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The Role of CCAAT Binding Factor in the Regulation of Catalase Gene Expression in *Candida albicans*

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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May 2022  
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This dissertation is approved for recommendation to the Graduate Council.

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## Abstract

*Candida albicans* is a fungal opportunistic human pathogen. Its infections range from surficial infections like skin rash to fatal systemic infections. Filamentation growth mode is associated with *C. albicans* virulence because it helps penetration of the host's epithelial cells. The CCAAT-binding factor (CBF) is a conserved heterooligomeric transcription factor found in 30% of eukaryotes genes. In *C. albicans* it is composed of 4 major subunits, including Hap2, Hap3, Hap4, and Hap5. Hap2 and Hap5 are essential for DNA binding and function. Hap4 has 3 homologous subunits: Hap41 and Hap42 are putative subunits of CBP. Hap43 is the only Hap4 subunit known to associate with the Hap 2/5/31 or 32 complex, and it is involved in gene regulation in iron-dependent genes. Catalase, encoded by the *CAT1* gene, is an antioxidant enzyme that detoxifies ROS produced by the host's immune system, allowing *C. albicans* to survive in the host cells. Iron is an essential element involved in several physiological processes, including *C. albicans* virulence. The *CAT1* promoter includes 5 putative CCAAT binding sites; the first aim of this work was to investigate the actual binding site that is involved in catalase gene expression. Therefore, I created several plasmids that carry the *CAT1* promoter fused to Renilla luciferase as a reporter gene and integrated in the genome. Renilla luciferase activity was measured in iron rich and iron low media, and in response to H<sub>2</sub>O<sub>2</sub> treatment. The two most proximal CCAAT sites were sufficient to provide regulation equivalent to the full promoter, excluding the 3 most upstream sites. Interestingly, both sites appear to cooperate in *CAT1* expression, since individual deletions showed partial loss of regulation in rich and low iron conditions. Reporter activity after H<sub>2</sub>O<sub>2</sub> treatment did not show any difference between the two proximal CCAAT sites tested, and increased enzymatic activity was observed in WT and *hap5ΔΔ* strains, suggesting an activation independent of CBF. We concluded that there might

be other transcription factors, such as *CAP1*, that contribute to catalase regulation under peroxide stress.

The second aim was to determine whether the glutamine rich domain present in the Hap5 C-terminus has a transcription activation role. Plasmids expressing wild type Hap5, a *Hap5* with a C-terminal truncation, and empty vector were integrated at the *ARG4* locus, and I measured *CAT1* mRNA expression. The Hap5 truncation had a basic expression in rich medium, confirming the Hap5 glutamine rich domain activation role.

Because the hyphal growth mode is involved in *C. albicans* virulence, the third aim was to study the possible role of Hap4 subunits in yeast to hypha transition. I monitored Hap 4 subunits' phenotypes in various filamentation-promoting media. The strain carrying a homozygous *hap41Δ/Δ* showed a clear hyphal defective growth similar to *hap5Δ/Δ* and *hap2Δ/Δ* strains in Spider and M Lee's media, indicating that Hap41 may associate with CBF under specific physiological conditions, allowing us to establish the parameters for further investigation.

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## 1- Literature review

*Candida albicans* is an opportunistic commensal pathogenic fungus. It has been known since 400 BC when a famous Greek physician described it as a microbe that causes a thrush [1,2]. Scientists started to research *C. albicans* in the 1970s, and by the 1990s, the *C. albicans* genomic library was established [1]. Approximately 75% of the oral cavity is harbored by *Candida* species that is harmless in individuals with normal or healthy immune systems [3]. It affects immunocompromised individuals such as cancer, organ transplants, and HIV patients whenever it acquires some macronutrients, including carbon or micronutrients such as iron [4,5]. *C. albicans* infections vary from superficial mucosal infections, including mouth or vaginal rash, to severe systemic infections that tend to be deadly [6]. The mortality rate of systemic infections could reach up to 50%, and 25% of that is related to cardiovascular and bloodstream diseases [1]. In addition, it may be transmitted to the newborn babies through the mother after birth [6]. About 75% of women experience vulvovaginal candidiasis at least once during their lifetime, while about (5-8%) could develop recurrent vulvovaginal candidiasis [1,3]. The main morphological growth modes in fungal organisms are budding, such as *Saccharomyces cerevisiae* and the filamentous growth such as *Aspergillus nidulans* [2,7]. *C. albicans* is a dimorphic fungus that can switch from budding to hyphae phase. It is unique because it has several morphological forms, including hyphae, pseudohyphae, budding yeast, opaque cells, and chlamydospores [1,3]. Hyphal growth forms are primarily responsible for its virulence [3]. In addition to the morphological growth, biofilm formation plays a vital role in *C. albicans* virulence [1,3].



## CCAAT-binding factor

Genes' expression is regulated by different transcription factors. Transcription factor binding proteins (TFBSs) work as enhancers or repressors binding to a specific DNA site on the gene promoter, and they can be located as close as 50 bp to 500bp upstream the transcription start site [2,8]. TFBSs include several proteins families such as the Helix loop Helix, Helix turn Helix (HTH), Leucine zipper, homeodomain, and zinc finger proteins [9,10].

*Cis*-acting elements are located upstream eukaryote promoters; they are specific DNA sequences that are recognized and bound by trans-acting factors like TFBs as heterodimers, homodimers, or heteromeric complexes [9,11]. Heteromeric complexes bind to the DNA as different polypeptides in a specific manner [9]. There are several *trans*-acting sequences, such as the herpes simplex virus activator VP16 that forms different complexes with Oct-1, GA binding protein complex is also requires for VP16 activation, and the core binding protein complex, which regulates T-cell specific expression [9,11,12].

The CCAAT-binding factor is a conserved *trans*-acting regulatory factor that binds to the promoter consensus sequence CCAAT [9,13,14]. It is found among most eukaryotes from fungi, plants, and animals and in about 30% of higher eukaryotes [14,15].

The *S. cerevisiae* CCAAT-binding factor comprises four subunits, including Hap2, Hap3, Hap4, and Hap5 [9,14]. In *C. albicans* CCAAT-binding factor is composed of Hap2, Hap3, composed of 2 subunits Hap31 and Hap32, Hap4 that contains three different subunits, including Hap41, Hap42, and Hap43, and Hap5. [16]. In *Aspergillus nidulans*, it is composed of HapB, HapC, HapE, and HapX [17]. The homolog of CCAAT-binding factor in *Schizosaccharomyces pombe* is Php and is composed of Php1p, Php4, and Php5p [9,13]. In humans, the CCAAT-binding factor ortholog is called nuclear factor-Y NF-Y, consisting of three subunits, NF-YA,

NF-YB, and NF-YC [15]. In *S. cerevisiae* and *S. pombe*, CCAAT-binding factor regulates the respiratory metabolism genes, while in *C. albicans* in addition to regulating genes involved in respiration such as *CYC1* and *COX5*, it regulates the expression of the oxidative stress response (OSR) enzymes such superoxide dismutase (*SOD*), catalase (*CAT1*), thioredoxin (*TXR*), and glutathione redoxin (*GRX*) [9,14,16,18]. Hap2 homolog is NF-YA in humans, Php1p in *S. pombe*, or HapB in *A. nidulan*. Hap3 homolog is NF-YB and HapC, whereas Hap5 is the homolog of NF-YC, Php5p, and HapE [15,17]. The homolog of Hap43 in *A. nidulans*, *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Cryptococcus neoformans* is Hap X, and in *S. pombe* is Php4 [16,18]. The N-terminal of NF-YA and the C-terminal of NF-YC are rich in glutamine and hydrophobic amino acids required for gene activation [19]. In addition, CCAAT-binding factor is involved in iron hemostasis in *C. albicans*, *C. neoformans*, and *A. fumigatus*. NF-Y in humans is involved in regulating different genes, including cell cycle, cell apoptosis, tumor suppression, cell self-renewal, and lipid depositions [15].

In *S. cerevisiae* Hap2/Hap3/Hap5 are essential for the binding to the DNA promoters, while Hap4, which has an acidic activation domain, binds to the previous trimeric complex Hap2/Hap3/Hap5 and regulate the gene's expression [9,14,20]. In *C. albicans*, it has been postulated that Hap2 and Hap5 bind to the promoter in conjunction with Hap31 or Hap32. Consistently, deletion of either Hap2 or Hap5 abolishes the activity of the CCAAT-binding factor [11,16].

Hap 4 in *C. albicans* is represented by three homologous proteins: Hap41, Hap42, Hap43 [21]. Hap41 and Hap42 contain the N-terminal putative interacting domain, but Hap43 is the only subunit that contains a b-Zip domain and is similar to HapX and Php4 [21]. HapX and Hap4 are similar; the Hap4-like domain includes 16 amino acids domains required for Hap5

binding, and the basic region leucine zipper bZIP DNA binding domain is similar to Yap1 [18,20]. Both HapX and Hap43 contain three cysteine-rich protein domains that play a role in iron sensing process [16,17].

### **Iron homeostasis**

Iron is an essential element for all organisms, and it is involved as a cofactor in many physiologic processes such as electron transport chain and respiration [21–23]. Maintaining iron homeostasis is essential because the high iron level can be toxic to the cells, whereas low levels affect the cell functions [21]. For example, the interaction between iron and hydrogen peroxide through the Fenton reaction produces highly toxic free radicals [23]. Iron is an essential element in *C. albicans*' pathogenicity and plays a critical role in its virulence [24]. By nature, humans have low free iron levels in the blood because it is mainly bound to heme. Hap31 interacts with Hap2 and Hap5 in iron-rich media, while Hap32 subunit interacts with Hap2 and Hap5 in iron-depleted medium [15]. Hap5 and Hap32 expression was increased in iron-depleted medium in Hap43/Cap2 dependent manner, whereas Hap31 expression was repressed, but it is not Hap43/Cap2 dependent [24]. *C. albicans* showed loss of growth in iron-depleted media when Hap43 was deleted, whereas Hap41 and Hap42 didn't show any significant difference [21]. Not only the deletion of Hap43 affected *C. albicans*' growth under iron-depleted conditions, but also it attenuated its virulence in vivo [21]. Indeed HapX in *A. fumigatus*, *C. neoformans* as well as Hap43 in *C. albicans* is associated with their virulence [15]. The research showed that Hap43 works as an activator under iron-rich conditions and as a repressor under iron-depleted conditions [16,21]. Some genes involved in *C. albicans* oxidative stress, including *TSA1*, *GRX1*, *SOD5*, and *GSH1*, are upregulated in iron-depleted medium, while *CAT1* and *GSH2* are upregulated in iron-rich medium [23]. Previous studies in our lab showed that *SOD1*, *SOD2*, and

*SOD3* were expressed differently depending on iron availability, where their transcription levels increased under iron-rich as well as iron-depleted conditions in *hap5Δ/Δ* strains. Both *GRX2* and *GRX5* are regulated by the CCAAT-binding factor differently depending on iron availability. *GRX2* is repressed under iron-rich and depleted medium, whereas *GRX5* is activated in iron-rich medium and repressed under iron-depleted medium [16]. In contrast to *GRX5*, *TRX1* is repressed under iron-rich medium and activated in iron-depleted medium [16]. In addition, cytochrome C (*CYC1*) gene expression is activated and suppressed by the CCAAT-binding factor depending on iron levels [16,25]. Moreover, iron plays a significant role in genes sensitive to pH and those involved in the regulation of changes in cell morphology [23]. In addition, genes involved in microbe adhesion and invasion to the host cell, such as secreted aspartyl proteinases and lipases, are affected by iron availability where they are upregulated under iron-depleted medium [23].

### **Iron uptake and regulation**

*C. albicans* is a commensal pathogen that is naturally found in the human flora where iron is abundant, but it also can cause life-threatening systemic infections where the iron is depleted in the bloodstream [22]. Iron is rarely found free in the human body, which challenges the human pathogen microbes to cause infections because they need iron to grow [23]. It is found as transferrin in the bloodstream, sequestered as ferritin in cells, and lactoferrin in secretions [23,26]. A high affinity iron uptake regulatory network is found in prokaryotes and eukaryotes [23].

There are three systems in *C. albicans* to regulate iron uptake, including the high-affinity reductive uptake, siderophore uptake, and the hemoglobin uptake [22,24]. In addition, *C. albicans* is able to acquire iron from ferritin with the assistance of Als3 protein [24]. The

reductive uptake starts with two reductases found on the cell membrane as b-type heme proteins called FRE1 and FRE2 that reduce ferric chelates to ferrous ions [27]. The reduced iron form is transported through the cell membrane via a protein complex which is a copper-dependent system composed of a multicopper oxidase or ferroxidase CaFet3, a polytopic membrane permease (CaFtr1) which converts the ferric chelates to ferrous, and a copper transporter (CaCcc2) [26–28]. *Candida* ferric reductase-like Cfl95 then reduces the ferric to ferrous form [27]. The siderophore uptake system is copper independent with a single siderophore permease (CaSit1) responsible for ferrichrome uptake [27,28]. The hemoglobin uptake depends on three transcription factors, including the GATA factor *Sfu1*, CCAAT-binding factor, and *Sef1* in iron-dependent manner [22,24]. In iron-rich environments such as the humans' gut, *Sfu1* represses *SEF1* and iron uptake genes leading to HAP43 repression [21,22]. *SEF1* is a gene required for *C. albicans*' growth in iron-depleted environment, and it is also essential as a virulence factor due to its significant role in iron uptake [22]. *Sef1* activates Hap43 and iron uptake genes in an iron-depleted environment, while Hap43 represses *Sfu1* [22]. Both *Sfu1* and *Sef1* genes are required for iron uptake, while CCAAT-binding factor is responsible for iron utilization [22]. Rbt5 is an extracellular membrane protein that binds to the iron in heme and transfers it to the cell through diffusion via the cell membrane [22,28]. The repression of *Sfu1* in iron-depleted medium is regulated by Hap43/Cap2, while *Sfu1* represses Cap2 expression in iron-rich medium. Hap43/Cap2, which contains Yap-bZIP like and Hap4 like domains, is required for *C. albicans* cell growth in iron-depleted medium and its virulence as well. The expression of Hap43/Cap2 is repressed in iron-rich medium [24].

## Catalase

The *CAT1* gene encodes catalase, an antioxidant enzyme that detoxifies hydrogen peroxide by converting it to water and oxygen. The host immune system attacks the invading microbes by phagocytosis [4]. The phagocytotic cells such as neutrophils and macrophages kill the invading microbes by producing ROS [4]. Catalase is an essential factor in *C. albicans* virulence and pathogenicity because it helps to detoxify the ROS produced by the host cells and allows the fungal cells to survive and grow within the host cells to cause infection [4]. *C. albicans* are more susceptible to phagocytosis by the immune host system after *CAT1* deletion, yet it doesn't affect its morphology [4,29]. Catalase expression in *C. albicans* is negatively affected by glucose presence, where high glucose levels lower catalase expression [4]. In addition, previous studies showed increased *CAT1* expression after hyperosmotic stress exposure [30]. Under iron-rich conditions, *hap31Δ/Δ hap32Δ/Δ* strains couldn't survive after hydrogen peroxide treatment, but they were able to survive under iron-depleted condition. The survival of the strains under iron-depleted conditions was associated with increased levels of *CAT1* mRNA, which was repressed under iron-rich medium [16,30]. Hap43 is the only Hap4 subunit that regulates *CAT1* expression under iron-depleted medium, while none of the other Hap4 putative subunits is associated with *CAT1* regulation under iron-rich medium [16].

Cap1 is a basic leucine zipper transcription factor binding protein that belongs to AP-1 transcription activators, and it is an ortholog to Yap1 in *S. cerevisiae*. Both of them are involved in ROS tolerance in a similar manner [31,32]. AP-1 transcription family may work as pro-apoptotic or apoptotic factors, but their role in *C. albicans* apoptosis is not well understood[31]. Yap1 in *S. cerevisiae* is associated with oxidative stress response including the response to diamide and H<sub>2</sub>O<sub>2</sub> [33]. Cap1 involvement in oxidative stress response is evident as the deletion

of Cap1 limits *C. albicans*' survival when exposed to polymorphonuclear (PMN) [33–35]. A previous study showed that Cap1 attenuates apoptosis in *C. albicans* because apoptosis increased in *cap1* $\Delta/\Delta$ , accompanied by decreased intracellular ATP levels [31]. In addition, *CAP1* deletion attenuates *C. albicans* virulence and prevents germ tubes formation in filamentous yeast [36]. *CAT1* was activated in *C. albicans* after 0.5mM -5mM hydrogen peroxide, yet the 50mM treatment lead to a significant reduction in *CAT1* activity due to severe cell damage [37].

Catalase can also contribute to antifungal resistance. For example, it has been proposed that cancer patients who are treated with 2-chloroethyl-L -nitrosourea are more vulnerable to develop candidiasis because the drug increases *C. albicans* catalase activity and develops antifungal medicine resistance [38].

### **Pathogenicity and morphogenesis**

Several factors contribute to *C. albicans*' pathogenicity, including iron availability, morphogenesis, and host immune system. Neutropenia, a condition with abnormal neutrophils present in the blood, was found to play a significant role in *C. albicans*' pathogenicity [29,32]. Polymorphonuclear leukocytes (PMNs) and monocytes produce ROS such as hydrogen peroxide to kill any invading fungus, including *C. albicans*, by phagocytosis [29]. Therefore, *C. albicans* builds up a defense system to survive those free radicals by expressing antioxidants enzymes to detoxify the radicals [29]. Some examples of antioxidants produced by *C. albicans* are catalase, thioredoxin, cytochrome C, and superoxide dismutases [30].

*C. albicans* is a dimorphic yeast that goes through different growth modes: budding, filamentous, and pseudohyphae depending on the cell needs [36,39]. The budding yeast is associated with normal gut flora, yet the hyphal mode is associated with infections because the hyphae help epithelial cells penetration to escape from phagocytosis [2,39]. *C. albicans* is

unique because it can switch from budding yeast in normal condition to hyphal mode once it gets the proper environment. *C. albicans* cell wall structure is vital to help the switch from budding to filamentous and initiate the hyphal formation to invade the host. The cell wall structure is essential in the host immune system recognition [40]. *C. albicans* cell wall is composed of 2 layers; the outer wall is composed of cell wall proteins (CWPs), and the inner wall is made of carbohydrates [39]. In addition, CWPs expression is regulated by HWP1, Hyr1, and Als3 during the transition of *C. albicans* from yeast to hyphae growth mode, which facilitates the host cells invasion. At the same time, Als3 helps with host cell invasion and adhesion [2,39]. HWP1 is responsible for cell adhesion to the host cell, and secreted aspartyl proteinase (SAP) expression is associated with *C. albicans* virulence [36]. The morphological switch of *C. albicans* depends on different signal pathways, including cyclic AMP, cAMP-dependent protein kinase A (PKA pathway), mitogen-activated protein kinase A (MAPK), high osmolarity glycerol (HOG), and Rim101 transcription factor pathway [2,36,39]. High levels of cAMP are associated with hyphae tube formation, and its level is lower in budding than pseudohyphae yeast [36,39]. Filamentous yeast can form germ tubes that will develop hypha when they mature [2]. Some evidence indicates an association between hyphae formation, ROS, and calcium gradients [2]. The transition of *C. albicans* from yeast mode to the filamentous mode depends on nutrient availability and environmental conditions. For example, the serum nutrients, CO<sub>2</sub> and hypoxia, neutral pH, and increased temperature above 37° C, are associated with the hyphal formation and the initiation of infection [2]. Some of those factors affect the cAMP–PKA signaling and Cyr1 directly, such as the CO<sub>2</sub>, yet other signals such as glucose and amino acids act on it indirectly by the activation of small GTPases, Ras1 and Ras2, G-protein coupled receptor (Gpr1) that is susceptible to available nutrients such as methionine [2,39]. Low nitrogen levels facilitate



hyphal development through the MAP signaling pathway [39]. The Ras1-Cyr1 pathway is sensitive to a temperature higher than 35° [2,39]. The Hog1 pathway works as a repressor of hyphal elongation, and it is activated by oxidative and heavy metal stress [2,37]. In addition to the previous stressors that affect the yeast to hyphal transition, extracellular pH is controlled by Rim101, which is activated by the neutral or alkaline environment, yet it is inactivated under acidic environment; thus, it develops hyphae under the neutral or alkaline environment, and any mutation in this pathway would attenuate its virulence [2,39]. In addition, hydrogen peroxide activates hyphal development through a different signaling pathways called the Rad53 checkpoint kinase [39].

## 2- Introduction

*Candida albicans* is a commensal opportunistic pathogen found in the gut of humans' normal flora. It can cause superficial mucosal infections such as mouth rash or vaginal rash in healthy individuals. However, it can turn into life-threatening systemic infections in patients with compromised immune response. Immunocompromised individuals, including cancer patients, those with organ transplants, human immunodeficiency virus (HIV) patients, and individuals with indwelling medical devices, are more susceptible to develop *C. albicans* infections [41]. *C. albicans* is a polymorphic fungus that can grow as budding yeast, pseudohyphae, and hyphal growth mode. The hyphal mode is associated with *C. albicans* virulence because it helps to penetrate and colonize the host cells and cause infections.

Different factors contribute to *C. albicans* virulence and hyphal formation. One crucial factor contributing to *C. albicans* virulence is the micronutrient availability, such as iron and zinc [42]. Iron is an essential micronutrient involved in many major physiologic processes such as carbon and nitrogen metabolism and DNA repair [43]. Iron is involved as a cofactor in hydrogen peroxide detoxication by catalase. Furthermore, iron plays a significant role in *C. albicans* virulence because *C. albicans* needs iron to induce infection where the iron is typically low in the human body [42]. Therefore, *C. albicans* uses three different systems of iron uptake and acquisition from the host, including the high-affinity reductive system, the siderophore, which is required for *C. albicans* pathogenicity, and the heme uptake that is suggested to play a role in its survival against the host immune system [42].

Zinc is another essential micronutrient limited in the human body and involved in *C. albicans* virulence [42]. *C. albicans* has developed a system similar to siderophore for iron

acquisition called zincophore. This system uses a cell surface protein called Pra1 that binds to the host's zinc and allows uptake into the fungal cells [42].

The CCAAT-binding factor is a conserved heterotrimeric transcription factor among eukaryotes that binds to the ubiquitous CCAAT promoter sequence. In *Saccharomyces cerevisiae*, it is comprised of three subunits: Hap2, Hap3, and Hap5. A fourth subunit, Hap 4, is only present in fungal species and associates with the Hap2/3/5 heterotrimer. In *Candida albicans*, the CCAAT-binding factor also comprises three different subunits, Hap2, Hap3, and Hap5. Hap2 and Hap5 are essential subunits for DNA binding [14,44]. The deletion of either or both Hap2 and Hap 5 abolishes CCAAT-binding factor activity in *C. albicans*. Hap3 is present as two homologous proteins encoded by two different genes, referred to as Hap31 and Hap32 [14], and they behave differently according to iron availability. Hap4 is represented by three putative subunits: Hap41, Hap42, and Hap43, yet Hap43 is the only subunit known to participate as a component of the complex. Hap43 is involved in iron regulation in *C. albicans* and contributes to *C. albicans* virulence because it regulates iron uptake system genes, including *SEF1* and *SFU*, because Hap43 represses *SFU1* leading to the repression of iron utilization genes [22]. At the same time, *SFU1* represses *SEF1* which is involved in *C. albicans* virulence and survival in the bloodstream because of its iron uptake gene repression, yet *SEF1* activates Hap43 and iron uptake genes [22]. In addition, Hap43 regulates about 16% of *C. albicans* ORFs in an iron-dependent manner [44]. Indeed, Hap43 deletion showed decreased resistance to DNA damaging substances, nitrogen uptake, oxidative stress sensitivity, abnormal life span, and smaller cells compared to normal cells [44]. The CCAAT-binding factor in *C. albicans* plays a significant role in gene expression because it can work as a repressor and activator of different genes. Indeed, the CCAAT-binding factor may function as a repressor or an activator of the

same gene, depending on the growth condition. For example, it represses catalase expression in an iron-replete medium while it activates its expression in an iron-deplete medium. Catalase is an antioxidant enzyme that detoxifies hydrogen peroxide into water and oxygen within 60 minutes in *C. albicans* [45]. The catalase's high basal expression increases peroxide resistance in *C. albicans* [45].

The host's innate immune system is the first defense line against any invading pathogen, including *C. albicans*. The host immune system defends the body against *C. albicans* by phagocytosis and reactive oxygen species (ROS) expression. Polymorphonuclear leukocytes (PMNL) play an essential role in host body defense against *C. albicans* [46]. PMNL are involved in *C. albicans* phagocytosis produce toxic components that can kill the invading pathogens, including *C. albicans*, and induce inflammatory cytokines production at the infection site [41,46].

The cell wall structure of *C. albicans* contributes to its virulence because each component triggers a different element of the host immune system [41]. *C. albicans* cell wall is composed of two layers; the outer layer comprises mannoproteins, while the inner layer comprises carbohydrates including  $\beta$ -1,3- and  $\beta$ -1,6-glucans, and chitin [41,47]. The different components of *C. albicans* cell wall trigger the host immune system signaling pathways starting with the innate immune system and end up with the adaptive with the immune system. They are recognized by either the pattern recognition receptors (PRRs) or by pathogen-associated molecular pattern (PAMPs) [41,42,47]. The PRRs are divided into different groups, including C-type Lectin receptors (CLRs) and Toll-Like Receptors (TLRs), which are involved in *C. albicans* recognition [41,48]. TLR1, TLR2, and TLR6 recognize the acylated lipoproteins, while TLR4 is associated with O-linked Mannan recognition [41]. All TLRs pathways activate

pro-inflammatory signaling pathways and cytokines production and adaptive immune system activation except TLR9 that reduces cytokines production [41,42,47,48]. On the other hand, the CLRs include Dectins1, 2, and 3, mannose receptor (MR), macrophage-inducible C-type lectin (Mincle), and dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) [41]. Dectin1 is associated with the recognition of *C. albicans* inner layer  $\beta$  glucans and induces pro-inflammatory and anti-inflammatory cytokines production [47]. Indeed, some studies found that Dectin1 interacts with TLR2 to trigger pro-inflammatory cytokine production [47]. Also, Dectin 2 and 3 are responsible for detecting the alpha-mannan and high mannose structures in hyphae [41,42]. In addition to Dectins, both MR and DC-SIGN recognize N-Linked mannan and mediate different cytokines' production [41]. DC-SIGN plays a significant role in *C. albicans* recognition and internalization in dendritic cells and inhibits ROS production and NADPH oxidative pathway [41]. MR is associated with oligosaccharides such as fructose, mannose, GlcNAc, and N-bound mannans recognition in *C. albicans* [47].

*C. albicans* is a unique organism because it has different growth phases from yeast budding to pseudohyphae, and hyphal growth mode. The hyphal stage is associated with *C. albicans* virulence [49]. Different genes are responsible for hyphal formation, including HYR1 and HWP1 [49]. Several environmental factors play a significant role in a phenotypic switch, such as pH [49]. Genes sensitive to pH are regulated by the Rim101 transcription factor, which binds to specific genes, including PHR1 and PHR2 [49]. When the cells are grown under pH 6 or higher, Rim101 activates PHR1 related to hyphal growth, while growing cells under pH <5 will activate PHR2 [49]. In addition to pH, nitrogen availability in the growth medium also contributes to *C. albicans* virulence and phenotypic switch [49]. In *C. albicans*, GATA-type factors including Gat1 and Gln3 regulate a gene that encodes ammonium permease MEP2 that

allows cell growth when ammonium is the sole nitrogen source in the growth medium [49,50]. The deletion of either Gat1 or Gln3 prevents *C. albicans* cell filamentation growth mode due to the reduced expression of MEP2 [50]. MEP2 gene's promoter has a *cis*-acting sequence activated under the nitrogen starvation growth medium [50]. The deletion of MEP2 won't affect the growth of the cells in medium containing serum or low ammonium levels. Yet, it won't allow it to switch or start the hyphal formation in a limited nitrogen growth medium [50]. The above examples illustrate the complexity of the regulatory circuitry of some of the genes that affect *C. albicans* virulence and host-pathogen interaction.

The *C. albicans* *CAT1* gene encodes catalase, the antioxidant enzyme that detoxifies hydrogen peroxide into water and oxygen. I focused my research on investigating the putative CCAAT binding site within the *CAT1* promoter and how the CCAAT-binding factor interacts with *CAT1* promoter. Previous research in our lab has shown that different factors such as iron availability play a major role in CCAAT-binding factor role as enhancer or repressor and that hydrogen peroxide influences CCAAT gene regulatory mechanism. Therefore, I analyzed the *CAT1* promoter sequence by generating different plasmids with different mutated putative CCAAT binding sites driving the expression of Renilla luciferase as a reporter gene. I measured Renilla luciferase activity for each plasmid in different conditions, including iron-rich medium, iron-depleted medium, and hydrogen peroxide pre-treatment. Understanding the mechanism of CCAAT binding to *CAT1* would be beneficial to understand how CCAAT regulates gene expression and how we can apply that mechanism to different antioxidant enzymes and develop antifungal treatments. I also investigated the role of the Hap5 subunit in transcriptional activation. Based on the structure of *C. albicans* Hap5, its extended C-terminus relative to the *S. cerevisiae* Hap5 suggest a possible transcriptional activation domain. I generated plasmids that

contain Hap5 and *hap5* $\Delta/\Delta$ , and Hap5 with a C-terminal truncation to assess the contribution of the Hap5 C-terminus domain to gene expression by the CCAAT-complex. Finally, I wanted to study the phenotypes of *C. albicans* with different *HAP4* mutated subunits. The Hap43 subunit is essential for the CCAAT-binding factor role in gene expression of iron-dependent genes; however, there is no clear function for the putative Hap41 and Hap42 subunits as components of the CCAAT-binding factor. *C. albicans* hyphal growth is associated with host invasion, a phenotype that can be easily identified in different types of growth media. I studied the phenotypic growth of *hap5* $\Delta/\Delta$ , *hap2* $\Delta/\Delta$ , and *hap4* subunits in different growth media and recorded their growth and their behavior in each media.

### 3- Materials and methods

#### Strains and media

The strains used in this study are listed in Table 1. Uppercase letters represent the wild type, while the lower-case letters represent the mutated alleles. All strains were grown in yeast extract-peptone dextrose (YPD) for normal culture growth or iron-rich medium, while bathophenanthroline disulfonate (BPS) (Sigma) was used for iron-depleted medium. Synthetic complete, lacking Arginine (SC-Arg) was used for the selection of strains transformed with plasmid DNA to be integrated at the *ARG4* locus.

For phenotypic studies, the cells were grown in 5ml Yeast-Peptone-Dextrose (YPD) overnight. For DNA transformation, synthetic complete (SC) lacking arginine was used. Different media were used for phenotypic analysis, including SLAD, modified Lee's, Spider, and Medium M199 containing Earle's salts and glutamine but lacked sodium bicarbonate (Gibco-BRL) and containing 150 mM HEPES adjusted to pH 7.5. Medium 199 was prepared using the M199 powder, 150 ml of 1M HEPES in 500 ml H<sub>2</sub>O, and pH was adjusted to 7.5. SLAD was prepared by adding 17g yeast nitrogen base without amino acids or ammonium sulfate, 20 g of glucose, 5ml of 10 mM ammonium sulfate solution in 500 ml of H<sub>2</sub>O. Spider was prepared by mixing 1L of H<sub>2</sub>O, 20 g nutrient broth, 20 g mannitol, 4 g of potassium diphosphate (K<sub>2</sub>HPO<sub>4</sub>), and 27g Bacto agar. Yeast nitrogen base medium (YNB) was prepared using 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco) and 0.1% amino acid dropout powder containing 20 amino acids buffered to the indicated pH with 150 mM HEPES buffer. Glucose or lactate was added to media at a 2% final concentration as indicated, and media were solidified with 1.5% agar as appropriate and adjusted the pH to 5.6 or 6.8. The plates were incubated at 37°C, and pictures were taken daily to study hyphal formation.



**Table 1: Strains and genotypes**

Strain	Genotype	Reference
DMC146 Wild type	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-HIS1</i> <i>arg4Δ::hisG/arg4Δ::hisG-ARG4-URA3</i>	[16]
DMC108 <i>hap5Δ/Δ</i>	<i>ura3 Δ::imm434/ura3 Δ::imm434 his1 Δ::hisG/his1 Δ::hisG arg4 Δ::hisG/arg4 Δ::hisG</i> <i>hap5 Δ::URA3/hap5 Δ::HIS1</i>	[11]
<i>hap31Δ/Δ</i>	<i>ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG hap31Δ::HIS1/hap31Δ::URA3</i>	[25]
<i>hap32Δ/Δ</i>	<i>ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG hap32Δ::HIS1/hap32Δ::URA3</i>	[25]
<i>hap31Δ/Δ/hap32Δ/Δ</i>	<i>ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG hap31Δ::hisG/hap31Δ::hisG</i> <i>hap32Δ::HIS1/hap32Δ::URA3</i>	[25]
<i>hap41Δ/Δ</i>	<i>ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG hap4.1::URA3/hap4.1Δ::HIS1</i>	[16]
<i>hap42Δ/Δ</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-HIS1</i> <i>arg4Δ::hisG/arg4Δ::hisG-ARG4 hap42Δ::hisG/hap42Δ::hisG</i>	[16]
<i>hap43Δ/Δ</i>	<i>ura3Δ::imm434/ura3 Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG hap4.3::URA3/hap4.3Δ::HIS1</i>	[16]
<i>hap41Δ/Δ</i> <i>hap42Δ/Δ</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG-ARG4 hap41Δ::URA3/hap41Δ::HIS1</i> <i>hap42Δ::hisG/hap42Δ::hisG</i>	[16]

**Table 1: Strains and genotypes (Cont)**

Strain	Genotype	Reference
<i>hap41Δ/Δ</i> <i>hap43Δ/Δ</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG ARG4</i> <i>hap41Δ::URA3/hap41Δ::HIS1 hap43Δ::hisG/hap43Δ::hisG</i>	[16]
<i>hap42Δ/Δ</i> <i>hap43Δ/Δ</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-HIS1</i> <i>arg4Δ::hisG/arg4Δ::hisG-ARG4 hap42Δ::hisG/hap42Δ::hisG</i> <i>hap43Δ::hisG/hap43Δ::hisG</i>	[16]
<i>hap41Δ/Δ</i> <i>hap42Δ/Δ</i> <i>hap43Δ/Δ</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG-ARG4 hap41Δ::URA3/hap41Δ::HIS1</i> <i>hap42Δ::hisG/hap42Δ::hisG hap43Δ::hisG/hap43Δ::hisG</i>	[16]
WT <i>CAT1-Rluc</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-HIS1</i> <i>arg4Δ::hisG/arg4Δ::hisG-CAT1-Rluc-ARG4</i>	[16]
<i>hap5Δ-CAT1-Rluc</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::CAT1-Rluc-ARG4 hap5Δ::URA3/hap5Δ::HIS1</i>	[16]
WT <i>CAT1</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-HIS1</i> <i>arg4Δ::hisG/arg4Δ::hisG-CAT1-ARG4</i>	This study
<i>hap5Δ-CAT1</i>	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::CAT1-ARG4 hap5Δ::URA3/hap5Δ::HIS1</i>	This study
DMC108 + pIP 280	<i>ura3 Δ::imm434/ura3 Δ::imm434 his1 Δ::hisG/his1 Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::pIP280-ARG4 hap5 Δ::URA3/hap5 Δ::HIS1</i>	This study
DMC108 + pDM583	<i>ura3 Δ::imm434/ura3 Δ::imm434 his1 Δ::hisG/his1 Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::pDM583-ARG4 hap5 Δ::URA3/hap5 Δ::HIS1</i>	This study

**Table 1: Strains and genotypes (Cont)**

Strain	Genotype	Reference
DMC108 + pDM590	<i>ura3 Δ::imm434/ura3 Δ::imm434 his1 Δ::hisG/his1 Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::pDM590-ARG4 hap5 Δ::URA3/hap5 Δ::HIS1</i>	This study

## Plasmid construction

All plasmids constructs are summarized in Table 2. Plasmid pDM657 was regenerated from two plasmids, pDM659 that contains the Renilla luciferase gene, and pDM692, which contains *C. albicans ARG4*; both plasmids were digested with PvuII and AatII, but pDM692 was also digested with KpnI. DNA fragments (AatII/PvuII and pDM 692 were cut under long UV wavelength, cleaned using a QIAquick gel extraction kit (Qiagen, California, USA) and ligated using T4 DNA ligase (Promega) to generate pDM657. Catalase promoter was amplified by PCR using oDM623 and oDM624 (Table 3). The PCR product and pDM657 were digested with BamHI and SphI, and both were ligated with T4 DNA ligase, and then introduced into *E. Coli* (DH5 $\alpha$ ) using the standard transformation procedure as described later.

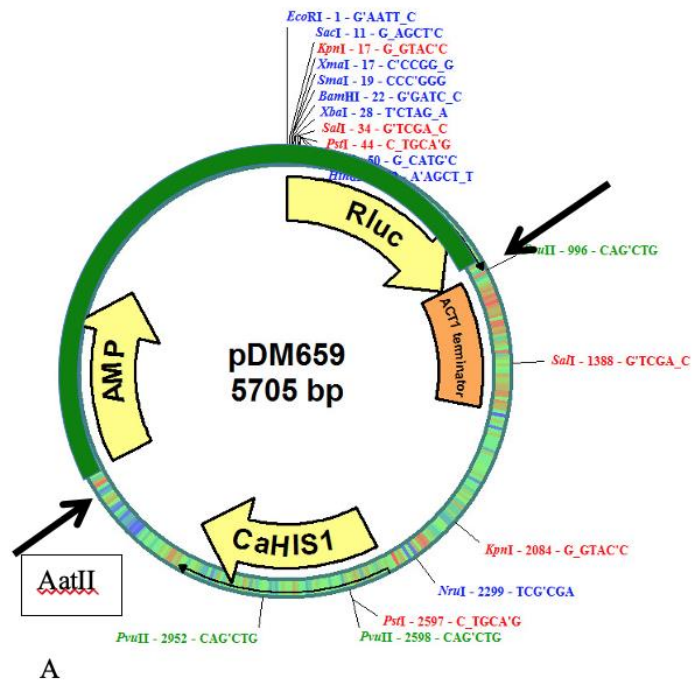


Figure 1:a is Plasmid

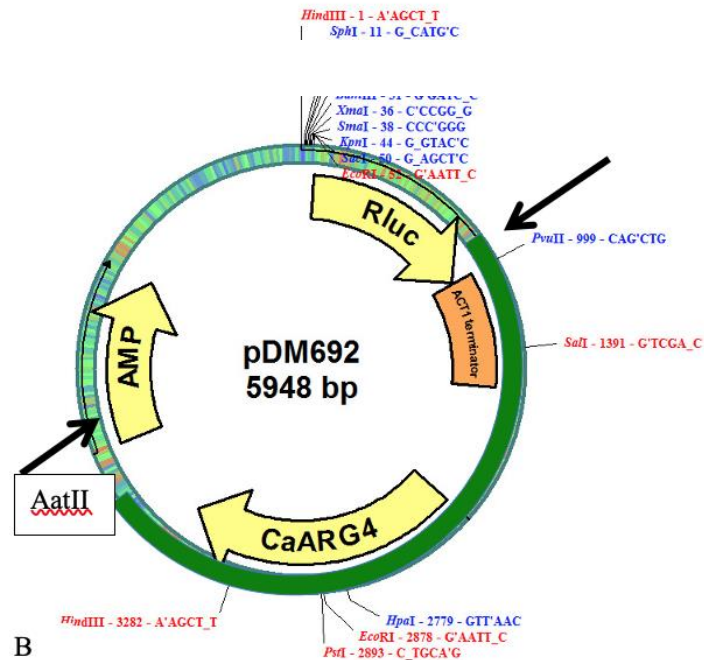


Figure 1: A) Plasmid 659 was used to excise Renilla luciferase promoter. B) Plasmid pDM692 was used to excise the *Arg4* locus and both pieces were ligated and cloned in *E. coli*.

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<Serial Cloner V2.5> -- <Jun 30, 2013 3:54 PM>
Restriction map of C albicans CTAl+1000 up
Showing restriction enzymes cutting maximum 1 time [using RELibrary as a Restriction Enzyme Library]
###

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10 20 30 40 50 60 70 80 90

taatgttgggaoaataaagttgattgotttttaoaoataototataotttttaogaoatgoataoataaaaaaaaoaaatgaototttoatta < 200
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110 120 130 140 150 160 170 180 190

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210 220 230 240 250 260 270 280 290

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410 420 430 440 450 460 470 480 490

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510 520 530 540 550 560 570 580 590

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610 620 630 640 650 660 670 680 690

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710 720 730 740 750 760 770 780 790

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810 820 830 840 850 860 870 880 890

taatottttttattaagaatatatattpotttatooatotoaattgatttagatotttttttttttttaatttoaattootatttatataaattattttatt < 1000
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910 920 930 940 950 960 970 980 990

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Figure 2: The full *CAT1* promoter in *C. albicans* showing all putative CCAAT binding sites in red boxes, and the putative TATA box is shown in a blue box.

pIP270 plasmid was generated by cutting both pDM657 and CAT1 with BamHI and SphI, excised, and cleaned using Qiagen gel extraction kit, and eluted DNA in H<sub>2</sub>O. The vector pDM657 and the CAT1 promoter were ligated using T4 DNA ligase. pIP271 plasmid, which contains 200 bp upstream of the catalase promoter, was generated by PCR amplification using oDM685, which includes BamHI site, and oDM624 that includes SphI cutting site. PCR product was digested by SphI, and pDM657 containing CAT1 was digested by BglII followed by DNA fragment ligation. pIP272 plasmid was generated by digesting pDM657 containing the CAT1 promoter by EcoRV and SmaI to generate a 500 bp upstream of the catalase promoter. To generate pIP273 plasmid, which contains 750 bp upstream of the catalase promoter, both oDM686 and oDM624, including BamHI and SphI cutting sites, were used, followed by ligation and *E. coli* transformation.

Next step was the generation of two plasmids with about 650 bp upstream of the catalase promoter one was with wild type pIP274, and the other one contained a mutated CCAAT binding site; pIP275. pIP274 was constructed using the forward oDM688 that contains BamHI cutting site and oDM624, while the mutated promoter, pIP275, was constructed using the mutagenetic forward primer oDM689 and oDM624. Both amplified PCR products were digested with BamHI and SphI.

pIP276 is similar to pIP273 but with a mutated CCAAT at -596 bp. Both oDM686 and oDM691 were used in the first PCR reaction to amplify the mega primer (183 bp) used in the second PCR reaction; then, the mutagenic primer oDM690 was used with oDM694 PCR reaction. Both PCR products were run on a gel and extracted, then cleaned using the Qiagen PCR gel extraction cleaning Kit. An extension cycle at 75°C for 15 minutes before running the regular second PCR cycle to ensure we had enough DNA extended after the first PCR. The PCR

program included initial denature cycle at 98°C for 30 seconds, followed 30 times repeated cycle of 98°C for 10 seconds, 56°C for 30 seconds and extension cycle at 72°C for 45 seconds, and final extension cycle at 72°C for 5 minutes.

Another mutated plasmid (pIP277) was constructed using the same previous method to induce a mutation of a potential CCAAT binding site in the catalase promoter at -240 bp using the following oligos oDM686 and the mutagens oDM693 at the first PCR cycle and the mutagens oDM692 and oDM694 at the second PCR cycle. The final PCR cycle used a 1:1 mix of the first and second PCR products as a DNA template with oDM686 and oDM694.

pIP278 include double CCAAT potential binding sites mutations at the catalase promoter were generated using pIP276 as the DNA template, and oDM686 that contain BamHI cutting site and oDM693 were used in the first PCR cycle, while oDM692 and oDM694, which include SphI cutting site, were used in the second PCR cycle to introduce the mutation at -240 bp of the catalase promoter. The final PCR reaction used a 1:1 mix of the 1<sup>st</sup> and the 2<sup>nd</sup> PCR products as the DNA template with oDM686 and oDM694 followed by an extension cycle at 75°C for 15 minutes then the regular PCR program 98° C for 30 sec and 29 repeated cycles as following 98°C for 10 sec, 56°C for 30 sec, 72°C for 45 seconds, and 72°C for 5 minutes. The final PCR product was cleaned and digested with BamHI and SphI.

pIP280 plasmid created a truncation of the C-terminal of *CaHAP5*. It was generated using oDM337 and oDM711, where oDM337 includes BamHI cutting site, and oDM711 is a reverse primer that creates a stop codon at amino acids 228 from ATG for *CaHAP5*. The first PCR reaction starts with an initial denaturation at 98° for 30 seconds and 30 cycles of 98° C 10 seconds, 56°C 30 seconds, 72°C 45°C 45 seconds, and 72°C for 5 minutes. The second PCR reaction follows the same program using oDM710, which creates a stop codon at amino acid 228



from *CaHAP5* ATG, and oDM712 makes XhoI at *CaHAP5*. The first and second PCR products were excised under the long UV wavelength, cleaned the gel with a Qiagen gel extraction kit, and eluted the DNA in H<sub>2</sub>O. The final PCR reaction used 1:1 of 1<sup>st</sup> PCR: 2<sup>nd</sup> PCR products were used with oDM337 and oDM712. Before running the previous PCR protocol, I ran an extension cycle at 75°C for 15 minutes. pIP280 was digested with BamHI and XhoI and ligated using ligase. To compare the results of *CaHAP5* mutation and the wild type results, I used pDM590, which includes the full *CaHAP5* and pDM583 that lacks *CaHAP5*.

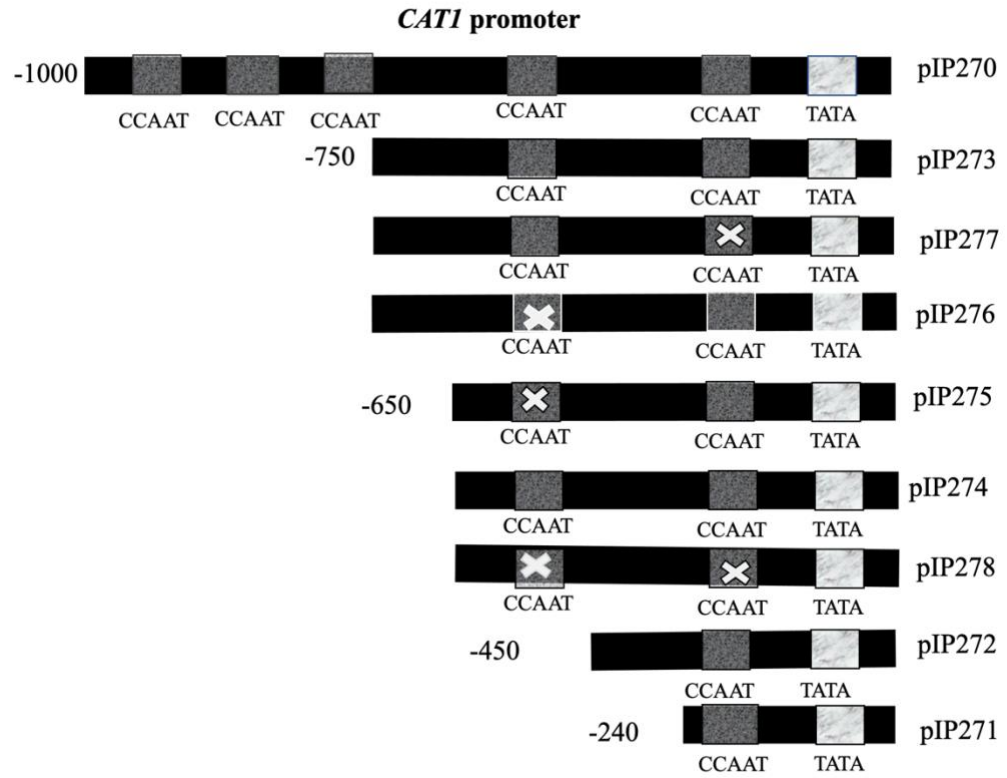


Figure 3: All plasmids used showing the putative CCAAT binding site and induced mutations sites.

**Table 2: Indicate the relevant features in each plasmid**

Plasmids	Relevant features	Backbone
pIP270	Contains the full <i>CAT1</i> promoter	pDM657
pIP271	Contains 240 bp upstream <i>CAT1</i> promoter	pDM657
pIP272	Includes 500bp was generated with EcoRV and SmaI	pDM657
pIP273	Includes 750bp of <i>CAT1</i> promoter	pDM657
pIP274	Includes 650bp of <i>CAT1</i> promoter (Wilde Type)	pDM657
pIP275	Includes 650bp of <i>CAT1</i> promoter (Mutated CCAAT)	pDM657
pIP276	Includes mutated CCAAT at -750bp of <i>CAT1</i> promoter.	pIP273
pIP277	Induced mutation in <i>CAT1</i> promoter at -240bp	pIP273
pIP278	Includes double CCAAT mutation at -750bp and -240bp of <i>CAT1</i> promoter	pIP276
pIP280	Includes the truncation in <i>CaHAP5</i> .	
pDM583	Vector used for HAP5 cloning.	
pDM590	Includes <i>HAP5</i>	

**Table 3: Used primers sequence, the highlighted regions indicate induced mutations sites**

Primer Name	Primer Sequence
oDM337	5'-GGCCGGATCCGGGTTGAAAGTCATTGCTGGTAG-3'
oDM512	5'-GGCCGGATCCCATGGAACAATCCGGAACAAC-3'
oDM513	5'-GGCCGGATCGCTGACCTCTTCTTCTTCTTC-3'
ODM623	5'- GGGGGATCCTAAACACAGAAATATGG-3'
oDM624	5'- GGGCGCATGCAATATATATAAATAGG-3'
oDM677	5'- GCCCAATCCAAAAGAGGTAT -3'
oDM678	5'- AGCTTCGGTCAACAAAACACTG3'
oDM685	5'-GGCCGGATCAATTCAATCTCTCTCCTTTGTAG-3'
oDM686	5'- GGCCGGATCCACATATATGAACTGGG-3'
oDM687	5'GTTATAACTATTTCGATTATTAACCTGG3'
oDM688	5'GGCCGGATCCCTGGGGAAGCCAAACAAAAAAAAAATTTCCAGCCA ATAATCGAATAGTT3'
oDM689	5'GGCCGGATCCCTGGGGAAGCCAAACAAAAAAAAAATTTCCAGTTAA TAATCGAATAGTT3'
oDM690	5' CAAAAAAAAAATTTCCAGTTAATAATCGAATAGTTATAACTAATAC 3'
oDM691	5'GTATTAGTTATAACTATTTCGATTATTAACCTGGAAATTTTTTTTTTG3'

**Table 3: Used primers sequence, the highlighted regions indicate induced mutations sites**

**(Cont)**

Primer Name	Primer Sequence
oDM692	5' AATACCCCTTGAAAGTTATTAATAAAATTTCAACAATTAC 3'
oDM693	5' GTAARRGTTGAAATTTTATTAATAACTTTCAAGGGGTATT 3'
oDM694	5' GGGCGCATGCAATAAATAATTTATATATAAATAGGAAATTG 3'
oDM710	5' AGAGAAGAGGAGAAACCTAAATAGTCCAATAAATCTGTCTG3'
oDM711	5' CGACAGATTATTGGACTATTAGGTTTCTCCTCTTCTCT3'
oDM712	5' GGCCctcgagGTGAGGAACTGAGAACTGATCTTACAAATAAGAGC3'

### ***E. coli* transformation**

Aliquots of 100  $\mu$ l of DH5 $\alpha$  CaCl<sub>2</sub> competent cells were mixed with 10  $\mu$ l DNA and mixed it gently. Incubated on ice for 20 minutes, followed by heat shock at 42°C for 2 minutes, and allowed them to recover on ice for 2 minutes before adding 1ml of LB. Incubated tubes for 30 minutes at 37°C, followed by a 30 second centrifuge spin. Finally, the pellet was resuspended in LB, and cells were plated on LB + ampicillin plates and incubated overnight at 37°C.

### ***Candida albicans* transformation**

All yeast transformation was done using the previously described lithium acetate transformation method [51]. Strains were linearized by HpaI to direct the plasmid integration into the *ARG4* locus. All new transformants were selected on a SC-Arg plates at 30°C. All plasmids were isolated with Qiagen plasmid Prep kit, and DNA was eluted in 50  $\mu$ l H<sub>2</sub>O.

### **Renilla Luciferase Assay**

I used the Renilla luciferase reporter assay system (Promega Corp., Madison, WI). All cell cultures were grown overnight in YPD to saturation, the next day transferred to a fresh media and incubated at 30°C. For iron-limited medium, 0.1 mM bathophenanthroline disulfonate (BPS) (Sigma) was added to the overnight culture and 0.2 mM BPS to the same day culture and both were incubated at 30°C until it reached 0.5 -1.0 OD at A<sub>600nm</sub>. For H<sub>2</sub>O<sub>2</sub> treatment, cells were treated with 50mM H<sub>2</sub>O<sub>2</sub> for 30- 45 minutes at 30°C, spun at max speed, and washed with water before starting the Renilla luciferase assay protocol. Then 1 ml of cells was centrifuged at 14,000 rpm for 1 minute, discarded the supernatant, and resuspended the cells in 100  $\mu$ l of 1X lysis buffer (Promega Corp.) 100  $\mu$ l of sterile glass beads were used to homogenize the cells. The samples were vortexed for one minute, cooled on ice for 30 seconds, vortexed for another minute, and then centrifuged at 14,000 rpm for one minute. A 10  $\mu$ l aliquot

was mixed with 100 µl of Renilla luciferase substrate, and luminescence was measured (Turner designs Luminometer model TD-20/20). The following formula was used to calculate the Renilla Luciferase activity:

$$RLA = \frac{RLU}{OD \times \frac{V_a \times V_c}{V_b}}$$

RLA is Renilla Luciferase activity, RLU is the initial Renilla Luciferase luminescence reading, OD is the optical density of the cell culture at A<sub>600nm</sub>, V<sub>a</sub> is the volume of the sample placed in the luminometer tube (0.01ml), V<sub>b</sub> is the volume of lysis buffer (0.1 ml), V<sub>c</sub> is volume taken from the original culture (1 ml).

Each experiment was conducted with a minimum of three independent colonies for each strain. The luciferase assay for each strain was repeated a minimum of three times.

### **RNA isolation**

All cells were grown at 30°C overnight in 5 ml YPD; then 200 µl of the overnight cells were transferred to a fresh 15ml YPD and allowed to grow until OD<sub>600</sub> reached 0.3. The cells were spun at 1500 rcf for 3 min, and frozen immediately at -80°C. Frozen pellets were treated with 800 µl Lysis buffer and 800 µl saturated acid phenol and incubated at 65°C for 45 min with multiple vortexing every 5 minutes. The mixtures were then centrifuged at max speed at 4°C for 10 minutes. The aqueous phase was transferred to a new tube with 800 µl saturated acid phenol, vortexed well, and then centrifuged at 5000 rpm for 5 minutes at room temperature. The aqueous phase was recovered to a new tube with 800 µl chloroform, vortexed well, and centrifuged at 500 rpm for 5 min at room temperature. The aqueous phase was transferred to a new tube, 75 µl of 3 M NaOAc (0.1 volumes) were added, and about 2.5 volume of 100% ethanol were added and allowed to precipitate at -20°C overnight. RNA was centrifuged at max

speed for 30 min at 4 °C, and the supernatant was discarded. Then the pellet was washed with 70% ethanol at max speed for 15 min at 4°C. Finally, pellets were resuspended in 100 µl TE, and RNA concentrations was measured using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) before they were frozen at -20°.

### **cDNA synthesis and Reverse Transcriptase Polymerase Chain Reaction (rt-qPCR)**

I used SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen- Life technologies. I added 1 µg of RNA to the provided dNTPs and primer, incubated the tubed at 65°C for 5 minutes, and then the tubes were placed on ice for 1 minute. The previous mix was added to the cDNA synthesis mix which includes 2 µl of 10X RT buffer, 4 µl of 25 mM MgCl<sub>2</sub>, 2 µl of 0.1 M DTT, 1 µ of RNAs OUT, and 1µL of super script (200 U/µL), then incubated for 10 minutes at 25°C followed by 50 minutes incubation at 50°C. The reaction was inactivated by placing the tubes at 85°C for 50 minutes and placed then placed on ice. 1 µl of RNase H was added and the tubes were incubated at 37°C for 20 minutes, cDNA was stored at -20°C.

### **qPCR**

I used Power Up SYBR Green Master Mix, Thermo Fisher Scientific. 1:10 dilution of cDNA was used, with 200 nM of Forward and Reverse primers and the SYBR Green Master mix for each reaction. All plates were sealed with clear BioRad tape, and a qPCR machine (BioRad CFX960) was used to run the cycles. For the housekeeping gene, I used *ACT1* with DM677 and oDM678 primers. For the catalase gene, I used oDM512 and oDM513. The program had initial denaturation at 90 °C for 30 seconds followed by 40 cycles of 90°C for 15 seconds, annealing at 57°C for 15 seconds and extension at 72°C for 30 seconds, and ended with a final extension at 72°C for 3 minutes.



Each experiment was conducted with at least three independent colonies per strain, with a minimum of two independent RNA extractions.

### **Statistical analysis**

Two sample  $t$  test was used for significance testing between individual mutants and wild-type or within strains under various treatments. Error bars represent standard errors of the means.

Statistically significant differences are depicted as asterisks above graph bars (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). Data were analyzed using GraphPad Prism.

### **Phenotypic analysis**

Light microscopy images of *C. albicans* colonies grown on different solid media were photographed using a Leica stereo microscope with a Canon camera. All images for the same set of media were taken at the same magnification. Each image is representative of colonies grown on at least two plates from independent batches of media.

## 4- Results

### *CAT1* promoter analysis

The aim of this project was to identify the CCAAT binding site (or sites) that may influence *CAT1* expression by the CCAAT-binding factor or Hap complex. I used plasmids containing the catalase promoter *CAT1* and Renilla luciferase as a reporter gene to measure catalase activity or expression. I measured the Renilla luciferase activity for *CAT1* in iron-rich medium (YPD), iron-deplete medium (BPS), and after hydrogen peroxide pre-treatment. It has been shown previously that the CCAAT-binding factor behaves on an iron-dependent manner in the regulation of genes that respond to iron availability. Since the full *CAT1* promoter contains 5 putative CCAAT binding sites, I generated different plasmids to identify which one is involved in catalase expression in *C. albicans*. Each plasmid was integrated at the *ARG4* locus in both wild-type and a *hap5Δ/Δ* *C. albicans* strains.

First, I measured Renilla luciferase activity in pIP270, which contains the full 1 Kb *CAT1* promoter, in wild type and *hap5Δ/Δ*. As shown in Figure 4, the activity was higher in wildtype compared to *hap5Δ/Δ* in YPD, indicating that the CCAAT-binding factor is working as an activator.

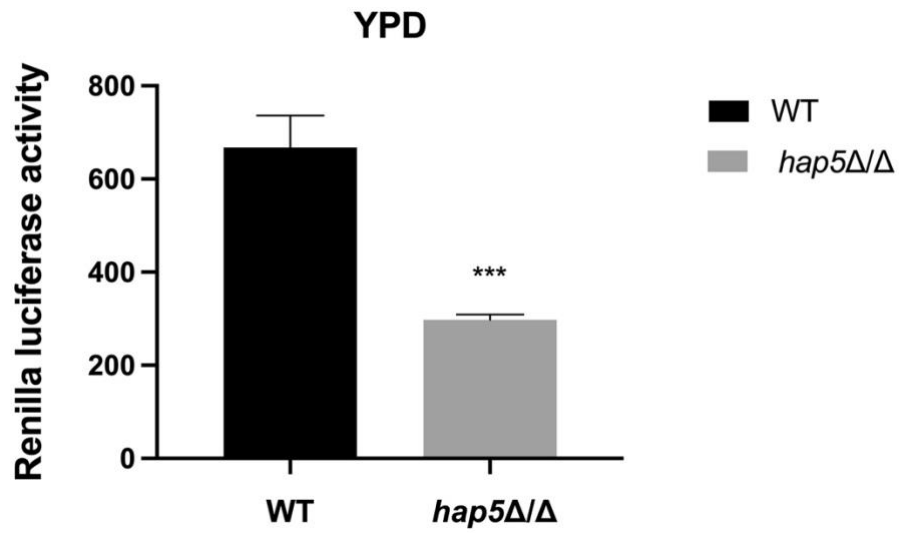


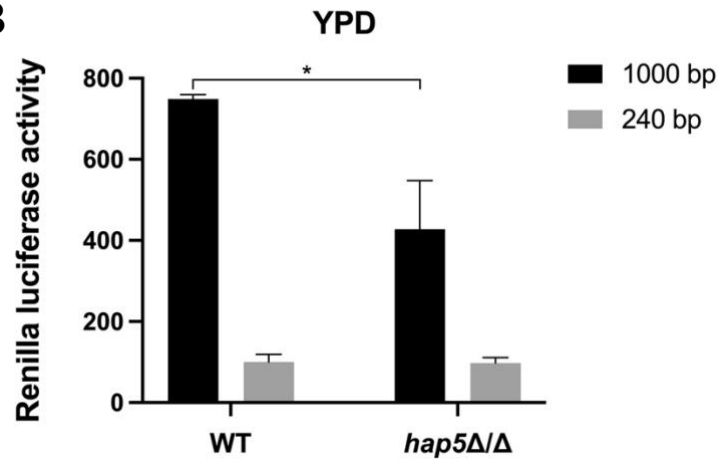
Figure 4: The Renilla luciferase activity for *CAT1* was measured in pIP270 in *C. albicans* wild type and *hap5Δ/Δ* in YPD (iron-rich) medium. The wildtype had higher activity than *hap5Δ/Δ* indicating that CCAAT binding complex works as an activator.

We hypothesized that the closest putative CCAAT binding site to the TATA box would be involved in catalase expression in *C. albicans*, so I generated pIP271 that contains 240 bp of *CAT1* promoter upstream of the TATA box. When I compared pIP270 and pIP271 Renilla luciferase activity in WT and *hap5Δ/Δ* strains in YPD (iron rich medium) and BPS (iron-deplete medium), pIP271 had a basic activity in both WT and *hap5Δ/Δ* in YPD. However, in BPS pIP270 showed a clear increased activity while pIP271 was close to its levels in iron-rich medium (Figure 5). Therefore, I concluded pIP270 provided the full promoter function for catalase expression. The wildtype strain showed that Hap5 works as an activator in iron-rich conditions and repressor in iron-deplete conditions, consistent with previous data. However, the strains with the pIP271 construct didn't show any change in Renilla luciferase activity in YPD. In contrast, in BPS, even though the levels of expression were significantly lower than those shown with the full-length promoter, the strains with the 240 bp promoter still showed derepression.

A



B



C

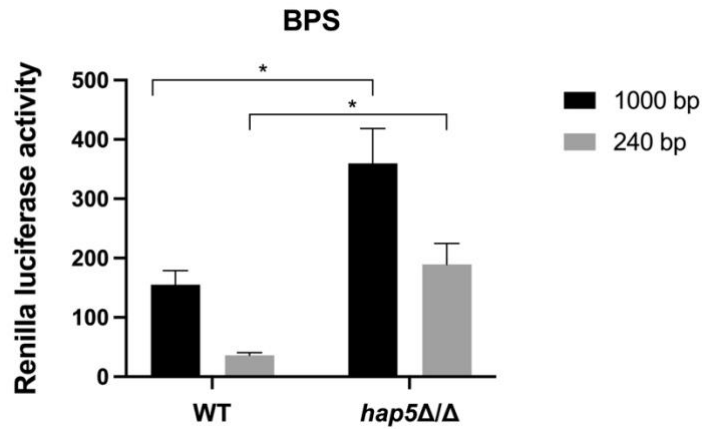
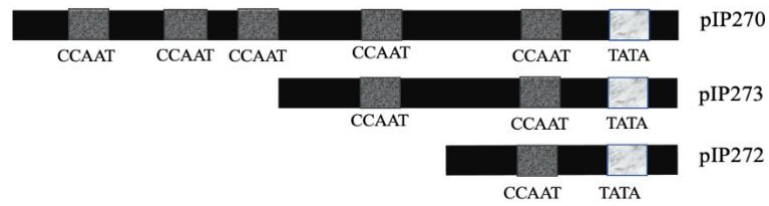


Figure 5: Renilla Luciferase activity for *CAT1* 1000 and 240bp in WT and *hap5Δ/Δ* in YPD and BPS. A) A diagram shows the plasmid used in this experiment. B) Renilla luciferase activity in YPD for the 1000bp was higher in WT and decreased in *hap5Δ/Δ*, while the 240bp activity was the same in WT and *hap5Δ/Δ*. C) The activity was repressed in WT and increased in *hap5Δ/Δ* for both 1000bp and 240bp.

Since the putative CCAAT binding site in pIP271 was impaired for catalase expression with respect to the 1 Kb promoter, I generated pIP272 that contains the first upstream putative CCAAT binding sites in *CAT1*, and pIP273 that contains the first 2 putative CCAAT binding sites excluding the 3 most upstream from the *CAT1* promoter. The strains with pIP272 and pIP271 had basic activity in YPD in WT and *hap5Δ/Δ*, while pIP273 was close to pIP270 (Figure 6). In BPS, the strains with pIP272 and pIP273 had basic activity levels in the WT, but the activity was increased in *hap5Δ/Δ* (Figure 7).

A



B

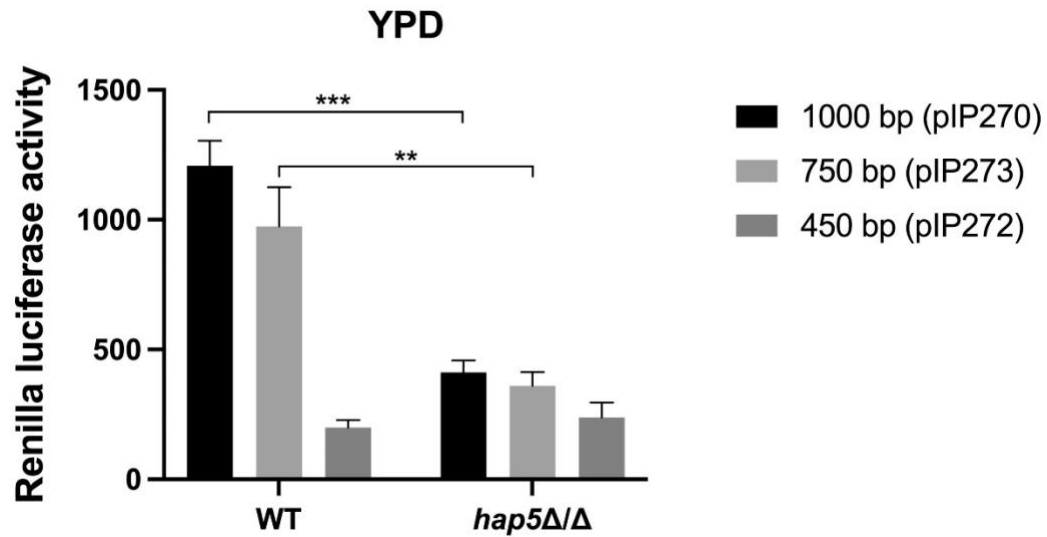
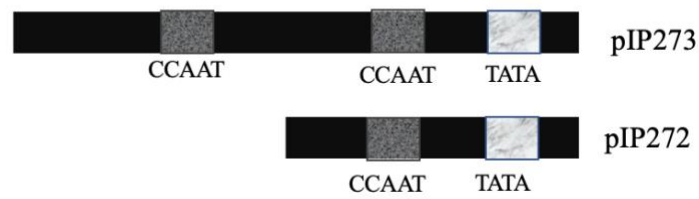


Figure 6: A) A diagram shows the plasmid used in this experiment. B) Renilla luciferase activity for pIP270, pIP272 and pIP273 in wild type and *hap5Δ/Δ* in YPD (iron-rich) medium. pIP270 and pIP273 behaved in similar manner in wild type and *hap5Δ/Δ* with higher activity in wild type and decreased activity in *hap5Δ/Δ* while pIP272 had basic activity in wild type and *hap5Δ/Δ*.

A



B

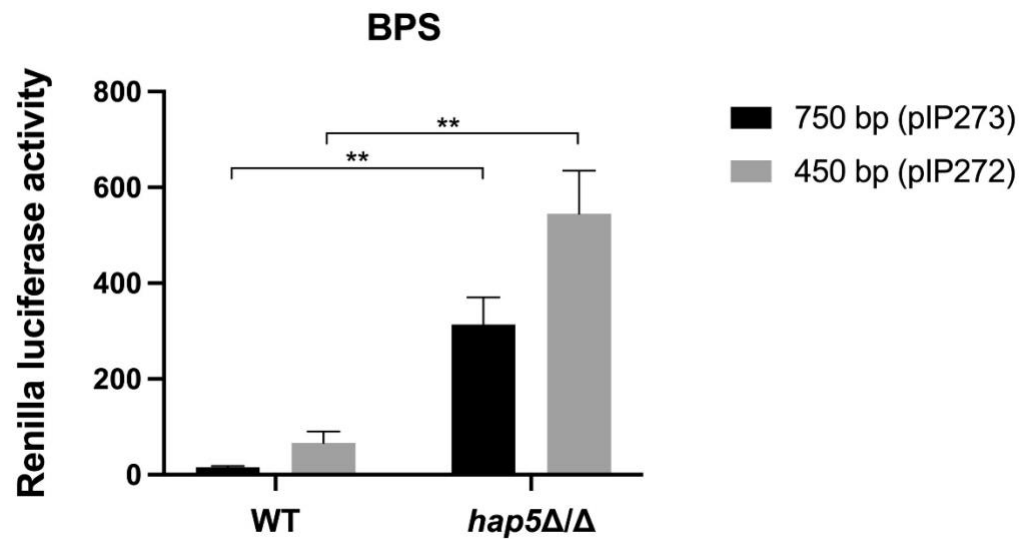
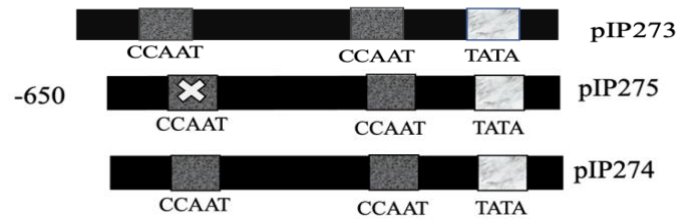


Figure 7: A) A diagram shows the plasmid used in this experiment. B) *CAT1* expression was measured by Renilla luciferase assay in BPS (iron-deplete) medium for pIP273 and pIP272 in wild type and *hap5Δ/Δ*. Both plasmids had low activity in wild type and increased activity levels in *hap5Δ/Δ*.

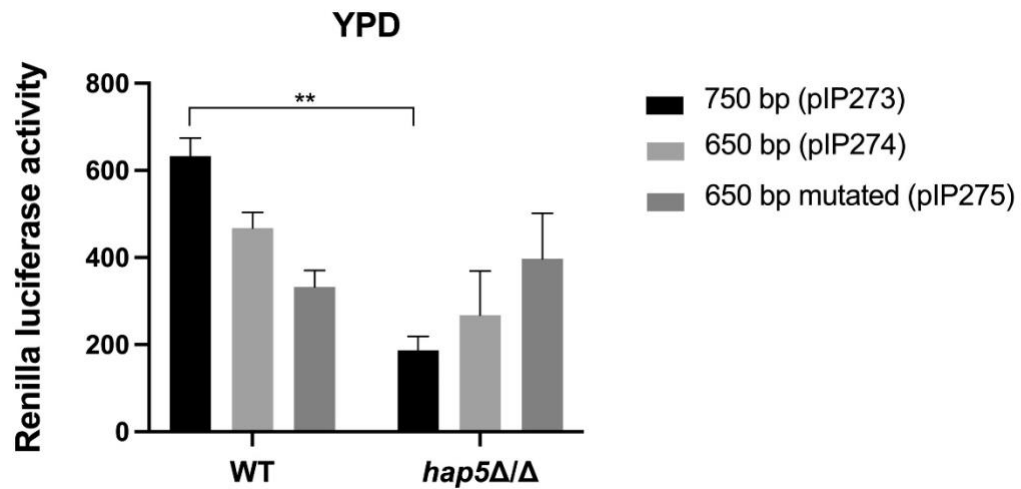


Next, I generated 2 plasmids containing 650 bp of *CAT1* promoter that included two CCAAT sequences, the most proximal to the ATG start codon and the second upstream, located at -596 bp. Plasmid pIP274 was the wild type, and pIP275 contains a mutated CCAAT (TTAAT) at -596 bp. Renilla luciferase activity in strains with pIP274 and pIP275 was measured in YPD and BPS in WT and *hap5Δ/Δ*. Both plasmids showed similar activity levels in YPD but lower than pIP273. Strains with pIP274 and pIP275 showed a slightly increased activity in *hap5Δ/Δ* strains compared to pIP273 but did not show decreased activity respect to the WT, indicating lack of activation in YPD. In BPS, strains with pIP273 and 274 showed derepression in iron-deplete medium when the Hap complex was absent. In contrast, strains with pIP275 showed a mild derepression but failed to be properly repressed in the WT strain (Figure 8), indicating that the CCAAT at -596 is functional for the binding of the Hap complex required for *CAT1* activation as well as repression.

A



B



C

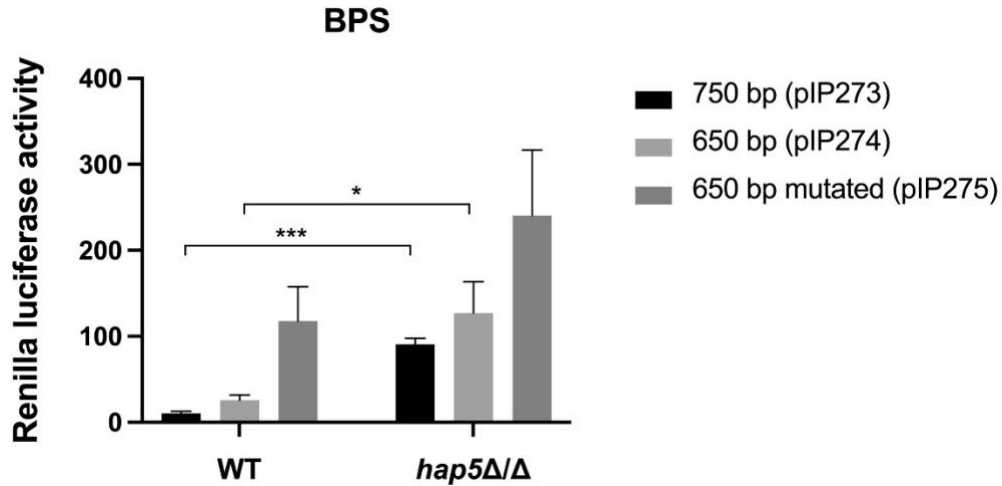


Figure 8:A) A diagram shows the plasmid used in this experiment. B) Renilla Luciferase activity in YPD for pIP273, pIP274, and pIP275 for WT and *hap5Δ/Δ*. pIP273 had a clear activity reduction in *hap5Δ/Δ*, while pIP274 and pIP275 activity was similar in WT and *hap5Δ/Δ*. C) Renilla luciferase activity in BPS for all plasmids were repressed in WT and increased in *hap5Δ/Δ*.

Later, I generated 2 plasmids with CCAAT induced mutations, pIP276 contained a mutation in the most upstream CCAAT binding site in pIP273 (-750), and pIP277 had a mutation in the CCAAT closest to the TATA box (-240). pIP278 contained double mutated CCAAT binding sites at -750 and -240bp. I compared Renilla luciferase activity for strains containing pIP276, pIP277, and pIP278 in WT and *hap5Δ/Δ*, in YPD and BPS. The individual mutants (pIP276 and pIP277) showed similar activity to the full-length promoter (pIP273) in the WT strain, but the double mutant was about half of the strain with pIP273. Minimal loss of activation is seen in the *hap5Δ/Δ* strain with pIP277, but the strain with both CCAAT boxes mutated shows expression levels independent of the presence or absence of the Hap complex. In BPS, Renilla luciferase activity for all plasmids in the WT strain had background level activity and increased activity in *hap5Δ/Δ* strains. Interestingly, pIP278 activity was close to pIP273 (Figure 9).

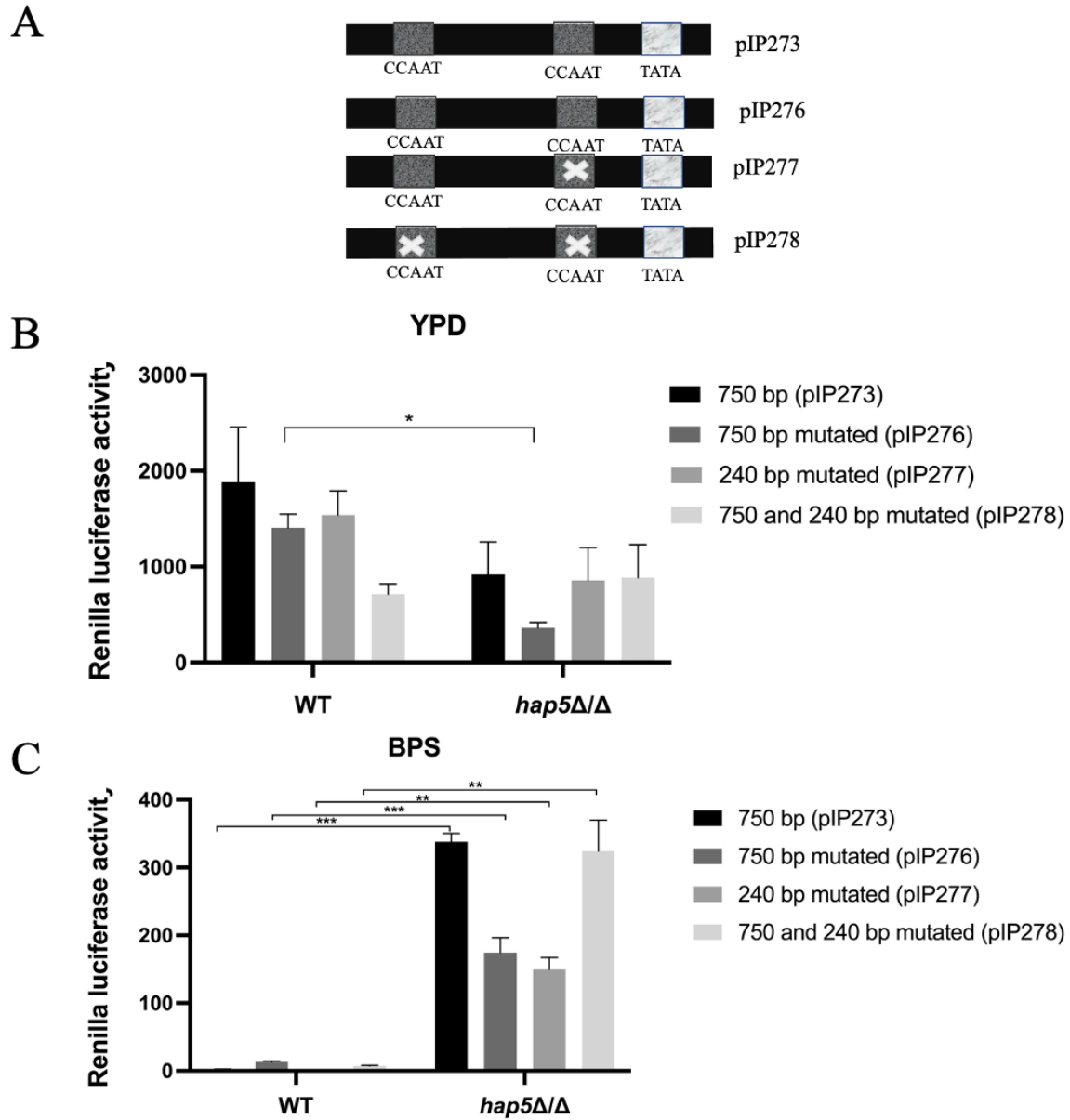


Figure 9: A) A diagram shows the plasmid used in this experiment. B) pIP273, pIP276, pIP277, and pIP28 in WT and *hap5Δ/Δ* Renilla luciferase activity for *CAT1* was measured in YPD, and pIP278 was the lowest while pIP276 and pIP277 were similar but lower than pIP273 in WT. In *hap5Δ/Δ* pIP273, pIP277, and pIP78 had similar activity levels, but pIP276 was the lowest. C) Renilla Luciferase activity in BPS. In WT all plasmids showed background level activity, and increased activity levels in *hap5Δ/Δ*.

### **Effect of hydrogen peroxide pre-treatment**

Because catalase detoxifies hydrogen peroxide, after I tested the plasmids' activity in YPD and BPS, I picked the plasmids where CCAAT boxes showed clear contribution to gene expression and tested them with hydrogen peroxide pre-treatment. Therefore, I used pIP273 and pIP272 in WT and *hap5Δ/Δ* (Figure10). In WT and *hap5Δ/Δ* both pIP272 and pIP273 showed increased Renilla luciferase activity after hydrogen peroxide treatment which indicates that the CCAAT-binding factor doesn't affect catalase expression in high hydrogen peroxide environment.

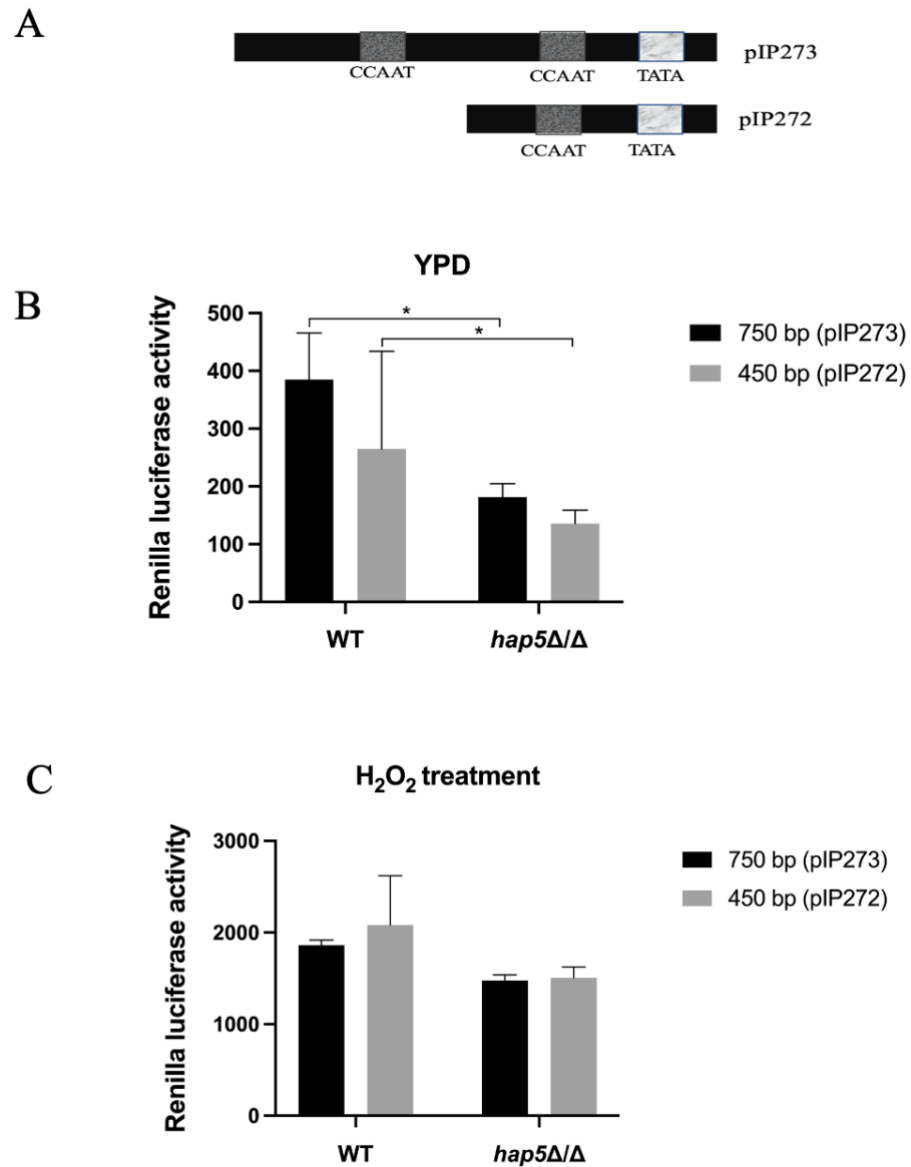


Figure 10: A) A diagram shows the plasmid used in this experiment. B) Renilla luciferase activity in YPD for pIP273 and pIP272 in WT and *hap5Δ/Δ*. Both plasmids had a clear reduction in *hap5Δ/Δ*. C) Renilla luciferase activity after 5mM  $H_2O_2$  for 30 minutes treatment showed similar activity in WT and *hap5Δ/Δ*.

### **Involvement of the Hap5 C-terminus in transcriptional activation**

I used 3 plasmids integrated at the *ARG4* locus of a *C. albicans* strain carrying a homozygous deletion of the *HAP5* gene (*hap5Δ/Δ*). I measured catalase expression using qPCR. Plasmid pDM590 contains wild type *HAP5*, pDM583 is the vector without *HAP5*, so the strain remains *hap5Δ/Δ*, and pIP280 contains a *HAP5* truncation at the 3'-end. We hypothesized that the C-terminal in Hap5 might have an activation role; therefore, catalase expression could decrease in the truncation, or it might be at similar levels to pDM583. The results proved our hypothesis correct, because pIP280 expression levels were almost identical to the *hap5Δ/Δ* in pDM583, and significantly lower compared to the WT pDM590 indicating that pIP280 failed to activate catalase expression in YPD (Figure 11).

Previous research in our lab showed that Hap5 works as a repressor in iron low medium (BPS), so we expected that catalase expression levels would increase in pDM583 while pIP280 should have a basic expression. The results showed that wild type pDM590 had a basic expression level as expected for repression in iron-depleted conditions, and *hap5Δ/Δ* pDM583 catalase expression was derepressed in the absence of *HAP5* (Figure 11). Interestingly, pIP280 behaved just like the WT, indicating that the Hap5 truncation was only affecting activation but not repression.

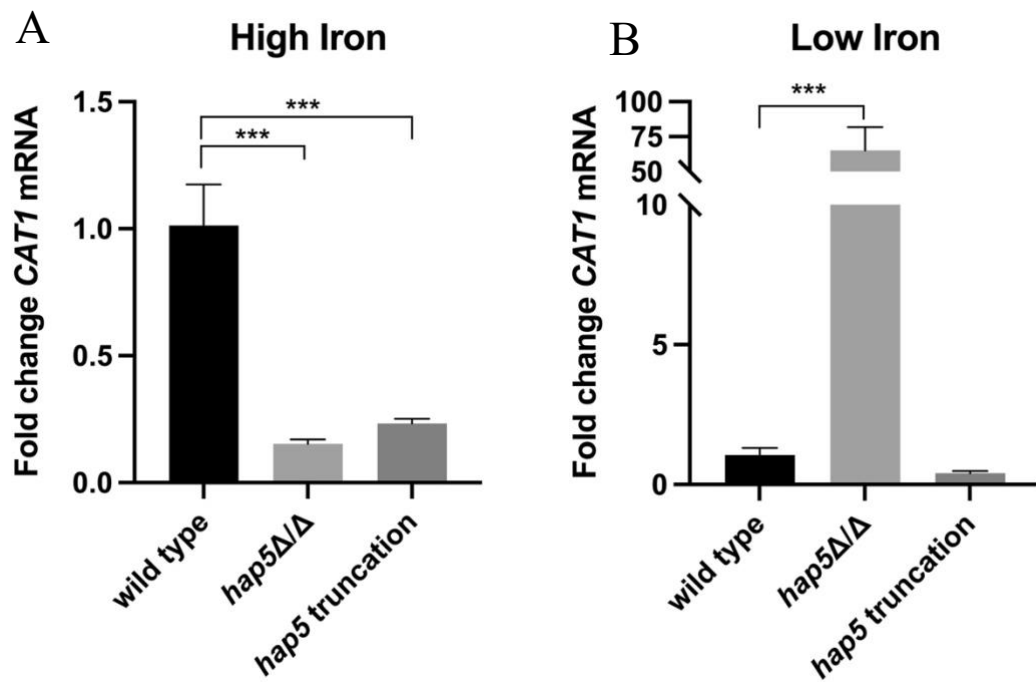


Figure 11: A) *CAT1* mRNA fold change in WT, *hap5Δ*, and *hap5* truncation in high iron was higher in wild type than *hap5Δ/Δ* and *hap5* truncation. B) *CAT1* mRNA fold change was extremely low in wild type and *hap5* truncation compared to *hap5Δ/Δ*.



Finally, I tested the effect of hydrogen peroxide in the regulation of catalase expression. There was no significant difference in *CAT1* mRNA expression between the *HAP5* WT, *hap5* $\Delta/\Delta$  and *hap5* truncation, indicating that activation by Hap5 is not required for the induction of *CAT1* mRNA in response to hydrogen peroxide treatment (Figure 12).

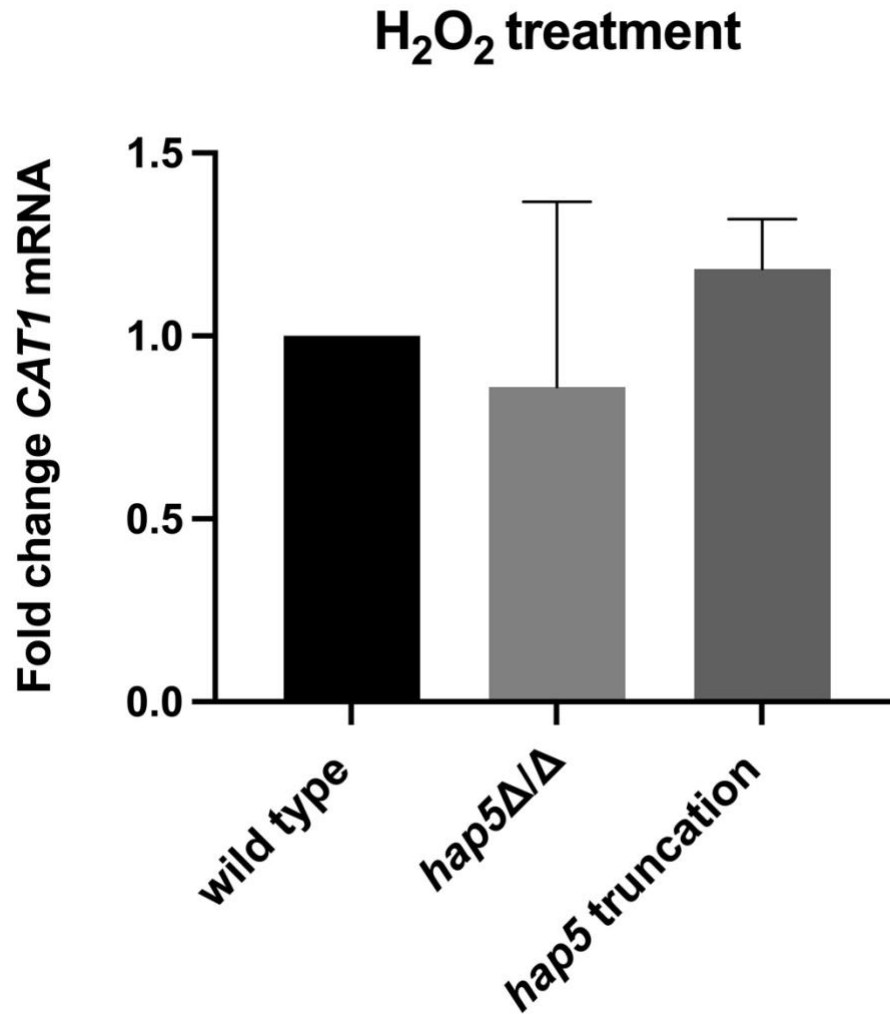


Figure 12: *CAT1* mRNA fold change for *HAP5* wild type, *hap5Δ/Δ* and *hap5* truncation in YPD and 5mM H<sub>2</sub>O<sub>2</sub> for 30 minutes. *CAT1* mRNA levels didn't show a clear difference between all strains which indicates that *CAT1* expression is not Hap5 dependent.

## Phenotypic analysis

*C. albicans* is known of its dual growth phenotypes because it can transit from yeast to hyphal mode. The hyphal growth mode is associated with infection initiation, and host's invasion. Hap4 has 3 subunits including Hap41, Hap42, and Hap43. Hap43 was the only subunit that was involved in iron response [21]. Hap41 and Hap42 haven't been studied well. Here we tried to study their role in yeast to hypha transition. I used 4 media that are known for inducing filamentation, including SLAD, M. Lee's, SPIDER, and M199 to investigate the role of Hap4 subunits, Hap2, and Hap5 in *C. albicans* and yeast to hypha transition. I grew the strains in SLAD plates that are low in nitrogen because it lacks amino acids, all strains showed slower growth and smaller sizes, yet all of them developed filamentation at day 9 except *hap5* $\Delta/\Delta$  (Figure 13).

The same strains were grown in Spider solid agar plates and incubated at 37°C for 6 days and only the wild type, *hap42* $\Delta/\Delta$  and *hap43* $\Delta/\Delta$  were able to form wrinkles while *hap42 43* $\Delta/\Delta$ , *hap41 42 43* $\Delta/\Delta$ , *hap2* $\Delta/\Delta$  and *hap5* $\Delta/\Delta$  developed filamentation but the colonies were smaller in size. *Hap41* $\Delta/\Delta$  was white and smooth (Figure 14).

M. Lee's induced filamentation in *HAP5* wild type, *hap42* $\Delta/\Delta$ , *hap43* $\Delta/\Delta$ . Even though *hap41 42* $\Delta/\Delta$  and *hap42 43* $\Delta/\Delta$  were smaller, they were able to develop filamentation, yet *hap41* $\Delta/\Delta$ , *hap41 43* $\Delta/\Delta$ , *hap41 42 43* $\Delta/\Delta$  and *hap5* $\Delta/\Delta$  were white and smooth with no hypha or wrinkled surface, which suggest that *HAP41* contributes to yeast to hypha transition in carbon source starvation (Figure 15).

M199 medium was able to induce hypha formation in all strains except *hap43* $\Delta/\Delta$ , but the wild type, *hap42* $\Delta/\Delta$  and *hap41 42* $\Delta/\Delta$  had regular wrinkled colonies while *hap 41* $\Delta/\Delta$ , *hap41*

*hap43Δ/Δ*, *hap43Δ/Δ*, and *hap41 42 43Δ/Δ* has a circular wrinkled ring. *Hap2Δ/Δ* was the smallest and slowest to grow while *hap43Δ/Δ* was white with no filamentation or wrinkles (Figure 16).

In addition to hyphal inducing media, I used YNB + AA at pH5.6 and pH6.8, YNB-glucose +AA at pH5.6 and pH 6.8, and YNB-Lactate +AA at pH5.6 and pH6.8 to investigate how the carbon source and the pH affect yeast to hypha transition. YNB +AA pH5.6 the wild type, *hap41Δ/Δ*, and *hap5Δ/Δ* were able to form hypha, yet *hap42Δ/Δ* and *hap43Δ/Δ* were smaller in size and less hyphae. *Hap42 43Δ/Δ* and *hap5Δ/Δ* were opaque with no filamentation indicating the importance of *HAP42* and *HAP43* in hyphal formation in this media (Figure 17).

However, when I grew the strains on YNB + AA at pH6.8 plates and incubated them for 5 days, they behaved differently where the WT had the most filamentation, followed by *hap41 42Δ/Δ*, *hap41 43Δ/Δ*. *Hap42Δ/Δ*, and *hap41 42 43Δ/Δ* were similar in size and filamentation formation. *Hap2Δ/Δ* didn't show any filamentation (Figure 18).

Later, we wanted to investigate if the carbon source would induce yeast-hyphal transition. Therefore, I grew the strains in YNB-Glucose +AA at pH5.6 and pH6.8 and YNB-Lactated at pH5.6 and pH6.8. Adding glucose to YNB +AA didn't rescue hyperfilamentation because all strains were white and smooth (Figure 19).

YNB-Glucose +AA at pH6.8 all strains were able to develop hypha and wrinkled except *hap41Δ/Δ* and *hap42Δ/Δ*; they grew slower with less filamentation and flat surface (Figure 20).

We wanted to test if the pH or carbon source affects the yeast to hypha transition. The poor carbon and acidic environment at pH5.6 defects hyperfilamentation in *hap41Δ/Δ*, *hap41 42Δ/Δ*, *hap41 43Δ/Δ*, *hap42 43Δ/Δ*, and *hap5Δ/Δ*. Only the wild type and *hap43Δ/Δ* developed hypha with wrinkles while *hap42Δ/Δ* and *hap41 42 43Δ/Δ* developed filamentation with a flat surface (Figure 21). YNB-Lactate +AA at pH6.8 induced filamentation in *hap41Δ/Δ*, *hap42Δ/Δ*,

suggesting that pH influences *hap41* and *hap42* filamentous growth. *Hap5Δ/Δ* was smaller than pH.5.6 but was able to develop filamentation (Figure 22).

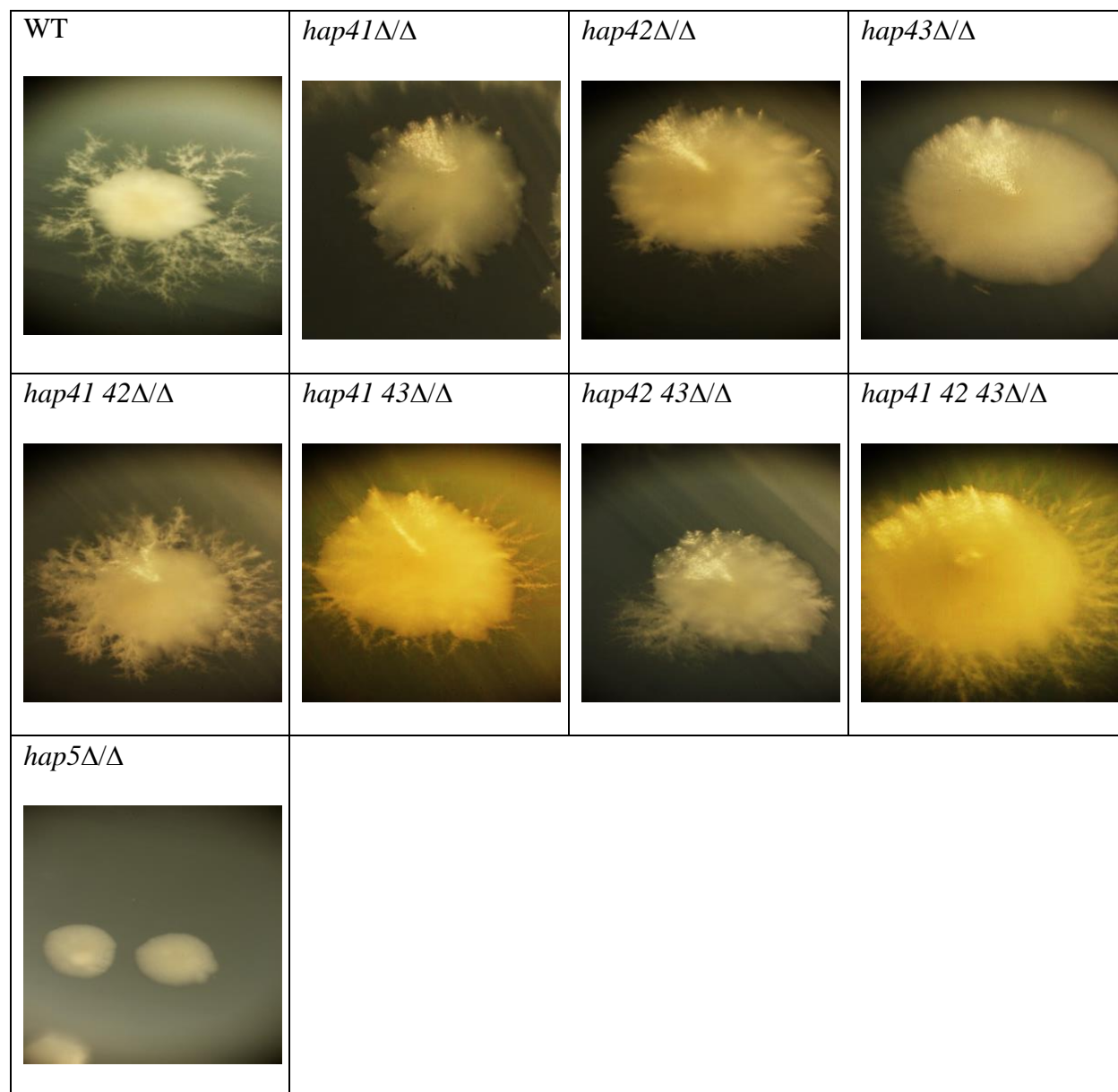


Figure 13: Wild type, *hap4* putative subunit mutants, and *hap5*Δ/Δ were grown in SLAD plates for 9 days at 37°C and pictures were taken at the same magnification. All strains developed hypha after 9 days except *hap5*Δ/Δ.

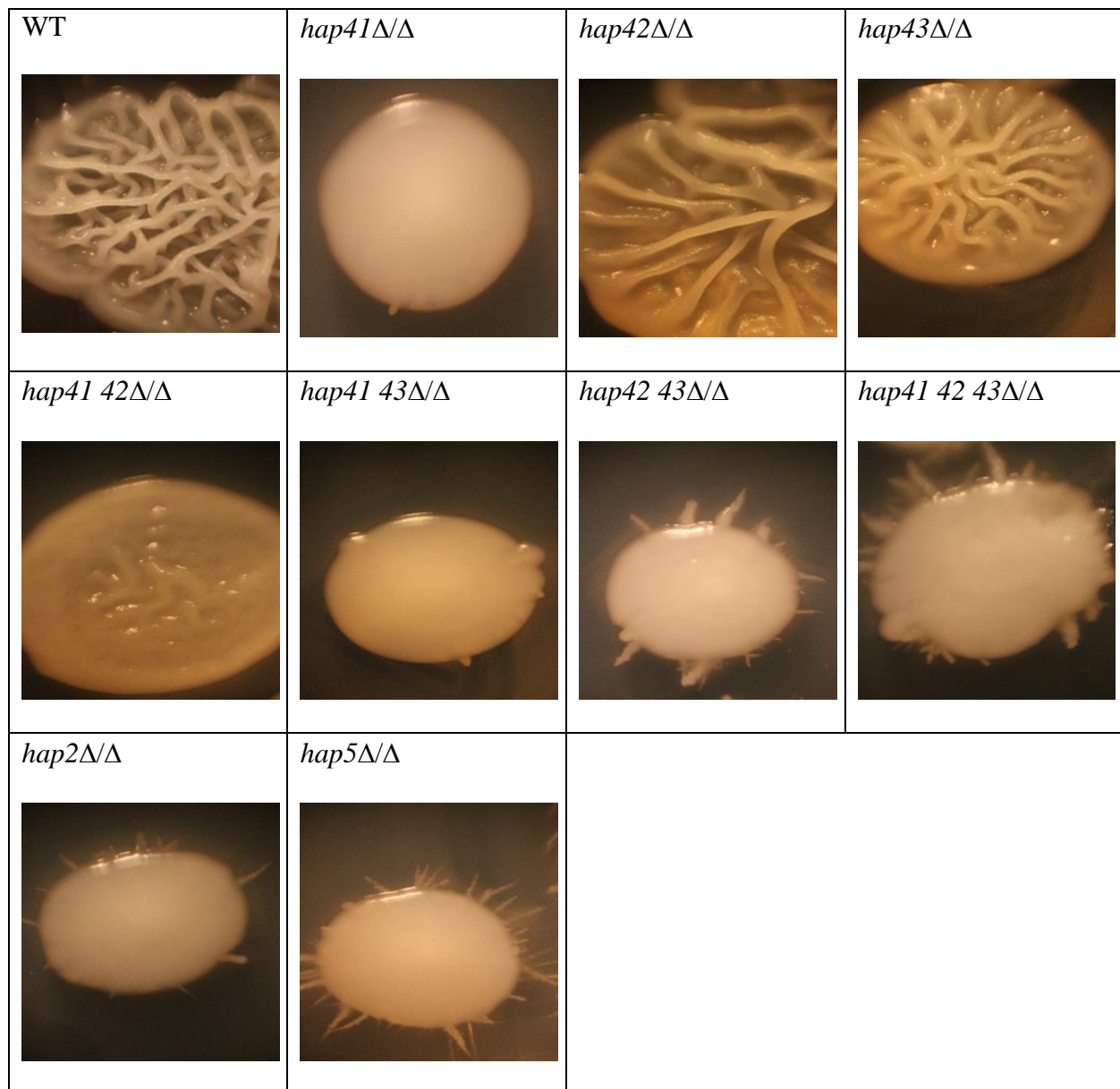


Figure 14: *HAP5* wild type, *hap2* $\Delta/\Delta$ , *hap5* $\Delta/\Delta$ , and *hap4* putative subunit mutants were grown in Spider and incubated at 37°C for 6 days and pictures were taken for all strains. Wild type, *hap41* $\Delta/\Delta$  and *hap43* $\Delta/\Delta$  formed hyphae. *Hap4* $\Delta$  was smooth with no hyphae or filamentation. *Hap41 42* $\Delta/\Delta$  formed hyphae but not as wild type or *hap42* $\Delta/\Delta$ . *Hap43* $\Delta$  was smooth with no filamentation but larger than *hap41* $\Delta/\Delta$ . *Hap 42 43* $\Delta/\Delta$ , *hap41 42 43* $\Delta/\Delta$ , *hap2* $\Delta/\Delta$  and *Hap5* $\Delta/\Delta$  were all similar in size and filamentation.

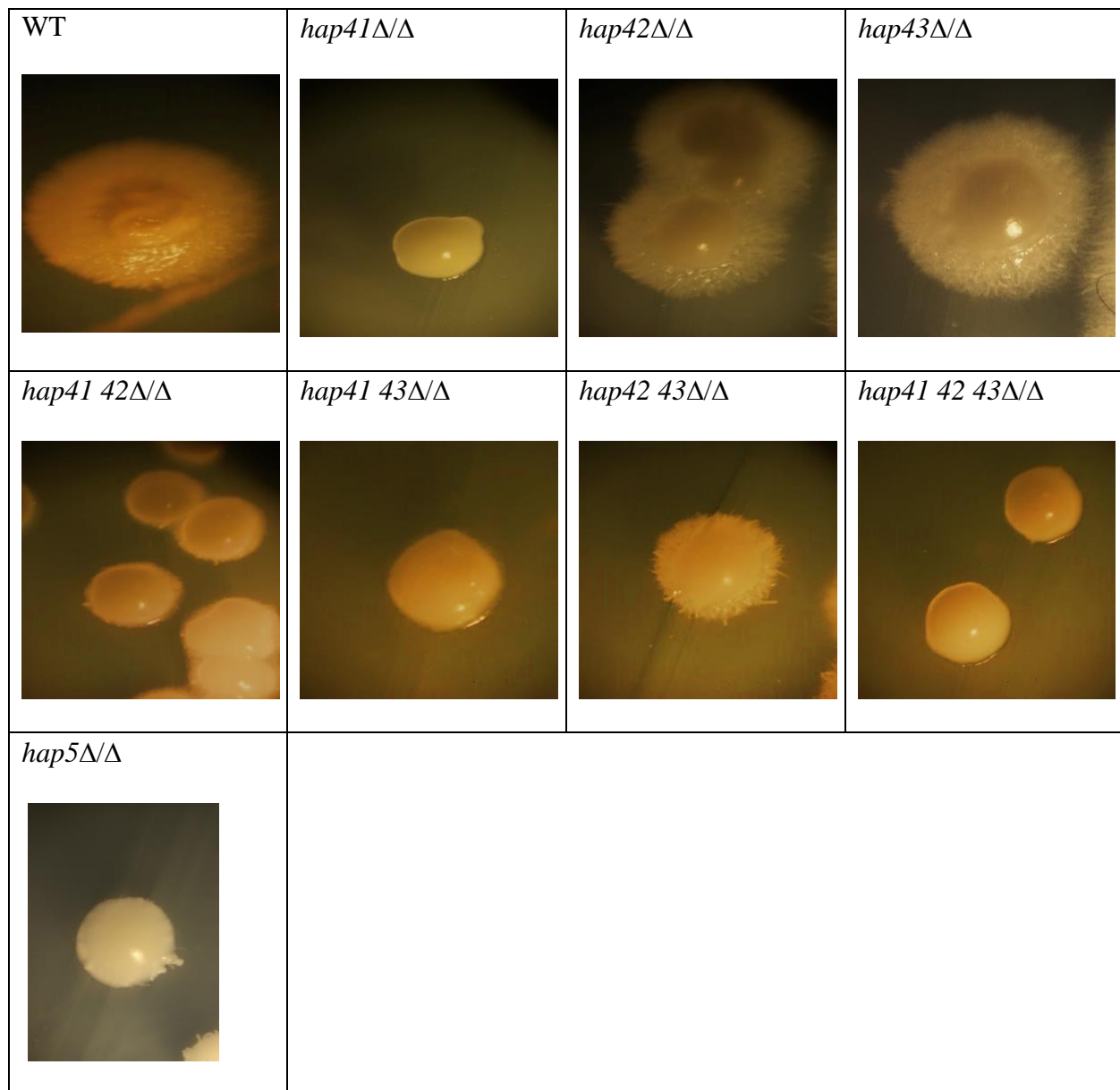


Figure 15: *HAP5* wild type, *hap2*Δ/Δ, *hap4* putative subunit mutants, and *hap5*Δ/Δ were grown on M. Lee's agar plates at 37°C for 7 days and pictures were captured at the same magnification. *Hap41*Δ/Δ, *hap43*Δ/Δ, *hap41 42 43*Δ/Δ and *hap45*Δ/Δ didn't form hypha and were white and smooth. Wildtype developed hypha with wrinkled surface, yet *hap42*Δ/Δ, *hap43*Δ/Δ, and *hap41 42*Δ/Δ developed hypha but not any wrinkles.



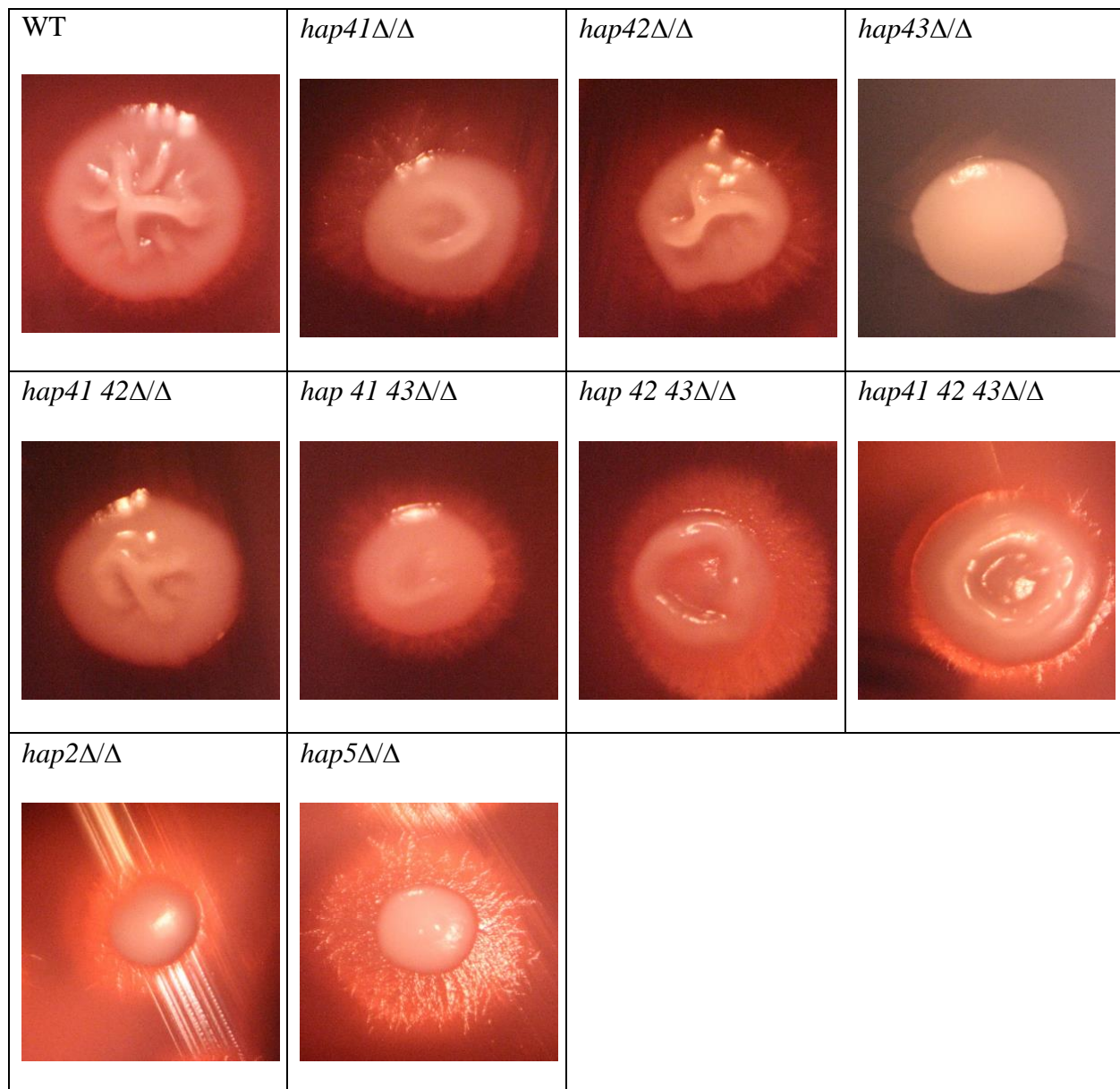


Figure 16: Wild type, *hap2* $\Delta/\Delta$ , *hap5* $\Delta/\Delta$ , and different *hap4* putative subunit mutants were grown in M199 agar plates, incubated at 37, and pictures were captured at day 4 at the same magnification for all the strains. The wild type, *hap42* $\Delta/\Delta$ , and *hap41 42* $\Delta/\Delta$  developed filamentation with wrinkles. *Hap41* $\Delta/\Delta$ , *hap41 43* $\Delta/\Delta$ , *hap 42 43* $\Delta/\Delta$  and *hap41 42 43* $\Delta/\Delta$  developed filamentation with a circular ring. *Hap43* $\Delta/\Delta$  was white and didn't form any hypha.

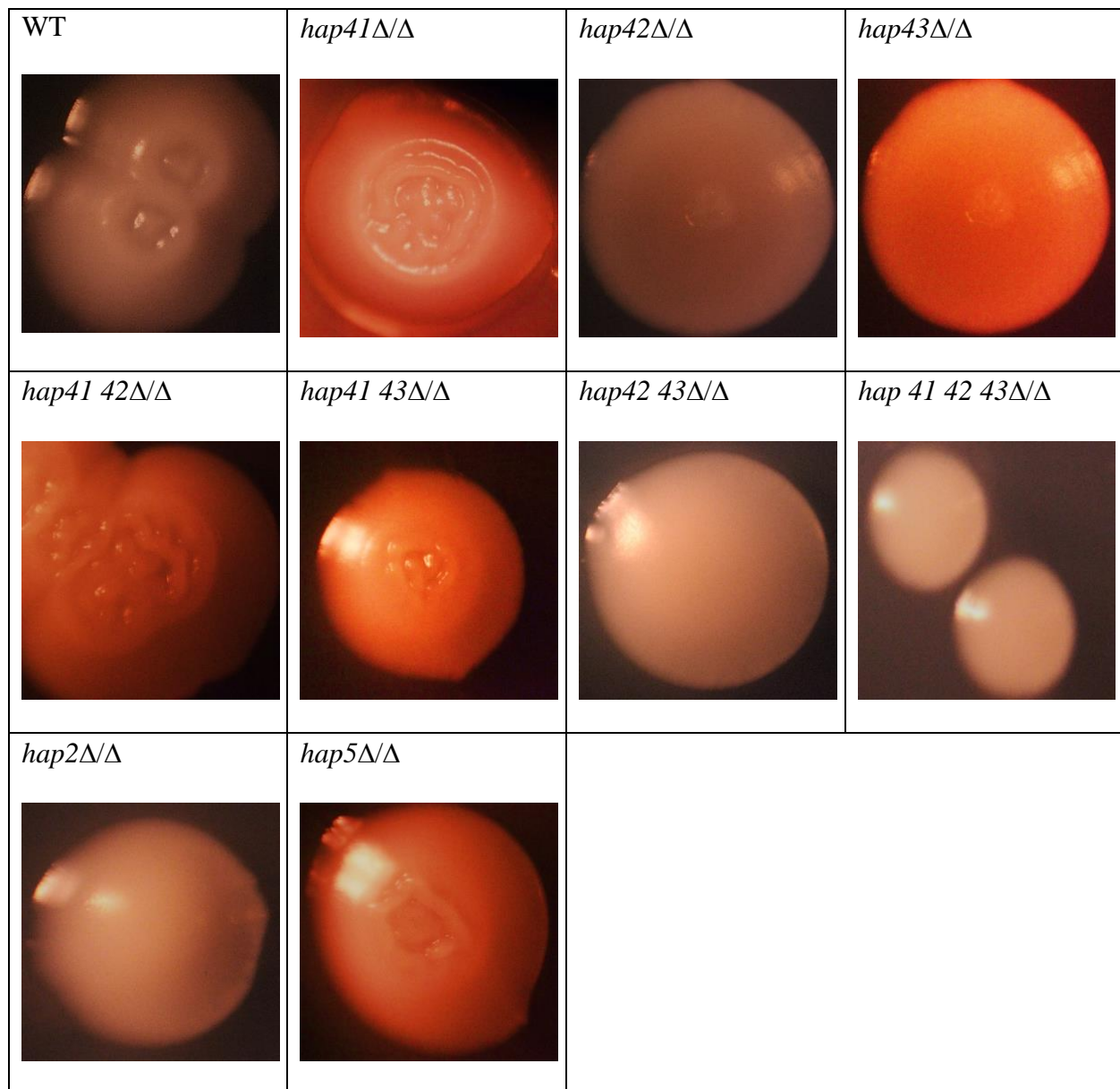


Figure 17: Wild type, *hap2* $\Delta/\Delta$ , *hap5* $\Delta/\Delta$ , and *hap4* putative subunit mutants were grown for 4 days on YNB + AA at pH5.6 and pictures were captured using the same magnification. Wild type, *hap41* $\Delta/\Delta$ , *hap41 42* $\Delta/\Delta$ , *hap41 43* $\Delta/\Delta$  were similar with a circular hypha. *Hap42 43* $\Delta/\Delta$ , *hap41 42 43* $\Delta/\Delta$ , and *hap2* $\Delta/\Delta$  were opaque.

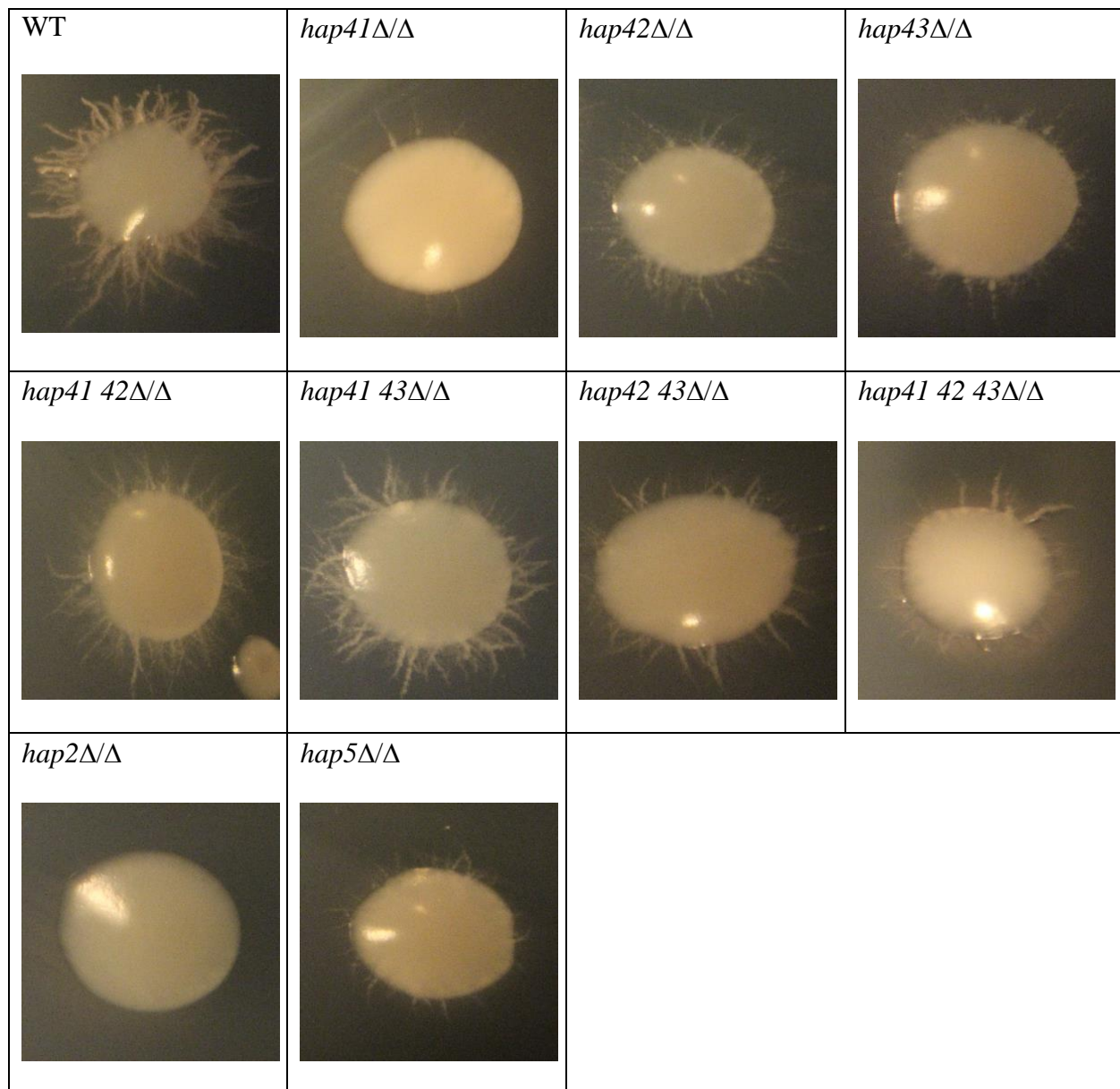


Figure 18: The wild type *hap2*Δ/Δ, *hap5*Δ/Δ, and *hap4* putative subunit mutants were grown on YNB+AA pH6.8 agar plates and incubated at 37°C for 5 days. All pictures were captured on day 5 at the same magnification. All strains were able to switch from yeast to hypha mode except *hap41*Δ/Δ, and *hap2*Δ/Δ were opaque.

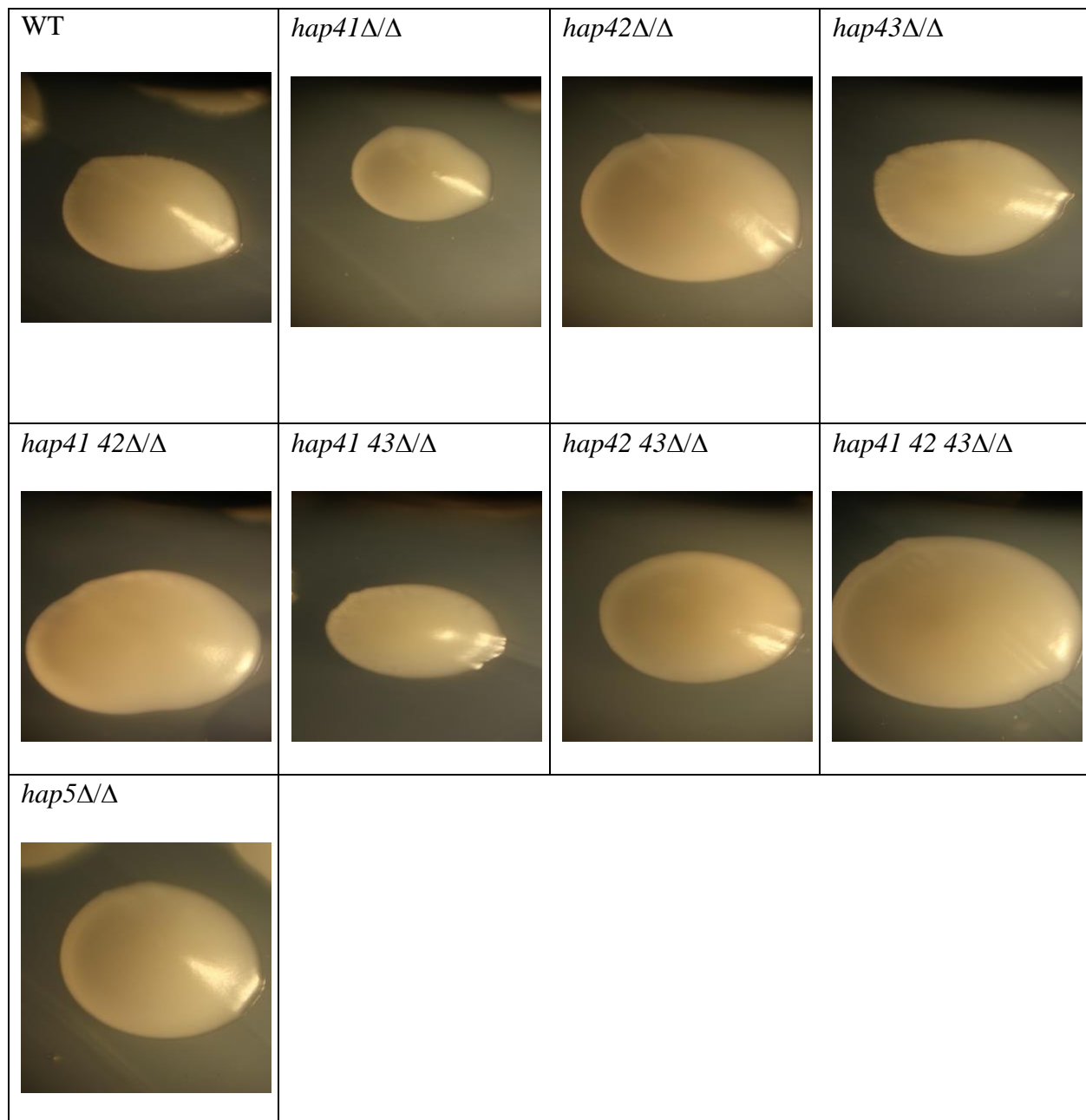


Figure 19: Wild type, *hap5*Δ/Δ, *hap4* putative subunit mutants were grown in YNB-Glucose +AA pH5.6 for 4 days. All strains were white and hyperfilamentation defective. *Hap41*Δ/Δ and *hap41 43*Δ/Δ were smaller in size and slower to grow.



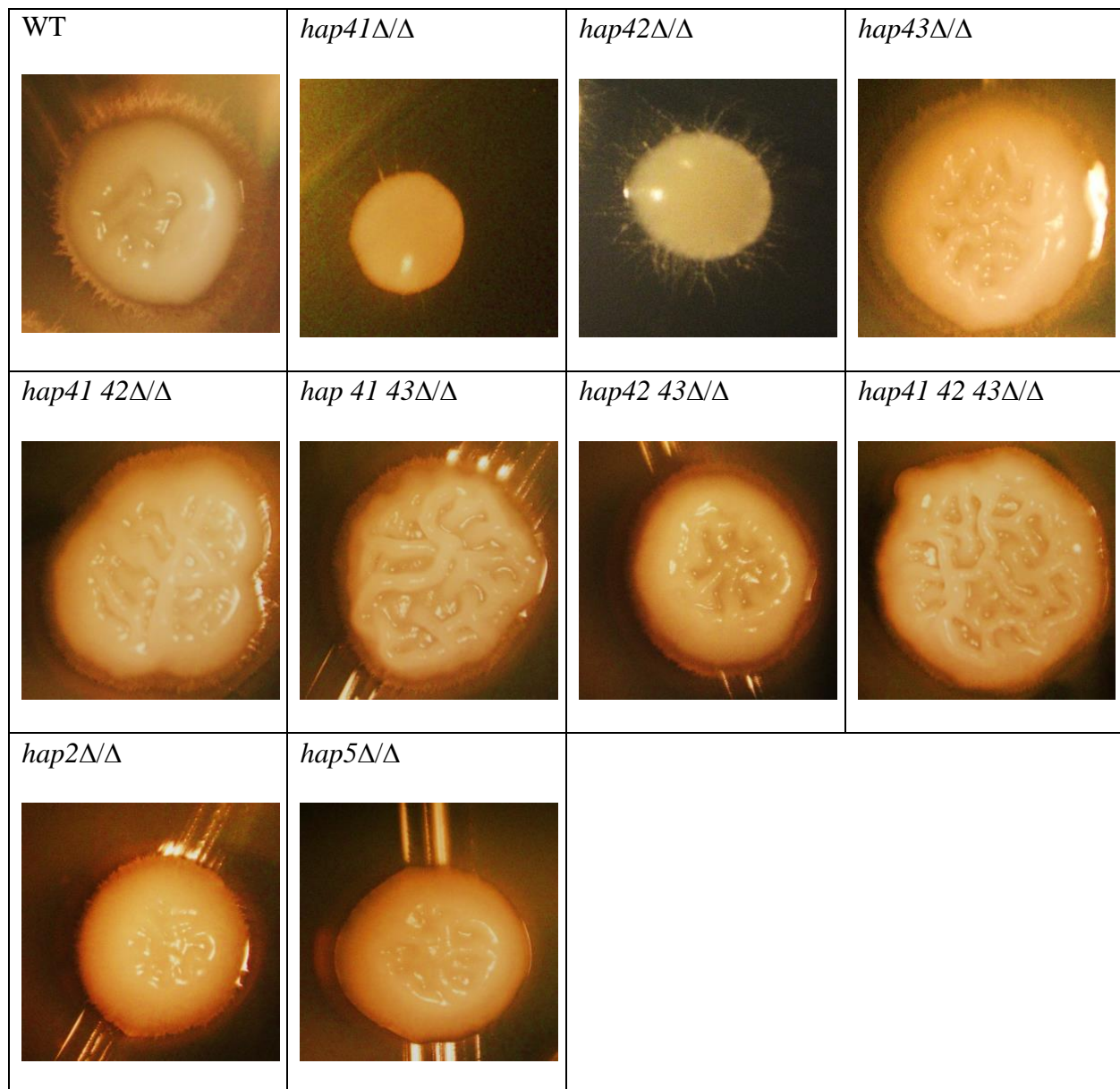


Figure 20: Wild type, *hap2* $\Delta/\Delta$ , *hap5* $\Delta/\Delta$  and *hap4* putative subunit mutants were grown on YNB-Glucose +AA at pH6.8 agar plates for 5 days. All pictures were captured using the same magnification. The wild type, *hap2* $\Delta/\Delta$ , *hap42 43* $\Delta/\Delta$  and *hap5* $\Delta/\Delta$  were similar in size and hyperfilamentation. *Hap41* $\Delta/\Delta$  was the smallest in size followed by *hap42* $\Delta/\Delta$  with less filamentation closer to opaque yeast cells.

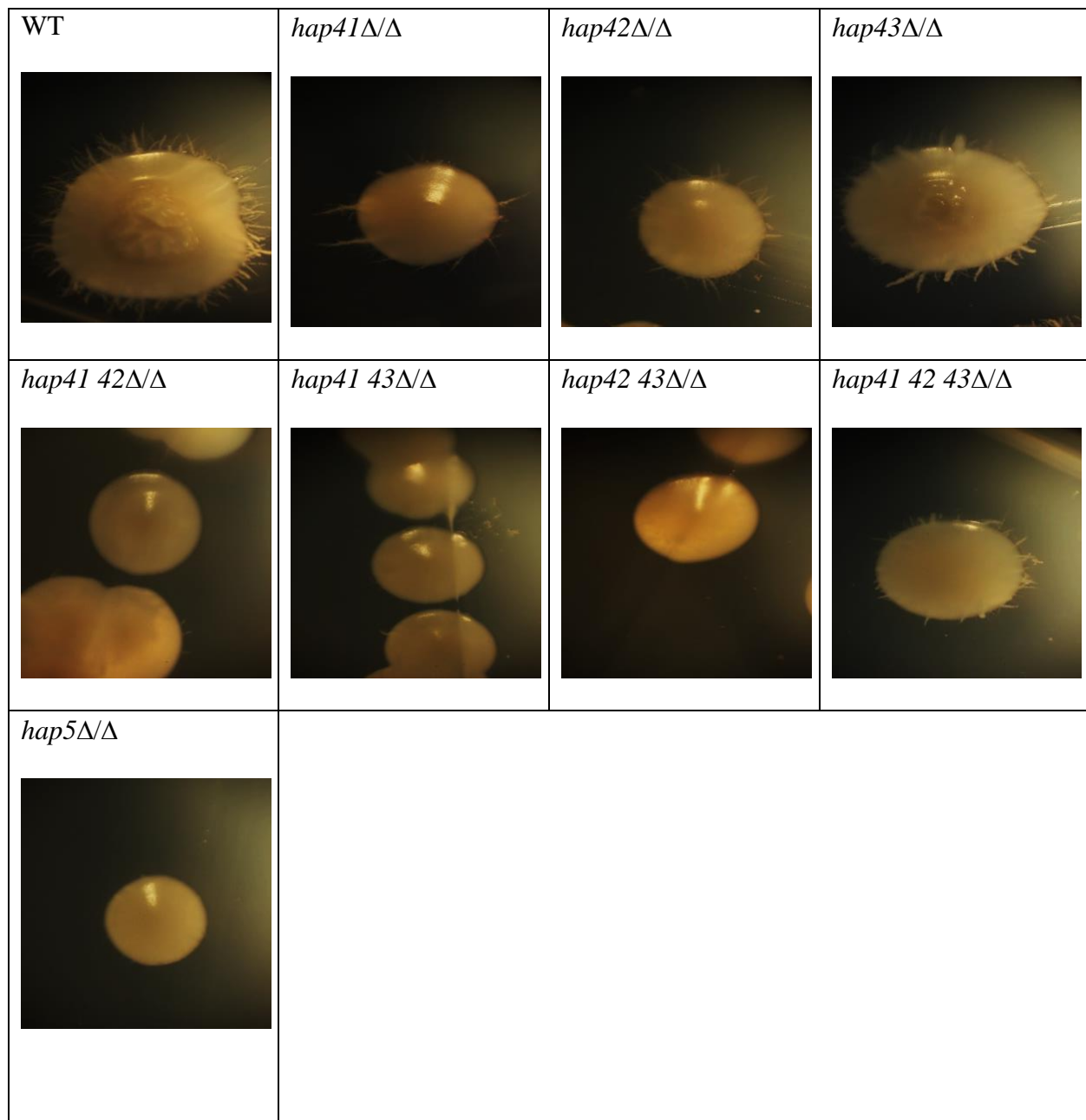


Figure 21: *HAP5* wild type, *hap5*Δ/Δ, *hap4* putative subunit mutants were grown in YNB-Lactate +AA pH5.6 for 7 days. All colonies showed a slow growth, and only the wild type and *hap43*Δ/Δ developed hypha with wrinkles. *Hap41*Δ/Δ, *hap42*Δ/Δ and *hap 41 42 43*Δ/Δ were similar in size and filamentation levels. *Hap41 42*Δ/Δ, *hap41 43*Δ/Δ, *hap42 43*Δ/Δ and *hap5*Δ/Δ were white, flat without any filamentation.

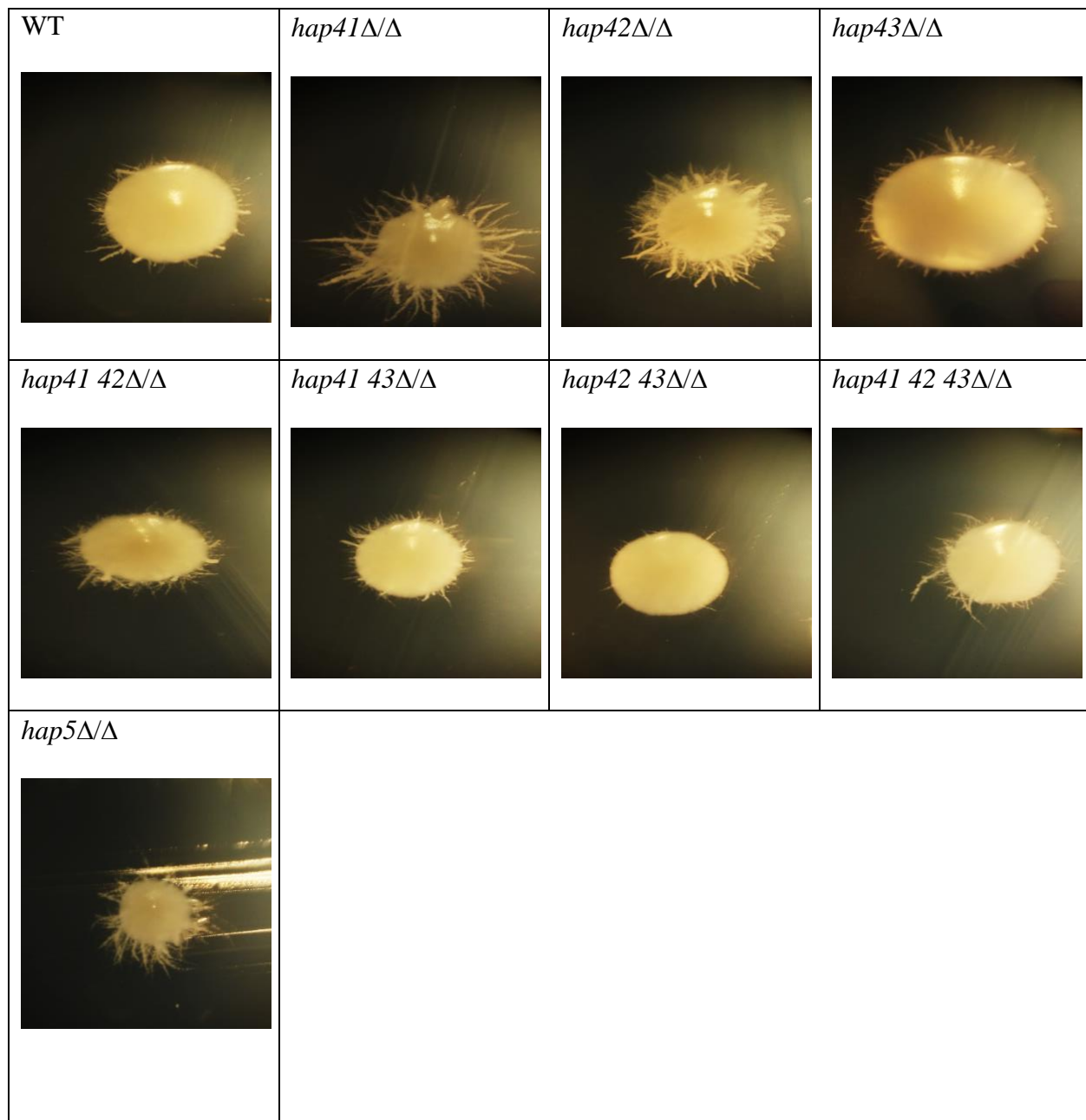


Figure 22: *HAP5* wild type, *hap5*Δ/Δ, *hap4* putative subunit mutants were grown on YNB-Lactate+ AA pH6.8 day 7, and then all the pictures were taken at the same magnification. They all developed hyperfilamentation but *hap41*Δ/Δ, *hap42*Δ/Δ, and *hap5*Δ/Δ had the most filamentation regardless of their small size compared to *hap43*Δ/Δ and wild type.

## 5- Discussion

### **CAT1 promoter functional CCAAT binding site**

*Candida albicans* is an opportunistic fungus found in human gut flora, yet it could cause infections whenever it finds a suitable environment. *C. albicans* infections range from surficial infections like mouth rash or skin rash to the severe lethal systemic infections. The host's immune system attacks the invading organisms using macrophages that produce toxins and reactive oxygen species. However, it is known that *C. albicans* and other yeast can develop antifungal resistance, which makes treating *C. albicans* challenging. *C. albicans* produces several antioxidant enzymes that are regulated by the CCAAT-binding factor, such as catalase, superoxide dismutases (SODs), thioredoxin (TRX), and glutaredoxins (GRXs) to survive the host's immune system [16]. Catalase is an antioxidant enzyme that detoxifies hydrogen peroxide to water and oxygen using iron as a cofactor. The CCAAT-binding factor is a conserved heterotrimeric transcription factor found in 30% of eukaryotic promoters [52,53]. In *Saccharomyces cerevisiae*, it is composed of 4 subunits, including Hap2, Hap3, Hap4 and Hap5, where Hap2, Hap3, and Hap5 bind to the promoter and Hap4 binds to that complex and activates the transcription [11,12]. In humans and murine species, the CCAAT-binding factor has 3 subunits which are NF-YA, NF-YB, and NF-YC [54]. In *Candida albicans* the CCAAT-binding factor is composed of 4 subunits Hap2, Hap3, Hap4, and Hap5, and previous research found that Hap2 or/and Hap5 deletion abolishes its activity [14]. Hap3 is composed of 2 homologs Hap31 and Hap32, while Hap4 is composed of 3 homologs which are Hap41, Hap42, and Hap43. Hap41 and Hap42 have not been characterized and remain putative subunits of the CCAAT binding factor. Hap43 is responsible of gene repression in iron deplete environments, and known to interact with Hap5 [16, 24]. In general, the CCAAT binding site is located as a



proximal promoter element in humans and mice and at a variable distance from the transcription start site in fungal species, typically ranging from 150-800 nucleotides from the transcription start site [52,55]. Since there are 5 putative CCAAT binding sites on the *CAT1* promoter in *C. albicans*, we hypothesized that the most downstream CCAAT sequence next to the putative TATA box would be the real CCAAT binding site. Therefore, we generated the plasmid pIP271 that includes the most downstream and excludes all other putative CCAAT binding sites and measured the Renilla luciferase activity. We found that pIP271 activity was minimal in YPD (iron-replete medium) but increased activity in BPS (iron-deplete medium), indicating that the activated levels in YPD were affected, but still able to derepress in BPS. These data suggest that this CCAAT site can bind the Hap complex but not sufficient for full activation, and it is involved in the response to limited iron conditions (Figure 5). To investigate more, I generated different plasmids targeting all putative CCAAT binding sites. Most plasmids showed minimal activity in YPD except pIP273 that removes the three most upstream putative CCAAT sites, leaving the -240 and -596 sites of the *CAT1* promoter (Figures 6-9). In BPS, all plasmids had a degree of increased activity in *hap5ΔΔ* regardless of their basic activity in a WT background. pIP273 (750 bp) behavior was similar to *CAT1* full promoter, indicating that both CCAAT sites are required for full catalase expression. This scenario suggests that there might be different proteins that cooperate with the CCAAT binding factor to regulate gene expression, like NF-Y. There are at least 3 known proteins that work with NF-Y, including RFX, SP1, and sterol regulatory element-binding protein-2 (SREBP-2) [56–58]. In *C. albicans* RFX2, which contains RFX motif, is found to be associated with DNA damage, hyperfilamentation, and virulence attenuation [59]. The RFX/NF-Y binding complex stabilizes the DNA binding complex and enhances the promoter activity; nevertheless, the RFX/NF-Y complex alone is not enough for

promoter activation, and there are other protein interaction that plays a role in promoter activation [60]. The decreased Renilla luciferase activity in YPD for the plasmids containing individually mutated CCAAT boxes suggests that each of the CCAAT binding sites is poorly bound by the Hap2/5/31 complex. Therefore, the CCAAT sites may contribute in an additive manner to full *CAT1* activation, perhaps but association with other factors that regulate binding to the promoter depending on the environment [54]. In a similar manner to our result, Xanthine oxidoreductase promoter in humans contains several putative CCAAT binding sites, and NF-Y binds to all of them with different activity levels ranging from the basic expression to high expression in each binding site, so it was suggested that some putative CCAAT binding sites would work as activators while other sites work as repressors for the same gene in the same promoter [61,62]. Human von Willebrand factor is another gene regulated by NF-Y where there are multiple CCAAT binding sites, and each putative binding site has a different effect on the gene's expression with the consideration of the environment and other cofactors proteins [62]. Interestingly, plasmids that contained one or both mutated CCAAT boxes showed repression under iron-rich environment (YPD) and de-repression under iron-deplete conditions (BPS), suggesting that Hap43 may bind efficiently to the mutated CCAAT sequences, in spite of poor binding of the Hap2/5/31. Therefore, we can conclude that both proximal CCAAT sites contribute to CCAAT binding factor activity, however, additional factors may interact with the CCAAT-binding factor on each binding site to regulate catalase expression.

In addition, the *CAT1* promoter contains a putative binding site for CAP1, which is a bZIP transcription factor in *C. albicans* that plays a vital role in reactive species oxygen (ROS) such as hydrogen peroxide detoxification [63,64]. CAP1 in *C. albicans* is similar to YAP1 in *S. cerevisiae*, PAP1 in *Saccharomyces pombe*, and AP-1 in mammalian cells [33,64]. In *S.*

*cerevisiae*, *YAP1* deletion increases the cells' sensitivity to hydrogen peroxide, whereas the *YAP1* overexpression increases hydrogen peroxide resistance [63]. *YAP1* in *S. cerevisiae* also is involved in *GSH* and *TRX 2* regulation, where *YAP1* deletion reduces *GSH* and *TRX2* expression, while it is induced in the presence of *YAP1* leading to hydrogen peroxide resistance in stressed conditions such as hydrogen peroxide and amide [63]. In *S. pombe*, catalase expression increased by *PAP1* in response to ROS such as hydrogen peroxide [4]. *CAP1* in *C. albicans* contributes to thioredoxin 2 (*TRX2*), superoxide dismutase 2 (*SOD2*), and glutathione reductase (*GLR1*) regulation [64]. *CAP1* deletion increases the cell sensitivity to hydrogen peroxide[32,33]. Previous data in our lab [16] showed that the CCAAT-binding factor contributes in *TRX1*, *SOD2*, and catalase (*CAT1*) regulation, so we may argue that the CCAAT-binding complex and *CAP1* both cooperate in catalase regulation depending on the environment conditions. Most of the research on *CAP1* showed that it regulates gene expression in a stressed environment such as hydrogen peroxide and diamide. That might explain the increased Renilla luciferase activity with hydrogen peroxide treatment, even for the plasmids with a mutated CCAAT box. The expression levels in *hap5Δ/Δ* strains indicated that the CCAAT-binding factor has a minimum, if any, role in catalase expression in response to hydrogen peroxide. The mechanism of this regulation is not clear yet, since we couldn't confirm a specific binding site that regulates hydrogen peroxide-induced *CAT1* expression. Future experiments should investigate *CAP1* contributions in *CAT1* regulation by determining the specific binding site for *CAP1* and establishing if the occupancy of the CCAAT sites by the CCAAT binding factor has any effect on *CAT1* expression under this stress condition.

### Catalase expression in Hap5 truncation

The C- terminal of Hap5 contains a glutamine rich domain which made us hypothesize that it might have an activation role [14]. The mammalian CCAAT-binding factor NF-Y B and NF-YC have serine/threonine and glutamine rich domain where the N- terminal of NF-Y B has 35% glutamine residues that is conserved between higher eukaryotes and lower eukaryotes such as yeast [65–67]. The deletion of either glutamine rich domain in NF-Y B or NF-YC led to 50% decrease in transcription activation while the deletion of both domains almost abolished its function [65,66]. SP1 is a transcription factor in eukaryotes that binds to the promoter GC boxes and contains a glutamine rich activation domain [67–69]. However, the activation domain doesn't affect DNA binding ability, but it influences its activation efficacy [69]. To investigate whether the glutamine rich domain in Hap5 has an activation role, I generated 3 plasmids contains the full *HAP5*, *hap5Δ/Δ*, and a *hap5* truncation and integrated them into a *hap5Δ/Δ* strain. I measured *CAT1* mRNA for all strains in YPD, BPS, and after hydrogen peroxide pre-treatment. The fold change of *CAT1* mRNA between *hap5* truncation and wild-type Hap5 in YPD was very similar to the decrease in expression observed in the *hap5Δ/Δ* strain. Supporting our argument that the glutamine rich domain in Hap5 plays a role in *CAT1* regulation (Figure 11). In BPS, since catalase expression in *hap5* truncation didn't increase like the *hap5Δ/Δ*, we can assume that the glutamine domain in *HAP5* does not affect the interaction with Hap43, allowing repression just like in the wild type strain. Hap5 truncation didn't show any significant fold change after H<sub>2</sub>O<sub>2</sub> treatment compared to the fold change in wild type (Figure 12), consistent with our finding of limited or no role for the CCAAT-binding factor in *CAT1* expression in response to hydrogen peroxide stress. Therefore, we conclude that *CAT1* expression in iron-rich media is influenced by the Hap5 glutamine rich domain. Moreover, this

domain does not affect the interaction of Hap43 with the CCAAT-binding factor, required for repression under iron limiting conditions.

### **Phenotypes of the putative Hap4 subunits**

We know that the hyphal growth mode is responsible for causing infection in *C. albicans* because it works on the host's cell penetration and adhesion. Hap4 in *C. albicans* is represented by 3 putative subunits: Hap41, Hap42 and Hap43. Hap43 is the only Hap4 subunit known to be involved in *C. albicans* virulence and iron-response, where Hap41 and Hap42 haven't been studied extensively [21]. Hyphal growth mode is responsible for *C. albicans* virulence, so we tested *hap* deletion strains in all Hap4 subunits and their combinations on several media that are used to induce yeast to hypha transition, such as Spider, M199, M. Lee's, and SLAD. SLAD, which is low in nitrogen, induced hyphal formation in all strains except *hap5Δ/Δ* (Figure 13). Three limited carbon source media were used (Spider, M. Lee's, and M199). In Spider and M. Lee's *hap41Δ/Δ* showed similar phenotypic growth to *hap5Δ/Δ* where in M199 *hap41Δ/Δ* showed phenotypic colonies similar to *hap5Δ/Δ* that was smooth colonies with hyper-filamentation (Figure 16). *Hap43Δ/Δ* in M199 was opaque with no wrinkles like those shown in the WT, neither with filamentation like *hap5Δ/Δ* and *hap2Δ/Δ* (Figure 16).

*MEP2* is a gene in *C. albicans* that encodes ammonium permease and it is responsible of yeast to hypha transition in low nitrogen levels [50,70]. Nitrogen starvation increases *MEP2* expression and induces filamentation through MAP kinase pathway that is regulated by CPH1, and cAMP pathway that is regulated by EFG1 and TEC1 [70]. *MEP2* is regulated by GATAA transcription factors which are Gln3 and Gat1, where the deletion of either one decreases *MEP2* expression and the deletion of both abolishes its expression [50]. Also, Gln3 deletion in *C.*

*albicans* failed to grow filamentation in low nitrogen levels, while Gat1p deletion affected *MEP2* levels but not the filamentation growth mode [50]. In addition, Rhb1 controls MEP2 pathways to induce yeast to hyphae transition in a nitrogen starvation environment [71]. Rhb1 is a small conserved G-protein that belongs to the GTPase family [71]. In *S. pombe*, Rhb1 is involved in amino acid uptake and stress response while *RHBA*, its ortholog in *Aspergillus fumigatus*, is activated in nitrogen starvation where *RHBA* deletion decreases *A. fumigatus* virulence [71]. The truncated *MEP2* in *C. albicans* failed to develop filamentation through cAMP and MAP-K pathways [71]. In *C. albicans* it has been shown that *Rhb1* overexpression decreases *MEP2* expression [71]. In *C. albicans* Rhb1 regulates TOR kinase pathway in nitrogen starvation environment through where TOR activates MEP2 expression by Gln3 and Gat1[71]. Hap43 is associated with Rhb1-TOR signaling pathway and MEP2 MAP-K pathway in low nitrogen, and untimely HAP43 influences filamentation growth in *C. albicans* [15]. Failure to develop filamentation in SLAD that is low in nitrogen for all *hap* deletion argues that limited nitrogen availability attenuates CCAAT binding complex in filamentation development and might affect nitrogen uptake and sensing; therefore, it may affect *C. albicans* virulence since it doesn't develop germ tubes to invade the host.

Media with limited glucose, such Spider and M199 that are known to induce hyphal growth, showed similar growth for *hap2Δ/Δ* and *hap5Δ/Δ* where both lost their ability to form hyphae but developed filamentation. However, they were smaller in M199 in size and slower to grow compared to the wild type, which indicated the importance of Hap2 and Hap5 in hyphae formation in glucose limited conditions. Regardless of the slower growth and the size of *hap43Δ/Δ* colonies in Spider, it was able to develop hyphae, yet it was opaque and smooth in M199. *Hap41Δ/Δ* alone failed to develop hypha, and the double mutants *hap41 42Δ/Δ* showed

slower growth compared to *hap42* $\Delta/\Delta$ . Interestingly, *hap41 43* $\Delta/\Delta$ , *hap42 43* $\Delta/\Delta$ , and *hap41 42 43* $\Delta/\Delta$  lost the hyphae growth, which suggest that Hap41 alone independently of Hap42 or Hap43 is involved in hyphal growth and hyper-filamentation in Spider or in the absence of glucose. In M199 *hap41* $\Delta/\Delta$  also showed a phenotype with slower growth and circular hypha ring that was similar to *hap42 43* $\Delta/\Delta$  and *hap41 42 43* $\Delta/\Delta$ .

Several factors contribute to yeast-hypha transition, such as pH, nitrogen, and carbon source. *C. albicans* cells grow as yeast in an acidic environment and switch to hyphal growth mode in basic environment [72]. Therefore, I used YNB + AA with no additional carbon source at pH5.6 and pH6.8, YNB-Glucose pH 6.8 + AA at pH5.6 and pH6.8, and YNB-Lactate (poor carbon source) + AA pH5.6 and pH6.8 to test the effect of carbon source and pH on yeast to hyphal transition. In YNB + AA at pH 5.6, all the colonies didn't develop filamentation but they developed filamentation at pH6.8 except for *hap2* $\Delta/\Delta$ . Adding glucose to YNB + AA at pH 5.6 didn't develop filamentation in *hap41* $\Delta/\Delta$  and *hap42* $\Delta/\Delta$  in pH5.6 and pH6.8 while the other strains developed filamentation at pH6.8 (Figure 20). This suggests that Hap41 and Hap42 are involved in carbon uptake and utilization but not in the pH response. Adding a poor carbon source such as lactate didn't rescue the filamentation for *hap41* $\Delta/\Delta$  and *hap42* $\Delta/\Delta$  at pH5.6, but it induced hyperfilamentation in *hap41* $\Delta/\Delta$ , *hap42* $\Delta/\Delta$ , and *hap5* $\Delta/\Delta$  at pH6.8 which indicates that Hap41 and Hap42 respond differently to carbon availability in different pH (Figures 21,22).

Hap41 and Hap42 putative subunits have not been well characterized. We know that Hap43 is responsible for CCAAT-binding factor repressive activity, but we don't know if Hap41 or Hap 42 are involved in CCAAT-binding factor transcriptional regulatory activity. Here, we have clear evidence that Hap41 plays a significant role in *C. albicans* yeast to hypha transition and likely in *C. albicans* virulence. Since *hap41* $\Delta/\Delta$  didn't develop filamentation in media that

are limited in carbon, Hap41 could be sensitive to carbon uptake and utilization. In addition, Hap41 different phenotypes in the same medium at different pH suggests that it could be sensitive to pH. Also, nitrogen assimilation could be involved in Hap41 activation. In all media *hap41Δ/Δ* colonies behaved like *hap5Δ/Δ*, so there is a chance that Hap41 binds to Hap5 forming a Hap5/Hap2/Ha31 or 32/Hap41 complex. Rim101 is a transcription factor that regulates hyphal growth in *C. albicans* in an alkaline environment, and a Rim101 deletion affected filamentation formation. Thus, we can argue that in a similar fashion, Hap41 bound to the CCAAT-binding complex might be involved in the pH response and yeast to hyphal transition [2,39]. The significance of this finding is that Hap41 could be involved in *C. albicans* virulence, and thus understanding Hap41 role in CCAAT-binding factor activation is worth further investigation. Potentially, these finding could lead to new antifungal drug development targeting *C. albicans* virulence not restricted to cell growth.

The CCAAT-binding factor represses *CYC1* and *COX5*, which are involved in respiratory metabolism and electron transport chain in low or no carbon source media, yet it activates them in the presence of glucose [14,16]. Hap4 in *S. cerevisiae* is involved in respiration and carbon metabolism, and its overexpression in media containing glucose contributes to mitochondrial genes regulation [73,74]. The *hap41Δ/Δ* strain in *C. albicans* showed a clear phenotype in response to the carbon source. Therefore, we propose that Hap41 in *C. albicans* could play a role similar to Hap4 in *S. cerevisiae*, involved in transcriptional activation. Future studies are needed to better understand the role of Hap41 in gene regulation and its association with the CCAAT- binding factor.



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