2003), heterogeneity of the morphological structures which leads to the varying susceptibility of those structures (Suci and Tyler 2002), and the biochemical makeup of biofilms which is composed mainly of carbohydrates and proteins (Baillie and Douglas 1999, Chandra *et al.* 2001, Douglas 2003).

The distinct morphology of *C. albicans* cells provide a simple mechanical way to overcome host defenses. This can be clearly seen when *C. albicans* yeast cells are engulfed by macrophages. The fungal cell goes through a morphological switch and begins to form hyphae. These hyphae are able to puncture the membrane of the macrophages, killing the macrophage and allowing the fungal cell to escape (Vazquez-Torres and Balish 1997).

Morphology is important in host cell invasion, overcoming host defenses, and drug resistance. This can be seen in biofilm drug resistance, macrophage destruction and host cell colonization. It is not a single morphological structure alone that allows infection, but the ability to transition between the yeast and hyphal forms that enables infection. Both yeast and hyphal cells have been found in infected tissue (Calderone 2002).

C. albicans is able to live as a commensal in warm blooded animals, which means that it is able to avoid immune response under normal circumstances. This microflora can become pathogenic when the host environment undergoes change including bacterial imbalance, use of antibiotics, or a compromised immune system. The signals for the actual morphological switch between yeast and hyphal growth is known to be caused by a change in iron availability and pH shift.

Molecular techniques

C. albicans ploidy and the lack of a true sexual cycle accounts for the inability to perform classical genetic studies. Gene discovery was mostly based on homology with *S. cerevisiae*. In 2004 the sequence of the *C. albicans* genome was published and subsequently an annotation of

the genome was released (Jones *et al.* 2004, Braun *et al.* 2005, Arnaud *et al.* 2007). Following these advances, the molecular techniques available for the manipulation of *C. albicans* have expanded greatly.

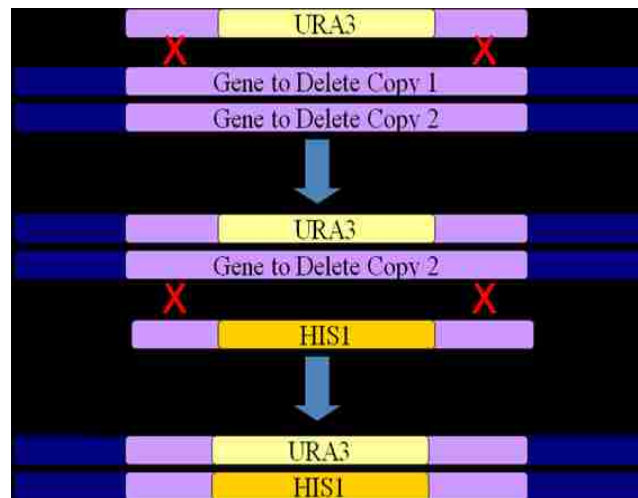
Originally, gene function for *C. albicans* was determined by a reverse genomic approach. A gene was identified by sequence and then deleted or mutated to determine an identifiable phenotype. This shot in the dark approach was realizable because of the homology between *C. albicans* and *S. cerevisiae*. Therefore, one way a gene was identified was by complementation analysis in *S. cerevisiae.* This involved the knowledge of *S. cerevisiae* genes and function. If a deletion in *S. cerevisiae* was complemented by a *C. albicans* sequence, then the possible function of the *C. albicans* gene could be determined. The next step would be to delete the sequence from *C. albicans* and determine if a similar phenotype occurred. Determination of complementation in *S. cerevisiae* was complicated by *C. albicans* alternative codon usage for CUG. The codon CUG in *C. albicans* is recognized to encode a serine residue, rather than a leucine typical of most organisms (Ohama *et al.* 1993, Santos *et al.* 1993).

Currently genes are still identified by sequence homology to *S. cerevisiae*, but complementation is not as widely used to determine function. Gene disruption in *C. albicans* utilizes homologous recombination, a normally occurring process. This method replaces the chromosomal copy of a gene with a linear segment of DNA that has regions homologous to the flanking regions of the target gene. This transformation process was adapted from previous methods (Gietz and Woods, 2002). Selectable markers are used to select for mutants that have undergone homologous recombination.

There are three main markers used in *C. albicans*, *URA3* (Fonzi and Irwin 1993), *HIS1* (Pla *et al.* 1995), and *ARG4* (Wilson *et al.* 1999) . These auxotrophic markers are mutated in *C. albicans* strain BWP17 (Wilson *et al.* 1999) and can therefore be used for selection. Because of *C. albicans* diploid genome, gene disruption involves mutagenesis of both copies of a gene. Therefore, it may be necessary to use all three markers to create a set of *C. albicans* deletion strains to evaluate the effect of one gene. Thus, this method limits the ability to mutagenize multiple genes in one strain. A recyclable *URA3* cassette (Alani *et al.* 1987, Fonzi and Irwin

1993, Pla *et al.* 1996) was developed that allowed the mutagenesis of more than one gene. It involves the use of hisG repeats and the counter selection of 5-FOA to encourage the recombination of the hisG repeats and the loss of the *URA3* marker.

In order to study gene function, two rounds of mutagenesis must be done to create a homozygous strain. This involves taking a wild type strain (+/+) and mutagenizing to create a heterozygote (+/-) and then one more round of mutagenesis to create a homozygote (-/-). If multiple genes are to be tested these steps must be repeated for each gene.



Additional tools have been developed for the study of genes in *C. albicans* including tools to develop conditional mutants. A set of constitutive and inducible promoters have been identified to allow the study of gene expression including *ADH1* (Bertram *et al.* 1996) and *ACT1*(Delbruck and Ernst 1993) which are constitutively produced; *GAL1* (Gorman *et al.* 1991), *PCK1* (Leuker *et al.* 1997), *MAL2* (Backen *et al.* 2000), and *MET3* (Care *et al.* 1999) that are inducible promoters. Also, a set of reporter genes have also been identified for use in *C. albicans*. They include lacZ (Uhl and Johnson 2001) and *Renilla reniformis* luciferase (Srikantha *et al.* 1996), used to monitor gene expression.

The CCAAT-binding factor

Transcriptional mechanisms and their effect on specific genes determine cellular responses to various stimuli resulting in normal or abnormal cellular phenotypes (Maniatis *et al.* 1987). In eukaryotes, the activation or repression of the transcriptional machinery is determined

by a number of diverse and highly specific processes. Among these processes are DNA-binding proteins that recognize specific DNA sequences in gene regulatory elements (Dynan and Tjian 1985, McKnight and Tjian 1986). These transcription factors bind DNA at specific sites and control the transcription of DNA to RNA; therefore, mediating the initial control over gene expression. These proteins can function alone or form complexes to facilitate or inhibit transcription.

Many of these transcription factors bind short sequences in the promoter region of a gene. The promoter region of a gene consists of upstream activation sequences (UASs) that promote the binding of the preinitiation complex (PIC) composed of the general transcription factors along with RNA polymerase, and other regulatory proteins. The promoter region generally consists of a TATA sequence (TATA box), a CCAAT sequence (CAT box) and a sequence of repeated GC nucleotides in eukaryotes. These sequences generally lie upstream about -25 to -90 base pairs away from the site of transcription initiation. The TATA sequence is important for the initiation of transcription through the TATA binding protein (TBP) or TFIID (Conaway and Conaway 1993, Travers 1996, Cler *et al.* 2009).

One such transcription factor is the heteromeric CCAAT-binding factor (CBF) that binds to CCAAT-containing sequences that are found upstream of the start of transcription in a number of eukaryotic promoters. Mutation of the CCAAT box results in a reduction or increase in the rate of RNA polymerase II binding to the promoter region and initiation of transcription (Connelly and Manley 1989, Kabe *et al.* 2005) . In humans, 30% of promoters include a CCAAT box, making it one of the most ubiquitous elements (Bucher and Trifonov 1988, Bucher 1990).

Most of what is known about the transcription factor that binds the CCAAT box is derived from studies in *S. cerevisiae* and humans. This heterooligomeric transcription factor was first identified in *Saccharomyces cerevisiae* and was designated the Hap complex for Heme Activated Protein (Forsburg and Guarente 1988). It was found to bind to the CCAAT sequences in both *S. cerevisiae* and *SchizoSaccharomyces pombe (McNabb et al. 1997).* Since that original discovery, this complex has been identified in all eukaryotes analyzed to date. Differing names have been given to the homologs of the Hap complex. It is identified as the Hap complex

in *S. cerevisiae* (Pinkham and Guarente 1985, McNabb *et al.* 1995), *C. albicans* (Johnson *et al.* 2005), *Arabidopsis thaliana* (Edwards *et al.* 1998), and *Kluyveromyces lactis* (Mulder *et al.* 1994), AnCF in *Aspergillus* species (Brakhage *et al.* 1999), Php in *S. pombe (McNabb et al. 1997)*, CBF (CCAAT binding factor)in rat (Sinha *et al.* 1995) and *Xenopus laevis* (Li *et al.* 1998) and NF-Y (nuclear factor Y) in mammals (Hooft van Huijsduijnen *et al.* 1990, Maity *et al.* 1990). Most of the genes encoding the subunits of the CCAAT-binding factor are highly evolutionarily conserved. The conserved portions of the subunits, which consist of the DNA binding and protein-protein interaction regions, contain a >70% identity among differing species, examples of primates, rodents, birds, frogs, lampreys, plants and fungi were tested (Li *et al.* 1992b).

Table 1-1. Percent homology to *C. albicans* subunit core domain[a].

Organism	Percent homology to		
	Hap2	*Hap3*	*Hap5*
S. cerevisiae	93%	92%	81%
S. pombe	87%	85%	82%
C. glabrata	93%	91%	80%
A. niger	86%	89%	84%
K. lactis	96%	88%	83%
N. crassa	84%	90%	89%
M. grisea	84%	90%	89%
A. thaliana	83%	88%	84%
M. musculus	79%	88%	75%
H. sapiens	79%	88%	75%

[a]Data assembled using NCBI database (http://www.ncbi.nlm.nih.gov/)

In *S. cerevisiae*, the DNA binding component that binds the CCAAT consensus sequence consist of the three subunits, Hap2p, Hap3p and Hap5p (Pinkham and Guarente 1985, Pinkham *et al.* 1987, Hahn and Guarente 1988, McNabb *et al.* 1995). In yeast and fungi, a fourth subunit, Hap4p, is required for regulating gene expression. Hap4p does not directly bind DNA, but interacts with the Hap2/3/5p complex and turns on transcription through an acid activation domain (Forsburg and Guarente 1989). In humans and other mammals, the transcription factor NF-Y consists of three components NF-YA, NF-YB, and NFY-C (Maity *et al.* 1992). CBF/NF-Y has been shown to possess two distinct activation domains, one in CBF-B (NF-YA) and one in CBF-C (NF-YC). (Li *et al.* 1992a, Coustry *et al.* 1995, Coustry *et al.* 1996, Coustry *et al.* 1996, Serra *et al.* 1998, Coustry *et al.* 2001).

The DNA-binding components of this transcription factor show similarities among eukaryotes over short domains (Maity *et al.* 1990, Vuorio *et al.* 1990). Human NF-Y factors A and B have been shown to be functionally interchangeable with *S. cerevisiae Hap2p* and *Hap3p* in vitro (Chodosh *et al.* 1988, Becker *et al.* 1991). Among identified CCAAT binding transcription factors these proteins show 66-74% identity and 80-86% similarity, respectively (Pinkham *et al.* 1987, Hahn *et al.* 1988, McNabb *et al.* 1995).

In *S. cerevisiae* Hap2p, Hap3p and Hap5p are required simultaneously to assemble and bind DNA. The Hap4p interaction with the complex requires that Hap2/3/5p already be bound to DNA (McNabb and Pinto 2005). In humans, the transcription factor is assembled in a stepwise fashion. NF-YB (HAP3) and NFY-C (HAP5) bind to form a stable dimer via a histone fold motif (Baxevanis *et al.* 1995, Zemzoumi *et al.* 1999, Romier *et al.* 2003). The heterodimer then serves as the surface for interaction with NF-YA (HAP2) which simultaneously assembles and binds DNA with a high affinity (Maity *et al.* 1992, Sinha *et al.* 1995, Sinha *et al.* 1996). The yeast Hap complex does not assemble in a stepwise manner, and requires all Hap2/3/4p subunits for stable complex formation (McNabb and Pinto, 2005). In mammals, all three DNA binding proteins are necessary for DNA binding and sufficient for transcriptional activity (Maity and de Crombrugghe 1998, Mantovani 1999).

In *S. cerevisiae* and other fermenting yeast, the Hap complex is required for growth on non-fermentable carbon sources such as glycerol as well as being involved in response to sugar signals and nitrogen metabolism (Guarente and Mason 1983, Guarente *et al.* 1984). The Hap complex has been shown to have a key role in the activation of genes involved in energy metabolism, respiration, and in cytochrome biogenesis (Mazon *et al.* 1982, Pinkham and Guarente 1985, McNabb *et al.* 1995, Dang *et al.* 1996, Gancedo 1998). The genes encoding Hap2p, Hap3p, and Hap5p are constitutively expressed, whereas Hap4p is repressed in the presence of glucose (Pinkham *et al.* 1987, Hahn *et al.* 1988, Forsburg and Guarente 1989, McNabb *et al.* 1995, DeRisi *et al.* 1997).

The Hap complex was first identified in *S. cerevisiae* as a regulator of the yeast *CYC1* (iso-1-cytochrome c) gene. Iso-1-cytochrome c facilitates the last steps of the electron transport

chain by transferring electrons, via a covalently attached heme group, from complex III to complex IV. In *S. cerevisiae, CYC1* was identified to contain two upstream activation sites, UAS1 and UAS2. UAS1 was found to be bound by the Hap1p transcription factor and it is responsive to the availability of heme (Guarente *et al.* 1984) . UAS2 is bound by the Hap2/3/4/5p complex and is regulated by carbon source availability. In the presence of glucose, CYC1 is repressed and the CCAAT-binding factor is inactive, while in the presence of a nonfermentable carbon source such as lactate the CCAAT binding factor is active and stimulates transcription (Guarente and Mason 1983, Guarente *et al.* 1984). The Hap2/3/4/5p complex was found to only be actively bound to UAS2 when *S. cerevisiae* is grown on non-fermentable carbon sources. When there is no heme available or the cells are grown in anaerobic conditions, UAS1 and UAS2 are both inactive (Hahn and Guarente 1988).

In *S. cerevisiae*, deletion of any subunit of the CCAAT-binding complex results in an inability to activate UAS2 and a global defect in respiratory metabolism (Pinkham *et al.* 1987, Hahn and Guarente 1988, Forsburg and Guarente 1989, McNabb *et al.* 1995). However, fusing *HAP2* to an activation domain bypasses the need for HAP4 (Olesen and Guarente 1990). *HAP2* or *HAP3* are not regulated by the Hap complex and overexpression of one does not compensate for the deletion of the other gene (Hahn and Guarente 1988, Hahn *et al.* 1988).

In *S. pombe*, a fission yeast, *PHP2* (*HAP2*) has been implicated in mitochondrial function (Olesen *et al.* 1991). Similar to *S. cerevisiae*, *hap2*Δ and *hap5*Δ mutants are defective in *CYC1* transcription (McNabb *et al.* 1997). In S. pombe Hap4p (Php4p) has been shown to play a role in regulation of gene expression in iron poor conditions. Hap4p (Php4p) was shown to be upregulated in response to iron deficiency (Mercier *et al.* 2006, Mercier *et al.* 2008). *Aspergillus nidulans* Hap complex appears to function similarly to that of S. pombe in regulating iron transport. HapXp (Hap4p) along with the DNA binding components have been shown to repress iron-dependent pathways in iron poor conditions (Hortschansky *et al.* 2007).

In *Kluyveromyces lactis*, which uses lactose as a carbon source (Bourgarel *et al.* 1999), a *HAP3* (*KlHAP3*) null mutant does not exhibit a respiratory defect and there is no effect on the expression of *CYC1* (*KlCYC1*) (Mulder *et al.* 1994). The Hap complex in *K. lactis* only plays a

minor role in activation during fermentative growth, but has a more major role in induction of genes during non-fermentative growth (Mulder *et al.* 1995). The complex has been linked to respiratory carbon and nitrogen assimilation in *K. lactis* (Bourgarel *et al.* 1999).

In mammals, the NF-Y transcription factor is an activator for over a hundred genes (Kim and Sheffery 1990, Ronchi *et al.* 1995, Sinha *et al.* 1995). This includes genes that encode proteins involved in a variety of housekeeping functions, developmental control, and responses to cellular conditions. Of important note is that this transcription factor has been implicated in coordinating gene expression with the cell cycle and is essential for cell proliferation (Mantovani 1998, Mantovani 1999, Hu *et al.* 2006).

In *S. cerevisiae*, Hap2p, Hap3p, Hap4p, and Hap5p are each encoded by a single unique locus. This is also true for the NF-Y complex in humans and mice (Maity and de Crombrugghe 1998). However, in *Arabidopsis thalania*, there is genomic redundancy for this transcription factor. There have been 36 genes identified so far that encode subunits of the CCAAT-binding factor (Riechmann and Ratcliffe 2000, Gusmaroli *et al.* 2001, Gusmaroli *et al.* 2002, Siefers *et al.* 2008). The identified genes can be divided amongst all three DNA binding components 10 NF-YA (HAP2-like), 13 NF-YB (HAP3-like) and 13 NF-YC (HAP5-like).

Three of the plant NF-Y subunits have been shown to complement the yeast HAPs in vivo (Masiero *et al.* 2002, Ben-Naim *et al.* 2006). The complex interaction between duplicate Hap proteins in plants has not been fully determined, but they appear to play a role in developmental control, drought stress, and photoperiod flowering time (Siefers *et al.* 2008). It has been shown that the closely related proteins NF-YB2 (HAP3b) and NF-YB3 (HAP3c) share similar function and are both necessary and appear to have an additive effect for flowering response to long-day photoperiodic conditions (Kumimoto *et al.* 2008). This leads to the suggestion that the multiplicity of these genes leads to combinatorial complexity in the association of the three components of this transcription factor (Siefers *et al.* 2008).

The Hap complex in C. albicans

In *C. albicans* as in *S. cerevisiae*, the Hap complex consists of the four subunits, Hap2p, Hap3p, Hap5p, and Hap4p. However, a different but parallel function for the complex has been established. *C. albicans* CCAAT-binding factor has been shown to function as a repressor of genes involved in respiration, as opposed to the activator function in *S. cerevisiae* (Johnson *et al.* 2005).

C. albicans Hap5p was found to have 71% identity and 81% similarity to *S. cerevisiae* Hap5p conserved regions involving the histone fold motif required for Hap5p's interaction with Hap2p and Hap3p along with the interaction domain required for complex formation with Hap4p. The *C. albicans HAP5* was shown to complement a *S. cerevisiae hap5Δ* mutant by restoring growth on the non-fermentable carbon source lactate. Moreover, mobility shift assays demonstrated that the C *albicans* CCAAT-binding factor could bind to the consensus CCAAT site derived from the *CYC1* promoter of *S. cerevisiae* (Johnson *et al.* 2005).

C. albicans hap5Δ/hap5Δ mutants are defective in the yeast-to-hyphal transition in vitro under several conditions and become hyperfilamentous when deprived of glucose as a sole carbon source (Johnson *et al.* 2005, McNabb and Pinto 2005). Moreover, the *hap5Δ/hap5Δ* mutant shows no CCAAT-binding activity, suggesting the loss of the CCAAT-binding factor alters the ability of cells to undergo the normal yeast-to-hyphal transition. Ca*CYC1* and Ca*COX5*, two genes that encode proteins involved in respiratory metabolism have been show to be regulated by the *C. albicans* Hap complex (Johnson *et al.* 2005).

The studies described in the following chapters will demonstrate the importance of the Hap complex subunits, including CaHap2p, CaHap31p, CaHap32p, and CaHap43p, in DNA binding and gene expression. Also, the effect of the complex under low iron conditions will be discussed. The duality of the Hap3p subunits will be shown along with the effect of the absence of both subunits on DNA binding and gene expression. The Hap4p subunits will also be discussed with a clear role of Hap43p in low iron conditions.

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Chapter 2. The role of Ca*HAP2* in the function of the CCAAT-binding factor and its effect on DNA-binding and gene expression.

Introduction

Candida albicans is an opportunistic pathogen that is found as a normal part of the mammalian microflora. This pathogen can sense environmental changes and respond by changing its morphology and physiology. Previous research has shown that the CCAAT-binding transcription factor (Hap complex) plays a role in environmental sensing in *C. albicans* (Johnson *et al.* 2005). This evolutionary conserved heterooligomeric transcriptional activator consists of three DNA-binding subunits, Hap2p, Hap3p and Hap5p. In fungi, a fourth subunit, Hap4p has been identified that binds to the complex and stimulates transcription (Forsburg and Guarente 1989b, McNabb and Pinto 2005).

In *Saccharomyces cerevisiae,* the CCAAT-binding factor has been shown to provide the regulatory switch between fermentation and respiration (Flores *et al.* 2000, Lascaris *et al.* 2004). *S. cerevisiae hap* mutants are unable to grow on nonfermentable carbon sources (Hahn *et al.* 1988, McNabb *et al.* 1995, McNabb *et al.* 1995), highlighting the importance of the Hap complex in respiration.

The three main components of the complex, Hap2p, Hap3p, and Hap5p, all contain essential core domains that have been evolutionary conserved from fungi to mammals (Mantovani 1999); however, the genes regulated by this complex vary among organisms. In *S. cerevisiae* the Hap complex has been shown to have a key role in the activation of genes involved in energy metabolism, respiration, and in cytochrome biogenesis (Mazon *et al.* 1982, Pinkham and Guarente 1985, McNabb *et al.* 1995, Dang *et al.* 1996, Gancedo 1998). In mammals, this transcription factor is a proximal promoter factor that acts as an activator for a large number of unrelated genes (Kim and Sheffery 1990, Ronchi *et al.* 1995, Sinha *et al.* 1995).

In this chapter, the characterization of the Hap2p component of the CCAAT-binding complex in *C. albicans* will be shown. The gene encoding Hap2p has been cloned and a *hap2Δ/hap2Δ* mutant generated. The phenotypes of the mutant have been examined and electrophoretic mobility shift assays performed to determine its effects on morphology and DNA-binding activity, respectively. These assays have shown that the Hap2p component of the

complex is required for DNA binding and that the *hap2Δ/hap2Δ* mutant is defective in hyphal formation similar to the previously described *hap5Δ/hap5Δ* (Johnson *et al.* 2005).

Materials and Methods

Yeast strains and growth conditions

Yeast strains used in these studies are listed inTable 2-1. Strains were cultured in yeast extract-peptone dextrose (YPD) medium (Guthrie and Fink 1991) supplemented with 80mg/L uridine as appropriate and incubated at 30ºC overnight. For selection of yeast transformants, synthetic complete media lacking the appropriate auxotrophic requirement was used (Guthrie and Fink 1991).

Table 2-1 *E. coli* and *C. albicans* strains

Strain	Genotype	Reference
E. coli		
DH5α	*F Φ80dlacZ-M15Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(r_{K-} m_{K+}), deoR thi1 supE44, λ⁻ gyrA96 relA1*	
C. albicans		
BWP17	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG*	(Romier *et al.* 2003)
DMC146	*ura3Δ::imm434/ura3 Δ::imm434 his1 Δ::hisG/his1 Δ::hisG-HIS1 arg4 Δ::hisG/arg4 Δ::hisG-ARG4-URA3*	(Johnson *et al.* 2005)
DMC245	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP2/hap2Δ::URA3*	
DMC249	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap2::URA3/hap2Δ::HIS1*	
DMCr29	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP2-ARG4 hap2::URA3/hap2Δ::HIS1*	
DMC120	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP5/hap5Δ::URA3*	(Johnson *et al.* 2005)
DMC117	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	(Johnson *et al.* 2005)
DMC126	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP5-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	(Johnson *et al.* 2005)

Media for hyphal analysis, including Lee's (Lee *et al.* 1975), modified Lee's (Liu *et al.* 1994), and Spider (Liu *et al.* 1994) medium, were prepared as previously described. Serum media consisted of YPD containing 10% newborn calf serum (Sigma). M199 medium was obtained from Gibco-BRL, containing Earle's salts and glutamine but lacking sodium bicarbonate and buffered with 150 mM HEPES adjusted to pH 4.5 or 7.5. SLAD is synthetic low ammonia medium containing 0.17% yeast nitrogen base w/o

amino acids and ammonium sulfate, 2% dextrose, and 50 μM ammonium sulfate. Yeast nitrogen base (YNB) medium was prepared using 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco). 0.1% w/v amino acids were added using an amino acid dropout powder containing the twenty amino acids at the described concentrations (Guthrie and Fink 1991), buffered to the indicated pH with 150 mM HEPES. Glucose or lactate was added to the media at a final concentration of 2% and solidified with 1.5% agar as appropriate.

For low iron media, bathophenanthroline disulfonate (BPS) (Sigma) was used to chelate iron. BPS was added to YPD at a concentration of 200μM in liquid media and for overnight cultures. BPS at a concentration of 0.1-0.25mM was added to solid YPD media. Hemin was added to solid YPD at a concentration of 50μM.

Yeast extract preparation and electrophoretic mobility shift assays.

Protein extracts were prepared as previously described (Johnson *et al.* 2005). The CCAAT probe was designed as described previously (Hahn and Guarente 1988) and is based on the sequence of the *CYC1* promoter from C. *albicans*. The DNA probe was end labeled by the Klenow fragment with [α-^{32}P]dATP (Amersham Corp.).

All binding reactions contained 20 to 30 μg of cell extract in DNA-binding buffer (20 mM HEPES-NaOH pH 8.0, 100 mM KCl, 1 mM EDTA, 10% glycerol), 0-1 μg of denatured salmon sperm DNA, 0-1 μg of poly(dI-dC), and 0.5 to 1.0 ng of radiolabeled probe in a final reaction volume of 20 μl. Reactions were incubated at room temperature for 30 to 45 min, and the protein-DNA complexes were resolved by gel electrophoresis (4 h at 300 V) on 5% polyacrylamide gels (acrylamide:bisacrylamide ratio, 29:1) in 0.5× Tris-borate-EDTA at 4°C. After electrophoresis, the gels were fixed, dried, and visualized with the Typhoon 9600. For the gel shift complementation assays, extracts were combined in equal concentrations prior to the addition of the labeled probe.

Microscopy and imaging.

Photographs of colony morphology were taken with a Nikon Biaphot microscope fitted with a high-resolution charge-coupled device camera and the AutoMontage imaging software package (Syncroscopy, Frederick, MD). Photographs of individual cells were done with a Zeiss Axioplan 2 microscope fitted with a high-resolution charge-coupled device camera and AutoMontage imaging software. Photographs of individual colonies were representative of the total population. The figures were prepared using Adobe Photoshop 7.0 and Microsoft PowerPoint 2007.

Northern blot analysis.

C. albicans strains were grown to saturation in YPD medium and subsequently inoculated into the indicated medium and grown until OD>0.6 at 30°C. The cells were harvested by centrifugation, and total RNA was prepared by the glass bead-acid phenol method as previously described (Ausubel *et al.* 1994). Approximately 20 μg of each total RNA sample was loaded, separated by formaldehyde-1% agarose gel electrophoresis, and transferred to GeneScreen Plus membranes (Dupont-NEN Research Products) according to the manufacturer's protocol. The membranes were hybridized and washed under standard high-stringency conditions (Sambrook *et al.* 1989).

The *CYC1*, *COX5*, and 26S rRNA probes for hybridization were obtained by PCR amplification from *C. albicans* genomic DNA using the primer pairs oDM0341/oDM342, oDM0449/oDM0450, and oDM0459/oDM0460, respectively Table 2-2. The probes were purified by agarose gel electrophoresis and GeneClean (Qbiogene, Inc.) and subsequently radiolabeled with [α-^{32}P]dATP (Amersham) by use of a random primer labeling kit (U.S. Biochemicals) according to the manufacturer's protocol. The transcript levels were quantified on the Typhoon 9600.

Table 2-2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5' – 3')
oDM0341………………	5'-GGCCGAATTCGGTGCCACTTTATTTAAAACTAGATG-3'
oDM0342………………	5'-GGCCGGATCCGCTTTCTTCAAATAAGTAACTAAATCG-3'
oDM0449………………	5'-GAGATCATTACAAAGAGCTGCCACTAAAGCC-3'
oDM0450………………	5'-GGAGTTAATTCTTGCCATGGTAATTCC-3'
oDM0459………………	5'-TAGCGGTTCTGACGTGCAAATCGATCGTCG-3'
oDM0460………………	5'-ACTAACACCTTTTGTGGTGTCTGATGAGCG-3'

Results

***HAP2* deletion causes a defect in morphogenic transition on solid media.**

The *C. albicans hap2Δ/hap2Δ* mutant is phenotypically analogous to *hap5Δ/hap5Δ* homozygote. To determine whether the *hap2Δ/hap2Δ* mutant displayed phenotypic defects similar to the *hap5Δ/hap5Δ* mutant, the appropriate strains were grown on media known to induce hyphal development. On solid media, the *hap2Δ* homozygous mutant displayed defective hyphal growth on all media examined. The heterozygote and marker rescued strains displayed phenotypes that were comparable to the wild-type strain (data not shown). The most prominent filamentation defects were seen on YPD containing 10% calf serum, SLAD, and Modified Lee's medium (Figure 2-1). The colonies from the *hap2Δ* homozygote grown on YPD + 10% serum or Modified Lee's medium did not produce the wrinkled colony appearance as compared to the wild type strain.

Growth on SLAD medium produced the most dramatic effect; the *hap2Δ* homozygote lacked any hyphal growth compared to the wild type strain. The *hap2Δ/hap2Δ* mutant displayed slow hyphal growth on Lee's media, and hyperfilamentation was seen on M199 pH 7.5 medium where the *hap2Δ/hap2Δ* mutant displayed little colony growth but an abundance of hyphal formation. On M199 medium the homozygous mutants began forming hyphae on day 2 when

grown at 37ºC. When the same strains were grown on M199 pH 4.5, no significant defects were observed (data not shown).

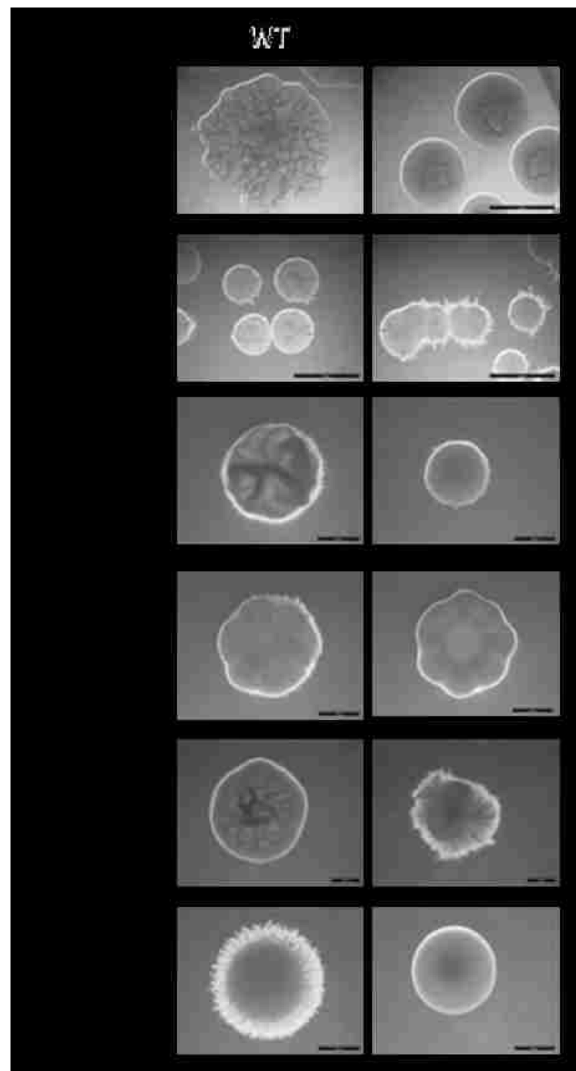

Figure 2-1. Defects in filamentation caused by loss of CCAAT-binding factor.
DMC146 (WT) and DMC249 (*hap2Δ/hap2Δ*) were grown on the indicated media for 4 days at 37ºC except for Spider and SLAD which were incubated for 6 days. Heterozygous and rescue strains were comparable to WT (data not shown).

In summary, the phenotypes observed on solid media for the *hap2Δ/hap2Δ* mutant are comparable to those seen with the *hap5Δ/hap5Δ* mutant (Johnson *et al.* 2005). The lack of hyphal production on SLAD and slow hyphal growth on Lee's along with lack of colony wrinkling on Modified Lee's and YPD + Serum are similar to *hap5Δ/hap5Δ* mutant. The hyperfilamentation on M199 medium pH 7.5 was also the same for both the *hap2Δ* and *hap5Δ* homozygotes.

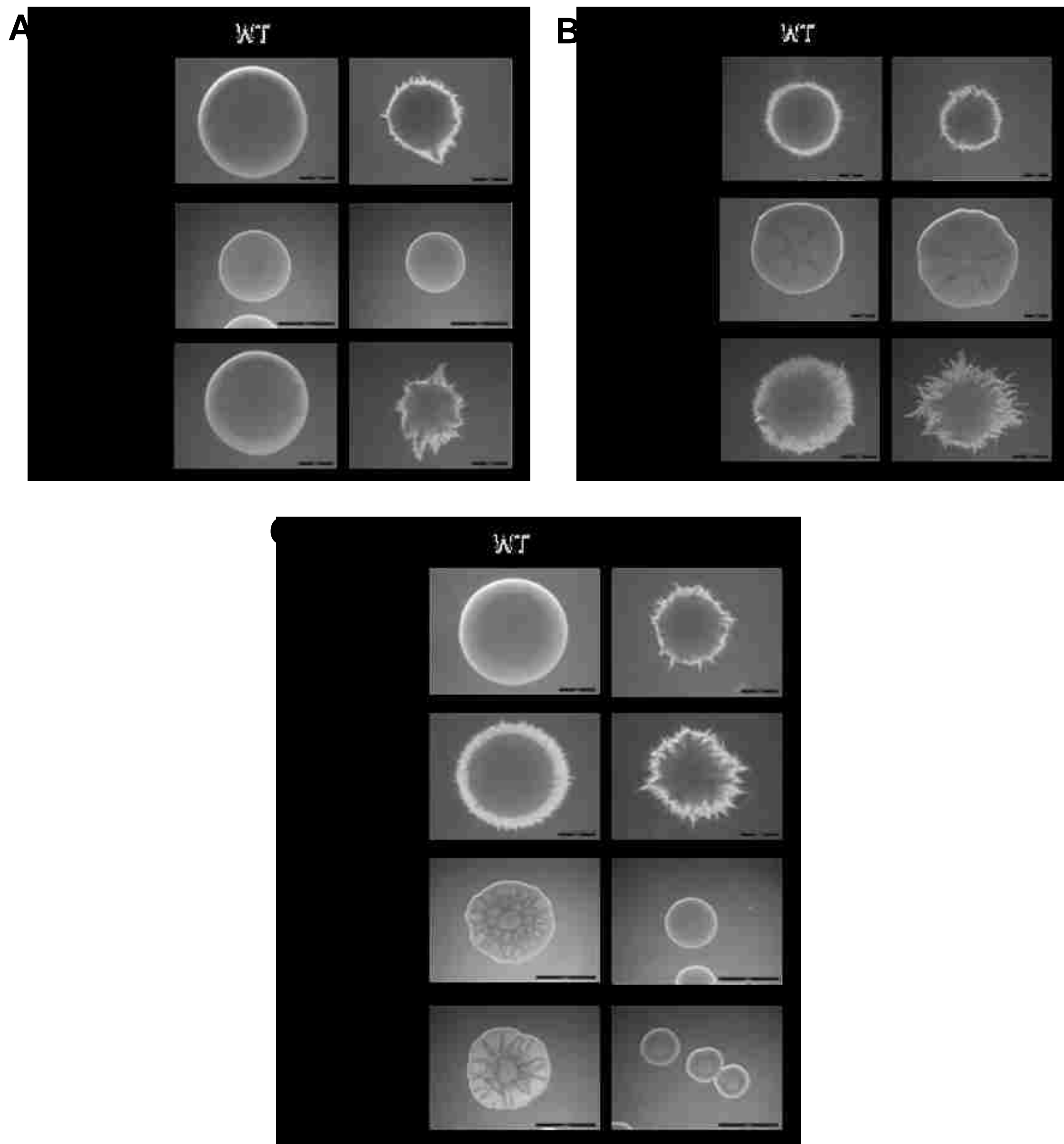

Figure 2-2. The hyperfilamentation of *hap2*Δ/*hap2*Δ is carbon source dependent. (A.)Strains DMC146 (WT) and DMC249 (*hap2*Δ/*hap2*Δ) were grown on indicated media at pH 5.6 for 5 days at 37ºC. Glucose or lactate (2%) was added to the medium as indicated. (B.) Strains grown on the indicated medium at pH 6.8. (C.) Strains grown on the indicated medium supplemented with NH_4, with and without glucose, at the indicated pH. Heterozygous and rescue strains were comparable to WT (data not shown).

Hyperfilamentation of the *hap2Δ/hap2Δ* mutant is dependent the absence of glucose. In *S. cerevisiae* and *S. pombe* the CCAAT-binding factor has been shown to regulate genes involved in respiratory metabolism (Forsburg and Guarente 1989a, Zitomer and Lowry 1992, McNabb *et al.* 1997, Brakhage *et al.* 1999, Liu and Butow 1999). The *hap2Δ/hap2Δ* mutant showed defects

on hyphae-inducing media that had little or no glucose (M199 pH7.5, Spider, and Modified Lee's media). Carbon utilization is effected in mutants that have defects in respiration. To investigate the morphological defects identified on hyphae-inducing media, the *hap2Δ/hap2Δ* mutant strains were grown in yeast nitrogen base with amino acids as the nitrogen source and either glucose, lactate, or no carbon source (other than the amino acids) at pH 5.6 and 6.8. In media where glucose was provided, regardless of pH, the *hap2Δ/hap2Δ* mutant grew comparable to the wild type strain (Figure 2-2).

In media where amino acids provided the only carbon source or with lactate (a nonfermentable carbon source) as the sole carbon source the homozygous mutant produced colonies that were predominantly hyphal. Both the *HAP2/hap2Δ* heterozygote and marker rescue strains produced colonies comparable to the wild-type strain on all media tested (data not shown). Since amino acids were the sole source of nitrogen for colony growth it is possible that nitrogen limitation was causing the hyperfilamentation. To address this question, NH_4 was added to the medium to determine whether it would rescue the phenotype of the *hap2Δ/hap2Δ* mutant. The *hap2Δ* homozygote still displayed the hyperfilamentation in the presence of $NH_{3,}$ suggesting that carbon source limitation was the cause of the phenotype. At higher pH the hyperfilamentation phenotype was enhanced (Figure 2-2).

The *hap5*Δ and *hap2*Δ homozygous mutants are unable to grow in limited iron media. The *S. cerevisiae CYC1* gene has been shown to be regulated by heme and the deletion of *C. albicans HAP5* causes a defect in the expression of *CYC1* (Johnson *et al.* 2005), suggesting the Hap complex in *C. albicans* may regulate similar genes in this organism. *CYC1* is involved in the final step of the mitochondrial respiratory chain and passes electrons via a covalently attached heme group from complex I to complex III of the respiratory chain. In *SchizoSaccharomyces pombe* and *Aspergillus nidulans* the Hap complex has been suggested to regulate genes in response to iron availability (Mercier *et al.* 2006, Hortschansky *et al.* 2007). Given the role of iron as an electron carrier for many proteins in the mitochondria and the respiratory phenotype seen with the *C. albicans hap2Δ* and *hap5Δ* homozygous mutants, it was reasonable to determine whether the Hap complex in *C. albicans* plays a role in iron metabolism. To test this hypothesis,

the appropriate strains were grown in YPD medium with and without the addition of the iron chelator bathophenanthroline disulfonate (BPS). Cells were initially grown overnight in YPD+200µM BPS to deplete the cells of stored iron. Cell concentrations were normalized prior to spot plating to ensure that equal colony forming units were plated (Figure 2-3).

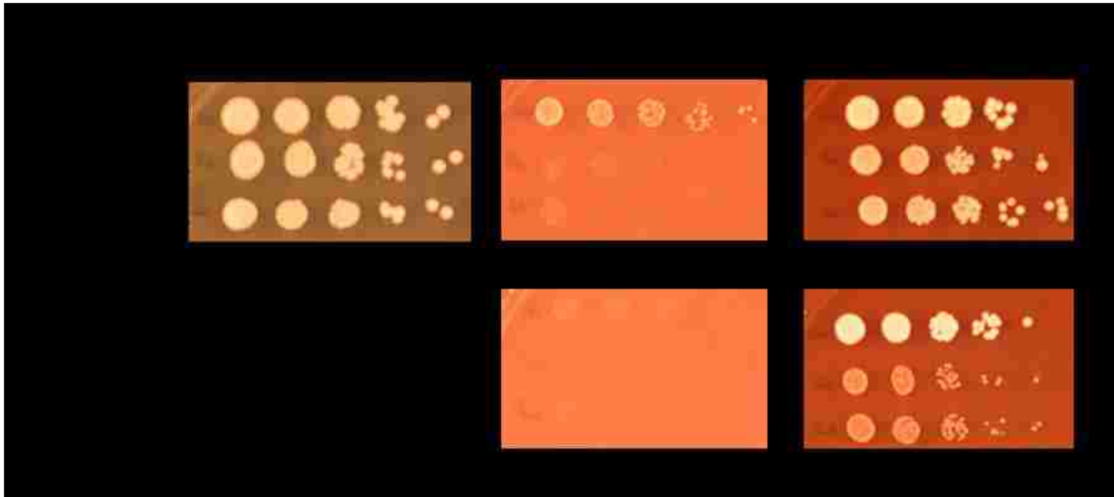

Figure 2-3. *hap2Δ* and *hap5Δ* homozygotes are unable to grow in low iron media. Strains with the indicated genotypes were grown overnight in YPD with 200µM BPS and spot plated onto YPD, YPD + .01 mM BPS, YPD + .25 mM BPS, YPD + .01 mM BPS + 50µM Heme, YPD + .25 mM BPS + 50µM Heme and grown at 30ºC for 3 days.

The *hap5Δ/hap5Δ* and *hap2Δ/hap2Δ* mutants were unable to grow in iron-limiting conditions (with BPS); whereas, strains grown in the presence of both BPS and heme grew normally. The *hap2Δ* heterozygote and *HAP2*-rescued strains grew comparable to the wild-type strain (data not shown). These data suggest that the defect in growth on iron-limiting media involves a defect in the utilization of the inorganic form of iron, but not the iron coupled to an organic carrier such as heme. Thus, these data indicate that the utilization of heme as a source of iron is not altered by mutations in the subunits of the CCAAT-binding factor.

Deletion of *HAP2* abolishes CCAAT-binding activity in *C. albicans*. In *S. cerevisiae*, the CCAAT-complex consists of three subunits, termed Hap2p, Hap3p, and Hap5p, that form the DNA-binding complex. It has been previously shown that CCAAT-binding activity is abolished in a *C. albicans hap5Δ/hap5Δ* mutant (Johnson *et al.* 2005). To determine whether *HAP2* is

required for CCAAT-binding in *C. albicans*, an electrophoretic mobility shift assay was performed. For the studies, the *C. albicans* wild-type strain, heterozygous mutant (*HAP2/hap2*Δ), homozygous mutant (*hap2*Δ*/hap2*Δ), and *HAP2*-rescued (*hap2*Δ*/hap2*Δ *ARG-HAP2*) strains were used. The strains were grown in YPD 30ºC to an OD_{600} of ~1, the cells were harvested and cell extracts were prepared as described in Materials and Methods. To assess CCAAT binding activity, a 37-bp double stranded DNA oligonucleotide derived from *C. albicans CYC1* CCAAT sequence was used. The wild-type and heterozygous strains displayed the same binding pattern, whereas the homozygous mutant showed no detectable DNA binding activity (Figure 2-4). Reintroduction of *HAP2* into the *hap2Δ* homozygote restored DNA binding activity (data not shown). In summary these data establish that Hap2p is required for the DNA-binding activity of the Hap complex.

Identification of multiple CCAAT binding complexes based on growth conditions. Since *C. albicans* strains containing mutations that abolish CCAAT-binding activity demonstrated both carbon- and iron-dependent phenotypes, we wanted to determine whether the multiple CCAAT-binding complexes previously observed (Johnson *et al.* 2005) varied based on the growth environment. Since there were multiple *HAP3* and *HAP4* genes, it seemed reasonable that changes in environmental conditions may regulate which of the Hap3p and Hap4p subunits are utilized by the cell. Thus, *C. albicans* wild-type, heterozygous mutant (*HAP2/hap2*Δ), homozygous mutant (*hap2*Δ*/hap2*Δ), and *HAP2*-rescued (*hap2*Δ*/hap2*Δ *ARG-HAP2*) strains were grown in YPD, and YPD with 200µM BPS and cell extracts prepared and used for gel shift studies (Figure 2-4) The extract prepared from the wild-type strain grown in YPD+BPS showed a shift in the abundance of different CCAAT-binding complexes compared to extracts from strains grown in YPD. For the wild-type strain grown in YPD, CCAAT-binding complexes I, II, IV were visible; whereas, the extracts from the same strain grown in YPD + BPS had more abundant amounts of complexes II, III, IV, and V. The most striking observation was the presence of an abundant complex II on YPD that was weak in strains grown in YPD + BPS. In addition, complex V was abundant on YPD + BPS, yet it was absent from strains grown in YPD.

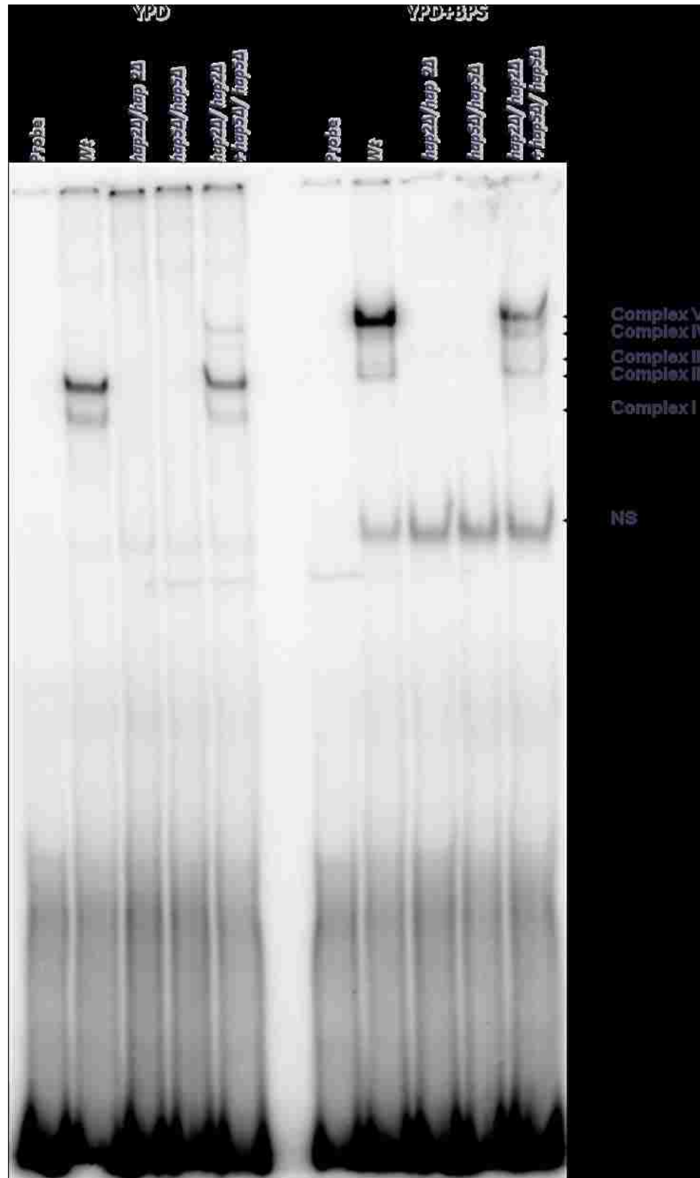

Figure 2-4. Deletion of Hap2p or Hap5p abolishes CCAAT-binding activity.
The electrophoretic mobility shift assay was performed with crude extracts prepared from the wild-type, *hap2Δ/hap2Δ*, and *hap5Δ/hap5Δ* strains grown in YPD or YPD + BPS (iron-limiting conditions) as indicated. Crude extracts from *hap2Δ/hap2Δ*and *hap5Δ/hap5Δ* strains were mixed prior to the addition of the probe. NS - Nonspecific Binding.

In addition to the studies described above, extracts were prepared from strains grown in YNB + amino acids + Glucose pH5.6, YNB + amino acids + Glucose pH6.8, and YNB + amino acids pH5.6. The gel shifts performed with crude extracts derived from strains grown in these media produced no dramatic changes in complex formation as compared to strains grown in YPD (data not shown).

In comparing all the different growth conditions examined, complex II is the most abundant under all growth conditions evaluated except YPD + BPS where complex V is the most abundant. These data show that there is a shift in CCAAT binding complexes based on environmental growth conditions, namely growth in the presence of abundant iron versus iron-limited conditions .

Addition of *HAP2* or *HAP5* rescues CCAAT-binding activity in deletion strains. To demonstrate that the CCAAT-binding factor could be reconstituted in the *hap2Δ* and *hap5Δ* homozygotes, extracts from the two mutants were mixed prior to assessing DNA-binding activity. As shown in Figure 2-4, this complementation assay restored DNA-binding activity, demonstrating that the other subunits of the CCAAT-binding factor are stable in cells and readily available for DNA-binding under the appropriate growth conditions.

The CCAAT binding factor regulates *CYC1* and *COX5* in both iron replete and iron poor conditions. Previously *C. albicans hap5Δ* homozygous mutants were shown to alter the expression of *COX5* and *CYC1* when grown in YNB + amino acids + glucose pH5.6 and YNB + amino acids pH5.6. *COX5* expression was reduced in the *hap5Δ* homozygous mutant in glucose media and up-regulated in the glucose-deficient media when compared to the wild-type strain. *CYC1* showed no difference in expression in the presence of glucose, but was up-regulated in the *hap5Δ* homozygous mutant as compared to the wild-type strain in glucose-deficient medium (Johnson *et al.* 2005). The *hap2Δ* homozygous mutant showed the same alteration as seen in the *hap5*Δ homozygous mutant for *COX5* and *CYC1* expression under the same growth conditions (data not shown). Given the changes in CCAAT-binding factor complex formation on YPD versus YPD + BPS it was reasonable to determine whether the expression of *COX5* and/or *CYC1* was altered in the *hap* mutants when cells were grown in low iron media. Thus, the strains were grown in YPD or YPD with 200µM BPS, RNA was isolated and northern blot analysis was preformed as described in Material and Methods (Figure 2-5). Both the *hap2Δ* and *hap5Δ* mutants showed changes in mRNA expression for both *CYC1* and *COX5* when compared to the

wild-type strain. These changes were visible for mRNA extracted from cells grown in either YPD or YPD + BPS.

In YPD medium, the expression of *CYC1* and *COX5* was significantly reduced in both the hap2Δ and hap5Δ homozygotes. For cells grown under iron limitation, the expression of *CYC1* and *COX5* was up-regulated as compared to the wild-type strain. These data suggest that the expression of *CYC1* and *COX5* is regulated by the Hap complex under both iron-replete and iron-limiting conditions. Thus, the CCAAT-binding complex must function in up-regulation of both *CYC1* and *COX5* in iron-replete conditions and in the down-regulation (repression) of these genes when iron is limiting.

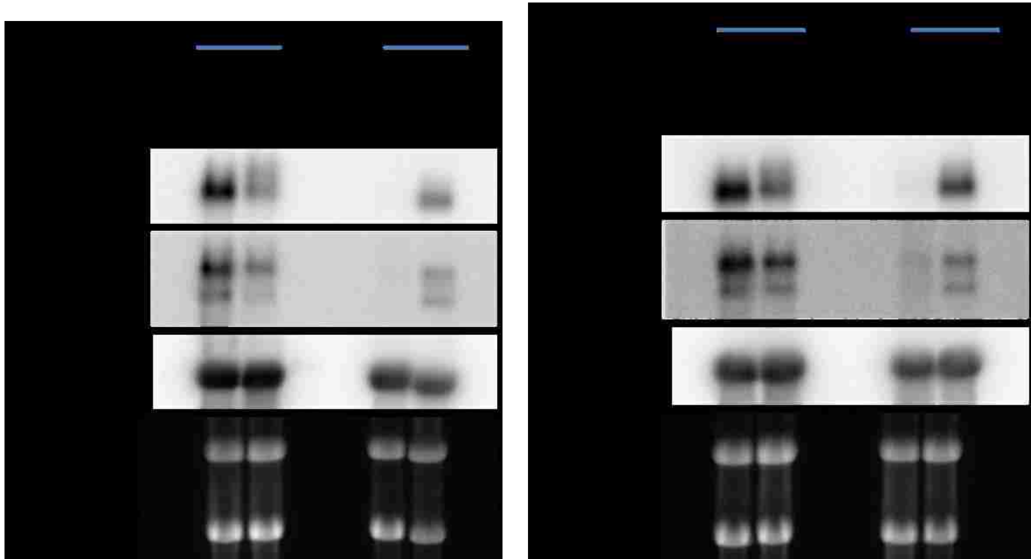

Figure 2-5. *C. albicans* CCAAT-binding factor regulates *CYC1* and *COX5* under both iron-replete and iron-limiting conditions. Northern blot analysis of total RNA isolated from the indicated *C. albicans* strains grown at 30ºC in YPD or YPD + BPS as shown. The membrane was hybridized with radiolabeled probes specific for *CYC1* and *COX5* as indicated. 26S rRNA was used to normalize results.

Discussion

The data shown in this chapter demonstrate that the cloned *C. albicans HAP2* gene encodes an authentic subunit of the CCAAT-binding factor. Deletion of *HAP2* caused the same defects in growth that were seen with the *hap5Δ* homozygous mutant and gel shift studies demonstrated that without Hap2p or Hap5p, DNA binding activity is abolished. Therefore, the *C.*

albicans CCAAT-binding complex requires both Hap2p and Hap5p for DNA-binding activity. These data are consistent with what has been previously observed with the CCAAT-binding factor in S. *cerevisiae* (McNabb and Pinto 2005).

The morphology of *C. albicans* colony growth is affected by carbon, pH, and nitrogen source. When *C. albicans hap2Δ* or *hap5Δ* homozygotes are grown on media lacking glucose, the cells become hyperfilamentous. The addition of NH_4 to the media lacking glucose delays hyperfilamentation, but does not rescue the phenotype, suggesting the availability of glucose as the carbon source is critical in the absence of the CCAAT-binding factor. In addition it was observed that the nonfermentable carbon source, lactate, failed to rescue the hyperfilamentation. It should also be noted that *hap* mutants grown on M199 medium, which contains a low amount of glucose (0.1%) and is rich in amino acids, show hyperfilamentation as well. These phenotypic data strongly suggest that the *hap* mutants have a defect in respiratory metabolism.

In SLAD medium, which was used to induce nitrogen starvation in the presence of glucose, loss of hyphal formation is observed. This indicates there is also a defect in nitrogen sensing/utilization at low concentrations in the *hap2Δ* and *hap5Δ* homozygotes. Overall the phenotypic data indicates that carbon and nitrogen source sensing/utilization is altered by the loss of CCAAT-binding activity.

In iron-limiting conditions both the *hap2Δ/hap2Δ* and *hap5Δ/hap5Δ* mutants are unable to grow (Figure 2-3). This suggests that the *C. albicans* Hap complex plays a role in iron acquisition/utilization. The addition of heme to the iron-limited media rescued colony growth for both mutants, suggesting that it is the metabolism of inorganic iron that is affected by the loss of the Hap complex. Therefore the Hap complex must play a role in iron acquisition and/or utilization. The Hap complex in *S. cerevisiae* is not affected by iron concentrations; however, in *SchizoSaccharomyces pombe* and *Aspergillus nidulans* the Hap complex has been shown to regulate genes in response to low iron conditions (Mercier *et al.* 2006, Hortschansky *et al.* 2007, Mercier *et al.* 2008). Thus, the CCAAT-binding factor in *C. albicans* seems to have evolved to regulated genes involved in respiratory metabolism like *S. cerevisiae*, but it also regulates genes involved in the uptake/utilization of iron similar to *S. pombe* and *A. nidulans*.

C. albicans has evolved several distinct pathways to acquire iron from an iron-limited host environment. The mechanisms include high-affinity transporters, a siderophore uptake system, and a heme uptake system (Howard 1999, Ramanan and Wang 2000, Heymann *et al.* 2002, Knight *et al.* 2002, Lesuisse *et al.* 2002, Pendrak *et al.* 2004, Knight *et al.* 2005). It has also been shown that iron acquisition through high affinity transporters and siderophores is required for pathogenesis (Ramanan and Wang 2000, Heymann *et al.* 2002, Pendrak *et al.* 2004, Haas *et al.* 2008).

The uptake of elemental iron involves a reductive copper-dependent iron uptake system where iron is acquired from transferin (Knight *et al.* 2002). This occurs via a two step process (Philpott and Protchenko 2008) were ferric iron is reduced to ferrous iron and then is transported into the cytosol via a high affinity, ferrous specific transport complex of Fet3p, a multicopper ferroxidase, and Ftr1p, a iron permease (Knight *et al.* 2005). A copper-independent siderophore transport system also exists for the uptake of iron (Lesuisse *et al.* 2002, Knight *et al.* 2005). *C. albicans* has also developed mechanisms for the uptake of heme, a characteristic of pathogenic fungi (Santos *et al.* 2003). Plasma membrane proteins have been identified that bind hemin and possess hemolytic activity (Santos *et al.* 2003).

It has been shown that the Hap complex is essential for the expression of Frp1p, a ferric reductase, in low iron conditions in *C. albicans* (Baek *et al.* 2008). Ferric reductases promote iron transport by acting with a multicopper oxidase and iron permease, *FTR1* (Dancis 1998, Kosman 2003). In addition, these gene products are required for the transport of internally stored iron (Singh *et al.* 2007). As mentioned previously, mutants defective in growth under iron-limited conditions are less virulent (Weinberg 2000) and are more susceptible to antifungal drugs (Ramanan and Wang 2000, Prasad *et al.* 2006).

The electrophoretic mobility shift assay shows that without Hap2p or Hap5p, the DNA-binding activity of the CCAAT-binding factor is abolished. Therefore, the *C. albicans* CCAAT-binding complex requires both Hap2p and Hap5p for DNA binding. There are five unique CCAAT-binding complexes observed when the extracts from the mutants were combined as compared to the wild type strain (Figure 2-4). These additional complexes can be observed in the wild-type

extracts when one compares the complexes derived from growth on YPD versus YPD + BPS. This suggests that there are various complexes that are formed based on environmental conditions. As previously published, there have been three genetic loci identified to encode Hap4p homologs and two genetic loci which encode Hap3p homologs (Johnson *et al.* 2005). This mixing of the various Hap3p and Hap4p homologs may explain the different CCAAT-binding factors. The composition of the complexes will be investigated further in later chapters.

The Hap complex in *S. cerevisiae* is regulated by heme and controls activation of genes encoding components of the electron transport chain, and many of these proteins contain iron as a cofactor. In *S. cerevisiae*, *CYC1* expression is low in low iron-conditions, but increases as the iron concentration increases (Protchenko and Philpott 2003). *CYC1* encodes cytochrome c which transfers electrons from ubiquinone-cytochrome c oxidoreductase to cytochrome c oxidase during cellular respiration (Voet and Voet 2004) and contains heme as a cofactor. However, the CCAAT-binding factor does not directly regulate genes involved in iron transport or utilization in *S. cerevisiae*.

C. albicans CYC1 is also transcriptionally regulated by iron. Transcription is high in iron replete conditions, and is repressed in iron-limiting conditions. In the *hap2Δ/hap2Δ* and *hap5Δ/hap5Δ* mutants, transcription is altered under these growth conditions. For both mutants, *CYC1* transcription is reduced under iron replete conditions and up-regulated in iron-limiting conditions as compared to the wild type strain. *COX5a* encodes a cytochrome c oxidase, a protein that is a terminal member of the electron transport chain (Voet and Voet 2004) In *S. cerevisiae COX5a* is predominantly expressed during aerobic growth (Hodge *et al.* 1989). In *C. albicans*, *COX5a* shows the same pattern of expression as *CYC1* in low iron versus iron replete conditions. This highlights the role of the Hap complex in respiration. Moreover, these data suggest that the regulation of some of the genes involved in iron uptake are coordinately regulated with genes involved in respiration.

The *C. albicans* CCAAT-binding factor plays a role in regulating genes under both iron replete and iron-limiting conditions. Deleting either *HAP2* or *HAP5* causes a loss of growth on low iron condition; however, at the level of transcription this alteration in gene expression extends

into the iron replete conditions since the transcription of *CYC1* and *COX5* are affected under both conditions. In addition, the CCAAT-binding factor also functions in the regulation of both CYC1 and COX5 is response to carbon source availability (Johnson et al. 2005). Thus, our data suggest that the CCAAT-binding factor is involved in the activation and repression of target genes depending on the environmental conditions. Understanding the molecular mechanisms of this dual function is the subject of subsequent chapters in this dissertation.

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Chapter 3. Duality of the Hap3p subunit and its effect on complex formation and DNA binding in *Candida albicans*.

Introduction

The CCAAT-binding factor is an evolutionarily conserved hetero-oligomeric transcription factor. In yeast, the DNA binding component that binds the CCAAT consensus sequence consist of the three subunits, Hap2p, Hap3p and Hap5p (Pinkham and Guarente 1985, Pinkham *et al.* 1987, Hahn and Guarente 1988, McNabb *et al.* 1995). In yeast and fungi, a fourth subunit, Hap4p, is required for regulating gene expression (Forsburg and Guarente 1989). In *Saccharomyces cerevisiae* Hap2p, Hap3p and Hap5p are required simultaneously to assemble and bind DNA (McNabb and Pinto 2005).

Candida albicans is a human opportunistic pathogen responsible for a variety of mucosal and systemic infections that result in significant morbidity and mortality, particularly in immunosuppressed individuals (Odds 1988, Pittet *et al.* 1997). *C. albicans* responds to environmental changes by altering its morphology between the yeast and hyphal forms during infection, and the ability to transition between the two forms is required for virulence (Lo *et al.* 1997, Navarro-Garcia *et al.* 2001, Saville *et al.* 2006).

In the mammalian host, iron is sequestered by ferritin causing the lack of free iron that could be used by a pathogen (Ratledge 2007). Therefore pathogens have developed several ways to acquire iron from the host. *C. albicans* has evolved several distinct pathways to acquire iron from an iron-poor host environment. The mechanisms include high-affinity transporters, a siderophore uptake system, and heme uptake system (Howard 1999, Ramanan and Wang 2000, Heymann *et al.* 2002, Knight *et al.* 2002, Lesuisse *et al.* 2002, Pendrak *et al.* 2004, Knight *et al.* 2005).

C. albicans hap5Δ/hap5Δ and *hap2Δ/hap2Δ*mutants are defective in the yeast-to-hyphal transition in vitro under several conditions and become hyperfilamentous when deprived of glucose as a sole carbon source (Johnson *et al.* 2005). Also, neither mutant is able to grow in iron poor conditions (Baek *et al.* 2008, Kroger-von Grote 2008). Moreover, both mutants show no CCAAT-binding activity in electrophoretic mobility shift assays (Johnson *et al.* 2005), suggesting the loss of the CCAAT-binding factor alters the ability of cells to undergo the normal yeast-to-hyphal transition and adequately acquire iron.

Additional Hap complexes were identified when the wild-type *C. albicans* strain was grown in iron replete verses iron poor conditions. This was also seen when the crude extracts from the *hap5Δ/hap5Δ* and *hap2Δ/hap2Δ*mutants were combined. It has been shown that *C. albicans* has two *HAP3* homologs (Johnson *et al.* 2005). This chapter will identify the possible function of the two distinct Hap3p proteins in the pathogenic commensal *C. albicans*.

Materials and Methods

Yeast strains and growth conditions.

Table 3-1. *E. coli*, *S. cerevisiae*, and *C. albicans* strains

Strain	Genotype	Reference
E. coli		
DH5α	*F Φ80dlacZ-M15Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(r_{K-} m_{K+}), deoR thi1 supE44, λ^- gyrA96 relA1*	
S. cerevisiae		
BWG1-7a	*Mata ura3-52 leu2-3,112 his4-519 ade1-100 36*	
SHY40	*Mata ura3-52 leu2-3,112 his4-519 ade1-100 hap3Δ::hisG*	
C. albicans		
BWP17	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG*	(Romier *et al.* 2003)
DMC146	*ura3 Δ ::imm434/ura3 Δ ::imm434 his1 Δ ::hisG/his1 Δ ::hisG-HIS1 arg4 Δ ::hisG/arg4 Δ ::hisG-ARG4-URA3*	(Johnson *et al.* 2005)
DMC245	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP2/hap2Δ::URA3*	
DMC249	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap2::URA3/hap2Δ::HIS1*	
DMCr29	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP2-ARG4 hap2::URA3/hap2Δ::HIS1*	
DMC120	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP5/hap5Δ::URA3*	(Johnson *et al.* 2005)
DMC117	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	(Johnson *et al.* 2005)
DMC126	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP5-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	(Johnson *et al.* 2005)
LBC001	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 hap31Δ::HIS1/hap31Δ::URA3*	
LBC001	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 hap32Δ::HIS1/hap32Δ::URA3*	

Yeast strains used in these studies are listed in Table 3-1. Strains were cultured in yeast extract-peptone dextrose (YPD) medium (Guthrie and Fink 1991) supplemented with 80mg/L uridine as appropriate and incubated at 30ºC overnight. For selection of yeast transformants,

synthetic complete media lacking the appropriate auxotrophic requirement was used (Guthrie and Fink 1991)For low iron media, bathophenanthroline disulfate (BPS) (Sigma) was used to chelate iron. BPS was added to YPD at a concentration of 200μM in liquid media and used for overnight cultures. BPS at a concentration of 0.1-0.25mM was added to solid YPD media. Hemin was added to solid YPD medium at a concentration of 50μM.

Imaging

The figures were prepared using Adobe Photoshop 7.0 and Microsoft PowerPoint 2007.

Yeast extract preparation and electrophoretic mobility shift assays.

Protein extracts were prepared as previously described (Johnson *et al.* 2005). The CCAAT probe was designed as described previously (Hahn and Guarente 1988) and is based on the sequence of CCAAT-binding consensus site from the *CYC1 promoter* of *C. albicans*. The DNA probe was end labeled by the Klenow fragment with [α-^{32}P]dATP (Amersham Corp.).

All binding reactions contained 20 to 30 μg of cell extract in DNA-binding buffer (20 mM HEPES-NaOH pH 8.0, 100 mM KCl, 1 mM EDTA, 10% glycerol), 0-1 μg of denatured salmon sperm DNA, 0-1 μg of poly(dI-dC), and 0.5 to 1.0 ng of radiolabeled probe in a final reaction volume of 20 μl. Reactions were incubated at room temperature for 30 to 45 min, and the protein-DNA complexes were resolved by gel electrophoresis (4 h at 300 V) on 5% polyacrylamide gels (acrylamide:bisacrylamide ratio, 29:1) in 0.5× Tris-borate-EDTA at 4°C. After electrophoresis, the gels were fixed, dried, and visualized with a Molecular Dynamics PhosphorImager.

For complementation assays, extracts were combined in equal concentrations prior to labeled probe addition. For denaturing assays, extracts were combined in equal concentrations and then treated with 3M Gu-HCl at 65°C for 30 min. The samples were then dialyzed overnight in DNA binding buffer (20 mM HEPES-NaOH pH 8.0, 100 mM KCl, 1 mM EDTA, 10% glycerol, beta-mercaptoethanol, 1 mM benzamidine, and 1 mM PMSF).

Northern blot analysis.

C. albicans strains were grown to saturation in YPD medium and subsequently inoculated into the indicated medium and grown until OD>0.6 at 30°C. The cells were harvested by centrifugation, and total RNA was prepared by the glass bead-acid phenol method as previously described (Ausubel *et al.* 1994). Approximately 20 µg of each total RNA sample was loaded, separated by formaldehyde-1% agarose gel electrophoresis, and transferred to GeneScreen Plus membranes (Dupont-NEN Research Products) according to the manufacturer's protocol. The membranes were hybridized and washed under standard high-stringency conditions (Sambrook *et al.* 1989).

Table 3-2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5' – 3')
oDM0341..................	5'-GGCCGAATTCGGTGCCACTTTATTTAAAACTAGATG-3'
oDM0342..................	5'-GGCCGGATCCGCTTTCTTCAAATAAGTAACTAAATCG-3'
oDM0449..................	5'-GAGATCATTACAAAGAGCTGCCACTAAAGCC-3'
oDM0450..................	5'-GGAGTTAATTCTTGCCATGGTAATTCC-3'
oDM0459..................	5'-TAGCGGTTCTGACGTGCAAATCGATCGTCG-3'
oDM0460..................	5'-ACTAACACCTTTTGTGGTGTCTGATGAGCG-3'

The *HAP31, HAP32,* and 26S rRNA probes for hybridization were obtained by PCR amplification from *C. albicans* genomic DNA using the primer pairs oDM0341/oDM342, oDM0449/oDM0450, and oDM0459/oDM0460, respectively (Table 3-2). The probes were purified by agarose gel electrophoresis and GeneClean (Qbiogene, Inc.) and subsequently radiolabeled with [α-^{32}P]dATP (Amersham) by use of a random primer labeling kit (U.S. Biochemicals) according to the manufacturer's protocol. The transcript levels were quantified on a Molecular Dynamics PhosphorImager.

Results

***C. albicans* has two distinct Hap3p proteins that functionally complement *S. cerevisiae hap3*Δ.** Two genetic loci have been identified that encode Hap3p homologs. The

orf19.517/orf19.8148 (318 nucleotides) has been designated *HAP31* and orf19.4647/orf19.12116 (990 nucleotides) designated as *HAP32* (Johnson *et al.* 2005). A computational approach to identifying introns determined that *HAP32* contains an intron of 119 base pairs in length (Mitrovich *et al.* 2007). *S. cerevisiae hap3*Δ mutants are defective in respiratory growth and are therefore unable to live on non-fermentable carbon sources (Hahn and Guarente 1988). To demonstrate that both *HAP31* and *HAP32* are functional homologs of the *S. cerevisiae HAP3*, they were expressed in a *S. cerevisiae hap3*Δ strain. SHY40 (*hap3*Δ) was transformed with either a low-copy (ARS/CEN) or high-copy (2 micron) vector containing *S. cerevisiae HAP3*, *C. albicans HAP31* or *HAP32* and as controls, *S. cerevisiae* BWG1-7a (*ScHAP3*) and SHY40 (*Schap3*Δ) were transformed with an ARS/CEN vector only. Transformants were selected on SC-Ura medium and subsequently replica plated on YPD or yeast extract-peptone-lactate (YPL) with glucose or lactate as the sole the carbon source and incubated at 30ºC (Figure 3-1).

Figure 3-1. *C. albicans* Hap3p homolog are capable of rescuing the respiration deficiency seen in *S. cerevisiae hap3*Δ mutant. *S. cerevisiae* strains containing the indicated genes on either a low copy (A/C) or high copy (2µm) plasmid were grown on either YPD and YPL to evaluate complementation of the respiratory deficiency.

The *hap3*Δ mutant was unable to grow on lactate as a carbon source, but both the *C. albicans HAP31* (McNabb, data not shown) and *HAP32* (Figure 3-1) complemented the respiratory phenotype of the *S. cerevisiae hap3Δ* strain when carried on either a low or high-copy number plasmid.

***hap3.2*Δ homozygous mutant has reduced growth on low iron media**. It has been shown that both *hap2*Δ and *hap5*Δ homozygous mutants are unable to grow in low iron conditions. With two identified genes encoding *HAP3*-like proteins that functionally complement *S. cerevisiae* it seemed likely that a gene knockout of at least one of these *HAP3* genes would share the iron deficiency phenotype of the *hap2*Δ and *hap5*Δ mutants. To determine whether either of the *C. albicans* Hap3p's was involved in growth under iron deficient conditions, cells were plated on YPD or YPD containing 0.10 or .25 mM bathophenanthroline disulfate (BPS) and grown for 3 days at 30ºC (Figure 3-2). The *hap31*Δ homozygous mutant showed no growth defect on iron-deficient medium; however, the *hap32*Δ mutants grew slower in the presence of the iron chelator BPS. The addition of heme to the YPD + BPS rescued the slow growth of the *hap3.2*Δ homozygous mutant, similar to the data observed with the *hap2Δ* and *hap5Δ* mutants. The *hap31Δ* and *hap32Δ* heterozygous strains as well as the homozygous mutants rescued by addition of the appropriate wild-type gene and were comparable to the growth of the wild-type strain (data not shown).

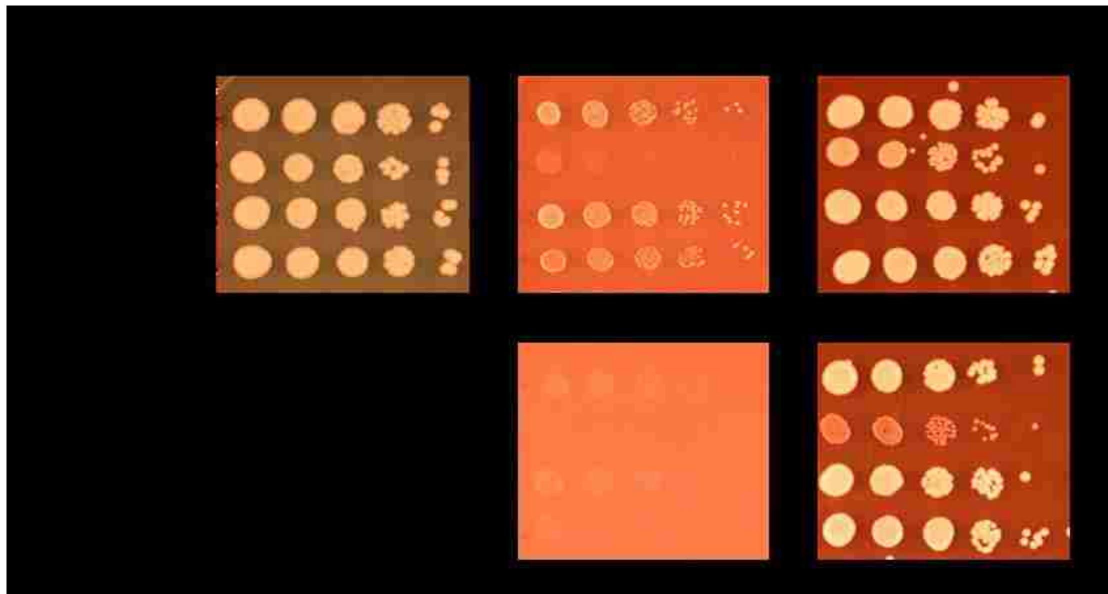

Figure 3-2. *hap32*Δ/*hap32*Δ mutant is affected by iron poor growth conditions. Strains with the indicated genotypes were grown overnight in YPD with 200μM BPS and spot plated onto YPD, YPD + .01 mM BPS, YPD + .25 mM BPS, YPD + .01 mM BPS + 50μM Heme, YPD + .25 mM BPS + 50μM Heme and grown at 30ºC for 3 days.

The CCAAT-binding complex involving Hap31p is functional in iron replete conditions, while Hap32p is important under iron poor conditions. Since there were two *HAP3*-like genes identified in *C. albicans* and the deletion of one of those genes resulted in slower growth on iron-deficient medium, it was suggestive that they might play differing roles in iron replete versus iron poor growth environment. Since, neither of the *hap3*-like mutants showed a complete loss of growth under iron-deficient conditions like the *hap2Δ* and *hap5Δ,* it was possible that the two gene products were, at least, partially compensating for each other in some way. To test this hypothesis, crude extracts were prepared from the appropriate strains grown in YPD or YPD with 200µM BPS media. An electrophoretic mobility shift assay was performed with crude extracts prepared from wild-type, *hap2Δ/hap2Δ*, *hap5Δ/hap5Δ*, *hap31Δ/hap31Δ*, and *hap32Δ/hap32Δ* strains.

As shown in Figure 3-3, there are 5 unique complexes visible in the gel shift based on the strain and the growth medium that was utilized. In YPD, the wild-type strain contained complexes I and II with complex II being the most abundant. Since both complexes are lost in the *hap31Δ/hap31Δ* mutant it seems likely that both complexes contain Hap31p. In both the *hap2Δ* and *hap5Δ* homozygous mutants, DNA binding has been abolished as previously shown (Chapter 2). In the *hap31Δ/hap31Δ* mutant, complex IV and V containing Hap32p are barely visible whereas, in the *hap32Δ/hap32Δ* mutant the abundance of complex I and II, containing Hap31p, are comparable to the wild-type strain.

In YPD+BPS, the wild-type strain contains complexes II, III, IV and V with complex V the most abundant (Figure 3-3). In both the *hap2Δ* and *hap5Δ* homozygous mutant lanes, DNA binding has been abolished as previously shown (Chapter 2). In the *hap31Δ/hap31Δ* mutant complex III and V are bound with complex V the most abundant, in the *hap32Δ/hap32Δ* mutant complex I and II are visible with complex II being the most abundant.

The DNA binding activity of the Hap complex does not require the presence of iron as a cofactor. This observation is based on the fact that crude protein lysates were prepared in the presence of EDTA; therefore, iron in the reaction would have been chelated. Moreover, electrophoretic mobility shift studies done in the presence of excess iron showed identical DNA

binding protein complexes as compared to reactions done in the presence of EDTA. Taken together, these data suggest that iron is not an essential cofactor for DNA binding activity (data not shown).

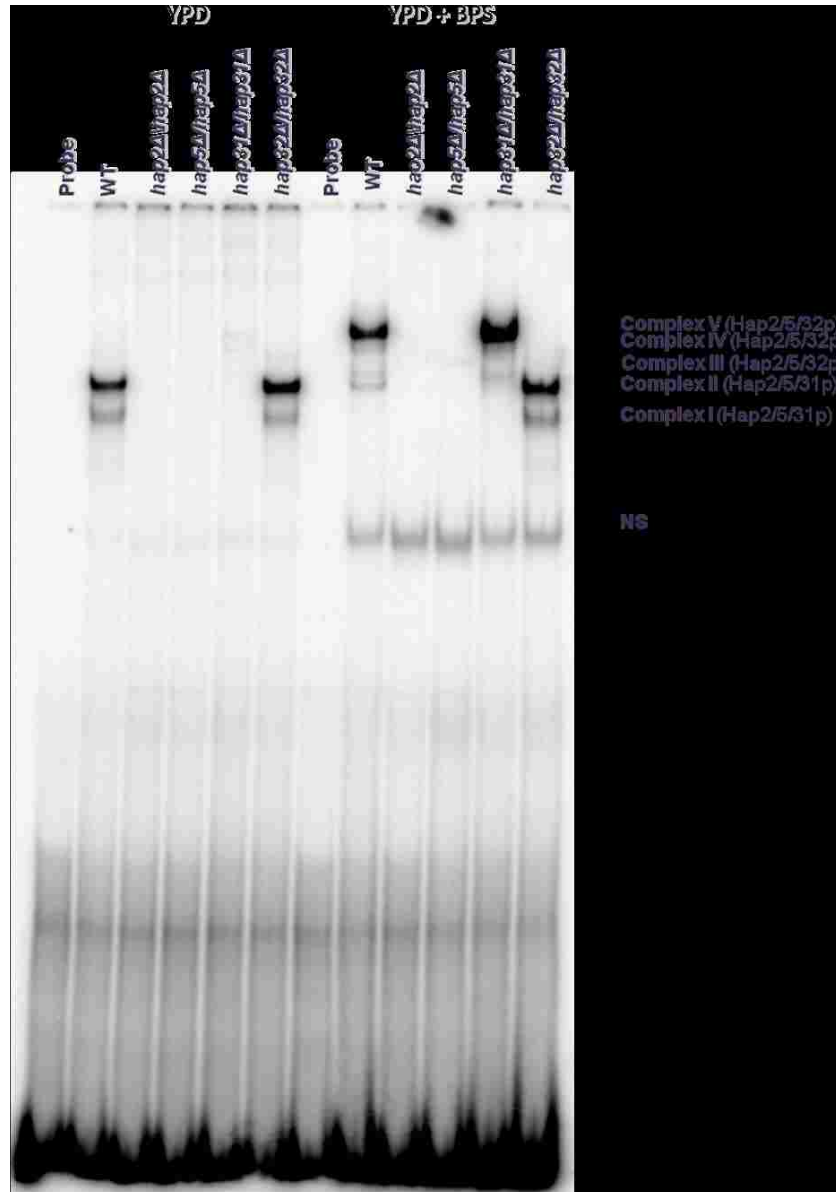

Figure 3-3. The CCAAT-binding complex differs based on environmental conditions. Crude extracts were prepared from strains grown in either YPD or YPD + BPS (low iron) medium. Electrophoretic mobility shift assay was performed with crude extracts prepared from wild type, hap5Δ/hap5Δ, hap2Δ/hap2Δ, hap31Δ/hap31Δ, and hap32Δ/hap32Δ strains as indicated. The putative identity of each complex is shown at the right of the figure. NS - Nonspecific Binding.

All the CCAAT-binding complexes contain Hap2p and Hap5p as seen by the loss of DNA binding in the mutant strains (Figure 3-3). Since the *hap31Δ/hap31Δ* mutant lacks Hap31p and

DNA binding still occurs, then Hap32p must be forming a complex with Hap2p and Hap5p, albeit very weak; likewise in the *hap32Δ/hap32Δ* mutant where Hap31p is forming a complex with Hap2p and Hap5p that replaces the loss of Hap32p Complex I and II are bound in the *hap32Δ/hap32Δ* mutant whereas complex III, IV, and V are bound in the *hap31Δ/hap31Δ* mutant. Therefore, we conclude that complex I and II are most likely to be formed by Hap2p/Hap5p/Hap31p. Using the same logic, complexes III, IV, and V are likely to be composed of Hap2p/Hap5p/Hap32p. Therefore, in the absence of Hap31p, the DNA binding complex that contains Hap32p is a weak replacement for Hap31p and in the absence of Hap32p, the DNA binding complex that contains Hap31p replaces Hap32p. In the latter case, Hap31p seems to be a better substitute for Hap32p than the reverse situation.

In the wild-type strain, complex II is the most abundant CCAAT complex observed in YPD medium; however, in YPD+BPS, complex V is the most abundant. Therefore, Hap31p is a part of the bound Hap complex in iron replete conditions (YPD); whereas, Hap32p is a component of the complex under iron-limiting growth conditions (YPD+BPS). Therefore, Hap31p and Hap32p are able to interchange in the complexes based on environmental factors.

***Hap31* and *Hap32* RNA transcripts are differentially expressed in response to environmental growth conditions.** To determine whether the mRNA expression of *HAP31* or *HAP32* was affected by the presence or absence of iron, northern blot analysis was performed. The wild type *C. albicans* strain was grown in either YPD or YPD with 200µM BPS, total RNA was isolated, separated by agarose gel electrophoresis, and northern blots performed as described in Material and Methods.

Both *HAP31* and *HAP32* mutants showed differences in expression in iron replete versus iron poor conditions (Figure 3-4 A). In the presence of iron (YPD) *HAP31* is highly expressed, but there was no expression in the absence of iron (YPD+BPS). *HAP32* shows two transcripts, a larger transcript that was weakly expressed when cells were grown in YPD medium and a more abundant, yet smaller transcript was observed when cells were grown in YPD+BPS. Interestingly the shift in the size of the *HAP32* mRNA transcript corresponds to the approximate size of the putative intron in *HAP32* (Mitrovich *et al.* 2007). Thus, it is plausible that the *HAP32* mRNA is

poorly expressed on YPD and is unspliced; whereas, the expression of the mature HAP32 transcript is transcriptionally induced in iron-limiting medium and is efficiently spliced. Importantly, the mRNA expression pattern observed with *HAP31* and *HAP32* corresponds with the gel shift studies shown in Figure 3-3; whereby the complex including the Hap31p subunit is present in iron replete conditions, and the Hap32p is present in iron-limiting conditions. Northern blot analysis performed with total RNA isolated from the *hap31Δ* or *hap32Δ* homozygotes grown in YPD or YPD + BPS demonstrated that *HAP31* is upregulated in a *hap32*Δ homozygous mutant grown in YPD + BPS medium (Figure 3-4 B) which is consistent with the gel shift data demonstrating that Hap31p functionally substitutes for Hap32p under iron-limiting conditions in a hap32Δ homozygote. .

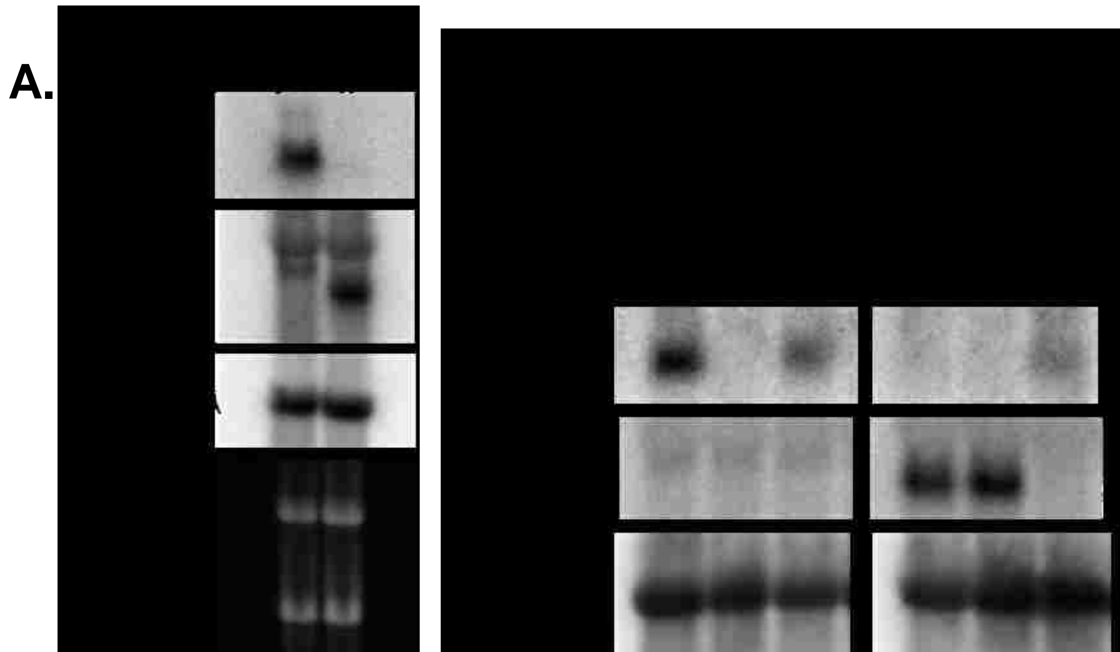

Figure 3-4 *HAP31* and *HAP32* are differentially expressed in response to iron availability. Northern blot analysis of total RNA isolated from *C. albicans* wild-type, *hap31Δ/hap31Δ,,* and *hap32Δ/hap32Δ* strains grown at 30ºC in YPD or YPD with 200µM BPS, as indicated. The membrane was hybridized with radiolabeled probes specific for *HAP31* and *HAP32* as indicated. 26S rRNA was used to normalize results.

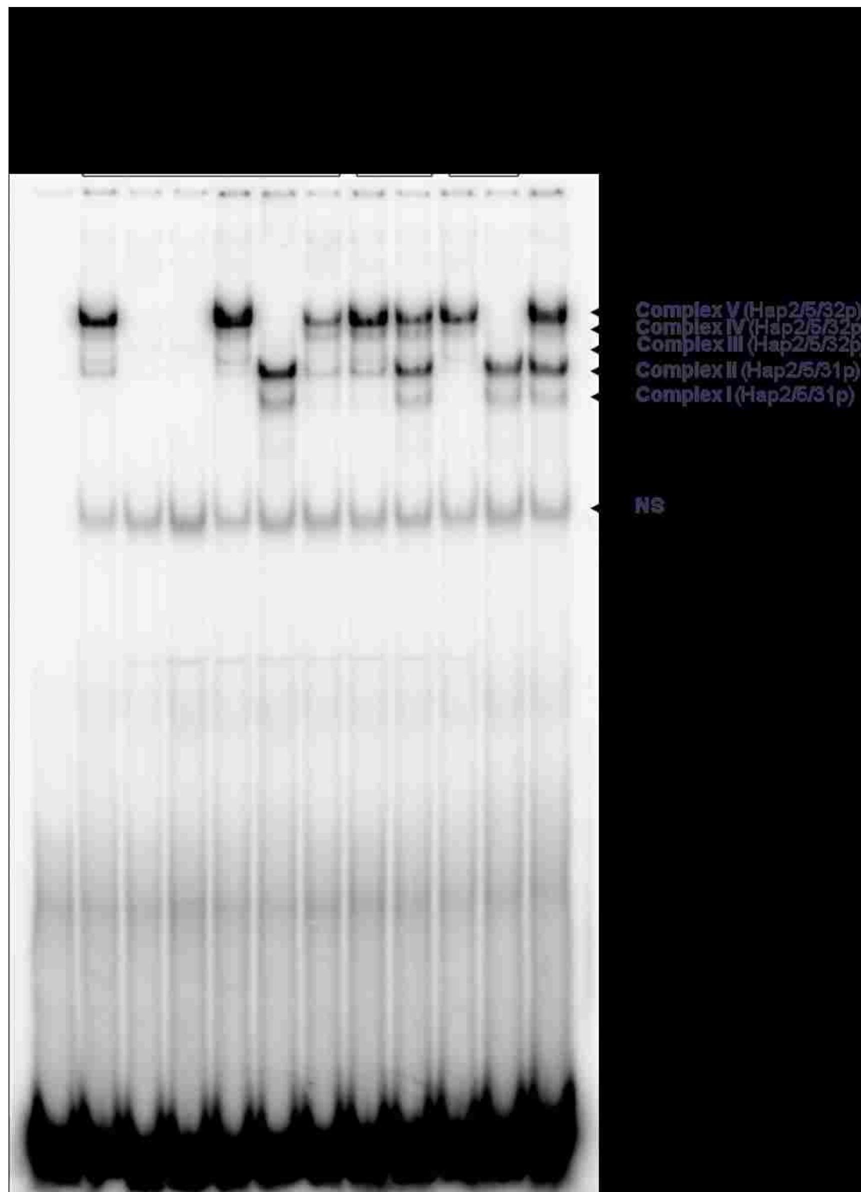

Figure 3-5. Hap31p/Hap5p and Hap32p/Hap5p form stable heterodimers. Mobility shift assay was performed with crude extracts prepared from strains grown in YPD + BPS. The *C. albicans* strains used included the wild-type (WT), *hap5Δ*/*hap5, hap2Δ*/*hap2Δ, hap31Δ/ hap31Δ,* and *hap32Δ/hap32Δ.* The crude extracts were mixed as indicated. The various CCAAT complexes are indicated at the right. NS - Nonspecific Binding.

There is no interchange between CCAAT complexes containing Hap31p and Hap32p.

Previous studies with the mammalian homologs of Hap3p and Hap5p, termed NF-YB and NFY-C, respectively, demonstrated that these subunits formed a stable heterodimer (Baxevanis *et al.* 1995, Zemzoumi *et al.* 1999, Romier *et al.* 2003). To determine whether *C. albicans* Hap31p and Hap32p subunits form a stable dimer with Hap5p, a mobility shift assay was performed with crude

extracts prepared from the wild-type, *hap5Δ/hap5Δ*, *hap2Δ/hap2Δ*, *hap31Δ/ hap31Δ*, and *hap32Δ/hap32Δ* strains grown under iron-limiting conditions. As indicated in Figure 3-5, the crude extracts were mixed in various combinations before the addition of the CCAAT site containing DNA probe.

In the wild-type strain grown in YPD + BPS, complex V is the predominant CCAAT-binding complex with a slight amount of complex II and III (Figure 3-5). In the *hap2*Δ and *hap5*Δ homozygous mutants, DNA binding was completely abolished as previously shown (Chapter 2). In the *hap31*Δ*/hap31*Δ mutant, complexes III and IV are weakly visible with complex V being the predominant form. In the *hap32*Δ*/hap32*Δ mutant, complex I and II are visible with complex II being the most abundant. These data are consistent with those previously shown (Fig 3-3). As expected, the combination of crude extracts from the *hap2Δ/hap2Δ* and *hap5*Δ*/hap5*Δ mutants restored the formation of the CCAAT complexes observed in the wild-type strain (Figure 3-5). When extracts from the *hap2Δ/hap2Δ* mutant are combined with the extracts from either the *hap31Δ* or *hap32Δ* homozygous mutant the various CCAAT-binding complexes were restored. In contrast, when the extracts from *hap5Δ/hap5Δ* mutant were combined with the extracts from the *hap31Δ* or *hap32Δ* homozygous mutants, the anticipated pattern of CCAAT binding complexes was not restored. These data suggest that Hap5p from each individual extract is unavailable to mix with the alternate Hap3p subunit from the other extract. One explanation for this observation would be that Hap5p is already bound to the particular Hap3p subunit present in the strain. These data suggests that Hap5p may form a stable heterodimeric complex with Hap31p or Hap32p.

Denaturation allows interchange of Hap5p/Hap31p and Hap5p/Hap32p. The data described above suggested that Hap31p/Hap5p or Hap32p/Hap5p dimers may form stable complexes that do not interchange in solution. To test this hypothesis, crude extracts were initially denatured with guanidine hydrochloride, then mixed and allowed to renature. If Hap31p/Hap5p and Hap32p/Hap5p formed stable dimers, under denaturing/renaturing conditions, one should see Hap31p and Hap32p exchange in the complexes. Thus, mobility shift assay was performed with

crude extracts prepared from strains grown in YPD + BPS. Extracts from the wild-type, *hap5Δ/hap5Δ*, *hap31Δ/ hap31Δ*, and *hap32Δ /hap32Δ* strains were combined in equal concentrations as indicated in Figure 3-6 and treated with 3M guanidine hydrochloride and placed at 65°C for 30 min. The samples were subsequently dialyzed overnight in DNA binding buffer to permit renaturation of the proteins and the formation of the CCAAT-binding complexes.

Figure 3-6. Denaturation-renaturation allows subunit exchange between CCAAT-binding complexes. Mobility shift assay was performed with treated crude extracts prepared from wild-type (WT), *hap5Δ*/*hap5Δ, hap31Δ/ hap31Δ,* and *hap32Δ/hap32Δ* strains. Extracts were combined in equal concentrations and treated with 3M guanidine hydrochloride at 65°C for 30 min. The samples were then dialyzed overnight in DNA binding buffer to renature the samples. Extracts were mixed as indicated and the various CCAAT-binding complexes are indicated at the right. NS - Nonspecific Binding.

As previously shown, the WT strain grown in YPD+BPS consists of complexes II, III, IV, and V with complex V the most abundant (Figure 3-6). In the *hap5*Δ homozygous mutant, DNA binding is abolished as previously shown (Chapter 2). In the *hap31Δ/hap31*Δ mutant complex III, IV and V are bound with complex V the most abundant, while in the *hap32Δ/hap32*Δ mutant complex I and II are visible with complex II being the most abundant.

Denaturation/renaturation of the wild-type extract did not change the binding pattern. When extracts from the *hap5Δ/hap5*Δ mutant are added to extracts from the *hap31Δ/hap31*Δ mutant, there is no change in complexes; however, it is important to note that Hap31p is not abundantly present in cells grown under iron-limiting conditions. More importantly, when extracts from the h*ap5Δ/hap5*Δ mutant are added to extracts from the *hap32Δ/hap32*Δ mutant, complexes I, II, IV and V are formed. Thus, there was interchange between the Hap5p found the in *hap32Δ/hap32*Δ extract with the Hap32p found in the h*ap5Δ/hap5*Δ extract. Thus, the denaturation-renaturation studies demonstrated that the exchange of Hap3p subunits could occur following denaturation. These data support the hypothesis that Hap5p is bound to either Hap31p or Hap32p to form a stable heterodimer.

Discussion

In *S. cerevisiae*, each component of the Hap complex is encoded at a single genetic locus. This is also true in humans and mice (Maity and de Crombrugghe 1998). However, in *Arabidopsis thaliana*, there have been 36 genes identified so far that encode subunits of the CCAAT-binding factor (Riechmann and Ratcliffe 2000, Gusmaroli *et al.* 2001, Gusmaroli *et al.* 2002, Siefers *et al.* 2008). While not as complicated as *A. thaliana*, *C. albicans* encodes two distinct *HAP3* subunits that leads to an increase in the number of CCAAT-binding complexes.

C albicans possesses two homologs of *HAP3*: *HAP31* and *HAP32*. These homologs are both capable of rescuing an *S. cerevisiae hap3*Δ respiratory deficient mutant. Electrophoretic mobility shift assays performed with crude extracts from *hap31Δ/hap31Δ* or *hap32Δ/hap32Δ* mutants show loss in DNA binding activity for non-overlapping CCAAT-binding complexes.

Therefore, Hap31p and Hap32p interact with Hap2p and Hap5p to form distinct DNA-binding protein complexes.

It has been previously shown that both the *hap2Δ/hap2Δ* (Chapter2) and the *hap5Δ/hap5Δ* mutants are unable to grow on low iron media, but the addition of hemin rescues this phenotype. While severity of the phenotype is not the same with the *HAP3* subunits, the *hap32Δ/hap32Δ* mutant did exhibit a slight defect in growth under iron-limiting conditions which was rescued by the addition of heme, whereas the *hap31Δ/hap31Δ* mutant shows no defect. When crude extracts were prepared from each mutant and examined by electrophoretic mobility shift assay, the iron defect was more profound. Iron availability affects the expression of the *HAP3* homologs. *HAP31* is expressed only in YPD and *HAP32* expression increases in BPS. *HAP32* also exhibits an alternative transcript that would correspond to a spicing of the computationally determined intron under low iron conditions.

Hap31p or Hap32p are differentially associated with the CCAAT-binding factor heterotrimer based on the amount of iron in the medium. Hap32p is associated in the absence of iron, whereas Hap31p is associated in iron replete conditions (Figure 3-7). When either Hap31p or Hap32p is deleted, the Hap complex will still bind DNA using the alternate Hap3 protein. This provides the explanation as to why the iron phenotype was not as strong with the *hap32Δ* homozygote as observed with the *hap2Δ/hap2Δ* and the *hap5Δ/hap5Δ* mutants. In iron-limiting conditions such as YPD with BPS, the DNA-binding complex consist of Hap2/5/32p bound to DNA. In iron replete conditions, Hap2/5/31p is bound to DNA. However, when the preferred Hap3 subunit is missing, the alternative Hap3 subunit is able to interact with Hap2p and Hap5p to create a DNA-binding complex that is at least partially active.

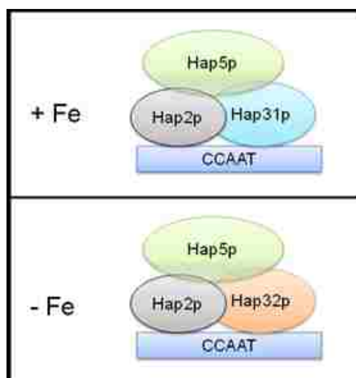

Figure 3-7. Proposed model for the CCAAT-binding complexes in *C. albicans*. In the absence of iron, the CCAAT-binding complex consists of Hap2p, Hap5p, and Hap32p. In the presence of sufficient iron, the CCAAT-binding complex consists of Hap2p, Hap5p, and Hap31p.

To determine whether the two distinct Hap3p proteins could freely interchange with Hap2p and Hap5p, the crude extracts were mixed prior to the addition of probe. It has been shown that the various subunits of the Hap complex are stable in the crude extracts from both the *hap2*Δ and *hap5*Δ homozygous mutant strains (see Figure 3-5 and Figure 3-8). Therefore, it was expected that the deletion of the genes encoding Hap31p or Hap32p would be provided in both the *hap2*Δ and *hap5*Δ homozygous mutant extracts. However, only the addition of *hap2*Δ extracts to the extracts of *hap31*Δ*/hap31*Δ or *hap32*Δ*/hap32*Δ mutant strains allowed the formation of missing complexes. Thus the Hap5p subunit was unable to interchange between individual extracts suggesting that it was irreversibly bound to other proteins. Given that previous studies demonstrated that the mammalian homologs of Hap3p and Hap5p formed a stable heterodimer (Baxevanis *et al.* 1995, Zemzoumi *et al.* 1999, Romier *et al.* 2003), it was reasonable to hypothesize that this was also true in C. *albicans*.

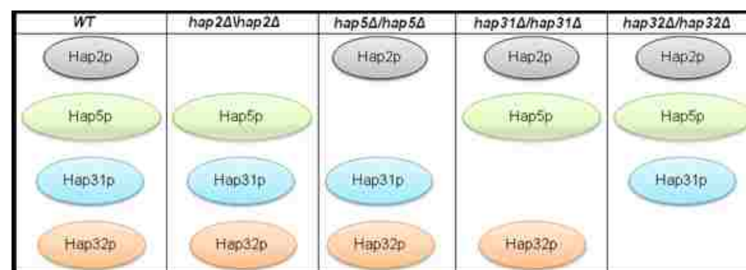

Figure 3-8. Availability of proteins in each *C. albicans* strain.

It was found that denaturing extracts and mixing prior to renaturing allowed the interchange of the Hap31p/Hap5p and Hap32p/Hap5p dimers. Thus, there appears to be no exchange of the HAP3p subunits when Hap5p is present. This leads to the model of *C. albicans* CCAAT-binding complex formation that is formulated based on the data previously published regarding the mammalian Hap homologs (Maity *et al.* 1992, Baxevanis *et al.* 1995, Sinha *et al.* 1995, Sinha *et al.* 1996, Zemzoumi *et al.* 1999, Romier *et al.* 2003). The Hap31p or Hap32p subunits initially bind Hap5p to form a stable heterodimer, then Hap2p interacts with the dimer to form an active DNA-binding complex (Figure 3-9).

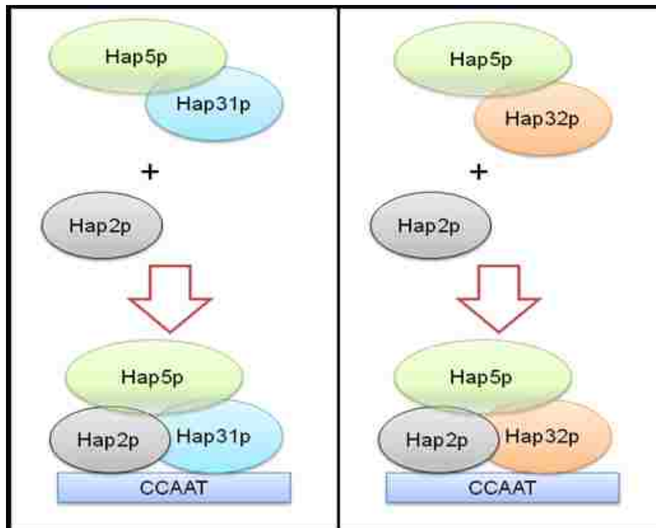

Figure 3-9. Proposed model for CCAAT-binding in *C. albicans*. Hap31p/Hap5p or Hap32p/Hap5p form a stable heterodimer that subsequently interacts with Hap2p before DNA binding at the CCAAT consensus site.

In *S. cerevisiae* Hap2p, Hap3p and Hap5p are required simultaneously to assemble and bind DNA (McNabb and Pinto 2005). Whereas with human NFY, NF-YB (Hap3p) and NFY-C (Hap5p) bind to form a stable dimer via a protein structure similar to a histone fold motif (Baxevanis *et al.* 1995, Zemzoumi *et al.* 1999, Romier *et al.* 2003). The heterodimer then serves as a surface for interaction with NF-YA (Hap2p) which assembles and binds DNA with a high affinity (Maity *et al.* 1992, Sinha *et al.* 1995, Sinha *et al.* 1996).

The *C. albicans* Hap complex possesses a duality that is not seen in *S. cerevisiae*. The duality found in the Hap3p homologs allows the opportunistic pathogen to respond to iron replete verses iron-limiting environmental conditions. Hap31p is bound to DNA as part of the Hap complex under iron replete conditions, whereas Hap32p is bound in iron-limiting conditions. It remains to be determined why *C. albicans* would need two distinct Hap3p subunits; however, one possibility is that Hap31p recruits a particular Hap4p subunit, while Hap32p recruits a different Hap4p. Previous studies have suggested that there are three different genetic loci that encode Hap4p homologs in *C. albicans*, and these Hap4p subunits may dictate how a gene is regulated in response to environmental growth conditions. Further studies will be required to understand how the various Hap4p subunits interact with the CCAAT-binding complexes containing either Hap31p or Hap32p to facilitate the correct program of gene expression.

Mammalian host systems sequester iron in such a way that there is virtually no free iron available (Ratledge and Dover 2000, Weinberg and Miklossy 2008). In low iron conditions, pathogens have developed many ways to capture iron from the host and survive in this environment. Iron availability has been shown to be a common virulence factor in pathogens (Howard 2004, Weinberg 2008) and the duality of the *C. albicans* Hap complex seems to be an adaptation to the host environment.

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Chapter 4. Deletion of *HAP31, HAP32* and *HAP31/HAP32*: the effect on the phenotype, DNA binding and gene expression in *Candida albicans*.

Introduction

Candida albicans is an opportunistic pathogen that is normally a part of the mammalian microflora and is found in more than 70% of the healthy population (Ruhnke and Maschmeyer 2002). Host immune system suppression allows *C. albicans* to invade and colonize host tissues, leading to significant morbidity and mortality (Odds 1988, Pittet *et al.* 1997). The attributed mortality of blood stream infections to *C. albicans* is estimated to be 30% (Odds 1988).

The CCAAT-binding complex is a heterooligomeric transcription factor that is evolutionarily conserved among eukaryotes (Mantovani 1999). This complex has been implicated in iron acquisition and glucose utilization in *C. albicans* (Lan *et al.* 2004, Hortschansky *et al.* 2007, Baek *et al.* 2008, Mercier *et al.* 2008). In humans, 30% of promoters include a CCAAT box, making it one of the most ubiquitous elements (Bucher and Trifonov 1988, Bucher 1990).

The *Candida albicans* CCAAT-binding complex is composed of the subunits Hap2p, Hap3p, Hap5p and Hap4p. Hap2/3/5p compose the DNA binding component of this complex. So far it has been shown that *hap2Δ/hap2Δ* and *hap5Δ/hap5Δ* mutants are unable to grow in iron poor conditions. The mutants also undergo hyperfilamentation when grown on media that lack glucose, and the deletion of either Hap2p or Hap5p abolishes DNA binding (Johnson *et al.* 2005).

In *S. cerevisiae* Hap2p, Hap3p and Hap5p are required simultaneously to assemble and bind DNA (McNabb and Pinto 2005). In humans the transcription factor is assembled in a stepwise fashion. NF-YB (HAP3) and NFY-C (HAP5) bind to form a stable dimer (Baxevanis *et al.* 1995, Zemzoumi *et al.* 1999, Romier *et al.* 2003), which then serves as a surface for interaction with NF-YA (HAP2), and the assembled complex binds DNA with a high affinity (Maity *et al.* 1992, Sinha *et al.* 1995, Sinha *et al.* 1996). *Arabidopsis thaliana*'s Hap complex consists of a combination of 36 genes (Riechmann and Ratcliffe 2000, Gusmaroli *et al.* 2001, Gusmaroli *et al.* 2002, Siefers *et al.* 2008) that can be divided into Hap2p-like, Hap3p-like and Hap5p-like. These components are involved in various roles in developmental control, drought stress, and photoperiod flowering time (Siefers *et al.* 2008).

There have been two Hap3p homologs identified in *C. albicans* (Johnson *et al.* 2005). Deletion of either of these genes does not cause a drastic growth phenotype. However when

crude extracts are examined by electrophoretic mobility shift assays, different CCAAT-binding complexes are bound to DNA based on the Hap3p homolog missing and the environmental growth conditions (Chapter3). Hap31p is the predominant CCAAT-binding subunit under iron replete conditions. In an iron-limiting growth environment, Hap32p is the predominant subunit and binds DNA under these conditions (Chapter3). In addition, the *HAP31* mRNA transcript is expressed in iron replete conditions, while *HAP32* mRNA is expressed under iron-limitation.

To understand the duality of the *HAP3* homologs in *C. albicans*, both have been deleted to generate a double mutant. This mutant is phenotypically the same as a *HAP2* or *HAP5* null mutant, suggesting that either Hap31p or Hap32p is required for CCAAT-binding factor assembly.

Materials and Methods

Yeast strains and growth conditions

Yeast strains used in these studies are listed in Table 4-1. Strains were cultured in yeast extract-peptone dextrose (YPD) medium (Guthrie and Fink 1991) supplemented with 80mg/L uridine as appropriate and incubated at 30ºC overnight. For selection of yeast transformants, synthetic complete media lacking the appropriate auxotrophic requirement was used (Guthrie and Fink 1991).

Media for hyphal analysis, including Lee's (Lee *et al.* 1975), modified Lee's (Liu *et al.* 1994), and Spider (Liu *et al.* 1994) medium, were prepared as previously described. Serum media consisted of YPD containing 10% newborn calf serum (Sigma). M199 medium was obtained from Gibco-BRL, containing Earle's salts and glutamine but lacking sodium bicarbonate and buffered with 150 mM HEPES adjusted to pH 4.5 or 7.5. SLAD is synthetic low ammonia medium containing 0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, 2% dextrose, and 50 µM ammonium sulfate. Yeast nitrogen base (YNB) medium was prepared using 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco). 0.1% amino acids were added using an amino acid dropout powder containing the 20 amino acids at the describe concentrations (Guthrie and Fink 1991), buffered to the required pH with 150 mM HEPES.

Glucose or lactate was added to the media at a final concentration of 2% and media was solidified with 1.5% agar as appropriate.

For low iron media, bathophenanthroline disulfate (BPS) (Sigma) was used to chelate iron. BPS was added to YPD at a concentration of 200μM in liquid media and used for overnight cultures. BPS (0.1 to 0.25mM) was added to solid YPD media. Hemin was added to solid YPD with BPS media at a concentration of 50μM.

Table 4-1. E. coli and C. *albicans* strains

Strain	Genotype	Reference
E. coli		
DH5α	*F Φ80dlacZ-M15Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(r_{K-} $_{mK+}$), deoR thi1 supE44, λ⁻ gyrA96 relA1*	
C. albicans		
BWP17	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG*	(Romier *et al.* 2003)
DMC146	*ura3Δ ::imm434/ura3Δ ::imm434 his1Δ ::hisG/his1Δ ::hisG-HIS1 arg4Δ ::hisG/arg4Δ ::hisG-ARG4-URA3*	(Johnson *et al.* 2005)
DMC245	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP2/hap2Δ::URA3*	
DMC249	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap2::URA3/hap2Δ::HIS1*	
DMCr29	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP2-ARG4 hap2::URA3/hap2Δ::HIS1*	
DMC120	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP5/hap5Δ::URA3*	(Johnson *et al.* 2005)
DMC117	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	(Johnson *et al.* 2005)
DMC126	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP5-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	(Johnson *et al.* 2005)
LBC001	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap31Δ::URA3/hap31Δ::HIS1*	This study
LBC002	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap32Δ::URA3/hap32Δ::HIS1*	This study
LBC003	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG hap31Δ::hisG/hap31Δ::URA3 hap32Δ::HIS1/hap31Δ::ARG4*	This Study

Construction of *C. albicans* strains.

The *hap31∆/hap31∆ hap32∆/hap32∆* double mutant strain LBC003 was generated as follows. Two consecutive rounds of transformation of the parent strain BWP17 were performed using the *hap31:URA3-hisG* cassette as described (Fonzi and Irwin 1993). Then two additional consecutive rounds of transformation were performed using the *hap32::URA3* and *hap32::HIS1* disruption cassettes (McCue and McNabb, unpublished). The cassettes were released from the plasmids by digestion with BamHI/HindIII and introduced into BWP17 using a lithium acetate transformation kit (QBiogene, Inc.), and transformants were selected on the appropriate selection

medium. To verify gene deletion, genomic DNAs were isolated from multiple transformants as described previously (Hoffman and Winston 1987), and PCR was used to confirm the appropriate recombination events. All strains used for phenotypic analyses were rescued to prototrophy as previously described (Johnson *et al.* 2005) , and subsequently confirmed by growth on synthetic minimal medium.

Yeast extract preparation and electrophoretic mobility shift assays.

Protein extracts were prepared as previously described (Johnson *et al.* 2005). The CCAAT oligonucleotide probe was designed similar to that described previously (Hahn and Guarente 1988) and is based on the sequence of CCAAT site from *CYC1* of *C. albicans*. The DNA probe was end-labeled by the Klenow fragment with [α-^{32}P]dATP (Amersham Corp.).

All binding reactions contained 20 to 30 µg of cell extract in DNA-binding buffer (20 mM HEPES-NaOH pH 8.0, 100 mM KCl, 1 mM EDTA, 10% glycerol), 0-1 µg of denatured salmon sperm DNA, 0-1 µg of poly(dI-dC), and 0.5 to 1.0 ng of radiolabeled probe in a final reaction volume of 20 µl. Reactions were incubated at room temperature for 30 to 45 min, and the protein-DNA complexes were resolved by gel electrophoresis (4 h at 300 V) on 5% polyacrylamide gels (acrylamide:bisacrylamide ratio, 29:1) in 0.5× Tris-borate-EDTA at 4°C. After electrophoresis, the gels were fixed, dried, and visualized with a Molecular Dynamics PhosphorImager.

Microscopy and imaging.

Photographs of colony morphology were taken with a Nikon Biaphot microscope fitted with a high-resolution charge-coupled device camera and the AutoMontage imaging software package (Syncroscopy, Frederick, MD). Photographs of individual cells were done with a Zeiss Axioplan 2 microscope fitted with a high-resolution charge-coupled device camera and AutoMontage imaging software. Photographs of individual colonies were representative of the total population. The figures were prepared using Adobe Photoshop 7.0 and Microsoft PowerPoint 2007.

Northern blot analysis.

C. albicans strains were grown to saturation in YPD medium and subsequently inoculated into the indicated medium and grown until OD>0.6 at 30°C. The cells were harvested by centrifugation, and total RNA was prepared by the glass bead-acid phenol method as previously described (Ausubel *et al.* 1994). Approximately 20 µg of each total RNA sample was loaded, separated by formaldehyde-1% agarose gel electrophoresis, and transferred to GeneScreen Plus membranes (Dupont-NEN Research Products) according to the manufacturer's protocol. The membranes were hybridized and washed under standard high-stringency conditions (Sambrook *et al.* 1989).

The *CYC1*, *COX5*, and 26S rRNA probes for hybridization were obtained by PCR amplification from *C. albicans* genomic DNA using the primer pairs oDM0341/oDM342, oDM0449/oDM0450, and oDM0459/oDM0460, respectively. The probes were purified by agarose gel electrophoresis and GeneClean (Qbiogene, Inc.) and subsequently radiolabeled with [α-^{32}P]dATP (Amersham) by use of a random primer labeling kit (U.S. Biochemicals) according to the manufacturer's protocol. The transcript levels were quantified on a Molecular Dynamics PhosphorImager.

Table 4-2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5' – 3')
oDM0341………………	5′-GGCCGAATTCGGTGCCACTTTATTTAAAACTAGATG-3′
oDM0342………………	5′-GGCCGGATCCGCTTTCTTCAAATAAGTAACTAAATCG-3′
oDM0449………………	5′-GAGATCATTACAAAGAGCTGCCACTAAAGCC-3′
oDM0450………………	5′-GGAGTTAATTCTTGCCATGGTAATTCC-3′
oDM0459………………	5′-TAGCGGTTCTGACGTGCAAATCGATCGTCG-3′
oDM0460………………	5′-ACTAACACCTTTTGTGGTGTCTGATGAGCG-3′

Results

Deletion of *HAP31/HAP32* abolishes CCAAT-binding activity in *C. albicans*. *C. albicans* has two distinct Hap3p proteins which functionally complement an *S. cerevisiae hap3*Δ mutant. Previous studies have shown that deletion of *HAP2* (Chapter 2) or *HAP5* (Johnson *et al.* 2005) causes a loss of CCAAT binding activity. To determine whether the deletion of both *HAP31* and

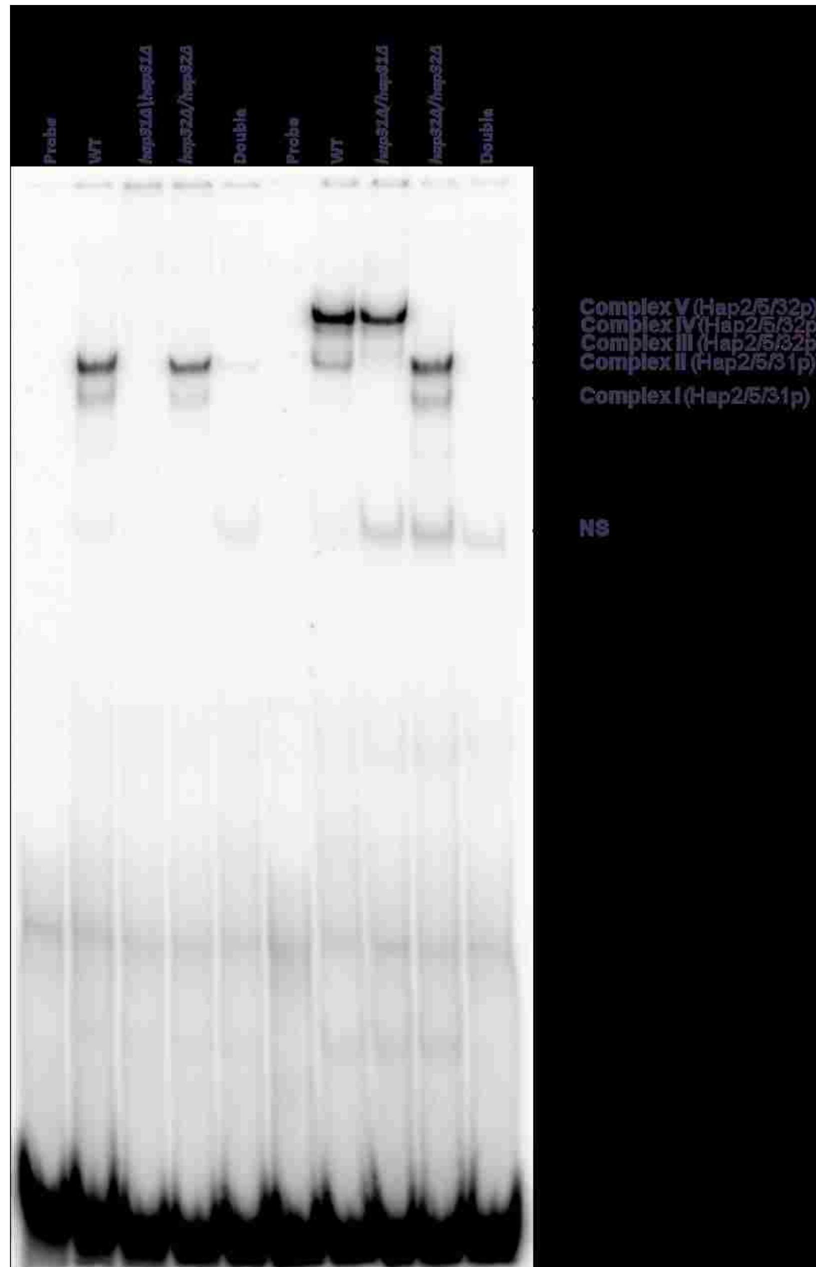

Figure 4-1. CCAAT-binding activity is abolished in a *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant. Crude extracts were prepared from cells grown in YPD or YPD with BPS. The electrophoretic mobility shift assay was performed with crude extracts prepared from the wild-type strain (WT), *hap31Δ/hap31Δ*, *hap32Δ/hap32Δ*, and the double mutant (double) as indicated. The various Hap complexes are indicated at the right. NS - Nonspecific Binding.

HAP32 would abolish CCAAT binding activity; a mutant strain containing the hap31Δ/hap31Δ hap32Δ/hap32Δ double knockout was created. The *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant was generated as outlined in Materials and Methods. The null mutations were confirmed by PCR (data not shown). *C. albicans* wild-type (WT) strain, homozygous mutants (*hap31Δ/hap31Δ* and *hap32Δ/hap32Δ*), and the double deletion (*hap31Δ/hap31Δ hap32Δ/hap32Δ*) strains were initially used to determine the effect of *hap31Δ/hap31Δ hap32Δ/hap32Δ* deletion on DNA binding. The strains were grown in YPD or YPD with 200μM BPS at 30ºC to an OD_{600} of ~1. The cells were harvested and cell extracts were prepared as described in Materials and Methods. To assess CCAAT binding activity, a 37-bp double stranded DNA oligonucleotide derived from *C. albicans CYC1* CCAAT sequence was used.

With strains grown in YPD, the wild-type strain contains complexes I and II with complex II being the most abundant (Figure 4-1). In the *hap31Δ/hap31Δ* mutant complex IV and V are weakly visible whereas, in the *hap32Δ/hap32Δ* mutant complex I and II are visible with complex II being the most abundant. In YPD+BPS, the wild-type strain contains complexes II, III, IV and V with complex V the most abundant. In the *hap31Δ/hap31Δ* mutant complex III, IV and V are bound with complex V the most abundant. In the *hap32Δ/hap32Δ* mutant complex I and II are visible with complex II being the most abundant. The double mutant showed no detectable DNA binding activity, similar to the *hap2Δ* and *hap5Δ* homozygous mutants (Figure 4-1). Therefore, like Hap2p and Hap5p, one of the Hap3p homologs must be present for DNA binding to occur.

The *C. albicans hap31Δ/hap31Δ hap32Δ/hap32Δ* mutant is phenotypically analogous to the *hap5Δ/hap5Δ* strain. To determine whether the single or double mutants displayed similar phenotypic defects to the *hap5Δ/hap5Δ* and *hap2Δ/hap2Δ* mutants, the appropriate strains were grown in several media known to induce hyphal development (Figure 4-2).

On solid media, the *hap31Δ/hap31Δ hap32Δ/hap32Δ* mutant displayed defective hyphal growth on all the media examined. The most prominent filamentation defects were seen on YPD containing 10% calf serum and SLAD. Hyperfilamentation was observed on M199 pH 7.5 media where the *hap31Δ/hap31Δ hap32Δ/hap32Δ* mutant displayed little colony growth but an

abundance of hyphal formation, analogous to the *hap5Δ/hap5Δ* strain. When the same strains were grown on M199 pH 4.5, no defect was seen other than a slight difference in colony size (data not shown). The *hap31*Δ homozygous mutant showed a more pronounced phenotype on all media, but not as obvious as the *hap31Δ/hap31Δ hap32Δ/hap32Δ* or *hap5*Δ mutants. The *hap32*Δ homozygote grew comparable to wild-type strain on all media examined. Thus, the *hap31Δ/hap31Δ hap32Δ/hap32Δ* mutant was phenotypically analogous to the *hap5Δ/hap5Δ* mutant which is consistent with the biochemical data demonstrating the complete loss of CCAAT-binding activity in this mutant.

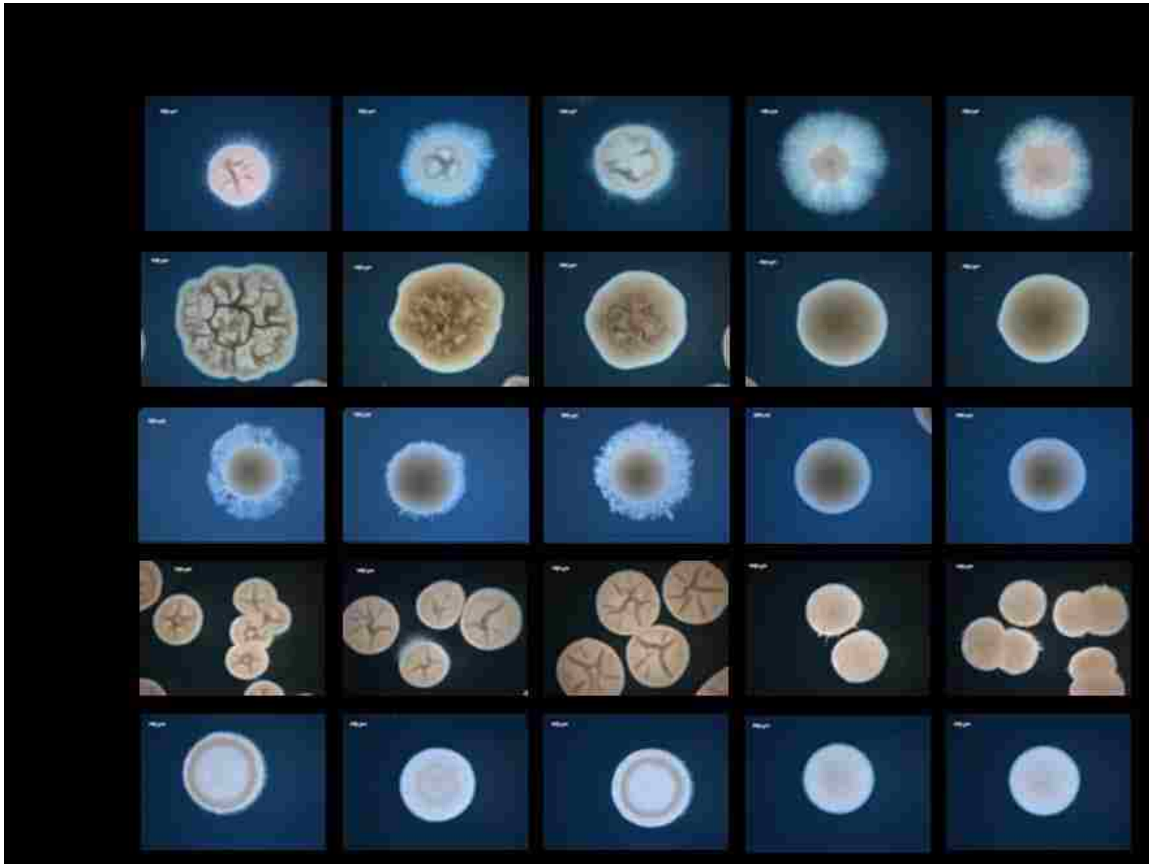

Figure 4-2. Defects in filamentation caused by loss of CCAAT-binding factor.
The wild-type strain (WT), *hap31Δ/hap31Δ*, *hap32Δ/hap32Δ*, *hap5Δ/hap5Δ*, and the *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant were grown on indicated media for 4 days at 37ºC except for Spider and SLAD media that was incubated until day 6.

Hyperfilamentation of the *hap31∆/hap31∆ hap32∆/hap32∆* mutant is carbon source-dependent. In *S. cerevisiae* and *S. pombe* the CCAAT-binding factor has been shown to regulate genes involved in respiratory metabolism (Forsburg and Guarente 1989, Zitomer and Lowry 1992, McNabb *et al.* 1997, Brakhage *et al.* 1999, Liu and Butow 1999). As previously stated, the *hap2∆* and *hap5∆* homozygous mutants were defective on hyphae-inducing media as were the *hap31∆/hap31∆* and *hap31∆/hap31∆ hap32∆/hap32∆* mutants. To further investigate the morphological defects identified on hyphae-inducing media, the double mutants were grown in yeast nitrogen base with amino acids as the sole nitrogen source and either glucose, lactate (data not shown), or no additional carbon source at pH 5.6 or 6.8 (Figure 4-3).

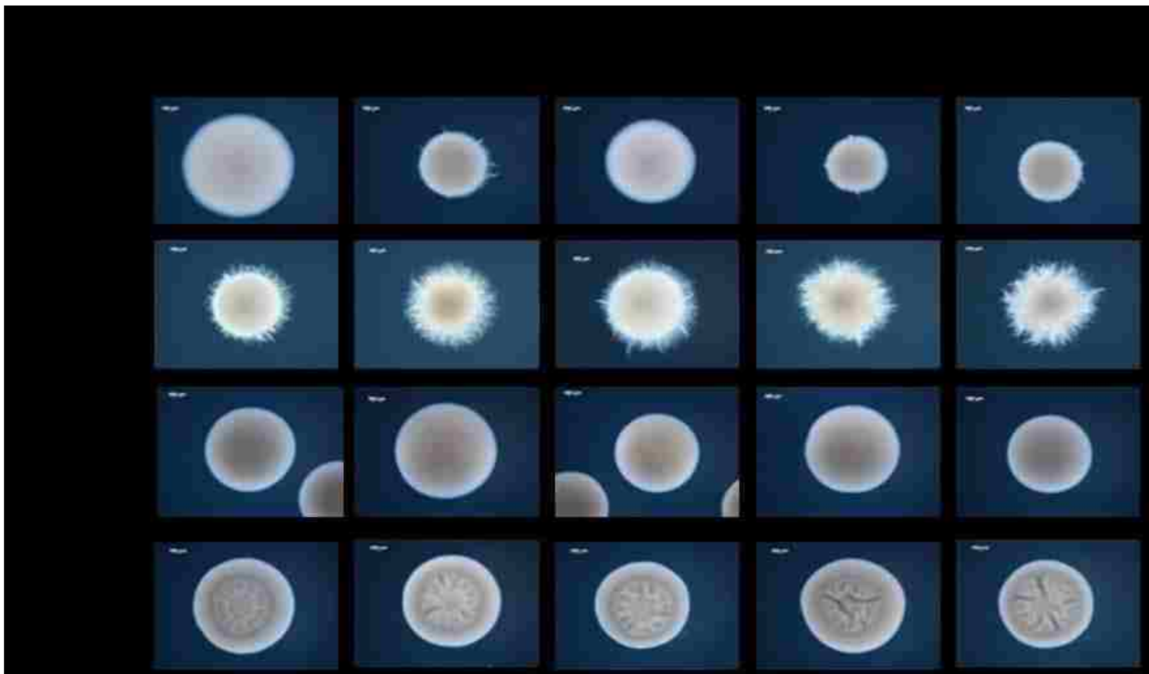

Figure 4-3. The hyperfilamentation of *hap31∆/hap31∆* and the *hap31∆/hap31∆ hap32∆/hap32∆* double mutant is carbon source dependant. Wild-type (WT), *hap31∆/hap31∆*, *hap32∆/hap32∆*, *hap5∆/hap5∆* strains and the *hap31∆/hap31∆ hap32∆/hap32∆* double mutant were grown on the indicated media buffered to pH 5.6 or pH6.8 with 150 mM HEPES for 5 days at 37ºC. Glucose (2%) was added to the medium as indicated.

In media where glucose was provided, regardless of pH, the mutant grew comparable to the wild-type strain (WT). In media in which the only carbon and nitrogen source was the amino acids or with lactate as the carbon source, the *hap31∆/hap31∆ hap32∆/hap32∆* mutant produced

colonies that consisted of mostly hyphae, a phenotype previously observed with the *hap5∆/hap5∆* mutant (Johnson *et al.* 2005). Since it is conceivable that YNB containing amino acids as the sole carbon and nitrogen source may result in limitation in carbon and nitrogen availability for growth, the mutants were further tested by the addition of NH_4 to the above media. The addition of NH_4 caused a slight delay in hyperfilamentation when glucose was absent from the media, but hyperfilamentation was still consistently observed (data not shown).

These data suggest that the various *hap* mutants have a defect in growth on nonfermentable carbon sources; hence, the mutants are likely to have defects in respiratory metabolism. In addition, the lack of hyphal development on SLAD medium further suggests that the mutants have some defect in nitrogen utilization; however, this could result from defects in pathways where carbon and nitrogen metabolism converge, such as the Krebs cycle.

hap31∆/hap31∆ hap32∆/hap32∆ **double mutants are unable to grow under iron-limiting conditions.** The *hap5∆* and *hap2∆* homozygous mutants have been shown to be unable to grow under iron-limiting conditions. The deletion of *HAP32* caused a slight growth defect on low iron medium, however, the deletion of *HAP31* showed no obvious growth phenotype on the same medium. To determine whether the deletion of both *HAP31* and *HAP32* resulted in a phenotype equivalent to *hap2Δ* and *hap5∆* homozygous mutants, the appropriate strains were grown in YPD with and without the addition of the iron chelator bathophenanthroline disulfate (BPS). Cell concentrations were normalized prior to spot plating to ensure that equal colony forming units were plated.

Both the *hap31∆/hap31∆ hap32∆/hap32∆* and *hap5∆/hap5∆* mutants were unable to grow in iron-limiting medium (Figure 4-4). The *hap32∆* homozygous mutant showed a slight retardation in growth on the iron poor media. The addition of hemin to the YPD + BPS medium rescued the growth defects demonstrating that the growth defect was related to the use of free iron. Importantly, the phenotype of the *hap31∆/hap31∆ hap32∆/hap32∆* mutant was identical to that observed with the *hap2Δ* and *hap5Δ* homozygous mutants.

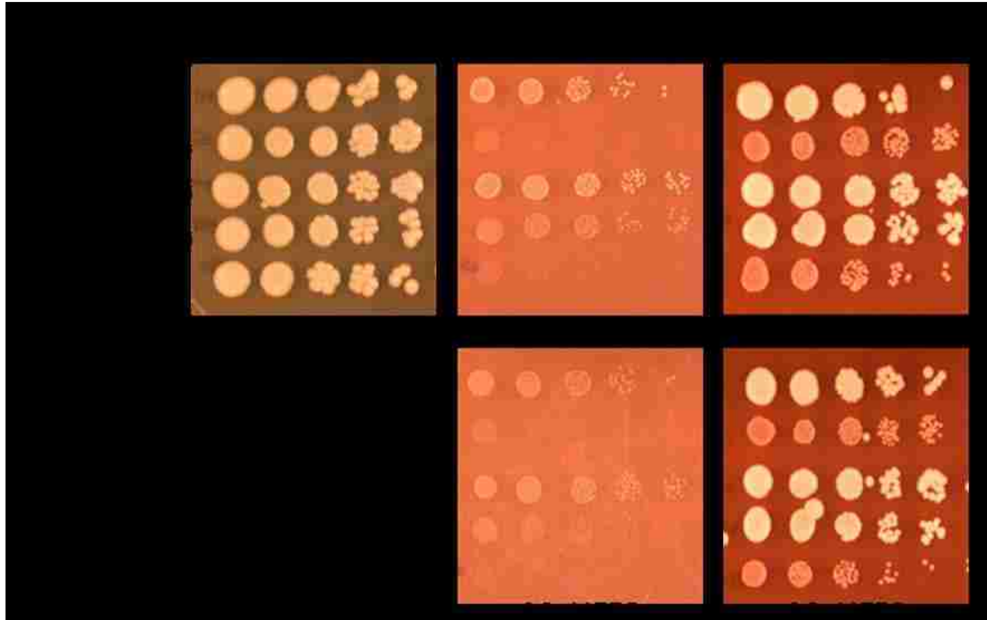

Figure 4-4. *hap31*Δ *hap32*Δ double mutant is affected by an iron-limited growth environment. Strains with the indicated genotypes were grown overnight in YPD with 200μM BPS, counted, normalized and then spot plated onto YPD, YPD + .01mMBPS, YPD + .25mM BPS, YPD + .01mMBPS + 50μM Heme, YPD + .25mM BPS + 50μM Heme and grown at 30ºC for 3 days.

The deletion of *HAP31* affects *CYC1* and *COX5*. *C. albicans hap5Δ/ hap5Δ* mutants have been shown to have altered expression of *COX5* and *CYC1* when cells are grown in YPD or YPD with 200μM BPS (Chapter 2). *COX5* and *CYC1* expression were both decreased in the *hap5Δ* homozygous mutant in the iron replete media and up-regulated in the iron deficient media when compared to the wild-type strain. The *hap2Δ* homozygous mutant showed a similar pattern of expression for these genes. To determine the effect of the *hap3* knockout mutations on the expression of *CYC1* and *COX5* the appropriate strains were grown in YPD and YPD containing BPS, RNA was isolated and northerns were performed as described in Material and Methods

Both mutants show differences in expression for both *CYC1* and *COX5* in YPD when compared to the wild-type strain. The expression of *CYC1* and *COX5* was reduced in the *hap31*Δ*/hap31*Δ mutant, but was up-regulated in the *hap32*Δ*/hap32*Δ mutant as compared to the wild-type strain. In YPD + BPS, the expression of *CYC1* and *COX5* is not affected; however, we have to consider that Hap31p and Hap32p may be compensating for each other under iron-limiting conditions as suggested by the phenotype of the strain on iron-limiting growth medium.

The expression of these genes in the *hap31Δ/hap31Δ hap32Δ/hap32Δ* mutant should clarify this issue.

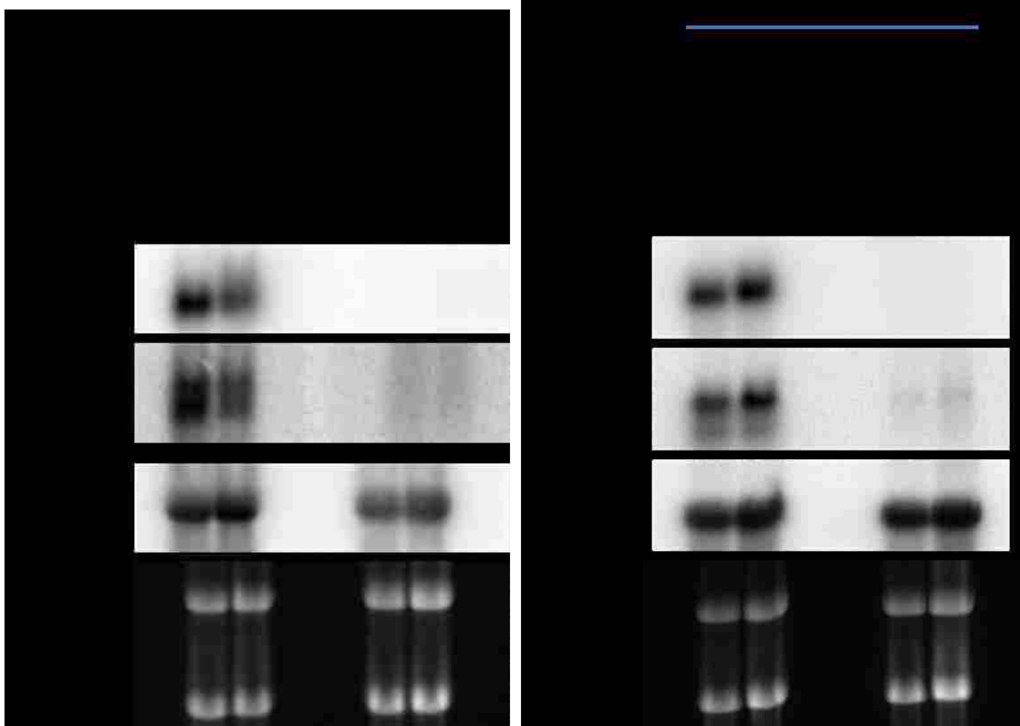

Figure 4-5. Deletion of *HAP31* affects the expression of *CYC1* and *COX5* in iron replete conditions. Northern blot analysis of total RNA isolated from *C. albicans* wild-type (WT), *hap31Δ/hap31Δ*, and *hap32Δ/hap32Δ* homozygous mutants grown at 30ºC in YPD and YPD with 200µM BPS, as indicated. The membrane was hybridized with radiolabeled probes specific for *CYC1* and *COX5* as indicated. 26S rRNA was used to normalize results.

Discussion

Previous data has presented the case that two functional *HAP3* homologs, named *HAP31* and *HAP32,* have been identified and cloned from *C. albicans.* Both proteins are able to functionally rescue the respiratory defects of a *S. cerevisiae HAP3* mutant. However, deletion of either gene singly in *C. albicans* does not result in the loss of complete DNA-binding activity, but instead, there is a loss of distinct CCAAT-binding complexes. To understand the multiple CCAAT-binding complexes and the role of the two Hap3p homologs, a double mutant was created where both *HAP31* and *HAP32* were deleted. The *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant resulted in a strain with the same growth defects as seen with both the *hap5Δ* and *hap2Δ* homozygous mutants. Therefore, Hap31p and Hap32p appear to be involved in the

regulation of genes analogous to Hap5p; however, Hap31p and Hap32p may play different roles in regulation or respond to different environmental signals. Nevertheless, electrophoretic mobility gel shifts showed that without both proteins, DNA binding is abolished Therefore, the *C. albicans* CCAAT-binding complex requires either Hap31p or Hap32p for DNA binding activity.

The morphology of *C. albicans* colonies are affected by carbon and nitrogen source. When *C. albicans hap31Δ* or *hap32Δ* homozygotes are grown on media where glucose is not provided, neither mutant is phenotypically analogous to the *hap2*Δ or *hap5*Δ homozygous mutants; however, the *hap31*Δ/*hap31*Δ mutant displayed a more pronounced filamentation defect similar to the *hap2*Δ or *hap5*Δ homozygous mutants.

The *hap31*Δ/*hap31*Δ *hap32*Δ/*hap32*Δ double mutant becomes hyperfilamentous in media where glucose is not provided. Hyperfilamentation in the double mutant is carbon source-dependent as has been shown previously with the *hap5*Δ/*hap5*Δ mutant (Johnson *et al.* 2005). The addition of NH_4 to the media lacking glucose delays hyperfilamentation, but does not rescue the phenotype (data not shown). This indicates that carbon and nitrogen source sensing/utilization is effected by the availability of the Hap complex. In M199 medium, which contains a low amount of glucose (0.1%) and is rich in amino acids, hyperfilamentation is also seen in the homozygous mutants. In SLAD medium, which was used to induce nitrogen starvation in the presence of glucose, loss of hyphal formation is observed. These data suggest that there is also a defect in nitrogen sensing/utilization at low concentrations in the *hap* mutants; however, we have not explored which genes may be involved. Overall the hyphal media indicates that carbon and nitrogen source sensing/utilization is regulated, either directly or indirectly, by the Hap complex.

In iron-limiting conditions the *hap2Δ/hap2Δ*,*hap5Δ/hap5Δ*, and *hap31Δ hap32Δ* homozygous double mutants are unable to grow. The *hap31*Δ homozygous mutant is unaffected by iron limitation; whereas, the *hap32*Δ/*hap32*Δ mutant displays a slightly retarded growth phenotype. This implicates the *C. albicans* Hap complex plays a role in iron acquisition/utilization and verifies that the Hap3p homologs are required for the complex to function. The addition of hemin to the low iron media rescued colony growth for all affected mutants suggesting that the

uptake and utilization of organic sources of iron are unaffected by the Hap complex, whereas the uptake of inorganic iron is altered.

It has been previously shown that the deletion of *HAP5* or *HAP2* leads to a defect in the expression of *CYC1* and *COX5.* In a *hap31*Δ homozygous mutant, the expression of these genes is only affected in iron replete conditions. This corresponds with the hyperfilamentation phenotype in media lacking glucose. The two identified Hap3p subunit of CCAAT-binding complex in *C. albicans* are involved in the differential expression of genes based on environmental iron conditions. However, both subunits are able to compensate for the deletion of the other as seen in the phenotypic assays (Figure 4-2, Figure 4-3, and Figure 4-4) where neither deletion was as severely affected as the wild-type and *hap31Δ hap32Δ* homozygous double mutant..

The duality of the *HAP3* genes and their ability to compensate under iron limitation indicates an importance of the CCAAT-binding factor. Given *C. albicans* role as a mammalian pathogen, this might be indicative of adaptation to the host environment. Mammalian host systems are an iron limiting environment (Ratledge and Dover 2000, Weinberg and Miklossy 2008) for microbial organisms. This duality of Hap3p subunits could allow *C. albicans* to better adapt to this environment by allowing the separate regulation of distinct genetic pathways by the various CCAAT-binding complexes in response to the different environmental niches of the human body which are infected by *C. albicans*.

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Chapter 5. The role of the *C. albicans* Hap4p subunits of the CCAAT binding complex: DNA binding and gene expression

Introduction

Candida albicans is an opportunistic fungal pathogen that can cause life threatening systemic infections in individuals with compromised immune systems. These individuals include premature infants, diabetics, surgical patients, organ recipients, and cancer patients. In United States hospitals, *Candida* species are the fourth most common cause of nosocomial blood stream infections (NNIS 1999).

C. albicans is a dimorphic fungus and it has been shown that the morphological switching between yeast and hyphal forms is essential for infection (Lo *et al.* 1997, Saville *et al.* 2003). Some of triggers for the formation of hyphae include temperature, pH, nutrient deprivation, and mammalian serum (Odds 1988, Sudbery *et al.* 2004, Whiteway and Bachewich 2007).

The CCAAT-binding factor, the Hap complex, has been identified in all eukaryotes to date and is composed of three DNA binding subunits. In *Saccharomyces cerevisiae,* Hap2/3/5p are required for DNA binding and an additional subunit unique to fungi, Hap4p, is required for transcriptional activation of gene expression (McNabb and Pinto 2005). In mammalian systems, the homologs of Hap2p and Hap5p both possess transcriptional activation domains (de Silvio *et al.* 1999, Mantovani 1999, Romier *et al.* 2003), and a homolog of Hap4p has not been identified in eukaryotes other than fungi.

Previous research has shown that the CCAAT-binding factor plays a role in environmental sensing in *C. albicans* (Johnson *et al.* 2005). In *C. albicans*, two Hap3p homologs have been identified and shown to be complementary in the function for the Hap complex (Chapter3, Chapter4). There are three Hap4p homologs that have been identified strictly by amino acid sequence homology to *S. cerevisiae* Hap4p (Johnson *et al.* 2005); however, the functional relevance of these proteins as subunits of the *C. albicans* CCAAT-binding factor has not been clearly demonstrated. In this chapter, the multiplicity of the Hap4p subunits will be examined.

Materials and Methods

Yeast strains and growth conditions

Yeast strains used in these studies are listed in Table 5-1. Strains were cultured in yeast extract-peptone dextrose (YPD) medium (Guthrie and Fink 1991) supplemented with 80mg/L uridine as appropriate and incubated at 30ºC overnight. For selection of yeast transformants, synthetic complete media lacking the appropriate auxotrophic requirement was used (Guthrie and Fink 1991).

Table 5-1: E. coli and *C. albicans* strains

Strain	Genotype	Reference
E. coli		
DH5α	*F Φ80dlacZ-M15Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(r_{K-} m_{K+}), deoR thi1 supE44, λ⁻ gyrA96 relA1*	
C. albicans		
BWP17	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG*	(Romier *et al.* 2003)
DMC146	*ura3 Δ ::imm434/ura3 Δ ::imm434 his1 Δ ::hisG/his1 Δ ::hisG-HIS1 arg4 Δ ::hisG/arg4 Δ ::hisG-ARG4-URA3*	(Johnson *et al.* 2005)
DMC120	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP5/hap5Δ::URA3*	(Johnson *et al.* 2005)
DMC117	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	(Johnson *et al.* 2005)
DMC126	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP5-ARG4 hap5Δ::URA3/hap5Δ::HIS1*	(Johnson *et al.* 2005)
DMC205	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP4.1/hap4.1Δ::URA3*	
DMC190	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap4.1::URA3/hap4.1Δ::HIS1*	
DMCr33	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP4.1-ARG4 hap4.1::URA3/hap4.1Δ::HIS1*	
DMC140	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP4.2/hap4.2Δ::URA3*	(Tuli 2004)
DMC134	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap4.2::URA3/hap4.2Δ::HIS1*	(Tuli 2004)
DMC130	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP4.2-ARG4 hap4.2::URA3/hap4.2Δ::HIS1*	(Tuli 2004)
DMC166	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1 arg4Δ::hisG/arg4Δ::hisG-ARG4 HAP4.3/hap4.3Δ::URA3*	(Pettway 2004)
DMC165	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-ARG4 hap4.3::URA3/hap4.3Δ::HIS1*	(Pettway 2004)
DMCR1	*ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG-HAP4.3-ARG4 hap4.3::URA3/hap4.3Δ::HIS1*	(Kroger-von Grote 2008)

Media for hyphal analysis, including Lee's (Lee *et al.* 1975), modified Lee's (Liu *et al.* 1994), and Spider (Liu *et al.* 1994) medium, were prepared as previously described. Serum media consisted of YPD containing 10% newborn calf serum (Sigma). M199 medium was

obtained from Gibco-BRL, containing Earle's salts and glutamine but lacking sodium bicarbonate and buffered with 150 mM HEPES adjusted to pH 4.5 or 7.5. SLAD is synthetic low ammonia medium containing 0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, 2% dextrose, and 50 mM ammonium sulfate. Yeast nitrogen base (YNB) medium was prepared using 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco). 0.1% amino acids were added using an amino acid dropout powder containing the 20 amino acids at the describe concentrations (Guthrie and Fink 1991), and buffered to the required pH with 150 mM HEPES. Glucose or lactate was added to the media at a final concentration of 2% and media was solidified with 1.5% agar as appropriate.

For low iron media, bathophenanthroline disulfate (BPS) (Sigma) was used to chelate iron. BPS was added to YPD at a concentration of 200μM in liquid media and used for overnight cultures. BPS at a concentration of 0.1-0.25mM was added to solid YPD media. Hemin was added to solid YPD with BPS media at a concentration of 50μM.

Yeast extract preparation and electrophoretic mobility shift assays.

Protein extracts were prepared as previously described (Johnson *et al.* 2005). The CCAAT DNA-binding probe was designed similar to that described previously (Hahn and Guarente 1988) and is based on the sequence of CCAAT site sequence from *CYC1* of *C. albicans*. The DNA probe was end labeled by the Klenow fragment with [α-^{32}P]dATP (Amersham Corp.).

All binding reactions contained 20 to 30 μg of cell extract in DNA-binding buffer (20 mM HEPES-NaOH pH 8.0, 100 mM KCl, 1 mM EDTA, 10% glycerol), 0-1 μg of denatured salmon sperm DNA, 0-1 μg of poly(dI-dC), and 0.5 to 1.0 ng of radiolabeled probe in a final reaction volume of 20 μl. Reactions were incubated at room temperature for 30 to 45 min, and the protein-DNA complexes were resolved by gel electrophoresis (4 h at 300 V) on 5% polyacrylamide gels (acrylamide:bisacrylamide ratio, 29:1) in 0.5× Tris-borate-EDTA at 4°C. After electrophoresis, the gels were fixed, dried, and visualized with a Molecular Dynamics PhosphorImager.

Microscopy and imaging.

Photographs of colony morphology were taken with a Nikon Biaphot microscope fitted with a high-resolution charge-coupled device camera and the AutoMontage imaging software package (Syncroscopy, Frederick, MD). Photographs of individual cells were done with a Zeiss Axioplan 2 microscope fitted with a high-resolution charge-coupled device camera and AutoMontage imaging software. Photographs of individual colonies were representative of the total population. The figures were prepared using Adobe Photoshop 7.0 and Microsoft PowerPoint 2007.

Northern blot analysis.

C. albicans strains were grown to saturation in YPD medium and subsequently inoculated into the indicated medium and grown until OD>0.6 at 30°C. The cells were harvested by centrifugation, and total RNA was prepared by the glass bead-acid phenol method as previously described (Ausubel *et al.* 1994). Approximately 20 µg of each total RNA sample was loaded, separated by formaldehyde-1% agarose gel electrophoresis, and transferred to GeneScreen Plus membranes (Dupont-NEN Research Products) according to the manufacturer's protocol. The membranes were hybridized and washed under standard high-stringency conditions (Sambrook *et al.* 1989).

Table 5-2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5' – 3')
oDM0341………………	5'-GGCCGAATTCGGTGCCACTTTATTTAAAACTAGATG-3'
oDM0342………………	5'-GGCCGGATCCGCTTTCTTCAAATAAGTAACTAAATCG-3'
oDM0449………………	5'-GAGATCATTACAAAGAGCTGCCACTAAAGCC-3'
oDM0450………………	5'-GGAGTTAATTCTTGCCATGGTAATTCC-3'
oDM0459………………	5'-TAGCGGTTCTGACGTGCAAATCGATCGTCG-3'
oDM0460………………	5'-ACTAACACCTTTTGTGGTGTCTGATGAGCG-3'

The *CYC1*, *COX5*, and 26S rRNA probes for hybridization were obtained by PCR amplification from *C. albicans* genomic DNA using the primer pairs oDM0341/oDM342, oDM0449/oDM0450, and oDM0459/oDM0460, respectively. The probes were purified by

agarose gel electrophoresis and GeneClean (Qbiogene, Inc.) and subsequently radiolabeled with [α-^{32}P]dATP (Amersham) by use of a random primer labeling kit (U.S. Biochemicals) according to the manufacturer's protocol. The transcript levels were quantified on a Molecular Dynamics PhosphorImager.

Results

Deletion of *HAP41* or *HAP42* causes a defect in morphogenetic transition on solid media. To determine whether the *hap41Δ/hap41Δ* or *hap42Δ/hap42Δ* mutants displayed similar phenotypic defects to the *hap5Δ/hap5Δ* mutant, the appropriate strains were grown on media known to induce hyphal development. The *hap41Δ/hap41Δ* and *hap42Δ/hap42Δ* mutants displayed various hyphal defects that were media-dependent (Figure 5-1), whereas the *hap2Δ* and *hap5Δ* homozygous mutants displayed defective hyphal growth on all media examined (Chapter 2, Johnson *et al.* 2005). Heterozygous and homozygous mutant strains rescued with the appropriate gene displayed phenotypes that were comparable to the wild-type strain (data not shown).

Neither *hap41Δ* nor *hap42Δ* homozygous mutants showed significant defects in hyphal growth on YPD containing 10% calf serum (Figure 5-1). The most prominent filamentation defects were seen on SLAD and Modified Lee's media. Both mutants were slow to initiate hyphal growth on SLAD medium and, the *hap41Δ/hap41Δ* mutants grown on Modified Lees medium lacked hyphal formation, while the *hap42Δ* homozygous mutant were slow to form hyphae. Neither strain produce wrinkled colonies as compared to the wild-type strain.

In general, the phenotype of the *hap41Δ* homozygous mutant was more severe than the *hap42Δ* homozygous mutant on hyphae-inducing. Interestingly, hyperfilamentation was observed with the *hap41Δ/hap41Δ* mutant on M199 pH 7.5 medium, similar to that previously seen with the *hap2Δ* and *hap5Δ* homozygotes; however, there was no phenotype seen with the *hap42Δ* homozygous mutant. When the same strains were grown on M199 pH 4.5, no defect was seen other than a slight size difference (data not shown). Thus, the *hap41Δ/hap41Δ* mutants

appeared to display phenotypes that were comparable to both *hap2Δ* and *hap5*Δ homozygous mutants. The phenotypes seen with the *hap42Δ* homozygous mutant were not as drastic as the *hap2Δ* and *hap5*Δ homozygous mutants. This could result from partial compensation by the other Hap4p homologs found in the strains, as previously seen with Hap31p versus Hap32p (Chapter 3, 4)

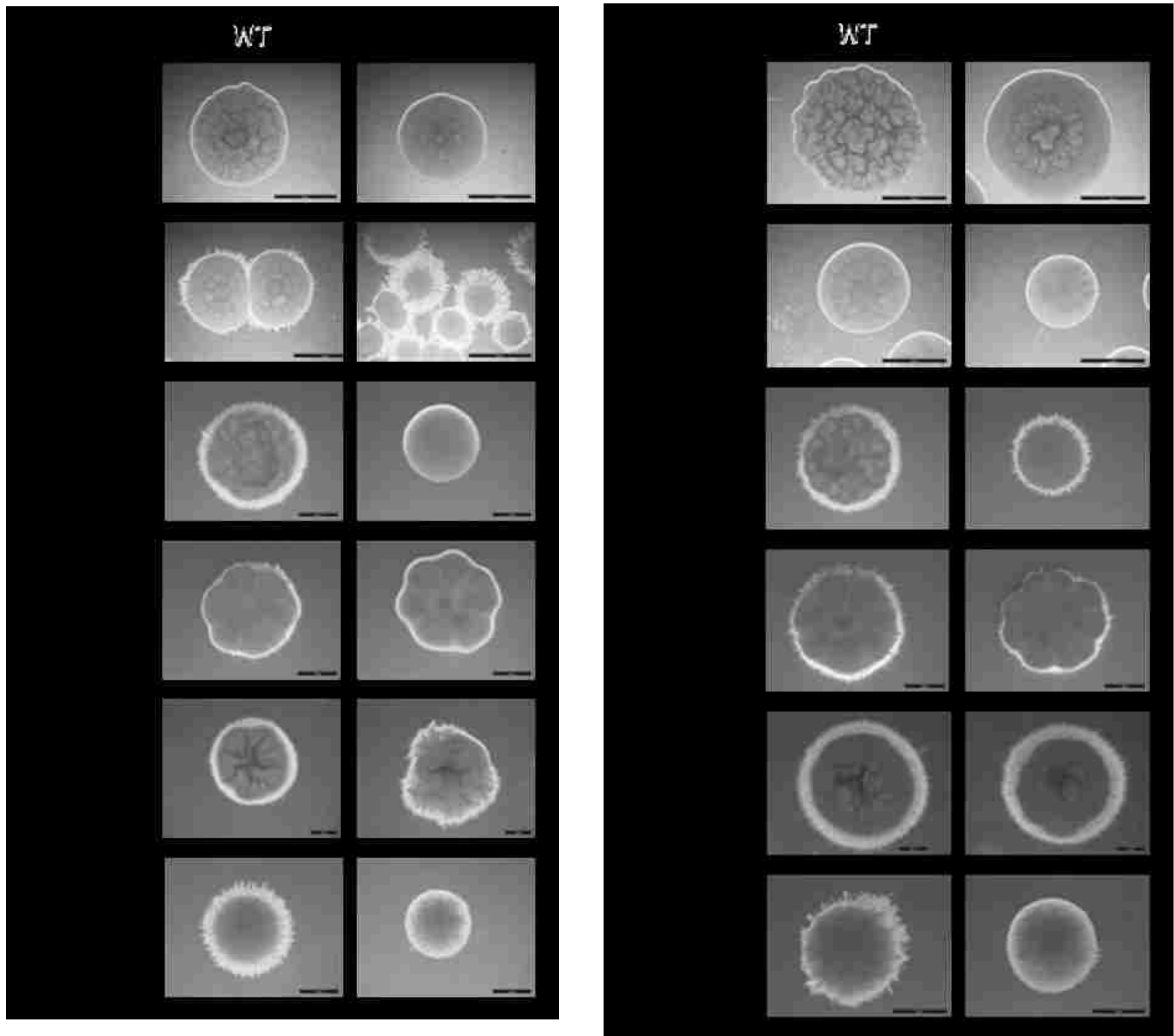

Figure 5-1. Effect of hyphae induction media on mutant growth.
Wild-type (WT), *hap41Δ/hap41Δ,* and *hap42Δ/hap42Δ* strains were grown on the indicated media for 4 days at 37ºC, except Spider and SLAD medium in which the incubation was extended until day 6.

The absence of glucose alters the growth of the *hap41Δ/hap41Δ* and *hap42Δ/hap42Δ* mutants. To determine whether the *hap41Δ* and *hap42Δ* homozygous mutants were affected by carbon source availability as observed with the *hap5Δ/hap5Δ* mutant, strains were grown in yeast nitrogen base with amino acids as the sole nitrogen source and either glucose, lactate, or no additional carbon source added and at either pH 5.6 or 6.8 (Figure 5-2).

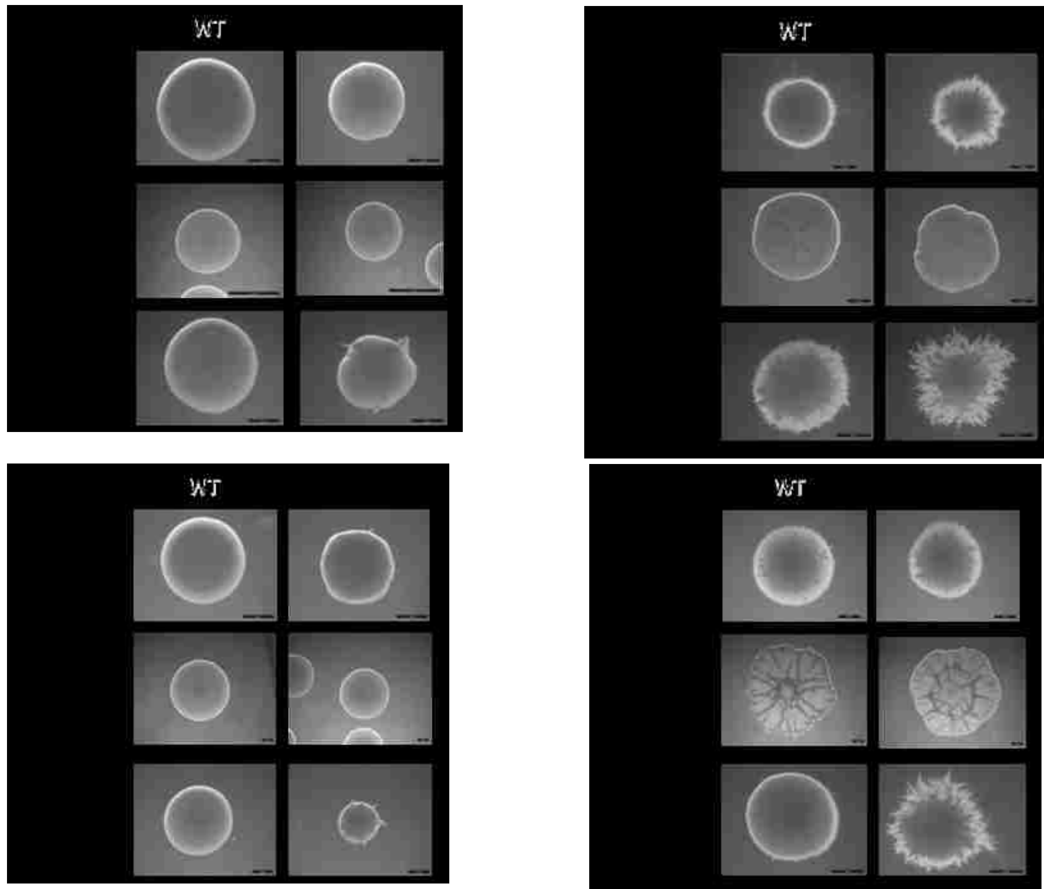

Figure 5-2. Hyperfilamentation of *hap41Δ/hap41Δ* and *hap42Δ/hap42Δ*is carbon source dependant. Wild-type (WT), *hap41Δ/hap41Δ* and, *hap42Δ/hap42Δ* strains were grown on YNB + AA media buffered to pH 5.6 or pH6.8 as indicated for 5 days at 37ºC. Glucose or lactate (2%) was added to the medium as indicated.

In media where glucose was provided regardless of pH, the *hap41Δ* and *hap42Δ* homozygous mutants grew comparable to the wild-type strain. In media where the amino acids provided the only carbon source or with lactate as the carbon source at pH 6.8 both *hap41Δ* and *hap42Δ* homozygous mutants produced colonies that consisted of predominantly hyphae (Figure

5-2). At pH 5.6, both mutants were smaller and produced hyphae earlier when compared to the wild-type.

The mutants were further tested by the addition of NH_4 to the above media. The addition of NH_4 delayed the hyperfilamentation when glucose was absent from the media. Cells grown at higher pH showed an increase in hyperfilamentation. In YNB + amino acid pH 6.8, the *hap41*Δ homozygous mutant showed an identical phenotype to *hap5*Δ, *hap2*Δ, and the *hap3*Δ double mutant. The *hap42*Δ homozygous mutant was affected, but not as severely. The *hap41*Δ and *hap42*Δ homozygous mutants were only slightly affected by the lack of glucose at pH 5.6 (YNB + amino acid pH5.6 and YNB + amino acid + Lactate pH 5.6), whereas, *hap5*Δ, *hap2*Δ, and the *hap3*Δ double mutant were hyperfilamentous under these conditions.

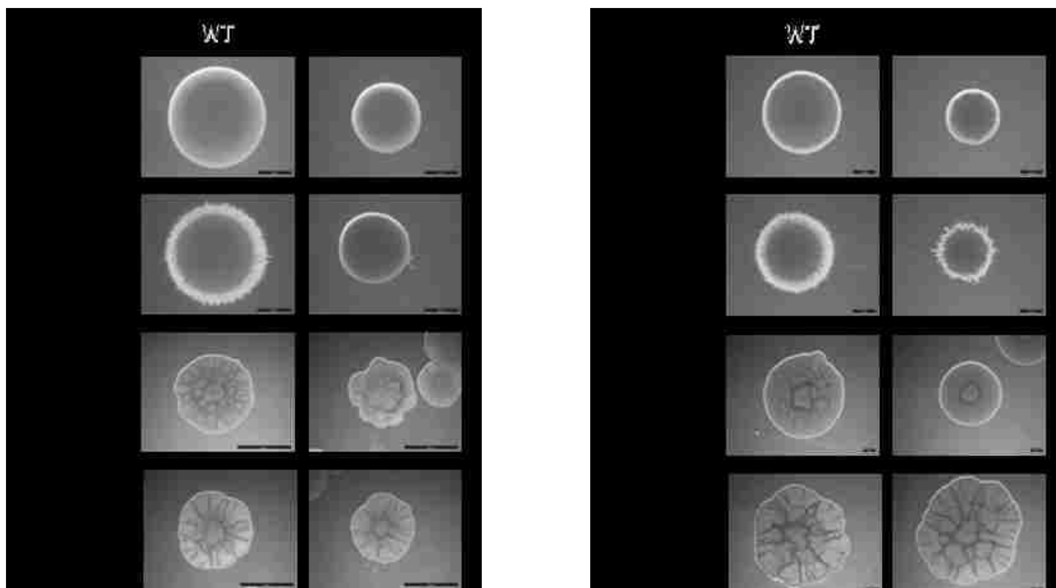

Figure 5-3. **NH_4 partially rescues hyperfilamentation.** Wild-type (WT), *hap41*Δ*/hap41*Δ and *hap42*Δ*/hap42*Δ strains were grown on YNB + AA + NH_4 with and without glucose buffered to pH 5.6 or pH 6.8 with 150 mM HEPES as indicated.

The *hap43/hap43*Δ mutant is unable to grow in low iron media. To determine whether any of the Hap4p homologs are involved in iron acquisition, the appropriate strains were grown in YPD with and without the addition of the iron chelator BPS (bathophenanthroline disulfate). Cells were grown overnight in YPD+200µM BPS, and cell concentrations normalized prior to spot plating to ensure that equal colony forming units were plated. Both the *hap41*Δ*/hap41*Δ and

*hap42*Δ/*hap42*Δ mutants were able to grow under iron limitation (Figure 5-4). In contrast, the *hap43*Δ/*hap43*Δ and the *hap5*Δ homozygous mutant could not grow under these conditions unless heme was added to the low iron media. The *hap41*Δ, *hap42*Δ, and *hap43*Δ heterozygotes and rescued strains grew comparable to DMC146 (wt) (data not shown). These data suggests that the defect in growth on iron-limiting media involves a defect in the utilization of free iron, but not iron coupled to heme. Since neither *hap41*Δ nor *hap42*Δ are affected under these conditions, *hap43*Δ must perform a function in iron acquisition/utilization.

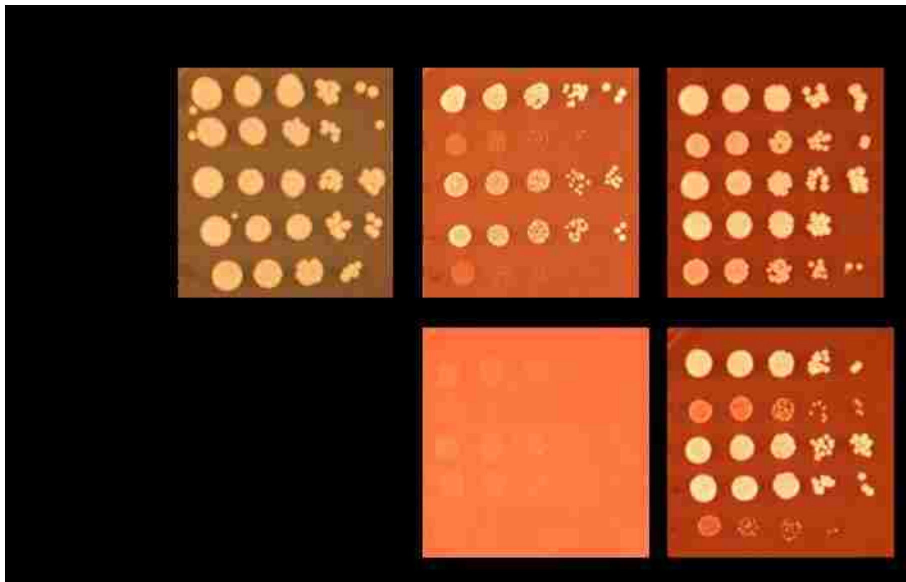

Figure 5-4. *hap43Δ* homozygote is unable to grow in low iron media.
Strains with the indicated genotypes were grown overnight in YPD with 200μM BPS, counted, normalized and then spot plated onto YPD, YPD + .01mMBPS, YPD + .25mM BPS, YPD + .01mMBPS + 50μM Heme, YPD + .25mM BPS + 50μM Heme and grown at 30ºC for 3 days.

The *HAP4* subunits regulate *COX5* under both iron replete and iron-limiting growth conditions. Previously *C. albicans hap5Δ* mutants were shown to effect the expression of *COX5* and *CYC1* when grown in YNB + amino acids + Glucose pH5.6 and YNB + amino acids pH5.6. *COX5* expression was reduced in the *hap5Δ* homozygous mutant in the glucose media and upregulated in the glucose deficient media when compared to the wild-type strain. *CYC1* showed no difference in expression in glucose, but was upregulated in the mutant when compared to the wild-type strain in glucose deficient media (Johnson *et al.* 2005). To determine whether the expression of these respiratory genes was affected by the deletion of the three *HAP4*-like genes

when cells were grown in iron-limiting media, the appropriate strains were grown in YPD or YPD with 200µM BPS, total RNA was isolated and northern blot analysis were preformed as described in Material and Methods.

The *hap41Δ/hap41Δ* mutant does not show any change in expression of *CYC1* and *COX5* when compared to the wild type (Figure 5-5). The *hap42Δ/hap42Δ* mutant showed a slight decrease in the expression of *COX5* mRNA when cells were grown in YPD medium, but there was no change under iron-limiting conditions when compared to the wild-type strain. There were no changes in *CYC1* under either condition. The *hap43Δ/hap43Δ* mutant showed the most dramatic change in mRNA expression when compared to the wild type in an iron-limiting growth medium. Both *COX5* and *CYC1* are up-regulated under iron limitation in a *hap43Δ* homozygous mutant. The up regulation under low iron conditions when *HAP43* is deleted is comparable to the *hap5Δ* homozygous mutant (Chapter 2); however, there is no change in mRNA levels when cells are grown in YPD medium which differs from that seen in the *hap5Δ* mutant. The altered expression of *CYC1* and *COX5* mRNA in the *hap43Δ/hap43Δ* mutant is consistent with the inability of the *hap43Δ* homozygote to grow on iron-limiting medium.

Deletion of *HAP43* alters CCAAT-binding activity in *C. albicans.* In *S. cerevisiae*, the CCAAT-binding complex consists of the Hap2p, Hap3p, and Hap5p subunits that form the DNA binding domain. It has been previously shown that CCAAT-binding activity is abolished in *C. albicans hap5Δ/hap5Δ* mutant (Johnson *et al.* 2005). To determine whether any of the Hap4p subunits is required for CCAAT-binding in *C. albicans*, an electrophoretic mobility shift assay was performed. *C. albicans* wild-type strain, *hap41Δ/hap41Δ*, *hap42Δ/hap42Δ*, and *hap43Δ/hap43Δ* homozygous mutant strains were used to determine the effect of these deletions on DNA binding. The strains were grown in YPD 30ºC to an OD_{600} of ~1, the cells were harvested and cell extracts were prepared as described in Materials and Methods. To assess CCAAT binding activity, a 37-bp double stranded DNA oligonucleotide derived from *C. albicans CYC1* CCAAT sequence was used.

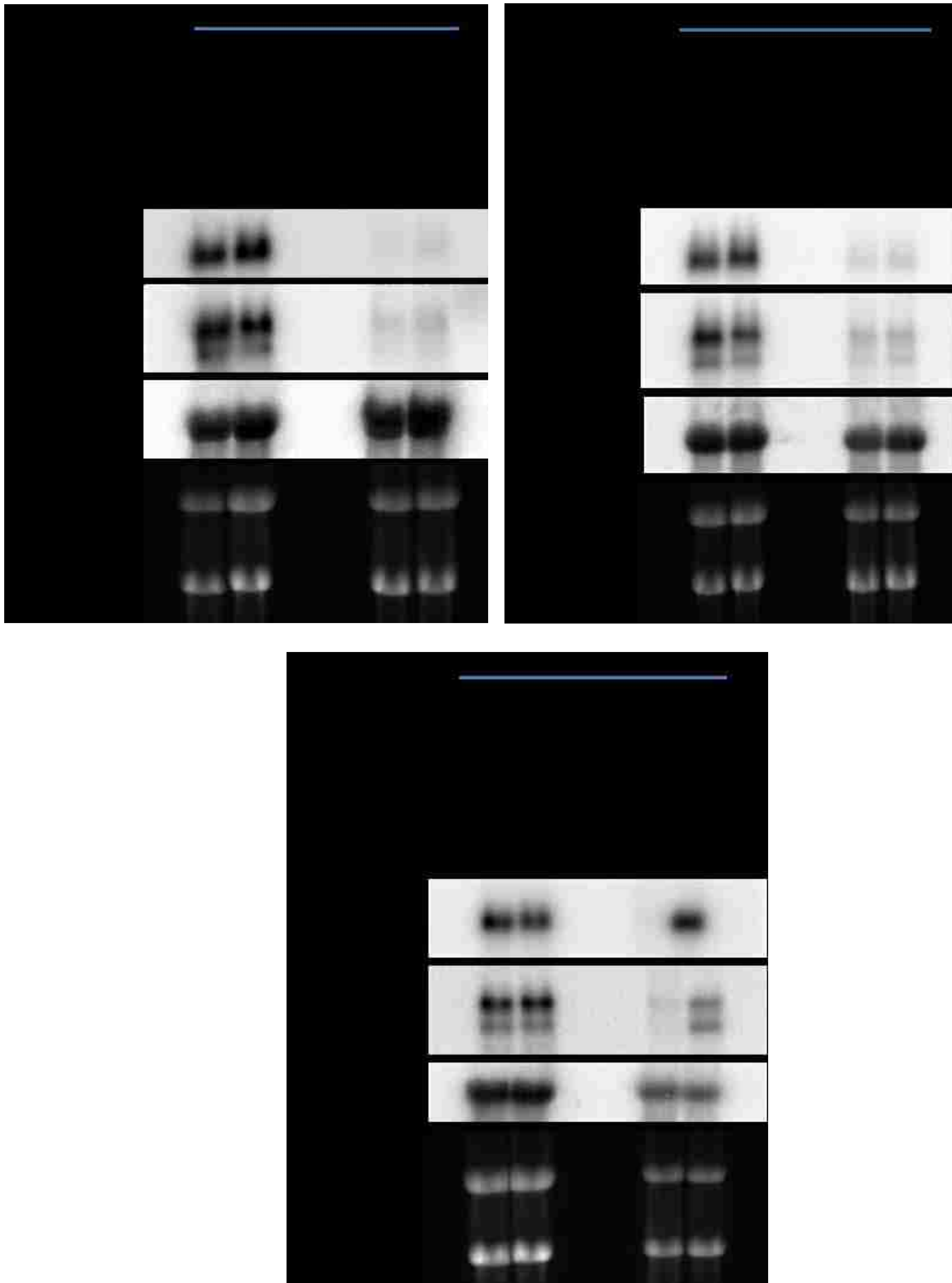

Figure 5-5. *C. albicans* CCAAT-binding factor regulates *CYC1* and *COX5* in iron replete and iron poor conditions. Northern blot analysis of total RNA isolated from *C. albicans* wild-type, *hap41Δ/hap41Δ*, *hap42Δ/hap42Δ*, and *hap43Δ/hap43* strains grown at 30ºC in YPD and YPD with 200µM BPS as indicated. The membrane was hybridized with radiolabeled probes specific for *CYC1* and *COX5* as indicated. 26S rRNA was used to normalize results.

As shown in Figure 5-6, there are 5 unique complexes visible in the gel shift depending on the strain and the media. In YPD, the wild-type strain contains complexes I, II, and a weakly

visible amount of complex V. In YPD the *hap41Δ*, *hap42Δ*, and *hap43Δ* homozygous strains show no major change in the predominant DNA binding activities observed.

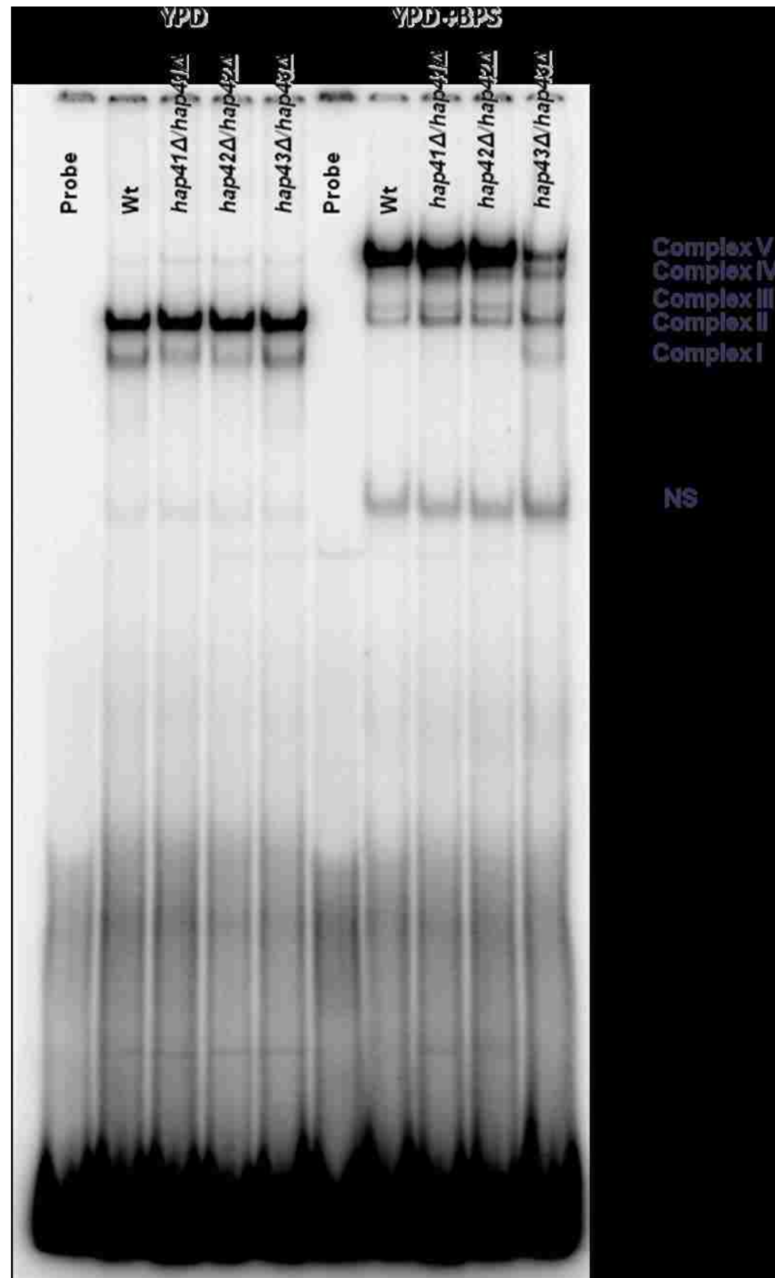

Figure 5-6 Deletion of *HAP43* alters CCAAT-binding activity.
Crude extracts were prepared from cells grown in YPD or YPD with BPS as indicated. The electrophoretic mobility shift assay was performed with crude extracts prepared from the wild-type, *hap41Δ/hap41Δ*, *hap42Δ/hap42Δ*, and *hap43Δ/hap43Δ* strains as indicated. The position of the various CCAAT-binding complexes is indicated at the right. NS - Nonspecific Binding.

In strains grown in YPD+BPS, the wild-type strain contains complexes II, III, IV and V with complex V the most abundant (Figure 5-6). The *hap41Δ* and *hap42Δ* homozygous mutants showed identical DNA-binding complexes as the wild type strain. The *hap43Δ/hap43Δ* homozygous mutant displayed the most significant effect on DNA binding activity under iron-limitation. There is a significant decrease in the binding complex V and a total loss of complex III, with the appearance of complex I.

Discussion

The data shown has presented the case that three *C. albicans HAP4* homologues encoding a subunit of the CCAAT-binding factor have been identified. Deletion of any of the three caused various defects in the growth of the strains. None of the defects except for the iron phenotype was as drastic as seen in either the *hap2*Δ or *hap5*Δ homozygous mutants. Therefore like the *HAP3* homologs, the *HAP4*-like proteins apparently act in a redundant manner. Gel shifts show only a difference in complex binding when Hap43p is not present unlike Hap2p or Hap5p, where DNA binding is completely abolished. Therefore, the *C. albicans* CCAAT-binding complex does not require any of the HAP4p's for DNA binding. This is similar to the Hap complex in *S. cerevisiae* where Hap2/3/5p are required to simultaneously bind DNA (McNabb and Pinto 2005) and Hap4p binds afterwards and is not require for binding. Northern blot analysis shows that the availability of iron plays a role in the regulation of *CYC1* and *COX5a* via the CCAAT-binding factor.

The morphology of *C. albicans* colonies are affected by carbon, pH, and nitrogen source. When *C. albicans hap41Δ* mutant is grown on media where glucose is not provided, the cells become hyperfilamentous. The homozygote switches to hyphal formation early in colony growth, before the wild type. Hyperfilamentation of the mutant is dependent on the absence of glucose. The addition of NH_4 to the media lacking glucose slows hyperfilamentation for the *hap41*Δ homozygous mutant. In M199, which contains a low amount of glucose (0.1%) and is rich in amino acids, hyperfilamentation is still seen in the *hap41*Δ homozygous mutant. In SLAD

medium which was used to induce nitrogen starvation in the presence of glucose, loss of hyphal formation is seen for both *hap41*Δ and *hap42*Δ homozygous mutants. This indicates there is also a defect in nitrogen sensing/utilization at low concentrations in the mutant. Overall the hyphal media indicates that carbon and nitrogen source sensing/utilization is effected by the Hap complex.

In an iron-limiting growth environment only the *hap43Δ/hap43Δ* mutants are unable to grow. This suggests a different roll for Hap43p that involves iron acquisition/utilization. The *hap43Δ/hap43Δ* mutant shows the same growth defect as *hap5Δ* and *hap2Δ* homozygous mutants and the *hap31Δ/hap31Δ hap32Δ/hap32Δ* double mutant. The addition of hemin to the iron poor media restored colony growth. This indicates that Hap43p must play a role in the acquisition of free iron versus iron from an organic source such as heme. We have not evaluated whether *C. albicans hap* mutants are defective in siderophore iron acquisition.

C. *albicans* has evolved several distinct pathways to acquire iron from an iron-poor host environment. The mechanisms include high-affinity transporters, a siderophore uptake system, and heme uptake system (Howard 1999, Ramanan and Wang 2000, Heymann *et al.* 2002, Knight *et al.* 2002, Lesuisse *et al.* 2002, Pendrak *et al.* 2004, Knight *et al.* 2005). It has been shown that iron acquisition through high affinity transporters and siderophores is required for pathogenesis (Ramanan and Wang 2000, Heymann *et al.* 2002, Pendrak *et al.* 2004, Haas *et al.* 2008).

The uptake of elemental iron involves a reductive copper-dependent iron uptake system where iron is acquired from transferin, a blood plasma protein (Knight *et al.* 2002). This occurs as a occurs in a two step process (Philpott and Protchenko 2008) were ferric iron is reduced to ferrous iron and then is transported into the cytosol via a high affinity, ferrous specific transport complex of Fet3p, a multicopper ferroxidase, and Ftr1p, a iron permease (Knight *et al.* 2005). A copper-independent siderophore transport system also exists for the uptake of iron (Lesuisse *et al.* 2002, Knight *et al.* 2005). *C. albicans* has also developed mechanisms for the uptake of heme, a characteristic of pathogenic fungi (Santos *et al.* 2003). Plasma membrane proteins have been identified that bind hemin and possess hemolytic activity. This process is oxygen dependant (Philpott and Protchenko 2008).

It has been shown that the Hap complex, with Hap43p, is essential for the expression of Frp1p, a ferric reductase, in low iron conditions (Baek *et al.* 2008). Ferric reductases promote iron transport by acting with a multicopper oxidase and iron permease (Dancis 1998, Kosman 2003). They also are required for the transport of internally stored iron (Singh *et al.* 2007). Mutants that are affected by low iron conditions are less virulent and if iron is depleted these mutants are more susceptible to drugs (Ramanan and Wang 2000, Prasad *et al.* 2006). It has been reported that pathogens that have impairment in iron uptake are not able to infect unless the host has an iron loading condition (Weinberg 2000).

Gel shifts show that without Hap2p or Hap5p, DNA binding is abolished (Johnson *et al.* 2005). Therefore, the *C. albicans* CCAAT-binding complex requires both Hap2p and Hap5p for DNA binding. There is no loss in overall binding when either Hap41p or Hap42p is deleted. However, when Hap43p is absent from cells grown under iron limitation, the overall DNA binding activity of complex V of the CCAAT-binding factor is decreased. .

The Hap complex in *S. cerevisiae* is regulated by heme and controls activation of genes encoding components of respiratory cytochromes, which are iron-rich protein complexes. *S. cerevisiae CYC1* expression is low in low iron-conditions, but increases as the iron concentration increases (Protchenko and Philpott 2003). *C. albicans CYC1* regulation is also transcriptionally regulated by the availability of iron. Transcription is high in iron replete conditions, and is down-regulated in low iron conditions. In the *hap2Δ/hap2Δ* and *hap5Δ/hap5Δ* mutants, transcription is not repressed under iron deficient conditions. For both mutants, *CYC1* is down-regulated in iron replete conditions and up-regulated in iron-poor conditions as compared to the wild type strain. *COX5* shows the same pattern in low iron versus replete conditions. Of the *hap4* deletions, only *hap43*Δ shows an obvious defect on *CYC1* and *COX5* expression in iron poor conditions.

In *S. cerevisiae* HAP4p encodes the activator subunit of the complex (Forsburg and Guarente 1989). In *C. albicans* there have been three Hap4p-like proteins identified. Mutations that abolish the activity of Hap41p or Hap42p display some of the phenotypes that have been identified with the loss of Hap5p activity; however there is no change in DNA binding activity in these mutants. For these strains, there has been no identification of a direct role in gene

expression. However, a role for Hap43p has been discovered. This Hap4p subunit is required for growth in iron deficient conditions and displays the same phenotype that is seen in a *HAP5* knockout strain. The *HAP43* knockout strain, when grown on solid YPD + BPS medium, is equivalent to a deletion of the Hap complex, however, a gel shift shows there is still DNA binding activity. This DNA binding is decreased overall in the *hap43*Δ homozygous mutant implicating it role in maintaining the stability of the complexes under low iron conditions.

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Chapter 6. Summary

Summary

In *S. cerevisiae*, each component of the Hap complex is encoded at a single unique genetic locus. This is also true in humans and mice (Maity and de Crombrugghe 1998). However, in *Arabidopsis thaliana*, there have been 36 genes identified so far that encode components of the CCAAT-binding factor (Riechmann and Ratcliffe 2000, Gusmaroli *et al.* 2001, Gusmaroli *et al.* 2002, Siefers *et al.* 2008). *C albicans* possesses two genes that encode homologs of Hap3p, *HAP31* and *HAP32* that are both capable of rescuing a *Schap3*∆ respiratory deficient mutant. In addition, *C. albicans* encodes three distinct homologs of *HAP4* (Johnson *et al.* 2005).

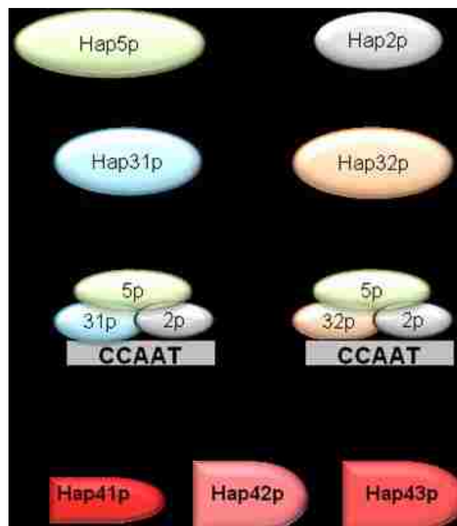

Figure 6-1. Multiplicity of the Hap complex. There are various possible combinations to form the CCAAT-binding complex.

The CCAAT binding complex in *C. albicans* consists of Hap2p, Hap5p, either Hap31p or Hap32p and either Hap41, Hap42p, or Hap43p (Figure 6-1). The composition of the complex is depended on environmental factors. Deletion of Hap2p, Hap5p or both Hap31p and Hap32p results in the loss of DNA binding. The *hap31Δ/hap31Δ* or *hap32Δ/hap32Δ* mutants show loss in DNA binding for non-overlapping CCAAT-binding complexes. This indicates that Hap31p and Hap32p interact with Hap2p and Hap5p to form separate complexes. Therefore, the *C. albicans* CCAAT-binding complex requires Hap2p, Hap5p and either Hap31p or Hap32p. The deletion of *HAP41* or *HAP42* did not show an effect on DNA binding under the growth conditions tested.

However, deletion of *HAP43* caused a decrease in overall DNA binding including both a loss and gain of complexes in extracts prepared from a hap43Δ homozygote grown under iron limiting conditions.

These complexes are differentially bound based on the environmental conditions. The most dramatic example of this observation is seen when cells are grown in iron replete (YPD) versus iron-deficient conditions (YPD + BPS). Hap32p is bound with Hap2p and Hap5p when iron is limiting; whereas Hap31p is preferentially bound under iron replete growth conditions. When either *HAP31* or *HAP32* is deleted, the Hap complex will bind DNA using the available Hap3 protein. This provides the explanation as to why the iron phenotype is not as strong with the *hap32Δ* homozygote as was seen in both the *hap2Δ/hap2Δ* and the *hap5Δ/hap5Δ* mutants. In iron-limiting conditions such as YPD with BPS, the complex with Hap2/5/32p is bound to DNA. In iron replete conditions Hap2/5/31p is bound to DNA. However, when Hap32p is absent, the Hap31p subunit is able to interact with Hap2p and Hap5p to create a functional CCAAT-binding complex. The reason for the redundancy in gene function for the Hap3p subunit remains unclear at present.

In *S. cerevisiae* Hap2p, Hap3p and Hap5p are required simultaneously for stable assembly (McNabb and Pinto 2005); whereas, the human NFY, NF-YB (Hap3p) and NFY-C (Hap5p) bind to form a stable dimer via a protein structure similar to a histone fold motif (Baxevanis *et al.* 1995, Zemzoumi *et al.* 1999, Romier *et al.* 2003). The NF-YB/C heterodimer then interacts with NF-YA (HAP2) and binds DNA (Maity *et al.* 1992, Sinha *et al.* 1995, Sinha *et al.* 1996). On the basis of our current data, the assembly of the CCAAT-binding complex in *C. albicans* mirrors that observed in the mammalian homologs. The Hap complex assembles first as a dimer consisting of Hap5p and Hap31p or Hap32p. Then Hap2p must bind to the dimer for DNA binding to occur.

The deletion of Hap43p caused a reduction in DNA-binding in iron deficient conditions as seen by electrophoretic mobility shift assays. There were three complexes that were clearly affected. Complex I that had been identified as containing Hap2/5/31p appeared in low iron conditions when Hap43p was not present. This complex was normally visible only in iron replete

conditions in the wild type strain. Complex III containing Hap2/5/32p disappeared in low iron conditions when Hap43p was not present, and the binding of complex V was dramatically reduced in the absence of Hap43p.

Northern blot analyses demonstrated that the Hap complex regulates expression of *CYC1* and *COX5a* and this is in part controlled by the availability of iron. *CYC1* transcription is high in iron replete conditions and is significantly reduced in iron deficient conditions. In the *hap2Δ/hap2Δ* and *hap5Δ/hap5Δ* mutants, transcription is affected in these conditions. For both mutants, *CYC1* is down-regulated in iron replete conditions and up-regulated in iron-poor conditions as compared to the wild type strain. *COX5* shows the same pattern in low versus iron replete conditions. In a *hap31*Δ homozygous mutant, the expression of these genes is only affected in iron replete conditions.

In the HAP4p deletions, only the *hap43*Δ mutant shows an obvious defect in *CYC1* and *COX5* expression. In iron-limiting conditions both *CYC1* and *COX5* are up regulated in the mutant when compared to the wild type. This corresponds to what is seen in the *hap2Δ/hap2Δ* and *hap5Δ/hap5Δ* mutants under these conditions; however, there is no effect under iron replete conditions.

The *C. albicans* CCAAT-binding factor plays a major role in the growth of cells under iron limitation. Deleting Hap2p, Hap5p, Hap43p or Hap31p and Hap32p causes a loss of growth on low iron medium. The addition of hemin to the iron-deficient media rescues growth. This indicates that the Hap complex must play a role in iron acquisition/utilization. The uptake and utilization of iron from an organic source, such as heme, is unaffected by the Hap complex, whereas the uptake of inorganic iron is affected. We have not evaluated whether the siderophore-dependent iron uptake is affected in these mutants.

The full effect of the multiple Hap4ps has not been seen, however, a direct effect of Hap43p has been established in iron-deficient environments. Since the two Hap3p subunits are able to compensate for each other under low iron conditions, the defining subunit in this growth condition appears to be Hap43p. Hap43p is required along with Hap2p and Hap5p for growth under iron limitation. Since Hap32p preferentially binds DNA under low iron conditions it must be

the subunit that preferentially interacts with Hap43p. Complex III has been identified as containing Hap2/5/32p. This complex is not seen when *HAP43* has been deleted from the strain; whereas, complex I (Hap2/5/31p) appears in the *hap43Δ* homozygous mutant. Loss of complex III is also seen in the *hap32Δ* homozygous mutant, along with an increase of complexes II and I. The *hap32Δ* homozygote is able to overcome iron-limiting growth conditions with only complexes II and I, whereas in the *hap43Δ* homozygous mutant these complexes are expressed at lower levels compared to the *hap32Δ/hap32Δ* mutant. Therefore, under iron-limiting conditions complex III (Hap32p) with Hap43p is optimal for growth; however when Hap32p is not available interactions between Hap43p and Hap31p can compensate. If Hap43p is absent neither Hap31p nor Hap32p can compensate, causing the inability of the strain to grow under iron limitation.

The decrease of the CCAAT-binding complexes bound to DNA in the absence of Hap43p implicates this component of the complex in maintaining either the stability of the complexes or the expression of the subunits. It is not currently known if the expression of *HAP31* or *HAP32* is affected in the absence of Hap43p.

The morphology of *C. albicans* colonies on solid medium are affected by carbon, pH, and nitrogen source. When *C. albicans hap2Δ, hap5Δ,* or *hap31Δ hap32Δ* homozygotes are grown on media where glucose is not provided, the cells become hyperfilamentous. The homozygotes switch to hyphal formation prematurely in colony growth before the wild type strain on the same media. Hyperfilamentation in these mutants is dependent on the absence of glucose.

The Hap complex has been shown in this research to be both an activator and a repressor. Under iron replete conditions, both *CYC1* and *COX5a* are normally up-regulated; however, in the absence of the Hap complex both of these target genes are down-regulated. Under iron deficient growth conditions, both *CYC1* and *COX5a* are normally down-regulated; however when the Hap complex is missing the genes are up-regulated.

Hap31p and Hap32p are both involved in the differential expression of *CYC1* and *COX5a* under iron replete conditions. Hap43p is involved in the differential expression of *CYC1* and *COX5a* under iron deficient conditions. Deletion of *HAP31* shows loss of activation: whereas deletion of *HAP32* shows the loss of repression. The deletion of Hap43p also shows the loss of

repression, leading to the hypothesis that Hap41p and/or Hap42p may be involved in transcriptional activation; however, that remains to be proven.

The Hap complex appears to function both in transcriptional activation and repression that is metabolically important under various environmental conditions or loss of virulence occurs. The presence of the multiple copies of subunits in *C. albicans* could explain this importance of function.

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