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Identification of novel factors that promote virulence of the human fungal pathogen Candida albicans

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Abstract of the Dissertation

Identification of novel factors that promote virulence of the human fungal pathogen Candida albicans

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Candida albicans is the most common human fungal pathogen and is capable of causing lethal systemic infections. Better therapeutic approaches are needed, as there is a mortality rate of about 40% for systemic candidiasis even with antifungal drug therapy. To help identify new avenues for therapeutic strategies, my research focused on two major virulence factors of *C. albicans*: the ability to grow invasively into tissues and the ability to resist oxidative attack by the immune system. The mechanisms that promote invasive growth include a switch in morphology from round budding cells to formation of elongated hyphal cells that grow invasively into tissues. To investigate the role of septin proteins in this switch, a strain carrying a temperature-sensitive mutation in the *CDC12* septin gene was created. Analysis of this $cdc12^{ts}$ mutant revealed that septins are needed for proper hyphal morphogenesis. Also, the $cdc12^{ts}$ mutant formed a second hyphal outgrowth in close proximity to the first, which limits the ability to disseminate invasive growth to new regions. To identify new mechanisms that promote *C. albicans* resistance to oxidative stress, I analyzed a family of four Flavodoxin-Like Proteins (FLPs), which are thought

to act as NAD(P)H quinone oxidoreductases. Interestingly, a quadruple mutant lacking all four FLPs (*pst1Δ pst2Δ pst3Δ ycp4Δ*) was more sensitive to a variety of oxidants in vitro. FLPs were detected at the plasma membrane in *C. albicans*, suggesting that they may act to reduce ubiquinone (coenzyme Q), which is known to act as an antioxidant in cellular membranes. The FLPs play an important role in vivo, as the quadruple mutant was avirulent in a mouse model of systemic candidiasis. Thus, these studies identified FLPs as a new antioxidant mechanism that is necessary for *C. albicans* virulence. Altogether, my dissertation research identified new roles for the septin proteins and FLP family quinone reductases in *C. albicans* virulence that will help to identify novel strategies for antifungal therapy.

Dedication

This page marks the beginning of the end of my PhD dissertation. This has been the most remarkable period of my life, up to now.

Some people say the *P* in PhD stands for perseverance. For a while, I thought it stood for persecution or punishment; but after all, it is just a letter in "philosophy", the first one though. PhD is a journey, just like life is. At a certain point in this journey, I realized it is not about how far I have gone, or what I have done, but about whom I have encountered during this journey. Family, friends, acquaintances or perfect strangers; people I enjoy and people that I don't. I thank God every day for putting every one of them in my life, because you all contributed to who I am now. I am glad how far I have come from who I was. I am glad I came this far from China to do my PhD. A lot of things happened during this very long period of PhD time (2/3 of a decade), which made me who I am now – Dr. Lifang Li.

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List of Abbreviations

CGD – Chronic granulomatous disease

CHP – Cumene hydroperoxide

CoQ – Coenzyme Q, also known as ubiquinone

FLP – Flavodoxin-like proteins

LNA – Linolenic Acid

MCC – Membrane Compartment occupied by Can1

MND – Menadione

NET – Neutrophil Extracellular Trap

OA – Oleic Acid

pBZQ – *p*-Benzoquinone

PM – Plasma membrane

PUFA – Polyunsaturated fatty acid

VVC – Vulvovaginal Candidiasis

Publications

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Li L., Zhang C., Konopka J.B. 2012. A *Candida albicans* temperature-sensitive *cdc12-6* mutant identifies roles for septins in selection of sites of germ tube formation and hyphal morphogenesis. Eukaryot. Cell. 11:1210-8.

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Chapter 1 – Introduction

1.1 Candida pathogenesis

Candida albicans is the most common human fungal pathogen, and can cause lethal systemic infections in both immune compromised and immune competent individuals. Better therapeutic strategies are needed to control candidiasis. Currently available antifungal drugs either commonly exhibit limited effectiveness due to drug resistance in *C. albicans* or severe side effects. Around 40% of patients with systemic candidiasis die, even with antifungal therapy (1). There is also no licensed anti-candida vaccine available to prevent disease (2). The estimated number of deaths caused by systemic candidiasis is above 15,000 per year in the United States (1), with annual costs more than one billion dollars (3). To develop new therapeutic approaches it is crucial to better understand the molecular mechanisms that promote the pathogenesis of *C. albicans* in vivo.

1.1.1 Overview of *C. albicans* infection

C. albicans is carried by most people as a harmless commensal organism, but under certain conditions it can cause severe infections (4). Most people are born with it on mucosal and skin surfaces, e.g. GI tract and mouth, and carry it throughout their life. The most common mucosal infections caused by *C. albicans* are oral infection and vulvovaginal candidiasis. Oral infection (thrush) is common in HIV patients, newborn babies and patients of oral cancer. Vulvovaginal candidiasis will occur in around 75% of women at least once in their lifetime (5).

Systemic candidiasis occurs when commensal *C. albicans* translocates into the bloodstream and disseminates to multiple internal organs. The primary target is the kidney, with spleen, lungs and brain being commonly infected organs as well (1,6). Indeed, *C. albicans* can

probably adapt to grow in every organ in the human body. Immunocompromised patients, such as cancer patients taking chemotherapy and organ-transplantation patients, are at high risk (7,8). However, immunocompetent individuals under certain conditions can also develop systemic candidiasis. Broad-spectrum antibiotics affect the balance of normal microbial flora, which can result in overgrowth of *C. albicans (9)*. Also *C. albicans* can adhere to medical devices, such as indwelling catheters and form biofilms (10). If clusters of cells break off a biofilm and enter the circulation, a disseminated infection can occur.

In both mucosal and systemic *C. albicans* infections, multiple morphologies are observed. There are three major growth forms of *C. albicans* in the human body: budding, pseudohyphae, and true hyphae (Fig. 1A-C). The roughly spherical budding cells are around 5 μm in diameter, which is similar in size to a red blood cell. This form allows the effective dissemination of *C. albicans* cells in the blood stream. Pseudohyphal cells are chains of elongated cells attached together. True hyphae are highly elongated cells with parallel cell walls. The true hyphal cells have a distinctive gene expression profile, including virulence genes that promote virulence, and altered cell wall structure and composition. All three forms of growth are expected and observed in commensal growth, mucosal infection and systemic infection. The ability of *C. albicans* to switch between morphologies is extremely important to its virulence, and will be discussed further in Section 1.2.

The host response to infection caused by *C. albicans* includes multiple defense mechanisms (11). The major host response comes from the innate immune system. Neutrophils are the key players. Macrophages and natural killer (NK) cells also play a role. Neutrophils can either phagocytose budding cells of *C. albicans*, or form neutrophil extracellular traps (NET) to kill the large filamentous cells, i.e. hyphae and pseudohyphae. Both mechanisms involve the

generation of reactive oxygen species (ROS) including superoxide, peroxide and hypochlorous acid (bleach).

The importance of the oxidative burst in controlling microbial infection in humans is manifested in patients with chronic granulomatous disease (CGD). CGD is a genetic disorder that affects the NAD(P)H oxidase complex-mediated oxidative burst, and is associated with an increased susceptibility to Candidiasis (12). Interestingly, mouse neutrophils have a lower efficiency oxidative burst, which correlates with their decreased ability to control fungal growth compared to human neutrophils ex vivo (13). Macrophages were previously thought not to play a key role in immune protection against *Candida* infection. However, recent studies demonstrated that bone marrow monocytes that are recruited to the *Candida* infection site as well as the kidney resident macrophages both exhibited significant candidacidal activity (14,15). NK cells also have anti-Candida function, both directly via perforin production and indirectly via priming neutrophil fungicidal activity (16,17). T cells are not important for systemic candidiasis, but are important for controlling mucosal infections, as HIV patients are prone to mucosal infection of *C. albicans*, but not systemic candidiasis. Recent data indicate that the Th17 cells are the most important T cell type for preventing mucosal infections of *C. albicans (18,19)*. The immune system can counteract a low level of infection but can be overwhelmed by a larger challenge, such as occurs when broad spectrum antibiotics induce overgrowth of *C. albicans* or when a biofilm forms on either a natural or inserted surface. Therefore, improvement of immune response can be a therapeutic strategy.

1.1.2 Virulence factors

Unlike most bacterial pathogens, which produce exotoxins, the major virulence factor of *C. albicans* is invasive growth into tissues, resulting in organ failure. *C. albicans* can grow in

practically every organ and tissue in the human body. The three major growth forms of *C. albicans* also help to disseminate and facilitate effective tissue invasion, which will be further discussed in the next section. Several families of secreted proteins have been shown to be critical for virulence, and will be discussed below.

Adhesin proteins are cell-surface proteins that mediate attachment to both epithelial cells and endothelial cells in the host to help initiate infection. They also promote adhesion of *C. albicans* to form biofilms. Several types of proteins have been implicated in promoting adhesion. The best studied of these are the Als (agglutinin-like sequence) proteins that are similar to the *S. cerevisiae* mating agglutinins (20). *C. albicans* encodes eight ALS genes, *ALS1, ALS2, ALS3, ALS4, ALS5, ALS6, ALS7,* and *ALS9,* all of which are GPI-anchored cell-surface proteins (20-23). *C. albicans* deletion mutants of *ALS2, ALS3,* and *ALS4* showed reduced adherence to epithelial cells (23). Another well-studied adhesion protein in *C. albicans* is Hwp1 (hyphal wall protein 1). Like the Als proteins, it is also a GPI anchored cell surface protein. Hwp1 enables adherence to the host epithelial cell by mimicking mammalian host cell proteins (24,25). A *C. albicans hwp1Δ* deletion mutant exhibited significantly reduced virulence in a mouse model of mucosal infection (26).

Secreted hydrolytic enzymes also contribute to virulence. The best-studied family of secreted hydrolytic enzymes are the SAPs (secreted aspartyl proteases). *C. albicans* encodes at least 10 SAP genes. Eight of the gene products (Sap1–8) are secreted extracellular proteins, while Sap 9 and Sap10 are GPI anchored proteins. They are thought to degrade host cells to release nutrients, and to degrade E cadherin connections between cells to make it easier for *Candida* cells to invade deeper into tissues. There is evidence that the SAPs degrade complement components to help *C. albicans* avoid innate immune attack. The roles SAPs play in *C. albicans*

virulence was first established when the proteinase inhibitor pepstatin A showed a protective effect against *C. albicans* infection in a mouse model (27). Other secreted hydrolytic enzymes, such as lipases and phospholipases, are thought to be important but have been less well studied. The only phospholipase that has been shown to be involved in virulence of *C. albicans* systemic infection is Plb1 (28).

1.1.3 Limitations of current antifungal therapies

Azole antifungal drugs, such as fluconazole, voriconazole, itraconazole and penconazole, are most commonly prescribed because they are tolerated well and can be taken as a pill, rather than intravenous injection. In *C. albicans*, azole drugs target *Erg11*, which is a smooth ER protein that is involved in the biosynthesis of ergosterol, the fungal form of cholesterol. Blocking ergosterol synthesis leads to the failure of fungal cell division. However, azole drugs are fungistatic, instead of fungicidal. Cells stop proliferating; existing fungal cells are not killed. Thus, long term treatment leads to emergence of drug resistance through mutation in *ERG11* that results in decreased affinity of azole binding, overexpression of *ERG11* and upregulation of drug efflux pumps (29). Azole-resistant clinical isolates of *C. albicans* are commonly observed to be multidrug resistant (30), which further complicates treatment.

The echinocandins, such as caspofungin, micafungin and anidulafungin, are a newer class of antifungal drugs that are used in the treatment of invasive fungal infections (31). They block cell wall synthesis by interaction with 1,3-β-D-glucan synthase (32). Drug resistance is emerging, through mutations in 1,3-β-D-glucan synthase and/or mutations that cause higher chitin levels in the cell wall to compensate for defects in beta glucan (33).

Polyene drugs, such as amphotericin B, nystatin and natamycin, act by binding ergosterol in the plasma membrane of fungal cells. Previously thought to act by forming pores (34),

more recent data indicates their major effect may be the sequestering of ergosterol and the perturbing of plasma membrane function (35). Amphotericin B, the most commonly prescribed polyene antimycotic, has broad spectrum antifungal activity against not only *Candida*, but also *Aspergillus* and *Cryptococcus* species. It is usually used as a second line of defense against uncontrolled candidiasis due to its toxicity. Amphotericin B binds to cholesterol, and therefore, displays dose-dependent deleterious side effects on the kidney, ultimately leading to kidney failure (36). Such side effects can be lethal, especially in weakened patients.

Despite the fact that we have gained a great deal of understanding about the pathogenesis of *C. albicans* and developed multiple antifungal drug therapies, there are still many drawbacks, such as drug resistance and severe side effects associated with the current therapeutic strategies for candidiasis. Therefore, better and safer drugs need to be developed to treat Candida infection. Better understanding of the pathogenesis of *C. albicans* is the key to identifying better drug targets.

The fact that current antifungal drugs target the plasma membrane (PM) shows it is a good drug target. Therefore, one major focus of the research in our lab is to study the PM and PM-associated protein function in *C. albicans* in order to identify good anti-*Candida* drug targets. The key factors that promote the success of *C. albicans* as a human fungal pathogen are its ability to disseminate *in vivo* by growing invasively into tissues and the ability to resist oxidative attack by the immune system. Therefore, I chose a group of PM-associated proteins from each of the two most important virulence factors of *C. albicans*, invasive growth and its ability to resist the immune attack, to study for my thesis research. In the subsequent sections, I will further introduce these two areas of my thesis studies.

1.2 Invasive growth and septin function

1.2.1 Role of the morphological switch in *C. albicans* virulence

Invasive growth has long been recognized as the key virulence factor of *C. albicans*. Morphological switching is crucial for invasive growth of *C. albicans*. As described above, *C. albicans* has three main growth forms, budding cells, pseudohyphae and true hyphae (Fig. 1A-C). All of these morphologies are normally observed in both mucosal and systemic infections. Budding cells, which are around 5 μm in diameter and similar in size to red blood cells, allow the effective bloodstream dissemination of *C. albicans* cells to occur; while the filamentous true hyphal cells and pseudohyphal cells are effective in tissue invasion. As expected, cells that cannot form hyphae are defective in invasive growth, both in vitro (agar invasion assay) and in vivo (tissue invasion) (37). As a result, they are significantly less virulent. Interestingly, cells that are locked into hyphal growth are less virulent too (38). This indicates that *C. albicans* cells must be able to freely switch between morphologies to be fully virulent.

Morphological switching of *C. albicans* cells is also crucial for biofilm formation. Biofilms are growth forms that associate with biological or artificial surfaces. *C. albicans* biofilms are a mixture of yeast cells, pseudohyphal, and hyphal cells. The biofilm formation of *C. albicans* in vitro can be summarized in three steps: adhesion of yeast cells to a surface, formation of a basal layer and growth of filamentous cells (pseudohyphal and hyphal cells) accompanied by formation of extracellular matrix (39). Morphological switching is thus intrinsically linked to biofilm formation. Biofilms are highly important for *C. albicans* virulence, not only because a significant amount of *C. albicans* cells are present in the biofilms, but also because *C. albicans* biofilms are more resistant to antifungal drugs.

1.2.2 Environmental cues and endogenous pathways that are responsible for the morphological switch in *C. albicans*

Studies that investigate the mechanisms that promote morphological switching in *C. albicans* have been going on for decades. A variety of stimuli induce *C. albicans* to form hyphae *in vitro*. The most powerful known hyphal inducing condition is the combination of serum and 37˚C. Other inducers include N-acetyl-D-glucosamine (GlcNAc), neutral or alkaline pH, 5% CO2, and contact with a solid matrix. Certain synthetic media, such as Lee's medium and Spider medium, can also induce *C. albicans* hyphal switching *in vitro*. A number of pathways involved in the induction of hyphal growth have been identified in the past few decades, with the most studied being the Protein kinase A (PKA) pathway. The Ras1/PKA pathway consists of five main gene members; *RAS1, CYR1, BCY1, TPK1, TPK2*. Ras1 is a small G-protein that binds to adenylyl cyclase (Cyr1) and activates it to produce cyclic-AMP (cAMP). The Protein Kinase A complex itself consists of either isoform of the catalytic subunit Tpk1 or Tpk2 bound to the regulatory subunit Bcy1. When cAMP binds Bcy1 it releases the catalytic subunit, which then becomes active. In *C. albicans*, activation of the PKA pathway results in phosphorylation of the hyphal transcription factor Efg1, release of the hyphal repressor Nrg1 from the promoters of hyphal-specific genes, and a switch to hyphal growth (40). Certain serum components known as MDPs (muramyl-dipeptides) and bicarbonate ion are known to directly bind Cyr1 adenylyl cyclase and cause hyphal growth in this manner (41,42).

Though it is clear that activation of PKA causes hyphal growth, the mechanism by which this occurs is unclear. It was previously thought that induction of hyphal-specific genes, which is understood at the level of promoter dynamics, causes hyphal growth (43). A clear mechanism was never established, however, and the problem was confounded by the observation that the

most highly induced hyphal-specific genes, such as *HWP1* and *ECE1*, were not necessary for hyphal growth (44). Our lab has recently demonstrated that induction of hyphal growth can occur without the obvious induction of hyphal-specific genes (44).

In addition to hyphal inducers, there are also molecules that are known to repress hyphal formation, and promote the budding morphology. An example of this is the quorum sensing factor farnesol (45). Farnesol is released by *C. albicans* cells and accumulated in the medium. It was found to repress hyphal growth by inhibition of Ras1 activation of Cyr1 adenylyl cyclase (46).

1.2.3 Septin family members and functions

To better define the mechanism that promotes hyphal formation, our lab has examined septin proteins. Septin proteins are PM associated GTP binding proteins that form cytoskeletal filaments. They were first identified in the yeast *Saccharomyces cerevisiae* as proteins that are needed for septum formation and cytokinesis (47,48). There are seven septin genes identified in *S. cerevisiae*. *CDC3* and *CDC12* are essential. *CDC10*, *CDC11*, and *SEP7* are not essential but contribute to proper septation and morphogenesis (49,50). *SPR3* and *SPR28* are expressed during sporulation in *S. cerevisiae.* Four of the septin proteins, Cdc3, Cdc10, Cdc11 and Cdc12 have been shown to make up a ring of 10-nm filaments (Fig. 1) on the inner surface of the plasma membrane at the bud neck in *S. cerevisiae* (47,51-53). Despite the fact that septins play important roles in septum formation and cytokinesis in *S. cerevisiae*, their functions are not limited to such roles. Our group previously discovered that septins also function in pheromone–induced mating projection formation, where the septins form a ring at the neck of the polarized growth that forms a conjugation bridge to connect the mating cells (54-56). The germ tube structure of *C. albicans*, which is the initial stage of the filamentous hyphal form shows certain

similarity to the polarized growth of *S. cerevisiae* mating cells. With a Cdc10-GFP fusion in *C. albicans*, our group showed that Cdc10 marked a faint mesh or cap-like structure at the site of germ tube emergence during the initial stage of germ tube formation (49). As the germ tube elongated, the Cdc10-GFP at the neck continued to appear more diffuse and less intense than the rings observed in buds or pseudohyphae. A previous report using immunostaining with antibody against *S. cerevisiae* Cdc11suggested that there was a septin ring at the neck of a germ tube that was similar to that of budding cells (57), which was also observed by live cell imaging with GFP tagged septin (49). Analysis of septin gene expression indicates that there does not appear to be a hyphal-specific septin gene. Thus, this change in septin localization is apparently due to changes in regulation of septin proteins during hyphal growth. This septin structure might play a crucial role in hyphal initiation and hyphal growth. Therefore, deletion mutants of *cdc10*Δ and *cdc11*Δ were made and their phenotype was examined (49). Both mutants displayed similar, but not identical, conditional defects in cytokinesis, cell wall chitin localization, and bud morphology. This indicates that these two proteins have distinct effects on septin function. Hyphal morphogenesis could be induced in the viable septin deletion mutants, but the resultant hyphae had abnormal curvature and differed in the selection of sites for the formation of subsequent hyphae. The *cdc11*Δ mutants were also defective for invasive growth. These results demonstrate the importance of these septins to the proper morphogenesis of both the budding and filamentous growth forms, further extending the known roles of these proteins (49).

As discussed above, there is a close connection between morphogenesis and virulence in *C. albicans*. Therefore, the virulence of the *C. albicans* non-essential *cdc10*Δ and *cdc11*Δ mutants were examined in a mouse model of systemic candidiasis (58). The *cdc10*Δ and *cdc11*Δ mutants were found to exhibit attenuated virulence in mice. However, mice infected with the

mutants displayed a similar level of fungal burden to mice infected with wild type *C. albicans* cells, without obvious symptoms of disease. Histological examination of infected kidneys revealed defects in organ invasion for both *cdc10*Δ and *cdc11*Δ mutants, which also displayed reduced tissue penetration and non-invasive fungal masses (58). This further established the connection between the septin proteins, invasive growth, and the pathogenesis of *C. albicans*.

To further investigate the potentially crucial roles that the essential septin genes play in the morphogenesis and virulence of *C. albicans*, a temperature sensitive mutant of *CDC12*, *cdc12-6*, was created in our lab, and its phenotype was examined. These results of this part will be presented in Chapter 3 - A *C. albicans* temperature-sensitive *cdc12-6* mutant identifies roles for septins in selection of sites of germ tube formation and hyphal morphogenesis.

1.3 Antioxidant pathways and *C. albicans* virulence

1.3.1 Overview of the effects of oxidation on *C. albicans*

Most, if not all, types of eukaryotic cells experience oxidative stress caused by reactive oxidative species (ROS). ROS can lead to severe damage of biomolecules. ROS have also been shown to play important roles in cell signaling and metabolism of both *S. cerevisiae* and *C. albicans* (59,60).

A major cellular source of oxidative stress is the leakage of electrons from the mitochondrial electron transport chain. This key source of cellular energy production, when it works imperfectly, allows electrons to escape and combine with oxygen to form superoxide. This initiates a series of redox reactions that results in the formation of subsequent ROS, such as hydrogen peroxide. Other cellular sources of ROS formation are the Fenton reaction and the Haber-Weiss reaction. They both utilize uncomplexed forms of metals (not complexed with

protein or other metals), such as iron or copper, that react with hydrogen peroxide to form the highly reactive hydroxyl radical.

In addition to the ROS produced endogenously by C. *albicans*, it is exposed to ROS released by immune cells. Indeed, this is the major source of oxidative stress *C. albicans* experiences during infection. As discussed in Section 1.1, the first line of defense against *C. albicans* in the human body is phagocytosis by innate immune cells, including macrophages and neutrophils. These phagocytes produce reactive oxygen species (ROS) through a process known as the oxidative burst, where superoxide is generated by the NADPH oxidase complex. Inside the neutrophil phagosomes, approximately 5 to 10 nmol of ROS is generated per second (61), and the estimated superoxide concentration in the phagocytic vacuole is approximately 4 M per bacterium engulfed (62). The superoxide is then converted to hydrogen peroxide by superoxide dismutase or to hydroxyl anions and hydroxyl radicals via the Haber-Weiss reaction.

Exposure to a high concentration of ROS leads to oxidation of biomolecules, such as DNA, proteins, saccharides, and lipids (63). Oxidative damage on DNA and proteins are better studied than such damage to saccharides and lipids. Oxidative damage to DNA causes alterations in DNA bases. If left unrepaired, the modifications of DNA bases in turn lead to mutations. Guanine is especially susceptible to oxidation. The oxidation product of guanine, 8-hydroxy-deoxyguanosine, has been commonly used as a biomarker to indicate oxidative damage to DNA (64). Protein oxidation can occur both at amino acid residues and the peptide backbone, both of which can cause to loss of function (65). Protein oxidation can also lead to malformation of protein complexes (66). If altered proteins or protein complexes accumulate in the cell, it may lead to death. Oxidation of DNA and proteins has long been studied and multiple mechanisms of controlling such damage have been identified, including DNA repair and protein degradation.

1.3.2 PM oxidation and lipid peroxidation

Like DNA and proteins, lipid molecules are abundant biomolecules that are susceptible to oxidative damage. The oxidation usually occurs on the fatty acid tails of the lipid molecules. Unsaturated fatty acid is more sensitive to oxidation than saturated fatty acid, and polyunsaturated fatty acid (PUFA) more sensitive than monounsaturated fatty acid.

PUFAs are very sensitive to peroxidation due to the ease with which the hydrogens can be abstracted from the methylene bridges (-CH_2-) that lie in between the double bonds (67,68). This leaves an unpaired electron on the carbon that can react with O_2 to form a peroxyl radical, which can in turn abstract the hydrogen from another PUFA to continue the cycle. Thus, lipid peroxidation starts a chain reaction that propagates to other lipids (Fig. 2A). The resulting oxidative damage can also spread to other cellular constituents, including proteins and DNA. Uncontrolled lipid peroxidation leads to cell death.

The membrane of *C. albicans* is extremely vulnerable to oxidation because it contains polyunsaturated fatty acids (PUFAs). Approximately 30% of the *C. albicans* fatty acids are polyunsaturated linoleic (18:2) or linolenic (18:3) acids (69,70). Therefore, we hypothesize it is extremely important for *C. albicans* to protect its membrane during infection. However, the mechanisms that protect the PM from oxidative damage are not nearly as well studied as other antioxidant mechanisms.

1.3.3 Known antioxidant mechanisms

Cap1 is the major transcription factor in *C. albicans* that regulates gene expression in response to oxidative stress, both *in vitro* and *in vivo* (71-73). The Cap1 transcription factor is in the same family as mammalian AP-1, *S. cerevisiae* Yap1 and *S. pombe* Pap1, which are all well-

characterized as master regulators of antioxidant pathways (74,75). Chromatin immunoprecipitation (CHiP) assays identified direct binding of Cap1 to promoters of multiple key antioxidant enzymes, such as *CAT1,* which encode catalase and *TRX1,* a gene encoding thioredoxine *(76)*. The consensus Cap1 binding sequence (76) has become a tool for identifying novel antioxidant proteins in *C. albicans*. The *cap1*Δ deletion mutant exhibited increased sensitivity to ROS and antifungal drugs (77-79) indicating its importance in fighting against the oxidative burst.

The first product of the oxidative burst is superoxide, which is a very reactive oxidant, as discussed in the subsection above. To detoxify it, *C. albicans* uses superoxide dismutase (SOD), an enzyme that converts superoxide to peroxide. There are six SOD genes in *C. albicans*, versus only two SODs in *S. cerevisiae*, a non-pathogenic yeast. SOD proteins are also well distributed through the cytoplasm, mitochondria and cell wall in the *C. albicans* cells, suggesting that multiple regions in the cells are potentially exposed to superoxide. Sod4–6 are GPI-anchored cell wall proteins. *SOD4* is expressed in both budding and hyphal cells of *C. albicans* (44), whereas *SOD5* is a hyphal-induced gene (51). The importance of cell-wall associated SOD proteins is manifested by the observation that deletion mutants of *sod4*Δ and *sod5*Δ exhibited increased susceptibility to both ROS induced killing in vitro and phagocyte mediated killing in vivo (23, 25).

Further demonstrating the importance of protecting the cell wall from oxidative stress is that catalase, the enzyme that converts peroxide to water, has also recently been found associated with the cell wall in *C. albicans* (54). This is not unexpected, since the cell wall is the outermost layer of a fungal cell and is therefore exposed first to oxidants from the environment. Also, protecting the cell wall from the oxidative damage helps prevent this highly toxic effect from

spreading further into the interior of the fungal cell. The plasma membrane is the next outermost layer, and must be protected from oxidative damage because it plays crucial roles in multiple functions in *C. albicans* including morphogenesis, metabolism, and virulence. It is also very prone to oxidative damage, as discussed above. However, to date, there has been no major antioxidant pathway that has been described that specifically targets the PM of *C. albicans*. We think our study will shine light on this understudied, yet very important area of *C. albicans* research, with details being further discussed in the section below.

1.3.4 Novel antioxidant pathways in the PM

As discussed above, the PM is susceptible to oxidative damage, which can occur on the associated proteins, carbohydrates, and lipids. To examine oxidative damage to the PM, our study focused on the oxidative damage that occurs to the lipid molecules. In particular, PUFAs are more susceptible than saturated lipids. Once oxidized, PUFAs initiate a chain reaction that propagates the oxidative damage to other lipids in a process known as lipid peroxidation (Fig. 2), which spreads the damage further to other membrane lipid molecules and also to proteins and DNA in other cell compartments. Therefore, for the *C. albicans* cells to survive, the cycle of lipid peroxidation has to be stopped. However, due to the hydrophobicity of the membrane lipids, the lipid peroxide is not easily accessible to the known antioxidant pathways in the cytoplasm. Therefore, something hydrophobic enough to be membrane associated is needed to prevent the chain reaction of lipid peroxidation that could potentially lead to cell death.

CoQ, also known as ubiquinone, is a likely source of reducing potential in the plasma membrane. CoQ is a well-known electron shuttle in the mitochondrial electron transport chain that has also been reported to have an antioxidant function by reducing peroxidized lipids. CoQ

is highly hydrophobic and has been shown to be abundant in a variety of cellular membranes, including the PM (80). The Clarke group showed that a *S. cerevisiae* mutant that cannot synthesize CoQ is much more sensitive to lipid peroxidation (81).

However, after CoQ reduces a lipid peroxide (Fig. 2B), it gets oxidized and becomes inactive. Enzyme(s) are then needed to reduce CoQ and reactivate it. It takes two electrons to fully reduce oxidized CoQ. If only one electron is donated to the oxidized CoQ, an intermediate known as semiquinone is formed (Fig.3). Semiquinone carries an unpaired electron, and therefore is a highly reactive free radical. To safely reduce CoQ, it requires the enzyme(s) to perform a concerted two-electron reduction. It is not clear what enzymes perform this function in yeast.

A mammalian NAD(P)H quinone oxidoreductase Nqo1 has been shown reduce CoQ in mammalian cells. Nqo1, formerly known as DT-diaphorase, safely carries out a two-electron reduction of ubiquinone and avoids semiquinone formation (82,83). However, there is no yeast orthologue of *NQO1* based on primary sequence analysis. Another family of NAD(P)H quinone oxidoreductases, namely the WrbA family of Flavodoxin-Like Proteins (FLPs) is found in bacteria, plants, and fungi, but surprisingly not in mammalian cells (84). They have been shown to carry out the safe, concerted two-electron reduction of quinones in vitro (85). However, the cellular function is unknown.

C. albicans contains four FLP genes, *PST1*, *PST2*, *PST3*, and *YCP4*. Their gene products are uncharacterized and functions are unknown. High throughput studies have shown that these genes are induced by oxidative stress (86), and contain consensus binding sequences in their promoter regions for Cap1 (77). The *S. cerevisiae* FLPs (Pst2, Rfs1, Ycp4) have been suggested to be involved in resistance to oxidative stress (87-89), although their physiological role is not

known (90). It is also interesting that the *C. albicans* FLPs are likely to act at the plasma membrane, since their orthologs in *S. cerevisiae* are associated with the plasma membrane. Furthermore, both *S. cerevisiae* and *C. albicans* Ycp4 have a lipid modification sequence at their C-termini. Therefore, *C. albicans* FLPs are highly likely to be PM associated.

Altogether, we hypothesize that *C. albicans* FLPs, which are putative NAD(P)H quinone oxidoreductases that perform safe concerted two-electron reductions of quinones and are likely to localize at the PM, and protect the PM of *C. albicans* from lipid peroxidation (Fig. 2C). This protection is mainly through reduction of CoQ, a known antioxidant in the PM. Chapter 4 will address our investigation into FLP functions *in vitro* and *in vivo*.

Figures

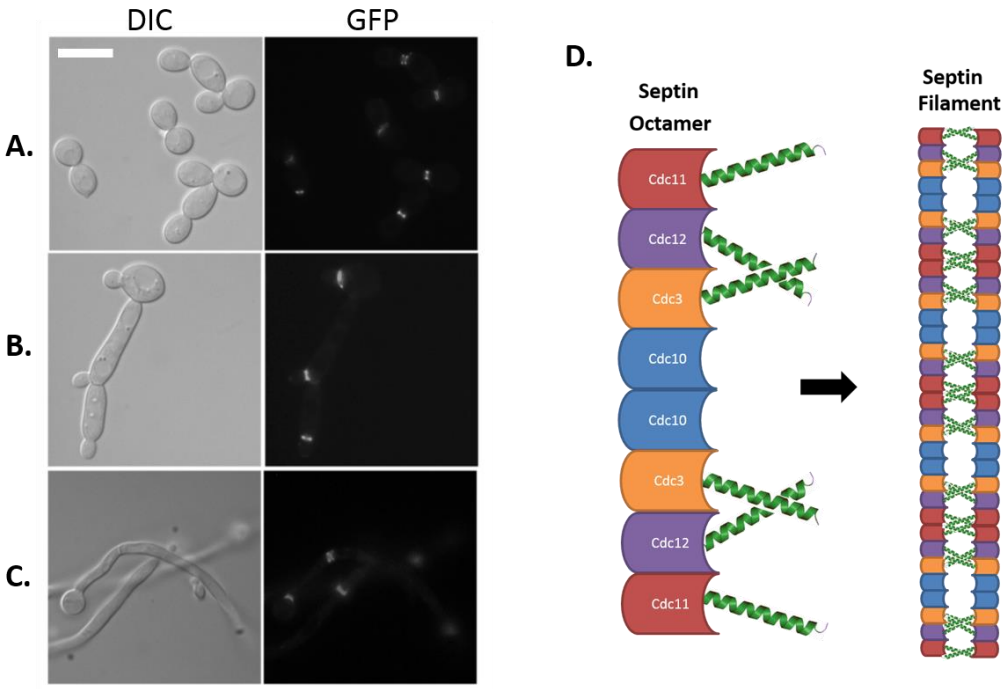

Fig. 1 – *C. albicans* morphology and septin localization.
C. albicans can grow in different morphologies including budding (A), pseudohyphae (B), and hyphae (C). Wild-type *C. albicans* cells engineered to produce Cdc10-GFP and Cdc11-GFP (LLF001) to enhance the GFP signal, were analyzed in either DIC channel or GFP channel, in the three growth forms. Bar, 10 µm. (D) A schematic diagram of a septin octamer and a septin filament, based on studies carried out with S. cerevisiae. Two copies of each of Cdc11, Cdc12, Cdc3 and Cdc10 form a septin octamer as indicated, which is the basic repeat of the septin polymer. The coiled coil domain of Cdc3 and Cdc12 mediate the pairing between the parallel octamers to form septin filaments.

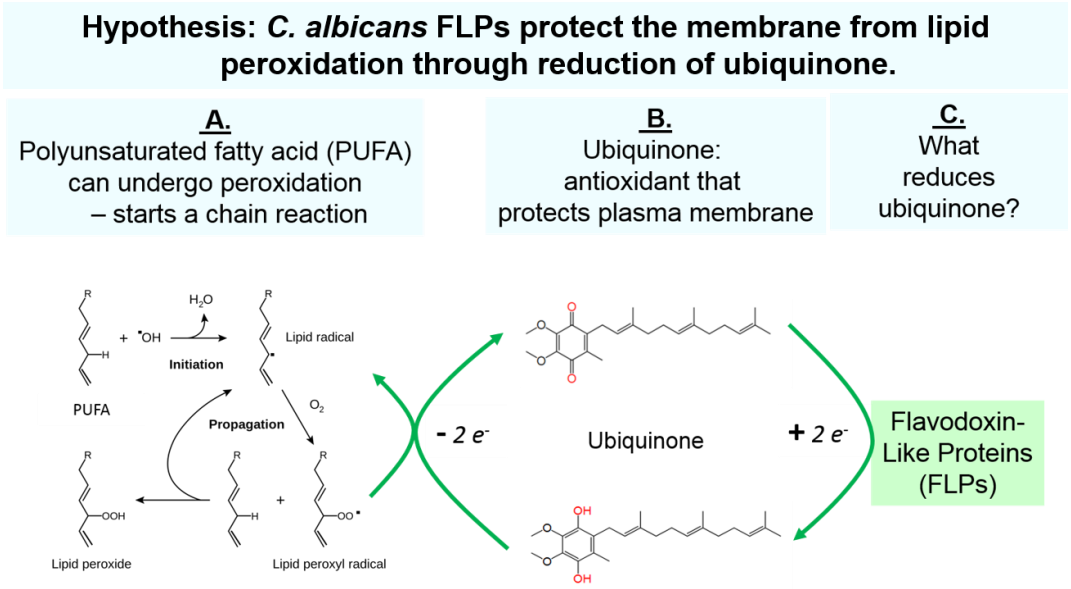

Fig. 2 Control of lipid peroxidation.
(A) Polyunsaturated fatty acids (PUFAs) are known to be prone to lipid peroxidation, which initiates a chain reaction and propagates the oxidative damage. (B) Ubiquinone, also known as CoQ, is known to reduce lipid peroxidation. (C) Our proposed mechanism for how *C. albicans* FLPs help protect the membrane lipids from peroxidation is that they promote the two-electron reduction of ubiquinone.

Fig. 3 Chemical structures of quinone.
Shown above are the three redox forms of a quinone molecule.
The chemical names corresponding to their redox status are:
Oxidized form – quinone; semi-reduced form – semiquione (a free radical); fully reduced form – quinol. Collectively, all three forms are often called quinones, when addressing redox status is not important. It is the basic chemical structure of the quinone family molecules. Various quinones can be derived from this basic structure by including a side chain of carbons. Refer to Fig. 2B for the chemical structure of ubiquinone.

Chapter 2 – Material and Methods

2.1 Material and Methods of the Septin Chapter

Strains and Media. The *C. albicans* strains used in this study are described in Table 1. The cells were propagated in rich YPD medium (2% glucose, 1% peptone, and 2% yeast extract) or SD (Yeast Nitrogen Base synthetic medium with dextrose) as described previously (91). 80 mg/liter uridine was added to cultures of *ura3Δ* cells.

A *C. albicans cdc12Δ/CDC12* heterozygous mutant was created in strain BWP17 by homologous recombination as described previously (49,92). In brief, a deletion cassette was constructed by PCR using primers that contain sequence homology to the 5' and 3' ends of the *CDC12* open reading frame to amplify a cassette containing the *ARG4* gene for use as a selectable marker to delete one copy of *CDC12*. The C-terminal coding sequences of the remaining *CDC12* allele were then replaced by an altered sequence that was patterned after the changes found in the *S. cerevisiae cdc12-6* mutant allele (Brian Haarer, personal communication). The *S. cerevisiae cdc12-6* allele contains mutations that result in a Lys to Asn change at position 391 followed immediately by a TAG stop codon, truncating the mutant protein by 16 amino acids. Amino acid sequence alignments indicated that the Lys-391 of Sc-Cdc12 corresponds to Ser-384 of Ca-Cdc12. Therefore, the analogous mutation was constructed by mutating one allele of *CDC12* to substitute Ser at position 384 with Asn and converting Leu at position 385 to the stop codon TAG (Fig. 1A). Oligonucleotide primers carrying the indicated changes were used to amplify the *URA3* gene using pGEM-URA3 as a template (92), and then the resulting cassette was used to select for integration of the *cdc12-6* allele into *C. albicans*.

The primer sequences were 5'-gactaatattattaatgaaagaaatagattgaatcaagacttggaagaaatgcaatcgaagttgaagaattagttcttgagtttgtaacagctgc-3' and 5'-gtaacccaaacaacagaaattaagtggaagtaatatattgctagcaaaaaaaaactaaaaaaatgttaatctctccccccttcacatttataat-3'. Similar temperature-sensitive phenotypes for budding and hyphal morphogenesis were observed for independent transformants that were *cdc12::ARG4/cdc12-6::URA3,* so one strain (CZ10) was then used for all subsequent studies. Strain CZ10 was transformed with a *HIS1* gene to complement all of the auxotrophies and create strain LLF016. The *HIS1* DNA fragment was isolated by PCR amplification using genomic DNA from *C. albicans* strain SC5314 as a template.

CDC10-GFP and *NOP1-GFP* fusion genes were created using PCR primers that contained 80 bp of sequence homologous to the region immediately upstream and downstream of the stop codon to amplify a *GFPγ::HIS1* cassette (93). This cassette contains the CaGFPγ version of GFP that is more photostable (93). Purified PCR product was transformed into the indicated strains and then the resulting colonies were screened to identify strains that produced the appropriate GFP fusion protein. Similar methods were also used to create a strain carrying a hyphal reporter gene *HWP1-GFP*, except that GFPγ sequences were used to replace the entire open reading frame of the hyphal-induced gene *HWP1*. The successful transformants were confirmed by microscopic examination of cells to demonstrate that that GFP expression was elevated in hyphal inducing condition.

Phenotypic analysis of the *cdc12-6* mutant. Temperature-sensitive growth properties of the *cdc12-6* mutant were demonstrated by spotting 3 μl of a series of 10-fold dilutions of cells on solid agar YPD medium, starting at a concentration of approximately 1×10^7 cells/ml. The

plates were then incubated at the indicated temperature for two days. Cells were also analyzed in liquid culture after shorter times of incubation at 37˚C. Liquid cultures were grown overnight to log phase at room temperature in rich YPD medium. The cultures were then split and then growth was then continued for the indicated time at room temperature or at 37˚C. Cell morphology was examined using DIC optics. Viability was determined by mixing cells with an equal volume of 0.4% Trypan Blue (Sigma-Aldrich Corp.), incubating for five min, and then cells were examined microscopically with visible light to detect intracellular staining indicate of plasma membrane lysis. GFP was analyzed directly in live cells without further processing using a fluorescent microscope equipped with a FITC filter set. This filter set was used to detect GFP, since it was easier to recognize the auto-fluorescence of dead cells as a different color from the true GFP signal. Chitin staining was performed by incubating cells with 10 μg/ml Calcofluor White in phosphate-buffered saline (PBS) for five min, followed by washing twice with PBS, and viewing the cells without fixation using a UV filter set. Filipin staining was carried out essentially as described previously (94,95). The cells were induced with or without serum for 2 h at 37˚, stained with 10 μg/ml filipin, and then analyzed immediately by fluorescent microscopy using a UV filter set. Cells were viewed on an Olympus BH-2 microscope and images were captured with an AxioCam digital camera (Carl Zeiss, Thornwood, NY) operated with Axiovision software. Fluorescence signal intensity for cells expressing *HWP1-GFP* was quantified using Axiovision software. The results represent the average of three independent experiments in which least 50 cells were counted each time.

Confocal microscopy was used to analyze septin ring structure in cells producing Cdc10-GFP. The cells were cultured overnight in log phase at room temperature and then aliquots were incubated for two hr at 23ºC or 37ºC. The aliquot incubated at 37ºC was further divided into two

tubes. One tube was incubated in rich YPD medium to promote growth of budding cells. Bovine calf serum (BCS) was added to the other tube to a final concentration of 20% to induce hyphal growth. Cells were then analyzed by fluorescence microscopy using a Zeiss LSM510 META NLO Two-Photon Laser Scanning Confocal Microscope at the Stony Brook University Central Microscopy Imaging Center.

2.2 Material and Methods of the FLP Chapter

Chemicals, strains and media

Oleic acid, linoleic acid, linolenic acid, α-tocopherol (vitamin E), hydrogen peroxide, tert-butyl hydroperoxide, cumene hydroperoxide, menadione, p-benzoquinone, thiobarbituric acid (TBA), hydrochloric acid, and 1,1,3,3-tetramethoxypropane were purchased from Sigma-Aldrich Corp. Trichloroacetic acid was from the Alfa Aesar Company, and nourseothricin from Werner BioAgents.

The *C. albicans* strains used in this study are described in Table 1. Cells were grown in SD medium (yeast nitrogen base synthetic medium with dextrose) (96). *C. albicans* deletion mutants were created in strain SN152 (*arg4Δ his1Δ leu2Δ*) by homologous recombination, as described previously (97). Mutant strains that carry homozygous deletion of *PST1*, *PST2, PST3, YCP4, or COQ3* were constructed with strain SN152 by the sequential deletion of both copies of the targeted gene. Gene deletion cassettes were generated by PCR amplification of the *LEU2* or *HIS1* selectable marker gene (97), using primers that also included ~80 bp of DNA sequence homologous to the upstream or downstream region of the targeted open reading frame (ORF). Cells that had undergone homologous recombination to delete the targeted gene were identified

by PCR analysis. A *pst3Δ ycp4Δ* double mutant strain was constructed by simultaneous deletion of both genes, taking advantage of the fact that they are adjacent in the genome. Homozygous triple and quadruple deletion mutation strains were then constructed by sequential deletion of both copies of the targeted gene using the *SAT1* flipper method to recycle the selectable marker (98). Similar phenotypes were observed for independent isolates. Deletion strains were then made prototrophic by transforming with the *ARG4* gene to correct the remaining auxotrophy.

Complemented strains, in which the wild-type FLP gene was reintroduced into the corresponding deletion mutant, were constructed by first using PCR to amplify the corresponding FLP gene plus 2000 base pairs (bp) upstream and 501 bp downstream of the *PST1* open reading frame (ORF), 811 bp upstream and 427 bp downstream of the *PST2* ORF, 1681 bp upstream and 427 bp downstream of the *PST3* ORF, or 1526 bp upstream and 310 bp downstream of the *YCP4* ORF. The DNA fragments were then inserted between the *Sac*I and *Sac*II sites in a derivative of plasmid pDDB57 (99) in which the *URA3* gene was replaced with *ARG4*. The resulting plasmids were then linearized by restriction digestion in the promoter region, and then transformed into the corresponding homozygous deletion mutant strains using *ARG4* for selection. The complementing plasmids were also transformed into the Δ/Δ/Δ/Δ mutant to create strains that express only a single FLP gene. A fully complemented quadruple mutant strain was constructed essentially as described above, except that both *PST1* and *PST2* genes were cloned in tandem into the *ARG4* plasmid. The plasmid was digested with *BspE*I to linearize it in the promoter region of the *PST1* gene, and then it was transformed into the quadruple mutant strain LLF054. The *PST3* and *YCP4* genes were cloned between the *Sac*I and *Apa*I restriction sites of a derivative of plasmid pDDB57 in which the *URA3* selectable marker was changed to the *SAT1* gene to confer nourseothricin resistance. Note that the *PST3* and *YCP4*

genes are adjacent in the genome in a head to head manner, such that the corresponding open reading frames are transcribed in a divergent manner. A PCR fragment that contains sequences between 1157 bp downstream of the *YCP4* ORF and 466 bp downstream of the *PST3* ORF was used to create the *PST3-YCP4* complementing plasmid. The resulting plasmid was digested with *Sna*BI to linearize it about 400 bp downstream of the *YCP4* open reading frame, and then the DNA was transformed into strain LLF078, a version of the Δ/Δ/Δ/Δ quadruple mutant in which the *PST1* and *PST2* genes were already introduced as described above.

The open reading frames for *E. coli wrbA* and rat *NQO1* were synthesized by GeneWiz Corp. so that the codons could be optimized and to avoid CUG codons that are translated differently in *C. albicans*. The *wrbA* and *NQO1* open reading frames were amplified by PCR and introduced downstream of the *ADH1* promoter and GFP in plasmid pND391 that carries the *ARG4* selectable marker. The resulting plasmids were then transformed into the Δ/Δ/Δ/Δ mutant strain LLF054 to create strains expressing *wrbA* (LLF074), *NQO1* (LLF076), or GFP (LLF080) as a control.

Growth assays to test sensitivity to oxidizing agents

Spot assays to test growth in the presence of oxidizing agents were carried out essentially as described previously (100,101). *C. albicans* mutant or wild type strains were grown overnight and then adjusted to 10^7 cells/ml. Serial 10-fold dilutions of cells were prepared, and three µl of each dilution was then spotted onto solid agar SD medium containing the indicated chemical. The plates were incubated at 37°C for 2 days and then photographed. Each assay was done at least three independent times.

Cells were also tested in liquid culture for sensitivity to oxidizing agents by assaying colony forming units (CFUs). *C. albicans* cells were grown in synthetic medium with 2% dextrose and without amino acids at 37˚C overnight. Cells were harvested at about 6-10 x 10^7 cells per ml, washed once, and resuspended in phosphate buffer (0.1M sodium phosphate, pH 6.2, 0.2% dextrose) at 10^7 cells per ml. Three ml was transferred to a 15 ml glass test tube and then fatty acids were added. Cells were then incubated at 37˚C on a tube rotator for the designated period of time. At the end of each treatment, cells were harvested by centrifugation and samples were plated to determine the number of viable CFUs. Results represent the average of 3-6 independent assays.

Detection of thiobarbituric acid reactive substances (TBARS)

The level of TBARS in yeast whole cell lysates was determined by a modification of a previously described procedure (102). At the end of the fatty acid treatment described above, 1.5 x 10^7 cells were harvested by centrifugation at 17,000 x g for 5 min, washed once with distilled water, and resuspended in 100 µl distilled water in a screw cap tube. 1ml of a freshly prepared solution of 0.375% thiobarbituric acid dissolved in 12% trichloroacetic acid and 0.5 M hydrochloric acid was added to each tube. After a 20-minute incubation at 90˚C, samples were allowed to cool down, and then the insoluble material was sedimented by centrifugation at 17,000 x g for 5 min. The absorbance of the supernatant was measured at 535 nm, and corrected by subtracting the nonspecific absorbance at 600 nm. The corrected absorbance was then compared with a standard curve created using 1,1,3,3-tetramethoxypropane treated under the

same conditions, which generates malondialdehyde (MDA). Results represent the average of 3-4 independent experiments.

Microscopic analysis of GFP fusion proteins

The GFPγ variant was fused to the 3' ends of the open reading frames for *PST1* and *PST3* using *HIS1* selection, in LLF018, as described previously (93). Strains were verified by PCR analysis and microscopic examination of GFP fluorescence. To add GFP at the 5' end of the open reading frames to create N-terminal fusions, GFPγ was introduced downstream of the *ADH1* promoter followed by the FLP gene and then the *ADH*1 terminator in pND397, which carries an *URA3* selectable marker gene (103). The plasmids of pADH1-GFPγ-PST1, pADH1-GFPγ-PST2, or pADH1-GFPγ-PST3 were also linearized with Not I, before being individually transformed into LLF089 using *URA3* as the selectable marker to create the strains LLF091, LLF092, and LLF093. The plasmid pADH1-GFPγ-YCP4 was linearized with *Not*I and transformed into LLF018 using *URA3* as the selectable marker to construct the strain LLF071. GFP fluorescence was analyzed directly in live cells without further processing using a Zeiss Axiovert 200M microscope equipped with an AxioCam HRm camera and Zeiss AxioVision software for deconvolving images.

Virulence assays

C. albicans strains were tested for virulence in a mouse model of hematogenously disseminated candidiasis similar to previous studies (104,105). *C. albicans* cells were cultured by growing

overnight at 30°C in YPD medium with 80 µg/ml uridine, reinoculating into fresh medium, and incubating again overnight at 30°C. Cells were prepared for infection assays by washing twice in phosphate-buffered saline (PBS), counting in a hemocytometer, and then diluting to 1.25 x 10^6 cells/ml with PBS. Female BALB/c mice were injected via the lateral tail vein with 2.5 x 10^5 cells, and then monitored at least twice a day for 28 days. Mice were considered to be moribund if food and water could no longer be accessed and then humane euthanasia was performed by carbon dioxide inhalation as per instructions from the Department of Laboratory Animals at Stony Brook University. All procedures were approved by the Stony Brook University IACUC Committee. Statistical analyses of the results for the survival studies were carried out using a log rank test (Mantel-Haenszel) with the Prism 6 software program (GraphPad Software, Inc., La Jolla, CA). To assess fungal burden, kidneys were excised, weighed, and then homogenized in 5 ml PBS for 30 s with a tissue homogenizer (Pro Scientific Inc.). The CFU per gram of kidney was determined by plating dilutions of the homogenates on YPD agar medium plates, and incubating for 2 days at 30°C. Statistical analysis of the CFU data was carried out with Prism 6 software using one-way analysis of variance with a nonparametric Kruskal-Wallis test and Dunn's post-hoc test.

Chapter 3 – A *C. albicans* temperature-sensitive *cdc12-6* mutant identifies roles for septins in selection of sites of germ tube formation and hyphal morphogenesis

Abstract

Septins were identified for their role in septation in *Saccharomyces cerevisiae*, and were subsequently implicated in other morphogenic processes. To study septins in *Candida albicans* hyphal morphogenesis, a temperature-sensitive mutation was created that altered the C terminus of the essential Cdc12 septin. The *cdc12-6* cells grew well at room temperature, but at 37˚ they displayed expected defects in septation, nuclear localization, and bud morphogenesis. Although serum stimulated the *cdc12-6* cells at 37˚C to form germ tube outgrowths, the mutant could not maintain polarized hyphal growth and instead formed chains of elongated cell compartments. Serum also stimulated the *cdc12-6* mutant to induce a hyphal reporter gene (*HWP1-GFP*) and a characteristic zone of filipin staining at the leading edge of growth. Interestingly, *cdc12-6* cells shifted to 37˚C in the absence of serum gradually displayed enriched filipin staining at the tip, which may be due to the altered cell cycle regulation. A striking difference from the wild type was that the *cdc12-6* cells frequently formed a second germ tube in close proximity to the first. The mutant cells also failed to form the diffuse band of septins at the base of germ tubes and hyphae, indicating that this septin band plays a role in preventing proximal formation of germ tubes in a manner analogous to bud site selection. These studies demonstrate that septins are not only important for cytokinesis, they also promote polarized morphogenesis and selection of germ tube sites that may help disseminate an infection in host tissues.

Introduction

The human fungal pathogen *C. albicans* is capable of causing severe systemic infections. Although immunocompromised patients are particularly at risk, immunocompetent individuals are susceptible to infection when the inoculum is high, which can occur under circumstances such as biofilm formation on medical devices. The major pathogenic effects of *C. albicans* are due to invasive growth into tissues, which is facilitated in part by the ability of *C. albicans* to switch between different morphologies (106,107). *C. albicans* can grow as budding cells, chains of elongated cells termed pseudohyphae, or as long filamentous cells with parallel walls called hyphae (107). The filamentous morphology promotes invasive growth into agar in vitro and has been linked to invasive growth into tissues in vivo (38,108). Previous studies also indicated that *C. albicans* must be able to switch between different morphologies to be fully pathogenic. Mutants that are locked in either the hyphal or budding form have been shown to be less virulent in models of hematogenously disseminated systemic candidiasis (5,38,109).

The septin family of cytoskeletal filament-forming proteins has been shown to contribute to morphogenesis in *C. albicans* (49,50,95,110). The septins were first identified in the yeast *Saccharomyces cerevisiae* as proteins that are needed for septum formation and cytokinesis (47,48). The septins localize to the bud neck where they form a scaffold to recruit other proteins that promote septum formation. They also act as a boundary domain to restrict proteins involved in septum formation to the proper position in the bud neck and also restrict certain proteins to the daughter cells (111,112). Deletion analysis of the seven different septin genes in *C. albicans* revealed that their relative importance is similar to the orthologous genes in *S. cerevisiae*. *CDC3* and *CDC12* are essential, whereas *CDC10, CDC11,* and *SEP7* are not essential but contribute to proper septation and morphogenesis (49,50). Mutation of the *C. albicans* orthologs of the *SPR3*

and *SPR28* septin genes that are expressed during sporulation in *S. cerevisiae* did not detectably affect *C. albicans* septation or morphogenesis (49).

Septins have also been implicated in other morphogenic events. For example, *S. cerevisiae* septins promote proper pheromone-induced morphogenesis, spore formation, and selection of the site of future bud formation (113). The possibility that the septins may also play special roles during *C. albicans* hyphal growth was suggested by studies that detected septins at sites distal to septum formation, including the base of the initial hyphal outgrowths (know as germ tubes) and at the growing hyphal tips (49,57). Consistent with this, *cdc10Δ* and *cdc11Δ* mutants exhibited partial defects in hyphal morphogenesis, including a greater frequency of curved hyphae, and slight inconsistencies in cell wall deposition (49). Significantly, the *cdc10*Δ and *cdc11*Δ mutants displayed defects in morphogenesis and invasive growth in a mouse model of systemic *C. albicans* infection (58). These mutants also had a related defect in the morphogenesis of the filamentous cells that produce chlamydospores (114).

In order to obtain a fuller understanding of the role of the *C. albicans* septins in hyphal growth, a new approach was necessary to study the essential septin genes *CDC3* and *CDC12*. Therefore, a temperature-sensitive *CDC12* mutant strain was constructed that was patterned after the well-studied *cdc12-6 S. cerevisiae* mutant. The *Ca-cdc12-6* strain grew well at room temperature but not at 37˚C, the temperature at which hyphal growth is induced. It also showed temperature-sensitive defects in septation and bud morphogenesis, similar to those reported for the analogous mutation of *S. cerevisiae* (115,116). Interestingly, the Ca-*cdc12-6* mutant also revealed important new roles for septins in maintaining highly polarized hyphal growth and for selection of the second site of germ tube formation.

Results

Construction of a *C. albicans* temperature-sensitive *cdc12-6* mutant. The essential septin gene *CDC12* was examined by creating a mutant allele based on the changes found in the well-studied temperature-sensitive *S. cerevisiae cdc12-6* mutant (Dr. Brian Haarer, personal communication). The mutation alters the C-terminal sequences of Cdc12 as described in Fig. 1A. These alterations may alter septin function because the C terminal region of Cdc12 contributes to the stability of septin structures by promoting interaction between two septin filaments (117-119). The other copy of *CDC12* was deleted so that only the mutant *cdc12-6* allele remained. The *C. albicans cdc12-6* strain displayed a strong temperature-sensitive phenotype (Fig. 1B). The *cdc12-6* strain grew as well as the wild type control strain or a *cdc12Δ/CDC12* heterozygote when spotted onto solid medium agar plates at temperatures up through 30˚, but it was not viable at 37 ºC. Thus, although the C termini of Cdc12 from *S. cerevisiae* and *C. albicans* are not identical, introduction of the analogous alterations of the *Sc-cdc12-6* allele into *Ca-CDC12* still resulted in a strong temperature-sensitive phenotype.

***cdc12-6* mutant phenotypes during budding.** The effects of the *cdc12-6* mutation on *C. albicans* morphogenesis were examined at room temperature (0 h) and at different times after shifting cells to 37˚C (Fig. 2). When grown at room temperature, the *cdc12-6* cells formed buds, albeit some with slightly abnormal shape. Many cells were also present in clusters, indicating a partial defect in septation and cytokinesis. After two h at 37˚C, *cdc12-6* cells formed elongated buds (Fig. 2B). After six h, continued growth of the mutant cells resulted in highly elongated filamentous cells. Around six h the *cdc12-6* cells began to lyse near the tips, as evidenced by intracellular staining with Trypan Blue (Fig. 2B). The highly elongated buds formed by the

cdc12-6 mutant are similar to the morphology of septin mutants in *S. cerevisiae*, which are thought to form due to activation of a cell cycle checkpoint pathway that prolongs apical growth of cells (112).

A hallmark of *S. cerevisiae* septin mutants is their defect in septation. To assay this in the *C. albicans cdc12-6* mutant, log phase cells shifted to 37˚C were stained with Calcofluor White to detect the ring of cell wall chitin that forms at the septum (120). At room temperature, both the wild type and the *cdc12-6* mutant showed typical Calcofluor White staining at bud necks (Fig. 3A). Similar results were observed for the wild type strain at 37˚C. By contrast, sites of septation were rarely detected in the *cdc12-6* mutant shifted to 37˚C (Fig. 3B). This was most evident after 6 h incubation at 37˚C; the *cdc12-6* cells formed chains of elongated pseudohyphal-type cells with few obvious septae (Fig. 3C). There were, however, patches of Calcofluor White staining that could represent aberrant attempts to form septae.

Septins also contribute to proper nuclear segregation in *S. cerevisiae* by interacting with microtubules to orient nuclear migration into the bud (121). The distribution of nuclei in *C. albicans* was therefore examined by monitoring the nuclear-localized protein Nop1-GFP. Wild-type cells typically contained one nucleus per cell, as expected. In contrast, the *cdc12-6* cell compartments frequently contained multiple nuclei or they lacked a nucleus, especially after the longer 6 h incubation at 37˚C, indicating a defect in nuclear segregation (Fig. 3C).

Septin localization in the *cdc12-6* mutant was analyzed by studying the Cdc10 septin fused to GFP. Wild-type cells grown at either room temperature or 37ºC exhibited the expected localization of Cdc10-GFP to rings at the bud neck (Fig. 3A). The *cdc12-6* cells grown at room temperature also showed bud neck localization of Cdc10-GFP (Fig. 3A). However, after a shift to 37˚C for two hours, approximately half of the *cdc12-6* cells lacked detectable Cdc10-GFP, and

the others primarily contained faint patches or rings of Cdc10-GFP towards the growing end of the elongated cell cluster (Fig. 3B). Thus, the septins are still capable of forming a complex at 37˚C, but it primarily appears near the leading edge of growth and does not stabilize at the pinched zones that correspond to bud necks. Cdc10-GFP was still showed a similar distribution in the *cdc12-6* mutant after 6 hr at 37˚C (Figure 3C). Although a majority of cells appeared to lack Cdc10-GFP, a patch or ring of septins was frequently detected at the leading edge of growth or at sites of budding off the main filamentous cell clusters.

The three dimensional structure of the Cdc10-GFP septin rings that formed in the *cdc12-6* mutants was analyzed by confocal microscopy (Fig. 4). Wild type cells grown at room temperature or 37˚C showed the expected Cdc10-GFP ring at the bud neck. In contrast, the Cdc10-GFP structures in the *cdc12-6* mutant were abnormal. At room temperature, Cdc10-GFP localized in a spectrum of patterns ranging from typical ring structures, to partial rings with a break in the continuity, and very faint rings (Fig. 4). Shifting the *cdc12-6* cells to 37˚C for 2 h resulted in much more severe defects in Cdc10-GFP localization. Cdc10-GFP most frequently appeared as a series of bars and did not form a contiguous ring. The Cdc10-GFP rings in cdc12-6 cells showed similar defects for cells grown in the presence or absence of serum. Some of these structures appeared to be similar to the types of septin rings seen in *S. cerevisiae* cells induced with mating pheromone or carrying a mutation in *GIN4* (122,123), and in *C. albicans* mutants with hyperactive Cdc42 (124).

Hyphal morphogenesis defects in *cdc12-6* mutant cells. The ability to undergo hyphal morphogenesis was examined by treating cells with 20% bovine calf serum at 37˚C. As expected, wild-type cells efficiently formed the initial polarized outgrowths termed germ tubes

that continued to elongate in a highly polarized manner to form filamentous cells with multiple cell compartments termed hyphae (Figs. 2 and 5). Serum also induced *cdc12-6* cells to form germ tubes at 2 h that were generally similar to the wild-type cells (Figs. 2 and 5). Serum clearly induced a distinct morphogenesis pathway in the *cdc12-6* mutant; most cell walls grew parallel and did not display the curvature that was seen in the absence of serum (Figs. 2 and 3). However, Cdc10-GFP localization was abnormal in the *cdc12-6* cells induced with serum. About half of the cell clusters lacked detectable Cdc10-GFP, and the Cdc10-GFP structures that were present were typically fainter (Fig. 5A). In addition, the septin rings that formed in *cdc12-6* cells had a wider diameter than those detected in the germ tubes and hyphae of wild type cells (Fig. 4). This is likely due in part to the continued expansion of the width of the *cdc12-6* germ tubes (see below). In addition, the Cdc10-GFP rings in *cdc12-6* cells typically had breaks in their continuity and some appeared as a series of bars as was seen for cells grown in the absence of serum (Fig. 4).

After 6 h incubation at 37˚C with 20% serum, the morphology of the *cdc12-6* cells was very distinct from the wild type (Fig. 5B). The *cdc12-6* cells formed filaments that were wider and curved, indicating the original germ tubes continued to grow in width, whereas new growth in wild-type cells is restricted to the apical tip. The hyphal inducing conditions did not appear to alter the viability of cells at 37˚C. Trypan Blue staining revealed that dead cells still began to accumulate by 6 h incubation (Fig. 2). The *cdc12-6* cells also showed frequent branching of new offshoots of filamentous outgrowth that was not seen for the wild type. Analysis of Nop1-GFP localization revealed many of these branched regions did not contain nuclei, whereas other regions contained multiple nuclei (Fig. 5). This indicates that nuclear division continued in the absence of septation, but that the nuclei were not segregating into the different cell

compartments. The *cdc12-6* cells rarely formed septae that could be detected by Calcofluor White staining, even at the sites where Cdc10-GFP was localized. Instead, patches of Calcofluor White staining were commonly detected in the new filamentous growth that may represent aberrant attempts to initiate septum formation. After 6 h there were patches or rings of Cdc10-GFP detected in about 30% of the cells. Interestingly, the Cdc10-GFP structures in the *cdc12-6* mutant were frequently detected in the middle of the elongating germ tube, as seen for wild type cells, and not at the tip of the filamentous cell as was seen for *cdc12-6* cells grown at 37˚C in the absence of serum. This suggests that septin localization is affected by the altered cell morphogenesis or by distinct signaling pathways activated in hyphae.

Hyphal-induced responses. The ability of the mutant cells to induce hyphal genes was assayed by quantifying the expression of a *HWP1-GFP* gene fusion. This reporter gene was constructed by placing *GFP* expression under control of the hyphal-induced *HWP1* promoter. Cells carrying this reporter gene were grown in the presence or absence of the hyphal inducers serum or GlcNAc, and the relative induction assessed by quantifying the signal intensity of GFP using fluorescence microscopy. Although the wild type and mutant cells strongly induced *HWP1-GFP* (Fig. 6A), the *cdc12-6* cells were slightly less efficient than the wild type ($P < 0.003$). Thus, septin function is not essential for induction hyphal genes.

Another hallmark of hyphal cells is that the apical region stains more readily with the ergosterol-binding agent filipin (125). As expected, essentially 100% of wild-type cells induced with serum showed increased staining with filipin at hyphal tips (Fig. 6B). Similar results were observed for *cdc12-6* cells induced with serum. Surprisingly, control studies showed that about 31.4% (n=191) of *cdc12-6* cells shifted to 37˚C for 1.5 h in the absence of serum also showed

stronger filipin staining at the tips. This increased over time to 41.8% at 2 h (n=110) and 63.3% at 3 h (n=128). These results for filipin staining contrasted with the expression of *HWP1-GFP*, which required serum to be induced. Thus, this characteristic of hyphae could be induced in *cdc12-6* cells in the absence of serum.

Altered position of second germ tubes in *cdc12-6* mutant. The *cdc12-6* cells induced to form hyphae for an extended time frequently formed a second germ tube very close to the first one, which was rarely observed in the wild type (Fig. 7 and ref. (126)). To quantify the difference, the relative positions of the sites where the first and second germ tubes initiated were scored as one of six patterns: 150˚-180˚ apart, 120˚-150˚ apart, 60-120˚ apart, <60˚ apart, two germ tubes in contact, or a second germ tube that emerged from the first germ tube rather than the mother cell. Interestingly, *cdc12-6* cells showed significantly increased frequency of cells forming a second germ tube proximal to the first (Fig. 7A). The majority of *cdc12-6* cells formed a second germ tube within 60˚ angle of the first, whereas essentially all of the wild type cells formed a second germ tube that was greater that 60˚ from the first. The c*dc10Δ* and *cdc11Δ* mutants occasionally formed proximal germ tubes (49), but this defect was more extreme in the *cdc12-6* cells.

The altered site selection for germ tube outgrowth in the *cdc12-6* mutant indicates that septins influence this process. An interesting possibility is that the basal band of septins may play a role in determining the site of the second germ tube (Fig. 7B). The basal band is a more diffuse type of septin ring that is located at the junction between the mother cell and the germ tube (49,57). The function of this basal band of septins is not clear, since cytokinesis does not occur at this site. However, its location suggests that the basal septin band may function in germ

tube site selection analogous to the role of the septin ring in bud site section (122). Consistent with this, the basal band of septins was not detected in *cdc12-6* cells (Fig. 7B). Thus, proper Cdc12 function is required to form both the basal septin band and the septin rings that form at sites of cytokinesis.

A relationship between bud site and germ tube site selection is also supported by previous studies which showed that the bud sites of wild-type *C. albicans* were clustered at an axial site at room temperature but were primarily not adjacent at 30˚ and 37˚C (126). The budding mode of *cdc12-6* cells could not be assessed at elevated temperature, which is not permissive for this mutant. Therefore, we analyzed the non-essential septin mutants *cdc10Δ*, *cdc11Δ*, and *sep7Δ*. These mutants also showed defects at 37˚C that prevented accurate assessment of bud site selection, but could be examined at 30˚. Interestingly, all three non-essential septin mutants budded primarily at a cluster of axial sites at room temperature (>90%; n = >200), similar to the wild type. In contrast, at 30˚ only 33% of wild-type cells budded in an axial manner (n = 202), whereas *cdc10Δ*, *cdc11Δ*, and *sep7Δ* all still budded primarily in an axial manner (>60%; n = >144). The effect was most obvious for the *cdc11Δ* mutant (76% axial budding at 30˚), consistent with *cdc11*Δ having the strongest morphogenesis phenotype of the three. This suggests that the temperature-related switch in bud-site selection underlies the mechanisms that promote germ tube formation at distal rather than axial sites.

Discussion

Temperature-sensitive septin mutants have played a valuable role in *S. cerevisiae* for identifying the function of septins in septation and other morphogenic events, including mating and sporulation (47,113). Although hyphal morphogenesis could not be examined in *S. cerevisiae*, studies of the non-essential septin genes *CDC10*, *CDC11*, and *SEP7* indicated that they are important for normal hyphal morphogenesis in *C. albicans* (49,50,110) and in filamentous fungi (127-129). Therefore, in this study a temperature-sensitive *cdc12-6* mutant was created to carry out the first analysis of an essential septin gene in *C. albicans*. Shifting the *C. albicans cdc12-6* strain to 37˚C caused a rapid defect in morphogenesis and septation, similar to those seen in *S. cerevisiae*. The *cdc12-6* mutation likely causes a Ts- phenotype because it alters the C terminal region of Cdc12 that is important for stabilizing connections between two septin filaments (117-119). Temperature-sensitive mutants are well suited for the study of *C. albicans* hyphal morphogenesis because cells can be grown at room temperature and then shifted to a non-permissive temperature of 37˚C, the optimal temperature for hyphal morphogenesis. The rapid inactivation of Cdc12 function at high temperature is therefore expected to reveal better insight into septin function than the use of a regulated promoter, which would require a longer incubation period to deplete the stable septin proteins. Thus, the new *cdc12-6* mutant represents an important new tool for *C. albicans* research.

Induction and maintenance of hyphal morphogenesis. The *cdc12-6* cells formed buds at room temperature and could be stimulated with serum to form germ tubes at 37˚C (Figs. 2 and 5). The initial germ tube outgrowths in the *cdc12-6* mutant cells at 2 h did not appear to be significantly more defective than the wild type, whereas bud morphogenesis was clearly affected by 2 h (Fig. 2). This suggests that septin function is not required to initiate germ tubes. Previous

studies showed that the *C. albicans cdc10Δ* and *cdc11Δ* mutants frequently formed curved germ tube necks (49,114), but this phenotype was not exacerbated in the *cdc12-6* mutant.

Although the *cdc12-6* mutant formed germ tubes, highly polarized hyphal morphogenesis was not maintained. The filamentous outgrowths became wider over time and took on the characteristics of pseudohyphal cells. The *cdc12-6* mutant phenotype was more extreme than the mutants lacking the nonessential septin genes *CDC10* and *CDC11*, which showed subtler defects in maintaining polarized morphogenesis (49). This indicates that the septins have a special function in maintaining highly polarized growth. Altered septin localization is therefore likely to contribute to the abnormal hyphal morphogenesis of mutants that display defects in targeting septins to appropriate sites (124,130,131). However, some phenotypes of *cdc12-6* cells may be due to activation of stress pathways. The defects of *cdc12-6* cells in septation and cell wall biogenesis (Figs. 2 and 5) should activate cell wall stress pathways that could indirectly affect actin localization and morphogenesis. Activation of stress pathways could also account from some of the altered patches of Calcofluor White staining in *cdc12-6* cells at 37˚C (Figs. 3 and 5). Unusual patches of Calcofluor White staining were also detected after treatment of cells with Caspofungin, an inhibitor of cell wall β-glucan synthesis (132).

Regulation of the Cdc11 septin by phosphorylation has also been implicated in proper *C. albicans* hyphal morphogenesis. Mutation of a site in Cdc11 to prevent phosphorylation by Cdc28 (S394A) caused a defect in maintaining highly polarized hyphal growth (110). This defect in maintaining polarized growth was more extreme than the defects seen for *cdc12-6* cells. Mutation of a different site in Cdc11 to prevent phosphorylation by Gin4 (S395A) caused cells to sequentially initiate multiple short germ tubes, suggesting a role for septins in stabilizing the active site of polarized morphogenesis (110). However, the *cdc10Δ*, *cdc11Δ*, *cdc12-6* mutants

rarely produce the multiple germ tube protuberances seen in the wild type or that were seen so frequently in the *cdc11-S395A* mutant (110). Thus, some phenotypes caused by mutating Cdc11 phosphorylation sites are likely due to the dominant activity of a mis-regulated septin, rather than the absence of septin function.

The *cdc12-6* mutant at 37˚C also showed more frequent branching of the filamentous outgrowths. This phenotype likely relates to altered cell cycle regulation due to the failure of the germ tubes to undergo septation to form hyphae with different cell compartments. In wild type cells, the mother cell vacuole swells to force most of the cytoplasmic constituents to the daughter cell compartment at the leading edge of growth (133). Consequently, the mother cells and subsequent subapical cells are delayed in initiating second germ tubes or branches until they can restore the cytoplasmic components. Consistent with this, septin rings persist in the hyphal cells at sites of septation, and do not disassemble quickly after septation as they do in budding cells (49,57). More frequent branching was also seen in septins mutants of the filamentous fungus *A. nidulans* (127,134).

Hyphal-induced gene expression and filipin staining. The ability of the *cdc12-6* mutant to induce hyphal responses was confirmed by showing that serum and GlcNAc also induced expression of the *HWP1-GFP* reporter gene (Fig. 6A). In addition, serum also induced a domain at the tips of the germ tubes in both the wild type and *cdc12-6* mutant that stained more readily with the ergosterol binding agent filipin (Fig. 6B). The increased filipin staining is indicative of an altered lipid content in the plasma membrane at hyphal tips (95). Surprisingly, a high proportion (63%) of the *cdc12-6* cells shifted to 37˚C in the absence of serum for 3 h also showed increased tip staining. This was unexpected because previous studies indicated that this filipin-staining domain was only detected in hyphal, not pseudohyphal cells (95). These results

could suggest that *cdc12-6* cells induce this response in the absence of hyphal inducers. However, enriched filipin staining is also transiently observed at sites of cytokinesis (94,95). Thus, a likely alternative possibility is that the filipin staining relates to altered cell cycle regulation in *cdc12-6* cells. In support of this latter possibility, enriched filipin staining of the tips of *cd12-6* cells increased over time, rather than coinciding with the induction of the initial germ tube outgrowth as seen with serum induction (95,135).

Site of second germ tube formation. A striking defect of the *cdc12-6* mutant is that the second germ tube usually formed proximal to the first germ tube (Fig. 7). This contrasts with wild-type cells in which the second germ tube emerges at a distal position. The basal band of septins that forms at the junction between the mother cell and the germ tube is therefore implicated in this process. The role of the basal band of septins is not well understood; septation does not occur at this site and the septins are detected in a more diffuse pattern than the septin ring seen at sites of septation (49,57,130). This raises the possibility that the function of the basal band is to prevent the initiation of second germ tubes proximal to the first. Consistent with this, the basal septin band is stably maintained after the germ tube has undergone cytokinesis and turned into a hyphal cell. Thus, it is stably maintained until later stages when the second gem tube initiates. The formation of the second germ tube at a distal site is significant, as it would help disseminate an infection by promoting growth in a new direction.

This role for septins in germ tube site selection is likely related to their role in bud site selection. In *C. albicans*, wild-type cells form buds in an axial manner at room temperature, but switch to forming buds at non-adjacent bipolar sites at 37˚C (126). In contrast, the *C. albicans* septin mutants primarily budded in an axial manner at 30˚C where wild-type cells had mostly switched to budding at bipolar sites. Thus, septins play roles in preventing the formation of a

new site morphogenesis adjacent to an existing bud or germ tube in *C. albicans*. In contrast, the *S. cerevisiae* septins are needed for proper axial budding of haploid cells (136), which means they act in a distinct manner to recruit the morphogenesis machinery to an adjacent site.

Altogether, these studies demonstrate that, in addition to their essential role in septum formation, the septins are needed for maintenance of the highly polarized morphology and proper selection of sites of germ tube formation. Both of these are important for dissemination of an infection in host tissues. These conclusions are supported by the defect of *cdc10Δ* and *cdc11Δ* mutants in invasive growth into tissues and virulence in a mouse model of candidiasis (58).

Acknowledgement

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TABLE 1. Strains used.

C. albicans strain	Parent strain	Genotype
BWP17	Sc5314	*ura3*Δ::λ*imm434/ura3* Δ::λ *imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*
DIC185	BWP17	*ura3*Δ::λ *imm434/URA3 his1*::*hisG/HIS1 arg4*::*hisG/ARG4*
YLS685	BWP17	*ura3*Δ::λ *imm434/URA3 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*
YAW12	BWP17	*cdc12::ARG4/CDC12 ura3*::λ*imm434/ura3* ::λ *imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*
CZ10	YAW12	*cdc12-6::URA3/cdc12Δ::ARG4 ura3*::λ*imm434/ura3*::λ *imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*
CZ11	CZ10	*cdc12-6* strain CZ10, except *CDC10-GFPγ::HIS1*
CZ14	CZ10	*cdc12-6 strain CZ10,* except *NOP1-GFPγ::HIS1*
LLF003	BWP17	BWP17, except *CDC10-GFPγ::HIS1*
LLF006	BWP17	BWP17, except *NOP1-GFPγ::URA3*
LLF009	LLF003	*CDC10-GFPγ* strain LLF003, except *URA3/ura3::λ imm434*
LLF010	CZ10	*cdc12-6* strain CZ10, except *HWP1-GFPγ::HIS1*

LLF012	YLS685	YLS685, except *HWP1-GFPγ::HIS1*
LLF016	CZ10	*cdc12-6 strain CZ10, HIS1/his1*::*hisG*

Figures

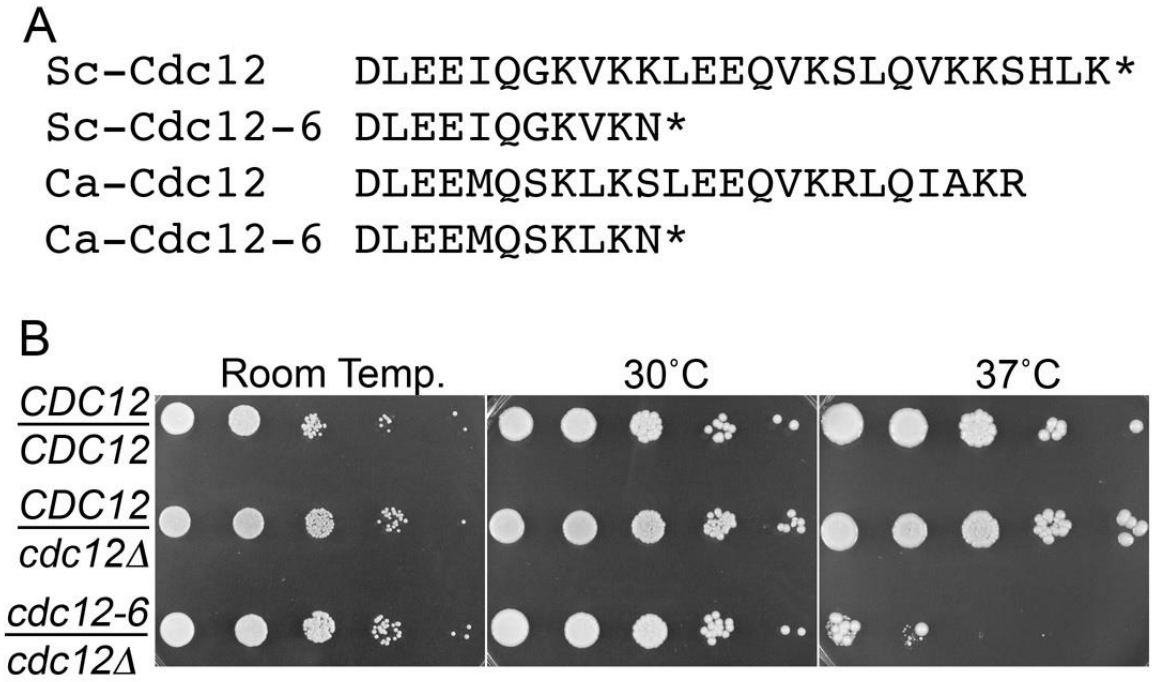


FIG. 1. The *C. albicans cdc12-6* septin mutant is temperature sensitive for growth.

(A) Alignment of the C terminal sequences of the *S. cerevisiae* and *C. albicans* Cdc12 proteins.

(B) Ten-fold dilutions of the wild-type control *CDC12/CDC12* (DIC185), *cdc12-6/cdc12Δ* (LLF016) and heterozygote *CDC12/cdc12Δ* (YAW12) strains were spotted onto rich-medium YPD plates. The plates were incubated for 48 hours at the indicated temperature and then photographed.

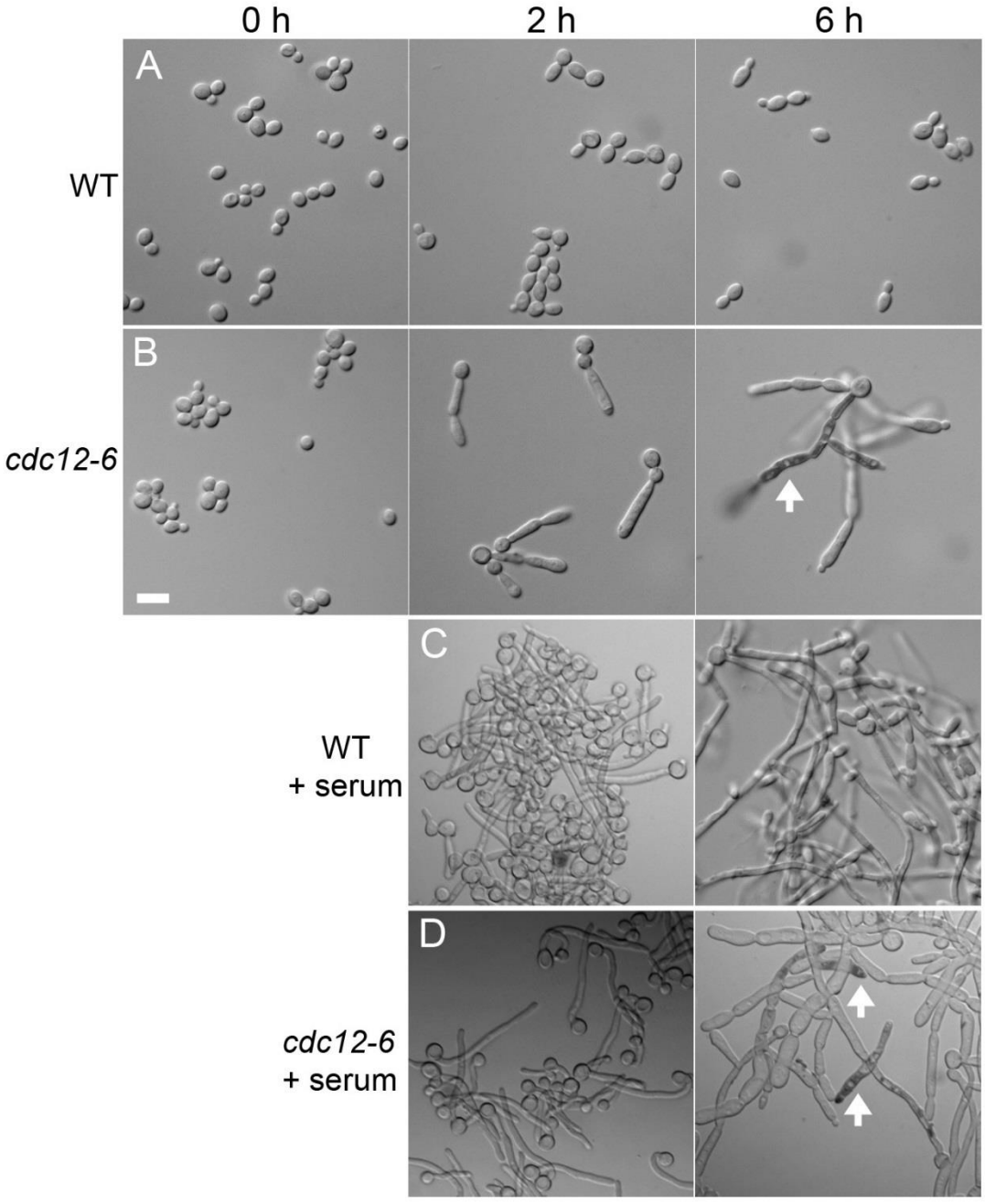

0 h
2 h
6 h
A
WT
B
cdc12-6
C
WT
+ serum
D
cdc12-6
+ serum

FIG. 2. Altered morphology and viability of *cdc12-6* cells after a shift to 37˚C.

Wild type (DIC185) and *cdc12-6* (LLF016) cells were cultured overnight in log phase in YPD medium at room temperature. The cells were then shifted to 37˚C in the absence (A, B) or presence (C, D) of 20% serum and incubated for the indicated time. Cells were stained with Trypan Blue and then examined by light microscopy with DIC optics. Examples of lysed cells that stain dark with Trypan Blue are indicated with an arrow. Bar, 10 µm.

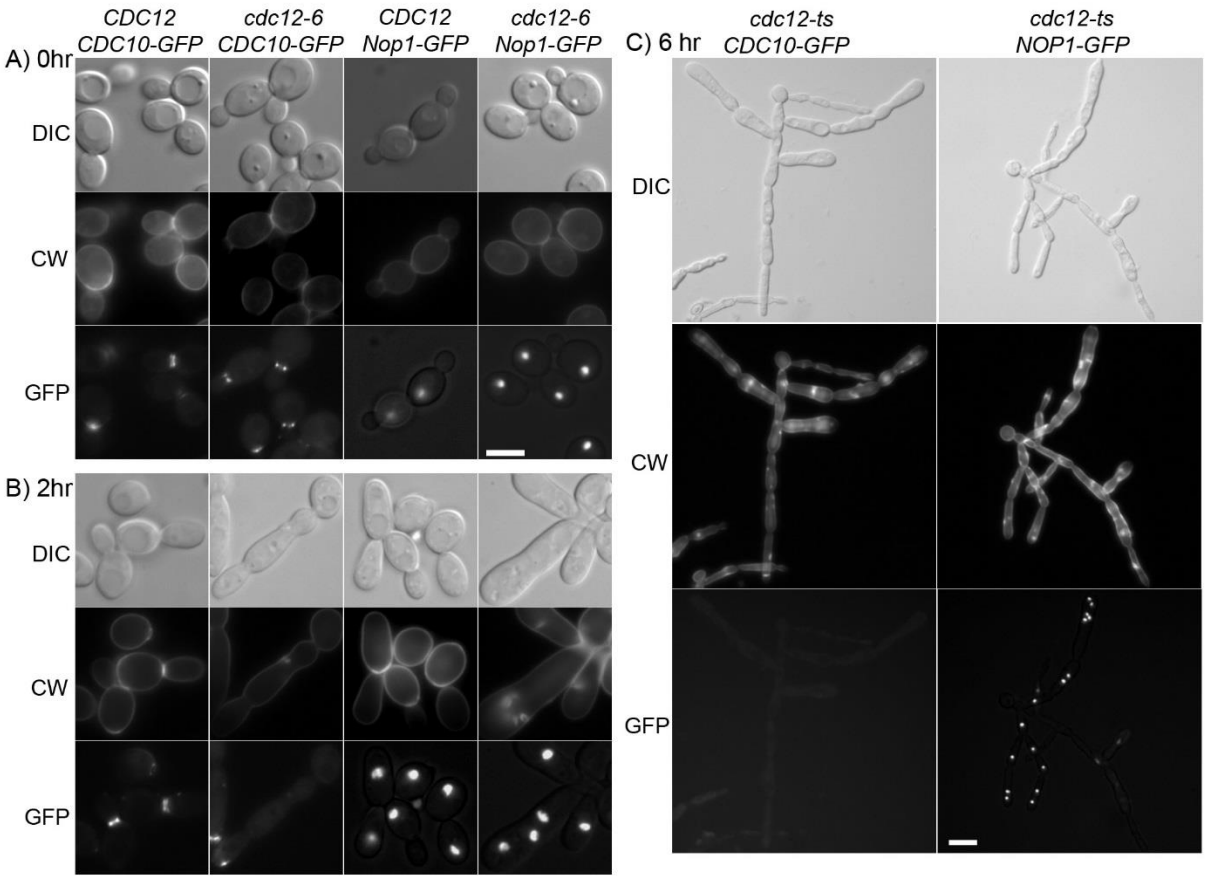

CDC12 CDC10-GFP
cdc12-6 CDC10-GFP
CDC12 Nop1-GFP
cdc12-6 Nop1-GFP
A) 0hr
DIC
CW
GFP
B) 2hr
DIC
CW
GFP
C) 6 hr
cdc12-ts CDC10-GFP
cdc12-ts NOP1-GFP
DIC
CW
GFP

FIG. 3. Altered localization of septins and nuclei in *cdc12-6* cells at 37˚C.

Wild type control and *cdc12-6* cells engineered to produce either Cdc10-GFP or Nop1-GFP were analyzed as indicated at the top of each column of panels. Cells were grown overnight to log phase at room temperature (A) or were shifted to 37˚C for 2 h (B) or 6 h (C). As indicated on the left, cells were analyzed using DIC optics to detect morphology, fluorescence microscopy to detect Calcofluor White (CW) staining of the cell wall chitin, and the localization of GFP-tagged proteins (GFP). Strains used were wild type cells carrying *CDC10-GFP* (LLF009) or *NOP1-GFP* (LLF006), and *cdc12-6* cells carrying *CDC10-GFP* (CZ11) or *NOP1-GFP* (CZ14). Photos taken at 0 h and 2 h using a 100 X objective, while those at 6 h were taken with a 40 X objective because of the larger cell size. Bars, 10 μm.

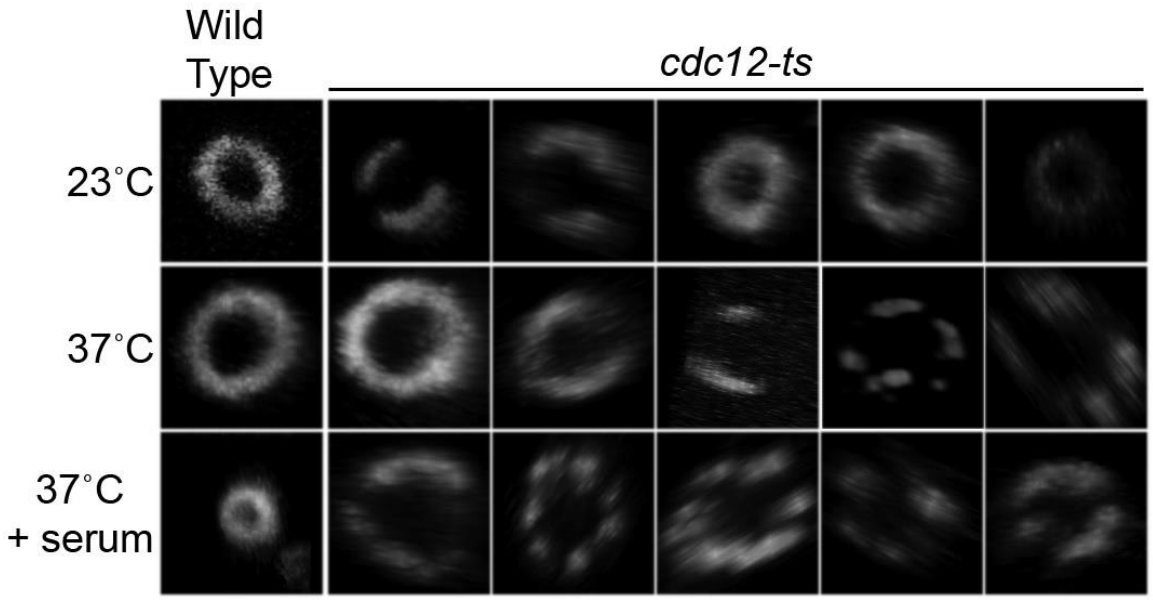


FIG. 4. Defects in septin ring formation by the *cdc12-6* mutant.

Wild type (LLF009) and *cdc12-6* (CZ11) cells engineered to produce Cdc10-GFP were grown at room temperature or shifted to 37˚C for 2 h in YPD medium in the presence or absence of 20% serum as indicated. Cdc10-GFP was analyzed by confocal microscopy to determine the three dimensional shape of the rings.

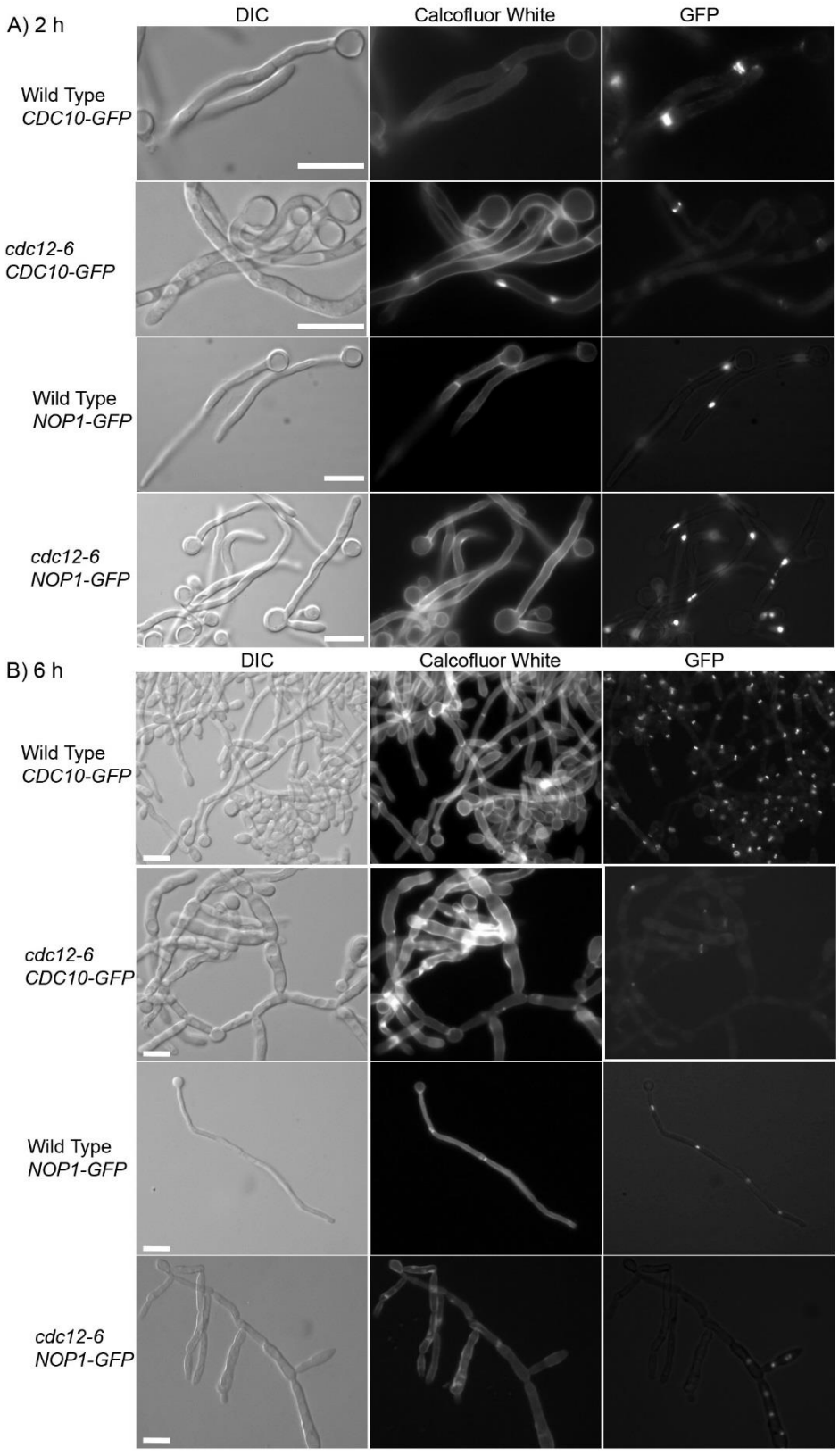
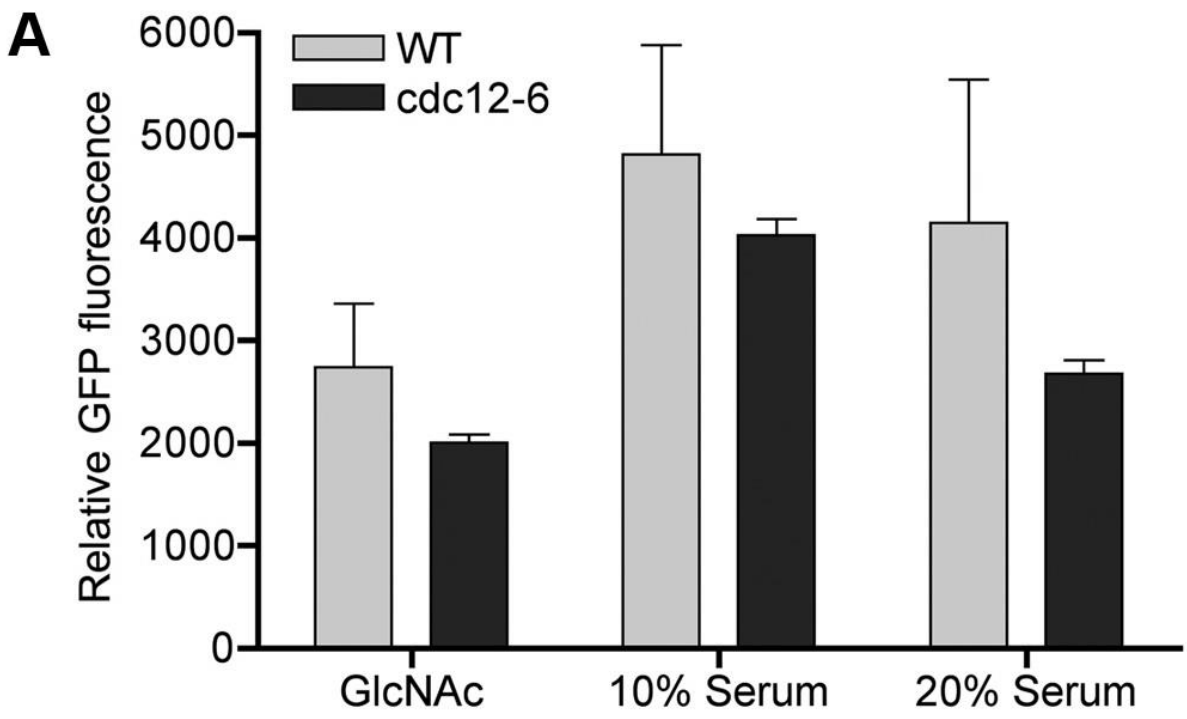

A) 2 h
DIC
Calcofluor White
GFP
Wild Type
CDC10-GFP
cdc12-6
CDC10-GFP
Wild Type
NOP1-GFP
cdc12-6
NOP1-GFP
B) 6 h
DIC
Calcofluor White
GFP
Wild Type
CDC10-GFP
cdc12-6
CDC10-GFP
Wild Type
NOP1-GFP
cdc12-6
NOP1-GFP

FIG. 5. Cell morphology, septin ring and nuclear localization are altered in *cdc12-6* cells under hyphal inducing conditions.

Cells were grown to log phase at room temperature, serum was added to 20% final concentration, and then cultures were shifted to 37˚C for 2h (A) or 6 h (B). As labeled at the top of each column of photos, cells were analyzed by light microscopy to detect morphology (DIC) or fluorescence microscopy to detect Calcofluor White staining of chitin (CW) or the GFP fusion protein (GFP) indicated on the left. Strains used were wild type cells carrying *CDC10-GFP* (LLF009) or *NOP1-GFP* (LLF006) and *cdc12-6* cells carrying *CDC10-GFP* (CZ11) or *NOP1-GFP* (CZ14). Bars, 10 µm.


A
6000
5000
4000
3000
2000
1000
0
Relative GFP fluorescence
WT
cdc12-6
GlcNAc
10% Serum
20% Serum

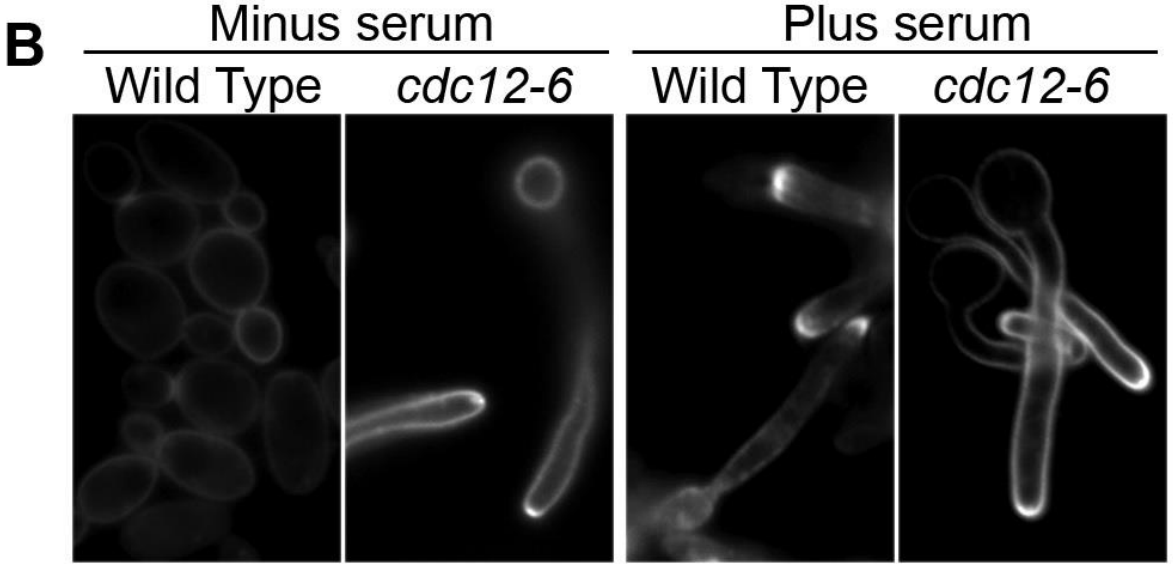

B
Minus serum
Plus serum
Wild Type
cdc12-6
Wild Type
cdc12-6

FIG. 6. Serum induces *cdc12-6* cells to express *HWP1-GFP* and to display increased filipin staining at hyphal tips.

(A) Expression of hyphal reporter gene *HWP1-GFP* in budding and hyphal conditions for wild type (LLF012) or *cdc12-6* cells (LLF010). Cells were grown in the presence or absence of the hyphal inducer serum for 2 h or GlcNAc for 3.5 h. The level of GFP was quantified by fluorescence microscopy. Cells incubated in the absence of serum or GlcNAc did not show GFP levels above the background.

(B) Wild-type (LLF009) and *cdc12-6* (CZ11) cells were incubated with or without serum for 2 h at 37˚C. The cells were then stained with 10 μg/ml filipin and analyzed by fluorescent microscopy.

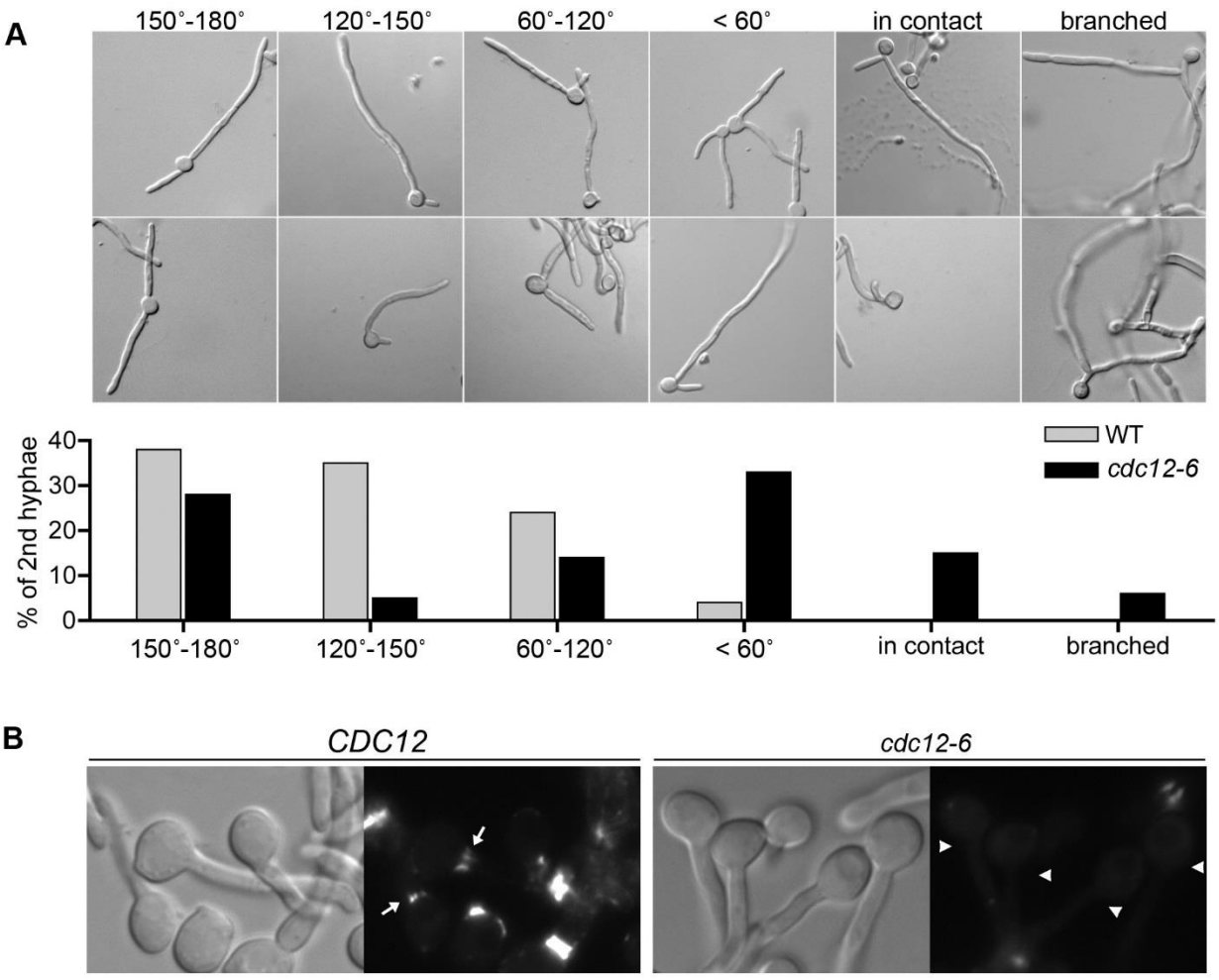

A
150˚-180˚
120˚-150˚
60˚-120˚
< 60˚
in contact
branched
% of 2nd hyphae
40
30
20
10
0
WT
cdc12-6
150˚-180˚
120˚-150˚
60˚-120˚
< 60˚
in contact
branched
B
CDC12
cdc12-6

Fig. 7. Position of the second germ tube is altered in *cdc12-6* cells.

(A) Wild type control (DIC185) and *cdc12-6* (LLF016) were shifted to 37˚C and induced with 20% bovine calf serum for 3 h. Representative images of cells are shown with different positions of second germ tubes. The degree of separation between the first and second germ tubes was quantified as shown in the table below. A total of 200 cells were counted from three independent hyphal induction experiments.

(B) Wild type control (LLF009) or *cdc12-6* (CZ11) cells engineered to produce Cdc10-GFP were induced with 20% serum for 2 h at 37˚C and then analyzed by fluorescence microscopy to detect the basal septin band at the junction between the mother cell and germ tube. Arrows point to the presence of the basal band in the wild type. Arrowheads point to the absence of the basal band in the *cdc12-6* mutant. Note that basal septin band is more diffuse than the tight septin ring seen at sites of septation.

Chapter 4 - Flavodoxin-like proteins protect *Candida albicans* from oxidative stress and promote virulence

Abstract

The fungal pathogen *Candida albicans* causes lethal systemic infections in humans. To better define how pathogens resist oxidative attack by the immune system, we examined a family of four Flavodoxin-Like Proteins (FLPs) in *C. albicans*. In agreement with previous studies showing that FLPs in bacteria and plants act as NAD(P)H quinone oxidoreductases, a *C. albicans* quadruple mutant lacking all four FLPs (*pst1Δ pst2Δ pst3Δ ycp4Δ*) was more sensitive to benzoquinone. Interestingly, the quadruple mutant was also more sensitive to a variety of oxidants. Quinone reductase activity confers important antioxidant effects because resistance to oxidation was restored in the quadruple mutant by expressing either *Escherichia coli wrbA* or mammalian *NQO1*, two distinct types of quinone reductases. FLPs were detected at the plasma membrane in *C. albicans*, and the quadruple mutant was more sensitive to linolenic acid, a polyunsaturated fatty acid that can auto-oxidize and promote lipid peroxidation. These observations suggested that FLPs reduce ubiquinone (coenzyme Q), enabling it to serve as an antioxidant in the membrane. In support of this, a *C. albicans coq3Δ* mutant that fails to synthesize ubiquinone was also highly sensitive to oxidative stress. FLPs are critical for survival in the host, as the quadruple mutant was avirulent in a mouse model of systemic candidiasis under conditions where infection with wild type *C. albicans* was lethal. The quadruple mutant cells initially grew well in kidneys, the major site of *C. albicans* growth in mice, but then declined after the influx of neutrophils and by day 4 post-infection 33% of the mice cleared the infection. Thus, FLPs and ubiquinone are important new antioxidant mechanisms that are

critical for fungal virulence. The potential of FLPs as novel targets for antifungal therapy is further underscored by their absence in mammalian cells.

Introduction

Oxidative stress poses a great threat to cells. Unchecked oxidative damage to DNA, proteins, and lipids causes disruption of physiological processes, harmful mutations, and cell death (137). In order to prevent these destructive effects, cells utilize a variety of mechanisms to protect against oxidation. These antioxidant mechanisms are especially important for pathogens to resist the oxidative attack by the immune system (138). As a result, the human fungal pathogen *Candida albicans* relies on several different mechanisms, such as extracellular, cytoplasmic, and mitochondrial forms of superoxide dismutases to break down superoxide radicals (139-141). Other intracellular mechanisms include catalase to detoxify H_2O_2 and glutathione to promote a reducing environment (60).

Cellular membranes require special protection from oxidation. The plasma membrane is particularly vulnerable because it directly faces oxidative attack by macrophages and neutrophils. Protecting the plasma membrane is critical for survival. In addition to forming a protective barrier around the cell, it functions in a wide range of essential processes including nutrient uptake, ion homeostasis, pH regulation, cell wall synthesis, and morphogenesis. This membrane is also vulnerable because it contains polyunsaturated fatty acids (PUFAs). Approximately 30% of the *C. albicans* fatty acids are polyunsaturated linoleic (18:2) or linolenic (18:3) acids (69,70). PUFAs are very sensitive to peroxidation due to the ease with which the hydrogens can be abstracted from the methylene bridges ($-CH_2-$) that lie in between the double bonds (67,68).

This leaves an unpaired electron on the carbon that can react with O_2 to form a peroxyl radical, which can in turn abstract the hydrogen from another PUFA to continue the cycle. Thus, lipid peroxidation starts a chain reaction that propagates to other lipids. The resulting oxidative damage can also spread to other cellular constituents, including proteins and DNA.

Several lines of evidence suggested that a family of four uncharacterized Flavodoxin-Like Proteins (FLPs) present in *C. albicans* could play a novel antioxidant role at the plasma membrane. The FLPs are encoded by *PST1*, *PST2*, *PST3*, and *YCP4*. These genes are induced by oxidative stress (86), and contain binding sites in their promoter regions for Cap1, a transcription factor that induces genes needed to combat oxidative stress (77). The *S. cerevisiae* FLPs (Pst2, Rfs1, Ycp4) have been suggested to be involved in resistance to oxidative stress (87-89), although their physiological role is not known (90). It is also interesting that the *C. albicans* FLPs are likely to act at the plasma membrane, since their orthologs in *S. cerevisiae* are associated with the plasma membrane (142).

The FLPs are highly conserved in bacteria, plants, and fungi, but surprisingly not in mammalian cells (84). Biochemically, the most well studied FLP is the *E. coli* WrbA protein. It uses flavin mononucleotide (FMN) as a cofactor and acts as a NAD(P)H quinone oxidoreductase (85,143,144). FLPs from fungi, plants and other bacteria have also been shown to act as NAD(P)H quinone oxidoreductases, indicating that this is a conserved feature of this family (145-149). A special feature of FLPs is that they carry out a two-electron reduction of a quinone to quinol (see structures in Fig. 1A). This converts both carbonyl groups on the benzoquinone

ring to hydroxyl groups. In contrast, other pathways that promote a one-electron reduction of quinone form a semiquinone intermediate that is a hazardous reactive oxygen species (67,68). Although the physiological role of WrbA is not known, there is suggestive evidence that it promotes resistance to oxidative stress (84,143,149).

Quinone reductases could promote resistance to oxidative stress in several ways. One is that they can reduce and detoxify small molecule quinones that are produced by some organisms for defense or created as benzene metabolites (150,151). In addition, they could act on endogenously produced quinones, such as ubiquinone (coenzyme Q), an isoprenylated benzoquinone. Ubiquinone is well known for its role in the mitochondrial electron transport chain, but it is also present in other cellular membranes, where it can undergo redox cycling to act as an antioxidant (80,81,152-154). Mammalian cells use the enzyme Nqo1 (NAD(P)H quinone oxidoreductase), formerly known as DT-diaphorase, to safely carry out a two-electron reduction of ubiquinone and avoid semiquinone formation (82,83). Nqo1 is analogous to FLPs in that it uses NAD(P)H for reducing potential, but it differs in amino acid sequence and in containing FAD as a cofactor (84). However, it is not known how fungal cells, including *C. albicans*, carry out this function since they lack an obvious ortholog of *NQO1*. Therefore, in this study we examined a quadruple mutant lacking all four FLP genes (*PST1*, *PST2*, *PST3* and *YCP4*). The results demonstrate that these proteins represent a new mechanism for protecting *C. albicans* against oxidative stress that is required for virulence in a mouse model of systemic candidiasis.

Results

C. albicans mutant lacking all four FLP genes is more sensitive to oxidation

Four FLPs were identified in *C. albicans* based on their high sequence identity (45-50%) and similarity (~65%) to the well studied *E. coli* WrbA. This type of enzyme is advantageous because it uses NAD(P)H to carry out a two-electron reduction of toxic quinones that avoids creation of the semiquinone radical (Fig. 1A) (143). The conserved residues are concentrated in the active site near the location of the FMN co-factor. The four *C. albicans* FLPs share a similar structure, although Ycp4 contains C-terminal extension of about 90 amino acids that ends in a CAAX box, indicating it is likely to be lipid modified. To examine their role in the diploid *C. albicans*, a quadruple mutant strain was constructed that lacks both copies of all four FLP genes. Fortuitously, *PST3* and *YCP4* are adjacent in the genome and were deleted simultaneously using the *HIS1* and *LEU2* selectable markers. Subsequent deletion of the *PST1* and *PST2* genes was carried out by successive use of the SAT Flipper that employs a recyclable *SAT1* selectable marker (98). For brevity, this *pst1Δ pst2Δ pst3Δ ycp4Δ* strain will be referred to as the Δ/Δ/Δ/Δ mutant. The sensitivity of this strain to quinones was tested by spotting dilutions of cells onto agar medium containing p-benzoquinone (BZQ) or menadione (MND), a heterocyclic napthoquinone (Fig. 1B). The growth of the Δ/Δ/Δ/Δ strain was clearly inhibited by these small molecule quinones, indicating it is more sensitive to quinones than either the wild type control or a complemented strain in which one copy of each of the FLP genes was reintroduced.

FLPs in bacteria and plants have also been suggested to have a role in fighting oxidative stress, but their physiological role is not known (84,143,147,149). Therefore, given the importance of antioxidant enzymes for microbial pathogens, we spotted the cells on medium

containing H_2O_2 and found that the Δ/Δ/Δ/Δ mutant was more sensitive to this oxidant (Fig. 1B). Since the FLPs are associated with the plasma membrane in *S. cerevisiae* (142), we further tested two other peroxides that are more hydrophobic. Interestingly, the Δ/Δ/Δ/Δ mutant was also very sensitive to tert-butyl hydroperoxide (TBHP) and cumene hydroperoxide (CHP), which are more hydrophobic than H_2O_2 and more likely to preferentially oxidize membranes.

The Δ/Δ/Δ/Δ mutant was next assayed for sensitivity to polyunsaturated fatty acids (PUFAs), which can auto-oxidize and initiate a chain reaction of lipid peroxidation (68,81). PUFAs are more readily oxidized because the presence of double bonds flanking a methylene group (-CH_2-) weakens the methylene C-H bond, making it much easier to abstract a hydrogen (67). This leaves a carbon with an unpaired electron that readily reacts with oxygen to form a peroxyl radical (LOO•). For example, linolenic acid, which has three unsaturated double bonds, is much more likely to auto-oxidize to form a peroxyl radical than is monounsaturated oleic acid. The peroxyl radical can then abstract a hydrogen from another PUFA to form a lipid peroxide (LOOH) and a new lipid radical that can further extend a chain reaction of lipid peroxidation (67,68). Linolenic acid was also used for this analysis because previous studies showed that it efficiently induced lipid peroxidation and cell death in *S. cerevisiae* (81). Interestingly, growth of the Δ/Δ/Δ/Δ mutant was strongly inhibited by the polyunsaturated linolenic acid (LNA; Fig. 1B). In contrast, the Δ/Δ/Δ/Δ mutant grew as well as the control cells in the presence of the monounsaturated oleic acid (OA). Taken together, these results indicate that the FLPs are needed for *C. albicans* to combat a variety of oxidative stresses.

FLPs are needed to prevent lipid peroxidation

The effects of linolenic acid on *C. albicans* were analyzed further in quantitative assays. A time course of cell death was assayed by incubating cells for different times in the presence of 0.5 mM linolenic acid followed by plating dilutions on agar medium to determine the viable colony forming units (CFUs). The results confirmed the spotting assays. The Δ/Δ/Δ/Δ mutant showed a significant trend toward decreased viability by 6-8 h that was not observed for the wild-type control or complemented strains (Fig. 2A). Analysis of the dose-response to incubation with linolenic acid for 6 h revealed a loss of viability starting at 0.25 mM that became more significant at 0.5 and 1.0 mM (Fig. 2C). In contrast, the cells remained viable after incubation in the monounsaturated oleic acid (data not shown).

To determine whether linolenic acid caused an increase in lipid peroxidation, cells were assayed for thiobarbituric acid reactive substances (TBARS) (81,155). This assay detects malondialdehyde (MDA), a common byproduct lipid peroxidation. As expected, both the Δ/Δ/Δ/Δ mutant and the control cells showed elevated TBARS after incubation for different times with linolenic acid (Fig. 2B). However, the Δ/Δ/Δ/Δ mutant showed a significantly higher level of TBARS than the control cells at 4 and 6 h. By 8 h, the results of the TBARS assays were quite variable. This may have been due to difficulties in dealing with the high fraction of dead cells during the analysis. Dose-response assays showed that the TBARS in the Δ/Δ/Δ/Δ mutant started trending upward at 0.25 mM and was significantly higher than control cells at 0.5 mM and 1.0 mM linolenic acid (Fig. 2D). These results demonstrate that linolenic acid stimulated higher levels of lipid peroxidation in the Δ/Δ/Δ/Δ mutant.

For comparison, mutants lacking a single FLP gene (*pst1Δ, pst2Δ, pst3Δ or ycp4Δ*), two genes (*pst3Δ ycp4Δ*), or three genes (*pst2Δ, pst3Δ ycp4Δ*) were also tested for their sensitivity to 0.5 mM linolenic acid (Fig. 2E,F). However, no significant changes in either CFU or lipid peroxidation level were detected compared to the wild type control. As will be described further below, this is consistent with redundancy of the different FLP genes in *C. albicans*.

To gain additional evidence that the effects of linolenic acid were due to oxidation, cells were incubated with α-tocopherol (vitamin E), a hydrophobic reducing agent that localizes to membranes and has been shown to prevent lipid peroxidation in other organisms (67,81). Treatment of cells with α-tocopherol alone had no detectable effects on CFUs or lipid peroxidation. In contrast, the addition of α-tocopherol significantly decreased the killing activity of linolenic acid in both WT and the Δ/Δ/Δ/Δ mutant (Fig 3A). Similarly, α-tocopherol reduced the levels of lipid peroxidation to below the limit of detection, as determined by the TBARS assay (Fig. 3B).

Heterologous expression of known NAD(P)H quinone oxidoreductases rescues the phenotypes of the Δ/Δ/Δ/Δ

To confirm whether quinone reductase activity is important to promote resistance to oxidative stress in *C. albicans*, the Δ/Δ/Δ/Δ mutant was engineered to express two distinct types of NAD(P)H quinone oxidoreductases: rat *NQO1* and *E. coli wrbA*. *NQO1* and *wrbA* were selected because their proteins have been well-studied biochemically (143,156,157). These genes were expressed under the control of the strong *ADH1* promoter. As a control, cells were also engineered to express GFP in a similar manner. Incubation of the cells in the presence of 0.5 mM linolenic acid for 6 h showed that expression of either *wrbA* or *NQO1* rescued the

viability of the Δ/Δ/Δ/Δ mutant (Fig. 4A). In contrast, the Δ/Δ/Δ/Δ mutant or the Δ/Δ/Δ/Δ mutant that expressed only GFP showed a significant drop in CFUs. Similarly, expression of *wrbA* or *NQO1*, but not GFP, diminished lipid peroxidation in cells that were exposed to linolenic acid (Fig. 4B). Growth assays on agar plates also showed that *wrbA* and *NQO1* could complement the increased sensitivity of the Δ/Δ/Δ/Δ mutant to H_2O_2, tert-butyl hydroperoxide, cumene hydroperoxide and menadione (Fig. 4C). The ability of two distinct quinone reductases to complement the Δ/Δ/Δ/Δ mutant phenotype demonstrates that this activity plays a key antioxidant role in *C. albicans*.

Functional differences between NAD(P)H quinone oxidoreductase homologues

The properties of the different quinone reductase homologues were examined by expressing individual genes in the Δ/Δ/Δ/Δ mutant. The *C. albicans* genes were reintroduced under control of their native promoters, whereas *wrbA* and *NQO1* were controlled by the *ADH1* promoter. Growth assays were performed to test the ability of cells carrying only one quinone reductase gene to resist different quinones and oxidants. All of the different quinone reductases were able to promote resistance to H_2O_2, tert-butyl hydroperoxide, and linolenic acid (Fig. 5A). However, some of the strains had differential ability to resist cumene hydroperoxide and the small molecule quinones: p-benzoquinone and menadione (Fig. 5A and summarized in Fig. 5B).

The strain expressing only *PST3* was very interesting in that it showed the strongest resistance to p-benzoquinone and menadione (Fig. 5A). Although several strains displayed obvious resistance to 75 µM p-benzoquinone, only the *PST3*-expressing strain was resistant to 100 µM p-benzoquinone. It grew remarkably better than the other strains, and nearly as well as

the complemented strain that carries one copy of each FLP gene. Similarly, it also grew better than the other strains on medium containing menadione. In contrast, the *PST3*-expressing strain did not show significant resistance to cumene hydroperoxide or linolenic acid, which are considered to be good inducers of lipid peroxidation. This strain was, however, more resistant than the Δ/Δ/Δ/Δ mutant to H_2O_2 and tert-butyl hydroperoxide, indicating that it can provide protection against some oxidants. Thus, it appears that Pst3 can preferentially act on small molecule quinones. In agreement with this, a *pst3Δ* strain was sensitive to the inhibitory effects of p-benzoquinone and menadione, whereas the *pst1Δ, pst2Δ* and *ycp4Δ* mutants were not (Fig. 5C).

Some of the other strains expressing a single quinone reductase showed the opposite phenotype of being more resistant to oxidants than to the small molecule quinones. For example, the strains expressing *PST2*, *YCP4*, *wrbA*, or *NQO1* all showed improved resistance to cumene hydroperoxide and linolenic acid compared to the Δ/Δ/Δ/Δ mutant, but were not significantly more resistant to the small molecule quinones under the conditions tested (Fig. 5A). The different phenotypes indicate that there are functional differences between the various quinone reductases.

Ubiquinone promotes resistance to oxidative stress, but not small molecule quinones

The major quinone found in cells, ubiquinone (coenzyme Q), is known to have two key functions. It plays a central role in the mitochondrial electron transport chain, and it is also present in other cellular membranes where it can function as an antioxidant (80,81,152-154). To investigate the relationship between ubiquinone and oxidative stress, both copies of *COQ3* were

deleted from *C. albicans* to prevent ubiquinone synthesis. As expected, a *C. albicans coq3Δ* mutant was not able to grow on glycerol, a carbon source that requires respiration to be utilized (Fig 6A). In contrast, the Δ/Δ/Δ/Δ mutant readily grew on glycerol and other non-fermentable nutrients (Fig. 6A and data not shown). Interestingly, the *coq3Δ* mutant was very sensitive to H_2O_2, even more so than the Δ/Δ/Δ/Δ mutant (Fig. 6A). Spot assays also showed that the *coq3Δ* mutant was more sensitive to linolenic acid than the Δ/Δ/Δ/Δ mutant. For comparison, two previously constructed mitochondrial mutants were examined that lack components of Complex I of the electron transport chain (158). Both *orf19.2570Δ* and *orf19.6607Δ* failed to grow on glycerol medium, as expected (Fig. 6A). However, they were not more sensitive to linolenic acid and showed perhaps only a minor increase in sensitivity to H_2O_2. This indicates that a mitochondrial defect does not account for the increased sensitivity to oxidation of the *coq3Δ* mutant, consistent with ubiquinone playing a major role as an antioxidant.

Analysis of cell viability after incubation with 0.5 mM linolenic acid for 6 h revealed a larger drop in CFUs for the *coq3Δ* mutant than for the Δ/Δ/Δ/Δ mutant (Fig. 6B). The *coq3Δ* mutant also displayed significantly higher levels of TBARS under these conditions (Fig. 6B). Similar results have been observed in *S. cerevisia*e, as a *coq3Δ* mutant in this yeast is also sensitive to oxidation and lipid peroxidation (81). These results demonstrate that ubiquinone plays an important role as an antioxidant to prevent lipid peroxidation and oxidative stress in *C. albicans*.

It is noteworthy that the *coq3Δ* mutant was not significantly more sensitive to p-benzoquinone and menadione, even though it was very sensitive to H_2O_2 and linolenic acid (Fig.

6A). This suggests that the FLPs in *C. albicans* can detoxify these small molecule quinones in the absence of ubiquinone, thereby prevent them from causing oxidative damage.

FLPs localize to the plasma membrane in *C. albicans*

The C termini of the *C. albicans* FLPs were fused to GFP to examine their subcellular localization. Unfortunately, Western blot analysis indicated that all four of the fusion proteins were subject to proteolytic cleavage, as a strong signal was detected at the size of free GFP and little or no full-length fusion protein was detected (not shown). In spite of this, Pst1-GFP and Pst3-GFP were detected at the plasma membrane by fluorescence microscopy (Fig. 7A). To improve detection, GFP was also fused to the N-termini of Pst2 and Ycp4, and the corresponding genes were introduced into *C. albicans* under control of the *ADH1* promoter. The GFP-Pst2 and GFP-Ycp4 fusion proteins showed obvious plasma membrane localization (Fig. 7B). The GFP-tagged FLPs showed a slightly patchy distribution in the plasma membrane, suggesting that they localize in part to the eisosome subdomains, as do their *S. cerevisiae* orthologs (142,159).

The FLP genes promote *C. albicans* virulence in mice

The role of the FLPs in virulence was examined using a mouse model of hematogenously disseminated candidiasis (160). After injection via the tail vein with 2.5×10^5 *C. albicans* cells, BALB/c mice infected with the wild type control strain succumbed to infection with a median time of 8 days (Fig. 8A). Similar results were observed for the complemented version of the Δ/Δ/Δ/Δ strain. In contrast, all mice infected with the Δ/Δ/Δ/Δ mutant survived to the end of the

experiment (Day 28). No CFUs were detected in the kidneys from these mice, indicating that they had cleared the infection (Fig. 8B).

To determine whether the Δ/Δ/Δ/Δ mutant failed to initiate an infection, or if it was cleared more rapidly, kidneys were examined at early times post infection. The kidney is a sensitive organ to test the ability of *C. albicans* to initiate an infection, since this fungus grows rapidly in the kidneys during the first two days after infection (105,161). At day 2 post infection, the wild type and Δ/Δ/Δ/Δ mutant both grew well, as indicated by similar high levels of CFU/g kidney (Fig. 8B). However, by the 4th day post infection, the median CFU/g kidney was 100-fold lower for mice infected with the Δ/Δ/Δ/Δ mutant than the wild type. Furthermore, 33% of the mice (3/9) had no detectable CFU/g kidney at day 4, indicating that they had cleared the infection. Thus, the FLPs are required for the persistence of *C. albicans* systemic infection.

Discussion

Cells utilize a variety of pathways to protect against oxidation (60,137,139,141). Cytoplasmic mechanisms include superoxide dismutase, catalase, thioredoxin, and glutathione. In addition, pathogens have also evolved extracellular mechanisms. For example, *C. albicans* produces three superoxide dismutases that are GPI-anchored and therefore on the cell surface or built into the cell wall (Sod4-6) (60,141). One of these, Sod5, was recently shown to have unique properties in that it uses copper as a co-factor, but not zinc (162). This appears to be designed to take advantage of the fact that copper is pumped into phagosomes but zinc is restricted as part of the antimicrobial attack by leukocytes. However, it is not as well understood how cellular membranes are protected from oxidation, particularly the fungal plasma membrane that is directly exposed to the oxidative attack by neutrophils and macrophages (138).

To better understand how the plasma membrane is protected against oxidation we examined four FLPs in *C. albicans* that are associated with the plasma membrane (Fig. 7). In agreement with their predicted role as NAD(P)H quinone oxidoreductases, a *C. albicans* Δ/Δ/Δ/Δ quadruple mutant lacking all four FLP genes (*PST1*, *PST2*, *PST3*, and *YCP4*) displayed increased sensitivity to p-benzoquinone and menadione, a napthoquinone (Fig. 1). Interestingly, the mutant cells were also more sensitive to a wide range of oxidants, indicating that they have a broader antioxidant function.

Consistent with the membrane localization of the FLPs, the Δ/Δ/Δ/Δ mutant was very sensitive to hydrophobic oxidants, including linolenic acid (Figs. 1 and 5). The increased

sensitivity to linolenic acid was particularly significant, since previous studies demonstrated that this PUFA auto-oxidizes and initiates a chain reaction of lipid peroxidation (81). In agreement with this, the Δ/Δ/Δ/Δ mutant showed elevated levels of TBARS (Fig. 2), a hallmark of lipid peroxidation (67,68). Furthermore, the effects of linolenic acid could be reversed by the hydrophobic antioxidant α-tocopherol (Vitamin E) (Fig. 3). Lipid peroxidation is likely to be a more serious problem for *C. albicans* than for *S. cerevisiae*, which lacks significant levels of PUFAs (81). About 30% of the fatty acids in *C. albicans* are polyunsaturated (69,70), which predisposes them to forming lipid peroxides (67,68). These PUFAs are typically found in more complex lipids, such as phospholipids, in addition to existing as free fatty acids. Taken together, the results identify FLPs as an important new set of antioxidant enzymes in *C. albicans*. These results also have broad significance for other pathogens, given that FLPs are induced by oxidative stress in diverse fungi (86,87,163-165) and there is suggestive evidence that they play an antioxidant role in bacteria (84,90,143,149).

NAD(P)H quinone oxidoreductases are critical for resisting oxidative stress

Biochemical studies of FLPs from bacteria, fungi, and plants have shown that they use NAD(P)H to reduce quinones in a manner that avoids creation of hazardous semiquinone intermediates (145-149). The Δ/Δ/Δ/Δ mutant was rescued by expression of *E. coli wrbA* (Fig. 4), confirming that NAD(P)H quinone oxidoreductase activity plays an important antioxidant role in *C. albicans*. Furthermore, heterologous expression of mammalian *NQO1* in the Δ/Δ/Δ/Δ mutant also rescued its sensitivity to oxidation and lipid peroxidation. Nqo1 does not share obvious sequence similarity with FLPs even though it carries out a similar enzymatic activity.

Although there are some underlying structural similarities between Nqo1 and FLPs, they are quite distinct (84). For example, Nqo1 binds FAD as a cofactor instead of FMN, and it forms dimers rather than tetramers as seen for wrbA. These observations provide strong support that the key function of the *C. albicans* FLPs is to act as quinone reductases.

Analysis of Δ/Δ/Δ/Δ cells engineered to express a single FLP gene indicated that they have overlapping but distinct functions. Pst3 provided the best protection against the small molecule quinones p-benzoquinone and menadione (Fig. 5). In agreement with this, a *pst3Δ* mutant was the only single FLP deletion mutant that more sensitive to the small molecule quinones (p-benzoquinone and menadione) (Fig. 5). In contrast, cells expressing only *PST3* were less able to resist other oxidants, such as linolenic acid or cumene hydroperoxide. These differences cannot simply be attributed to variation in the level of expression, and instead are consistent with different functional properties.

Ubiquinol is an important antioxidant in *C. albicans*

The most likely target for the quinone reductase activity of FLPs in *C. albicans* is ubiquinone (coenzyme Q). Ubiquinone has a benzoquinone head group and a hydrophobic isoprenylated tail that localizes it to membranes (153,166). Analogous to its well-known role as an electron carrier in the mitochondria, ubiquinone is present in other cellular membranes where its reduced form (ubiquinol) can act as an antioxidant (80,81,152-154). In particular, ubiquinol is thought to be able to reduce lipid radicals, which would otherwise propagate a chain reaction of lipid peroxidation to cause more extensive damage (67,68). To determine if ubiquinol plays

an important antioxidant role in *C. albicans*, *COQ3* was deleted to block its synthesis. The *coq3Δ* mutant was found to be very sensitive to oxidative stress and also displayed increased levels of lipid peroxidation in response to linolenic acid (Fig. 6). In further support of the conclusion that FLPs act on ubiquinone, rat *NQO1*, which is known to reduce ubiquinone (82,83), can rescue the defects of the Δ/Δ/Δ/Δ mutant (Figs. 4 and 5).

There were interesting differences between the Δ/Δ/Δ/Δ mutant and the *coq3Δ* mutant that reveal insights into their roles. Whereas the *coq3Δ* mutant was highly sensitive to oxidizing conditions promoted by peroxides or PUFAs, it was not significantly altered in sensitivity to p-benzoquinone and menadione (Fig. 6). This indicates that the FLPs can reduce quinones in the absence of ubiquinol. The *coq3Δ* mutant was also much more sensitive than the Δ/Δ/Δ/Δ mutant to H_2O_2 and linolenic acid. One possibility is that other reductases can contribute to reduction of ubiquinone in the absence of the FLPs. However, if these enzymes use a one-electron mechanism for reduction of quinones, they will generate deleterious semiquinone radicals that would contribute to the phenotype of the Δ/Δ/Δ/Δ mutant.

FLPs are required for virulence and represent novel drug targets

The FLPs were required for virulence in a mouse model of hematogenously disseminated candidiasis (Fig. 8A). Whereas the median survival time was 8 days for mice injected with 2.5 x 10^5 wild type *C. albicans*, all of the mice infected with the Δ/Δ/Δ/Δ mutant survived to the end of the experiment (day 28). Thus, the Δ/Δ/Δ/Δ mutant appears to have a stronger virulence defect

than was reported for other *C. albicans* oxidation sensitive mutants including a *cat1Δ* catalase mutant (167), a *sod1Δ* or *sod5Δ* superoxide dismutase mutant (168,169), a *grx2Δ* glutathione reductase mutant (168), or a *tsa1Δ* thioredoxin peroxidase mutant (170).

Interestingly, the Δ/Δ/Δ/Δ mutant could initially grow in the kidney essentially as well as a wild type strain (Fig. 8B). However, by day 4 there was about a 100-fold decrease in median CFUs and 3 out of 9 mice cleared the infection. This decline in CFUs for the Δ/Δ/Δ/Δ mutant correlates with the expected influx of neutrophils that peaks about day 2 of infection (105,161). By day 28, all of the mice infected with the Δ/Δ/Δ/Δ mutant lacked detectable CFU and appear to have cleared the infection. Generally similar results were reported for a *C. albicans cat1Δ* catalase mutant that also grew well initially but then CFUs declined in most infected mice (167). In this regard it is also significant that a *wrbAΔ* mutant of the bacterial pathogen *Yersinia tuberculosis* can initiate an infection but is defective in establishing a persistent infection (171).

This key role in virulence for the FLPs indicates they have strong potential to serve as novel targets for antifungal therapy. New therapeutic approaches are needed; ~40% of patients with systemic candidiasis succumb to the infection even with current antifungal therapy (172,173). This outcome is likely to worsen, as drug resistance is a growing problem for two of the three most commonly used antifungal drugs (174,175). An important advantage of targeting FLPs is that they do not have orthologs in humans. The analogous NAD(P)H quinone oxidoreductases in mammals, Nqo1 and Nqo2, are very different (84).

Pharmacological studies on Nqo1 have identified multiple ways that quinone reductases can be targeted. One approach is to identify inhibitors, such as dicoumarol that blocks the Nqo1 activity (176). In addition, the ability of Nqo1 to reduce small molecule quinones has been studied as a basis for cancer chemotherapy. The fact that many cancer cells overexpress *NQO1* has been exploited to develop novel therapies in which quinone compounds are reduced by Nqo1 to convert them into a toxic form that preferentially kills cancer cells (83,177). Similarly, Nqo1 has also been shown to reduce benzoquinone-containing ansamycin drugs, which makes these compounds more potent inhibitors of the Hsp90 chaperone (178). This suggests yet another way drugs targeting FLPs could be useful, since Hsp90 inhibitors can prevent the emergence of drug resistance in *C. albicans* (179). Thus, the important roles of FLPs in oxidative stress response and virulence, combined with their absence in mammalian cells, identifies them as important new targets for therapeutic strategies aimed at combating fungal and bacterial pathogens.

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Figures

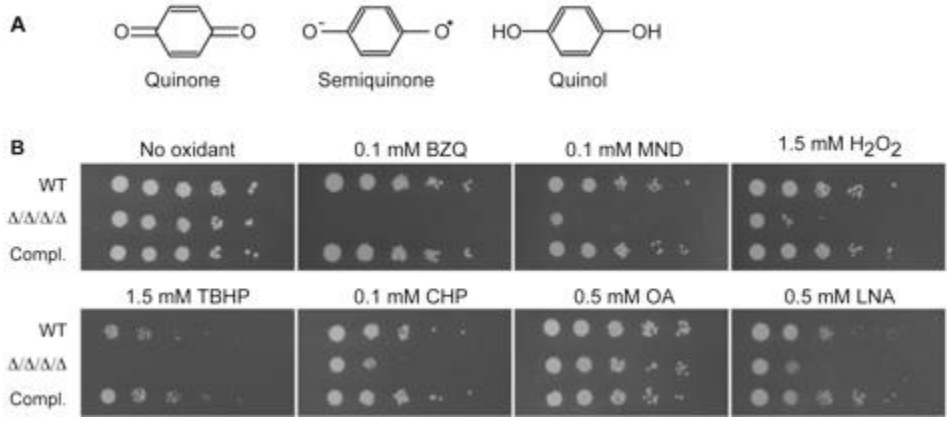

Fig. 1. *C. albicans* FLP genes are needed for resistance to oxidative stress.

Serial 10-fold dilutions of cells were spotted onto synthetic medium agar plates containing the indicated chemical and then incubated at 37°C for 48 h. Quinones tested included BZQ (p-benzoquinone) and MND (menadione). Oxidants included H_2O_2, as well as more hydrophobic peroxides that target the membrane: TBHP (tert-butyl hydroperoxide) and CHP (cumene hydroperoxide). In addition, monounsaturated OA (oleic acid) was used as a control for the polyunsaturated LNA (linolenic acid), which is known to induce lipid peroxidation. Strains used were the wild type strain LLF100, Δ/Δ/Δ/Δ strain LLF060 (*pst1*Δ *pst2*Δ *pst3*Δ *ycp4*Δ), and the complemented strain LLF079 in which one copy of each FLP gene was introduced into the Δ/Δ/Δ/Δ strain (*pst1*Δ *pst2*Δ *pst3*Δ *ycp4*Δ *PST1 PST2 PST3 YCP4*).

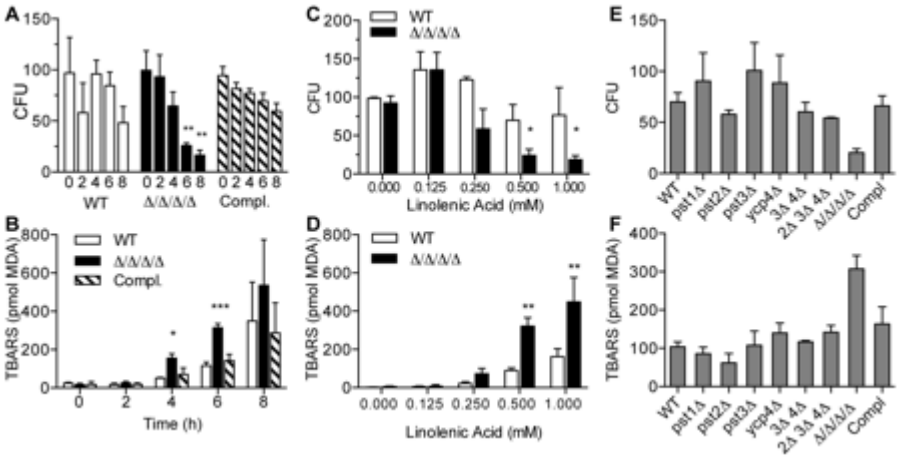

Fig. 2. The Δ/Δ/Δ/Δ mutant strain is more sensitive to linolenic acid-induced cell death and lipid peroxidation.

(A) *C. albicans* strains were incubated with 0.5 mM linolenic acid (LNA) at 37˚C for the indicated time, and then dilutions of cells were plated to determine the viable colony forming units (CFU). (B) Cells were incubated with 0.5 LNA for the different times and then thiobarbituric acid reactive substance (TBARS) assays were carried out to detect malondialdehyde (MDA), a byproduct of lipid peroxidation.

(C) Cells were exposed to different concentrations of LNA for 6 h, and then CFUs were determined.

(D) TBARS assays to detect lipid peroxidation in cells treated with different concentrations of LNA for 6 h.

(E) CFU analysis and (F) TBARS assays of the indicated FLP mutant strains. Note that in contrast to the Δ/Δ/Δ/Δ quadruple mutant strain LLF060, the single, double and triple FLP deletion mutant strains did not display increased sensitivity to LNA. Error bars indicate SE. *= $p<0.05$, **=$p<0.01$, ***=$p< 0.001$ by ANOVA. Strains used included the wild type strain LLF100, Δ/Δ/Δ/Δ strain LLF060, and the complemented strain LLF079 in which one copy of each FLP gene was introduced into the Δ/Δ/Δ/Δ strain.

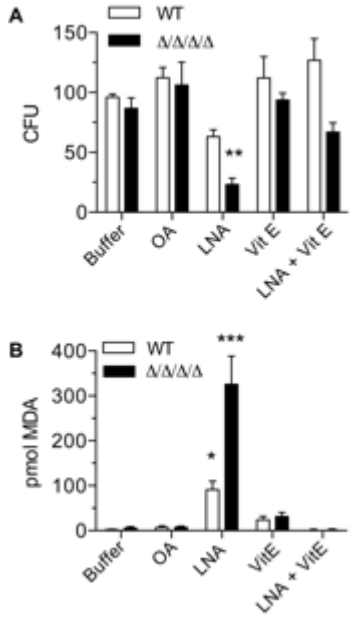

Fig. 3. Antioxidant Vitamin E blocks the effects of linolenic acid.

Cells were suspended in phosphate buffer (0.1M Na_2HPO_4, 0.2% dextrose) containing 0.5 mM oleic acid (OA), 0.5 mM linolenic acid (LNA), 0.5 mM vitamin E (α-tocopherol; Vit E), or 0.5mM LNA + 0.5 mM Vit E. After incubation at 37˚C for 6 h,

(A) cells were plated to determine CFU or

(B) they were assayed for TBARS. The wild type strain was LLF100, the Δ/Δ/Δ/Δ strain LLF060, and the complemented strain was LLF079 in which one copy of each FLP gene was introduced into the Δ/Δ/Δ/Δ strain. Error bars indicate SE. *= $p<0.05$, **=$p<0.01$, ***=$p<0.001$ by ANOVA.

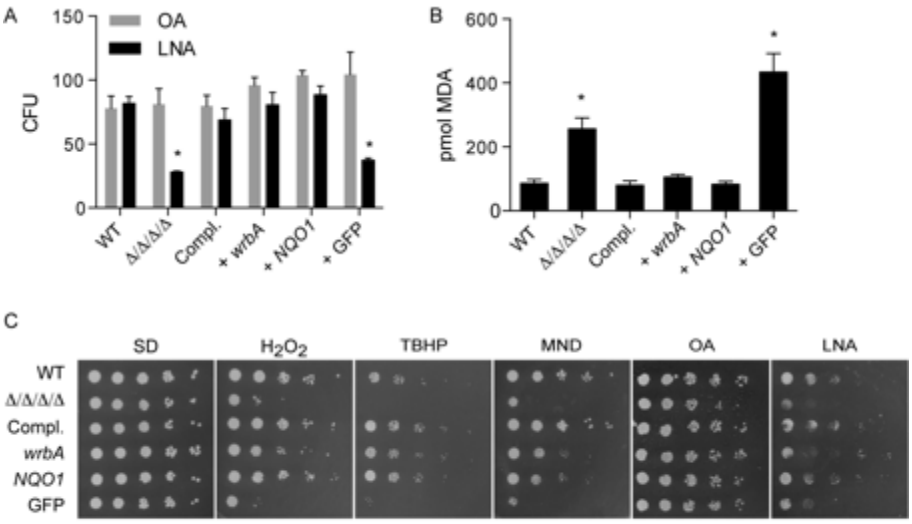

Fig.4. Heterologous expression of *E. coli wrbA* or rat *NQO1* rescues the sensitivity of Δ/Δ/Δ/Δ mutant to oxidants.

The distinct NAD(P)H quinone oxidoreductase genes *wrbA* and *NQO1* were expressed in the Δ/Δ/Δ/Δ strain under control of the *ADH1* promoter. A control strain was also constructed in a similar manner that expressed GFP. The cells were then incubated with 0.5 mM linolenic acid (LNA) for 6 h at 37˚C and then

(A) assayed for viable CFUs, or

(B) assayed for TBARS as an indicator of lipid peroxidation.

(C) Dilutions of cells were spotted onto different agar plates containing synthetic medium and the indicated oxidant, and then incubated at 37˚C for 2 d. The plates contained H_2O_2, tert-butyl hydroperoxide (TBHP), cumene hydroperoxide (CHP), menadione (MND), monounsaturated oleic acid (OA), and polyunsaturated linolenic acid (LNA). Strains used included the wild type control strain LLF100, Δ/Δ/Δ/Δ strain LLF060, the complemented strain LLF079, and the Δ/Δ/Δ/Δ strain in which *E. coli wrbA* (LLF074), rat *NQO1* (LLF076) or GFP (LLF080) was expressed under control of the constitutive *ADH1* promoter. Error bars indicate SE. *, $p < 0.05$ by ANOVA.

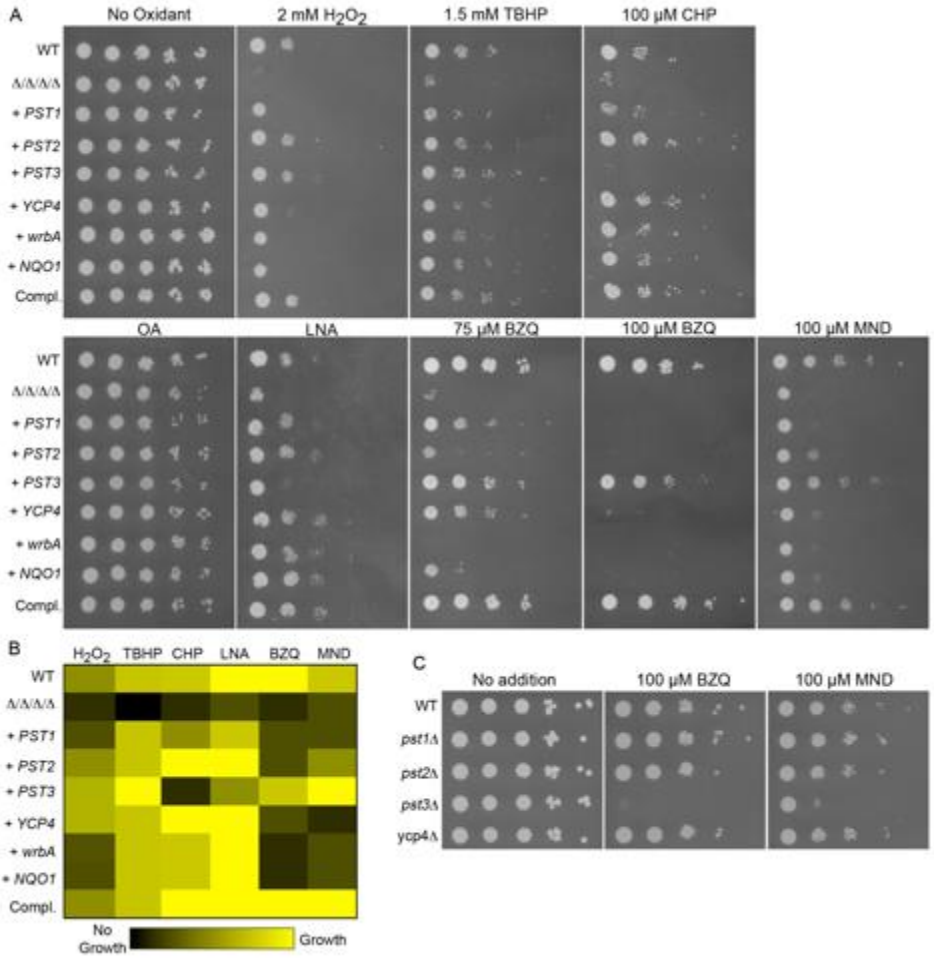
A
No Oxidant
2 mM H2O2
1.5 mM TBHP
100 µM CHP
WT
Δ/Δ/Δ/Δ
+ PST1
+ PST2
+ PST3
+ YCP4
+ wrbA
+ NQO1
Compl.
OA
LNA
75 µM BZQ
100 µM BZQ
100 µM MND
B
H2O2 TBHP CHP LNA BZQ MND
No Growth
Growth
C
No addition
100 µM BZQ
100 µM MND
pst1Δ
pst2Δ
pst3Δ
ycp4Δ

Fig. 5. Differential sensitivity to oxidants for *C. albicans* strains expressing a single FLP gene or the analogous *NQO1* gene.

Strains expressing individual *C. albicans* FLP genes under control of their native promoter, or the *E. coli wrbA* or rat *NQO1* gene under control of the *ADH1* promoter were created by introducing the corresponding genes into the Δ/Δ/Δ/Δ mutant strain LLF054.

(A) Serial 10-fold dilutions of cells were spotted onto agar plates containing synthetic medium and the indicated chemical. Plates were incubated at 37°C for 48 h and then photographed.

(B) Summary of mutant phenotypes shown in panel A. A brighter yellow color indicates better cell growth of under the specified condition.

(C) Deletion mutant strains lacking a single FLP gene were spotted onto medium containing the indicated quinones. Note that the *pst3Δ* strain was more sensitive.

Strains used included the wild type control strain LLF100, Δ/Δ/Δ/Δ strain LLF060, and the complemented strain LLF079. Also, the Δ/Δ/Δ/Δ strain engineered to express *E. coli wrbA* (strain LLF074), rat *NQO1* (strain LLF076), *GFP* (strain LLF080), *PST1* (LLF064), *PST2* (LLF081), *PST3* (LLF066) or *YCP4* (LLF082). Single mutant deletion strains used in panel C were *pst1Δ* (LLF052), *pst2Δ* (LLF059), *pst3Δ* (LLF036), and *ycp4Δ* (LLF037).

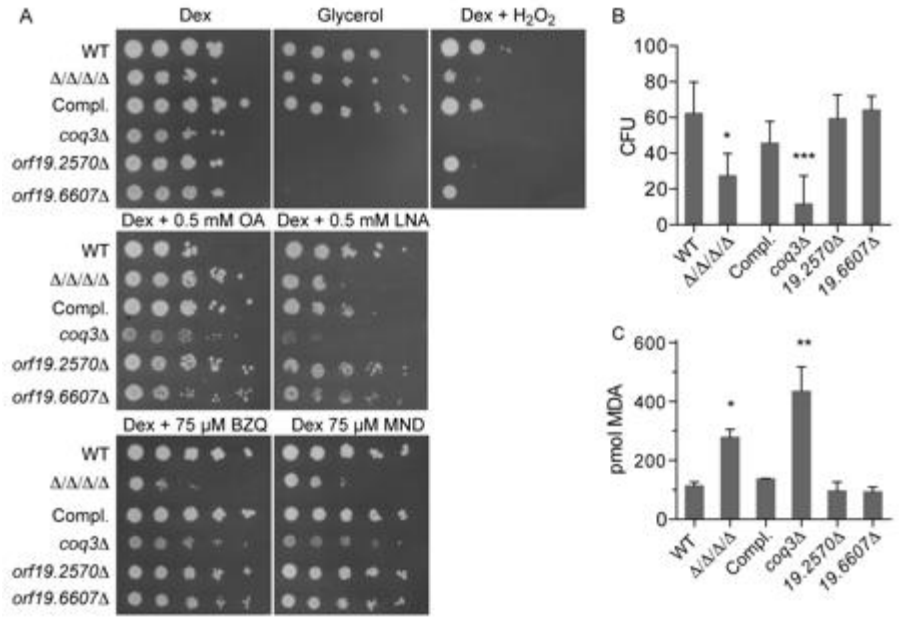

Fig. 6. Comparison of the Δ/Δ/Δ/Δ mutant with a *coq3Δ* mutant that fails to produce ubiquinone.

The Δ/Δ/Δ/Δ mutant was compared with a *coq3Δ* mutant (LLF088) and two representative mitochondrial mutants, *orf19.2570Δ,* and *orf19.6670Δ.*

(A) Serial 10-fold dilutions of cells were spotted onto agar plates containing synthetic medium and the indicated carbon source, or with dextrose plus H_2O_2. Plates were incubated at 37°C for 48 h and then photographed.

(B) Cells were treated for 6 hours in the presence of 0.5mM LNA, and then assayed for viable CFUs, or

(C) assayed for TBARS as an indicator of lipid peroxidation. Error bars indicate SE.

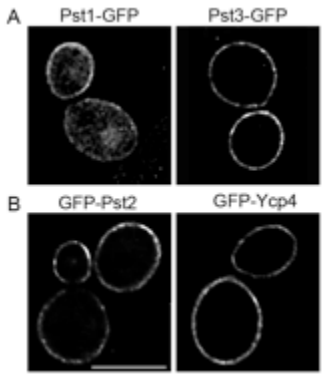

Fig. 7. Plasma membrane localization of the *C. albicans* FLP proteins.

Fluorescence microscopy of *C. albicans* cells producing (A) Pst1-GFP and Pst3-GFP fusion proteins in which GFP was added to the C terminus. (B) Cells producing GFP-Pst2 and GFP-Ycp4 fusions in which GFP was added to the N terminus. The GFP fusion genes in panel A were regulated by their endogenous promoters, whereas the fusion genes in panel B were expressed using the *ADH1* promoter.

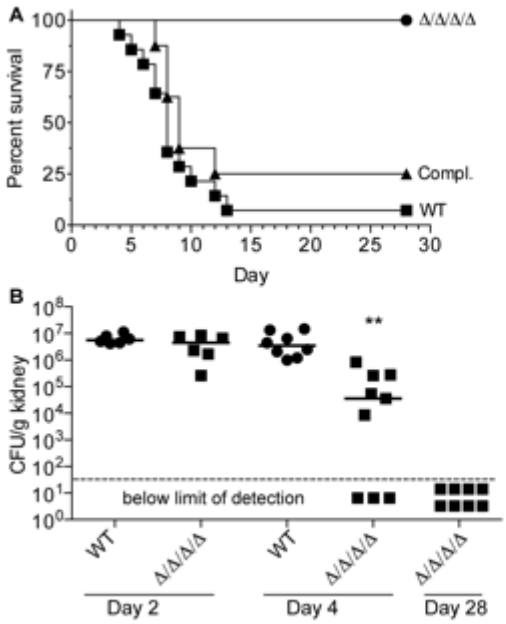

Fig. 8. The Δ/Δ/Δ/Δ mutant is avirulent in a mouse model of systemic candidiasis.

(A) 2.5 x 10^5 *C albicans* cells were injected via the tail vein into 8-week old female Balb/c mice. The mice were then monitored for survival over the next 28 d.

(B) Mice were infected as described above and then CFU per g kidney was determined at day 2, 4 or 28 post infection. **, $p < 0.01$ by ANOVA.

Table 1. Strains used

C. albicans strain	Parent	Genotype
SN152	SC5314	*arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm IRO1/iro1Δ::imm*
LLF100	SN152	(prototrophic wild type control) *arg4Δ/ARG4 leu2Δ/LEU2 his1Δ/HIS1 URA3/ura3Δ::imm IRO1/iro1Δ::imm*
LLF052	SN152	(*pst1*Δ) *pst1Δ::HIS1/pst1Δ::LEU2 ARG4/arg4Δ*
LLF059	SN152	(*pst2*Δ) *pst2Δ::HIS1/pst2Δ::LEU2 ARG4/arg4Δ*
LLF036	SN *pst3*Δ	(*pst3*Δ) *pst3Δ::HIS1/pst3Δ::LEU2 ARG4/arg4Δ*
LLF037	SN *ycp4*Δ	(ycp4Δ) *ycp4Δ::HIS1/ycp4Δ::LEU2 ARG4/arg4Δ*
LLF025	SN152	(*pst3Δ ycp4*Δ) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2*
LLF034	LLF025	(*pst3Δ ycp4*Δ) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 ARG4/arg4Δ*
LLF032	LLF025	*(pst2Δ pst3Δ ycp4Δ) pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt*
LLF063	LLF032	*(pst2Δ pst3Δ ycp4Δ) pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt ARG4/arg4Δ*
LLF054	LLF032	(Δ/Δ/Δ/Δ) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt arg4Δ/arg4Δ*
LLF060	LLF054	(Δ/Δ/Δ/Δ) *LLF054 except for ARG4/arg4Δ*
LLF079	LLF054	(Compl.) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt PST2-PST1::ARG4 arg4Δ/arg4Δ PST3-YCP4::SAT1*

LLF074	LLF054	(+*wrbA*) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt pADH1-GFP$_\gamma$-EcWrbA::ARG4 arg4Δ/arg4Δ*
LLF076	LLF054	(+*NQO1*) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt pADH1-GFP$_\gamma$-rat NQO1::ARG4 arg4Δ/arg4Δ*
LLF080	LLF054	(+GFP) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt pADH1-GFP$_\gamma$::ARG4 arg4Δ/arg4Δ*
LLF064	LLF054	(+*PST1*) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt PST1::ARG4 arg4Δ/arg4Δ*
LLF081	LLF054	(+*PST2*) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt PST2::ARG4 arg4Δ/arg4Δ*
LLF066	LLF054	(+*PST3*) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt PST3::ARG4 arg4Δ/arg4Δ*
LLF082	LLF054	(+*YCP4*) *pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt YCP4::ARG4 arg4Δ/arg4Δ*
LLF083	SN orf19.2570Δ	*orf19.2570Δ::HIS1/ orf 19.2570Δ::LEU2 ARG4/arg4Δ*
LLF084	SN orf19.6607Δ	*orf19.6607Δ::HIS1/ orf 19.6607Δ::LEU2 ARG4/arg4Δ*
LLF088	SN152	(*coq3Δ*) *coq3Δ::HIS1/coq3Δ::LEU2 ARG4/arg4Δ*
BWP17	SC5314	*ura3Δ::λimm434/ura3 Δ::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*
LLF018	BWP17	*LSP1/LSP1-mCherry::ARG4 ura3Δ::λimm434/ura3 Δ::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*
LLF098	LLF018	*PST1/PST1-GFPγ::HIS1 LSP1/LSP1-mCherry::ARG4 ura3Δ::λimm434/ura3 Δ::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*
LLF090	LLF018	*PST3/PST3-GFPγ::HIS1 LSP1/LSP1-mCherry::ARG4 ura3Δ::λimm434/ura3 Δ::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*

LLF071	LLF018	*pADH1-GFPγ-YCP4::URA3 LSP1/LSP1-mCherry::ARG4 ura3*Δ::λ*imm434/ura3* Δ::λ *imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*
LLF089	BWP17	*pADH1-mCherry-YCP4::SAT1 ura3*Δ::λ*imm434/ura3* Δ::λ *imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*
LLF092	LLF089	*pADH1-GFPγ-PST2::URA3 pADH1-mCherry-YCP4::SAT1 ura3*Δ::λ*imm434/ura3* Δ::λ *imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*

Chapter 5 – Future directions

I investigated two main areas for my dissertation study on *Candida albicans* morphogenesis and antioxidant defense. Through analysis of a temperature-sensitive mutant of an essential septin gene, the *cdc12*^{ts} mutant, we demonstrated that septins are needed for the proper maintenance of highly polarized hyphal morphogenesis. Also, in contrast to wild type cells, the *cdc12*^{ts} mutant formed a second hyphal outgrowth in close proximity to the first, which limited the ability to disseminate to new regions. To better understand how *C. albicans* resists oxidative stress, I analyzed a family of four Flavodoxin-Like Proteins (FLPs). Previous studies showed that FLPs in other organisms influence redox pathways by acting as NAD(P)H quinone oxidoreductases. Interestingly, a quadruple mutant lacking all four FLPs (Δ/Δ/Δ/Δ) was more sensitive to a variety of oxidants *in vitro*. The key role of quinone reductase activity was confirmed by showing that the resistance to oxidants was restored to the quadruple mutant by expressing either of two very different types of quinone reductases: *Escherichia coli wrbA* or mammalian *NQO1*. FLPs were detected at the plasma membrane in *C. albicans*, suggesting that they may act to reduce ubiquinone (coenzyme Q or CoQ), which is known to act as an antioxidant in cellular membranes. In agreement with this, a *C. albicans* mutant that does not synthesize ubiquinone (*coq3Δ*) was very sensitive to oxidation. The FLPs play an important role *in vivo*, as the Δ/Δ/Δ/Δ mutant was avirulent in a mouse model of systemic candidiasis. Thus, these studies identified FLPs as part of a new antioxidant mechanism that is necessary for *C. albicans* virulence.

5.1 Future directions for *C. albicans* septin research

5.1.1 Which is important for the hyphal morphological switch, septin proteins or septin ring structure?

As discussed in Section 1.2 and demonstrated in Chapter 3, there is a close connection between septin ring formation and hyphal morphogenesis in *C. albicans*. However, despite the fact that hyphal structure is impaired to different degrees in the septin deletion mutants, all these septin mutants can initiate hyphal formation. The observation that the *cdc12-ts* mutant exhibits widened germ tubes and widened septin rings at the same time suggests that septin rings guide the formation of germ tubes. Another observation is that despite the fact that all of the septin mutants have defective septin ring structures, the septin ring is present in all of these mutants, which suggests the structural importance of the septin ring. An interesting question is whether complete absence of septin ring structure will abolish the hyphal formation in *C. albicans*.

5.1.2 What is the connection between bud-site selection genes and septin genes in second germ tube site selection?

One observation we made was that the *cdc12-6ts* mutant has a defect in site selection for the second germ tube. While the wild-type cells typically form the second germ tube far from the first, the *cdc12-6* cells frequently formed a second germ tube in close proximity to the first. The *cdc10*Δ and *cdc11*Δ mutants occasionally formed proximal germ tubes as well (49). The *C. albicans* septin mutants we examined also exhibited a defect is bud site selection at 30˚C, as discussed in Chapter 3. While the wild-type cells budded predominantly in a bipolar manner at 30˚C, *cdc10*Δ*, cdc11*Δ*,* and *sep7*Δ mutants all still budded primarily in an axial manner.

Such similarity in defects for selection of sites for the second germ tubes and buds suggests a connection between the two site selection mechanisms. It has been previously reported that septins play important roles in bud site selection in *S. cerevisiae* (122). Our data that the nonessential septin deletion mutants of *C. albicans* exhibit defects in bud site selection indicates that septins play an important role in *C. albicans* bud site selection as well. An interesting future direction would be to study the second germ tube selection phenotype of the mutants of bud site selection genes. If these mutants show similar phenotypes as the septin mutant in germ tube selection, the next step would be to investigate the interaction between septins and the Bud proteins to define the mechanisms for how these proteins determine the spatial location of future sites of polarized morphogenesis.

5.2 Future direction for *C. albicans* FLP research

5.2.1 Is PM localization crucial for FLPs to protect the PM from lipid peroxidation?

The deconvolution microscopy data revealed that the FLPs are localized to the PM and are enriched in a punctate pattern in *C. albicans*, which likely correlates with the MCC/eisosome domains of the PM. As discussed above, the PM of *C. albicans* is prone to oxidation due to its high PUFA content. It has been reported that PUFA are further enriched in areas of membrane curvature, as it makes the packing of the lipids into the curved area more efficient due to the change in lipid structure caused by the unsaturated bonds of the PUFA (180). Since MCC/eisosome domains form membrane furrows (181), there are two areas of intense curvature that correspond to the rim and the apex base of the furrow. If PUFAs are enriched at such areas then MCC/eisosomes would be expected to be more prone to lipid peroxidation and, therefore, need extra reducing potential to protect the PUFAs. Co-localization of FLPs and eisosomal

markers can be performed to confirm this prediction. Although *C. albicans* FLPs localize to PM, it may not be essential for their function. One FLP, Ycp4 is predicted to have a lipid modification at its C-terminus and is likely to anchor the protein at the membrane. Ycp4 could act as an anchor to recruit other FLPs, since crystal structures clearly demonstrated that members of the WrbA protein family of flavodoxin-like proteins from many organisms form tetramers (144,182,183). Yeast two-hybrid data indicate that *S. cerevisiae* Pst2 interacts with both itself and the other FLP orthologues. Therefore, it is very likely that the four *C. albicans* FLP proteins form a heterotetramer. Our unpublished data also suggest *C. albicans* Ycp4 is responsible for bringing Pst2 to the PM. In the *C. albicans ycp4*Δ deletion mutant, the rest of the FLPs are also likely to be mislocalized, yet the deletion mutant of *ycp4*Δ did not phenocopy the quadruple FLP deletion. This is not too surprising, since mammalian Nqo1, which has been reported to affect the redox status of CoQ and perform functions similar to FLPs, is a cytoplasmic protein (184). The heterologous expression of mammalian *NQO1* in the *C. albicans* quadruple FLP deletion mutant restored its resistance to the lipid peroxidation-inducing agent LNA. Taken together this strongly suggests that PM localization is not crucial for FLP cellular function. However, further research investigating the relationship between the cellular localization and function of the *C. albicans* FLPs, should be carried out.

5.2.2 Do *C. albicans* FLPs act as NAD(P)H quinone oxidoreductases in vitro?

We did not perform biochemical studies to confirm the enzymatic activity of these *C. albicans* FLPs *in vitro*, as our primary interest was to study their physiological function. Therefore, we used genetic means to probe function; namely, heterologous expression of a known NAD(P)H quinone oxidoreductase. However, confirming the NAD(P)H quinone

oxidoreductase activity by a direct *in vitro* assay would be worthwhile. The four *C. albicans* FLP proteins share 43%-49% identity and 59%-66% similarity with the *E. coli* WrbA, which is a well characterized NAD(P)H quinone oxidoreductase (143) and founding member of the family of flavodoxin-like proteins, so they are likely to have the same activity. Patterning after the biochemical test of *E. coli* WrbA, the protein function of purified *C. albicans* FLPs can be tested *in vitro*, with the *E. coli* WrbA as a positive control.

5.2.3 Is the enzymatic activity of FLPs essential for protecting the PM from lipid peroxidation?

Our data highly suggest that *C. albicans* FLPs function as NAD(P)H quinone oxidoreductases *in vivo,* and that they reduce CoQ, a major cellular antioxidant that prevents lipid peroxidation in the PM. Therefore, the enzymatic activity of FLPs is likely to be essential for their cellular functions. To confirm this, one can perform site-directed mutagenesis to selectively abolish the quinone reductase activity while leaving the rest of the protein scaffold relatively unchanged. Such mutant proteins can be first tested *in vitro* to confirm they lack enzymatic activity before being expressed *in vivo* to assay functionality.

The information necessary for the site-directed mutagenesis is available due to the structural studies of the members of the WrbA family of FLPs in bacteria. The crystal structures revealed conserved residues that are crucial for enzymatic activity through either cofactor (FMN) binding or substrate binding among members of the WrbA family of FLPs (144,182,183). FMN binding might be important for the protein folding and structure, though this is less likely so for substrate binding, i.e. NAD(P)H and quinone binding. Therefore, site-directed mutagenesis of the amino acid residues for the substrate binding can be introduced to test protein function *in vitro* and *in vivo*. As published structures of WrbA in complex with benzoquinone and NAD(P)H suggest a sequential binding mechanism for both molecules in the catalytic cycle (143), by mutating

residues only crucial for NAD(P)H binding or quinone binding, or both, one can potentially create mutants that lack enzymatic activity but through different mechanisms. This would allow the question to be answered more precisely. The recently developed CRISPR system for *C. albicans* would greatly expedite these site directed mutagenesis experiments (185).

5.2.4 Do different *C. albicans* FLPs have different substrate specificities in vitro?

Analysis of *C. albicans* FLP mutants suggested the four FLP proteins exhibit different substrate specificities (Chapter 4). Pst3 appears to be specialized to detoxify small molecule quinones. A *pst3*Δ mutant was the only single FLP mutant to show sensitivity to small molecule quinones. Also cells expressing *PST3* as the only FLP gene in the cell were resistant to small quinones, but sensitive to other oxidizing conditions. This test of the substrate specificity can be an extension of the experiments described in Section 5.2 where each of the FLP proteins can be tested with a range of quinones, e.g. small molecule, short-chain, and long-chain quinones.

5.2.5 Do *C. albicans* FLPs reduce CoQ?

Another important discovery we made was that a *C. albicans* mutant that fails to synthesize CoQ was found to have increased sensitivity to lipid peroxidation induced by PUFA, the same as was observed in *S. cerevisiae* (81). However, the *S. cerevisiae* work never explored which enzyme(s) act to reduce oxidized CoQ. Our work strongly suggests it biochemically with purified *C. albicans* FLPs. This is an extension of the substrate specificity studies described above.

We would also like to examine the redox status of CoQ in *C. albicans* cells in relationship to FLPs. One approach could be to use hexane to extract CoQ from the PM, as

described previously (186). HPLC can then distinguish the mass of the reduced form of CoQ and oxidized form due to the two extra hydrogen atoms on the reduced form.

Inducible promoters can be introduced into the genome to control the expression of wild type FLPs and catalytically dead forms (as discussed in Section 5.3). Redox status of CoQ will be measured in the presence and absence of either wild type FLP or the catalytic dead FLP.

5.2.6 Do *C. albicans* FLPs represent potential new targets for antifungal and antibacterial drugs?

The *C. albicans* quadruple FLP deletion strain was avirulent in the mouse model of candidiasis. Inhibitors that block the function of the *C. albicans* FLP proteins are therefore good potential therapeutics. Given that there are no mammalian orthologues by sequence homology, such drugs would likely be safe for human patients. Evidence that drugs specific for *C. albicans* FLPs can be obtained comes from the observation that dicumarol, the classic inhibitor of mammalian Nqo1, does not inhibit *E. coli* WrbA (84). As discussed above, *E. coli* WrbA shares significant sequence homology with *C. albicans* FLPs. The four *C. albicans* FLPs share high sequence similarity with each other, thus one drug may be able to inhibit all four. If this is not the case, a cocktail of compounds is another solution.

A drug screen can be performed both *in vitro* and *in vivo*. The virulence function of *C. albicans* FLPs is likely the reduction of CoQ. If this is confirmed as per the experiments described in the section above, a compound that blocks the reduction of CoQ by FLPs *in vitro* will be a good candidate to be further tested.

Amino acid sequence comparison revealed multiple orthologues of *C. albicans* FLPs in not only other *Candida* species, such as *C. dubliniensis, C. maltose, C. parapsilosis, and C.*

tropicalis, but also other pathogenic fungal species, including *Cryptococcus* species, e.g. *C. neoformans* and *C. gattii*; *Aspergillus* species, e.g. *A. fumigatus, A. nidulans, A. ochraceoroseus, A. terreus, A. clavatus, A. kawachii, A. parasiticus, A. niger, A. ruber, A. oryzae*, and *A. flavus*; as well as *Histoplasma capsulatum*. In addition, as discussed in earlier sections, orthologues of FLPs are also present in bacteria are present in *E. coli* and other pathogenic bacterial species as well, including both *Salmonella* species, *S. enterica and S. bongori; Francisella* species, *F. tularensis* and *F. guangzhouensis*. These proteins are mostly labeled in the databases as unnamed proteins or listed as putative NADH-quinone oxidoreductases as they have not been studied. The results presented in this thesis indicate that these orthologous proteins are likely to perform important cellular functions as antioxidants in other bacterial and fungal pathogens. Therefore, drugs that target FLPs have potential to be useful as broad-spectrum antibiotic drugs.

5.3 Concluding comments.

In conclusion, both aspects of my dissertation research led to significant new insights in controlling the infection due to the most common fungal pathogen *C. albicans* and potentially other pathogenic fungi and bacteria. Septin proteins were found to play important roles in the ability of *C. albicans* to undergo hyphal morphogenesis and grow invasively. Further studies in this field may lead to new therapeutics designed to prevent biofilm formation and/or dissemination of *C. albicans* into tissues. *C. albicans* FLPs were found to play an important antioxidant role and were required for the virulence in mice. FLPs are conserved in a most, if not all, of the major species of pathogenic bacterial and fungi. Although the enzymatic activity of FLPs has been well studied in *E. coli* and other bacteria (85,143,149), the physiological function has not been discovered. The studies on FLPs in *C. albicans* provided the best insight

to date into the physiological role of FLPs for a pathogen. These results will therefore help to understand the physiological functions of FLPs in bacteria and other fungal pathogen. Thus, studies on FLPs in *C. albicans* and in bacteria have synergized to better understand the functions of this novel family of NAD(P)H quinone oxidoreductases. Lack of mammalian orthologues makes FLPs a promising new drug target for both antibacterial and antifungal therapies.

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