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REGULATION OF ALTERNATIVE CARBON METABOLISM IN *CANDIDA ALBICANS*

by

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A

THESIS

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by

Arely Yhedid Gonzalez, B.S.

Houston, Texas

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REGULATION OF ALTERNATIVE CARBON METABOLISM IN *CANDIDA ALBICANS*

Publication No._____*

Arely Yhedid Gonzalez

Supervisory Professor: Michael C. Lorenz, Ph.D.

Candida albicans is the most important fungal pathogen of humans. Transcript profiling studies show that upon phagocytosis by macrophages, *C. albicans* undergoes a massive metabolic reorganization activating genes involved in alternative carbon metabolism, including the glyoxylate cycle, β-oxidation and gluconeogenesis. Mutations in key enzymes such as ICL1 (glyoxylate cycle) and FOX2 (fatty acid β-oxidation) revealed that alternative carbon metabolic pathways are required for full virulence in *C. albicans.* These studies indicate *C. albicans* uses non-preferred carbon sources allowing its adaptation to microenvironments were nutrients are scarce. It has become apparent that the regulatory networks required for regulation of alternative carbon metabolism in *C. albicans* are considerably different from the *Saccharomyces cerevisiae* paradigm and appear more analogous to the *Aspergillus nidulans* systems. Well-characterized transcription factors in *S. cerevisiae* have no apparent phenotype or are missing in *C. albicans*.

CTF1 was found to be a single functional homolog of the *A. nidulans* FarA/FarB proteins, which are transcription factors required for fatty acid utilization. Both *FOX2* and *ICL1* were found to be part of a large CTF1 regulon. To increase our understanding of how CTF1 regulates its target genes, including whether regulation is direct or indirect, the *FOX2* and *ICL1* promoter regions were analyzed using a combination of bioinformatics and promoter deletion analysis. To begin characterizing the *FOX2* and *ICL1* promoters, 5' rapid amplification of cDNA ends (5'RACE) was used to identify two transcriptional initiation sites in *FOX2* and one in *ICL1*. GFP

reporter assays show *FOX2* and *ICL1* are rapidly expressed in the presence of alternative carbon sources. Both *FOX2* and *ICL1* harbor the CCTCGG sequence known to be bound by the Far proteins, hence rendering the motif as a putative CTF1 DNA binding element. In this study, the CCTCGG sequence was found to be essential for *FOX2* regulation. However, this motif does not appear to be equally important for the regulation of *ICL1*. This study supports the notion that although *C. albicans* has diverged from the paradigms of model fungi, *C. albicans* has made specific adaptations to its transcription-based regulatory network that may contribute to its metabolic flexibility.

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Chapter 1. Introduction

BACKGROUND

Infection and pathogenesis of *C. albicans*

<u>**Clinical manifestations of *C. albicans***</u>

C. albicans is considered the major fungal pathogen of humans [1]. Clinical

manifestations of *C. albicans* infections can be divided into two broad categories: relatively

benign superficial infections and severe, often life-threatening invasive infections. Susceptibility

to a particular type of *Candida* infection is frequently dictated by the immunologic status of the

host, with invasive infections almost always affecting immunocompromised patients. However,

invasive candidiasis is becoming an increasing concern in critically ill, non-immunosuppressed

patients [2,3].

<u>**Superficial candidiasis**</u>

According to Pfaller and Diekema, 2010 [4], *C. albicans* is the etiological agent of more

than 90% of cases of superficial infections of cutaneous and mucosal membranes. Cutaneous

candidiasis is frequently seen at the intertrigos (i.e. bending creases between toes or fingers,

under the breast or armpit) and is triggered by maceration of the skin, heat, humidity and

obesity [5]. In healthy neonates and young infants, diaper dermatitis is the main clinical

manifestation caused by *Candida.*   Clinical manifestations in mucosal surfaces primarily include

oropharyngeal candidiasis (oral thrush) and vulvovaginal candidiasis (VVC) in women. Oral

infection with *Candida* is associated with several risk factors. Among these are the two

extremes of age (neonates and people older than 65 years), the use of broad-spectrum

antibiotics and prosthetic dentures [6]. Systemic conditions including diabetes mellitus,

immunosuppressive therapy and radiation therapy for head and neck cancer are known to be

predisposing factors for oropharyngeal candidiasis [7-9].   In addition, patients who have

suppressed T-cell function are at high risk for oropharyngeal candidiasis. This is indicated by

the fact that oropharyngeal candidiasis is observed in 7%–48% of people with human

immunodeficiency virus-positive (HIV+) infection and in over 90% of AIDS patients [10,11] [12].

Infection of vaginal mucosa with *Candida* is associated with pregnancy and the use of contraceptives [13], as well as non-hormonal factors such as antibiotic use and diabetes mellitus [14] . Studies indicate up to 75% of women suffer from vulvovaginitis [15]. Only those that experience recurrent vulvovaginal candidiasis (RVVC) are thought to have an underlying innate immune deficiency [16]. Chronic mucocutaneous candidiasis is one of the most serious superficial infections involving colonization of both mucosal and skin surfaces by *Candida* spp. (especially *C. albicans*) and it develops in patients with impaired T-cell function where inadequate production of cytokines leads to inefficient cell mediated immunity [17].

Invasive candidiasis

Invasive candidiasis refers to *Candida* infections that occur once epithelial tissues are breached. Blood-stream infections known as candidemia can lead to disseminated or systemic candidiasis, which is characterized by the hematogeneous spread to one or more vital organs such as the kidney and liver [18]. In fact, disseminated candidiasis is the most severe clinical manifestation of *Candida* species [4,19]. *Candida* species are recognized as the fourth leading cause of nosocomial bloodstream infections in the U.S. and are surpassed only by *Staphylocci* [19]. Mortality rates attributed to candidemia or invasive candidiasis range from 10% to 49% [4]. In the United States, *C. albicans* causes most candidemias (50-60%), followed by *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* [20]. Risk factors for invasive candidiasis are diverse [21] and include candidal colonization [22], prolonged intensive care unit (ICU) stay [19], broad-spectrum antibiotics, chemotherapy-induced neutropenia [23,24], extensive surgery, organ transplantation, and prolonged use of intravascular catheters, which are substrates for biofilm formation [4,25].

Diagnostic methods to detect candidiasis

Early diagnosis of invasive candidiasis is difficult due to the non-specific symptoms presented by fungal infections, which are often mistaken to be of bacterial origin. Slow diagnosis is mainly attributed to the lack of sensitive and specific diagnostic methods [2,21,26].

Traditional microbiological methods including microscopic detection of *Candida* and its isolation from a normally sterile site such as blood have been used. However, early diagnosis using these methods has been hampered by the inability to differentiate between different *Candida* species and false-negative blood culture results that only become positive late in the course of infection. Although a histopathological approach is a reliable method to diagnose systemic infection, it does not differentiate between species and the requirement of tissue biopsy is not feasible in some critically ill patients.

In an attempt to circumvent these problems, researchers have also developed non culture-based methods that target fungal metabolites, antigens, antibodies and nucleic acid. Serological tests have been reported to be successful at detecting several antigens including mannan (the major cell wall protein of *C. albicans*) and the fungal specific β-1,3-glucan (required for cell wall synthesis). However, it may be difficult to interpret serological results since the levels of circulating antigens can vary based on the host's immunologic status, and in some cases antigens are detected in healthy individuals due to *Candida* colonization. Detection of fungal antibodies as a criterion for disease has been less useful also due to *Candida* colonization in healthy individuals, as well as to the absence of detectable antibody in immunocompromised patients with candidemia or invasive candidiasis. Although, the fungal metabolite D-arabinitol produced by most medically important *Candida* species can be successfully identified in serum or urine of some patients, metabolites similarly to antibodies are rapidly cleared from circulation and may not be readily detected. Molecular biological methods such as PCR-based assays that identify fungal nucleic acid have proved to be useful for early and accurate disease diagnosis. However, most molecular biological methods have not been standardized and are not yet available in all clinical settings. Although, some diagnostic methods have been proven better than others at detecting candidemia or invasive candidiasis, all pose limitations. Therefore a combination of microbiological, serological, histopathological, and molecular biological tests may be required to have an early and accurate diagnosis [26].

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Current Antifungal agents

In addition to the limitations posed by the current diagnostic tools, *Candida* bloodstream infections remain problematic in clinical settings due to a lack of treatment options, a delayed administration of therapy, inadequate dose or duration of treatment, as well as drug-related toxicity and the use of a drug to which the *Candida* species is resistant [4]. The administration of proper antifungal treatment early in the course of infection is essential to decrease the mortality associated with invasive candidiasis in clinical settings. For example, a study done by Garey *et al.*, 2006, showed the mortality rate in patients with candidemia was less than 15% in patients treated within 24 hours of a positive blood culture, whereas a mortality rate of 41% was observed for patients that received treatment on day 3 or later [27]. In the ICU, *Candida* species are the third most common cause of nosocomial bloodstream infections [19]. This is in part due to the lack of effective antifungal agents currently available. Antifungal drugs can be grouped into four main categories: polyenes, ergosterol biosynthesis inhibitors, echinocandins, and nucleic acid synthesis inhibitors [28]. Polyenes are designed to target ergosterol, a sterol that is absent in humans, but is essential for fungal cell membranes and growth. The *modus operandi* of polyenes is to bind ergosterol and create pores in the plasma membrane which allow cell contents to leak, thereby resulting in cell death. The azoles, which are the most important class of ergosterol biosynthesis inhibitors, function by inhibiting the Erg11 enzyme which leads to the depletion of ergosterol in the cell membrane. Echinocandins have been recently developed and belong to a category of antifungal drugs that work by inhibiting the β-1,3-glucan synthase, an enzyme that is required for cell wall biosynthesis. Finally, the last class of antifungal drugs which inhibits nucleic acid synthesis is represented by 5-flucytosine. Cytosine deaminase (absent in mammalian cells) converts 5-flucytosine to 5-fluorouracil, which specifically interferes with fungal nucleic acid synthesis [28].

Therapeutic options

Treatment options for fungal infections vary substantially. Current treatments have to take into consideration the site and type of infection, the patient's immune status, underlying disease, and risk factors for infection, as well as the specific species of *Candida* responsible and whether it exhibits antifungal drug resistance. Superficial infections are usually self-limited in non-immunocompromised patients and are readily treated with basic hygiene measures [2] and topical antifungal treatment (i.e. clotrimazole, miconazole, or nystatin), whereas persistent and refractory infections are treated with systemic treatment (i.e. ketoconazole, fluconazole, or voriconazole) [5,29-31]. Current treatment options for candidemia include the azoles (fluconazole and triazoles), echinocandins and the polyenes [32,33]. Treatment with amphotericin B has been the gold standard for years, yet some studies have shown large doses of amphotericin B are associated with nephrotoxicity [34]. When required, the more expensive amphotericin B lipid formulations are used. Caspofungin, micafungin and anidulafungin are the only echinocandin agents approved for clinical use [33] and are currently recommended for the treatment of candidemia or invasive candidiasis. However, echinocandins have also been associated with hepatic toxicity [35].   Fluconazole is considered the first-line agent in non-neutropenic patients with candidemia or invasive candidiasis as it is conveniently administered and has few side effects [32].

Antifungal resistance

As mentioned previously, the use of antifungal agents can also be limited by antifungal resistance. Antifungal resistance is a complex phenomenon that involves several mechanisms. For example, resistance to azole drugs such as fluconazole has been well documented and can be caused by alterations in sterol biosynthesis, mutations in the drug target enzyme, sterol 14α-demethylase (14DM), increased expression of the *ERG11* gene which encodes 14DM, or overexpression of genes that code for membrane transport proteins of the ABC transporter (*CDR1/CDR2*) as well as the major facilitator (*MDR1*) superfamilies [28,36]. Resistance to 5-

flucytosine is known to occur as a result of specific mutations in cytosine deaminase, whereas resistance to Amphotericin B is less clear [28]. Administration of ineffective antifungal therapy can greatly impact the outcome of *Candida* infections leading to an increase in hospital mortality. As mentioned above, the efficacy of antifungal treatments has been complicated by multiple factors including inadequate treatment, drug-related toxicity and increasing antifungal resistance. Therefore, the development of novel effective antifungal agents is needed.

Commensal colonization

C. albicans is an opportunistic pathogen with no known environmental reservoir, and instead is found as a harmless commensal of the mammalian microflora [1]. Skin and mucosal surfaces including the gastrointestinal tract and oropharynx, as well as the vaginal canal and vulva of healthy women, are commonly colonized with *Candida*. *C. albicans* has been reported as the predominant *Candida* species, and as an opportunistic pathogen it has the potential to cause infection at any anatomical site [1]. Different studies have revealed oral asymptomatic *Candida* carriage rates vary among different age groups, and range between 45-65% in healthy children [1], 68% in healthy adults [37] and up to 88% in the elderly [38]. Asymptomatic *Candida* carriage rates have been estimated to be over 50% in the gastrointestinal tract and up to 25% for vaginal areas [1].

Virulence factors of *C. albicans*

The ability of *C. albicans* to transition from a harmless commensal of the microflora to a pathogen depends on *C. albicans* classical and non-classical virulence factors, as well as a competent host immune system [39]. The initial colonization of mucosal and cutaneous membranes requires classical virulence factors, including adhesins and extracellular hydrolytic enzymes [40,41]. Adhesins such as the agglutinin-like proteins (Als family of proteins) [42] and Hwp1 (hyphal wall protein 1) [43] are not only involved in adhesion and colonization of host tissues, but are also important for biofilm formation [44,45]. The best characterized extracellular hydrolytic enzymes are the secreted aspartyl proteinases (SAPs), which are known to be

differentially expressed based on the host niche encountered and the stage of infection [46,47]. For example, *SAP1*, *SAP3*, and *SAP6-SAP8* expression is correlated with adhesion to the vaginal epithelium during vaginitis, whereas *SAP1*, *SAP3*, *SAP4*, *SAP7*, and *SAP8* expression were found to be relevant in the oral cavity during oropharyngeal candidiasis [46]. Phenotypic switching is a phenomenon that is also believed to contribute to *C. albicans* pathogenesis. According to Soll, 2002 [48], phenotypic switching is a process that affects different cellular properties including morphology [49], adhesion [50] and antigenicity [51] among others. The white–opaque system in strain WO-1 in which smooth, white colonies switch to flat, opaque colonies [52] is the most studied type of phenotypic switching and has been observed in strains isolated from patients with *Candida* infections including invasive candidiasis [53]. Host factors that confer protective immunity against pathogens are essential to maintain *C. albicans* as a harmless commensal and although phagocytes are usually effective at clearing fungal infections, several studies have revealed *C. albicans* has the ability to overcome host defenses by immune evasion or modulation strategies that allow intracellular survival of the fungus [54]. For example, a study using mutant strains that are lacking O-linked and N-linked mannans demonstrated the importance of cell surface glycosylation to avoid phagocytosis of *C. albicans* [55]. If phagocytosis cannot be avoided, *C. albicans* resorts to other strategies including the manipulation of phagosome maturation and the ability to survive and replicate intracellularly [56]. One of the key virulence factors of *C. albicans* is its ability to switch from yeast to filaments in the form of pseudohyphae or hyphae, which allow macrophage destruction and the subsequent escape of the fungus [57,58]. The importance of this morphological transition to establish infection has been further highlighted by mice studies, which show that non-filamentous *C. albicans* mutants are avirulent [58].

Interaction of *C. albicans* with the host's immune response

Host innate and adaptive immune responses are responsible for conferring protective immunity against *C. albicans* infections [25]. Adaptive immune responses counteract infections

at specific sites and consist of humoral and cell-mediated immunity. For example, humoral immune mechanisms are activated in response to cutaneous infections, whereas cellular immune responses are required against mucosal infections such as oropharyngeal candidiasis [10]. In contrast, the predominant protective mechanism against invasive candidiasis is the innate branch of the immune system. Innate immunity consists of physical barriers such as the skin and mucous membranes, as well as professional phagocyte populations including neutrophils, monocytes/macrophages and dendritic cells whose primary function is to eliminate pathogens [59]. The fungicidal effects of these cells not only depend on the site of infection, but also on the type of cell encountered [59,60]. Phagocytes engulf pathogens forming an intracellular vesicle or phagosome that progressively fuses with early and late endosomes, and the lysosome to form the phagolysosome. Inside the phagolysosome, pathogens are exposed to an acidic environment, microbicidal molecules and limited nutrients [54]. Once within the phagolysosome, the pathogen is killed via oxidative (e.g. reactive oxygen species generated by NADPH-oxidase and myeloperoxidase) [61] and non-oxidative mechanisms.

Types of phagocytes

Neutrophils are the first immune cells to be recruited from the bloodstream to sites of infection, and their main function is to kill invading pathogens via intracellular and extracellular mechanisms [62]. Studies have shown that neutrophils prevent the transition of yeast to hyphae [63] and are the only cell type in the bloodstream that inhibits *C. albicans* germ tube formation [64]. Furthermore, activated neutrophils kill *C. albicans* yeast and hyphal forms using neutrophil extracellular traps (NETs), which are fibers that consist of granule proteins and chromatin [65]. Based on the importance of neutrophils in clearing infections, it is not surprising that patients with reduced levels of neutrophils (i.e. neutropenia) are more susceptible to disseminated candidiasis [23,24,66]. Unlike neutrophils, monocytes are recruited to infected tissues where they differentiate into macrophages and dendritic cells. Although it appears the microbicidal potential of macrophages is less elaborated than that seen in neutrophils [67], macrophages

also have the ability to act as antigen presenting cells and act in concert with neutrophils to clear infections [68]. Recently, dendritic cells have been shown to be important for phagocytosis of both yeast and hyphal cells of *C. albicans* leading to the activation of the adaptive immune response depending on the morphotype encountered [69]. In response to hyphal forms, murine dendritic cells lead to the activation of the TH2 responses, whereas TH1 responses were activated as a result of yeast cells [69]. Therefore, dendritic cells are important for linking innate immunity to adaptive immune responses.

Transcriptional responses to phagocytes

A plethora of studies have focused on virulence factors such as adhesins and hydrolytic enzymes, as they are important for the establishment of *C. albicans* infections. However, less emphasis has been given to the acquisition of essential nutrients required for growth and survival within diverse host niches. Colonization of the gastrointestinal tract has been considered as a pre-requisite for *Candida* infection and it is believed that infection arises as a result of changes in the host's microflora and immune system [24]. Although *C. albicans* encounters an abundant source of nutrients within the human microflora, nutrient acquisition is limited by competing microorganisms. In order for *C. albicans* to grow and maintain systemic infections, it must be able to undergo metabolic adaptations to different changing micro-environments within the host. According to Koh *et al.*, 2008 [24], alterations in the microflora as a result of antibiotic treatment and chemotherapy-induced neutropenia allows epithelial invasion and dissemination of *C. albicans* cells. The ability of *C. albicans* to disseminate via the blood-stream is in part due to the acquisition of nutrients available in the blood. Glucose is the preferred carbon source for yeast including *C. albicans.* Blood glucose levels within the host range between 6 and 8 mM [70] offering the nutrition required for *C. albicans* to grow and maintain systemic infection. However, nutrient availability is scarce once *C. albicans* cells are internalized by phagocytic cells. Several genomic transcript profiling studies have revealed *C. albicans* uses complex transcriptional programs that are significantly different in response to

blood [64,71], tissue [72] and upon phagocytosis by neutrophils [73] or macrophages [57].

These studies indicate that glucose, which is the preferred carbon source for *C. albicans*, is the

major carbon source in plasma and tissue, but is limiting inside the phagosome. In order to

survive intracellularly, *C. albicans* must be able to assimilate non-preferred carbon sources.

Differential display technology was used by Prigneau *et al.*, 2003 to identify *C. albicans* genes

that are differentially expressed in response to phagocytosis [74] and found most of the genes

induced were involved in carbon metabolism. A transcript profiling approach was used by

Lorenz *et al.*, 2004 to study the interaction of *C. albicans* with mammalian macrophages [57].

The transcription profile observed is similar to nutrient starvation, where induction of alternative

carbon metabolism with a concomitant downregulation of translation and glycolysis takes place

suggesting *C. albicans* is able to use multiple non-preferred carbon sources during macrophage

infection [57]. This study revealed that upon phagocytosis, *C. albicans* displays a complex

transcriptional response characterized by an early and a late phase (Figure 1-1). During the

early phase (within 1 hr), *C. albicans* undergoes a remarkable reprogramming of transcription

(>500 genes regulated) [57]. In addition to the carbon metabolic response, activation of *C. albicans*-specific genes, oxidative stress responses, DNA damage repair, peptide utilization,

and arginine biosynthesis was seen during the early phase. The metabolic shift early in the *C. albicans*-macrophage interaction allows the morphogenetic switch from yeast to hyphae, which

promotes escape into the bloodstream. In the late phase, *C. albicans* successfully resumes

rapid glycolytic growth [57].

Figure 1-1. *C. albicans* **interaction with macrophages.**

Upon phagocytosis, *C. albicans* survives inside macrophages, transitions from yeast to hyphae, and pierces the macrophage allowing its escape. Genetic reprogramming of transcription analogous to changes seen during carbon starvation is part of the early response against host innate immunity. Images courtesy of M.C. Lorenz.

Figure 1-1. *C. albicans* **interaction with macrophages.**

Phagocytosis	Survival	Yeast-hyphae transition	Destruction of macrophage	Escape

1 hr	2 hr	3 hr	4 hr	6 hr
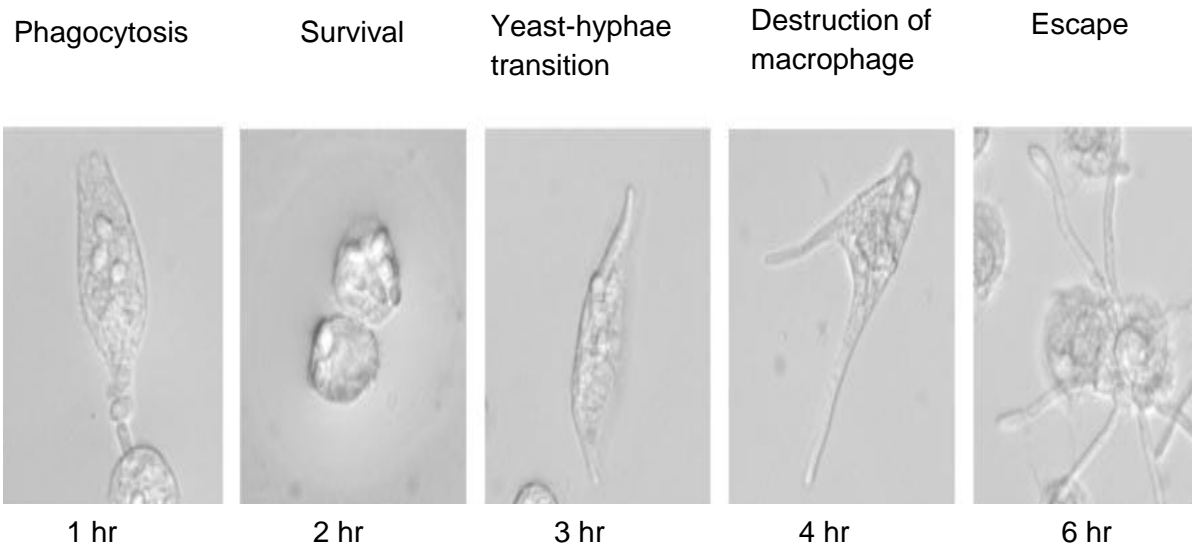
Alternative carbon metabolism in *C. albicans*

Several studies have indicated that the metabolic flexibility displayed by *C. albicans* is important for its ability to adapt to diverse host niches. Therefore, it is important to increase our understanding of the regulatory networks governing carbon metabolism in *C. albicans*. Survival within the phagosome requires the production of energy and catabolites necessary for the synthesis of complex macromolecules**.** In the absence of glucose, *C. albicans* represses glycolysis and activates alternative carbon metabolic pathways. A description of alternative carbon metabolic pathways which include the glyoxylate cycle, gluconeogenesis, and β-oxidation is shown in Figure 1- 2 [75]. Fatty acids such as oleate (an unsaturated 18-carbon fatty acid) are degraded by a series of β-oxidation steps that result in the production of the key intermediate acetyl-CoA [76-78]. The glyoxylate cycle, which bypasses the tricarboxylic acid (TCA) cycle, is required for the net conversion of acetyl-CoA to oxaloacetate allowing the generation of glucose via gluconeogenesis [79]. Two carbon compounds such as acetate, ethanol, lactate, and some amino acids are converted to acetyl-CoA and incorporated via the glyoxalate cycle. Compounds such as malate, citrate, and succinate can replenish TCA cycle intermediates. Furthermore, additional substrates such as glycerol and other amino acids are assimilated through gluconeogenesis. According to Hynes *et al*., 2007, filamentous fungi use a vast array of gluconeogenic substrates in comparison to *S. cerevisiae* [11].

Several lines of evidence indicate glucose availability is limited within the phagosome, and the utilization of multiple non-fermentable carbon sources is critical for *C. albicans* growth and survival. This observation is supported by microarray data generated by our laboratory [57]. The transcript profiles of phagocytosed cells show induction of genes encoding key enzymes involved in alternative carbon metabolic pathways. For example, *ICL1* (glyoxylate pathway isocitrate lyase) and *FOX2* (multifunctional protein of β-oxidation) are induced 33.6- and 44-fold, respectively (Figure 1-2). [57]. Mutations affecting key enzymes of alternative carbon metabolic pathways have varying degrees of influence on *C. albicans* virulence. In

2001, using whole-genome microarray analysis, Lorenz and Fink showed that *C. albicans* glyoxylate cycle genes (isocitrate lyase, *ICL1* and malate synthase, *MLS1*) are induced during phagocytosis, and that disruption of *ICL1* decreased *C. albicans* virulence in a mouse model of disseminated candidiasis [80]. Other independent studies have agreed with this finding [81,82]. In addition, Ramirez and Lorenz, 2007 demonstrated that strains deficient in *FOX2* show a moderate, but significant decrease in *C. albicans* virulence [75]. It is clear that upon phagocytosis, *C. albicans* encounters multiple non-preferred carbon sources such as acetate and fatty acids. However, the exact range of substrates available during infection is unknown. In 2003, Barelle *et al.* used green fluorescent protein (GFP) fusions to examine the activity of key genes involved in glycolysis, gluconeogenesis and the glyoxylate cycle [81]. *In vitro* and *ex vivo* experiments revealed genes required for gluconeogenesis (*PCK1*) and glyoxylate cycle (*ICL1*) had a prominent role during macrophage and neutrophil infection, whereas the subsequent progression of systemic disease was dependent upon the activation of glycolytic genes [81]. In addition, by using an *in vivo* infection model, they were able to show that within infected tissues, different subpopulations of cells encounter distinct micro-environments that vary on nutrient availability.   Altogether these studies showed that *C. albicans* regulates central carbon metabolism based on the host niche encountered and on the stage of infection. Overall, it was concluded that the metabolic flexibility displayed by *C. albicans* is critical for survival and disease progression.

Figure 1-2. Induction of alternative carbon metabolism in *C. albicans*

Upon phagocytosis of *C. albicans* by macrophages there is an upregulation of key genes involved in alternative carbon metabolism. These metabolic pathways converge on the key intermediate, acetyl-CoA to provide sufficient energy and catabolite products allowing the survival and escape from phagocytic cells (Ramirez and Lorenz, 2007).

Figure 1-2. Induction of alternative carbon metabolism in *C. albicans*
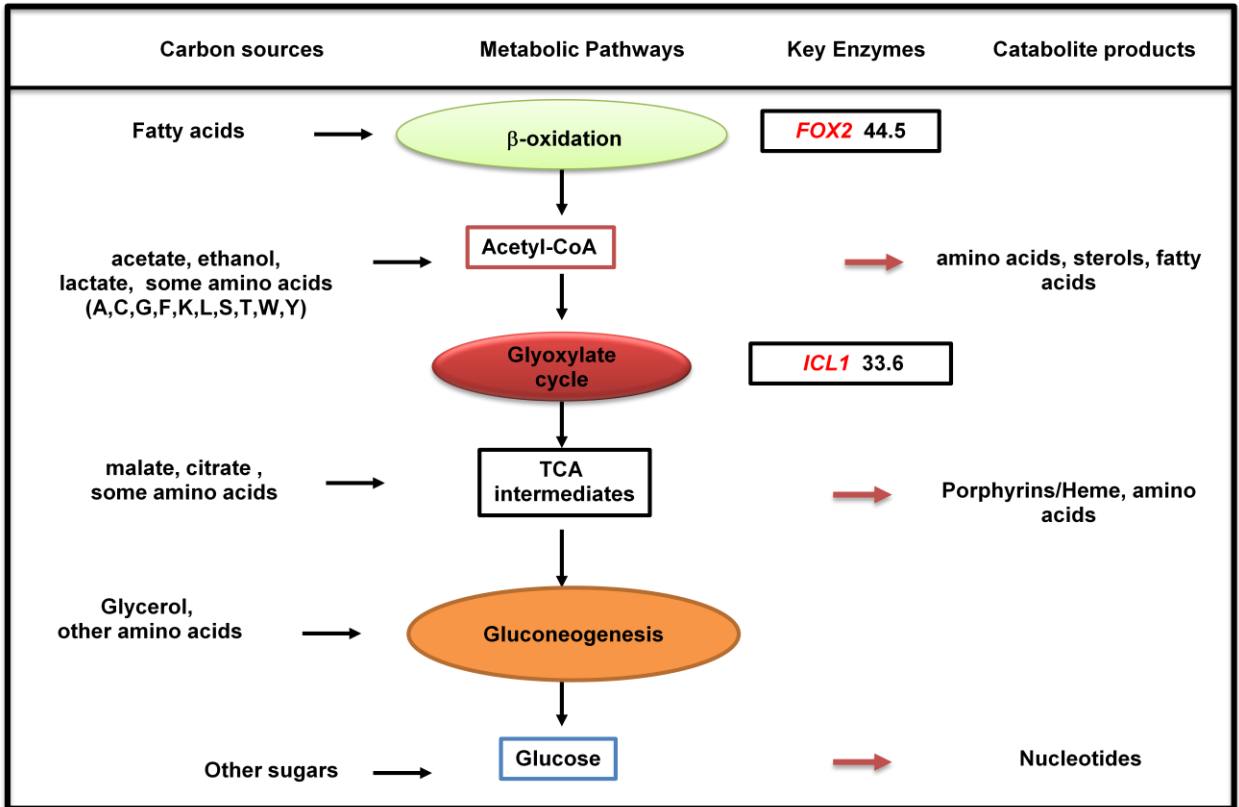
Regulation of alternative carbon metabolism in yeast, filamentous fungi and *C. albicans*

A search for carbon regulators in *C. albicans* was done in our laboratory through sequence and homology studies based on two model fungi: *Saccharomyces cerevisiae* and the filamentous fungi, *Aspergillus nidulans* [75,83]. It has become apparent that the transcriptional regulatory networks governing alternative carbon metabolic pathways in *C. albicans* are considerably different from *S. cerevisiae*, and appear more analogous to *A. nidulans*. The major regulators of carbon metabolism have been characterized in *S. cerevisiae* (reviewed in reference [84]). Many of them belong to the Gal4 family and are zinc binuclear cluster proteins [85] characterized by the presence of a fungal-specific zinc cluster motif, Zn(II)2Cys6 (Cys-X_2Cys-X_6Cys-X_{5-12}Cys-X_2Cys-X_{6-8}Cys) that is found within the DNA-binding domain [86]. Regulation of carbon metabolism involves glucose repression and induction by specific carbon sources. In the presence of glucose, Mig1, a C2H2 zinc finger protein [87], targets the global repressor complex Tup1p/Ssn6p to the promoters of genes involved in alternative carbon metabolism. In conditions were glucose has been depleted, Mig1 targets are derepressed allowing expression at basal levels. This derepression is mediated by the Snf1 kinase, which phosphorylates Mig1 promoting its export from the nucleus to the cytoplasm [88], thus allowing positive regulators such as Cat8, Sip4, Adr1, Oaf1 and Pip2 to induce expression of alternative carbon metabolic genes. Cat8 is a transcription factor regulated by gluconeogenic substrates such as acetate, glycerol, and fatty acids. It activates genes involved in the glyoxylate cycle (e.g. Icl1) [89] and gluconeogenesis (e.g. Fbp1) [90] by binding to a carbon source-responsive element (CSRE) found in their promoters. Expression of the transcription factor, Sip4 is dependent on Cat8 activation [91], and in turn Sip4 contributes to the activation of genes involved in the glyoxylate pathway and gluconeogenesis by binding to their CSRE [92]. The transcription factors Oaf1 and Pip2 bind as a heterodimer to oleate response elements (OREs) found within the promoters of β-oxidation and peroxisomal genes [77,93]. However, there is evidence that some targets (e.g Fox2) are regulated by Oaf1 homodimers in the absence of

Pip2 [94]. The direct binding of oleate to the ligand binding domain of Oaf1 leads to its activation, whereas activation of Pip2 is mediated by the Oaf1/Pip2 heterodimer [95]. In addition to its role as an alcohol dehydrogenase regulator [96], Adr1 activates Pip2 heterodimer for maximal inducibility of target genes in response to oleate [97].

In *A. nidulans*, glucose repression is mediated by CreA, the Mig1 homologue [98]. FacB is a major regulator of genes that encode enzymes of acetate metabolism including isocitrate lyase (*acuD*) and malate synthase (*acuE*) [99,100]. Sequence analysis revealed FacB is similar to Cat8 and Sip4 of *S. cerevisiae*, but genetic studies showed *facB* mutations affect growth on acetyl-CoA sources, but not on gluconeogenic substrates indicating that FacB is a specific regulator of the glyoxylate cycle and not gluconeogenesis [101,102]. To activate genes involved in gluconeogenesis, *A. nidulans* instead employs two transcription factors, AcuK and AcuM [103,104]. Mutations in AcuK and AcuM affect growth on gluconeogenic substrates and result in loss of induction of the gluconeogenic genes acuF, encoding phosphoenolpyruvate carboxykinase, and *acuG*, encoding fructose-1,6-bisphosphatase[103,104]. Furthermore, *A. nidulans* lacks the Oaf1/Pip2 heterodimer which is important for activation of β-oxidation and peroxisome biogenesis in *S. cerevisiae*. In *A. nidulans*, the FarA and FarB proteins are responsible for activating not only β-oxidation and peroxisomal genes, but also those involved in the glyoxylate cycle [105]. In addition, Northern blot analysis has shown that deletion of the *farA* gene abolishes induction by fatty acids, both short-chain (e.g. butyrate) and long-chain (e.g. oleate), while deletion of the *farB* gene eliminates only short-chain fatty acid induction [105].

Regulatory networks governing alternative carbon metabolic pathways in *C. albicans* have diverged from *S. cerevisiae* and appear to be more similar to those seen in *A. nidulans*. There are clear homologs of Snf1, Mig1, Cat8 and Adr1 in *C. albicans*. In *C. albicans*, SNF1 is essential [106], and although there are some regulatory differences, overall MIG1 has a similar function in both species [107]. Furthermore, data from our lab show the roles of Cat8 and Adr1 are not conserved in *C. albicans* despite having high sequence homology to the *S. cerevisiae*

counterparts [83]. *C. albicans* is analogous to *A. nidulans* in that it also lacks Oaf1 and Pip2, and instead CTF1 is a single functional ortholog of the FarA/FarB proteins [83]. CTF1 was initially isolated from the plant pathogen *Fusarium solani* for its ability to induce cutinase genes in the presence of cutin monomers (hydroxylated fatty acids), and was designated as cutinase transcription factor 1α [108]. Studies done in *Fusarium* revealed that a palindrome rich site with a 6-bp core (CCTCGG) is required for DNA binding by CTF1α in the presence of fatty acids [108]. A single CCTCGG motif was shown to be required for binding of both FarA and FarB of *A. nidulans* [105]. In the presence of inducer, FarA/B positively regulates genes involved in fatty acid catabolism. FarA was also shown to have a repressor function in the absence of inducer. The exact molecular mechanisms have not been elucidated, but competition for the binding motif by the induced and non-induced FarA as well as a possible post-translational regulation has been suggested. According to Hynes *et al.*, 2006, the CCTCGG motif is also conserved in other fungal species, but it is not conserved in genes involved in *S. cerevisiae* fatty acid catabolism [105]. *C. albicans* CTF1 shares 39.1% identity with FarA, 27.4% with FarB, and 28% with FsCTF1α [83]. Phenotypic assays of a strain lacking CTF1 show a considerable growth defect in various fatty acids including oleate indicating that CTF1 is required for fatty acid degradation. In addition, several key enzymes including ICL1 and FOX2 are under positive CTF1 regulation. Although these data indicate CTF1 is a regulator of fatty acid degradation, knowledge of how CTF1 directly regulates its target genes is limited.

SIGNIFICANCE OF THE STUDY

C. albicans is typically carried as a relatively harmless commensal, but has the potential to cause a broad range of infections. Superficial mucocutaneous infections can affect relatively healthy individuals, whereas systemic infections pose a higher risk to immunodeficient individuals or critically ill patients. *C. albicans* is the causal agent of more than 50% of candidemias and is considered the major fungal pathogen of humans [1]. It can infect virtually any anatomical site, reflecting its remarkable plasticity in adapting to diverse niches within its host. The high mortality associated with disseminated candidiasis is attributed to several factors including poor diagnosis and inadequate or delayed treatment. In addition, the management of *Candida* infections in clinical settings is hindered by drug toxicity and increasing drug resistance. Therefore, there is a need for novel therapeutic approaches that target unique aspects of fungal biology. Ideally, new antifungal agents should target processes that are not present in humans (*e.g.* glyoxylate cycle) or are highly divergent between the host and the pathogen (*e.g.* fatty acid β-oxidation)[77]. Most antifungal drugs currently available target the fungal cell membrane and cell wall, but metabolic pathways are also suitable candidates for the development of new drugs [109,110]. The knowledge about nutrient acquisition and essential metabolic pathways used by pathogenic fungi is critical for the development of novel disease control strategies.

Several studies indicate that there are sufficient levels of glucose in the bloodstream, which allows rapid growth and dissemination, whereas *C. albicans* intracellular survival is dependent upon the utilization of alternative carbon sources such as acetate and fatty acids. The ability to use a wide variety of gluconeogenic substrates may be an important aspect of *C. albicans* pathogenicity. Increasing evidence indicates that upon phagocytosis, *C. albicans* undergoes a complex transcriptional response that leads to the upregulation of genes involved in alternative carbon metabolism, including the glyoxylate cycle, gluconeogenesis and β-oxidation, and a downregulation of the translational machinery [57,64,71]. Mutations affecting

key enzymes of alternative carbon metabolic pathways attenuate the virulence of *C. albicans*, highlighting the importance of central carbon metabolism for the establishment *of C. albicans* infections. Evidence from genome-scale analyses in multiple fungi suggests metabolic processes are under a global coordination that is mediated in great part by transcriptional regulation [111]. This underscores the significance of elucidating transcriptional regulatory networks that govern alternative carbon metabolism.   However, knowledge of the strategies employed by *C. albicans* to control the expression of carbon metabolic genes is limited. Studies by Ramirez and Lorenz, 2009 [83], have started to elucidate the role of CTF1 in *C. albicans*. CTF1 is required for fatty acid degradation and is required for the co-regulation of fatty acid β-oxidation, *FOX2* and glyoxylate cycle, *ICL1* genes. Microarray data indicate CTF1 has a large regulon (217 genes), and many of the genes under CTF1-dependent regulation have unknown functions [Ramírez, MA and Lorenz, MC unpublished data]. The fact that CTF1 regulates so many genes underscores its importance as a transcriptional regulator and warrants further studies on its mode of regulation. The expression pattern of genes is in great part determined by short promoter sequences or motifs that are binding elements for specific transcription factors. Hence, identifying and characterizing regulatory motifs within CTF1-dependent genes, *FOX2* and *ICL1* is central in understanding regulatory networks that control alternative carbon metabolism.

Chapter 2. Materials and Methods

Strains, growth, and transformation

Table 2-1 lists all the *C. albicans* strains used for these studies, which are based on the parent wild-type strain SC5314 and its auxotrophic derivative CAI4-F2 (URA-) [112]. The strains were maintained on standard yeast media [113], including YPD (1% yeast extract, 2% peptone, 2% dextrose) and YNB (0.17% yeast nitrogen base, 0.5% ammonium sulfate). To manipulate the expression of target proteins, YNB was supplemented with 2% glucose, 2% potassium acetate, or oleic acid solubilized with Tween 20 (0.5% oleic acid, 1% Tween 20). CAI4-F2 and *ctf1Δ/Δ* (MRC39) strains were transformed using the electroporation method as described by Reuss *et al.*, 2004 [114].

Table 2-2 lists all the plasmids used for these studies. DNA manipulations including plasmid isolation, PCR, restriction digestion, cloning, and gel electrophoresis were performed by standard methods [115]. Genomic DNA was isolated by using the glass bead disruption method [116]. Automated DNA sequencing was performed by Genewiz Inc. (Southplain, NJ). *Escherichia coli* DH5α strain was used as a host for plasmid constructions and propagation. *E. coli* was grown on selective Ferric Broth (FB) liquid or agar growth media containing the appropriate antibiotics, ampicillin (100 µg/ml) or nourseothricin (100 µg/ml) at 37°C. A total of 5 µl of ligation products or purified plasmid DNA was used to transform chemically competent JS238 *E. coli* cells [115].

Construction of *C. albicans* strains

The *FOX*2 and *ICL1* promoters were dissected via promoter deletion analysis using the yeast enhanced green fluorescent protein (GFP) based on the pGFP vector described by Barelle *et al.*, 2004 [117]. Constructs for GFP transcriptional reporters were made in which the expression of *GFP* is driven by 1000 bp promoter fragment or by systematic 5' promoter deletions immediately upstream of the translational start codon (ATG). To create the GFP reporter constructs, promoter-specific primer pairs containing a *Kpn*I site in the 5' oligo and an *Xho*I or *Hind*III site in the 3' oligo were designed. The oligonucleotides listed in table 2-3 were

used to PCR amplify each DNA fragment to be inserted into the pGFP vector previously

digested with *KpnI/ Xho*I or *HindIII*. A promoter-less pGFP vector lacking GFP activity was used

as a negative control, whereas a 1000 bp ACT1 promoter- pGFP vector driving constitutive GFP

expression was used as a positive control. Each plasmid was linearized with *Stu*I, gel purified,

and transformed via the *Candida* electroporation protocol [114] to the wild-type strain (CAI4-F2)

and the *CTF1* mutant strain, *ctf1Δ/Δ*. Transformants were selected on SD-URA plates and

grown at 30°C for 3 days. All constructs were incorporated at the phenotypically neutral *RPS10*

locus and correct integration was confirmed by PCR analysis of genomic DNA [75,118,119].

Overlap extension PCR

Overlap extension PCR as described by Heckman and Pease, 2007 [120] was used for

deletion and site-directed mutagenesis of DNA sequences. Briefly, two separate PCRs that

create overlapping gene segments were performed using internal primers designed to delete

the 6 bp core CCTCGG motif from the *FOX2* promoter, as well as to introduce nucleotide

substitutions in the same motif (CCTCGG → CATCAG). In a subsequent PCR, flanking primers

with KpnI/ XhoI were used to generate the full-length product. The product was then inserted

into the pGFP vector (KpnI/ XhoI) and incorporated into the CAI4-F2 and *ctf1Δ/Δ* as described

previously.

RNA extraction and Northern Blot Analysis

C. albicans strains were grown overnight in YNB media supplemented with 2% glucose

and subcultured to an OD_{600} of ~ 0.5 in the same media. The cultures were grown at 30°C to

mid-log phase (OD_{600} of ~1.0), collected by centrifugation and washed twice with double

deionized water (ddH_2O). The cells were resuspended in YNB media supplemented with one of

three carbon sources: glucose (2%), potassium acetate (2%) or 0.5% oleic acid, 1% Tween 20

and incubated for 1 hour at 30°C. The cells were again collected by centrifugation and washed

twice with ddH_2O. The cell pellet was frozen in dry ice and ethanol for storage at -80 °C. Total

RNA was extracted from *C. albicans* using the hot acid phenol method with glass bead disruption [121].

For Northern Blot analysis, twenty micrograms of total RNA was resolved by electrophoresis using a 1% agarose/1X MOPS gel with formaldehyde. The RNA was then transferred to a nylon membrane and was probed using gene-specific probes that were radioactively labeled at the 5'end with [γ-^{32}P] ATP via the action of Polynucleotide kinase (Roche, Manheim Germany). Probes were purified using Roche Quick Spin columns and quantified using a scintillation counter. Blots were incubated in prehybridization solution containing 6X SSC, 10X Denhardt's solution and 0.1% SDS for 1 h at 42°C followed by overnight hybridization with the 5'end labeled probe. Images were processed using a Storm PhosphorImager and exposed to film for autoradiography. Ribosomal RNA (rRNA) visualized by ethidium bromide staining was used as a loading control.

GFP reporter assays and fluorescence microscopy

Reporter strains were grown as described for Northern Blot analysis. After growing reporter strains for 1 hr in YNB media plus one of three carbon sources: glucose (2%), potassium acetate (2%) or oleate (0.5% oleic acid, 1% Tween 20), *C. albicans* cells were harvested and visualized using an Olympus IX81–ZDC confocal inverted microscope (Tokyo, Japan). All images were obtained using a 60X immersion oil objective, as well as the following filters and exposure times: DIC (100 ms), FITC for GFP (100 ms) or TRITC for mCherry (500 ms). Subsequently, SlideBook 5.0 digital microscopy software (Intelligent Imaging Innovations, Inc., Denver, CO, USA) was used to process DIC, FITC, and TRITC images. Quantification of GFP fluorescence intensity was done essentially as described by Brothers *et al*., 2011 [122], with some modifications. To quantify the level of fluorescence in individual cells on saved image files, the SlideBook 5.0 circle tool was used to trace at least 50 cell segments (yeast, hyphae and pseudohyphae), and a single area of background fluorescence. Each selected region was converted to a mask object and the mask statistic mean function was then used to generate the

mean FITC (green) and TRITC (red) fluorescence intensity values. After subtracting the

background value, the data were graphed using Excel as the mean green to red fluorescence

ratio ± S.D.

5'- RNA Ligase Mediated Rapid Amplification of cDNA Ends (5'-RLM-RACE) Analysis of

FOX2* and *ICL1

To map transcription initiation sites within the *FOX2* and *ICL1* promoters, 5' Rapid

amplification of complementary ends was performed using the FirstChoiceR RLM-RACE Kit

(Ambion, USA). RNA was extracted as described for Northern Blot analysis. Ten micrograms of

total RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) and Tobacco Acid

Pyrophosphatase (TAP) to remove both free 5'-phosphates and the cap structure before the

ligation of a 5'-RLM-RACE adapter to the mRNA. The processed mRNA was then reverse

transcribed to cDNA, which served as the template for an outer PCR using a 5'-RLM-RACE

outer primer (Ambion) and a gene-specific outer primer. The outer PCR product was then

amplified by 5'-RLM-RACE inner primer (Ambion, USA) and a 5' gene-specific inner primer. The

resulting PCR products were analyzed by agarose gel electrophoresis and sequencing.

Table 2-1: Lists of *C. albicans* strains used for this study

Strain	Relevant genotype	Complete genotype	Source/ Reference
SC5314	Wild-type prototroph		[112]
CAI4-F2	Wild-type auxotroph (*URA3⁻*)	*ura3::λimm434/ura3:: λimm434*	[112]
AGC8	*Promoterless-GFP/WT*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: GFP-URA3-HIS1*	This study
AGC10	*ACT1p-GFP/WT*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: ACT1p-GFP-URA3-HIS1*	This study
AGC22	*FOX2p(1000)-GFP*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: FOX2p(1000)-GFP-URA3-HIS1*	This study
AGC24	*FOX2p(500)-GFP*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: FOX2p(500)-GFP-URA3-HIS1*	This study
AGC26	*FOX2p(400)-GFP*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: FOX2p(400)-GFP-URA3-HIS1*	This study
AGC28	*FOX2p(300)-GFP*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: FOX2p(300)-GFP-URA3-HIS1*	This study
AGC30	*FOX2p(200)-GFP*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: FOX2p(200)-GFP-URA3-HIS1*	This study
AGC32	*FOX2p(100)-GFP*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: FOX2p(100)-GFP-URA3-HIS1*	This study
MLC61	*ICL1p(1000)-GFP*	*ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG RPS10/rps10:: ICL1p(1000)-GFP-URA3-HIS1*	This study

AGC12	*ICL1p(500)-GFP*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(500)-GFP-URA3-HIS1*	
AGC14	*ICL1p(400)-GFP*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(400)-GFP-URA3-HIS1*	
AGC16	*ICL1p(300)-GFP*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(300)-GFP-URA3-HIS1*	
AGC18	*ICL1p(200)-GFP*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(200)-GFP-URA3-HIS1*	
AGC20	*ICL1p(100)-GFP*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(100)-GFP-URA3-HIS1*	
AGC96	*Promoterless-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: GFP-URA3-HIS1* *ADH1/adh1::yCherry-SAT1*	This study
AGC97	*ACT1p-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ACT1p-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC98	*FOX2p(1000)-* *GFP/ctf1Δ/Δ,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *ctf1::HIS1/ctf1::hisG* *RPS10/rps10:: FOX2p(1000)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC99	*ICL1p(1000)-* *GFP/ctf1Δ/Δ,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *ctf1::HIS1/ctf1::hisG* *RPS10/rps10:: ICL1p(1000)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC100	*FOX2p(1000)-GFP,*	*ura3::λimm434/ura3:: λimm434*	This study

	ADH1p-mCherry	*his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(1000)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	
AGC10	*FOX2p(1000)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(1000)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC102	*FOX2p(500)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(500)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC103	*FOX2p(500)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(500)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC104	*FOX2p(400)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(400)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC105	*FOX2p(400)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(400)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC106	*FOX2p(300)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(300)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC107	*FOX2p(300)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(300)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC108	*FOX2p(200)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(200)-GFP-URA3-*	This study

		HIS1 ADH1/adh1:: yCherry-SAT1	
AGC109	*FOX2p(200)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(200)-GFP-URA3-* *HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC110	*FOX2p(100)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(100)-GFP-URA3-* *HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC111	*FOX2p(100)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: FOX2p(100)-GFP-URA3-* *HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC112	*ICL1p(1000)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(1000)-GFP-URA3-* *HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC113	*ICL1p(500)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(500)-GFP-URA3-* *HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC114	*ICL1p(500)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(500)-GFP-URA3-* *HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC115	*ICL1p(400)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(400)-GFP-URA3-* *HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC116	*ICL1p(400)-GFP,* *ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(400)-GFP-URA3-* *HIS1* *ADH1/adh1:: yCherry-SAT1*	This study

AGC117	*ICL1p(300)-GFP, ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(300)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC118	*ICL1p(300)-GFP, ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(300)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC119	*ICL1p(200)-GFP, ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(200)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC120	*ICL1p(200)-GFP, ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(200)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study
AGC121	*ICL1p(100)-GFP, ADH1p-mCherry*	*ura3::λimm434/ura3:: λimm434* *his1::hisG/his1::hisG* *RPS10/rps10:: ICL1p(100)-GFP-URA3-HIS1* *ADH1/adh1:: yCherry-SAT1*	This study

Table 2-2: Lists of plasmids used for this study

Plasmid	Description	Source/ Reference
pGFP	Promoterless *GFP* in CIp10, URA3 selection marker, ampicillin resistance	[117]
pADH1-mCherry	Construct to integrate *mCherry* at the *ADH1* locus, driven by the *ADH1* promoter. Nourseothricin selection marker	[122]
pAG3-1	*ICL1p(1000)-pGFP*	This study
pAG4-1	*ICL1p(500)-pGFP*	This study
pAG5-1	*ICL1p(400)-pGFP*	This study
pAG6-1	*ICL1p(300)-pGFP*	This study
pAG7-1	*ICL1p(200)-pGFP*	This study
pAG8-1	*ICL1p(100)-pGFP*	This study
pAG9-1	*FOX2p(1000)-pGFP*	This study
pAG10-1	*FOX2p(500)-pGFP*	This study
pAG11-1	*FOX2p(400)-pGFP*	This study
pAG12-1	*FOX2p(300)-pGFP*	This study
pAG13-1	*FOX2p(200)-pGFP*	This study
pAG14-1	*FOX2p(100)-pGFP*	This study

Table 2-3: List of oligonucleotides used for this study

Gene name	Oligonucleotide (oligo)	Description	Sequence[a]
Promoter deletion analysis			
FOX2	MLO882	5' oligo -1000 bp relative to ATG	CCAGGTACCAATCAACAATTTGAGGTTTGT
FOX2	MLO883	5' oligo -500 bp	CCAGGTACCTTGCTGTTGGCATTGTGTAATAAT
FOX2	MLO884	5' oligo -400 bp	CCAGGTACCTCGTTGTGTTATGTTAAAGTGGAA
FOX2	MLO885	5' oligo -300 bp	CCAGGTACCTAATACACACGACTTTTCTCCAG
FOX2	MLO886	5' oligo -200 bp	CCAGGTACCACAACAAATGATATGAATGAATATTTA
FOX2	MLO887	5' oligo -100 bp	CCAGGTACCCATCTGAAATTTATTTAATTGAAA
FOX2	MLO1022	3' oligo -1 bp	AGTAAGCTTGGAGGAAAGTATTATGTTTGA
FOX2	MLO1125	5' oligo for deletion of CCTCGG	GGAATTCCCACTTTTTATTTCATCTTGGTTGAAGATG
FOX2	MLO1126	Reverse complement of MLO1125	CATCTTCAACCAAGATGAAATAAAAAGTGGGAATTCC
FOX2	MLO1127	5' oligo for site-directed mutagenesis of CCTCGG motif (C**A**TC**A**G)	GAATTCCCACTTTTTATTTCATCCATCAGTTGGTTG
FOX2	MLO1128	Reverse complement of MLO1127	CAACCAACTGATGGATGAAATAAAAAGTGGGAATTC
ICL1	MLO888	5' oligo -1000 bp	CCAGGTACCCCAGCGTGGTCATGGAATCGT
ICL1	MLO889	5' oligo -500 bp	CCAGGTACCGTCAGCTTTTCTACTCAATCC
ICL1	MLO890	5' oligo -400 bp	CCAGGTACCGAATCAAAATAGATGTAATGTGATG
ICL1	MLO891	5' oligo -300 bp	CCAGGTACCGGATCAGTGGAAGATTGCGAG
ICL1	MLO892	5' oligo -200 bp	CCAGGTACCGTCCGATTACAATATTTGGCCC
ICL1	MLO893	5' oligo -100 bp	CCAGGTACCAAATTTCATTCTTTTTTAATACCC
ICL1	MLO1015	3' oligo -1 bp	AGTAAGCTTGGTAGATATTATTAATGTTTATTCTT
Northern Blot Analysis			
FOX2	MLO329	3' oligo +1700 bp	ACAGTGGTGTGTTCATCGTG

ICL1	MLO66	3' oligo +2153 bp	TAAGCCTTGGCTTTGGATTCT
GFP	MLO1094	3' oligo +536 bp	TACCAGCAGCAGTAACAAATTCT
5'RACE Analysis			
FOX2	MLO1261	Gene specific oligo +103 bp	GTTGTTGTCAACGATTAGGAGGT
FOX2	MLO1262	Gene specific inner oligo +197 bp	ACCACCGTTCTTGGTAATTTCA
FOX2	MLO1263	Gene specific outer oligo +247 bp	TCAACAATTTTGGCACCATCCA
ICL1	MLO1264	Gene specific oligo +77 bp	GGTCTGAACCAAGATGGAGAAA
ICL1	MLO1265	Gene specific inner oligo +228 bp	AACAGTCTTGTCAGCATCGTGT
ICL1	MLO1266	Gene specific outer oligo +283 bp	AGTACTTGGCCATTTGAGCAACGT
[a] The restriction sites introduced into the primers are underlined; KpnI (GGTACC), HindIII (AAGCTT)			

Chapter 3. Identification of *cis-acting* elements in the promoters of CTF1 co-regulated genes, *FOX2* and *ICL1*

Introduction

Increasing evidence indicates the ability to use a wide variety of gluconeogenic substrates may be an important aspect of *C. albicans* pathogenicity. As mentioned previously, *C. albicans* undergoes a dramatic reprogramming of transcription in order to adapt to changing host microenvironments. Elucidating transcriptional regulatory networks that govern alternative carbon metabolism is important in order to gain insight into *C. albicans* pathogenic characteristics. However, there is a gap in knowledge of how *C. albicans* controls the expression of carbon metabolic genes. Much of what we know about the transcriptional regulatory networks governing alternative carbon metabolic pathways is based on the paradigms seen in *S. cerevisiae* and *A. nidulans.* Several studies have revealed significant differences between *C. albicans* and *S. cerevisiae*, and increasing evidence shows these processes are more analogous to those employed by *A. nidulans.* For example, in *A. nidulans* and *C. albicans*, β-oxidation and peroxisome biogenesis are regulated by the orthologues FarA/FarB and CTF1, but the unrelated transcription factors Oaf1/Pip2 in yeast [83,93,105]. Work published by our laboratory [75,83], showed a *ctf1Δ/Δ* mutant strain in a mouse model of disseminated candidiasis showed a mild, but significant attenuation in virulence. The results were comparable to a *fox2Δ/Δ* mutant which showed a mean time to death of 6.9 days, compared to 4.8 days for the wild-type strain [75,83]. Because CTF1 is also relevant *in vivo*, it is important to gain insight on how CTF1 regulates target genes (e.g. *FOX2* and *ICL1*). Northern blot analysis of strains grown in glucose and the non-fermentable carbon sources, acetate and oleate was used to assess the endogenous expression of *CTF1*, *FOX2* and *ICL1* genes [83]. Several observations were made based on Northern blot analysis. First, the absence of *CTF1* mRNA transcripts in the tested conditions other than oleate indicates *CTF1* is regulated by carbon source. Second, CTF1 positively regulates the expression of *FOX2* and *ICL1* only in the presence of oleate [83]. Third, *ICL1* is expressed in a CTF1-independent manner in the presence of acetate, suggesting additional transcription factor(s) must be involved [83]. Recent

microarray analysis derived from wild-type vs. *ctf1Δ/Δ* mutant strains grown in glucose or oleate revealed a large CTF1-dependent regulon (217 genes) [Ramírez, MA and Lorenz, MC unpublished data]. The results show CTF1 regulates genes required for fatty acid catabolism, gluconeogenesis, and the glyoxylate cycle, as well as glucose transport and a few genes involved in other cellular processes. Surprisingly, 60% of the genes in this regulon lack homology to those found in other organisms or their homologs have not been characterized. *C. albicans* is highly specialized to exist within its host as a commensal, and when given the opportunity (e.g. defects in host immunity) it can alter its transcriptional response to adapt to changing host microenvironments, causing disease. Although CTF1 itself has a partial contribution to *C. albicans* virulence, CTF1-dependent genes that are transcriptionally upregulated in response to fatty acids and are fungal specific may represent genes that are important for *C. albicans* pathogenicity making them potential candidates for development of antifungal drugs. Genetic data indicate *FOX2* and *ICL1* are regulated by carbon source and that CTF1 plays a major role in the regulation of these genes. Previous studies clearly highlight the importance of CTF1 as a regulator of fatty acid degradation, yet there is limited knowledge on how CTF1 regulates its targets. Of the CTF1 regulon I have focused on *FOX2* and *ICL1* in this study as model targets. The analysis of *FOX2* and *ICL1* promoter regions will help understand the mechanism of regulation by CTF1 of target genes, including whether regulation is direct or indirect. The knowledge resulting from understanding the mechanisms that govern gene expression of key metabolic enzymes can be used for designing novel antifungal drugs.

Results

Endogenous *FOX2* and *ICL1* are rapidly expressed in alternative carbon sources

The expression pattern of *FOX2* and *ICL1* from a wild-type background was determined over a three-hour period following transfer to media containing glucose, acetate or oleate as the sole carbon source (Figure 3-1 and 3-2). As demonstrated previously by our lab [75,83], Northern blot analysis probing for the endogenous *FOX2* and *ICL1* genes show their expression is completely repressed in the presence of glucose. Transcripts for both *FOX2* and *ICL1* genes were detected within 30 minutes of incubation in the presence of acetate and oleate indicating a rapid cellular response to changing carbon conditions. Interestingly, high *FOX2* expression levels are maintained throughout the three hours in oleate, but an apparent decline is seen after two hours in acetate. Based on these data, full induction of these genes should be observed within one hour. Therefore, a one hour timepoint was chosen for later experiments.

Identification of *cis-acting* elements involved in the regulation of *FOX2* and *ICL1* genes

The expression pattern of a gene is in great part determined by *cis*-acting elements or motifs that are binding sites for specific transcription factors. Hence, the analysis of promoter regions to identifying regulatory motifs is important to understand regulation of specific genes. To identify *cis*-acting sequences important for the regulation of *FOX*2 and *ICL1*, a combination of bioinformatic analysis and promoter deletion analysis was employed. Initially to dissect *FOX*2 and *ICL1* promoters, we used the LacZ reporter system. However, inconsistent results were obtained upon analyzing β-galactosidase activity. Instead, we choose to use GFP as a reporter of transcription. The GFP reporter is a good alternative to enzymatic reporters. For example, the GFP fluorescence signal from a single cell or a population of cells can be analyzed via fluorescence microscopy without the need to disrupt cells [123]. The GFP reporter system was constructed as described in Chapter 2 and as shown in figure 3-3. Briefly, constructs consisting of 1000 bp 5' of the translational start of both genes were cloned upstream of the *GFP* ORF. I also made 5' sequential deletions at 100 bp intervals upstream of the translational start codon of

each gene to isolate promoter fragments with relevant *cis*-acting sequences**.** To make *C. albicans* GFP reporter strains, constructs were used to transform a wild-type strain (CAI4-F2) or *ctf1Δ/Δ* mutant strain (MRC39). Integration at the desired *RPS10* locus was confirmed by PCR. Reporter strains were grown as described in Figure 3-1, and cells were subsequently washed and analyzed by fluorescence microscopy. Initial attempts to quantify expression were complicated by inter-experimental variability, compromising interpretation of the data. Therefore, a different approach described by Brothers *et al.*, 2011 [122] was used to quantify GFP fluorescence intensity. This new approach requires the introduction of an additional expression plasmid encoding the red fluorescent protein (*ADH1-mCherry*) at the ADH1 locus. The constitutive mCherry expression serves as an internal fluorescence standard.

<u>Figure 3-1. Diagram for growth of strain in glucose or non-fermentable carbon sources</u>

The wild-type (SC5314) strain was grown overnight in YPD, collected by centrifugation, washed with water and diluted in YNB supplemented with 2% glucose and grown to log-phase. Cells were collected by centrifugation, washed and grown in YNB with 2% glucose, 2% potassium acetate or 0.5% oleic acid/1% tween 20 for 1 hr at 30°C. RNA was extracted for Northern Blot analysis as described in chapter 2.

Figure 3-1. Diagram for growth of strain in glucose or non-fermentable carbon sources

Grow overnight in YPD

↓

Dilute to OD ~ of 0.5 in YNB and grow 3 hours at 30°C to OD~1

↓

collect, wash and resuspend in YNB plus:

| GLUCOSE (G) | ACETATE (A) | OLEATE (O) |

↓

Grow 1 hour at 30°C

↓

Collect, wash and freeze cell pellets
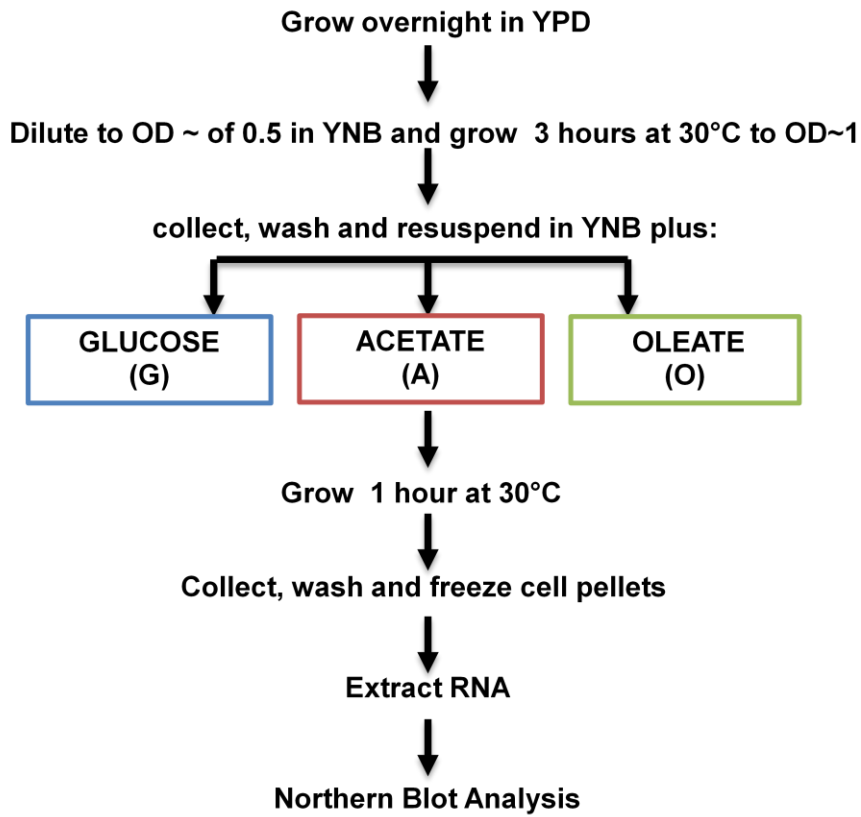
↓

Extract RNA

↓

Northern Blot Analysis

Figure 3-2. *FOX2* **and** *ICL1* **expression is rapidly induced by alternative carbon sources**

The wild-type (SC5314) strain was grown overnight in YPD, collected by centrifugation, washed with water and diluted in YNB supplemented with 2% glucose and grown to log-phase. Cells were collected by centrifugation, washed and incubated at 30°C for a three-hour period in YNB media containing 2% glucose, 2% potassium acetate or 0.5% oleic acid/1% tween 20. Endogenous *FOX2* A) and *ICL1* B) gene expression was detected via Northern blot analysis using gene-specific probes. Ethidium bromide staining of the rRNA was used as a loading control.

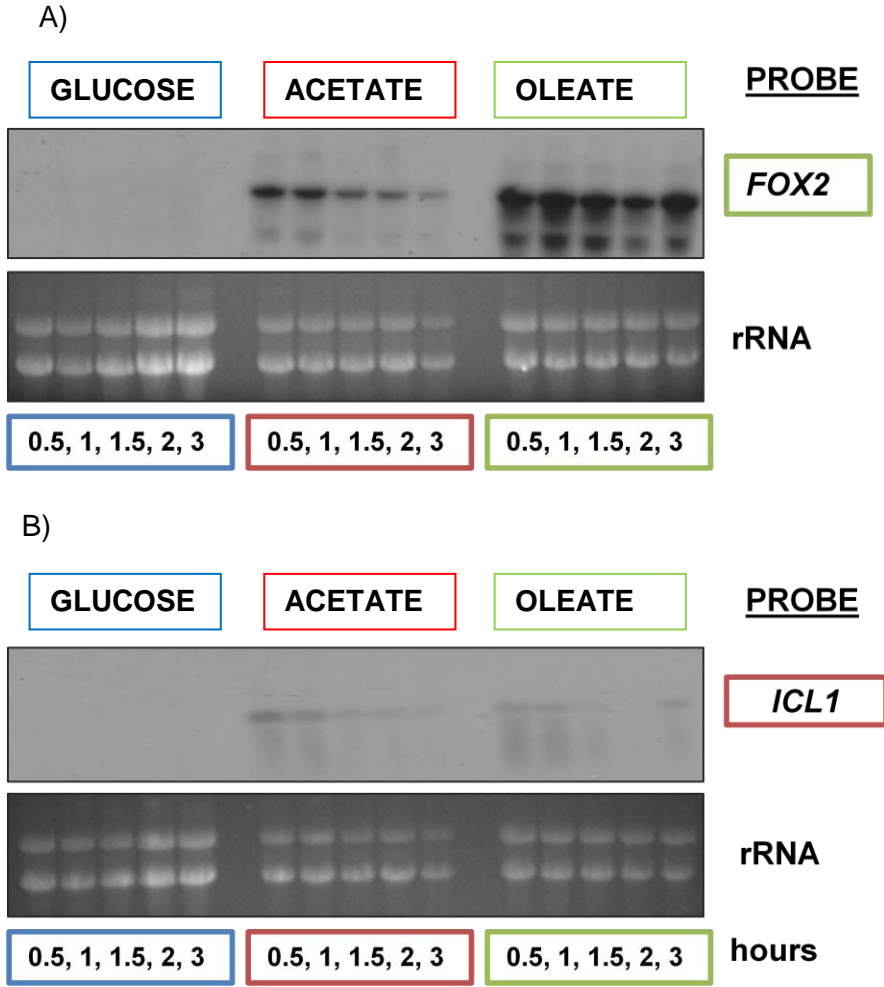**Figure 3-2.** *FOX2* and *ICL1* expression is rapidly induced by alternative carbon sources

A)

B)

Figure 3-3. **Schematic representation of reporter strains used for promoter deletion analysis**

1000 bp 5' of the translational start and systematic 100 bp deletions of the *FOX2* or *ICL1* promoters were fused to *GFP* and chromosomally integrated into a wild-type strain (CAI4-F2) or *ctf1Δ/Δ* mutant strain (MRC39) at the phenotypically neutral *RPS10* locus. The constitutive mCherry expression of the reporter strains serves as an internal reference. At least two independent strains were tested.

Figure 3-3. Schematic representation of reporter strains used for promoter deletion analysis

Reporter strains contain both *GFP* and *mCherry*
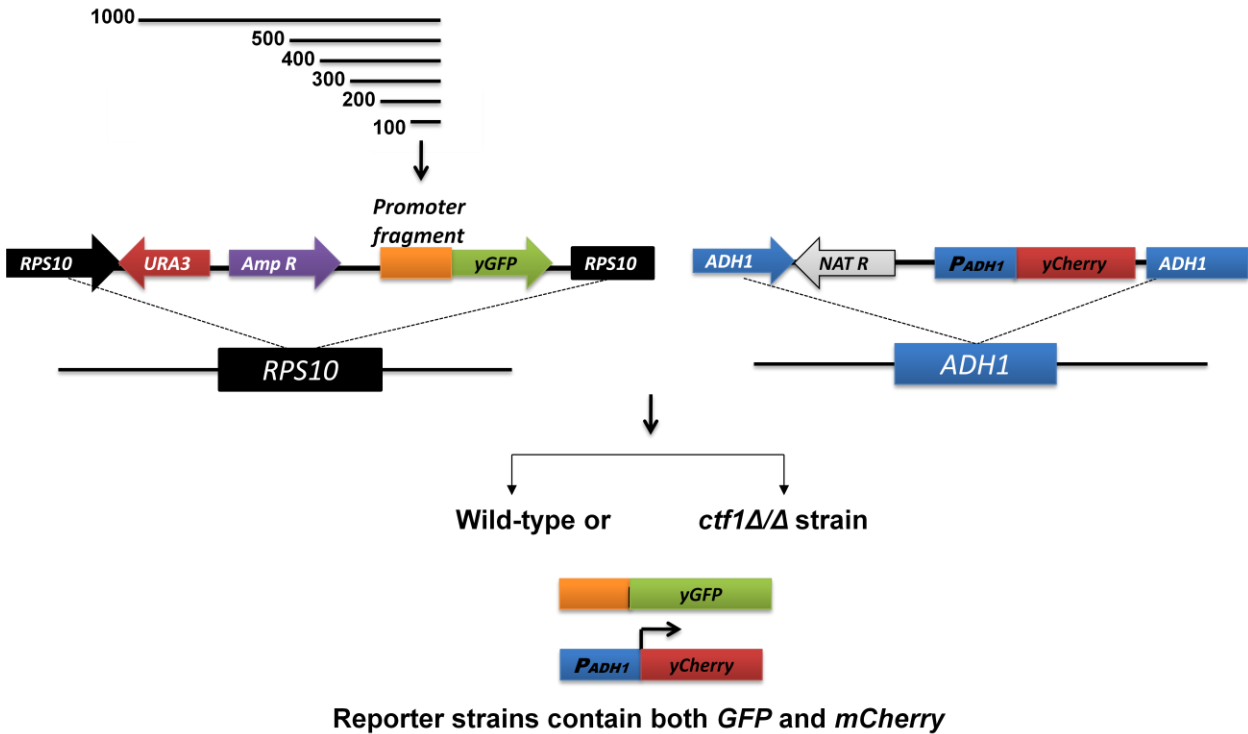
CTF1 regulates the *FOX2* and *ICL1* promoters in non-fermentable carbon sources
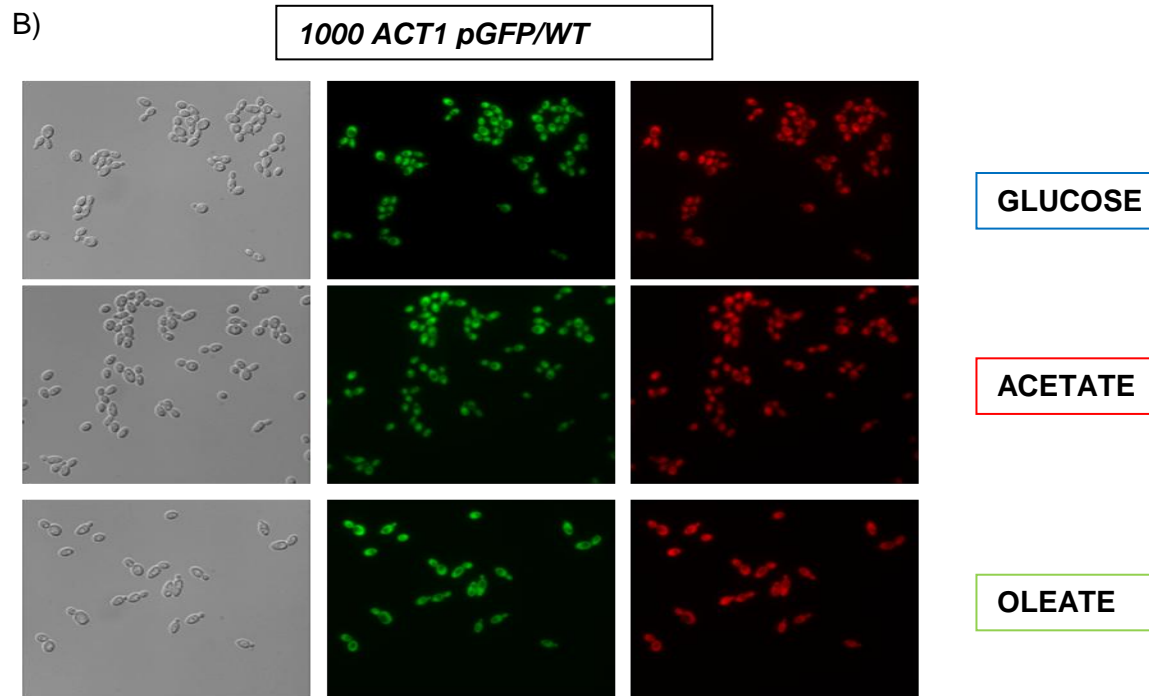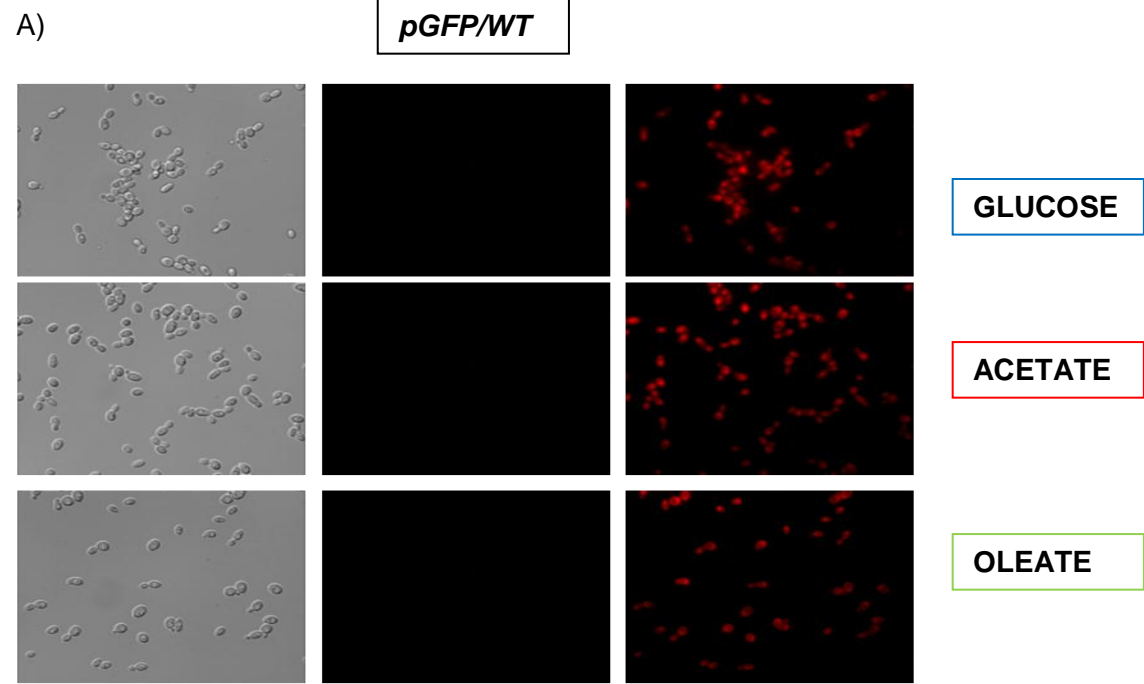
The GFP reporter system has been employed in this study to gain insight into how CTF1 regulates its targets, *FOX2* and *ICL1*. The expression of 1000 bp *FOX2-GFP* and *ICL1-GFP* reporter strains, as well as the appropriate control strains was assessed using fluorescence microscopy as described in Chapter 2. The representative images are shown in figure 3-4. Because all the strains exhibit constitutive mCherry expression, the ratio of GFP to mCherry fluorescence was generated to effectively normalize the GFP fluorescence intensity throughout the different reporter strains and conditions tested (Figure 3-5). Overall, GFP reporter results in a wild-type background correspond to previous Northern blot data obtained by our lab [83]. Fluorescence microscopy analysis of control strains shows that regardless of the conditions tested, high GFP fluorescence intensity is detected in the positive, but not the negative control strain. Consistent with *FOX2* and *ICL1* being glucose-repressed, expression of these genes was not detected in glucose-containing media. On the other hand, *FOX2-GFP* and *ICL1-GFP* reporter strains were fluorescent in media containing acetate or oleate, indicating carbon-source dependent expression. These results are consistent with previous Northern analysis.

To determine the contribution of CTF1 in regulating *FOX2* and *ICL1*, I analyzed the 1000 bp *FOX2-GFP* and *ICL1-GFP* reporters in a *ctf1Δ/Δ* mutant background (MRC39). In the absence of CTF1, the *FOX2* reporter still shows some GFP activity in the presence of acetate and oleate that is apparent in the Northern Blot data and upon quantification of GFP fluorescence. This indicates that in the absence of CTF1, *FOX2* is no longer induced, but it is still subject to derepression. In contrast, minimal GFP activity is seen for the *ICL1* reporter in the same conditions suggesting CTF1 is required for *ICL1* expression even in the presence of acetate. As mentioned previously, Northern blot data generated by Ramirez and Lorenz, 2009 [83] showed CTF1 is not required for the expression of *ICL1* in acetate. The current *ICL1* reporter data in a *ctf1Δ/Δ* mutant background contradicts their data. This discrepancy may be as a result of variations in the strains and requires further analysis.

Figure 3-4. **CTF1 regulates the *FOX2* and *ICL1* promoters in non-fermentable carbon sources**

Fluorescence microscopy analysis of empty-*GFP*/*WT* (A) and *ACT1-GFP*/*WT* (B). Fluorescence microscopy analysis of *FOX2-GFP*/*WT* (C), *FOX2-GFP*/*ctf1Δ/Δ* (D), and *ICL1-GFP*/*WT* (E), *ICL1-GFP*/*ctf1Δ/Δ* (F). *C. albicans* wild-type cells expressing *GFP* reporters at the *RPS10* locus, as well as *ADH1-mCherry* at the *ADH1* locus were harvested and visualized using an Olympus IX81–ZDC confocal inverted microscope (Tokyo, Japan). All images were obtained using a 60X immersion oil objective and the following channels and exposure times: DIC (100 ms), FITC for GFP (100 ms) or TRITC for mCherry (500 ms). SlideBook 5.0 (Intelligent Imaging Innovations, Inc., Denver, CO, USA) was used to analyze all images.

Figure 3-4. CTF1 regulates the *FOX2* and *ICL1* promoters in non-fermentable carbon sources

A)

| pGFP/WT |

GLUCOSE

ACETATE

OLEATE

B)

| 1000 ACT1 pGFP/WT |

GLUCOSE

ACETATE

OLEATE

C)

1000 FOX2-pGFP/WT

GLUCOSE

ACETATE

OLEATE

D)

1000 FOX2-pGFP/ctf1Δ/Δ

GLUCOSE

ACETATE

OLEATE

E)

1000 Icl1-pGFP/WT

GLUCOSE

ACETATE

OLEATE
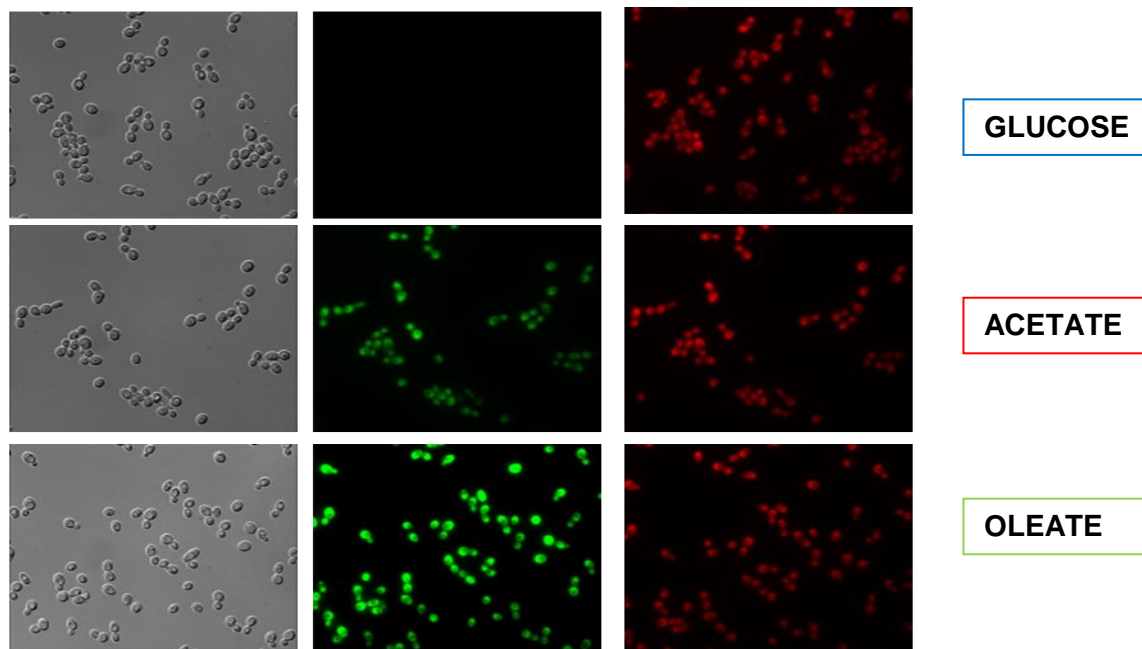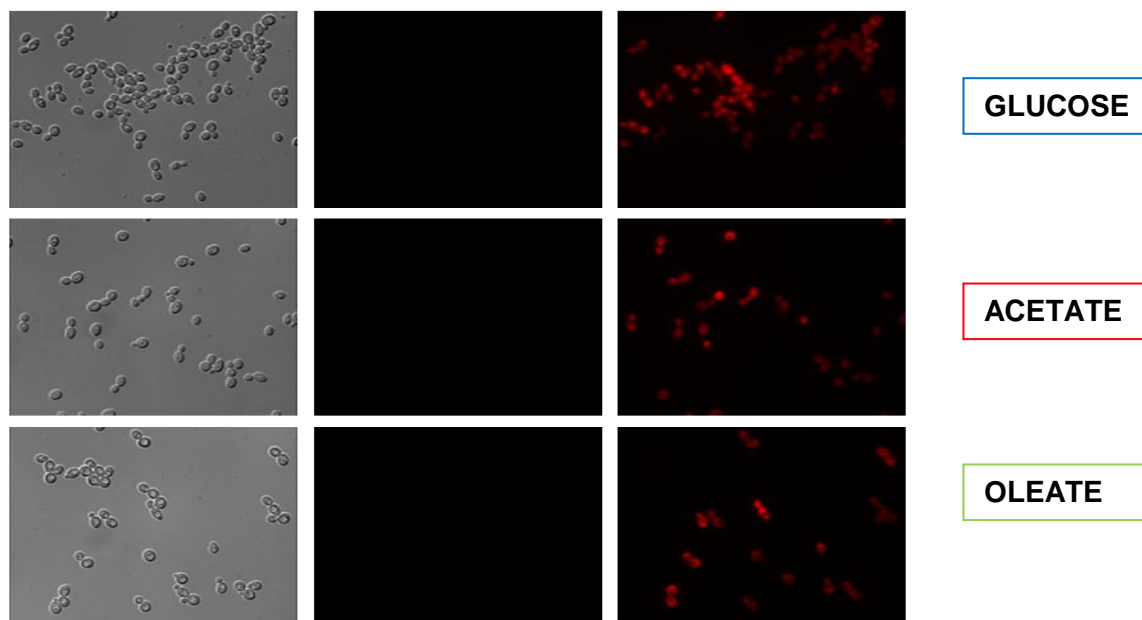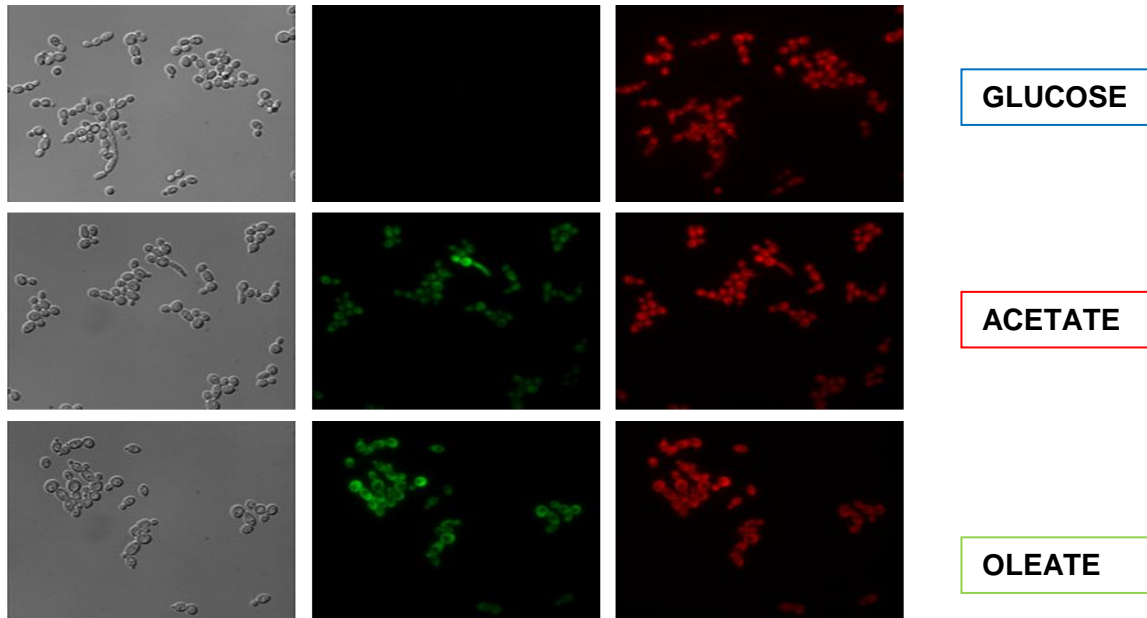
F)

1000 ICL1-pGFP/ctf1Δ/Δ

GLUCOSE

ACETATE

OLEATE
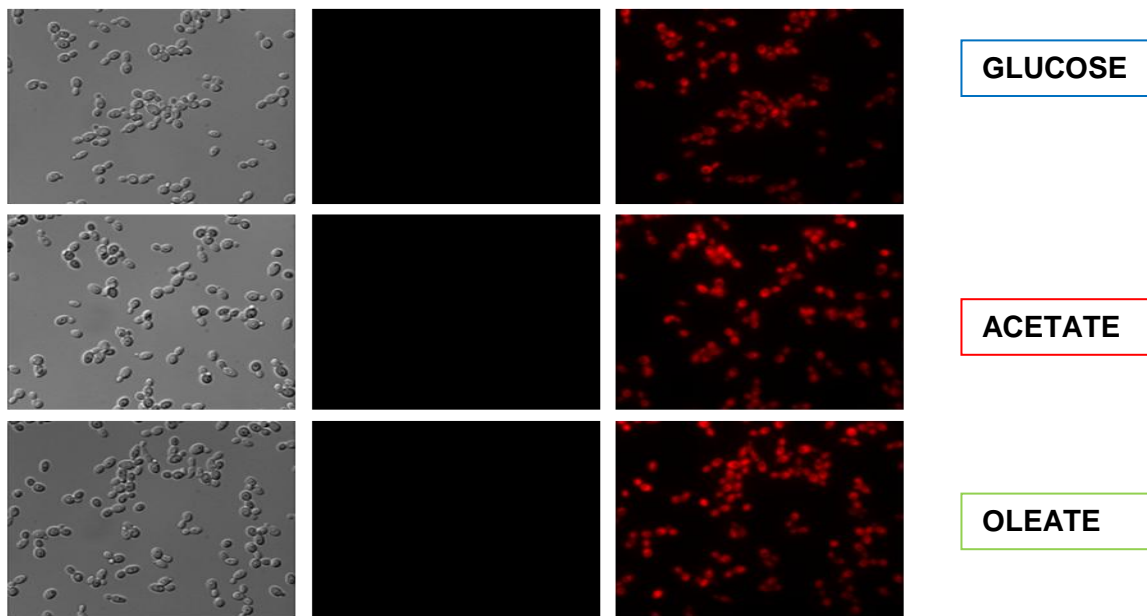
Figure 3-5. Quantification of GFP fluorescence intensity

To quantify GFP fluorescence intensity, the ellipse tool from SlideBook 5.0 was used to select at least 50 cell segments (yeast, hyphae and pseudohyphae), and a single area of background fluorescence [122]. Each selected region was converted to a mask object and the mask statistic mean function was then used to generate the mean FITC (green) and TRITC (red) fluorescence intensity values. After subtracting the background value, the data were graphed using Excel as the mean green to red fluorescence ratio ± S.D.

Figure 3-5. Quantification of GFP fluorescence intensity
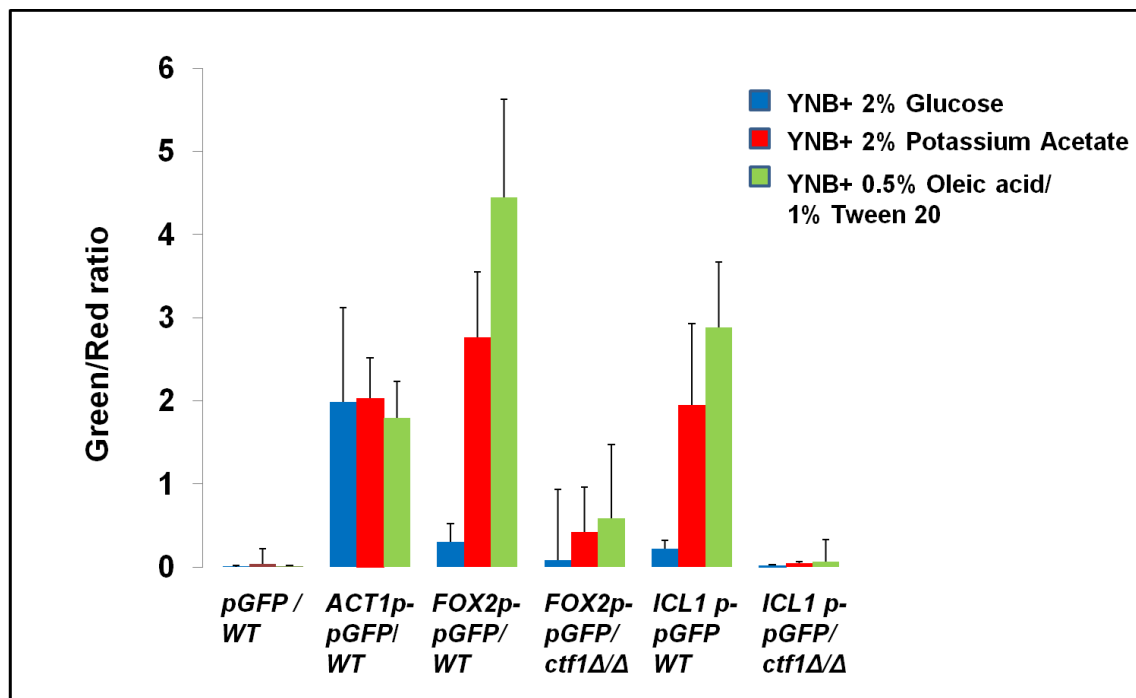
Several transcription initiation sites are located within the *FOX2* and *ICL1* promoters

To identify *cis*-acting sequences within the *FOX2* and *ICL1* promoters, the next step was to perform promoter truncation analysis. Before proceeding, it was important to ensure that any loss of GFP activity was not due to deletion of transcription initiation sites within proximal promoter regions. Transcription initiation sites had not been previously mapped for *FOX2* and *ICL1*. Therefore, 5'- RNA Ligase Mediated Rapid Amplification of cDNA Ends (5'-RLM-RACE) was used to determine transcription initiation sites within the *FOX2* and *ICL1* promoters. The 5'-RLM-RACE PCR reactions for each promoter resulted in multiple bands. However, sequencing of the PCR fragments revealed the location of the transcription initiation sites within the *FOX2 and ICL1* promoters (Figure 3-6). The results showed that the *FOX2* promoter contains two transcription initiation sites at positions -100 and -74 relative to the translation start codon, whereas the *ICL1* promoter contains only one transcription initiation site located at position -95. As expected, both the *FOX2 and ICL1* promoters contain at least one TATA box consensus sequence (TATAWAWR, W indicates A/T; R indicates A/G) [124] within a reasonable distance upstream of the transcription initiation sites (Figure 3-6). The *FOX2* promoter has two overlapping putative TATA boxes between positions – 168 and -161, and -164 and -157. On the other hand, only one TATA box can be found in the *ICL1* promoter between positions – 140 and -133. TATA-containing genes are known to be involved in response to environmental stress such as heat and starvation [124]. The presence of putative TATA boxes within these promoters agrees with the involvement of *FOX2 and ICL1* in the assimilation of alternative carbon sources.

<u>Figure 3-6.</u> Several transcription initiation sites are located within the *FOX2* and *ICL1* promoters

To map transcription initiation sites within the *FOX2* and *ICL1* promoters, 5'RACE PCR analysis was performed using both 5'RACE primers and gene-specific primers. Processed mRNA harboring a 5'RACE adapter was reverse transcribed to cDNA and used as the PCR template. The location of transcription initiation sites within the *FOX2* and *ICL1* promoters was then revealed via agarose gel electrophoresis and sequencing analysis of the resulting 5'RACE PCR products.

Figure 3-6. Several transcription initiation sites are located within the *FOX2* and *ICL1* promoters

A)

-180
ATATGAATGAA**TATTTATATATA**TAAATATATCTTAATCCCCCTTTTCCTTATTTCCATC
-100
-74
TGAAATTTATTTAATTGAAA**T**TTTTCTTTTTCTTTTTATTCTTTTT**C**TTTTTCCTTTTAA

TCCTTTGAAATATTCTATTTTATTAACTTCAAACATAATACTTTCCTCCCCCCCCCCATT **ATG** FOX2

B)

-180
AAAAAATGAGTCGTTACTCCAAGTGGTCAATGACTTTGC**TATAAATA**TTGCCAAGTTTCT
-95
CCTTTTTCAAATTTCATTCTTTTTT**A**ATACCCTTTTTCTTTTTCTTTTTTCCTTCTTTCC

TCTATACTTATACCTTTTATTCTAATATAAATTAAAGAATAAACATTAATAATATCTACC **ATG** ICL1
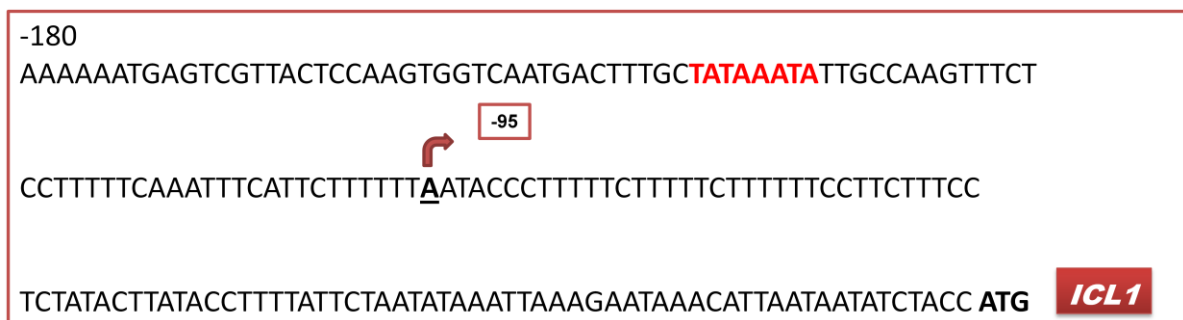
Putative DNA binding sites within the *FOX2* and *ICL1* promoters

In parallel with promoter deletion analysis we began a search for putative DNA binding sites within the *FOX2* and *ICL1* promoters that are regulated by carbon source through a survey of the literature. Transcription factors with zinc binuclear clusters can recognize regulatory elements that contain trinucleotide sequences (e.g CGG) in single or repeat forms, in either a symmetrical or an asymmetrical format [125]. In addition to binding DNA as monomers, these transcription factors can function as homodimers or as heterodimers [126]. DNA binding specificity is largely determined by the orientation of trinucleotide sequences and the nucleotide spacing between the triplets [127]. In the plant pathogen *Fusarium solani*, the CTF1 orthologue, CTF1α binds a GC-rich palindrome with an oppositely oriented 5'-GCC(n2)GGC to induce the cutinase gene in the presence of fatty acids [108]. In *A. nidulans*, both FarA and FarB positively regulate genes involved in fatty acid catabolism by binding sequences containing the 6-bp core CCTCGG motif [105]. According to Hynes *et al*., 2006, the CCTCGG motif is overrepresented in the 5' regions of genes predicted to be induced by fatty acids in filamentous fungi, but not in *S. cerevisiae* [105].

Guided by the studies performed in other fungi including *F. solani* and *A. nidulans* we decided to scan 1000 bp upstream of the translational start codon for exact matches to the CCTCGG sequence using pattern match software (Patmatch) from the *Candida* genome database (http://www.candidagenome.org/cgi-bin/PATMATCH/nph-patmatch). Patmatch analysis revealed one or more copies of the CCTCGG motif are present in the promoters of CTF1 target genes including *FOX2* and *ICL1* (Figure 3-7). A single copy of the CCTCGG motif was found between -350 and -355 relative to the translational start codon in *FOX2*. On the other hand, *ICL1* contains two regions with the CCTCGG motif positioned between -618 and -623, and its complement, CCGAGG positioned between -333 and -338, respective to the translational start codon. A more extensive Patmatch analysis showed the motif was also conserved in numerous other *C. albicans* genes that have a known role in alternative carbon

metabolism. A total of 252 genes containing the CCTCGG motif and its complement within 1,000 bp 5' of the translational start codon were analyzed by the Gene Ontology (GO) term mapper from the *Candida* genome database (http://www.candidagenome.org/cgi-bin/GO/goTermMapper) (Table 3-1) [Lorenz, M. C., unpublished results). The GO term mapper results for these genes show an overrepresentation of genes that are induced by fatty acids, such as those involved in β-oxidation and peroxisome processes. For example, the fatty acid oxidation GO term shows that the CCTCGG motif and its complement are upstream of 9 out of 12 genes annotated to that term (Table 3-1).

Transcript profiling studies performed by Murad *et al.*, 2001 [107] identified both *FOX2* and *ICL1* as targets for MIG1 repression in *C. albicans*. Nehlin and Rohne, 1990 [87] previously identified the sequence 5'-(G/C)(C/T)GG(G/A)G or SYGGRG as the DNA binding element for Mig1 in *S. cerevisiae*. Because *FOX2* and *ICL1* are under glucose repression it is possible that they harbor a Mig1 site that allows their expression to be repressed. To determine if *FOX2* and *ICL1* harbor this putative Mig1 binding site, I scanned the promoters using Patmatch and found both promoters carry multiple copies within 1000 bp upstream of the translational start codon. Putative Mig1 sites were found within the following regions: -865 to -860, -623 to -618, -282 to -277, and -260 to -255 for the *FOX2* promoter, whereas *ICL1* harbored two putative sites within -747 to -742 and-252 to -247 regions (Figure 3-7). The relevance of the putative DNA binding sites found by bioinformatic analysis in *C. albicans* needs to be assessed in future studies.

Figure 3-7. **Putative DNA binding sites within the *FOX2* and *ICL1* promoters**

Pattern match software (Patmatch) from the *Candida* genome database was used to find

putative DNA binding sequences within the *FOX2* A) and *ICL1* B) promoters. Putative DNA

binding sequences include the fatty acid response element, CCTCGG ■ , as well as the more

degenerate SYGGRG sequence ■ required for Mig1 repression.

Figure 3-7. Putative DNA binding sites within the *FOX2* and *ICL1* promoters
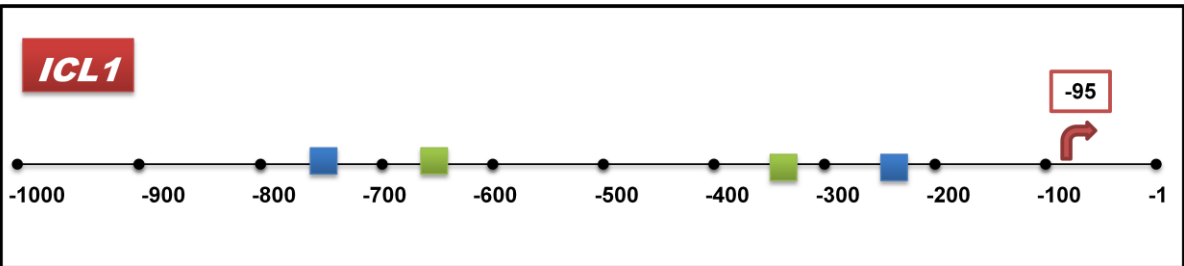
A)
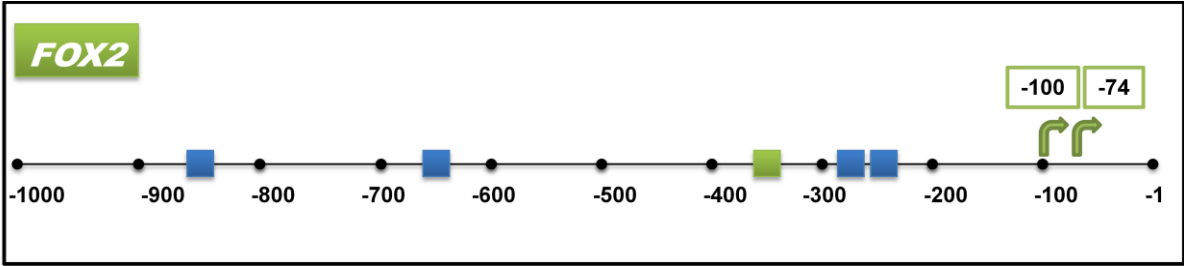
B)

Table 3-1. GO-enriched terms for genes with CCGAGG/CCTCGG within 1,000 bp 5' of the translational start codon

Process GO terms	Cluster Frequency (252 genes)	Background Frequency (6514 genes)	Corrected P-value	False Discovery Rate	Gene(s) annotated to the term
fatty acid oxidation	9, 3.6%	12, 0.2%	1.60E-08	0.00%	PEX11:FOX2: POX1-3:PXP2: orf19.4122: CAT2:PEX5: POX1:ANT1
lipid oxidation	9, 3.6%	12, 0.2%	1.60E-08	0.00%	PEX11:FOX2: POX1-3:PXP2: orf19.4122: CAT2:PEX5: POX1:ANT1
fatty acid β-oxidation	8, 3.2%	10, 0.2%	9.03E-08	0.00%	FOX2:POX1-3: PXP2:orf19.41 22:CAT2:PEX5 :POX1:ANT1
lipid modification	10, 4.0%	30, 0.5%	4.55E-05	0.00%	PEX11:FOX2: POX1-3:PXP2: orf19.4122: CAT2:orf19.48 65:PEX5: POX1:ANT1
cellular lipid catabolic process	9, 3.6%	28, 0.4%	0.00029	0.00%	FOX2:POX1-3: PXP2:orf19.41 22:CAT2:PEX5 :POX1:ANT1: PLB3
fatty acid metabolic process	12, 4.8%	58, 0.9%	0.00075	0.00%	PEX11:FOX2: POX1-3:PXP2: CRC1:orf19.41 22:CAT2:PEX5 :POX1:ANT1: PEX13:PEX7
mono-carboxylic acid metabolism	14, 5.6%	89, 1.4%	0.00319	0.00%	PEX11:FOX2: POX1-3:PXP2: CRC1:orf19.41 22:CAT2:MLS1 :PEX5:POX1: ANT1:ICL1: PEX13:PEX7
lipid catabolic process	9, 3.6%	40, 0.6%	0.00766	0.00%	FOX2:POX1-3: PXP2:orf19.41 22:CAT2:PEX5 :POX1:ANT1:

					PLB3
hydrogen peroxide catabolism	3, 1.2%	3, 0.0%	0.02712	1.09%	CAT1:TSA1B: TSA1
glyoxylate cycle	3, 1.2%	3, 0.0%	0.02712	1.00%	FOX2:MLS1: ICL1
glucose transport	6, 2.4%	22, 0.3%	0.06644	1.14%	HGT6:SHA3: HGT17:HGT19 HGT10:HGT13
peroxisomal transport	5, 2.0%	15, 0.2%	0.08637	1.20%	PEX4:PEX5: PEX1:PEX13: PEX7
protein targeting to peroxisome	5, 2.0%	15, 0.2%	0.08637	1.12%	PEX4:PEX5: PEX1:PEX13: PEX7
Component GO_terms					
peroxisome	17, 6.7%	48, 0.7%	9.91E-11	0.00%	PEX11:orf19.1 64:POX13:PX P2:PEX4:orf19 .4122:CAT2: MLS1:PEX5: POX1:CAT1: ANT1:PEX1: orf19.6591: ICL1:PEX13: PEX7
peroxisomal part	7, 2.8%	22, 0.3%	0.00147	0.00%	PEX11:orf19.1 64:POX1-3: PEX5:CAT1: ANT1:PEX13

The CCTCGG motif is required for *FOX2* regulation

      To identify promoter regions that harbor relevant *cis*-acting sequences, I tested the 5'

sequential deletions at 100 bp intervals upstream of the start codon of each gene and analyzed

them in a wild-type background using Northern Blot and Fluorescence microscopy. Northern

Blot analysis of the 100 bp-*FOX2* deletions showed that in contrast to the 1000 and 500 bp-

FOX2-GFP promoter fragments, there is a significant reduction in expression for the 400 bp and

300 bp fragments (Figure 3-8 A). These results indicated that the region between -500 and -300

is important for fatty acid regulation. However, fluorescence microscopy analysis demonstrated

that *cis*-acting sequences under positive fatty acid regulation are more likely to be located

between -400 and -300 (Figure 3-8 B). Based on these results and the fact that the CCTCGG

motif is found at position -350 within the *FOX2* promoter, I hypothesized that this motif is the

relevant *cis*-acting sequence under positive fatty acid regulation. To test this, I made additional

reporter constructs that consisted of 1000 bp upstream of the start codon with either a deleted

motif or a motif with a specific-site mutation (CCTCGG → CCTCAG). The relevance of the

CCTCGG motif was analyzed using fluorescence microscopy (Figure 3-9). The results show a

significant reduction in GFP fluorescence upon deleting the CCTCGG motif. A disadvantage of

deleting the motif is that a new sequence is generated and it is often necessary to perform site-

specific mutations to maintain intact flanking sequences. Changing a guanine to an adenine

residue in the CGG triplet showed a reduced GFP activity indicating that the second guanine

residue is important for positive fatty acid regulation. Surprisingly, a reduction in GFP

expression is seen in acetate. It is possible that changing the guanine residue may lead to a

change in DNA conformation that allows a subtle Mig1-dependent or –independent repression.

Overall, these results are consistent with other studies performed in related fungi were the

importance of the CCTCGG motif for carbon source regulation has been shown. In particular,

the second guanine residue in the CGG triplet was determined to be the dominant contact for

Gal4 binding in *S. cerevisiae* [128], suggesting that a similar scenario may occur in *C. albicans*.

<u>Figure 3-8.</u> *FOX2* promoter contains one *cis*-acting element under fatty acid regulation

A) The wild-type (SC5314) strain and *FOX2-GFP* reporter strains harboring 5' 1000 bp or

systematic 100 bp deletions (see table 2-1) were grown overnight in YPD, collected by

centrifugation, washed with water and diluted in YNB supplemented with 2% glucose and grown

to log-phase. Cells were collected by centrifugation, washed and incubated at 30°C for a one-

hour period in YNB media containing 2% glucose, 2% potassium acetate or 0.5% oleic acid/1%

tween 20. Expression of endogenous *FOX2* gene and the *GFP* gene was detected via Northern

blot analysis using gene-specific probes. Ethidium bromide staining of the rRNA was used as a

loading control. B) Quantification of *FOX2-GFP* reporter strains was performed as described

previously chapter 2.

Figure 3-8. *FOX2* promoter contains at least one *cis*-acting element under fatty acid regulation

A)

B)
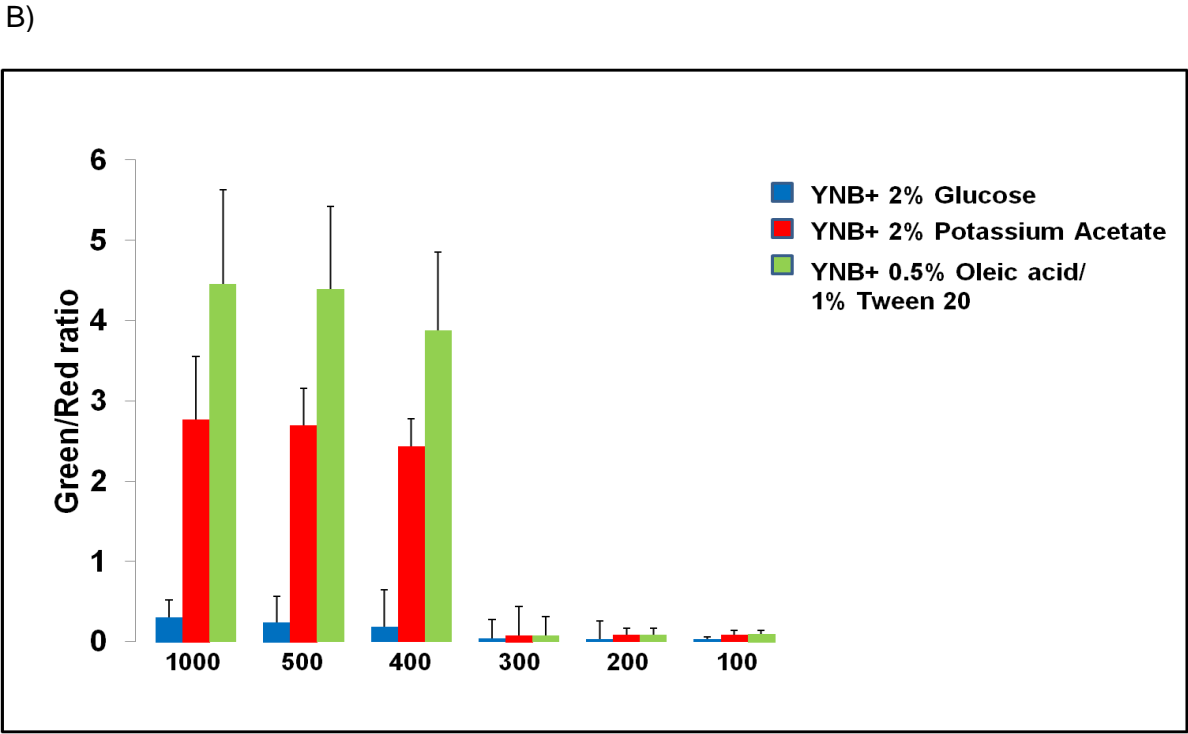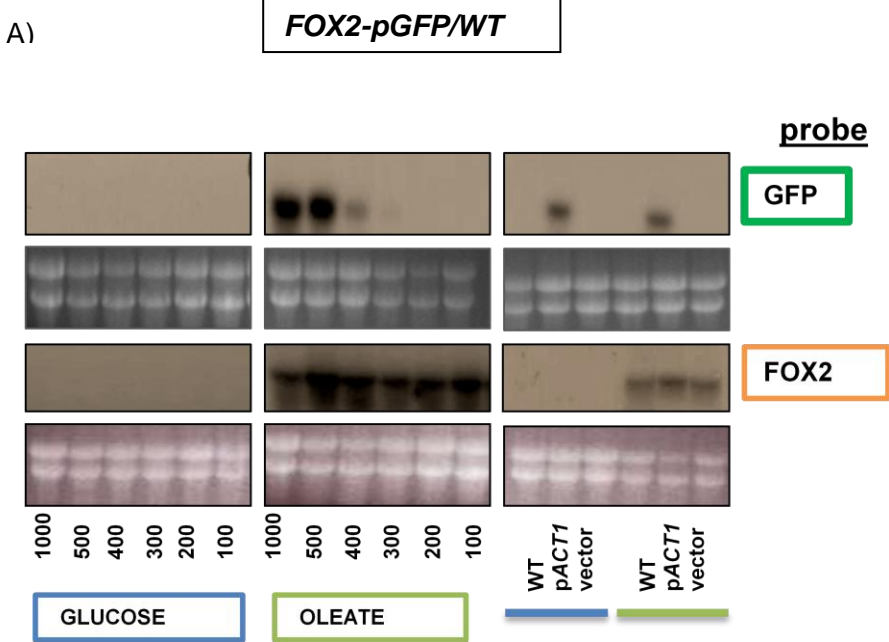
Figure 3-9. The CCTCGG motif is required for *FOX2* regulation

Overlap extension PCR was used to delete the entire CCTCGG motif from the *FOX2* promoter or to mutate key residues using site-directed mutagenesis (SM) (CCTCGG → CCTCAG). *FOX2-GFP* reporter strains lacking the CCTCGG motif or containing the mutagenized version (see table 2-1) were grown overnight in YPD, collected by centrifugation, washed with water and diluted in YNB supplemented with 2% glucose and grown to log-phase. Cells were collected by centrifugation, washed and incubated at 30°C for a one-hour period in YNB media containing 2% glucose, 2% potassium acetate or 0.5% oleic acid/1% tween 20. Quantification of reporter strains was performed as described previously.

Figure 3-9. The CCTCGG motif is required for *FOX2* regulation
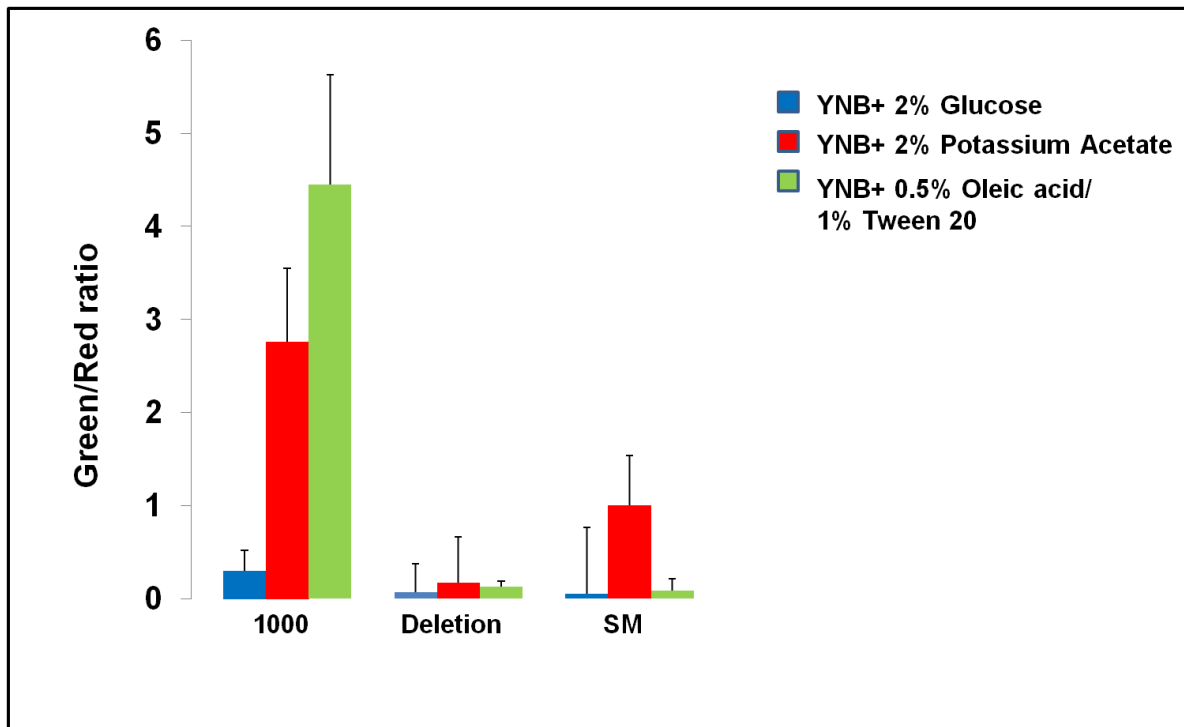
The CCTCGG motif does not appear to be relevant for *ICL1* regulation, whereas additional unidentified elements seem to be important

The 5' sequential deletions at 100 bp intervals upstream of the start codon of *ICL1* were also analyzed in a wild-type background using Northern Blot and Fluorescence microscopy. Northern Blot analysis of the 100 bp-*ICL1* deletions showed the *ICL1* promoter contains one or more positively regulated sites between -1000 and -500 relative to the translational start (Figure 3-10). As shown previously in figure 3-7, the *ICL1* reporter harbors a CCTCGG motif between -623 to -618 and its complement between -338 to -333 relative to the translational start codon. Therefore, I hypothesized that the CCTCGG motif found between -623 to -618 in the *ICL1* promoter was a relevant *cis*-acting sequence for *ICL1* regulation. Overlap extension PCR was used to delete or mutagenize this motif using the 1000 bp-GFP reporter construct as a template. The goal was to delete or alter each motif as done previously for *FOX2*. Initial PCR products were obtained and used as templates to create a final PCR product. However, even after multiple attempts I was unable to generate a final PCR product. As a result, the relevance of the CCTCGG motif for ICL1 regulation could not be assessed. Therefore, I decided to create additional 5' systematic deletions at 100 bp intervals between the -1000 and -500 promoter region relative to the translational start. Quantification of GFP fluorescence of the *ICL1* reporter in a wild-type background suggests *ICL1* contains one or more positively regulated sites between -1000 and -900 relative to the translational start codon. Overall, these results suggest that it is unlikely that the CCTCGG motifs found at positions -618 and the complement, CCGAGG at position -333 are relevant for *ICL1* regulation. Conclusions and a discussion of these findings are addressed in the next chapter.

Figure 3-10. *ICL1 cis*-acting elements are found upstream of -900 position and CCTCGG motif does not appear to be relevant for *ICL1* regulation.

A) The *ICL1-GFP* reporter strains harboring 5' 1000 bp or systematic 100 bp deletions (see table 2-1) were grown overnight in YPD, collected by centrifugation, washed with water and diluted in YNB supplemented with 2% glucose and grown to log-phase. Cells were collected by centrifugation, washed and incubated at 30°C for a one-hour period in YNB media containing 2% glucose, 2% potassium acetate or 0.5% oleic acid/1% tween 20. Expression of *ICL1-GFP* was detected via Northern blot analysis using a GFP-specific probe. Ethidium bromide staining of the rRNA was used as a loading control. B) Quantification of *ICL1-GFP* reporter strains was performed as described previously.
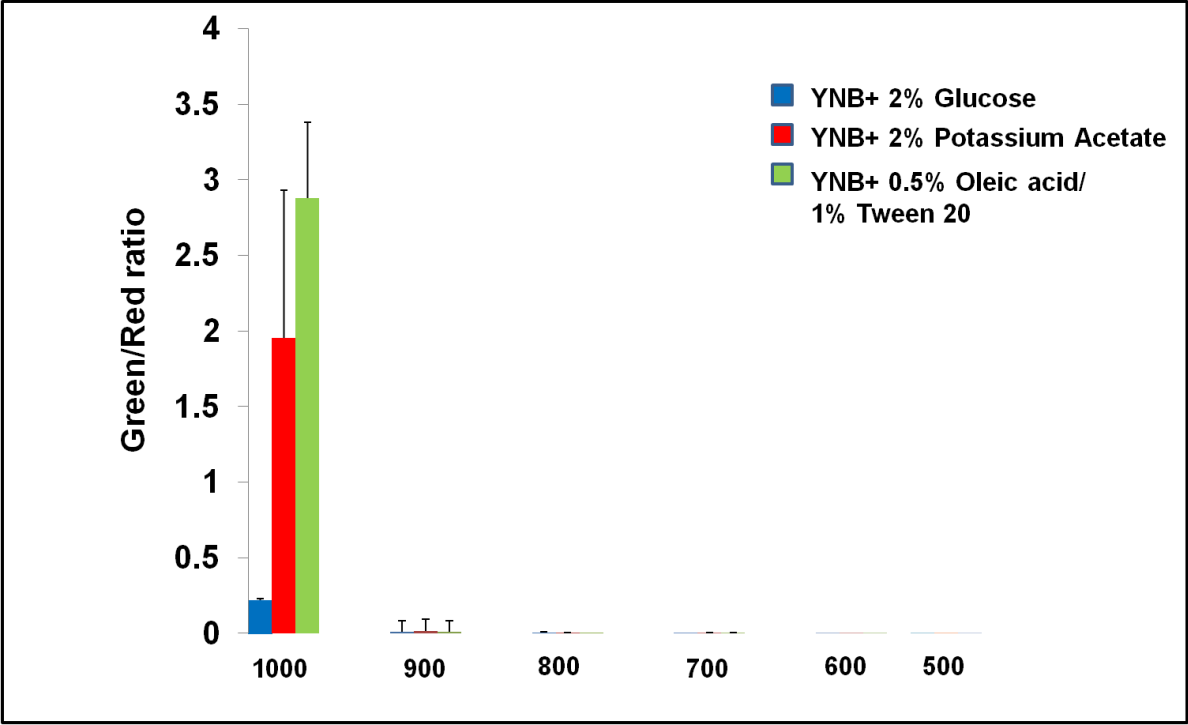
Figure 3-10. *ICL1 cis*-acting elements are found upstream of -900 position and CCTCGG

motif does not appear to be relevant for *ICL1* regulation.
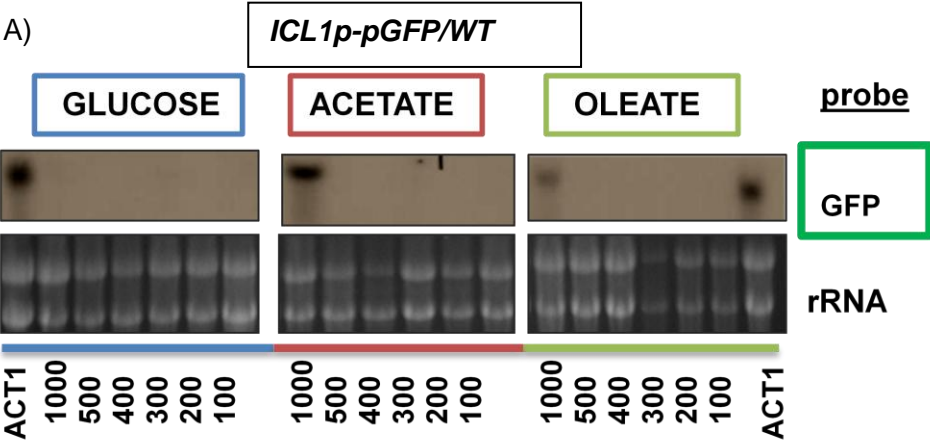
Chapter 4. Conclusions and Perspectives

In the past two decades, there has been an increase in infections caused by opportunistic fungal pathogens. The rise of hospital-acquired fungal infections has been attributed in part to an increase in immunocompromised and non-immunocompromised, critically ill patients. In addition, the management of invasive candidiasis is complicated by several factors including poor diagnosis, inadequate or delayed treatment, drug-related toxicity and increasing drug resistance. Under predisposing conditions, *C. albicans* can switch from a commensal to a pathogenic state, causing a broad range of infections which manifest as superficial or systemic infections. Because *C. albicans* is considered the predominant cause of invasive candidiasis and is associated with high morbidity and mortality, it has become the subject of extensive research. However, most studies have focused on classical virulence factors such as adhesins and hydrolytic enzymes involved in persistence of colonization. Less emphasis has been placed on the metabolic requirements of *C. albicans* during infection.

C. albicans has the ability to survive in a variety of harsh micro-environments within its mammalian host. Its remarkable plasticity in adapting to diverse host niches is reflected by its ability to invade virtually any site of the human body. The nutrient composition in different anatomical sites varies throughout the body, and some micro-environments lack preferred carbon sources that allow fungal growth and survival. For example, glucose, the preferred carbon source for *C. albicans* appears to be scarce inside phagocytic cells, but is accessible in the bloodstream. Several research groups, including ours have focused on the interaction of *C. albicans* with host innate immune cells, which serve as the first line of antifungal defense. Phagocytic cells such as neutrophils and macrophages play a key role in preventing disease progression by confining *C. albicans* to site of colonization or infection, and in preventing dissemination to the bloodstream and tissue invasion. Upon phagocytosis, *C. albicans* undergoes a complex transcriptional response that leads to the upregulation of genes involved in alternative carbon metabolism, including the glyoxylate cycle, gluconeogenesis and β-oxidation [57,64,71]. It has been proposed that *C. albicans* upregulates these pathways in order

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to use a wide variety of non-preferred carbon sources such as fatty acids, acetate and other gluconeogenic substrates encountered upon phagocytosis. Without the acquisition of essential nutrients required for growth, *C. albicans* would not be able to maintain infection. The importance of the metabolic flexibility displayed by *C. albicans* is evident, since mutations affecting key enzymes of alternative carbon metabolic pathways attenuate the virulence of *C. albicans*. Due to the importance of alternative carbon metabolic pathways during infection, these pathways are promising candidates for the development of new anti-fungal drugs [109,110].

The regulation of alternative carbon metabolism in *C. albicans* appears to be a combination of the regulatory systems in *S. cerevisiae* and *A. nidulans*

To enhance our understanding on how *C. albicans* may regulate alternative carbon metabolic pathways, homologs of the well-characterized transcription factors in the non-pathogenic yeast, *S. cerevisiae* and the filamentous fungus, *A. nidulans* have been identified in our lab. Although the metabolic machinery is essentially conserved among different yeast species, variations in the regulatory networks confer phenotypic differences between them [129]. Current data indicates the mode of transcription-based regulation in *C. albicans* is significantly different from yeast and although some aspects appear more analogous to filamentous fungi, specific adaptations in *C. albicans* may contribute to its ability to survive in harsh microenvironments. *C. albicans* has homologs of the *S. cerevisiae* CAT8 and ADR1 proteins, which correspond to FacB and AmdX in *A. nidulans* and are known to regulate gluconeogenesis, the glyoxylate cycle, and ethanol utilization. In contrast to *S. cerevisiae* and *A. nidulans*, deleting these genes in *C. albicans* does not confer a phenotype [83]. Furthermore, the transcription factors Oaf1p and Pip2p, which regulate genes involved in fatty acid β-oxidation and peroxisome biogenesis in *S. cerevisiae* are lacking in both *A. nidulans* and *C. albicans*. In *A. nidulans*, the FarA and FarB proteins are responsible not only for regulating fatty acid β-oxidation and peroxisome biogenesis, but also the glyoxylate cycle. It has become

apparent that *C. albicans* has a more integrated regulatory network than *S. cerevisiae* and

displays more similarities to regulatory networks in *A. nidulans.* According to Ramirez and

Lorenz, 2009 [83], *C. albicans* cannot afford to have periods of metabolic inactivity if it is to

prevail against the host's immune response. In order to counteract immune attack, it must be

able to rapidly adapt to hostile environments such as those provided by the macrophage

intracellular milieu, where there it encounters limited nutrients. Being able to rapidly assimilate

multiple non-preferred carbons sources may make the difference between life and death. The

different environmental pressures faced by the *S. cerevisiae* and *C. albicans* may have resulted

in metabolic gene expression differences between species.

CTF1 regulates fatty acid metabolism and glyoxylate cycle genes

Recently, the *C. albicans* transcription factor CTF1 has been identified and characterized

based on its homology to the known *A. nidulans* transcripiton factors FarA and FarB [83]. Gene

deletion of *CTF1* results in a mild, but significant decrease in virulence. Northern blot analysis

revealed CTF1 is required for the regulation of genes involved in fatty acid β-oxidation, the

glyoxylate cycle, and peroxisome biogenesis [83]. In *S. cerevisiae*, the transcription factors Oaf1

and Pip2 control the expression of broad range of genes that are involved β-oxidation or

peroxisomal biogenesis. Although CTF1 is significantly different from Oaf1 and Pip2, it has a

similar function to these factors since it regulates a wide array of genes required for fatty acid

degradation. Based on Northern Blot analysis, the induction of some of these genes in the

presence of fatty acids has been shown to be either partially dependent or entirely dependent

on CTF1 regulation [83]. Global transcription profiling using microarray is a powerful approach

to gain insight into the mode of regulation of a transcription factor under different conditions,

such in the presence of different carbon sources. Microarray analysis derived from wild-type vs.

ctf1Δ/Δ mutant strains grown in glucose or oleate revealed CTF1 coordinates the expression of

a large set of genes (217 genes) [Ramírez, MA and Lorenz, MC unpublished data]. This large

CTF1 regulon consists of genes required for fatty acid catabolism, gluconeogenesis, and the

glyoxylate cycle, as well as glucose transport and a few genes involved in other cellular processes including the stress response. Consistent with previous Northern blot analysis, some of the genes were identified as being partially dependent or entirely dependent on CTF1 regulation. It was surprising to find that 60% of the genes either lack homology to those found in other organisms or their homologs have not been characterized. Many of these genes are fungal specific and may represent genes that are important for *C. albicans* pathogenicity making them potential candidates for development of antifungal drugs.

To gain insight into the interplay between transcription factors and *cis*-acting sequences that equip *C. albicans* with the metabolic flexibility necessary for disease progression, we have focused on CTF1 and two of its regulatory target genes, *FOX2* and *ICL1*. Identifying and characterizing regulatory motifs within CTF1 co-regulated genes, *FOX2* and *ICL1* is important to increase the current knowledge on the regulatory mechanisms that govern alternative carbon metabolism at the transcriptional level. Previous Northern blot results generated in our lab [75,83], have been confirmed in this study. The endogenous expression of *FOX2* and *ICL1* genes is repressed in the presence of glucose, whereas their expression in the presence of acetate and oleate is relatively rapid. To identify *cis*-acting sequences important for the regulation of *FOX*2 and *ICL1*, a combination of bioinformatic analysis and promoter deletion analysis was employed. 1000 bp 5' of the translational start and systematic 100 bp deletions of each gene were fused to the GFP reporter and analyzed for reporter expression in wild-type strains and strains lacking CTF1 using Northern blot analysis and fluorescence microscopy. *FOX2-GFP* and *ICL-GFP* reporter expression was not detected in glucose-containing media. These data confirms previous Northern blot [75,83] and transcript profiling results [107] that identified both *FOX2* and *ICL1* as glucose-repressed genes. In addition, a carbon-source dependent expression of *FOX2* and *ICL1* was evident since *FOX2-GFP* and *ICL1-GFP* reporter strains were fluorescent in media containing acetate or oleate. In the absence of CTF1, *FOX2* is no longer induced, but it is still subject to derepression. These results are consistent with

previous Northern analysis [75,83]. However, the results obtained for *ICL1-GFP* reporter strains are less clear. Northern blot data generated in our lab [83] showed CTF1 is not required for the expression of *ICL1* in the presence of acetate. Therefore, it was surprising to find that the *ICL1-GFP* reporter expression was significantly reduced in the absence of CTF1. Future experiments are needed to resolve the discrepancies seen between previous Northern results and current GFP reporter data for *ICL1*.

The CCTCGG motif is a *cis*-regulatory element important for control of the fatty acid β-oxidation gene, *FOX2*

In *A. nidulans*, the *cis*-acting sequence (CCTCGG) has been shown to be required for fatty acid regulation and binding of both FarA and FarB target genes. This motif is present within the *FOX2* and *ICL1* promoters and is a candidate sequence for fatty acid CTF1-dependent regulation and binding.   To determine its relevance in *C. albicans*, the CCTCGG motif found within the *FOX2* promoter was deleted or mutagenized and fused to the GFP reporter for fluorescence analysis. In this work, we have identified the CCTCGG motif as necessary for control of the fatty acid β-oxidation, *FOX2* gene. Previous Northern analysis of wild-type strains grown in glucose, acetate and oleate showed expression of CTF1 only in the presence of oleate. This suggests *FOX2* is only induced by CTF1 in the presence of oleate. However, deleting or mutagenizing the CCTCGG motif results in a significant reduction in *FOX2-GFP* reporter regardless of whether it is grown in acetate or oleate. These results indicate additional mechanisms are involved. For example, deleting the motif may cause spacing alterations that disrupt regulation at the chromatin level.

Contrary to what is seen for *FOX2*, the CCTCGG motif does not appear to be relevant for *ICL1* regulation. Positively regulated site(s) between -1000 and -900 relative to the translational start appear to be more relevant for *ICL1* regulation. Based on the *ICL1* promoter truncation analysis performed in this study, there is no role for one specific motif, but there could be close mismatches elsewhere that are important for *ICL1* regulation. The significance of the

CCTCGG motif and its complement for *ICL1* regulation remains questionable since their position does not coincide with previously identified regions. Although this study has helped increase the current knowledge on the regulatory mechanisms that govern alternative carbon metabolism at the transcriptional level, several unanswered questions remain. 1) What are the additional *cis*-acting sites involved in *FOX2* and *ICL1* regulation? and 2) Is CTF1 regulation direct or indirect?

What are the additional *cis*-acting sites involved in the regulation of *FOX2* and *ICL1*?

Promoter regions are likely to harbor multiple elements that act in a combinatorial manner thereby complicating the identification of discrete elements. The mechanisms by which *FOX2* and *ICL1* are activated by CTF1 seem to be different. Bioinformatic analysis of the upstream region of *FOX2* and *ICL1* genes revealed their promoters contain several putative cis-regulatory elements for transcription factors found in *S. cerevisiae* (Sc) and *A. nidulans* (An). These include putative sites for ScMig1, ScCat8/ AnFacB, ScAdr1, and AnFarA/FarB. Consistent with *FOX2* being under Mig1 repression and its role in fatty acid metabolism, the *FOX2* promoter harbors several putative Mig1 sites and a single copy of the CCTCGG motif. In *A. nidulans,* Hynes *et al*., 2006 [105] predicted that there are three classes of genes, class I: genes induced specifically by acetate, class II: genes that are induced by both acetate and fatty acids and class III: genes that are only induced by fatty acids. Based on our results and the putative sites found within FOX2 promoters, it appears that FOX2 might be a class III gene, since it is highly induced in the presence of oleate and is required for fatty acid catabolism.

Increasing evidence indicates that although there is conservation of *cis-* and *trans-*regulatory systems across different fungal species, extensive transcriptional reorganization or rewiring can lead to highly plastic regulatory networks [130,131]. These can form as a result of many types of modifications including mutations in *cis-* regulatory sequences. Changes in transcription factors, including binding specificity, trans-activating potential, and/or cooperative binding characteristics can also contribute to transcriptional flexibility [129]. In addition, some

transcription factors with identical binding specificity and kinetics can regulate genes that are considerably different, thereby changing the members of a particular regulon [132]. Evidence of transcriptional rewiring across species has been provided by several studies. For example, Martchenko *et al.*, 2007, showed that Gal4, a transcription factor that is required for galactose utilization in *S. cerevisiae* is instead required for glycolysis in *C. albicans* [133]. Studies in our lab have demonstrated that *CAT8* and *ADR1* have no apparent phenotype in *C. albicans* despite having significant sequence similarity to their counterparts in *S. cerevisiae.* In *C. albicans*, CTF1 is the single functional ortholog of the *A. nidulans* FarA/FarB proteins, which are known to bind the CCTCGG motif [83]. However, DNA sequences that are relevant in related fungi may not be functional in *C. albicans.* The *in vivo* function of putative *cis*-acting element across multiple fungal species can be influenced by several factors including site degeneracy, a requirement for specific adjacent sequences and changes in transcription binding specificity. Although the CCTCGG motif is present in both *FOX2* and *ICL1*, it is not equally important for the regulation of both genes. It is possible that the identification of regulatory sites within the *ICL1* promoter may have been complicated by the presence of multiple regulatory sites. Bioinformatic analysis of the *ICL1* promoter showed multiple putative sites for Mig1, Cat8/ FacB, and AnFarA/FarB. Many of these sequences were found at overlapping positions. Based on this, I speculate that the regulation of *ICL1* is most likely as a result of multiple inputs, unlike *FOX2*, whose induction is dependent on the presence of fatty acids. Although, 5' promoter deletion analysis is useful in defining the boundaries of a regulatory region, a more refined deletion and substitution analysis is required to identify all the *cis*-acting elements involved in carbon based source regulation.

Is CTF1 regulation direct or indirect?

Genetic data indicates CTF1 is a regulator of fatty acid degradation, yet knowledge of whether CTF1 regulation is direct or indirect is limited. Since, the CCTCGG motif is a *cis*-regulatory element important for control of the fatty acid β-oxidation, *FOX2* gene, gel shift

assays can be used to determine if *FOX2* is a direct target of CTF1. Gel shift assays are particularly useful for analyzing DNA-protein interactions when using short probes with well-defined elements. Therefore, using a short *FOX2* probe containing the CCTCGG motif can be used. In addition, competition assays using specific competitors (unlabeled wild-type and mutant probes) can be employed to effectively determine CTF1 binding. I have performed preliminary gel shift assays using *C. albicans* whole cell lysates and purified recombinant CTF1 protein from *E. coli*. A faint band shift was observed when *C. albicans* lysates were tested suggesting a binding event is taking place. Additional attempts to detect CTF1 specific binding events were not successful and further optimization is required. Specific binding events may have been missed when using protein from *C. albicans* lysates due to insufficient CTF1 protein levels. To detect potential specific CTF1 binding events, gel shift assays using a purified CTF1-TAP protein can be performed in future experiments. I have created two independent strains that express CTF1 with the TAP tag at the C-terminus using PCR-mediated homologous recombination [7] and efforts to purify the protein using the TAP method as described by Lavoie *et al.*, 2008 [7] are underway in our lab.

Although *in vitro* approaches performed in *F. solani* and *A. nidulans* have been successful in detecting binding to the CCTCGG motif, this may not be equally feasible in *C. albicans*. It is possible that CTF1 itself is regulated by phosphorylation in a context-dependent manner. Studies in *F. solani* indicate that phosphorylation correlates with FsCTF1α DNA binding and transcriptional activity [134]. *In vitro* gel shift assays and transcription assays revealed the phosphorylation was required for binding and transactivation capacity of FsCTF1α. *In silico* analysis of the CTF1 amino acid sequence revealed multiple putative serine, threonine, and tyrosine phosphorylation sites. Based on these findings, it is possible that phosphorylation of CTF1 influences DNA binding and activation of *FOX2* and *ICL1*. In addition, DNA topology and other transcription factors may influence binding, therefore hindering detection of CTF1-DNA

complexes *in vitro*. Chromatin immunoprecipitation followed by high throughput sequencing

(ChIP-Seq) may be required to actually detect *in vivo* CTF1 binding events.

Promising strategies for the development of antifungal drugs

Several strategies have been proposed for the development of more effective antifungal

drugs. Transcription factor-based therapeutic approaches include creating artificial transcription

factors and the use of small molecules that disrupt DNA binding. However, these approaches

have inherent disadvantages and targeting a particular carbon metabolic pathway as a whole

may be more effective. For example, Simm *et al.*, 2011 [135] have used a cell-based screen to

identify new antifungal drugs that target fungal zinc homeostasis. A similar approach that

targets fatty acid β-oxidation in *C. albicans* could be used instead. This would involve the use of

a strain that monitors changes in fatty acid utilization. In this study, we have shown that wild-

type cells transformed with the *FOX2-GFP* reporter is repressed in the presence of glucose and

induced in acetate and oleate. Because FOX2 is a key enzyme required for the assimilation of

fatty acids, a cell-based assay using the *FOX2-GFP* reporter can be used to identify compounds

that inhibit fatty acid β-oxidation. For this assay, reporter cells would be grown in oleate and

treated with test compounds. Fluorescence microscopy analysis would then allow the detection

of compounds that inhibit GFP fluorescence. To facilitate high through-put screening, a multi-

well format (e.g. 384 micro-titer plates) would be used in a primary screen of compound libraries

along with positive and negative controls. GFP fluorescence intensity would then be measured

using an automated plate reader. Cells treated with a known compound that inhibits fatty acid β-

oxidation in *C. albicans* such as 2-methoxy-4-thia fatty acid (±)-2-methoxy-4-thiatetradecanoic

acid [136] can be used as a positive control, whereas a negative control would consist of

untreated samples. Additional positive controls such as including untransformed wild-type cells

or the addition of glucose to the wells would increase the confidence of the screen. Compounds

that significantly reduce GFP expression would be candidates for further validation tests. A

secondary screen for growth inhibition would allow the identification of potent and specific

inhibitors of fatty acid β-oxidation and would give insight into compounds' mode of action. For the secondary screen, reporter cells treated with candidate compounds would be grown in glucose, acetate or oleate as the sole carbon source. Compounds found to inhibit *FOX2-GFP* expression in the primary screen are expected to inhibit growth in oleate. Growth inhibition in acetate would suggest the test compound does not directly disrupt fatty acid β-oxidation, but may be targeting a downstream pathway or enzyme. Additional studies such as microarray analysis of treated versus untreated cells can be performed to determine the identity of their targets.

In this study, a combination of bioinformatics and promoter analysis was employed to determine how CTF1 regulates its target genes *FOX2* and *ICL1* involved in alternative carbon metabolism. This study has revealed the CCTCGG motif is relevant for CTF1-dependent regulation of FOX2, but not for *ICL1*. However, it is unknown whether CTF1 binds the CCTCGG motif and further studies are required to determine if CTF1 regulation is direct or indirect. *C. albicans* has adapted its metabolic regulatory repertoire resulting in a dynamic response that allows it to thrive within nutrient poor microenvironments. This study provides insight into the adaptation strategies at the transcriptional level used by *C. albicans*. Understanding how these strategies lead to the metabolic flexibility displayed by *C. albicans* will be a cornerstone for future advances in anti-fungal treatments against systemic candidiasis.

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