WPI

MAJOR QUALIFYING PROJECT

CHARACTERIZATION OF ZCF TRANSCRIPTION FACTORS THAT ARE EXPANDED IN THE HUMAN PATHOGEN *CANDIDA ALBICANS*

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May 1, 2014

CHARACTERIZATION OF ZCF TRANSCRIPTION FACTORS THAT ARE EXPANDED IN THE HUMAN PATHOGEN *CANDIDA ALBICANS*

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ABSTRACT

An opportunistic pathogen, *Candida albicans*, is commonly found in humans as a commensal organism populating the skin, oral cavity, gastrointestinal, and urogenital tract. However in immunocompromised patients, *C. albicans* colonizes epithelial and non-epithelial tissues, giving rise to mucosal and bloodstream infections. The pathogenesis of *C. albicans* has not been fully understood and the available treatments for *Candida* infections are still limited. Previous studies have shown that a subset of genes encoding putative $Zn(II)_2Cys_6$ transcription factors, *ZCF*, is only expanded in pathogenic yeast and might be involved in *C. albicans*'s virulence. This study utilizes *ex vivo* and *in vitro* approaches to characterize select *ZCF*s in macrophage cells and various stress environments. The findings suggest that these *ZCF*s may be key pathogenic determinants in *C. albicans* and may provide potential fungal-specific drug targets.

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INTRODUCTION

Pathogenicity of *Candida albicans*

Candida albicans is a polymorphic fungus commonly found as a commensal organism in the human microbiome. In healthy individuals, it populates many areas of the body including the skin, oral cavity, gastrointestinal, and urogenital tract (Noble *et al.* 2010). However, when the host's immunity is compromised, *C. albicans* can become pathogenic and cause a variety of diseases. The diseases range from superficial, non-life threatening infections such as oral thrush and vaginal infections to fatal bloodstream infections (candidemia) in severe cases of immunodeficiency, it may potentially invade all body sites and important organs like the kidney and brain (Vandeputte *et al.* 2011). The following groups of people are the most susceptible to *C. albicans* infections due to their weakened immune systems: AIDS/HIV-infected individuals, patients undergoing anticancer treatment, patients having transplantation therapies and individuals who have implanted medical devices (Fox and Nobile 2012). Along with other *Candida* species, *C. albicans* is notorious for being the fourth most common cause of nosocomial bloodstream infections. According to Brown *et al.* (2012), the mortality rates for worldwide case of *Candida* infections or candidiasis range between 46-75% in infected populations.

Gow and Calderone (2002) states that *Candida* infection occurs as the results of weakening of host defenses and invasiveness of *C. albicans*. When the host's immune system becomes deficient, *C. albicans* utilizes several key virulence factors to invade and infect the host. Cell surface adhesion proteins called adhesins assist *C. albicans* to establish contact with and colonize the host's epithelial cells. *C. albicans* also secretes hydrolytic enzymes such as aspartyl proteinases and phospholipases by digesting the host's cell membrane for tissue invasion and degrading host's surface molecules to reinforce adhesion (Hube and Naglik 2002). It is believed that morphogenesis benefits *C. albicans* during invasion and dissemination. *C. albicans* exists as a hyphal form for tissue invasion and switches to its yeast form when disseminating (Brown 2002). The phenomenon of phenotype switching involves the changes in the *C. albicans'*s expression of cell surface antigens, tissue affinities of the organism, enzyme production, and drug sensitivity to adapt to a range of physiological extremes within the host (Calderone and

Fonzi 2001). In addition, *C. albicans* also produces biofilm and secondary metabolic signals such as farnesol as virulence factors (Ghosh *et al.* 2010).

One reason that accounts for the high cases of *Candida* infection is the limited number of ways to treat these infections. The current attempts to treat candidiasis and candidemia involve the use of antifungal agents that target *C. albicans*'s cell structure. The antifungal agents are divided into four major compound classes: polyenes (amphotericin B and nystatin), azoles (fluconazole, itraconazole, and voriconazole), pyrimidine analogues (5-fluorocytosine), and echinocandins (caspofungin) (Vandeputte *et al.* 2011). Their functions and mode of actions are shown in Figure 1. Azole drugs, like fluconazole (FLU) in Figure 1A, target the biosynthesis of ergosterol, which is the main component of plasma membrane structure specific to yeast (MacPherson *et al.* 2005). Polyenes, in Figure 1B, bind to ergosterol and compromise the plasma membrane integrity, allowing 5-fluorocytosine drugs to enter and inhibit the nucleotide biosynthesis (Mukherjee *et al.* 2005). Echinocandins, in Figure 1D, act by inhibiting the synthesis of 1,3-β-D-glucan, a component of fungal cell wall, so to allow other antifungal agents to enter the cell (Barker and Rogers 2006).

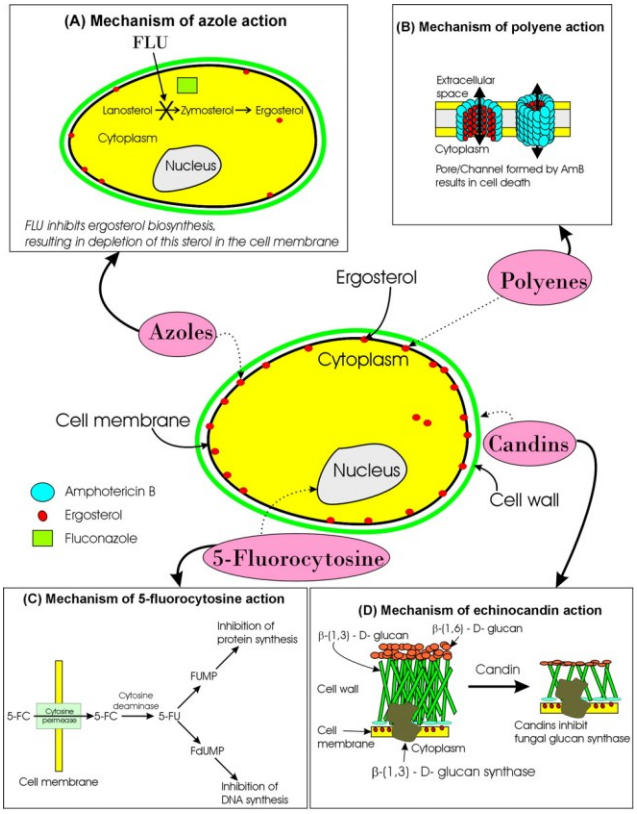

Figure 1. The mode of actions of antifungal drugs. Reprinted from Mukherjee *et al.* (2005)

In general, azoles are preferred over other antifungal agents to treat *C. albicans* infections due to their lower toxicity to the host, improved efficacy, and availability in both topical and oral form (Znaidi *et al.* 2009). However, the current antifungal treatments only target limited parts of *C. albicans* and do not guarantee full recovery for the infected individuals. Moreover, repeated and prolonged use of antifungal agents leads to resistance. In a recent report from the US Centers for Disease Control and Prevention (2013), an increasing incidence of *Candida* infections was reported from resistance of azole and echinocandins drugs. This issue has gathered attention and has become a serious public health threat. Due to these reasons, it is important to seek alternate ways to treat *Candida* infections and gain a better understanding of *Candida*'s pathogenesis, especially the pathogenesis of *C. albicans.*

Transcription Factors and Zinc Finger Protein

Several studies have been conducted to determine other possible *C. albicans*'s virulence factors; this includes a previous screening done by Chiasson *et al.* (2010) that identified four novel genes as potential virulence factors, namely orf19.6713, orf19.1219, *DOT4*, and *ZCF15*. The last kind, *ZCF15* particularly is interesting since it is a transcription factor that can be implicated in various metabolic pathways. Transcription factors (TFs) use signals from their surrounding and send adapted responses by modulating the gene transcription (Vandeputte *et al.* 2011). They can be associated with various metabolism processes such as DNA replication, repair, and transcription gene control.

TFs are categorized into different families based on their DNA binding domains. Some of these DNA binding domains are fungal helix-loop-helix (HLH), helix-turn-helix (HTH), high mobility group (HMG) box, basic region-leucine zipper (bZIP), MADS box, TEA/ATTS domain, copper DNA binding, and heat shock transcription factor (HSTF) (Maicas *et al.* 2005). One particular subfamily of TFs, zinc finger proteins, utilizes zinc atoms for DNA binding. These zinc-binding proteins form one of the largest families of transcriptional regulators in eukaryotes due to their variable secondary structures and functional diversity (MacPherson *et al.* 2006).

The motif of zinc finger protein was first identified in *Xenopus'*s TFIIIA in 1985. When viewed on its 3D protein structure, a "finger-like" structure can be seen in the DNA binding domain, hence the term "zinc finger" protein is introduced (MacPherson *et al.* 2006). Cysteine or histidine residues coordinate the zinc atoms in this TF to stabilize the domain and contribute

to proper protein structure and function. Other than binding to DNA, zinc finger proteins also bind to RNA for physiological processes such as mediating protein-protein interactions, chromatic remodeling, protein chaperoning, lipid binding, and zinc sensing (MacPherson *et al.* 2006). Based on the zinc-binding motifs, the DNA- and RNA-binding zinc finger proteins can be classified into three classes:

Zinc finger class	Subclass(es)	Consensus amino acid sequence	Example
I (C_2H_2)	FOG (C_2HC)	Cys-X_{2-4}-Cys-X_{12}-His-X_{3-5}-His	*Xenopus* TFIIIA
II (C_4)	GATA, nuclear receptors, LIM (C_3H)	Cys-X_2-Cys-X_n-Cys-X_2-Cys-X_n-Cys-X_2-Cys-X_n-Cys-X_2-Cys	Glucocorticoid receptor
III (C_6)		Cys-X_2-Cys-X_6-Cys-X_{5-12}-Cys-X_2-Cys-X_{6-8}-Cys	*S. cerevisiae* Gal4p

Table 1. Different classes of zinc finger proteins. Reprinted from MacPherson *et al.* (2006)

Compared to the other classes, the class III zinc finger proteins are special because they are only specific to fungi. The DNA-binding domain (DBD) has highly conserved the cysteine motif that was first discovered in *Saccharomyces cerevisiae* (Maicas et al. 2005). The motif comprises of six cysteine residues bound to two zinc atoms with the pattern of Cys-X_2-Cys-X_6-Cys-X_{5-16}-Cys-X_2-Cys-X_{6-8}-Cys, hence marked the origin of the name zinc cluster/zinc binuclear cluster/$Zn(II)_2Cys_6$/Zn_2C_6 proteins (MacPherson *et al.* 2006). For the purpose of this study, this motif will be referred as $Zn(II)_2Cys_6$. As can be seen in Figure 2, the six cysteine residues maintain the structure of the protein and coordinate the folding of the DBD by binding with two zinc atoms in the first and fourth cysteine residue, Cys_1 and Cys_4 that form a metal-binding domain. The other cysteine residues are present as terminal ligands (Maicas *et al.* 2005). In the sequence motif, each cysteine residue is separated by "X", which symbolizes amino acid residues. The subscripts that follow "X" account for the amount of amino acid residue. For example, Cys_1 and Cys_2 are separated by two amino acid residues and is written like Cys_1-X_2-Cys_2; Cys_2 and Cys_3 by six residues, Cys_2-X_6-Cys_3, and so on. Cys_3-Cys_4 and Cys_5-Cys_6 have variable separation of 5-16 and 6-8 amino acid residues respectively (Maicas *et al.* 2005).

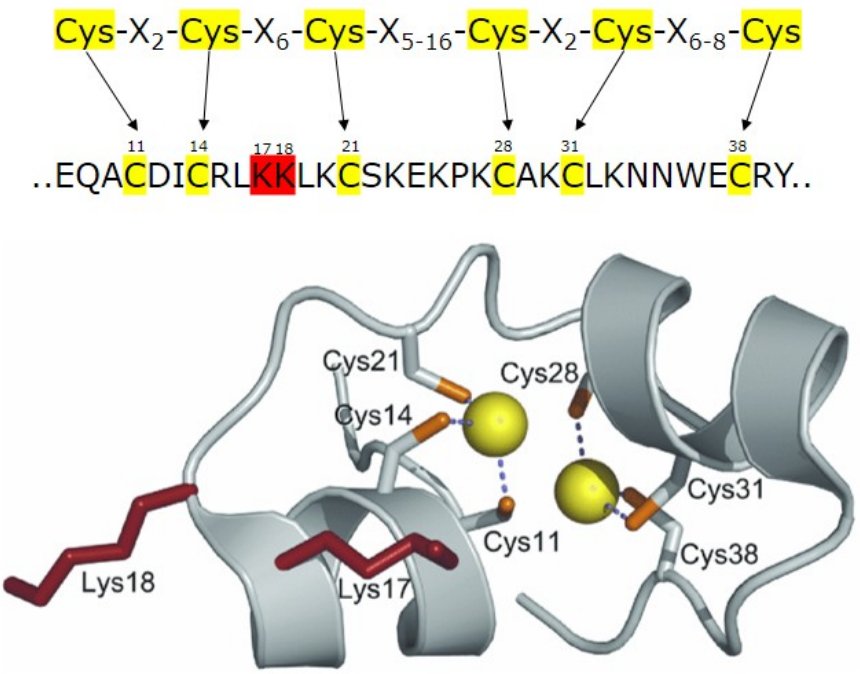

Figure 2. $Zn(II)_2Cys_6$ motif in *S. cerevisiae*'s Gal4p. The yellow highlights in the amino acid sequence show the cysteine residues conserved in the motif. The red highlights show lysine residues that bind with DNA. Adapted from Campbell *et al.* (2008)

S. cerevisiae's Gal4p is the best-studied member of this protein class that acts as a transcription gene activator for galactose catabolism (Schillig and Morschhauser 2013). It has lysine residues located at positions 17 and 18 that bind with DNA in the recognition site (Campbell *et al.* 2008). A full genome analysis on *S. cerevisiae* had revealed 55 TFs having the $Zn(II)_2Cys_6$ motif, making this class as one of the largest in yeast (MacPherson *et al.* 2006). In *C. albicans*, the $Zn(II)_2Cys_6$ TFs control the expression of certain virulence factors and may allow for the development of resistance to antifungal drugs (Schillig and Morschhauser 2013). The $Zn(II)_2Cys_6$ TFs can also be found in other fungal organisms such as *Kluyveromyces lactis*, fission yeast *Schizosaccharomyces pombe*, and another human pathogen *Aspergillus nidulans* (MacPherson *et al.* 2006).

$Zn(II)_2Cys_6$ TFs must be localized to the nucleus to perform their functions as transcriptional regulators either to activate or repress transcription of certain genes (MacPherson *et al.* 2006). The proteins can be classified based on their initial location within the cell. The first group is permanently present in the nucleus and constitutively binds to the target promoters. Its activity is controlled by the direct interaction of surrounding molecules, creating

"condition-invariant" regulators (MacPherson *et al.* 2006). The second group is present in cytoplasm and must be imported into the nucleus upon activation. The nuclear import involves many exclusive pathways and the transport is mediated through nuclear pores in which soluble transport receptors bind to nuclear localization signals on target molecules (MacPherson *et al.* 2006).

Zinc Cluster Transcription Factor (ZCF)

Schillig and Morschhauser (2013) analyzed the *C. albicans* genomes and identified 80 TFs with $Zn(II)_2Cys_6$ motif that might be involved in cellular processes such as metabolism of sugar and amino acids, multi-drug resistance, control of meiosis, cell wall architecture, regulation of invasive filamentous growth, and phenotypic switching (Maicas *et al.* 2005). These TFs are still yet to be characterized, but after looking at sequence comparisons with known transcriptional regulators in model yeast *S. cerevisiae,* it reveals that there are many close orthologs between them (MacPherson *et al.* 2006). Some of these TFs are only specific to *C. albicans* and it is suspected that these TFs may be responsible for *C. albicans* pathogenicity. An extensive transcription rewiring might have taken place in which the roles of homologous TFs have diverged in *S. cerevisiae* and *C. albicans*, making *C. albicans* a pathogenic fungus (Schillig and Morschhauser 2013). The identification and characterization of $Zn(II)_2Cys_6$ TFs in *C. albicans* have just started and so far, only some have been well studied.

Some of the known TFs that have been fully characterized in *C. albicans* and have orthologs in the model yeast *S. cerevisiae* are Fcr1p, Suc1p, Czf1p, and Cwt1p (Maicas et al. 2005). Suc1p is the first $Zn(II)_2Cys_6$ TF identified in *C. albicans* and it affects an inducible α-glucosidase for sucrose utilization (Kelly and Kwon-Chung 1992). In *S. cerevisiae,* the gene *CZF1* confers moderate pheromone resistance and the structure of the protein, Czf1p, has glutamine-rich region in the central part and cysteine-rich region at the C-terminus (Whiteway *et al.* 1992). Cwt1p is required for cell wall integrity in *C. albicans,* and Maicas *et al.* (2005) has predicted a presence of PAS, a C-terminal motif, that might be involved in eukaryotic signal transduction or dimerization events. *FCR1* gene encodes $Zn(II)_2Cys_6$ protein that is homologous to *S. cerevisiae*'s TFs Pdr1p and Pdr3p and it behaves as a negative regulator of drug resistance in *C. albicans* (Maicas et al. 2005).

Among those 80 TFs that have been found in *C. albicans*, a subset of $Zn(II)_2Cys_6$ TFs are still poorly characterized and do not have homologs in other fungi. These poorly characterized TFs are called ZCF (**Z**inc **C**luster Transcription **F**actor) and their functions and structures have not yet been fully elucidated. As shown in Figure 3, some ZCFs are expanded in *C. albicans* and other emerging pathogenic fungi but poorly conserved in nonpathogenic fungi. This may suggest that this family of TFs might have important roles in virulence that are evolutionarily conserved in pathogenesis.

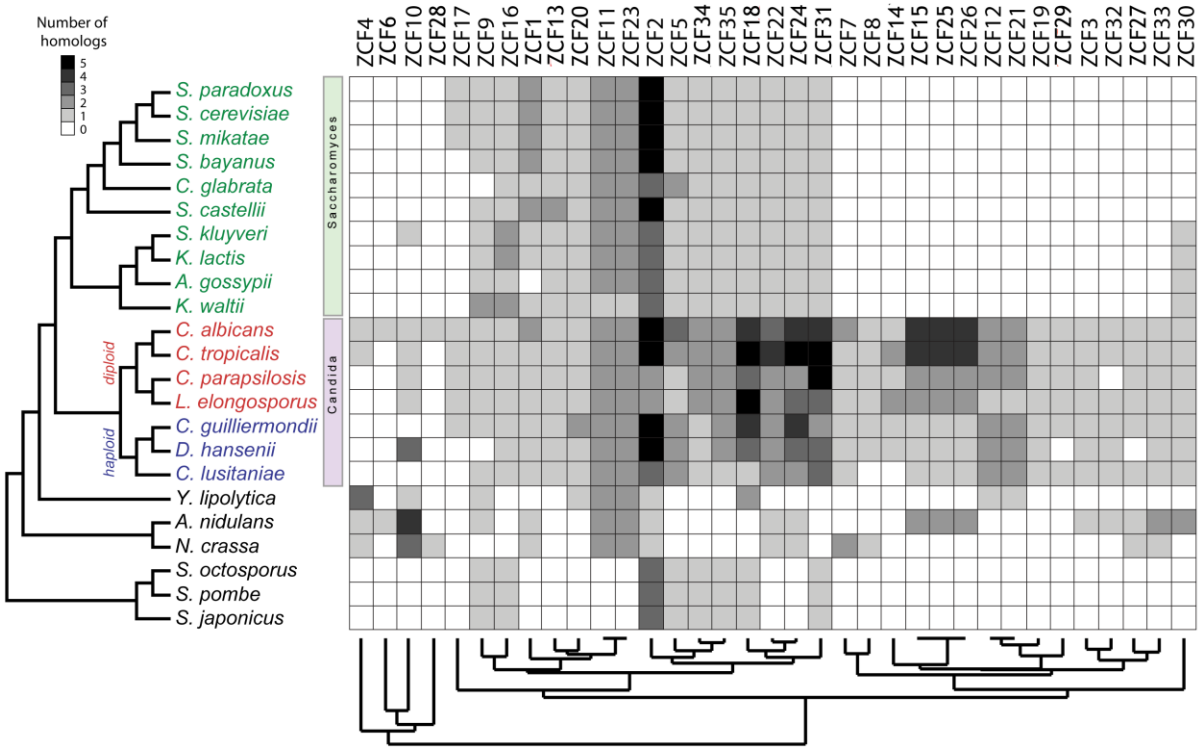

Figure 3. Certain *ZCF*s are only expanded in pathogenic fungi

To date, the characterization and functions of these *ZCF*s have begun to unveil slowly. The Candida Genome Database records 39 *ZCF*s in their hits but four of them have been comprehensively investigated and have been named differently, previously known as *ZCF12, ZCF33, ZCF36,* and *ZCF37*. *ZCF12* is now known as *ECM22* and it is implicated in the regulation of genes involved in ergosterol synthesis and azole resistance. This *ZCF* has a homolog in *S. cerevisiae* and it is highly related to Upc2p (MacPherson *et al.* 2005). *ZCF33*/*WOR2* is a regulator of white-opaque switching and it is a required gene to maintain the opaque state (Hernday *et al.* 2013) while *ZCF36*/*MRR1* controls the transcription of the multidrug transporter gene *MDR* in

C. albicans (Lohberger et al. 2014). Another regulator in the white-opaque switching, *ZCF37/AHR1* specifically acts as an activator of white cell formation and a repressor of the opaque state (Wang *et al.* 2011).

Since *ZCF*s encode for TFs, they can be involved in many different metabolic pathways. In fact, Khamooshi *et al.* (2014) has illustrated how *ZCF* can affect different pathways. Figure 4 summarizes the pathways of *GOA1,* a gene that has a role in cell energy production, cross-talk among mitochondria and peroxisomes, non-glucose energy metabolism, maintenance of stationery phase growth, and prevention of premature apoptosis. *GOA1* is positively controlled by the transcriptional regulators *RBF1*, *HFL1*, and *DPB4* (Khamooshi *et al.* 2014). The blue arrows in Figure 4 represent biological processes that are resulted when the genes *GOA1, RBF1*, *HFL1*, and *DPB4* were knocked out. The deletion of these four genes leads to the down-regulation of the TFs in green font color and concurrent up-regulation of genes in red. For example, the deletion of either *RBF1* or *GOA1* causes *ZCF15* to be down-regulated and *ECM22* to be up-regulated. This indicates that *RBF1* or *GOA1* could be involved in the same pathway. As TFs, *ZCF* could also have effects in the protein repertoires and pathway orchestration.

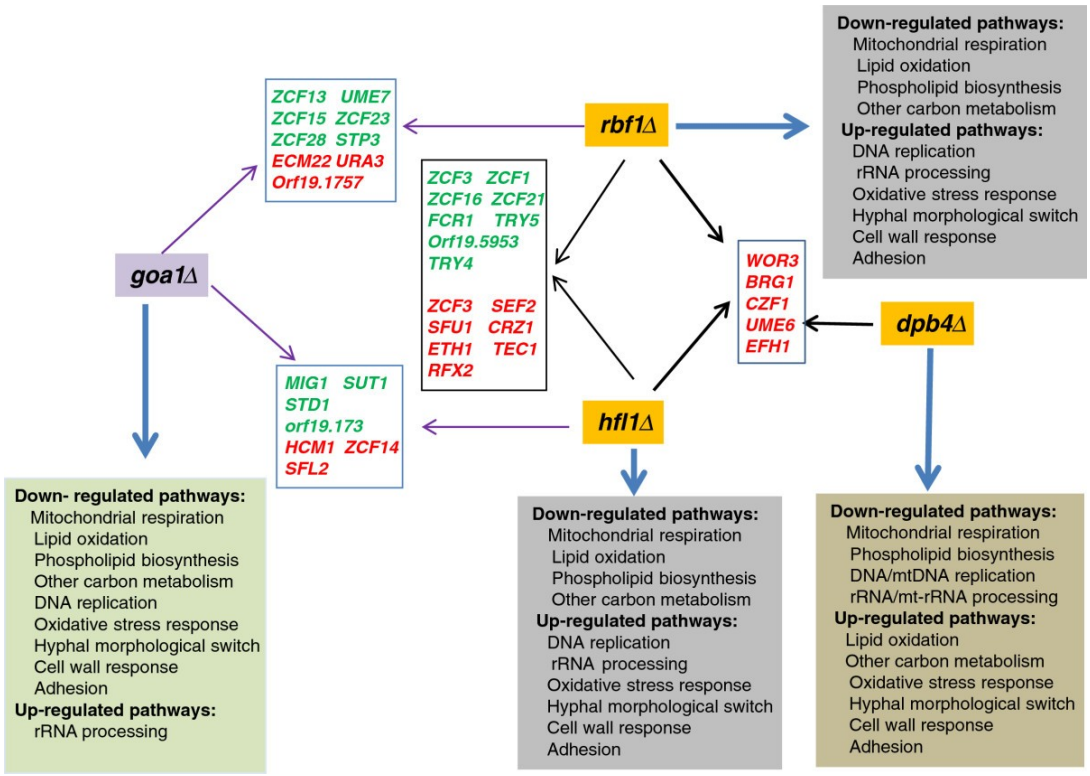

Figure 4. Some of the *ZCF*s are involved in various pathways. The red color of the fonts indicates up-regulation of the gene while green means down-regulation. Reprinted from Khamooshi *et al.* (2014)

All the *ZCF*s that have been characterized are implicated in different pathways for virulence factors. For the purpose of this study, ten *ZCF*s have been selected based on interesting characterizations that have appeared in previous studies. Throughout this research, analysis of these *ZCF*s was executed to discern whether they take parts in *C. albicans'*s pathogenesis. These *ZCF*s are summarized in Table 2 along with their reported phenotypes from Homann *et al.* (2009), Candida Genome Database's descriptions, and a summary of their evolutions. Two of the *ZCF*s, *ZCF6* and *ZCF28*, are unique because they are only present in *C. albicans* and not in other *Candida* species, potentially regulating pathways that differentiate *C. albicans* from other *Candida* species.

In order to characterize some of these *ZCFs,* the dual approach will be used. First, the evaluation of the ability of individual *ZCF* deletion strains to survive murine macrophage killing will be carried out *ex vivo*. Second, testing the ability of the same deletion strains to resist various chemical stimuli *in vitro* will be conducted. The macrophage assay can show the contribution of *ZCF*s in resisting one of the most important components of the host's immune system while the *in vitro* phenotypic profiling will determine some insight on the possible mechanisms of action of these *ZCF*s.

Gene	ORF	*In vitro* Phenotype from Homann *et al.* (2009)	Candida Genome Database's Description	Evolution
ZCF1	orf19.255		$Zn(II)_2Cys_6$ transcription factor; transcript regulated during hypha formation; 5'-UTR intron; mutants show decreased colonization of mouse kidneys; flow model biofilm induced; spider biofilm induced	*ZCF*1 has 1 homolog in *C. albicans* 19.254. *ZCF1* is present in other *Candida* species as well as *S. cerevisiae* but the homolog orf19.254 is not present in many other *Candida* species.
ZCF6	orf19.1497		Putative transcription factor with zinc cluster DNA-binding motif	*ZCF6* ONLY found in *C. albicans* no homologs or orthologs in other *Candida* or *S. cerevisiae*.
ZCF13	orf19.2646		Predicted $Zn(II)_2Cys_6$ transcription factor; similar to but not the true ortholog of *S. cerevisiae*'s *Hap1*; mutants display decreased colonization of mouse kidneys	*ZCF13*, NO homologs in *C. albicans.* Present but NOT expanded in most other *Candida* and NOT present in *S. cerevisiae*.
ZCF15	orf19.2753		Predicted $Zn(II)_2Cys_6$ transcription factor of unknown function; rat catheter biofilm induced	*ZCF15* has 4 homologs in *C. albicans*. Present in other pathogenic *Candida* (including *C. tropicalis* and *C. dubliniesis*) but not *S. cerevisiae*. 1 homolog is unknown and other 2 homologs are *ZCF25* and *ZCF26*.
ZCF24	orf19.4524		Predicted $Zn(II)_2Cys_6$ transcription factor; caspofungin induced; *Hap43*-repressed	*ZCF24*, 4 homologs in *C. albicans*. Present in most other *Candida* but not *S. cerevisiae*. Homologs are *TCC1* that regulates filamentation and interacts with *TUP1*. Other 2 homologs are unknown.
ZCF25	orf19.4568		Putative $Zn(II)_2Cys_6$ transcription factor	*ZCF25*, 4 homologs in *C. albicans*. Present in other pathogenic *Candida* (including *C. tropicalis and C. dubliniesis*) but not *S. cerevisiae*. 1 homolog is unknown and other 2 homologs are *ZCF15* and *ZCF26*.
ZCF28	orf19.4767		$Zn(II)_2Cys_6$ transcription factor; required for yeast cell adherence to silicone substrate; spider biofilm induced	*ZCF28*, only present in *C. albicans*. NOT in any other *Candida* or *S. cerevisiae*.
ZCF29	orf19.5133	ΔΔorf19.5133 strains exhibited strong sensitivity to caffeine and menadione and strong resistance to fenpropimorph.	$Zn(II)_2Cys_6$ transcription factor; mutants sensitive to caffeine and menadione, resistant to fenpropimorph; *Hap43*-repressed; mutants have decreased CFU in mouse kidneys and defects in filamentous growth; spider biofilm induced	*ZCF29*, No homologs in *C. albicans*. Present but NOT expanded in most other *Candida* and NOT present in *S. cerevisiae*.
ZCF31	orf19.5924	Strong resistance to calcofluor white, strong sensitivity to copper and SDS, and weak sensitivity to fluphenazine, fenpropimorph, caffeine, and low pH.	$Zn(II)_2Cys_6$ transcription factor of unknown function; mutant is sensitive to copper and SDS, and resistant to calcofluor white; required for yeast cell adherence to silicone substrate	*ZCF31*, 4 homologs in *C. albicans*. Present in other pathogenic *Candida* (including *C. tropicalis* and *C. dubliniesis*) but not in *S. cerevisiae*. 1 homolog is *BBC1* and other 2 homologs are *PGA55* and *PGA58*, *GPI* anchored proteins, involved in filamentation.
ZCF34	orf19.6182	Strong sensitivity to fluconazole and weak resistance to fenpropimorph.	$Zn(II)_2Cys_6$ transcription factor; null mutant displays fluconazole sensitivity; *Hap43*-repressed gene; mutants display decreased colonization of mouse kidneys; required for yeast cell adherence to silicone substrate	*ZCF34*, 2 homologs in *C. albicans*. *POP2* is involved in antifungal resistance. Present in most other *Candida* and NOT present in *S. cerevisiae*.

Table 2. List of ZCFs being studied

MATERIALS AND METHODS

To improve the efficiency of the experiments, all of the protocols have been optimized for the specific strains used in this study. The complete protocols can be found in the Appendix section.

Strains and Growth Conditions

C. albicans ZCF deletion strains were obtained from the Fungal Genetic Stock Center (http://www.fgsc.net/) created by Oliver Homann as reported in his study (Homann *et al.* 2009). The deletion strains were grown in complete medium YPD (2% yeast extract, 1% peptone, and 2% glucose) at 30°C incubator. The wild type strain used for this study was SN250 constructed from Noble *et al.* (2010).

Mouse macrophage leukemic cell line RAW264.7 was retrieved from Professor Reeta Rao lab's frozen stock in liquid nitrogen for macrophage invasion study. Cells were quickly thawed and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100units/mL penicillin G, and 100µg/mL streptomycin sulfate (Pen/Strep). This medium combination shall be termed DMEM+10% FBS+1% PenStrep. Cells were propagated at 37°C incubator with 5% CO_2 in sterile 75cm^2 tissue culture flasks.

For transformation of *C. albicans*, the vector plasmid pSN105 (provided by Suzanne Noble) was grown in Luria-Bertani broth with 100µg/mL ampicillin at 37°C. *Saccharomyces cerevisiae* BY4741 was thawed and propagated in YPD at 30°C, the same growth condition as *C. albicans*.

Strain	Phenotype	Genotype
C. albicans SN250	Reference strain Arg$^-$	*his1Δ/his1Δ, leu2Δ::C. dubliniensis HIS1 /leu2Δ::C. maltosa LEU2,arg4Δ /arg4Δ, URA3/ura3Δ::imm434, IRO1/iro1Δ::imm434*
S. cerevisiae BY4741		*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*

Table 3. Strains used in this study

Macrophage Invasion Assay

A 75cm^2 tissue culture flask (T75) of macrophage RAW264.7 cells was prepared and grown up to at least 90% confluency. Meanwhile, an overnight culture of *C. albicans ZCF* deletion strain was subcultured 1:50 in fresh YPD and incubated for 5-6 hours. The old medium

from the T75 flask was removed and replaced with the same volume of fresh medium. The macrophage cells that attached to the surface of the flask were gently scraped using a cell scraper and transferred to a conical tube. A 100µL of the macrophage cell suspension was removed and mixed with 100µL Trypan Blue for cell counting.

The macrophage cells were plated in the three wells of a 6-wells plate at a density of 2 x 10^6 cells per well while the remaining wells were filled with just media. The plate was incubated at 37°C for 6 hours to let the macrophage cells adhere to the surface of 6-wells plate. While waiting, the diluted overnight *C. albicans* culture was centrifuged and re-suspended in DMEM+10% FBS+1% PenStrep. For counting purposes, a small aliquot of the *C. albicans* suspension was diluted 1:100. The *C. albicans* suspension was diluted with the appropriate volume of DMEM+10% FBS+1% PenStrep to give 13.3 x 10^4 cells per well. The final ratio of the *Candida*:macrophage had to be 15:1. The *C. albicans* dilutions were added to all wells of the 6-wells plate with the macrophage and incubated at 37°C for 16 hours.

The next day, all the wells were scraped using cell scrapers and the cells were lysed by vortexing with 0.05% Triton X-100 for 90 seconds. Aliquot of the lysed product was serially diluted 1:10 and plated at dilution 1:1,000 in YPD plates. The YPD plates were incubated in 30°C for 2 days for colony counting. A survival rate was generated from the ratio of macrophage-invaded and non macrophage-invaded *C. albicans*.

Phenotypic Profiling in Stress Media

Cultures of deletion strains were grown overnight in YPD at 30°C. The cultures were diluted 1:10 with sterile water and the optical density at 600nm of each strain was measured using a spectrophotometer. The cultures were diluted with appropriate volume of sterile water to make a final OD_{600} of 1 for each strain. In a 96-well plate, a 1:5 serial dilution was performed and 10µL of final dilution was spotted on YPD plates containing stress agents. For YPD plates with 0.04% SDS, only 3µL of each dilution was spotted. Most of the plates were incubated at 30°C unless indicated otherwise. *C. albicans* grown in Spider media (1% mannitol, 1% nutrient broth, 0.2% K_2HPO_4, adjust to pH 7.2 prior to autoclaving) was incubated at 37°C for 24 hours. The cells grown in other stress conditions were observed for a few days and any noticeable observations were recorded and scored compared to the wild type strain.

RESULTS

ZCF13 and *ZCF15* Aids in *C. albicans*'s Survival within Phagosomes

From the macrophage experiment, the *C. albicans* deletion strains' ability to survive macrophage killing was compared to the wild type SN250 (Figure 5). The survival rate for wild type SN250 was normalized to 100% while deletion strains in general were neutralized by macrophage's candidacidal activity. The results revealed seven deletion strains with lower survival rates and they were *zcf6-/-, zcf13-/-, zcf15-/-, zcf24-/-, zcf28-/-, zcf29-/-,* and *zcf31-/-*. However, out of those mentioned seven strains, only *zcf13-/-* and *zcf15-/-* were statistically significant when their survival rates were compared to that of wild type via student's t-test (P-value < 0.05). The survival rates for *zcf13-/-* and *zcf15-/-* were reduced by more than 60%. Deletion of *ZCF13* and *ZCF15* genes made *C. albicans* more susceptible to macrophage killing, suggesting a role of these genes in resistance to mammalian host's immunity.

In contrast, there were three deletion strains with higher survival rates than the wild type, which were *zcf1-/-, zcf25-/-,* and *zcf34-/-*. The survival rate for *zcf34-/-* increased by 50% and has a statistical significance. The survival rates for *zcf1-/-* and *zcf25-/-* were not statistically significant and their rates only increased by 10-20%. *C. albicans* had a better survival without the *ZCF34* gene, indicating that *ZCF34* might be a potential repressor of an important pathway for resistance to mammalian immunity.

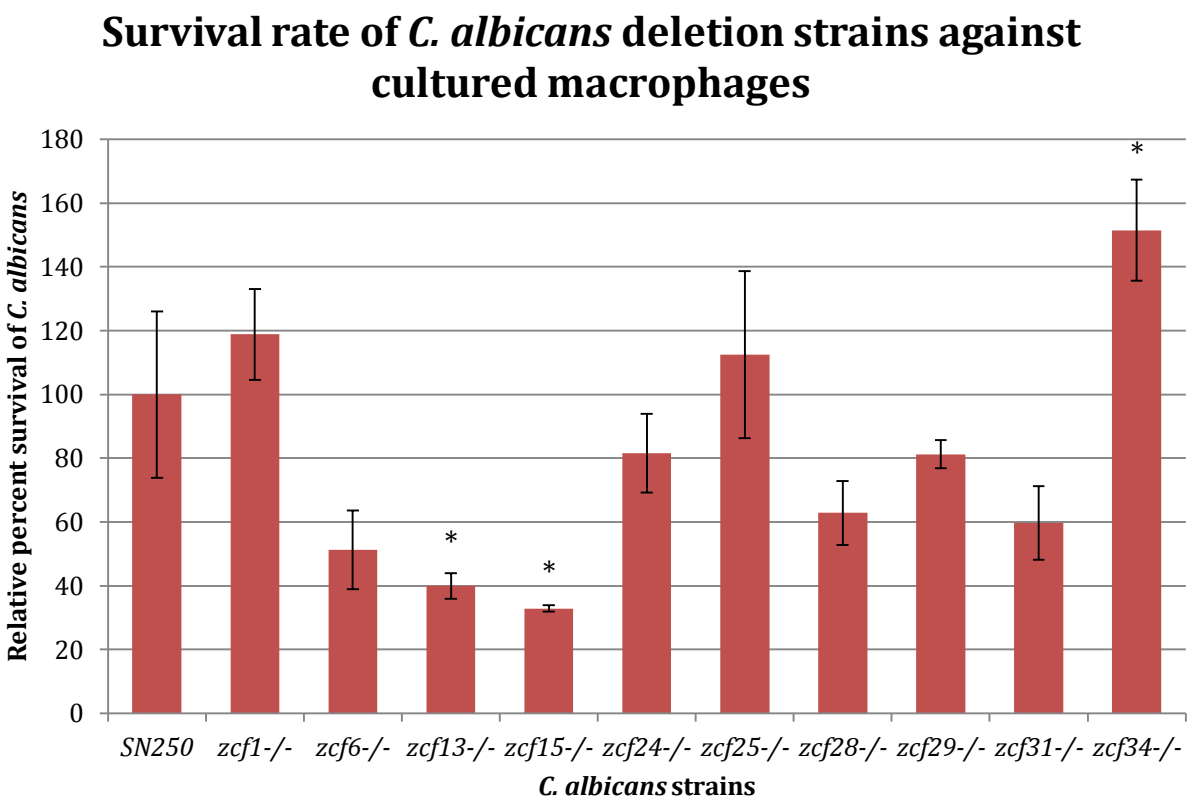

Figure 5. The relative survival rates of *C. albicans* deletion strains generated from macrophage invasion assay normalized to wild type SN250. The asterisks indicate P-value < 0.05

Validation of Phenotypes in Response to Stress Agents

Based on the phenotype screening reported by Homann *et al.* (2009), it is important to validate the findings and confirm the deletion strains that developed certain phenotypes different from the *C. albicans* wild type SN250. The *C. albicans* wild type and the deletion strains were grown in media added with various stress agents to assess their sensitivity and resistance. After a few days, the phenotypes were observed and compared with the wild type. The deletion strains showing similar phenotypes as the wild type was given a score of 0. If there were extreme reduction in the cell growth, they were scored negatively up to -4 for most extreme. An increase in growth gave positive score up to +4 for hyper-resistance. All the scores were put together to build a heatmap for a better visualization in Figure 6. The data shown are results of two sets of repeats or more. The results obtained by this study discover more phenotypes than Homann *et al.* (2009) had found.

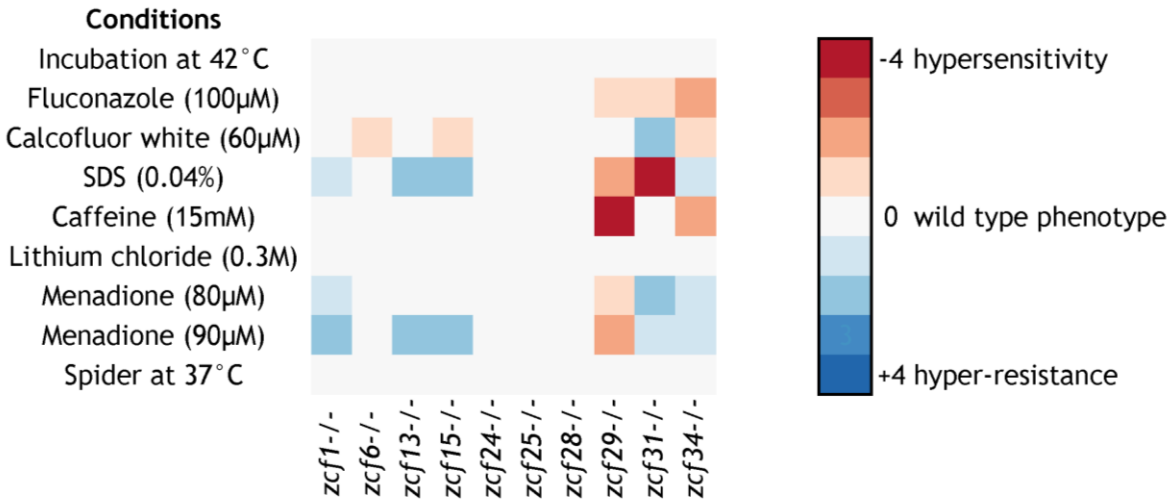

Figure 6. Heatmap summarizing the phenotypes of *ZCF*-deficient *C. albicans*

No Significant Phenotype in Lithium Chloride, High Temperature, and Spider Media

From Figure 6, all the deletion strains displayed similar phenotypes as the wild type when grown in media containing 0.3M lithium chloride (LiCl), media incubated at 42°C, and Spider media. The addition of LiCl usually induces osmotic stress and toxicity to *C. albicans* because the Li^+ ions can inhibit some important metabolic pathways (Martins *et al.* 2008). None of the strains were affected by LiCl, which indicates that the deletion of *ZCF* genes did not impair their ability to neutralize the effects of LiCl.

Growing *C. albicans* at higher temperature of 42°C would normally affect its membrane fluidity, protein denaturation, and cause up-regulation of heat shock proteins. In *S. cerevisiae,* the heat-induced toxicity is most likely contributed by denatured protein and protein aggregates by loss of function and the inhibition of ubiquitin-proteasome system (Riezman 2004). None of the deletion strains also showed sensitivity or resistance to high temperature, meaning that none of *ZCF* genes are involved in the regulation of heat shock proteins.

The Spider medium is a rich medium that strongly stimulates the hyphal growth and colony wrinkling through carbon starvation (Homann *et al.* 2009). Figure 7 summarizes the phenotypes exhibited by a negative control, positive control, wild type SN250, and *C. albicans* deletion strains. All the deletion strains displayed the same phenotypes as the wild type SN250 as illustrated by some of the deletion strains. The cells had grown a few filaments around the

edges of the cell but their edges were not completely smooth compared to the negative control *edt1-/-/cph1-/-*. The strain *edt1-/-/cph1-/-* does not form any filaments since the gene responsible for filamentation on this strain has been knocked out. The positive control isolate 4639 was obtained from a clinical strain where it expressed resistance mechanisms even during carbon starvation and it had been characterized with abundant filaments in Spider media (Fazly *et al.* 2013). Hyphal formation in *C. albicans* is associated with pathogenesis (Pukkila-Worley *et al.* 2009) and since none of the deletion strains' phenotypes were different from the wild type's then, these evidences propose that the *ZCF* genes might not be involved in morphogenesis pathway.

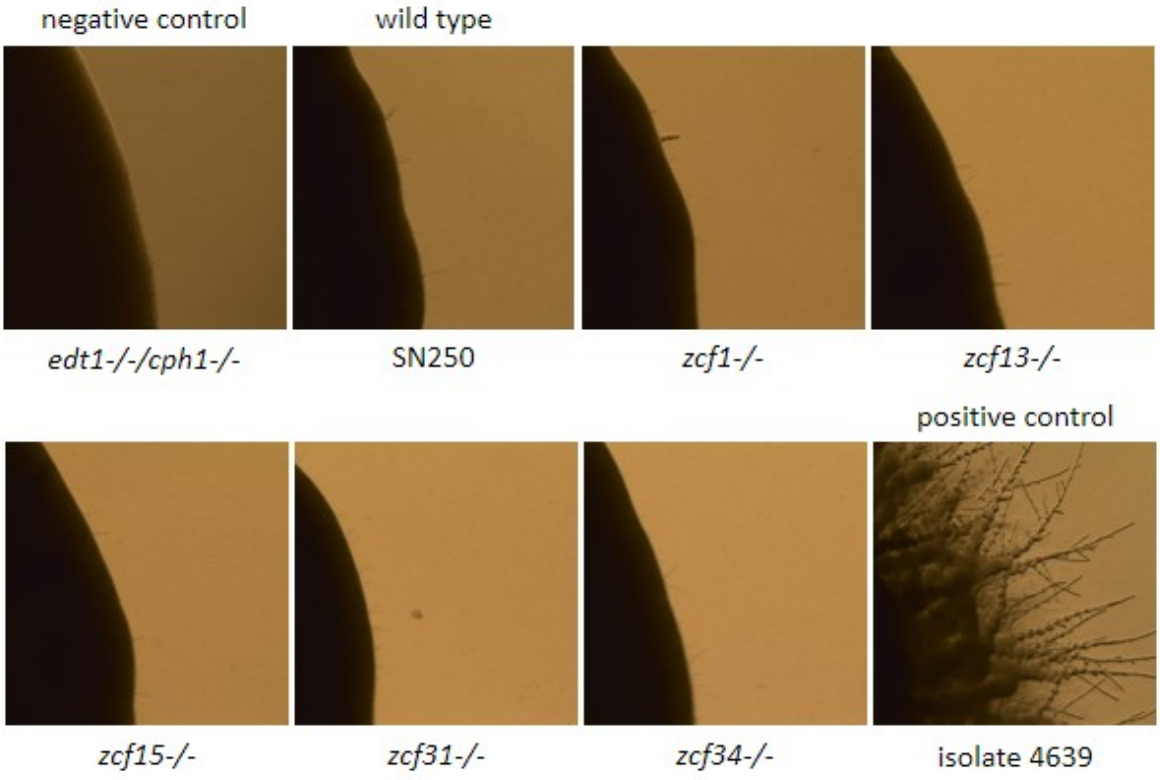

Figure 7. *C. albicans* grown in Spider medium.

Sensitivity to Fluconazole

Fluconazole is an antifungal drug that inhibits ergosterol biosynthesis and interferes with fungal cell membrane (Homann *et al.* 2009). The addition of 100μM of fluconazole in the media resulted in the reduction of growth of *C. albicans zcf29-/-, zcf31-/-,* and *zcf34-/-*. Although not extreme, the result from *zcf34-/-* confirmed the phenotype reported by Homann *et al.* (2009) as

the growth reduced by 50% (see Figure 8). This finding confirms *ZCF29, ZCF31,* and *ZCF34*'s roles to help *C. albicans* survive against fluconazole's mechanism of action.

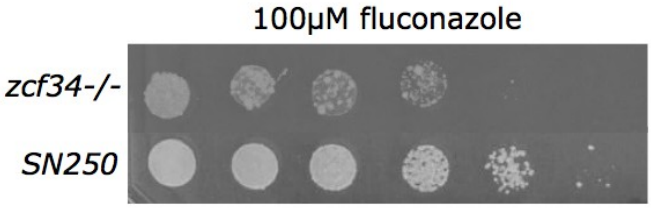

Figure 8. Phenotype of wild type SN250 and *zcf34-/-* in 100μM fluconazole

Phenotype to Calcofluor White

Calcofluor white is a cell wall staining agent that has high affinity to chitin and tends to bind to chitin component of the fungal cell wall. It is often used to detect any defect in cell wall integrity pathways and hypersensitivity that is caused by mutations affecting chitin synthesis and deposition (Kachurina 2012). *zcf6-/-, zcf15-/-,* and *zcf34-/-* were slightly sensitive to calcofluor white and these genes might be involved in chitin synthesis. However, *zcf31-/-* grew better in the presence of calcofluor white (see Figure 9) and this slight resistance also confirm Homann *et al.*'s claim.

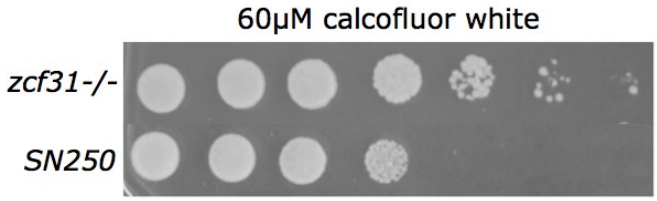

Figure 9. Phenotype of wild type SN250 and *zcf31-/-* in 60μM calcofluor white

Phenotype to Sodium Dodecyl Sulphate (SDS)

As a detergent, SDS causes damages to cell membrane, alters metabolism of carbon and induces oxidative stress response (Kachurina 2012). Some of deletion strains show slight resistance to SDS such as *zcf1-/-, zcf13-/-, zcf15-/-,* and *zcf34-/-* but the most extreme phenotype was observed in *zcf31-/-*, where none of the colonies grew (see Figure 10). *zcf29-/-* also got weakened by more than 50%. The hypersensitivity of *zcf31-/-* is confirmed by Homann *et al.* and *ZCF31* and *ZCF29* might be implicated in cell membrane formation and survival against SDS.

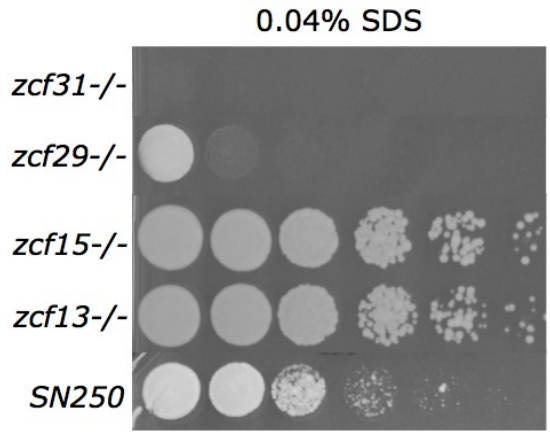

Figure 10. Phenotype of wild type SN250, *zcf13-/-*, *zcf15-/-*, *zcf29-/-* and *zcf31-/-* in 0.04% SDS

Sensitivity to Caffeine

A purine analogue, caffeine impairs the response to nutrient signaling and extensions of lifespan by interfering with the Target of Rapamycin (TOR) pathway by direct inhibition (Homann *et al.* 2009). Other than that, caffeine also affects variety fungal cellular processes. At concentration of 15mM of caffeine, *zcf29-/-* completely disappeared and *zcf34-/-* had reduced growth by about 50% (see Figure 11). This strongly suggests that *ZCF29* might be involved in regulation of caffeine pathway. Homann *et al.* also reported *zcf29-/-*'s strong sensitivity to caffeine however his claim about slight sensitivity in *zcf31-/-* is questionable since the *zcf31-/-* phenotype shown in Figure 11 look similar to the wild type's phenotype.

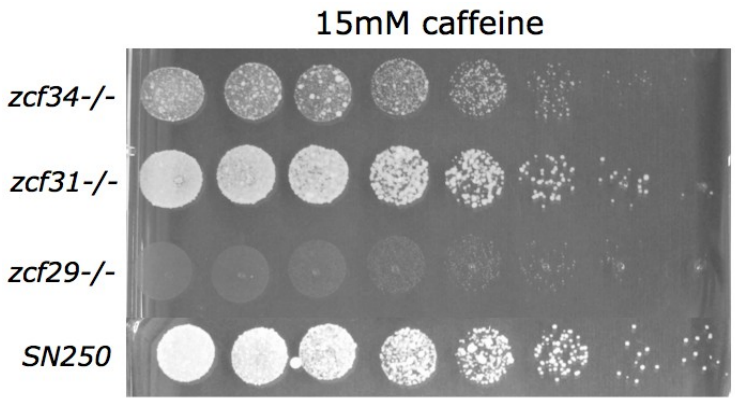

Figure 11. Phenotype of wild type SN250, *zcf29-/-*, *zcf31-/-*, and *zcf34-/-* in 15mM caffeine

Phenotype to Menadione

Menadione is a quinone molecule that induces the production of reactive oxygen species through redox cycling (Homann *et al.* 2009). At concentration of 90μM, the deletion strains generally exhibit more extreme phenotypes (see Figure 6). For example at concentration of 80μM, *zcf1-/-* was slightly resistant but with increased concentration of menadione, the resistance also increased. The same applies for *zcf29-/-*, where it was slightly sensitive at 80μM and became more sensitive at 90uM. Homann *et al.* (2009) also reported a similar observation from *zcf29-/-* and it seems that the *ZCF29* might provide protection against oxidative stress. The opposite trend was observed in *zcf31-/-* in which the resistance was reduced when the concentration of menadione was increased. *zcf34-/-* exhibited similar level of resistance at both concentrations, indicating the resistance might be independent from the amount of menadione in the media.

C. albicans Transformation

Because *C. albicans* is a diploid fungus, inserting a copy of the *ZCF* genes back to the deletion strains can potentially rescue the wild type phenotype. This approach can be used to further characterize the *ZCF* genes and their functions. As a part of this study, *C. albicans* transformation protocols had been developed and optimized. A number of sources including Zeng *et al.* (2014) has reported *C. albicans'*s low transformation efficiencies and to address this issue, the transformation was done in multiple steps using *S. cerevisiae* and *Escherichia coli* to improve the success. The flowchart of the protocols can be viewed in Figure 12. The flowchart summarizes the complete *C. albicans* transformation protocols that can be found in the Appendix section. The insertion of *ZCF* genes was aided with the vector plasmid pSN105 obtained from Suzanne Noble. This plasmid contains uracil, arginine, and ampicillin-resistance markers that are useful for selecting cells that have fully integrated the genes into the plasmid. The plasmid construction process is shown in Figure 13.

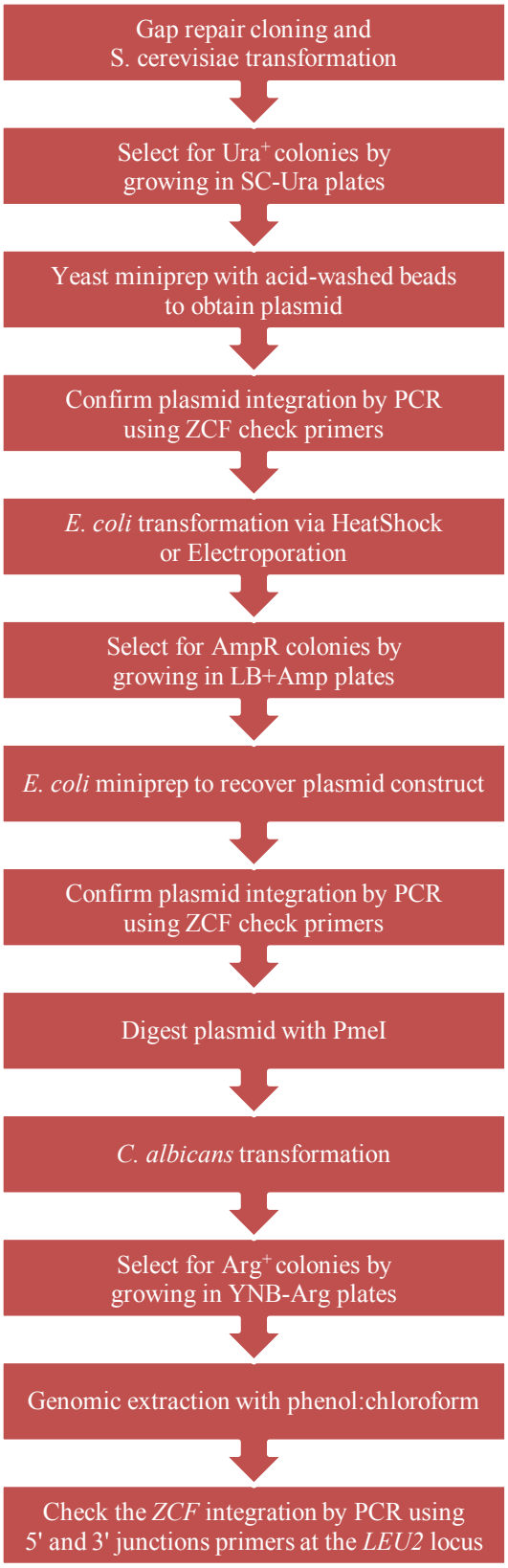

Figure 12. Flowchart summarizing *C. albicans* transformation processes

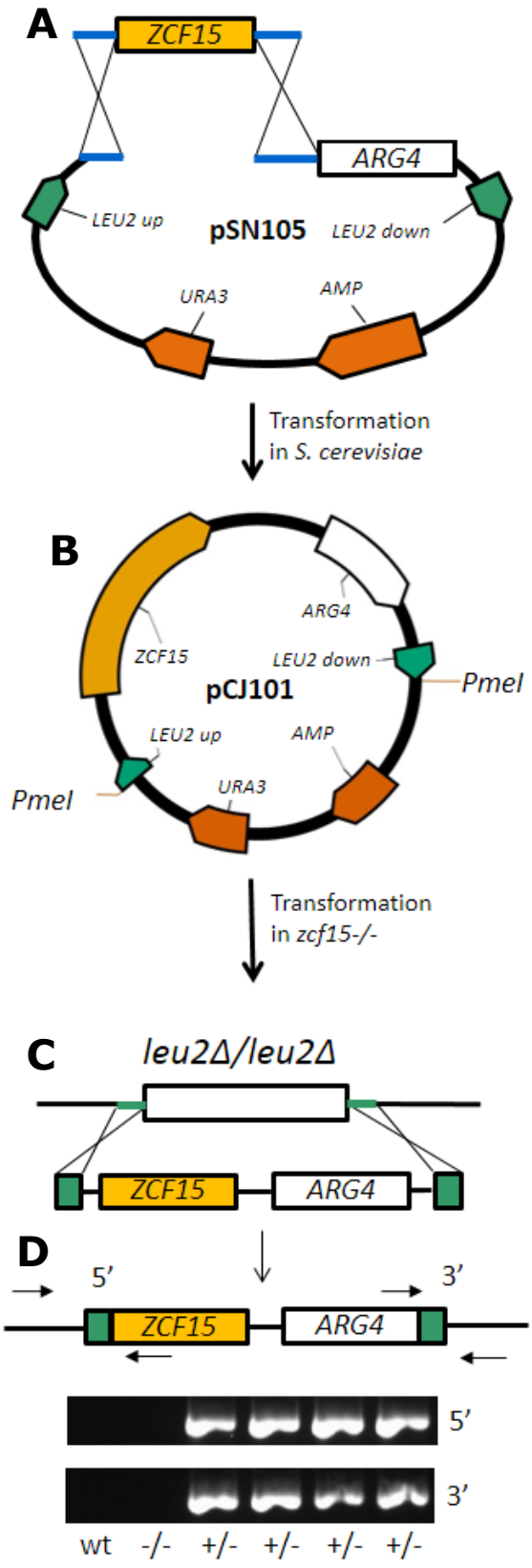

Figure 13. Construction of plasmid for *C. albicans* transformation

First, the desired *ZCF* gene is amplified by PCR using SN250 as the template. The PCR product will contain the *ZCF* gene and extra nucleotides upstream and downstream of the gene for homologous recombination. The plasmid pSN105 then is digested with restriction enzyme BmgBI to cut the plasmid at the site where homologous recombination will occur (see Figure 13A). The amplified *ZCF* gene and the cut plasmid are incubated together with the *S. cerevisiae* BY4741 cells and single-strand salmon sperm DNA for transformation. Gap-repair cloning method is particularly used for this transformation in order to improve efficiency and integration (Gerami-Nejad *et al.* 2013). The successful integration of plasmid in *S. cerevisiae* can be detected by growth of colonies in SC-Ura plates.

The integrated plasmid is renamed to pCJ### (list of plasmids is available in Appendix) and isolated for further cloning in electrocompetent cell *E. coli DH5*α by electroporation. If the *E. coli* transformation is successful, colonies will grow in LB plates supplemented with ampicillin. The plasmid from *E. coli* is recovered by miniprep and cut with restriction enzyme PmeI to isolate the *ZCF* plus *ARG4* gene (see Figure 13B) that have extra nucleotides upstream and downstream that are homologous to the *leu2* gene locus of the *C. albicans* deletion strains (see Figure 13C). The transformation in *C. albicans* integrates the *ZCF* gene into the deletion strain and the colonies are selected by growing the cells in Yeast Nitrogen Base plates without arginine (YNB-Arg). The integration can be confirmed by PCR to check the presence of genes in 5' junction of the *leu2* locus and 3' junction of the *ARG4* (see Figure 13D).

This transformation protocols had been done successfully to restore *ZCF1, ZCF13* and *ZCF15* back to their respective deletion strains and the success of the transformation had been confirmed through PCR. Unfortunately, these *ZCF*s do not have defined phenotypes between the deletion strains and wild type therefore it is difficult to perform complementation analysis and assess if the single copy gene can rescue the wild type phenotype.

DISCUSSION

From all the results of the experiments done in this study, only certain deletion strains displayed interesting phenotypes and characteristics. The data from the macrophage killing suggest that *ZCF13* and *ZCF15* genes might play an important role for *C. albicans*'s survival against the host's immunity since the deletion strains became more susceptible by at least 2-fold. On the other hand, *ZCF34* might be a potential repressor for *C. albicans*'s survival since the deletion strain showed a much higher survival rate than of the wild type. The macrophage invasion assay is an effective approach to study macrophage's action against *C. albicans*. However, this assay is limited to the candidacidal action of macrophage. The invasion assay could be extended to using neutrophil as the main challenger of *C. albicans* instead of macrophages since neutrophil makes up the most of human's innate immune system and the results can simulate the host-pathogen interaction better.

The results from this macrophage assay also require more repeats to confirm the mentioned findings since this experiment was only carried out once due to time constraint. The initial protocol of the assay had slightly modified steps in which after the 16-hours incubation, the cells were lysed with 0.02% Triton X-100 without vortexing and washed with water for two times. The cell washing with water generated problems during the experiment since the pellets from centrifugation did not clump together. As a consequence, some of the cells got discarded with the supernatants and the number of colonies varied greatly after serial dilution, creating a high technical "noise" for the data.

In an effort to solve this issue, different macrophage killing protocols were reviewed and it was found that by eliminating the cell-washing step, the variability or the noise could be reduced. The optimized macrophage killing protocol removed the cell-washing step and added a step of vortexing the cells with 0.05% Triton X-100 to improve cell lysis as recommended by Lopes da Rosa *et al.* (2010). Compared to the data from the initial macrophage assay protocol, the optimized protocol produced more consistent results and less noise. However the similar trends were still observed, supporting the results of *ZCF13, ZCF15*, and *ZCF34*. The macrophage invasion assay should be repeated at least three times for each strain to increase the confidence of the results.

The *in vitro* phenotypic profiling has confirmed most of the Homann *et al.*'s report. The data show how *C. albicans* deletion strains performed in various stress conditions and also

provide hints about the *ZCF* genes involvement in certain pathways. *zcf29-/-* completely lost its ability to form colonies in the presence of caffeine, meaning in wild type, *ZCF29* helps *C. albicans* to metabolize caffeine and neutralize caffeine's toxic effect. In addition, *zcf31-/-* also showed the same phenotype as *zcf29-/-* except it was in SDS. *ZCF31* potentially is involved in cell membrane formation and neutralization of SDS molecule. Both *zcf29-/-* and *zcf31-/-* exhibited the most extreme phenotypes in this experiment and further studies are encouraged to investigate their functions. If these genes are important for *C. albicans*'s survival or pathogenesis, this information can be useful for designing a drug that targets *ZCF29* and *ZCF31*.

ZCF6 and *ZCF28* were initially selected in this study as potential candidates who might have diversified *C. albicans* from other *Candida* species since they are only present in *C. albicans*. However, the results from this study have not provided enough evidence to confirm this hypothesis and it seems that these genes might not be directly involved in virulence. The data from the macrophage invasion assay reveal that *zcf6*- and *zcf28*-deficient *C. albicans* were susceptible to macrophage but the survival rates are not statistically significant. From the phenotype characterization, *zcf6-/-* growth was slightly reduced in the presence of calcofluor white however *zcf28-/-* did not show any distinct phenotype from the wild type. The other fungal organism that shares the same *ZCF6* gene is *Aspergillus nidulans*, a non-pathogenic filamentous fungus and a model organism, while *C. albicans* shares *ZCF28* with *Neospora crassa*, an ascomycete known as a red bread mold. This might indicate that *ZCF6* and *ZCF28* are not involved with pathogenesis pathways but again, further studies are needed to confirm this.

With the optimization of the transformation protocols, the complementation analysis will be a promising prospect for further *ZCF* characterization. Complementation analysis will be beneficial in determining if a single copy of the *ZCF* genes will produce similar phenotype as the wild type. Since *ZCF* encodes for fungal-specific transcription factors, investigating their gene functions and involvement in biochemical pathways in *C. albicans* can improve the current understanding of *C. albicans'*s pathogenesis and potentially provide new drug targets that will help treat those who suffer from *Candida* infections. Some other assays that can be useful to determine the *ZCF*'s roles in metabolic pathways include RNA and ChIP sequencing. Both are novel technologies that have been widely used to reveal gene functions.

APPENDIX A: MACROPHAGE INVASION ASSAY PROTOCOL

Protocol adapted by Jain (2014) from Lopes da Rosa *et al.* (2010)

1. Start 5mL overnight cultures of *C. albicans* deletion strains in YPD at 30^0C.
2. On the day of the experiment, dilute 100µl of the culture in 5mL of fresh YPD and let them grow for 4-5 hours.
3. Aspirate the used media from the RAW264.7 macrophage flask and add fresh DMEM supplemented with 10% FBS, 100U/mL penicillin and 100µg/mL streptomycin. Add about 15mL for one T75 flask.
4. Scrape the cells from the flask using a scraper and pipette the macrophage up and down a few times to separate any clumps of cells.
5. Transfer to a 50mL tube and take 100µl of the suspension to an Eppendorf tube.
6. To this Eppendorf tube, add 100µl of 0.4% Trypan Blue and mix.
7. Clean a hemocytometer with 70% ethanol. Put a clean coverslip on it and add 10µL of macrophage-trypan blue mix to one side of the hemocytometer.
8. Pick three large squares that best represent the population number of the cells and count the cells in those squares.
9. Calculate the concentration of cells by using this formula
10. Concentration of cells = Average of cells in 3 squares x 10,000 x 2
11. Determine the volume of macrophage suspension to get 2×10^6cells/well for each well in the 6-wells plate. Do this for as many wells as you need.
12. Volume of suspension needed = $\frac{2 \times 10^6 \text{ cells/well}}{\text{concentration of cells}}$ x number of wells to be filled
13. Separate the suspension needed in a 15mL conical tube. Centrifuge at 2000 rpm for 5 minutes.
14. Remove supernatant and re-suspend in the fresh medium for 1mL/well. For example, if adding macrophages to 3 wells, then re-suspend pellets in 3mL of fresh medium.
15. Add 1mL/well of fresh medium to control wells (without macrophages).

16. Incubate all the plates at 37^0C and 5% CO_2 for 6 hours so that the cells can adhere to the surface.

17. A little before the 5 hours are up, take the *C. albicans* cultures and centrifuge them for 5 minutes at 4000 rpm.

18. Re-suspend cultures in the same volume of fresh DMEM.

19. Count the number of cells/mL for the *C. albicans* strains using the hemocytometer. Make a 1:100 dilution to obtain countable number of cells.

20. Add the appropriate volume of the candida culture to get 13.3×10^4cells/well (in both with and without macrophages) since you want the ratio of the *Candida*:macrophage to be 1:15, and to make up the final volume of 2mL in each well.

21. Incubate all the plates at 37^0C and 5% CO_2 for 16 hours.

22. On the next day, scrape the cells using cell scrapers.

23. Add the 2mL from the plate to 50mL conical tube containing 18mL 0.05% Triton X-100.

24. Wash each plate twice with 2mL of 0.05% Triton X-100 each and add to the respective tube to get the final volume of ~24mLs.

25. Prepare a 96-well plate for 1:10 serial dilution and add 270µL of sterile water to all columns except for column 1.

26. Vortex the tubes for 90 seconds and transfer 200µL of vortexed suspension to the column 1 of 96-well plate. Quickly make a serial dilution by taking 30µL of the suspension to the next row, pipet up and down, and take 30µL for the next row. Do the same thing until dilution 10^3.

27. Repeat for all the tubes.

28. Plate 100µL of dilution 10^3 in YPD and incubate for 2 days at 30°C.

29. Count the colony forming units (cfu) on each plate and calculate the % survival by taking the ratio of cfu obtained from *Candida* and macrophage to cfu obtained for *Candida* alone.

APPENDIX B: TRANSFORMATION PROTOCOLS

The following protocols have been designed and optimized for strains and conditions mentioned in this study. For the outline of the transformation process, please revise the steps in Figure 12. For *E. coli* miniprep, restriction enzymes, and PCR, please follow the protocols attached with the kit and the manufacturer instruction.

S. cerevisiae Transformation and Gap-Repair Cloning

Protocol adapted from Gerami-Nejad *et al.* (2013)

1. Pick a colony of *S. cerevisiae* strain BY4741 and grown in 5mL of YPD overnight at 30°C.
2. Inoculate 50mL of fresh YPD with 1.5mL of the overnight culture and incubate at 30°C for 5-6 hours in shaker or until the OD_{600} reaches 1-1.5. (Initial OD_{600} for the 50mL culture should be around 0.1).
3. Centrifuge cultures at room temperature in clinical centrifuge at 3000 rpm for 5 minutes.
4. Wash with 5mL LiOAc mix (1 x TE pH 8, 100mM lithium acetate).
5. Centrifuge at room temperature in clinical centrifuge at 3000 rpm for 5 minutes.
6. Re-suspend in 1mL LiOAc.
7. Mix 100µL cells with 10µL single-strand salmon sperm DNA, 1µL of BmgBI-digested pSN105 and 10µL PCR product of desired *ZCF*.
8. Add 500µL PEG mix (1 x TE pH 8, 100mM lithium acetate, 40% PEG 3350).
9. Mix well by pipetting and incubate for 30 minutes at room temperature.
10. Heat shock at 37-42°C for 5 minutes.
11. Centrifuge at approximately 3600 rpm for 3 minutes, at room temperature.
12. Remove PEG mix manually with pipette. Wash gently with 0.5mL YPD.
13. Re-suspend in 100µL water and plate in SC-Ura.
14. Grow for 48 hour at 30°C incubator.

Isolation of Plasmid DNA from Yeast Using the QIAprep Spin Miniprep Kit

Protocol adapted from Jones (2001)

1. Inoculate a single colony into 5mL of SC-Ura liquid medium and grow the culture overnight (16-24 hours) at 30°C.

2. Harvest the cells by centrifugation for 5 minutes at 5000 x *g* and re-suspend cells in 250µL Buffer P1 containing 0.1mg/mL RNase A. Transfer the cell suspension to a 1.5mL microcentrifuge tube.

3. Add 100µL of acid-washed glass beads (Sigma G-8772) and vortex for 5 minutes. Let stand to allow the beads to settle. Transfer supernatant to a fresh 1.5mL microcentrifuge tube.

4. Add 250µL lysis buffer P2 to the tube and invert gently 4–6 times to mix. Incubate at room temperature for 5 minutes.

5. Add 350µL neutralization buffer N3 to the tube and invert immediately but gently 4–6 times.

6. Centrifuge the lysate for 10 minutes at maximum speed in a tabletop microcentrifuge (13,000 rpm or ≥10,000 x *g*). Meanwhile, place a QIAprep Spin Column in a 2mL collection tube.

7. Transfer the cleared lysate from step 6 to QIAprep Spin Column by decanting or pipetting.

8. Centrifuge for 1 minute at maximum speed. Discard flow-through.

9. Wash QIAprep Spin Column by adding 0.75mL of Buffer PE and centrifuging 1 minute at maximum speed.

10. Discard flow-through and centrifuge for an additional 1 minute to remove residual wash buffer.

11. Place QIAprep Spin Column in a clean 1.5mL microcentrifuge tube. To elute DNA, add 25µL of Buffer EB (10mM Tris·Cl, pH 8.5) or water to the center of each QIAprep Spin Column, let stand for 1 minute, and centrifuge for 1 minute.

High Efficiency Electrotransformation of *E. coli* (Electroporation)

Protocol adapted from Bio-Rad (n.d.) and Invitrogen (2013)

1. Thaw the electrocompetent cells on ice. For each sample to be electroporated, place a 1.5mL microfuge tube and a 0.2cm electroporation cuvette on ice.

2. In a cold, 1.5mL microfuge tube, mix 25µL of the cell suspension with 5µL of DNA (DNA should be in a low ionic strength buffer such as TE). Mix gently with pipette tip and incubate on ice for ~1 minute. Do not mix by pipetting up and down.

3. Set the MicroPulser to "Ec2".

4. Transfer the mixture of cells and DNA to a cold electroporation cuvette and tap the suspension to the bottom.

5. Place the cuvette in the chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber. Pulse once.

6. Remove the cuvette from the chamber and immediately add 1mL of SOC medium to the cuvette. Quickly but gently re-suspend the cells with a sterile Pasteur pipette.

7. Transfer the cell suspension to microfuge tube and incubate at 37°C for 1.5 hour, shaking at 225 rpm.

8. Warm LB+Amp plates at 37°C for 30 minutes.

9. Spread 100µL of cell suspension in LB+Amp plates.

High Efficiency Chemical Transformation of *E. coli* (Heat Shock)

Protocol adapted from Bio-Rad (n.d.)

1. Centrifuge the vials containing the ligation reaction briefly and place on ice.
2. Thaw, on ice, one 50µL vial of One Shot® cells for each transformation.
3. Pipet 5µL of DNA directly into the vial of competent cells and mix by tapping gently. Do not mix by pipetting up and down.
4. Incubate the vials on ice for 30 minutes.
5. Incubate for exactly 30 seconds in the 42°C bath and place them on ice.
6. Add 1mL of SOC medium to each vial.
7. Incubate tubes at 37°C for 1.5 hour, shaking at 225 rpm.
8. Warm LB+Amp plates at 37°C for 30 minutes.
9. Spread 100µL of cell suspension in LB+Amp plates.

Transformation of *C. albicans*

Protocol adapted from Hernday *et al.* (2010)

1. Grow one colony of the knockout *C. albicans* strain in 5mL of YPD overnight at 30°C.

2. Inoculate 50mL of fresh YPD with 1.5mL of the overnight culture and incubate at 30°C for 5-6 hours in shaker or until the OD_{600} reaches 1-1.5 (Initial OD_{600} for the 50 mL culture should be around 0.1).

3. Centrifuge for 2 minutes at 1000 g and discard supernatant.

4. Re-suspend in 900μL LiOAc/TE and transfer to a microcentrifuge tube.

5. Pellet for 1 minute at 1000 g and discard supernatant.

6. Wash two more times with 900μL LiOAc/TE then re-suspend in 400μL final volume with LiOAc/TE.

7. In a separate microfuge tube mix (in order) the following:
    a. 10μL of 10mg/mL denatured Salmon Sperm DNA (prepared by boiling 2 minutes then snap cooling in ice water)
    b. 1μg of *E. coli* miniprep
    c. 200μL washed cells in LiOAc/TE
    d. 1mL PEG mix

8. Incubate overnight at room temperature.

9. Heat shock at 44°C for 15 minutes.

10. Pellet for 1 minute at 1000g and discard supernatant.

11. Wash one time with 1mL sterile water.

12. Re-suspend in 150μL final volume with sterile water.

13. Plate on YNB-Arg media and incubate at 30°C for 2–3 days.

APPENDIX C: PLASMIDS CONSTRUCTED IN THIS STUDY

Plasmid	Database No.	Description	Host Organism
pCJ101	F190	Gap repair pSN105 digested with BmgBI and MscI + *ZCF15*	*S. cerevisiae*
pCJ102	F191	Gap repair pSN105 digested with BmgBI + *ZCF15*	*S. cerevisiae*
pCJ103	F192	Gap repair pSN105 digested with BmgBI and MscI + *ZCF1*	*S. cerevisiae*
pCJ104	F193	Gap repair pSN105 digested with BmgBI + *ZCF1*	*S. cerevisiae*
pCJ105	F194	Gap repair pSN105 digested with BmgBI + *ZCF13*	*S. cerevisiae*
pCJ106	F195	*ZCF15* cloned in pSN105 via gap repair colony 1	*E. coli*
pCJ107	F196	*ZCF15* cloned in pSN105 via gap repair colony 2	*E. coli*
pCJ108	F197	*ZCF13* cloned in pSN105 via gap repair colony 1	*E. coli*
pCJ109	F198	*ZCF13* cloned in pSN105 via gap repair colony 2	*E. coli*
pCJ110	F199	*ZCF1* cloned in pSN105 via gap repair colony 1	*E. coli*
pCJ111	F200	*ZCF1* cloned in pSN105 via gap repair colony 2	*E. coli*
pCJ112	F201	*ZCF15*+/- (obtained by transforming *zcf15-/-* with PmeI digested pCJ106)	*C. albicans*
pCJ113	F202	*ZCF13*+/- (obtained by transforming *zcf13-/-* with PmeI digested pCJ108 colony 1)	*C. albicans*
pCJ114	F203	*ZCF13*+/- (obtained by transforming *zcf13-/-* with PmeI digested pCJ108 colony 2)	*C. albicans*
pCJ115	F207	*ZCF1*+/- (obtained by transforming *zcf1-/-* with PmeI digested pCJ110)	*C. albicans*
pCJ116	F210	Gap repair pSN105 digested with BmgBI + *ZCF29*	*S. cerevisiae*
pCJ117	F211	Gap repair pSN105 digested with BmgBI + *ZCF31* colony 1	*S. cerevisiae*
pCJ118	F212	Gap repair pSN105 digested with BmgBI + *ZCF31* colony 2	*S. cerevisiae*
pCJ119	F213	Gap repair pSN105 digested with BmgBI + *ZCF34* colony 1	*S. cerevisiae*
pCJ120	F214	Gap repair pSN105 digested with BmgBI + *ZCF34* colony 2	*S. cerevisiae*
pCJ126	F224	Gap repair pSN105 digested with BmgBI + *ZCF6*	*S. cerevisiae*
pCJ127	F225	Gap repair pSN105 digested with BmgBI + *ZCF24*	*S. cerevisiae*
pCJ128	F226	Gap repair pSN105 digested with BmgBI + *ZCF28*	*S. cerevisiae*
pCJ129	F227	Gap repair pSN105 digested with BmgBI + *ZCF34*	*S. cerevisiae*
pCJ130	F228	Gap repair pSN105 digested with BmgBI + *ZCF25*	*S. cerevisiae*
pCJ131	F229	Gap repair pSN105 digested with BmgBI + *ZCF31*	*S. cerevisiae*
pCJ132	p277	*ZCF6* cloned in pSN105 via gap repair	*E. coli*
pCJ133	p278	*ZCF24* cloned in pSN105 via gap repair	*E. coli*
pCJ134	p279	*ZCF34* cloned in pSN105 via gap repair	*E. coli*
pCJ135	F229	Gap repair pSN105 digested with BmgBI + *ZCF29*	*S. cerevisiae*
pCJ136	p280	*ZCF25* cloned in pSN105 via gap repair	*E. coli*
pCJ137	p281	*ZCF28* cloned in pSN105 via gap repair	*E. coli*
pCJ138	p282	*ZCF29* cloned in pSN105 via gap repair	*E. coli*
pCJ139	p283	*ZCF31* cloned in pSN105 via gap repair	*E. coli*

APPENDIX D: PRIMERS USED IN THIS STUDY

Database No	Oligonucleotides	Sequence
602	3''junc compl LEU2 R REV	CAATAACTACCTCGGCACCTT
601	3'junc compl LEU2 F FWD	GTATCGCCTTTGGGATGTCTAT
600	5' junc Leu2 forward	GTAACGACGCCAAATCTCAATAC
584	Check 5' jun +/- REV	GGATTGTGCGTCGATCAATAA
569	F ZCf15 check	GGCAACCACCAAGTCATCTA
629	Rev 5' ZCF15-/- pro	ATCTTAACCTTCTAGTCCACGTAAA
590	Zcf 1 FWD	TCGACAGTGATGCACCTAATC
591	Zcf 1 REV	CCCAATTTCTCCTCCACTACTAC
603	Zcf1 check qPCR rev	ACTGCAGCTGCCATTCTT
592	zcf1 F gap repair (BmGBI)	GTAAAATTACAATTGGTATTTTCAACCAATATTACCACACTGTTCAAGTCTGTCACTTCC
594	zcf1 gap repair (BmgBI reverse)	CTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACCATCAACAAGAGGGTCCAAG
619	ZCF6 BmgBI cloning FWD	GTAAAATTACAATTGGTATTTTCAACCAATATTACCACACCACCACCGATAGCAACTCAA
620	ZCF6 BmgBI cloning REV	CTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACATGGACGACCCAAATTCACA
636	ZCF6 check FWD	CCCACCTGTTAAACGACCAA
637	ZCF6 check REV	CTGCTGCTACTGGTGCTATT
598	Zcf13 check	CCTTCTGTAACGACACCATCTT
599	Zcf13 check	GCCATTGGCGTTTCCATTAC
595	Zcf13 for gap (BmgBI)	GTAAAATTACAATTGGTATTTTCAACCAATATTACCACACCCATCTTCACAGGCCAATCT
597	zcf13 repair (reverse BmGBI)	CTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACAGTCCGCACTGAGACAATTC
604	zcf13 Rev qPCR	TAGTTGGTGACAACACTCCATC
570	ZCf15 check REV	CTGTGGTACATTGCTGCTACT
587	ZCF15 gap for (BmgBI)	GTAAAATTACAATTGGTATTTTCAACCAATATTACCACACAGGAGGAGGAGGACAAGAAA
589	zcf15 gap rev Bmgl	CTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACATCAAACATCAGCAGGAGGA
623	ZCF24 BmgBI cloning FWD	GTAAAATTACAATTGGTATTTTCAACCAATATTACCACACCGCAACACCAGGGACAATTA
624	ZCF24 BmgBI cloning REV	CTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACTGCCACACAAAGCAGAATCA
630	ZCF24 check FWD	GGTGGGTTTCCGTGTCTAAA
631	ZCF24 check REV	GGAACTCCAACACGAGGTAA
625	ZCF25 BmgBI cloning FWD	TTGTAAAATTACAATTGGTATTTTCAACCAATATTACCACACTTGCGGAACCGGAATTGT
626	ZCF25 BmgBI cloning REV	CTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACAGCAGCTACTGGGTCTAACT
632	ZCF25 check FWD	ACAGTCTCAGGACCCAGTT
633	ZCF25 check REV	TGCTTGCCGTCTTGAGAAA
621	ZCF28 BmgBI cloning FWD	GTAAAATTACAATTGGTATTTTCAACCAATATTACCACACGGTAGCAACGTGTGGTAGTT
622	ZCF28 BmgBI cloning REV	CCTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACTCCTCTTCGGGTGGTGTAT
638	ZCF28 check FWD	TGACACTACACTTGGGTTTG
639	ZCF28 check REV	GCTGGTGATTCATCGTAGTT
615	ZCF29 BmgBI cloning FWD	GTAAAATTACAATTGGTATTTTCAACCAATATTACCACACAGTATCGCCACTTCACCAA
616	ZCF29 BmgBI cloning REV	CTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACCACCACCAACAAACAGAACA

617	ZCF29 check FWD	AGCACTGGTGTCAGCATAAA
618	ZCF29 check REV	GTGTTTGTGAGCGAAGGAATAAA
609	ZCF31 check FWD	GCCTCCAAGTATCCCAACAA
610	ZCF31 check REV	CGGTAGACGCCAATTCTTCA
607	ZCF31 cloning BmgBI FWD	GTAAAATTACAATTGGTATTTTCAACCAATATTACCACACCGGAATCACATCACCCAAGA
608	ZCF31 cloning BmgBI REV	CCTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACAACCCTCAACCACCACAAA
613	ZCF34 check FWD	GTGCCTCATGCCACAAATC
614	ZCF34 check REV	GGCTCGGTATGTATGCTTCT
611	ZCF34 cloning BmgBI FWD	TGTAAAATTACAATTGGTATTTTCAACCAATATTACCACACGCTGGACCTTGGACTTTGA
612	ZCF34 cloning BmgBI REV	CTATCTCTCTTTTTTTTGCCCATTTGAGTTAGTGCATGACCGGCTTTGCTTGAACTTACG

REFERENCES

Barker, K., and P. D. Rogers, 2006 Recent insights into the mechanisms of antifungal resistance. Current Infectious Disease Reports **8:** 449-456.

Bio-Rad, Micropulser Electroporation Apparatus Operating Instructions and Applications Guide, pp., edited by B.-R. Laboratories.

Brown, A. J. P., 2002 Expression of Growth Form-Specific Factors during Morphogenesis in *Candida albicans*, pp. 87 in *Candida and Candidiasis*, edited by R. A. Calderone. ASM Press, Washington, D.C.

Brown, G. D., D. W. Denning, N. A. Gow, S. M. Levitz, M. G. Netea *et al.*, 2012 Hidden killers: human fungal infections. Sci Transl Med **4:** 165rv113.

Calderone, R. A., and W. A. Fonzi, 2001 Virulence factors of Candida albicans. Trends Microbiol **9:** 327-335.

Campbell, R. N., M. K. Leverentz, L. A. Ryan and R. J. Reece, 2008 Metabolic control of transcription: paradigms and lessons from Saccharomyces cerevisiae. The Biochemical journal **414:** 177.

Chiasson, M., B. Landry, K. McCannell and K. Pastor, 2010 A Pathogenesis Screen Using *Caenorhabditis elegans* to Identify Virulence Factors in Candida albicans, pp. Worcester Polytechnic Institute, Worcester, MA.

Fazly, A., C. Jain, A. C. Dehner, L. Issi, E. A. Lilly *et al.*, 2013 Chemical screening identifies filastatin, a small molecule inhibitor of Candida albicans adhesion, morphogenesis, and pathogenesis. Proceedings of the National Academy of Sciences **110:** 13594-13599.

Fox, E. P., and C. J. Nobile, 2012 A sticky situation: untangling the transcriptional network controlling biofilm development in Candida albicans. Transcription **3:** 315-322.

Gerami-Nejad, M., L. F. Zacchi, M. McClellan, K. Matter and J. Berman, 2013 Shuttle vectors for facile gap repair cloning and integration into a neutral locus in Candida albicans. Microbiology **159:** 565-579.

Ghosh, S., N. Howe, K. Volk, S. Tati, K. W. Nickerson *et al.*, 2010 Candida albicans cell wall components and farnesol stimulate the expression of both inflammatory and regulatory cytokines in the murine RAW264.7 macrophage cell line. FEMS Immunol Med Microbiol **60:** 63-73.

Gow, N. A. R., and R. A. Calderone, 2002 Host Recognition by *Candida* species in *Candida and Candidiasis*, edited by R. A. Calderone. ASM Press, Washington, D.C.

Hernday, A. D., M. B. Lohse, P. M. Fordyce, C. J. Nobile, J. L. DeRisi *et al.*, 2013 Structure of the transcriptional network controlling white-opaque switching in Candida albicans. Molecular Microbiology **90:** 22-35.

Hernday, A. D., S. M. Noble, Q. M. Mitrovich and A. D. Johnson, 2010 GENETICS AND MOLECULAR BIOLOGY IN CANDIDA ALBICANS, pp. 737-758. ELSEVIER ACADEMIC PRESS INC, SAN DIEGO.

Homann, O. R., J. Dea, S. M. Noble and A. D. Johnson, 2009 A phenotypic profile of the Candida albicans regulatory network. PLoS Genet **5:** e1000783.

Hube, B., and J. Naglik, 2002 Extracellular Hydrolases, pp. 107 in *Candida and Candidiasis*, edited by R. A. Calderone. ASM Press, Washington, D.C.

Invitrogen, 2013 One Shot® TOP10 Competent Cells, pp., edited by L. Technologies.

Jain, C., 2014 Macrophage assay, pp., Worcester, MA.

Jones, M., 2001 User Developed Control: Isolation of plasmid DNA from yeast using the QIAprep® Spin Miniprep Kit, pp. Chugai Institute for Molecular Medicine, Ibaraki, Japan.
Kachurina, N., 2012 Phenotypic analysis of a family of transcriptional regulators in the human fungal pathogen *Candida glabrata*, pp. 98 in *Department of Medicine, Division of Experimental Medicine*. McGill University, Montréal, Canada.
Kelly, R., and K. J. Kwon-Chung, 1992 A zinc finger protein from Candida albicans is involved in sucrose utilization. Journal of Bacteriology 174**:** 222-232.
Khamooshi, K., P. Sikorski, N. Sun, R. Calderone and D. Li, 2014 The Rbf1, Hfl1 and Dbp4 of Candida albicans regulate common as well as transcription factor-specific mitochondrial and other cell activities. BMC Genomics 15**:** 56.
Lohberger, A., A. T. Coste and D. Sanglard, 2014 Distinct Roles of Candida albicans Drug Resistance Transcription Factors TAC1, MRR1, and UPC2 in Virulence. Eukaryotic Cell 13**:** 127-142.
Lopes da Rosa, J., V. L. Boyartchuk, L. J. Zhu and P. D. Kaufman, 2010 Histone acetyltransferase Rtt109 is required for Candida albicans pathogenesis. Proceedings of the National Academy of Sciences 107**:** 1594-1599.
MacPherson, S., B. Akache, S. Weber, X. De Deken, M. Raymond *et al.*, 2005 Candida albicans zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob Agents Chemother 49**:** 1745-1752.
MacPherson, S., M. Larochelle and B. Turcotte, 2006 A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol Mol Biol Rev 70**:** 583-604.
Maicas, S., I. Moreno, A. Nieto, M. Gomez, R. Sentandreu *et al.*, 2005 In silico analysis for transcription factors with Zn(II)(2)C(6) binuclear cluster DNA-binding domains in Candida albicans. Comp Funct Genomics 6**:** 345-356.
Martins, L. F., M. Montero-Lomelí, C. A. Masuda, F. S. A. Fortes, J. O. Previato *et al.*, 2008 Lithium-mediated suppression of morphogenesis and growth in Candida albicans. FEMS Yeast Research 8**:** 615-621.
Mukherjee, P. K., D. J. Sheehan, C. A. Hitchcock and M. A. Ghannoum, 2005 Combination Treatment of Invasive Fungal Infections. Clinical Microbiology Reviews 18**:** 163-194.
Noble, S. M., S. French, L. A. Kohn, V. Chen and A. D. Johnson, 2010 Systematic screens of a Candida albicans homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat Genet 42**:** 590-598.
Pukkila-Worley, R., A. Y. Peleg, E. Tampakakis and E. Mylonakis, 2009 Candida albicans Hyphal Formation and Virulence Assessed Using a Caenorhabditis elegans Infection Model. Eukaryotic Cell 8**:** 1750-1758.
Riezman, H., 2004 Why Do Cells Require Heat Shock Proteins to Survive Heat Stress? Cell Cycle 3**:** 60-62.
Schillig, R., and J. Morschhauser, 2013 Analysis of a fungus-specific transcription factor family, the Candida albicans zinc cluster proteins, by artificial activation. Mol Microbiol 89**:** 1003-1017.
Vandeputte, P., F. Ischer, D. Sanglard and A. T. Coste, 2011 In vivo systematic analysis of Candida albicans Zn2-Cys6 transcription factors mutants for mice organ colonization. PLoS One 6**:** e26962.
Wang, H., W. Song, G. Huang, Z. Zhou, Y. Ding *et al.*, 2011 Candida albicans Zcf37, a zinc finger protein, is required for stabilization of the white state. FEBS Lett 585**:** 797-802.

Whiteway, M., D. Dignard and D. Y. Thomas, 1992 Dominant negative selection of heterologous genes: isolation of Candida albicans genes that interfere with Saccharomyces cerevisiae mating factor-induced cell cycle arrest. Proceedings of the National Academy of Sciences **89:** 9410-9414.

Zeng, G., Y.-M. Wang, F. Y. Chan and Y. Wang, 2014 One-step targeted gene deletion in Candida albicans haploids. Nat. Protocols **9:** 464-473.

Znaidi, S., K. S. Barker, S. Weber, A. M. Alarco, T. T. Liu *et al.*, 2009 Identification of the Candida albicans Cap1p regulon. Eukaryot Cell **8:** 806-820.