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I am submitting herewith a dissertation written by Ying-Lien Chen entitled “The Roles of Phospholipid Biosynthesis in *Candida albicans* Virulence”. I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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THE ROLES OF PHOSPHOLIPID BIOSYNTHESIS IN *CANDIDA ALBICANS* VIRULENCE

A Dissertation
Presented for the
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Degree
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Ying-Lien Chen
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DEDICATION

To
my father and mother,

Hong-Ming Chen and Mei Hsueh,

who made my dreams come true.

And also to

my wife and kids,

Shu-Chen Tsai, Wei-Shin Chen, and Expected little Chen

who made my life cheerful.

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ABSTRACT

Candida albicans is a major human fungal pathogen. A primary goal in studying *C. albicans* virulence is actually to find the ways to undermine it and prevent disease. The best drug targets are proteins or molecules that are not conserved in humans. For most antimicrobials against bacteria, fungi, or protozoans, the targets are not virulence factors, but rather enzymes that are important for basic growth or survival. It is not imperative that the target be required in all growth conditions, but rather just in the host. An area of research that has shown promising potential for the discovery of new drug targets in several microbal pathogens is in phospholipid biosynthesis. In this dissertation, there are three projects that revolve around studying proteins that may be potential drug targets for *C. albicans*. 1) inositol biosynthesis was examined. It has been shown in the pathogens *Mycobacterium tuberculosis* and *Trypanosoma brucei* that *de novo* inositol biosynthesis is required for virulence or growth. Therefore, the enzyme required for *de novo* inositol synthesis was examined for a role in virulence. 2) The phosphatidylserine (PS) synthase gene (*CHO1*) of *S. cerevisiae* is conserved in other fungi, and is not conserved in humans. Given that PS is an abundant phospholipid in yeasts, it was hypothesized that it would be important for virulence in the host. 3) Fungi carry homologs of a fungal-specific transcription factor, called Opi1p, that has been shown to be involved in regulating filamentous growth and phospholipid biosynthesis in *S. cerevisiae*. Its homolog was examined in *C. albicans* to determine if it regulated these phenotypes in this pathogen because filamentous growth is an important virulence factor. If the CaOpi1p protein were essential for filamentous growth then it might be good drug target as it is not conserved in humans. All three of these projects revolve around phospholipid biosynthesis in *C. albicans* and each has revealed interesting and sometime surprising aspects of the biology and virulence of this fungus.

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Chapter 1: Background and Introduction

CLINICAL SIGNIFICANCE OF CANDIDIASIS

Candida albicans is a dimorphic yeast that can exist in a human host as either a harmless commensal, or as an opportunistic pathogen when the host's immune system is impaired. *C. albicans* is capable of causing host damage (or disease) leading to oral, vaginal, cutaneous, or systemic candidiasis (20). In patients that are neutropenic, *Candida* species are associated with severe and deadly systemic candidiasis with a high mortality rate (31.8%)(27, 116). In fact, *Candida* species are the fourth most common cause of catheter-related bloodstream infections in hospitalized patients in the intensive care unit, and *C. albicans* is the most commonly isolated species from these infections, giving rise to enormous associated personal and economic costs (19).

The rise of immunocompromised individuals in our society has provoked a significant emergence in the number of patients affected by *Candida* species. About one-third of episodes of candidaemia occur in the intensive care unit (ICU). Although non-albicans *Candida* species, such as *C. glabrata*, *C. krusei*, and *C. parapsilosis* are observed and some have increased resistance to common antifungals, *C. albicans* was still the predominant species, causing up to two-thirds of all cases of invasive candidiasis (166). In invasive candidiasis, a delayed treatment initiation is associated with an increased mortality, thus empirical therapy strategies become vitally important.

Two decades ago, the therapeutic possibilities for invasive candidiasis were limited to amphotericin B and azoles, but with the advent of new antifungal agents, such as echinocandins (e.g. caspofungin), other therapeutic options have become available (148). Though only a few caspofungin resistant cases have been identified so far, this indicates that *Candida* species could develop more wide-spread resistance to this agent over time. This dissertation will discuss a new class of proteins that may serve as a source (i.e. phospholipid biosynthesis) for urgently needed new antifungal drug targets in *C. albicans*.

VIRULENCE FACTORS OF C. *ALBICANS*

Morphogenetic conversions

C. albicans is a polymorphic organism that is capable of converting between yeast, pseudohyphal, and hyphal forms (Fig 1-1, all tables and figures are in the appendix D). The conventional view has been that yeast forms are associated with passage through the blood stream or commensal carriage, whereas hyphal forms were associated with tissue invasion and developing disease. This was based on evidence showing that mutant forms

of *C. albicans* that were locked into the yeast form are avirulent (98). However, this concept has been challenged by Braun et al., who found that a *TUP1*-deficient *C. albicans* strain that is constitutively hyperfilamentous is also avirulent in a murine model of systemic candidiasis (15). So far, the precise nature of the association between fungal morphogenesis and host invasion is still in debate. However, it is now widely accepted that the ability to undergo morphogenetic conversion, rather than the morphological form itself, is the primary determinant of pathogenicity (57).

Adhesion to host cells

Adherence of *C. albicans* to host cells is seen as an essential early step in establishing disease. Moreover, *C. albicans* can also adhere to the surfaces of medical devices and form biofilms, which are relatively resistant to many common antifungal agents. The ability to form biofilms exhibits a positive association with the degree of virulence. There are several adhesion molecules (adhesins) in *C. albicans*. These adhesins are part of a structurally related superfamily of fungal adhesins, and in *C. albicans* include Hwp1p, Eap1p and the eight known members of the agglutinin-like sequence (ALS) family of adhesins (72, 94, 157). All of these adhesins are glycosylphosphatidylinositol (GPI)-anchored proteins that are believed to be cell wall proteins (GPI-CWPs). The GPI-CWPs all have N-terminal signal peptides for translocation into the ER, and C-terminal signals that mediate GPI membrane anchor addition (171). The GPI-anchor is believed to mediate attachment to cell wall glucan (164). One protein that is not in the GPI-CWP class that was initially described as an adhesin, Int1p (49, 50), is described in this chapter as well.

Agglutinin-like sequence (ALS) family

The ALS family of *C. albicans* includes eight genes (A*LS1-ALS7,* and *ALS*9) that encode large cell-surface GPI-CWP adhesins that are primarily involved in host-pathogen interactions and appear to have differing roles in adhesion and transmigration (72). ALS3 and ALS8 represent a single locus (186). Several of the ALS genes have been characterized and appear to play roles in virulence. Evidence shows that *ALS1*-transformed *Saccharomyces cerevisiae* exhibits up to 100-fold greater adherence to endothelial cells (156). In addition, Als1-deficient *C. albicans* hyphae exhibit reduced adhesion to endothelial cells (186). Delayed filamentation of the *als1*Δ*/als1*Δ mutant and the flocculation of the strain overexpressing Als1p led to the conclusion that Als1p is similar to Flo11p of *S. cerevisiae* (164). Similarly, *S. cerevisiae* transformed with *ALS3* shows increased adhesion (156), while Als3-deficient hyphal forms of *C. albicans* exhibit

defective adhesion to endothelial cells (186). *ALS3* is also necessary for biofilm formation in vitro and it mediates endocytosis by epithelial cells (127, 138). In contrast, mutational analysis has shown that deletion of *ALS5*, *ALS6*, or *ALS7* results in increased adhesion of yeast forms of *C. albicans* to endothelial cells, suggesting an antiadhesive role for these proteins (187).

Hyphae cell wall protein (*HWP1*)

HWP1 encodes a major *C. albicans* hyphae cell wall protein which is a substrate of mammalian transglutaminases and could promote the cross-link of the fungus to epithelial cells. Hwp1p is an important developmentally regulated adhesin and found on surfaces of germ tubes, but not yeast or pseudohyphal forms of *C. albicans*. Surface localization, expression in host tissues and importance in systemic candidiasis have been rigorously demonstrated for Hwp1 (157, 158, 164). *C. albicans hwp1*Δ*/hwp1*Δ mutants exhibited avirulence whereas *HWP1/hwp1*Δ or wild-type exhibietd full virulence (164). Hwp1p is also involved in mating and biofilm formation in vitro and in vivo (35, 127).

Integrin-like protein (*INT1*)

Int1p is an integrin-like protein not in the GPI-CWP class, but implicated in adhesion properties which binds to host extracellular matrix protein. Although Int1p has motifs reminiscent of mammalian integrins, it has been shown to be similar to Bud4p of *S. cerevisiae* in primary amino acid sequence, in co-localizing with septins and in functioning in bud site selection (49, 50). The *int1*Δ*/int1*Δ mutant exhibited reduced mortality compared to the wild-type strain, but increased *C. albicans* persistence in the kidney (8).

Extracellular hydrolases

Hydrolase production is known to play a central role in the pathogenicity of bacteria, protozoa, and pathogenic fungi (150). The production of hydrolases may play more direct role in contributing to host tissue invasion by digesting host cell membranes and molecules of the host immune system to enhance invasion and to avoid antimicrobial attack, respectively (20). *C. albicans* secretes three major hydrolytic enzymes: secreted aspartyl proteases (Sap), phospholipases, and lipases. Glucanases and chitinases have also been identified, but not linked to virulence (150).

Secreted aspartyl proteases (SAPs)

The Sap family contains 10 members, SAP1-10, that are all found extracellularly. Sap1-8 are secreted into the media, but proteases SAP9 and SAP10 are GPI-anchored and associated with the cell periphery. Interestingly, all secreted *Candida* proteases belong to

the same class of aspartic proteases. Serine-, metallo-, or cysteine- proteases have not been identified in pathogenic *Candida* (20). Saps have been one of the most comprehensively studied virulence factors. Of the SAPs, Sap2, has been the most extensively studied. Sap2p has been crystallized, and it is the most abundant secreted protein *in vitro* when grown in the presence of protein as the sole source of nitrogen (33, 73). Sap1 to Sap3 are preferentially expressed in acidic pH (also their pH optima for proteolytic activity), while Sap4 to Sap6 are preferentially made in neutral environments and are believed to be corregulated with filamentous growth. Sap1 to Sap3p are associated with mucosal infection while Sap4 to Sap6 are associated with invasive disease, although both of the subgroups also are known to have overlapping functions (123).

C. albicans isolates from patients with vaginal candidiasis were significantly more proteolytic than isolates from asymptomatic vaginal carriers (37). *C. albicans* isolates from HIV-positive women with vaginitis also produced significantly higher levels of protease than did *C. albicans* strains isolated from HIV-positive asymptomatic carriers or HIV-negative subjects with candidal vaginitis (39).

Phospholipase

To date, only two secreted phospholipases have been identified. These phospholipases are both of the B subfamily and are encoded by the genes *PLB1* and *PLB2*. In a mouse model of systemic candidiasis, the virulence of *C. albicans plb1*Δ*/plb1*Δ mutant, which had only 1% of the extracellular phospholipase B activity of the wild-type strain, was shown to be significantly attenuated (90). Importantly, immunogold electron microscopy studies showed that *C. albicans* secretes phospholipases during the infectious process (54). The function of phospholipases during *C. albicans* infections are not precisely known, but they have been implicated in host cell penetration and may interact with host signal transduction pathways (150).

Lipase

The first lipase gene *LIP1* was cloned in *C. albicans* in 1997 (47). Nine further members of this lipase gene family (*LIP2* to *LIP10*) were subsequently cloned and characterized by Hube and his colleagues (75). These secreted lipases may have functions other than solely providing nutrients for the cells. However, the roles and functions of lipases during *C. albicans* infections remain to be elucidated (20). The *LIP8* gene has been shown to be required for virulence in a mouse model of systemic candidiasis based on the phenotype of the *lip8*Δ*/lip8*Δ mutant (48).

ANTIFUNGALS AGAINST *C. ALBICANS* AND OTHER HUMAN PATHOGENS

There are five major classes of antifungals used against fungal infections (43, 53) (Fig 1-5). The total number of approved systemic antifungal drugs was only 14 in 2004 (43). The relatively few classes of antifungal drugs will restrict clinicians' therapeutic choices and these choices are further reduced by the emergence of drug resistance (21). The development of new class of drugs will provide combination therapy more flexibility and may provide better treatment (78). Therefore, new drug targets are urgently needed.

Polyenes

In the 1950s, Amphotericin B (AmB) was released and has been served as the standard systemic antifungal therapy (51), but it is often poorly tolerated and associated with nephrotoxicity. The function of polyene agents is to directly bind to ergosterol which is the fungal equivalent to cholesterol, and is unique in the fungal cell membrane. Binding of ergosterol by polyenes alters cell permeability resulting in cell death. The lipid derivatives of amphotericin B, particularly liposomal amphotericin B (LAmB) may be less nephrotoxic while maintaining a broad antifungal spectrum.

Flucytosine

In the early 1970s, flucytosine (Ancobon) was released by Valeant Pharmaceuticals Inc. and became an alternative systemic therapeutic option in addition to AmB. Flucytosine is a pyrimidine analog that inhibits both DNA synthesis and protein synthesis in the fungal cell (175). However, the toxicity of this agent and the rapid development of resistance resulted in limited use for treatment (139).

Azoles

In 1979, the first systemic azole antifungal drug, ketoconazole, was released. Azoles exert their antifungal activity by blocking the demethylation of lanosterol, thereby inhibiting ergosterol synthesis (60). Ketoconazole was also used for topical agent because shampoo and cream formulations of ketoconazole have been available for many years (151). Ketoconazole was followed chronologically by fluconazole and itraconazole. Each agent offers a specific antifungal spectrum. Although fluconazole's target Erg11p or CYP51 in *C. albicans* has human homolog huCYP51, the fluconazole was developed as a drug (136). However, azoles exhibit fungistatic activity and drug resistance, a growing problem among fungal pathogens including *C. albicans*. It is particularly problematic in some other *Candida* species such as *Candida glabrata* (89).

Allylamines

This class includes naftifine and terbinafine, and inhibit ergosterol synthesis at the level of squalene epoxidase. They are selective for the fungal enzyme and have a minimal effect on mammalian cholesterol synthesis. Naftifine, the original member of the allylamine series, possesses only topical activity, whereas the naftifine analog terbinafine is active both topically and orally (12). Terbinafine is active against a wide variety of dermatophytes, molds, and dimorphic fungi in which it exerts a fungicidal action. However, terbinafine exhibits fungistatic activity against *C. albicans* and drug resistance has been observed (113).

Echinocandins

Echinocandins form the newest class of antifungals. Caspofungin (Cancidas) was released by Merck Inc. in 2001. Micafungin and anidulafungin were released afterward (43). The spectrum of activity is limited to pathogens that rely on these glucan polymers and is less than the spectrums of the polyenes or azoles. Echinocandins are semisynthetic lipopeptides, which inhibit synthesis of β-1,3-glucan, an essential and major fungal cell wall component (80). Because there is no cell wall in mammalian cells, the echinocandins are less toxic than polyenes antifungals. For example, caspofungin has been emerging as first-line therapy for invasive candidiasis due to less toxicity and fungicytotic activity (184). Caspofungin can kill most *Candida* and *Aspergillus* species (Table 1-1) and only rare resistant strains have been identified. Moreover, *Cryptococcus neoformans* is naturally resistant to caspofungin (105)**.** It is highly possible that susceptible fungi will develop resistance over time as this has been observed to a limited extent.

PHOSPHOLIPID BIOSYNTHETIC PATHWAYS MAY SERVE AS A GOOD SOURCE FOR NOVEL ANTIFUNGAL TARGETS IN *C. ALBICANS*

Phospholipid biosynthesis in yeast

Phospholipid composition

The major phospholipids found in growing cells of *S. cerevisiae* and *C. albicans* are PC, PE, PI, and PS. Phospholipid composition can vary dramatically when culture conditions (i.e. inositol supplementation or starvation) are altered (81). The total lipid content of *C. albicans* was 5% of the dry weight while phospholipids and sterols represented 1.1% and 1.2% of the dry weight, respectively (104). In an azole-sensitive *C. albicans* strain, PE (26.2%) constitutes the major phospholipid, then PS (21.1%), PC

(15.5%) and PI (7.9%) (100). In the plasma membrane of *S. cerevisiae*, PS (34%) constitutes the major phospholipid, then PE (20%), PI (18%), PC(17%) while in the mitochondria, PC (51%) is major phospholipid, then PE (25%), PI (13%), and PS (1%) (13).

Genes and enzymes involved in phospholipid biosynthesis

The genes involved in *C. albicans* phospholipid biosynthesis have not been studied, however, this fungus has homologs of the genes found to be required for phospholipid biosynthesis in *S. cerevisiae*, therefore *S. cerevisiae* is used as a model with which to explore the process in *C. albicans*. There are two main pathways for generating the aminophospholipids phosphatidylethanolamine (PE), and phosphatidylcholine (PC), and one pathway each for generating phosphatidylserine (PS) and phosphatidylinositol (PI) (Fig 1-2). Of all of these enzymes, the PS synthase Cho1p is one of the only ones that is not conserved in humans. Cho1p is localized to the endoplasmic reticulum (ER), and catalyzes the formation of PS from the precursors serine and cytidine diphosphate-diacylglycerol (CDP-DAG) along with the release of CMP (Fig 1-3A). PS serves as a precursor for the synthesis of PE and ultimately PC by the *de novo* aminophospholipid pathway (Fig 1-2). First PS is decarboxylated to PE by one of two enzymes, Psd1p and Psd2p (Fig 1-3B). Psd1p and Psd2p are mainly localized in the inner mitochondrial membrane and Golgi complex membrane, respectively. In the *de novo* pathway, PE is then used as a substrate to form PC by two phospholipid methyltransferases, Cho2p and Opi3p. Three methylations of PE are required to form PC, and Cho2p transfers the first methyl group to PE to form phosphatidylmonomethylethanolamine (PMME). PMME is then converted by Opi3p to phosphatidyldimethylethanolamine (PDME) and then PC via the addition of a second and third methyl groups, respectively. As an alternative mechanism for making PE and PC, the cells can rely on the Kennedy pathway so long as exogenous choline or ethanolamine are present as substates (Fig 1-2). The Kennedy pathway has two branches: CDP-ethanolamine and CDP-choline, which produce PE and PC, respectively by utilizing exogenous ethanolamine and choline. In the CDP-ethanolamine pathway, exogenous ethanolamine is converted to phospho-ethanolamine (Etn-P) by ethanolamine kinase (Eki1p). The Etn-P is converted to CDP-ethanolamine by phosphoethanolamine cytidylyltransferase (Ect1p). The CDP-ethanolamine is then finally converted to PE by ethanolaminephosphotransferase (Ept1p). In CDP-choline pathway, exogenous choline is converted to phospho-ethanolamine (Etn-P) by ethanolamine kinase (Eki1p). The Etn-P is converted to CDP-ethanolamine by

phosphoethanolamine cytidylyltransferase (Ect1p). The CDP-ethanolamine then is converted to PE by ethanolaminephosphotransferase (Ept1p). Though Kennedy pathways are not major ways of PE and PC production in yeast, they do play roles for phospholipid biosynthesis.

The phospholipid PI is formed from the precursors inositol and CDP-DAG by phosphatidylinositol synthase (*PIS1*) (Fig 1-2). Although there is only one enzyme in yeast to generate PI, the inositol precuror may come from one of two sources. It can either be made *de novo* via a cyclization reaction in which glucose-6-phosphate is converted to inositol-3-phosphate by the Ino1p enzyme. Inositol-3-phosphate is then dephosphorylated to inositol by an inositolmonophosphatase encoded by *INM1* or *INM2*. Alternatively, inositol may be imported from the extracellular environment via the inositol transporters Itr1p or Itr2p.

Regulation of phospholipid biosynthesis in yeast

The regulation of phospholipid biosynthetic enzymes by inositol has been extensively studies in *S. cerevisiae*. The factors that regulate phospholipid biosynthesis include choline, ethanolamine, and lipids (e.g. phosphatidic acid) (23, 58, 99), but inositol has the strongest effect. Inositol is the rate limiting reagent in PI biosynthesis and *de novo* synthesis rates of inositol do not supply as much inositol for PI biosynthesis as imported inositol when concentrations are high (*i.e* 75μM). Thus, addition of inositol to the medium results in an increase in PI biosynthesis and subsequent decreases in levels of PC, PE, and PS (23). This is because both PI and PS are made from a common pool of CDP-DAG. As PI synthesis increases and CDP-DAG is consumed more rapidly for this branch of the pathway, PS synthesis is decreased and as a result the downstream aminophospholipids PE and PS are also decreased.

The regulation of the inositol level in the cell is very important, and the rates of synthesis of most phospholipid biosynthetic genes are controlled by a set of transcription factors that themselves are regulated by inositol levels. These transcription factors regulate *de novo* inositol biosynthesis by controlling expression of the inositol-3-phosphate synthase gene, *INO1*, as well as other genes (24). Ino2p and Ino4p activate the expression of the genes encoding for inosiol-3-phosphate synthase (*INO1*) and other phospholipid biosynthesis genes including *CHO1*, *CHO2*, and *OPI3*, while Opi1p represses the expression of these genes (Fig 1-2). The Opi1p repressor was named by its mutant phenotype of overproduction of inositol (59). Opi1p is a leucine zipper protein with two poly-glutamine rich domains but there is no evidence that this protein binds to

DNA directly. In the absence of exogenous inositol, Opi1p binds to phosphatidic acid (PA) in the endoplasmic reticulum (ER), hence its ability to translocate to the nucleus to interact with Ino2p is limited (99). In the presence of inositol, PA levels are decreased because PA is a precursor for PI biosynthesis, hence Opi1p is released from the ER and rapidly translocates to nucleus where it inhibits transcription of genes such as *INO1*, *CHO1*, *CHO2*, and *OPI3*. It inhbits transcription by binding to the Ino2p component of the Ino2-Ino4p heterodimeric transcriptional activator, which is bound at the upstream activating sequence for inositol biosynthesis (UAS_{INO}) located on the promoters of the above target genes (58). Opi1p has an Ino2p interacting domain, and Ino2p has an Opi1p interacting domain, and these have been demonstrated by *in vitro* co-purification experiments (67, 174).

Moreover, inositol phosphorylceramide (IPC) synthase which utilizes PI to synthesize sphingolipids, is also regulated by inositol. IPC synthase activity is elevated in wild-type cells supplemented with inositol (85).

Acquisition of phospholipid metabolite inositol in *C. albicans* and other human pathogens

In order to cause an infection, microbes must be able to live and grow inside the host. This necessitates the acquisition of essential nutrients by the pathogen. The pathogen must either obtain these nutrients from the host or synthesize the nutrients de novo from more basic compounds. The biosynthesis of phospholipids is an area that has received considerable attention both in terms of the insight it may give us regarding host pathogen interactions as well as the possibility that an understanding of phospholipid metabolism may help in the identification of new drug targets (172). Phospholipids are complex molecules that are essential for membrane integrity and intracellular signaling. The biosynthesis of these compounds requires a complex ordered set of biochemical steps involving a number of enzymes (58, 169, 172). An area of particular interest to pathogenic microbiologists is understanding how pathogens acquire basic molecules for the synthesis of phospholipids (172) such as myo-inositol, serine, choline or ethanolamine. In the last several years a number studies have been performed to elucidate the roles that proteins involved in myo-inositol acquisition play in controlling virulence and/or viability in a several divergent pathogens (25, 108, 109, 118). Several of these pathogens' acquisition systems are summarized in Fig 1-3.

Since the roles of virulence of inositol acquisition was described in *C. albicans* by our published work (25), we will here briefly review the roles of inositol biosynthesis in

virulence of *Mycobacterium tuberculosis* and *Trypanosoma brucei*. The prokaryotic pathogen *M. tuberculosis*, the causative agent of most cases of tuberculosis, can acquire inositol via de novo biosynthesis or import from environment. The *Mtino1* mutant exhibited inositol auxotrophy, but only high concentration of inositol (77mM) could rescue mutant's growth to wild-type level of growth. In addition, *Mtino1* mutant exhibited avirulence in SCID mice (118). In the parasite *T. brucei*, which causes fatal sleeping sickness, the *TbINO1* gene was shown to be necessary for inositol biosynthesis, efficient GPI biosynthesis, and even viability *in vitro*. The *Tbino1* mutants are inviable due to insufficient GPI production (108, 109).

Since these two very different pathogens both require *de novo* inositol biosynthesis to cause disease or grow, it suggests the possibility that other pathogens will have similar requirements. Thus, in chapter two we will describe a study that explores whether the fungal pathogen *Candida albicans* also requires *de novo* inositol biosynthesis to cause disease.

Phospholipid biosynthetic enzymes as drug targets in human pathogens

Effective drugs to treat various infectious diseases commonly work by targeting a pathway or activity that is crucial to the pathogen but not to the host. The target needs to be essential for pathogen's survival, and its inhibition should cause a cytotoxic effect rather than a cytostatic effect. Several groups have been identified phospholipid biosynthetic pathways as novel drug targets in fungal and parasitic pathogens (102, 182) (Table 1-2). Hence, the phospholipid biosynthetic pathway might serve as an Achilles' heel of human pathogens.

This part of the introduction will revolve around examples of phospholipid biosynthetic enzymes in bacteria, fungi, and parasites that may one day serve as drug targets. They include IPC synthase, serine-decarboxylase phosphoethanolamine methyltransferase (SDPM), PC synthase, and PS synthase, which are critical for pathogens' growth and have no homologs in human.

<u>Inositol phosphorylceramide (IPC) synthase in fungi and parasite</u>

Sphingolipids play roles as structural components of eukaryotic cellular membranes. In yeast, as in mammalian cells, the first and apparently rate-limiting step in sphingolipid metabolism involves the condensation of serine and palmitoyl-CoA in the endoplasmic reticulum (ER) by serine palmitoyl transferase (SPT) (Fig 1-6). SPT was shown to have two homologous subunits *LCB1* and *LCB2*, both of which are required for its activity. A third gene required for optimal SPT activity was identified as *TSC3*. After

the initial condensation of serine and palmitoyl-CoA into 3-keto dihydrosphingosine, it is converted to dihydrosphingosine by the enzyme 3-keto reductase. The reductase was also identified as *TSC10.* The next step in yeast sphingolipid synthesis appears to diverge between yeast and mammalian cells. In yeast, dihydrosphingosine (DHS) is hydroxylated into phytosphingosine, whereas in mammalian cells, dihydrosphingosine appears to be acylated to dihydroceramide before its reduction. The next step in yeast sphingolipid synthesis involves the acylation of the long chain base (LCB), phytosphingosine to phytoceramide. Ceramides in yeast are next incorporated into complex sphingolipids that are different from mammalian sphingolipids in that the head group is comprised of an inositol phosphate (IP) instead of a choline phosphate. PI acts as the donor for inositol phosphate. The enzyme involved in this step is encoded by Aur1p, an essential protein in yeast (122). This enzyme is also a target for another fungal toxin called aureobasidin, and because it is not present in mammalian cells, it has emerged as a potentially important target for anti-fungal drugs. IPC is further mannosylated in the Golgi to MIPC and MIP_2C by the enzymes Csg1p, Csg2p, and Ipt1p (41).

The fungal inositolphosphotransferase (Ipt1p), which is required for biosynthesis of mannosyl diinositol phosphorylceramide MIP_2C, was first characterized in *S. cerevisiae* (42). *CaIPT1*, a *ScIPT1* ortholog encoding the $M(IP)_2C$ synthase in *C. albicans*, was characterized, but the mutant's effect on virulence was not assessed (142). Since *CaIPT1* has no mammalian ortholog (145), it could be a potential phospholipid drug target if *CaIPT1* is demonstrated to be required for virulence.

The Del Poeta group demonstrated that the *Cryptococcus neoformans* IPC synthase mutant (*ipc1*Δ) exhibited reduced virulence in a rabbit animal model of cryptococcal meningitis (102). Meanwhile, Denny et al. isolated a functional ortholog of *AUR1* in parasite *Leishmania major*, causative agent of Leishmaniasis. Expression of this gene in a mammalian cell line led to the synthesis of an IPC-like species, strongly indicating that IPC synthase activity was reconstituted. The identification and characterization of this protozoan IPC synthase, an enzyme with no mammalian equivalent, will raise the possibility of developing anti-protozoal drugs with minimal toxic side affects (40).

Serine Decarboxylase Phosphoethanolamine Methyltransferase (SDPM) in parasite

Parasitic protozoa are surrounded by membrane structures that have different proportions of phospholipids relative to the membranes of the host. It was shown that some parasites like *Plasmodium falciparum* have unique phospholipid metabolic pathways (172). PC biosynthesis in *P. falciparum* is of particular interest since it is the most abundant lipid, accounting for half of the total PLs in parasite membranes. PC and PE comprise 40-50% and 35-45%, respectively, of the parasite's total plasma membrane phospholipid content (172). This is quite different from the plasma membrane of red blood cells in which it resides, where the concentrations of PC and PE are 30.3% and 26.9%, respectively (141).

Salom-Roig and Vial identified that rationally designed choline analogs can inhibit *Plasmodium de novo* PC biosynthesis, and exhibit antimalarial effect. These compounds also interact with malarial pigment, which could enhance the antimalarial effect. It is likely that they are dual molecules, exerting their antimalarial activity via two simultaneous toxic effects on the intracellular intraerythrocytic parasites (172). However, a potential problem with choline analogs might arise because choline is the precursor of the neurotransmitter acetylcholine. As an alternative approach, the Mamoum group has identified a PC biosynthetic enzyme as a possible anti-malarial drug target: serine decarboxylase phosphoethanolamine methyltransferase (SDPM) encoded by *PfPMT* gene (182).

PfPMT is required for optimum PC biosynthesis, but performs an enzymatic activity that is not conserved in mammals. The synthesis of PC in *P. falciparum* takes place via the Kennedy pathway, but phosphocholine for this pathway is supplied by importing it from the host or by making phosphocholine via the serine-decarboxylase phosphoethanolamine methyltransferase (SDPM) pathway. The SDPM pathway depends on the *PfPMT* enzyme which methylates phosphoethanolamine to phosphocholine (Fig 1-7). This differs from mammals and yeast where PC is made by the Kennedy pathway only from imported choline or by the *de novo* pathway through methylating PE (Fig 1-2). Disruption of *PfPMT* function results in a complete loss of the SDPM pathway. This drop in phosphocholine compromises PC biosynthesis and results in defects in parasite growth, multiplication, and viability, suggesting this gene plays an important role in the pathogenesis of intraerythrocytic *Plasmodium* parasites (182). *PfPMT* is a member of PEAMT family of phosphoethanolamine methyltransferase and has no mammalian homologs. The restricted phylogenetic distribution of this class of enzymes suggests that

they could be potential targets for the development of drugs to treat malaria and other parasitic diseases.

Phosphatidylcholine synthase in bacteria

The prokaryotic phospholipid biosynthesis pathway is quite different from eukaryotic cells. For example, the eukaryotic PC biosynthesis and salvage pathways were missing in most prokaryotic species. In addition, phospholipid biosynthesis in prokaryotic cells is divergent among species. For example, there is no salvage pathway for PC production in *E. coli*, but a salvage pathway for PC production does exist in *Brucella abortus* (30)(Fig 1-8). However, the salvage pathway for PC production between mammalian cells and *B. abortus* cells is dramatically different. The mammalian cells use the Kennedy pathway, where choline is converted to CDP-choline and then DAG and CDP-choline are used to make PC and CMP. In *B. abortus* choline and CDP-DAG are used to generate PC and CMP (Fig 1-8) in the choline dependent pathway. The *PcsA* mutant, which lacks this activity, exhibits defective mouse spleen colonization (30). Therefore, the PC synthase (*PcsA*) of the *B. abortus* choline dependent pathway could be a potential drug target. In fact, there is no *PcsA* homolog in mammalian cells based on BLAST searches of the PcsA protein against human proteins. Moreover, phospholipid methyltransferase (*PmtA*) of *B. abortus* which methylates PE to form PC also has no mammalian homolog. The *PmtA* mutant also exhibits defective mouse spleen colonization (30). Conover et al. have demonstrated a similar story in *Legionella pneumophila*, a gram-negative respiratory bacteria causing severe pneumonia. *L. pneumophila* exhibited two pathways to synthesize PC either by phospholipid *N*-methyltransferase (*PmtA*) or phosphatidylcholine synthase (*PcsA*), but the latter pathway was demonstrated predominate. The *LpPcsA* mutant exhibited defective growth in marcrophages (31).

Phosphatidylserine synthase in fungi and bacteria

Our lab has demonstrated that phosphatidylserine synthase (*CHO1*) in *C. albicans* is essential for virulence and cell wall integrity (Chapter 3). The Cho1p orthologs in other organisms were obtained (Table 1-3), but there are no human or mammalian orthologs. Therefore, Cho1p could be potential drug target.

The biological function of PS has been analyzed in *S. cerevisiae*. Although PS is a quantitatively minor phospholipid in most biological membranes, it is important for specific cellular functions in addition to its structural role in membrane. In addition to the PE precursor, PS is an enzyme cofactor. The best known role of PS as an enzyme

cofactor is as a specific activator of protein kinase C (PKC). PKC plays crucial roles in diverse signal transduction pathways (170). PKC contains a C1 and C2 domain, both are required for translocation of kinase from cytoplasm to the inner leaflet of the plasma membrane. The C2 domain is responsible for the binding of PKC to PS in a calcium-dependent manner and the specificity for recognition of PS by the enzyme requires the lipid second messenger diacylglycerol. Another protein that requires PS for its function is cRaf1 protein kinase. Interaction of Raf-1 with PS promotes the translocation of the protein to the plasma membrane as well (121).

Politino and Kim demonstrated that PS physically interacted with calcineurin, a immunosuppressive reagent. The interactions with PS and calcineurin were enhanced by Ca^{2+} which implicates a regulatory role for the Ca^{2+}-binding subunit in this process. This also suggests that Ca^{2+}- and calmodulin-stimulated interactions of calcineurin with acidic phospholipids may play a role in regulating the substrate specificity of this multifunctional phosphatase (140). A study also highlighted the importance of lipid signaling molecules in the development and pathogenicity of clinically important fungi. In *Cryptococcus neoformans*, sphingolipid-derived diacylglycerol has been shown to induce the transcription of the putative virulence factor App1, which inhibits the phagocytosis of fungal cells by alveolar macrophages, as well as to activate the protein kinase C Pkc1, which promotes cell-wall stability and increased melanin production (153).

Recently, Bukata et al. demonstrated that phosphatidylserine synthase (PssA) is required for optimal virulence in *B. abortus*. The *BaPssA* mutant exhibited defective mouse spleen colonization (18). There is no *BaPssA* ortholog found in mammalian cells. Taken together, the results show that phosphatidylserine synthase in fungi, and bacteria could be potential antimicrobial drug targets.

APPENDIX A: TABLES AND FIGURES

Table 1- 1. Antifungal spectrum of activity against common fungi.

Table adapted from (43).

	Antifungal agent								
Organism	AmB[a]	Flu	Itr	Vor	Pos	Anidulafungin	Caspofungin	Micafungin	Flucytosine
Aspergillus species	+	−	+	+	+	+	+	+	−
A. flavus	±	−	+	+	+	+	+	+	−
A. fumigatus	+	−	+	+	+	+	+	+	−
A. niger	+	−	±	+	+	+	+	+	−
A. terreus	−	−	+	+	+	+	+	+	−
Candida species	+	+	+	+	+	+	+	+	+
C. albicans	+	+	+	+	+	+	+	+	+
C. glabrata	+	±	±	+	+	+	+	+	+
C. krusei	+	−	±	+	+	+	+	+	±
C. lusitaniae	−	+	+	+	+	+	+	+	+
C. parapsilosis	+	+	+	+	+	±	±	±	+
C. tropicalis	+	+	+	+	+	+	+	+	+
Cryptococcus neoformans	+	+	+	+	+	−	−	−	+
Coccidioides species	+	+	+	+	+	±[b]	±[b]	±[b]	−
Blastomyces	+	+	+	+	+	±[b]	±[b]	±[b]	−
Histoplasma species	+	+	+	+	+	±[b]	±[b]	±[b]	−
Fusarium species	±	−	−	+	+	−	−	−	−
Scedosporium apiospermum	±	−	±	+	+	−	−	−	−
Scedosporium prolificans	−	−	−	±	±	−	−	−	−
Zygomycetes	±	−	−	−	+	−	−	−	−

Table 1- 2. Phospholipid biosynthetic genes as potential drug targets in pathogenic microbes.

Fungus			
C. albicans: Candidiasis, Vaginitis, Oral Thrush			
gene	product	Mutant virulence	Reference
CHO1	Phosphatidylserine synthase	Avirulence in mouse model of systemic infection	Unpublished
IPT1	Inositol phosphoryl transferase	N/A (hyphal growth abnormal)	(142)
***C. neoformans*: Meningitis and Meningo-encephalitis**			
IPC1	Inositol phosphoryl ceramide synthase	Reduced virulence in a rabbit animal model of cryptococcal meningitis	(102)
A. nidulans*: Aspergillosis*			
BarA	acyl-CoA-dependent ceramide synthase	N/A (Hyphal growth abnormal)	(95)
LagA	Ceramide synthase	N/A (Hyphal growth abnormal)	(95)
Parasite			
***Leishmania major*: Leishmaniasis**			
IPCS	Inositol Phosphorylceramide Synthase	N/A	(40)
***Plasmodium falciparum*: Malaria**			
PMT	Phosphoethanolamine methyltransferase	Defects of parasite growth, multiplication, and viability in erythrocytes	(182)
Bacterium			
***Brucella abortus*: Brucellosis**			
PssA	Phosphatidylserine synthase	Defective for mouse spleen colonization	(17)
PmtA	Phospholipid N-methyltransferase	Defective for mouse spleen colonization	(30)
Pcs	Phosphatidylcholine synthase	Defective for mouse spleen colonization	(30)
Legionella pneumophila			
PcsA	Phosphatidylcholine synthase	Defective growth in macrophage	(31)

Table 1- 3. Cho1p amino acid identity between *C. albicans* and other organisms.

Organism	Gene	%	E value
Candida albicans SC5314	phosphotidylcholine synthase	100%	1e-157
Pichia stipitis CBS 6054	phosphotidylcholine synthase	81%	1e-122
Lodderomyces elongisporus NRRL YB-4239	CDP-diacylglycerol-serine O-phosphatidyltransferase	76%	6e-113
Debaryomyces hansenii	DEHA2B15686p	73%	4e-109
Pichia guilliermondii ATCC 6260	hypothetical protein PGUG_02313	72%	5e-104
Kluyveromyces lactis	hypothetical protein	65%	9e-92
Ashbya gossypii ATCC 10895	AER357Cp	61%	1e-87
Vanderwaltozyma polyspora	hypothetical protein Kpol_1035p53	62%	2e-87
Saccharomyces cerevisiae YJM789	phosphatidylserine synthase	61%	5e-87
Candida glabrata	**hypothetical protein**	**60%**	**3e-86**
Yarrowia lipolytica	YALI0D08514p	58%	4e-75
Penicillium marneffei ATCC 18224	phosphatidylserine synthase	57%	7e-67
Schizosaccharomyces pombe	CDP-diacylglycerol--serine O-phosphatidyltransferase	54%	3e-64
Sclerotinia sclerotiorum 1980	hypothetical protein SS1G_10973	62%	4e-64
Botryotinia fuckeliana B05.10	hypothetical protein BC1G_11334	61%	3e-63
Neurospora crassa OR74A	hypothetical protein NCU02381	55%	8e-63
Pyrenophora tritici-repentis Pt-1C-BFP	phosphatidylserine synthase	54%	2e-62
Schizosaccharomyces japonicus yFS275	CDP-diacylglycerol-serine O-phosphatidyltransferase	53%	6e-62
Podospora anserina	unnamed protein product	54%	7e-62
Phaeosphaeria nodorum SN15	hypothetical protein SNOG_12017	51%	2e-61
Magnaporthe grisea 70-15	hypothetical protein MGG_03550	52%	5e-61

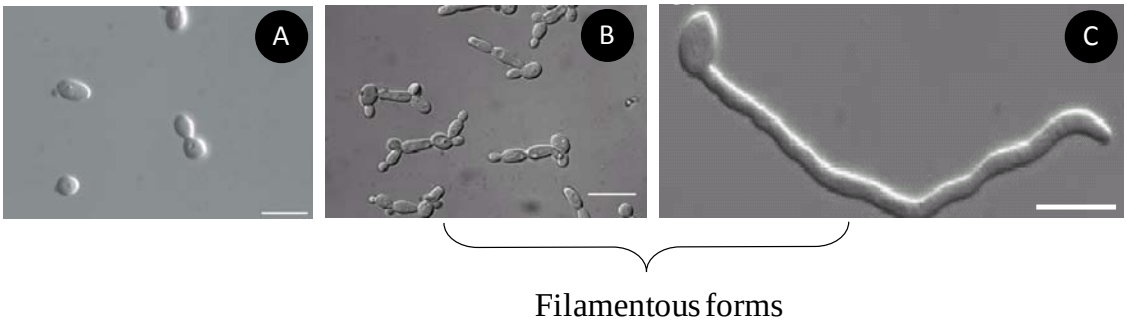


Fig 1- 1. *Candida albicans* can grow in at least three different morphologies.

(A) Yeast. (B) Pseudohyphae. (C) Hypha.

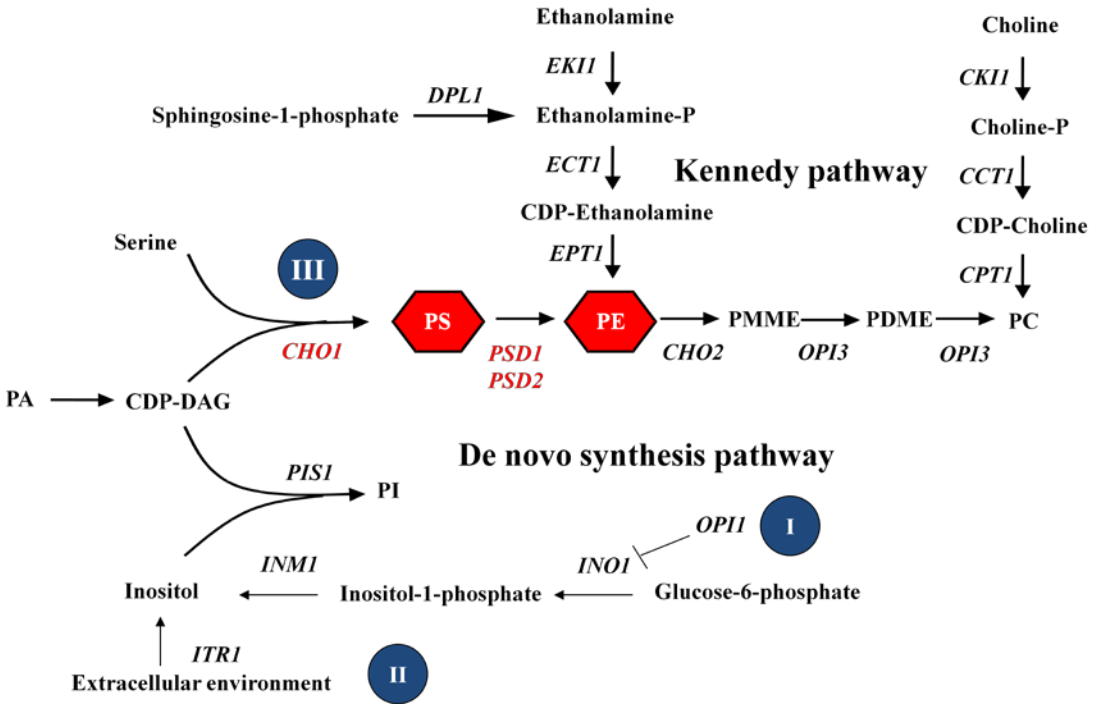


Fig 1- 2. Phospholipid biosynthetic pathways in *S. cerevisiae* are used as a model to explore phospholipid biosynthesis in *C. albicans*.

The *CHO1* gene encodes the only phosphatidylserine (PS) synthase in yeast, and it forms PS *de novo* from serine and CDP-diacylglycerol (CDP-DAG). As the *de novo* pathway continues PS is decarboxylated by the enzymes *PSD1* or *PSD2* to generate phosphatidylethanolamine (PE). PE is methylated by the enzymes *OPI3* and *CHO2* to make phosphatidylcholine (PC). Phosphatidylinositol (PI) is made in a separate *de novo* pathway by *PIS1* from CDP-DAG and inositol. The cell may also generate PE and PC from imported exogenous ethanolamine (Etn) or choline (Cho) and DAG via the Kennedy Pathway. *S. cerevisiae* and *Candida albicans* has two equally effective mechanisms for obtaining inositol while in the host. It can either generate inositol *de novo* through *INO1*, or it can import it from the host through *ITR1*.

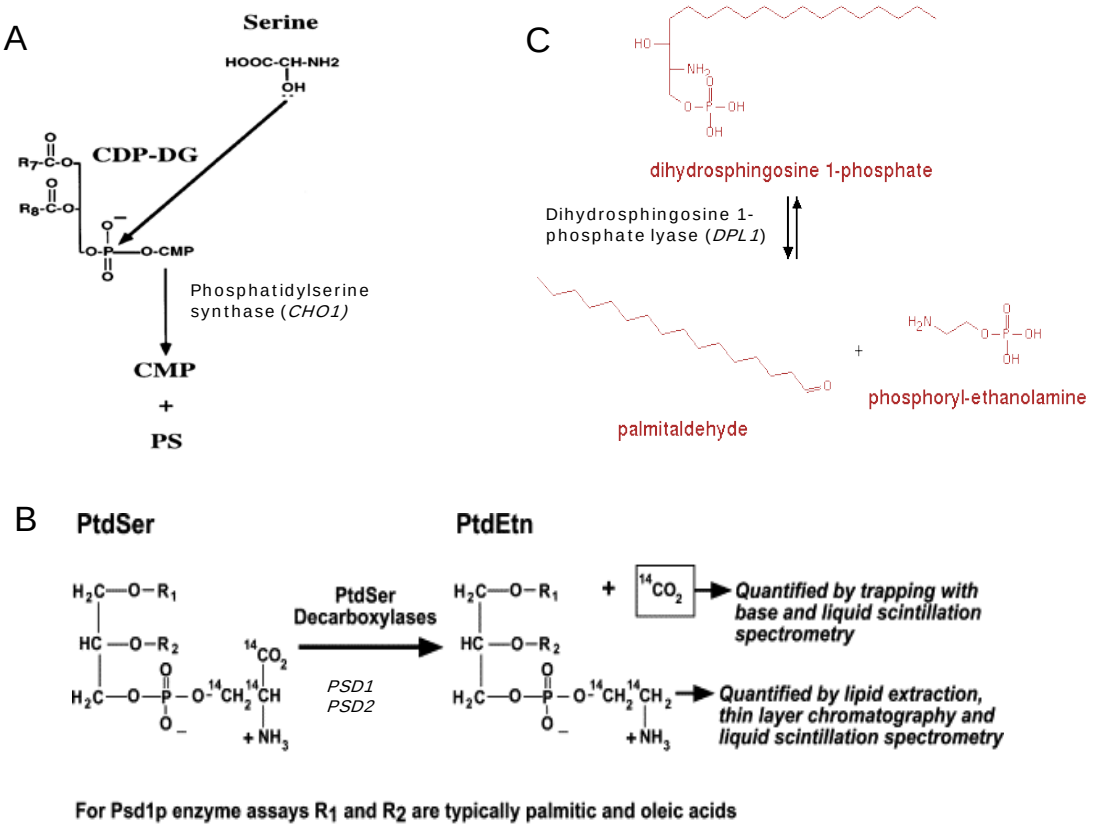
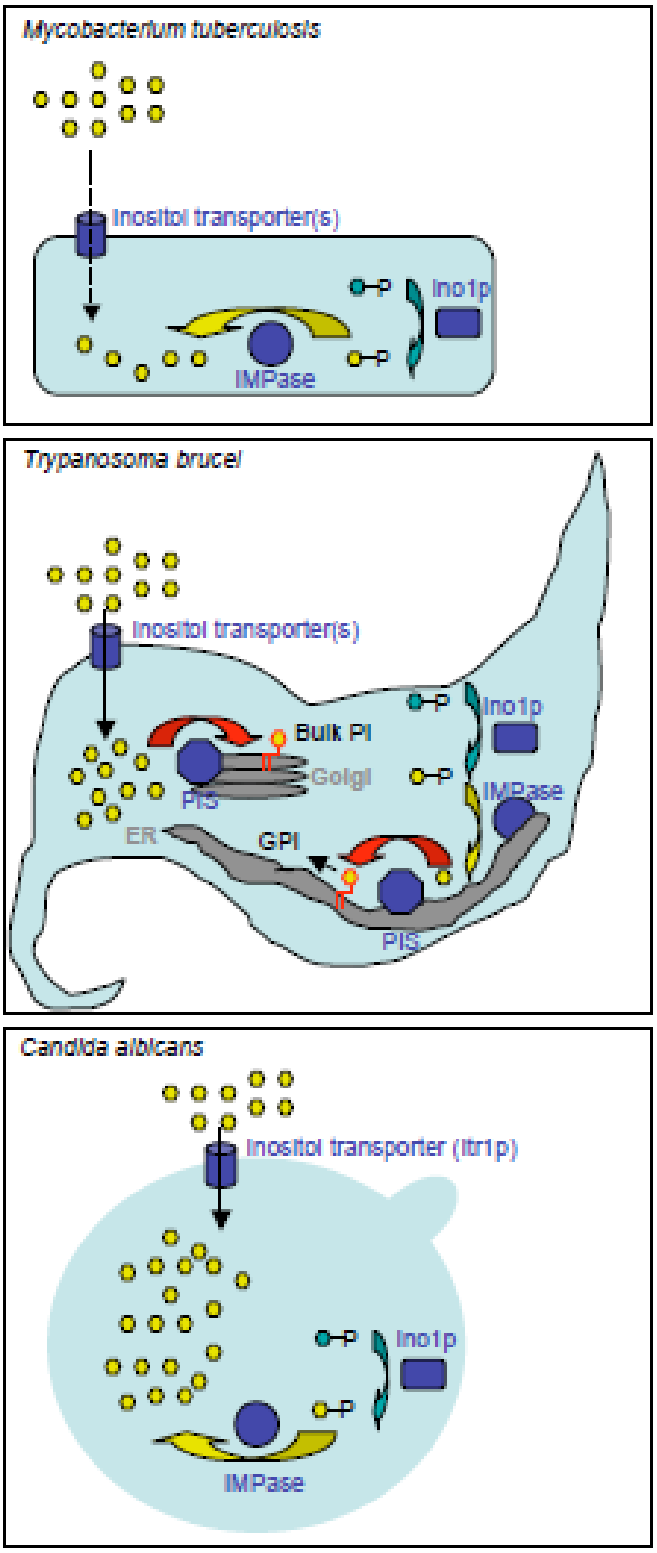


Fig 1- 3. Biosynthesis of phospholipids. (A), phosphatidylethanolamine (B), and phosphoryl-ethanolamine (C).

Refer texts for details. Figures modified from (27, 165) and SGD (http://www.yeastgenome.org/).

Fig 1- 4. Different pathogens utilize diverse strategies for inositol acquisition.

All three microbes represented in this figure are able to generate inositol (yellow hexagon) *de novo* by first converting glucose-6-phosphate (green hexagon-P) to inositol-3-phosphate (yellow hexagon-P) via the enzyme inositol-3-phosphate synthase (Ino1p). Next, an inositol monophosphatase (IMPase) dephosphorylates inositol-3-phosphate to make inositol. All three microbes have at least some level of inositol transport activity through one or more inositol transporters. *Mycobacterium tuberculosis*: This prokaryotic pathogen is able to both synthesize inositol *de novo* as well as import inositol from the environment. However, transport may not be very efficient (as indicated by the dotted arrow passing through the inositol transporter) since the *Mtino1* mutant must be grown in 77mM inositol to allow wild-type levels of growth, and mutants lacking the *MtINO1* gene are avirulent. *Trypanosoma brucei*: This eukaryotic parasite is able to import inositol from the environment or synthesize it *de novo*. However, according to the current model, inositol imported from the environment is utilized primarily in bulk phosphatidylinositol (red and yellow phospholipid) production via a phosphatidylinositol synthase (PIS) localized to the Golgi complex. Inositol synthesized *de novo* is primarily used to generate phosphatidylinositol that is used for production of glycosylphosphatidylinositol (GPI). The *de novo* inositol is believed to be utilized mostly for GPI production because the IMPase that converts inositol-3-phosphate to inositol is localized to the ER where GPI synthesis occurs. Mutants lacking *TbINO1* are inviable because of diminished GPI production. *Candida albicans*: This fungal pathogen can acquire inositol by *de novo* synthesis or by importing it, and either mechanism is sufficient to support wild-type growth in vitro and full virulence. Figure adapted from Reynolds' unpublished data.


Mycobacterium tuberculosis
Inositol transporter(s)
Ino1p
IMPase
Trypanosoma brucei
Inositol transporter(s)
Ino1p
Bulk PI
Golgi
PIS
IMPase
ER
GPI
PIS
Candida albicans
Inositol transporter (itr1p)
Ino1p
IMPase

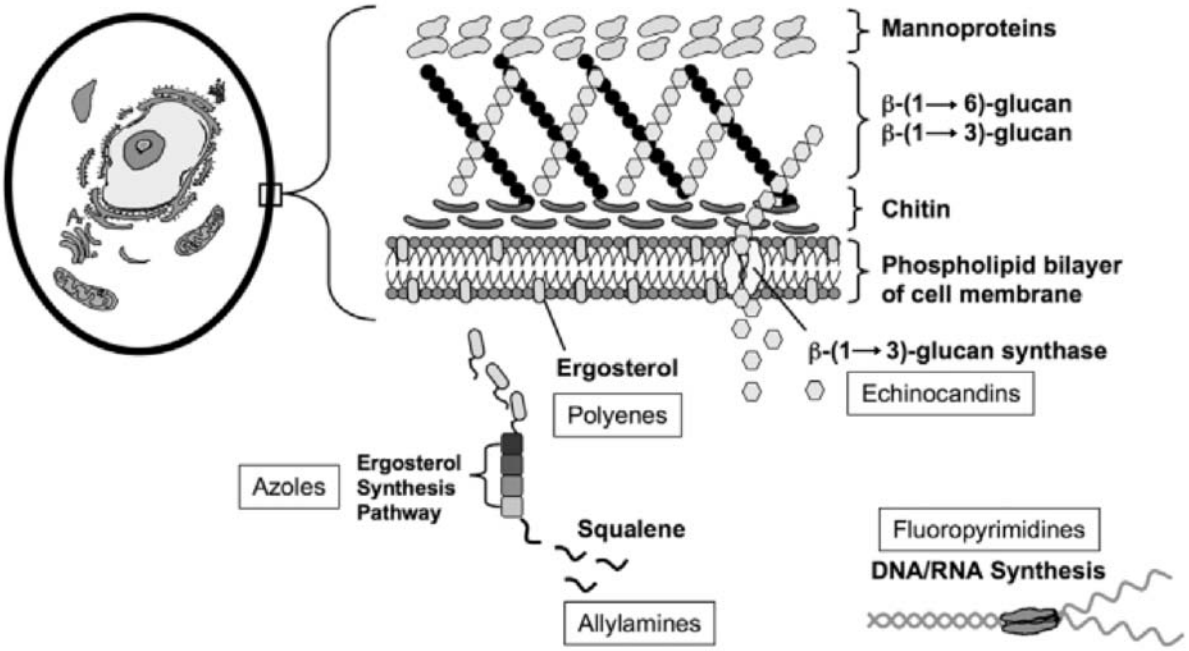


Fig 1- 5. Targets of systemic antifungal agents. Refer texts for details.

Figure adapted from (43).

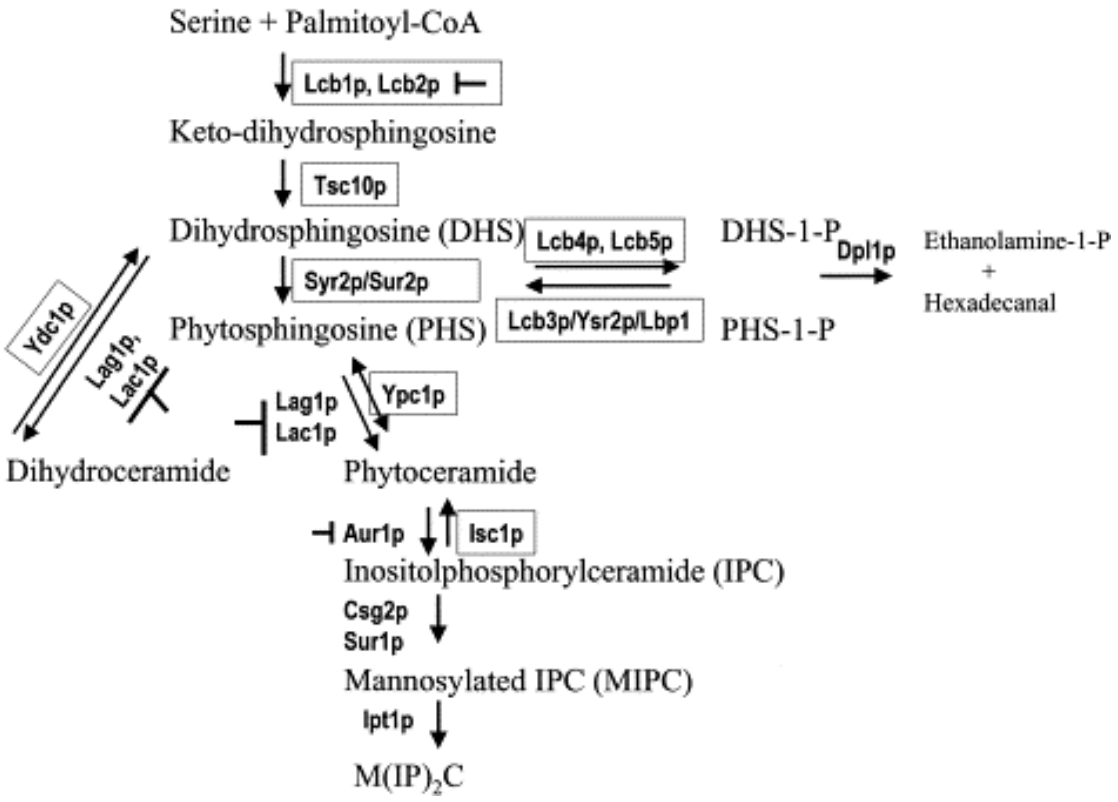


Fig 1- 6. Outline of sphingolipid metabolism in Saccharomyces cerevisiae.

Refer texts for details. Figure adapted from (130).

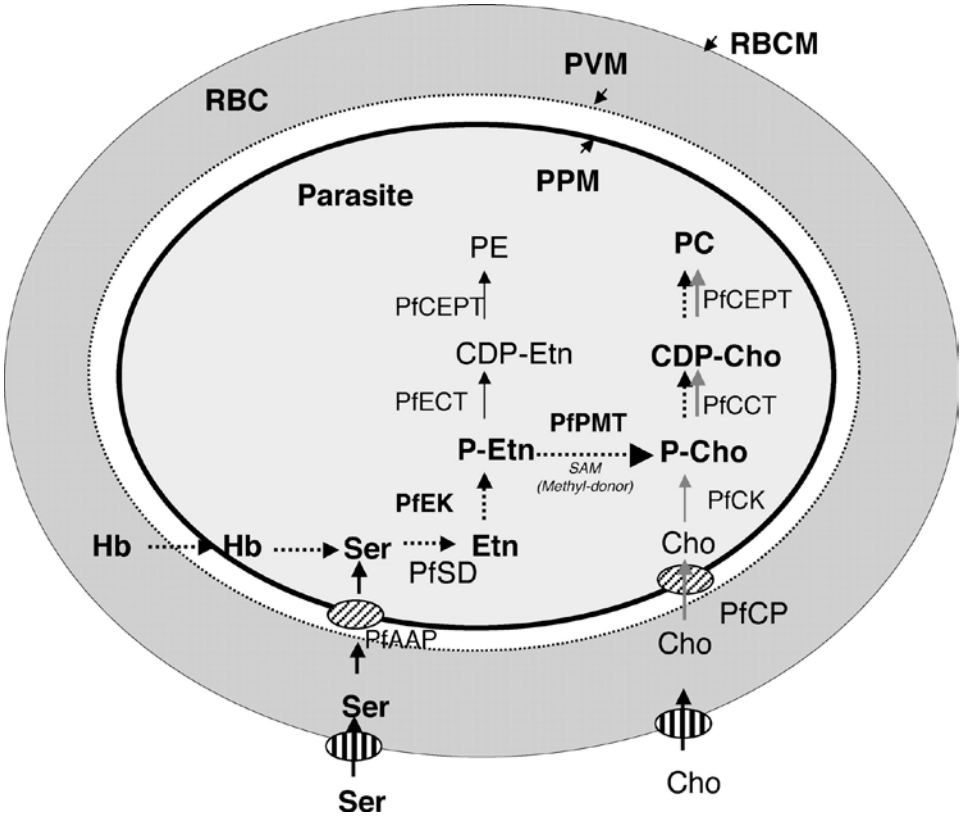


Fig 1- 7. Pathways for the biosynthesis of phosphatidylcholine in *P. falciparum*.

The CDP-choline pathway is shown with *gray arrows*. The SDPM pathway is depicted by *dotted arrows*. *PfAAP*, amino acid permease; *Cho*, choline; *CDP*, cytidine diphosphate; *CDP-cho*, CDP-choline; *CDP-Etn*, CDP-ethanolamine; *Hb*, hemoglobin; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PfCCT*, *P. falciparum* CTP phosphocholine cytidylyltransferase; *PfCEPT*, *P. falciparum* choline/ethanolamine-phosphate transferase; *PfCK*, *P. falciparum* choline kinase; *PfCP*, *P. falciparum* choline permease; *PfECT*, *P. falciparum* CTP phosphoethanolamine cytidylyltransferase; *PfEK*, *P. falciparum* ethanolamine kinase; *PfPMT*, *P. falciparum* phosphoethanolamine methyltransferase; *PPM*, parasite plasmamembrane; *PVM*, parasitophorous vacuolar membrane; *RBC*, red blood cell; *RBCM*, red blood cell membrane; *PfSD*, serine decarboxylase; *Ser*, serine; *SAM*, *S*-adenosyl-L-methionine. Figure adapted from (182).

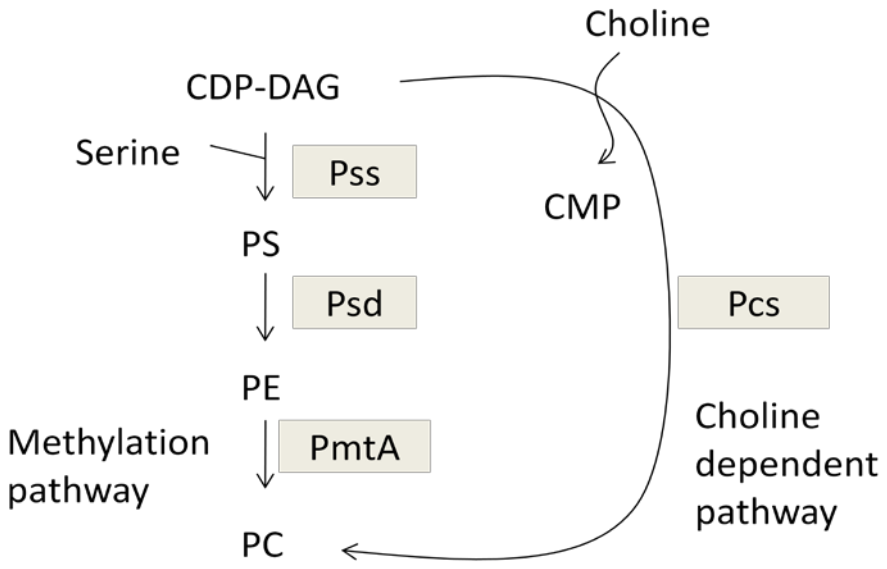


Fig 1- 8. Possible phospholipid biosynthetic pathways in prokaryotic *Brucella abortus* and *Legionella pneumophila*.

Chapter 2: *Candida albicans* uses multiple mechanisms to acquire the essential metabolite inositol during infection

This work is lightly adapted from Publication citation: Infection and Immunity. 2008. June p.2793-2801 coined by **Ying-Lien Chen, Sarah Kauffman and Todd B. Reynolds**
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ABSTRACT

Candida albicans is an important cause of life-threatening systemic bloodstream infections in immunocompromised patients. In order to cause infections *C. albicans* must be able to synthesize the essential metabolite inositol or acquire it from the host. Based on its similarity to *Saccharomyces cerevisiae* it was predicted that *C. albicans* may generate inositol *de novo*, import it from the environment, or both. The *C. albicans* inositol synthesis gene *INO1* and inositol transporter gene *ITR1* were each disrupted. The *ino1Δ/ino1Δ* mutant was an inositol auxotroph, and the *itr1Δ/itr1Δ* mutant was unable to import inositol from the medium. Each of these mutants were fully virulent in a mouse model of systemic infection. It was not possible to generate an *ino1/ino1Δ itr1Δ/itr1Δ* double mutant suggesting that in the absence of these two genes *C. albicans* could not acquire inositol and was inviable. A conditional double mutant was created by replacing the remaining wild-type allele of *ITR1* in an *ino1Δ/ino1Δ itr1Δ/ITR1* strain with a conditionally expressed allele of *ITR1* driven from the repressible *MET3* promoter. The resulting *ino1Δ/ino1Δ itr1Δ/*P_{MET3}*::ITR1* strain was found to be inviable in medium containing methionine and cysteine (which represses the P_{MET3} promoter), and it was avirulent in the mouse model of systemic candidiasis. These results suggest a model in which *Candida albicans* has two equally effective mechanisms for obtaining inositol while in the host. It can either generate inositol *de novo* through Ino1p, or it can import it from the host through Itr1p.

INTRODUCTION

Candida albicans is a dimorphic yeast that can exist in a human host as either a harmless commensal, or as an opportunistic pathogen when the host's immune system is impaired (20). In patients that are neutropenic, *Candida* species are associated with severe and deadly systemic bloodstream infections with a high mortality rate (31.8%) (116). In fact, *Candida* species are the fourth most common cause of catheter-related blood stream infections in hospitalized patients in the intensive care unit, and *C. albicans* is the most commonly isolated species from these infections (19).

The ability of any pathogenic microbe to cause an infection depends on the organism's ability to acquire or generate essential nutrients during residence within the host. There are several examples of auxotrophies that compromise virulence in *C. albicans* (82). The most notable example is the defect in uracil biosynthesis caused by a mutation in *URA3*. Homozygous *URA3* mutants (*ura3*Δ*/ura3*Δ) block the ability of *C. albicans* to grow without uridine supplementation and lead to avirulence. In fact, the level of *URA3*-encoded orotidine 5'-monophosphate decarboxylase activity can be correlated with growth and virulence (88). Homozygous mutations in the *ADE2* gene, which blocks the ability of *C. albicans* to grow in minimal medium or serum in the absence of exogenous adenine also diminish virulence (45). The homozygous mutations in the *HEM3* gene leading to heme auxotrophy compromise virulence (82). In addition, an induced temperature-sensitive serine auxotroph was significantly less virulent than the parent strain (106).

In contrast to these results, auxotrophies for a number of amino acids including serine, lysine, leucine, histidine, and arginine do not appear to compromise virulence (106, 128, 155). For example, Noble and Johnson (128) demonstrated that *C. albicans his1*Δ/*his1*Δ, *his1*Δ/*his1*Δ *leu2*Δ/*leu2*Δ, and *his1*Δ/*his1*Δ *arg4*Δ/*arg4*Δ auxotrophic mutants exhibited wild-type virulence in a mouse model of systemic candidiasis, whereas *his1*Δ/*his1*Δ *leu2*Δ/*leu2*Δ *arg4*Δ/*arg4*Δ triply auxotrophic strains were only mildly attenuated in virulence compared to the wild-type. These results suggest that there are some metabolites such as amino acids that *C. albicans* can scavenge from the host during an infection, while there are other metabolites that it must make *de novo* such as uracil, adenine, and heme.

Myo-inositol (referred to here as inositol) is essential for the growth of all eukaryotes, and is needed by some bacteria (63). Inositol is involved in many intracellular processes including growth regulation, membrane structure, osmotolerance,

signal transduction, and the formation of glycosylphosphatidylinositol (GPI)-anchored proteins, which are themselves essential (11, 24, 133, 161). Inositol has been implicated in the pathogenicity of *C. albicans* because it is an essential precursor for phospholipomannan, a GPI-anchored glycolipid on the cell surface of *Candida* which is involved in pathogenicity (115).

There are three potential sources of inositol available to *C. albicans* based on work that has been done in the related yeast *S. cerevisiae*: 1) *de novo* biosynthesis, where glucose 6-phosphate is converted to inositol 1-phosphate by the inositol-1-phosphate synthase enzyme; 2) inositol import from the extracellular environment by an inositol transporter; and 3) recycling of inositol from the dephosphorylation of inositol polyphosphate products (24, 125, 161).

In *S. cerevisiae*, *de novo* inositol biosynthesis and import have both been well studied. The *S. cerevisiae* inositol 1-phosphate synthase is encoded by the *INO1* gene (44). The Ino1p enzyme converts glucose-6-phosphate to inositol-1-phosphate, and then a second enzyme, inositol monophosphatase, encoded by the *INM1* gene, dephosphorylates inositol-1-phosphate to create inositol (101). *S. cerevisiae* carries two distinct inositol transporter genes, *ScITR1* and *ScITR2*, which are both capable of importing inositol, but at different efficiencies (126).

C. albicans carries a putative homolog of *INO1* based on sequence similarity which was identified and sequenced by Klig *et al* (Genbank accession #L22737) (83, 84). *C. albicans INO1* shows 64% identity to *S. cerevisiae INO1* at the amino acid level (84). In addition, it has been shown that *C. albicans* has a potent inositol transporter activity (77). It was demonstrated that the inositol transporter in *C. albicans* has an apparent Km value of 240±15 μM, which appears to be active and energy-dependent (77). *C. albicans* inositol transport activity differs in substrate and cotransporter specificity from the human inositol-Na^+ transporter. However, the inositol transporter gene in *C. albicans* was not identified in this previous study.

Recently, the importance of inositol biosynthesis in some human infectious agents was demonstrated by reports in two unrelated pathogens, *Mycobacterium tuberculosis* and *Trypanosoma brucei*. In *T. brucei* its *INO1* homolog was shown to be necessary for inositol biosynthesis, efficient GPI synthesis, and growth *in vitro* (108, 109). In *M. tuberculosis*, it was found that its *INO1* homolog was required for *de novo* inositol biosynthesis and full virulence in a mouse model of infection (118).

It is unknown what roles inositol import or biosynthesis play in the virulence of *C.*

albicans. This fungus may be able to both synthesize and import inositol. It became of interest to determine if *C. albicans* would behave like *M. tuberculosis* and *T. brucei* and require *INO1* function for virulence/viability, or if in contrast to these pathogens, *C. albicans* would be able to utilize either *de novo* synthesis or inositol import to support virulence.

MATERIALS AND METHODS

Strains and growth media

C. albicans strains and plasmids used in this study are shown in Table 2-1 and Table 2-2 (see all tables and figures in the appendix A). Media used in this study include YPD (yeast extract-peptone-dextrose, 1% yeast extract, 2% peptone, 2% glucose) (163), defined medium 199 (M199, Invitrogen), yeast carbon base-bovine serum albumin (YCB-BSA) (154), and minimal medium (MM, 0.67% Difco yeast nitrogen base without amino acids, 2% glucose) (163). MM medium was supplemented with 75 μM inositol for supporting the growth of the *C. albicans ino1*Δ/*ino1*Δ mutant and 2.5mM methionine and cysteine for *MET3* promoter shutoff assays (22). Inositol-free media (163) was made for testing the phenotype of the *ino1*Δ/*ino1*Δ mutant. Agar plates were solidified with 2% agar (granulated, Fisher) for YPD and with 2% BactoAgar (contains no residual inositol) for MM and inositol-free media.

Strain construction

The *C. albicans INO1* gene (*INO1*) was disrupted by using the *CaNAT1-FLP* cassette (154), whereas, the *C. albicans ITR1* gene (*ITR1*) was disrupted by using the *SAT1* flipper (143).

For the *INO1* disruption construct, the 564 basepair (bp) 5' non-coding region (NCR) of *INO1* was amplified with primers JCO39 and JCO40 (Table 2-3), which introduced *Kpn*I and *Apa*I restriction sites, and was cloned into pJK863 5' of the *CaNAT1-FLP* cassette (Fig 2-1A). The 583bp 3' NCR of *INO1* was amplified with primers JCO41 and JCO42 which introduced *Sac*II and *Sac*I sites, and was cloned into pJK863 3' of the *CaNAT1-FLP* cassette (Fig 2-1A). This created the *INO1* knock out construct plasmid pYLC94 (Table 2-2, Fig 2-1A), which was cut with *Kpn*I and *Sac*I to release the disruption construct which was transformed into the wild type SC5314 strain by electroporation as described previously (36, 143). The disruption construct was used to sequentially disrupt both alleles of *INO1* as previously described (154). The *INO1* reconstitution construct was made by amplifying a 2.1 Kb fragment containing the *INO1*

ORF and 5' NCR from SC5314 genomic DNA using primers (JCO39 and JCO47) that introduced *Kpn*I and *Sal*I sites. This fragment was ligated into the pRS316 vector along with a 1.7 Kb fragment containing the *NAT1*-3'*INO1*^{NCR} amplified from plasmid pYLC94 using primers JCO50 and JCO42 which introduced *Sal*I and *Sac*I sites. This resulted in the *INO1* reconstitution plasmid pYLC119 (Table 2-2, Fig 2-1B). The 3.8Kb *Kpn*I-*Sac*I fragment from pYLC119 was transformed into the *ino1*Δ/*ino1*Δ mutant (YLC113) in order to create the reconstituted *ino1*Δ/*ino1*Δ::*INO1* strain (YLC120).

A similar approach was used to knock out *ITR1* with the *SAT1* flipper plasmid pSFS2A (143). Approximately 500 bp 5' and 3' NCRs from *ITR1* were amplified using the primer pairs JCO89-JCO90 and JCO93-JCO94, respectively, each of which introduced restriction sites into the corresponding fragments. The *Apa*I-*Xho*I 5'*ITR1*^{NCR} and *Not*I 3'*ITR1*^{NCR} fragments were ligated into pSFS2A resulting in the *ITR1* knock out construct plasmid pYLC164 (Table 2-2, Fig 2-2A). The 5Kb *Apa*I-*Sac*II fragment from pYLC164 was electroporated into SC5314 and used to disrupt both copies of *ITR1* by sequential disruptions (117, 143). For the reconstituted construct, JCO89 and JCO111 primers, which added *Apa*I and *Eco*RI sites, were used to amplify a 2Kb fragment containing the 5'*ITR1*^{NCR} and *ITR1* ORF region, which was used to replace the *Apa*I-*Eco*RI fragment (containing 5' *ITR1*^{NCR}-P_{MAL2}-*FLP*) in the pYLC164 vector resulting in pYLC208 (Table 2-2, Fig 2-2B). The 5Kb *Apa*I-*Sac*II fragment from pYLC208 was used to reconstitute *ITR1* in the *itr1*Δ/Δ mutant (YLC196) strain creating the *itr1*Δ/Δ::*ITR1* strain (YLC211).

The *ITR1* conditional mutant was made as follows. Using SC5314 genomic DNA as template, primers JCO118 and JCO119 (introduced *Pst*I and *Not*I sites) were used to amplify a 1362bp fragment containing the *CaMET3* promoter (22), and primers JCO120 and JCO121 (introduced *Not*I and *SacII* sites) were used to amplify the *ITR1* ORF. These fragments were used to replace the *Pst*I-*Sac*II fragment in pYLC164, which deleted the 3' flanking FRT site, resulting in the conditional *ITR1* expression construct plasmid pYLC229 (Table 2-2, Fig 2-2C). The 7.6 Kb *Apa*I-*Sac*II fragment from pYLC229 was transformed into the *ino1*Δ/*ino1*Δ *itr1*Δ/*ITR1* strain (YLC184) to create the *ino1*Δ/*ino1*Δ *itr1*Δ/P_{MET3}::*ITR1* conditional strains (YLC261 and YLC266).

Northern blot analysis

Northern blotting for *ITR1* expression was performed as described (144) with the following exceptions. Strains grown in liquid medium 199 at 37°C for 2 hrs were collected for total RNA extraction by the hot phenol method (144). A PCR product

containing bps 24-750 of the *ITR1* ORF (primers JCO102 and JCO103) was used as a probe. Expression was normalized against *C. albicans ACT1* gene expression probed on the same membrane. The *ACT1* probe was generated with the primers JCO48 and JCO49.

Southern blot analysis

Hybridization conditions for the Southern blot analysis were similar to those for Northern blot analysis, except that the Techne Hybrigene oven was set to 60°C for the incubation step, and 42°C and 60°C for washing steps. The cells were grown in liquid YPD at 30°C overnight. The genomic DNA was extracted using the Winston-Hoffman method (70) and 20µg of genomic DNA were subjected to Southern blotting. The genomic DNA of *ino1* mutants was cut by *Afl*II and *Sph*I restriction enzymes while the genomic DNA of *itr1* mutants was cut by *Pst*I. PCR products containing the ~500bp 3' NCR of *INO1* (primers JCO41 and JCO42) and 3'NCR of *ITR1* (primers JCO93 and JCO94) were used as probes.

Inositol uptake assays

The inositol uptake assay protocol was adapted in part from a protocol of Jin and Seyfang (77). The wild type, *itr1*Δ/*ITR1*, *itr1*Δ/*itr1*Δ, and *itr1*Δ/*itr1*Δ::*ITR1* strains were grown in YPD liquid cultures overnight at 30°C. Cells were diluted to 1 ^{OD600} in YPD, grown at 30°C, and collected at 5 ^{OD600} by centrifugation at 2,600g for 5 min. Cells were then washed twice with water at 4°C and resuspended in 2% glucose to a final concentration of $2x10^8$ cells/ml as determined by a hemacytometer. From this time point on, cells were kept on ice until used for the actual assay. For the uptake assay, the reaction mixture (250µl) contained 2% glucose, 40mM citric acid/KH_2PO_4 (pH5.5), 0.15µM [2-^{3}H] inositol (1 µCi/µl, MP Biomedicals), and 200µM unlabeled inositol (Alexis Biomedicals). Equal volumes of the reaction and cell mixtures (60µl each) were warmed to 30°C and mixed for the uptake assay which was performed for 10 minutes at 30°C. As negative controls, mixtures were kept at 0°C (ice) during the 10 minute incubation. One hundred µl aliquots were removed and transferred to pre-wetted metricel filters on a vacuum manifold. The filters were washed 4 times each with 1ml ice cold water. The washed filters were removed and added to liquid scintillation vials for measurements on a Perkin Elmer TRI-CARB 2900TR scintillation counter. The uptake of radiolabelled inositol over 10 minutes was calculated and plotted as a function of incubation temperature.

Mouse infection studies

Five- to six-week-old male CD1 mice (18 to 20g) from Charles River Laboratories were used in this study. Mice were housed at five per cage. For infection, colonies from each *C. albicans* strain were inoculated into 20ml of YPD or MM media. Cultures were grown overnight, washed twice with 25ml of sterile water, counted by hemacytometer, and resuspended at 10^7 cells per ml in sterile water. The cells were then plated on YPD to determine the viability. Mice were injected via the tail vein with 0.1ml of the cell suspension (10^6 cells) (176), and the course of infection was monitored for up to 30 days. At least two independent infections were performed for each strain. Survival was monitored twice daily, and moribund mice were euthanized. All experimental procedures were carried out according to the NIH guidelines for the ethical treatment of animals.

Statistics

The statistical analysis was done using Prism 4.0 software (GraphPad Software). For the mouse model of systemic infection, Kaplan-Meier survival curves were compared for significance using the Mantel-Haenszel log rank test. Tests for significance of differences between inositol uptake strains were determined using the two-tailed unpaired *t* test. Statistical significance was set at $P < 0.05$.

RESULTS

The *INO1* gene in *C. albicans* is required for growth in the absence of exogenous inositol

The *C. albicans* homolog of the *S. cerevisiae INO1* gene (*ScINO1*) identified by Klig *et al* (84) was disrupted in order to determine if it was required for inositol biosynthesis in *C. albicans*. The *C. albicans INO1* homolog (*orf19.7585*, which we refer to as *INO1* in this communication) was disrupted by sequentially replacing both alleles of the gene with the *NAT1-FLP* cassette which contains the nourseothricin resistance gene (154). The *INO1* disruption construct is diagrammed in Fig 2-1A. The *INO1* gene was reintegrated into the *INO1* locus of the homozygous mutant (*ino1*Δ/*ino1*Δ) using the construct shown in Fig 2-1B to verify linkage of any resulting phenotypes with the genotype. Wild-type (*INO1*/*INO1*), heterozygous (*ino1*Δ/*INO1*), homozygous (*ino1*Δ/*ino1*Δ), and reconstituted (*ino1*Δ/*ino1*Δ::*INO1*) strains were analyzed by PCR (data not shown) and Southern blotting (Fig 2-1C) to confirm the deletion and reintegration of the correct genes. The wild-type, *ino1*Δ/*INO1*, *ino1*Δ/*ino1*Δ, and

*ino1*Δ/*ino1*Δ::*INO1* strains were then compared for growth on medium lacking inositol (163) to determine if an *ino1*Δ/*ino1*Δ mutation would compromise the ability of the strain to grow in the absence of inositol. The *ino1*Δ/*ino1*Δ strain was unable to grow on inositol free medium, while the wild-type, heterozygous, and reconstituted strains grew equally well. As expected, the *ino1*Δ/*ino1*Δ strain was able to grow as well as the wild-type on identical solid or liquid media with inositol added (Fig 2-1D and data not shown, respectively).

The *INO1* gene is not required for virulence in *C. albicans*

The *INO1* gene does not appear to be required for virulence in a mouse model of disseminated candidiasis. The wild-type, *ino1*Δ/*INO1*, *ino1*Δ/*ino1*Δ::*INO1*, and two separately derived *ino1*Δ/*ino1*Δ strains were tested for virulence in the mouse disseminated infection model (176). These tests revealed no difference in virulence between the wild type and *ino1* mutant strains (Fig 2-5A).

The *ITR1* gene is required for inositol transport in *C. albicans*

The fact that the *ino1*Δ/Δ mutant can grow on medium containing inositol supports previous studies that have shown that *C. albicans* harbors an inositol transporter (77). A *C. albicans* homolog of the *S. cerevisiae ITR1* and *ITR2* transporters was identified by BLASTing the ScItr1p and ScItr2p protein sequences against the *Candida* Genome Database (CGD, http://www.candidagenome.org). This homolog was *orf19.3526* and is referred to as *HGT15* in the CGD, but its alias in CGD is *ITR2* . This gene has never been characterized functionally. Based on our BLAST search using CGD, *C. albicans orf19.3526* is the closest homolog to *ScITR1* and *ScITR2* and is 51% identical to both *S. cerevisiae* transporter genes over its full length (data not shown). *C. albicans orf19.3526* also contained the critical inositol transporter motif (D/E)(R/K)ϕGR(R/K) (152). Based on the results described below we will refer to *orf19.3526* as *ITR1* for the rest of this paper.

Both alleles of the *ITR1* gene were sequentially disrupted by replacing each ORF with the nourseothricin resistance cassette from the *SAT1* flipper (143). The *itr1*Δ disruption construct is diagrammed in Fig 2-2A. The *ITR1* gene was reconstituted in the *itr1*Δ/*itr1*Δ strain using the reconstitution construct shown in Fig 2-2B. Gene deletions and replacements were checked for accuracy using PCR (data not shown) and Southern blotting (Fig 2-2D). As a further test, Northern blotting revealed that the *ITR1* transcript could only be detected in wild-type (*ITR1*/*ITR1),* heterozygous *itr1*Δ/*ITR1,* and reconstituted *itr1*Δ/*itr1*Δ::*ITR1* strains, but not in the *itr1*Δ/*itr1*Δ strain (Fig 2-2E).

Expression was lower in the *itr1*Δ*/ITR1 and itr1*Δ*/itr1*Δ::*ITR1* strains presumably because they contain only one allele of the gene.

Analysis of inositol uptake in the wild-type, *itr1*Δ*/ITR1, itr1*Δ*/itr1*Δ and *itr1*Δ*/itr1*Δ::*ITR1* strains revealed that *ITR1* is required for inositol uptake (Fig 2-3). Inositol uptake was not detectable at 0˚C [(77) and Fig 2-3], hence this was used as a control. Inositol uptake was 58-fold higher at 30˚C than at 0˚C in the wild type. In contrast, inositol uptake in the *itr1*Δ*/itr1*Δ mutant was only 4-fold higher at 30˚C than at 0˚C, but a two-tailed paired *t* test analysis revealed that there was no significant difference in uptake between these two temperatures (*P*=0.34). The *itr1*Δ*/ITR1* mutant showed decreased inositol import compared to the wild type, and the *ITR1* reconstituted strain restored inositol import to a level similar to that seen in the wild-type.

Inositol uptake in the wild type strain in 200μM inositol was found to be 288 pmol per $5x10^7$ cells during a 10 minute uptake period. Jin and Seyfang showed approximately 60 pmol per 5 x 10^7 cells during a single minute uptake (77). The difference between their data and ours is probably due to saturation of uptake over a 10 minute time course (77).

The *ITR1* gene is not required for virulence in *C. albicans*

The *itr1*Δ*/itr1*Δ strain does not appear to be any less virulent than the wild type in a mouse model of systemic candidiasis. The wild-type, *itr1*Δ*/ITR1, itr1*Δ*/itr1*Δ, and *itr1*Δ*/itr1*Δ::*ITR1* strains were compared in the mouse model for disseminated infection. There was not a significant difference between the virulence of the wild-type and the *itr1*Δ*/itr1*Δ strains (Fig 2-5B, *P*=0.95). The *itr1*Δ*/ITR1* mutant behaved like the wild-type (Fig 2-5B) as well. In the reintegrant strain (*itr1*Δ*/itr1*Δ::*ITR1*) only half of the mice succumbed to the infection. This may be due to technical error during the injection or an uncharacterized mutation within the strain. The fact that the wild-type, *itr1*Δ*/ITR1*, and *itr1*Δ*/itr1*Δ strains were all similarly virulent strongly indicates that a lack of *ITR1* does not impair virulence.

The *INO1* and *ITR1* genes show synthetic defects in growth and virulence

Although neither *ITR1* nor *INO1* were required for virulence in a mouse model of systemic candidiasis, it was possible that each could compensate for loss of the other during infection since it has been shown that rat serum contains 20-100μM inositol (76, 134) and mouse liver contains approximately 100μM inositol (10). In order to test this hypothesis an attempt was made to disrupt both genes simultaneously. One allele of *ITR1* was disrupted in the *ino1*Δ/Δ strain. However, it was not possible to disrupt the other

allele of *ITR1* suggesting that a double mutant consisting of *ino1*Δ*/ino1*Δ and *itr1*Δ*/itr1*Δ was synthetically lethal. Therefore, in the *ino1*Δ/*ino1*Δ *itr1*Δ*/ITR1* strain the promoter of the remaining wild-type *ITR1* allele was replaced with the *CaMET3* conditional promoter (22) using the construct described in Fig 2-2C. When the *CaMET3* promoter (P_{MET3}) is used to replace the promoter of a target gene, it represses transcription of that gene in the presence of methionine and cysteine in the medium. The correct insertion of the P_{MET3}::*ITR1* allele into the chromosome was confirmed by PCR analysis (data not shown) and Southern blotting (lane 7, Fig 2-2D). The resulting *ino1*Δ/*ino1*Δ *itr1*Δ*/*P_{MET3}::*ITR1* strain was tested for growth in minimal medium containing 75μM inositol and either 0 or 2.5mM methionine and cysteine. It was found that unlike the wild-type or the *ino1*Δ/Δ *itr1*Δ*/ITR1* strains, two separately derived *ino1*Δ/Δ *itr1*Δ*/* P_{MET3}::*ITR1* strains failed to grow in the presence of 2.5 mM methionine and cysteine (Fig 2-4).

The *ino1*Δ/*ino1*Δ *itr1*Δ*/*P_{MET3}::*ITR1* strain was tested to determine whether its virulence was affected in a mouse model of systemic candidiasis. Previous work had indicated that the presence of the P_{MET3} promoter on the essential gene *CaFBA1* could compromise virulence (147). The methionine in the mouse bloodstream is presumably able to prevent expression of the *CaFBA1* gene sufficiently to compromise growth and virulence in the mouse. The wild-type, *ino1*Δ/*ino1*Δ *itr1*Δ*/ITR1* and two *ino1*Δ/*ino1*Δ *itr1*Δ*/*P_{MET3}::*ITR1* strains were compared in the mouse model of systemic candidiasis. This experiment revealed that the *ino1*Δ/Δ *itr1*Δ*/ITR1* strain was attenuated for virulence, and the *ino1*Δ/*ino1*Δ *itr1*Δ*/*P_{MET3}::*ITR1* strains were avirulent (Fig 2-5C).

DISCUSSION

The mechanism by which *C. albicans* acquires the essential metabolite inositol during an infection has not been explored previously. We have found that *C. albicans* is able to generate inositol *de novo* via the *INO1* gene product or import inositol from the environment via the *ITR1* gene product with efficiencies that allow it to establish an infection regardless of which mechanism is employed. This conclusion only applies to bloodstream infections as this was the only model tested. This result implies that the bloodstream content of inositol in mice is sufficient to support an infection even if *C. albicans* must acquire inositol solely by importing it. Although our search of the literature did not reveal the estimated inositol content of mouse serum, the inositol levels found in rats is 20-100μM (76, 134) and may be comparable to mice and is similar to that found in humans (61.0±12.4 μM) (86).

The results found with *C. albicans* are in contrast to what has been observed in two important human pathogens, *M. tuberculosis* and *T. brucei*, which require *de novo* inositol biosynthesis via *INO1* homologs in order to be fully virulent (*Mt*) in mouse models or viable (*Tb*) (109, 118). Both of these pathogenic microbes are capable of importing inositol, but in neither case is import sufficient to allow viability in *T. brucei* or virulence in *M. tuberculosis.* In the case of *T. brucei*, *de novo* synthesized inositol is used to make GPI-anchored proteins, while imported inositol is used very inefficiently for this purpose (108). GPI-anchored proteins are required for viability in *T. brucei* (97), so disruption of *INO1* compromises viability. In the case of *M. tuberculosis* the inositol transporter is too inefficient to import inositol from the host in order to support infection (118).

Unlike either of these pathogens *Candida albicans* possesses an inositol transporter, encoded by *ITR1*, that is capable of transporting inositol efficiently enough to allow full virulence in a mouse model of systemic candidiasis even in the absence of *de novo* inositol biosynthesis. In the case of both *M. tuberculosis* and *T. brucei* it has been suggested that the development of selective inhibitors of Ino1p homologs might be an effective way to generate antimicrobials (108, 118). The data reported in this communication indicates that this would not be effective in *C. albicans* as it is fully virulent even in the absence of its Ino1 enzyme. As an alternative approach, it has been suggested that toxic inositol analogs that are selectively taken up by the *C. albicans* inositol transporter but not by the human inositol-Na^+ transporter, could be effective drugs (77). This may be a possibility, although it needs to be determined which of these two mechanisms, *de novo* biosynthesis or import, is used by wild-type *C. albicans* during an infection. If import is used extensively, then this approach might work, however since *C. albicans* is fully virulent in the absence of *ITR1*, the development of resistant mutants lacking Itr1p transporter function could be a problem. Nonetheless, a toxic analog might be useful in combination with drugs affecting other targets such as an azole or polyene (2).

Based on our results it appears that Itr1p is the primary and perhaps sole inositol importer in *C. albicans* both *in vitro* and during infection. Disruption of *ITR1* greatly inhibits inositol uptake *in vitro* (Fig 2-3), and in the absence of *INO1* a strain carrying only the P_{MET3}::*ITR1* allele of *ITR1* cannot grow in medium containing cysteine and methionine, even in the presence of 75 µM extracellular inositol (Fig 2-4). The amount of methionine and cysteine in mouse serum is apparently sufficient to decrease expression

from the *MET3* promoter as well, which is consistent with results using a *MET3* driven form of the *C. albicans FBA1* gene (147). Our conclusion that *ITR1* is the primary or sole inositol transporter is consistent with a previous study of *C. albicans* inositol transport which concluded that there was one inositol transporter in *C. albicans* that was responsible for all, or at least the vast majority of, inositol transport activity (77).

The situation in *C. albicans* contrasts with *S. cerevisiae*, which has two inositol transporters that are expressed at widely different levels (126). In *S. cerevisiae*, the ScItr1p transporter is the most highly expressed of the two transporters and accounts for most of the transport activity. The residual transport activity in *S. cerevisiae* is carried out by ScItr2p, which is expressed at much lower levels. Based on BLAST searches of the *Candida* Genome Database using *ScITR1* or *ScITR2* as queries, the *C. albicans ITR1* gene was the closest homolog to the *S. cerevisiae* inositol transporters showing 51% identity over the whole sequence to either *ScITR1* or *ScITR2*. *C. albicans* carries at least one other homolog of the *S. cerevisiae* inositol transporters that is predicted to have 12 transmembrane domains and the (D/E)(R/K)øGR(R/K) motif typical of inositol transporters. The predicted protein sequence of this gene (*orf19.5447*, *HGT19*) bears 26% and 27% identity to ScItr1p and ScItr2p, respectively. It is possible that *orf19.5447* encodes an inositol transporter, but our results suggest that if so, this other transporter is expressed at too low of a level to transport inositol efficiently or it is expressed in different conditions than those tested in our experiments. Alternatively, it is a very low affinity transporter like that seen in *M. tuberculosis* (118) or it transports molecule other than inositol.

Taken together our results indicate that either Ino1p or Itr1p can supply the inositol requirement during an infection, but a number of questions remain to be answered. For wild-type *C. albicans* it is not known whether *de novo* inositol biosynthesis or import is utilized during an infection or a combination of both are in operation. It may be variable depending on the distribution of *C. albicans* cells within the host. In addition, it is not known whether *de novo* synthesis or import is favored during growth in other host niches such as the gut, the oral mucosa, and the vaginal tract. This again may be variable depending on the nutrient conditions of the particular host niche and location of cells within those host sites. Further studies will be required to answer these questions.

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APPENDIX B: TABLES AND FIGURES

Table 2- 1. *C. albicans* strains

Strain	Parent	Genotype	Source of reference
SC5314 (wild-type)	Clinical isolate	Prototrophic wild type	(55)
YLC100	SC5314	*ino1*Δ::*NAT1-FLP*/*INO1*	This study
YLC105	YLC100	*ino1*Δ/*INO1*	This study
YLC111	YLC105	*ino1*Δ/*ino1*Δ::*NAT1-FLP*	This study
YLC113	YLC111	*ino1*Δ/*ino1*Δ	This study
YLC120	YLC113	*ino1*Δ/*ino1*Δ::*INO1*	This study
YLC101	SC5314	*ino1*Δ::*NAT1-FLP*/*INO1*	This study
YLC108	YLC101	*ino1*Δ/*INO1*	This study
YLC126[a]	YLC108	*ino1*Δ/*ino1*Δ::*NAT1-FLP*	This study
YLC176	SC5314	*itr1*Δ::*SAT1-FLP*/*ITR1*	This study
YLC185	YLC176	*itr1*Δ/*ITR1*	This study
YLC192	YLC185	*itr1*Δ/*itr1*Δ::*SAT1-FLP*	This study
YLC196	YLC192	*itr1*Δ/*itr1*Δ	This study
YLC211	YLC196	*itr1*Δ/*itr1*Δ::*ITR1*	This study
YLC180	YLC113	*ino1*Δ/*ino1*Δ *itr1*Δ::*SAT1-FLP*/*ITR1*	This study
YLC184	YLC180	*ino1*Δ/*ino1*Δ *itr1*Δ/*ITR1*	This study
YLC261	YLC184	*ino1*Δ/*ino1*Δ *itr1*Δ/*SAT1-*P_{MET3}*-ITR1*	This study
YLC181	YLC113	*ino1*Δ/*ino1*Δ *itr1*Δ::*SAT1-FLP*/*ITR1*	This study
YLC187	YLC181	*ino1*Δ/*ino1*Δ *itr1*Δ/*ITR1*	This study
YLC266[b]	YLC187	*ino1*Δ/*ino1*Δ *itr1*Δ/*SAT1-*P_{MET3}*-ITR1*	This study

[a] another separately derived *ino1*Δ/*ino1*Δ homozygous mutant.

[b] another separately derived *ino1*Δ/*ino1*Δ *itr1*Δ/P_{MET3}::*ITR1* conditional strain.

Table 2- 2. Plasmids

Plasmids	Characteristics	Source of reference
pJK863	*CaNAT1-FLP* cassette carrying nourseothricin resistance gene	(154)
pSFS2A	*SAT1* flipper carrying nourseothricin resistance gene	(143)
pYLC94	pJK863 flanked 5' and 3' $INO1^{NCR}$ for *INO1* gene knock out	This study
pYLC119	*INO1* reconstitution construct	This study
pYLC164	pSFS2A flanked 5' and 3' $ITR1^{NCR}$ for *ITR1* gene knock out	This study
pYLC208	*ITR1* reconstitution construct	This study
pYLC229	*ITR1* conditional construct controlled by *CaMET3* promoter	This study

Table 2- 3. PCR primers

Primer	Use	Sequence (5' → 3')
JCO39	Disrupt *INO1*	AAAAAAGGTACCGGGATCAAACAATCTAGACTCAC
JCO40	Disrupt *INO1*	AAAAAAGGGCCCAGTTATTTGTTTGTGAAGGAGAT
JCO41	Disrupt *INO1*	AAAAAACCGCGGTGTTGCTTTATAGTAATATCGCT
JCO42	Disrupt *INO1*	AAAAAAGAGCTCCGACAGCCCATATATTTTAATCG
JCO47	Restore *INO1*	AAAAGTCGACTGATTATTTGAGAATTCTTTC
JCO50	Restore *INO1*	AAACGTCGACACTGGATGG
TRO369	Confirm *ino1*Δ	GCACGTCAAGACTGTCAAGG
JCO105	Confirm *ino1*Δ	TTATCTATTGTCAATTTCGCC
JCO106	Confirm *ino1*Δ	TGGGAGTTTAGTGTTTGAGC
JCO89	Disrupt *ITR1*	AAAAAAGGGCCCCTCAACAAATTGTCGATTAT
JCO90	Disrupt *ITR1*	AAAAAACTCGAGTTCCCTCAAATCAATACACT
JCO93	Disrupt *ITR1*	AAAAAAGCGGCCGCCTCAGTCTAGTATACTAAAT
JCO94	Disrupt *ITR1*	AAAAAAGCGGCCGCTGAAATACTTGAACTGTGTGA
JCO95	Confirm *itr1*Δ	GATTATTAGTTAAACCACTGC
JCO96	Confirm *itr1*Δ	TGAAGGGGGAGATTTTCACT
JCO100	Confirm *itr1*Δ	AAACCCCCACTTGAGTCTAA
JCO101	Confirm *itr1*Δ	TTGATCATTTGACCTCGGCA
JCO111	Restore *ITR1*	AAAAAAGAATTCGAGCTATACGGTTGGTTTCGA
JCO118	*ITR1* conditional construct	AAAAAACTGCAGAAAACTACGAACAATTGTC
JCO119	*ITR1* conditional construct	AAAAAAGCGGCCGCGTTTTCTGGGGAGGGTATTT
JCO120	*ITR1* conditional construct	AAAAAAGCGGCCGCATGGGAAGTTCAACCAATAA
JCO121	*ITR1* conditional construct	AAAAAACCGCGGCTATACGGTTGGTTTCGATT
JCO102	*ITR1* Northern blot probe	ACAATCAAAAGCTACCCCCA
JCO103	*ITR1* Northern blot probe	TGGTGTATCTGGTAAAAACCA
TRO562	*INO1* Northern blot probe	GAAAACTCTGTTGTTGAAAAAGATG
TRO563	*INO1* Northern blot probe	TTGTTGGCACGTTCACTTTG
JCO48	*CaACT1* Northern blot probe	CCAGCTTTCTACGTTTCC
JCO49	*CaACT1* Northern blot probe	CTGTAACCACGTTCAGAC

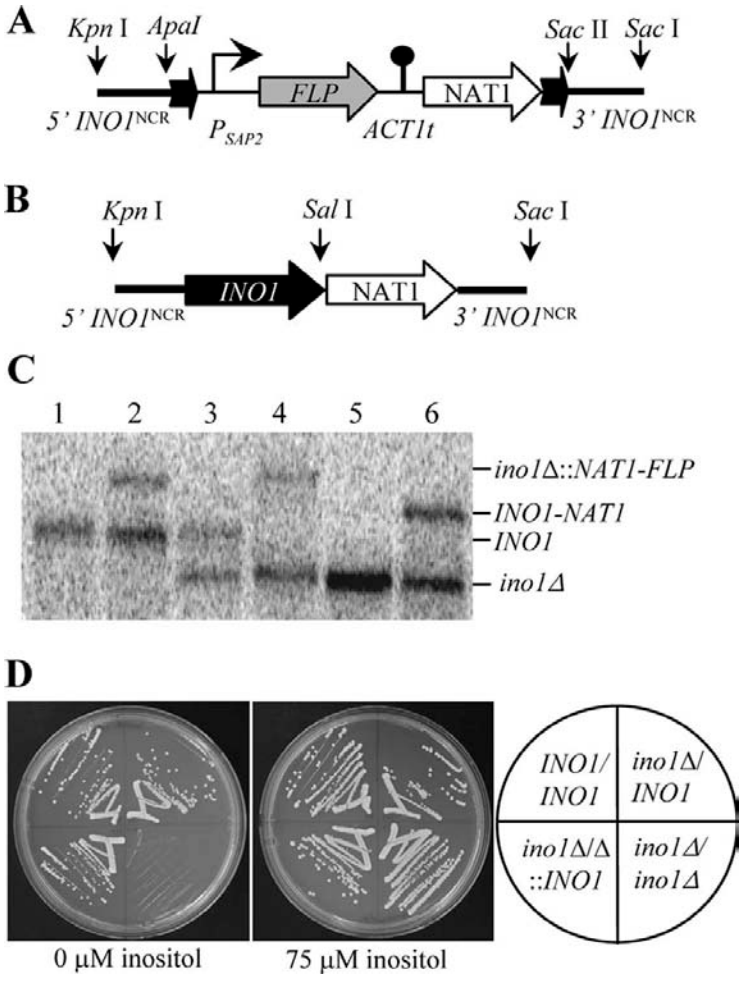


Fig 2- 1. The *INO1* gene was disrupted in *C. albicans*.

(A) Structure of the *INO1* disruption construct. Approximately 500 base pairs of non-coding DNA flanking the 5' and 3' ends of the *INO1* gene (5'-and 3'-$INO1^{NCR}$, respectively) were cloned onto either flank of the *CaNAT1-FLP* construct (154). The thick dark arrows represent the FRT target sights of the *FLP* recombinase. The ball and stick represents the *ACT1* terminator (*ACT1t*) and the thinner arrow on a raised line represents the *SAP2* promoter (P_{SAP2}). (B) The *INO1-NAT1* construct used to reintegrate *INO1* ORF into the *ino1*Δ/Δ mutant. (C) Southern blotting was used to confirm the *INO1* disruptions. Lane 1 (wild-type); lane 2 (*ino1*Δ::*NAT1-FLP*/*INO1*), lane 3 (*ino1*Δ/*INO1*); lane 4 (*ino1*Δ/Δ:: *NAT1-FLP*); lane 5 (*ino1*Δ/Δ); lane 6 (*ino1*Δ/Δ::*INO1*). (D) The *INO1* gene is required for growth in the absence of exogenous inositol. The cells were streaked onto medium containing either 0 or 75μM inositol and grown for 2 days at 30°C.

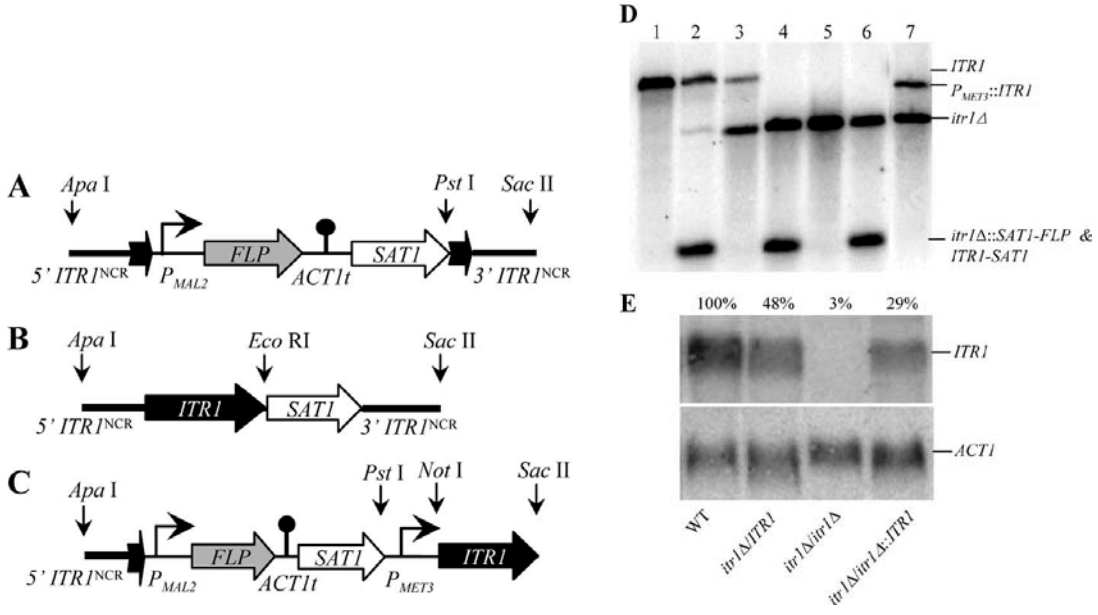


Fig 2- 2. The *ITR1* gene was disrupted in *C. albicans*.

(A) Structure of the *ITR1* disruption construct. Approximately 500 base pairs of non-coding DNA flanking the 5' and 3' ends of the *ITR1* gene (5'-and 3'-*ITR1*^{NCR}, respectively) were cloned into the pSFS2A plasmid such that they flanked the *SAT1* flipper cassette. The thick dark arrows represent the FRT target sights of the *FLP* recombinase. The ball and stick represents the *ACT1* terminator (*ACT1t*) and the thinner arrow on a raised line represents the *MAL2* promoter (P_{MAL2}). (B) The *ITR1-SAT1* construct used to reintegrate *ITR1* ORF into the *itr1*Δ/Δ mutant. (C) The P_{MET3}::*ITR1* construct used to replace the *ITR1* allele in the *ino1*Δ/Δ *itr1*Δ*/ITR1* strain to generate the *ITR1* conditional allele. (D) Southern blotting was used to confirm the *ITR1* disruptions. Lane 1 (wild-type); lane 2 (*itr1*Δ::*SAT1-FLP*/*ITR1*); lane 3 (*itr1*Δ/*ITR1*); lane 4 (*itr1*Δ/Δ::*SAT1-FLP*); lane 5 (*itr1*Δ/Δ); lane 6 (*itr1*Δ/Δ::*ITR1-SAT1*); lane 7 (*ino1*Δ/Δ *itr1*Δ*/*P_{MET3}::*ITR1*). The *itr1*Δ::*SAT1-FLP* band (disrupted allele prior to "flipping out" the *SAT1-FLP* construct) and *itr1*Δ*/itr1*Δ ::*ITR1-SAT1* band (reintegrated *ITR1* marked with *SAT1*) ran together on the gel. (E) Northern blotting revealed that the expression of *ITR1* was lost in the *itr1*Δ*/itr1*Δ mutant, but not the wild-type, heterozygous, and reconstituted strains. Strains were grown in defined medium 199 for 2hrs at 37°C, and RNA was isolated, Northern blotted, and probed for *ITR1* expression. *ITR1* expression was normalized to *ACT1* expression on the same blot.

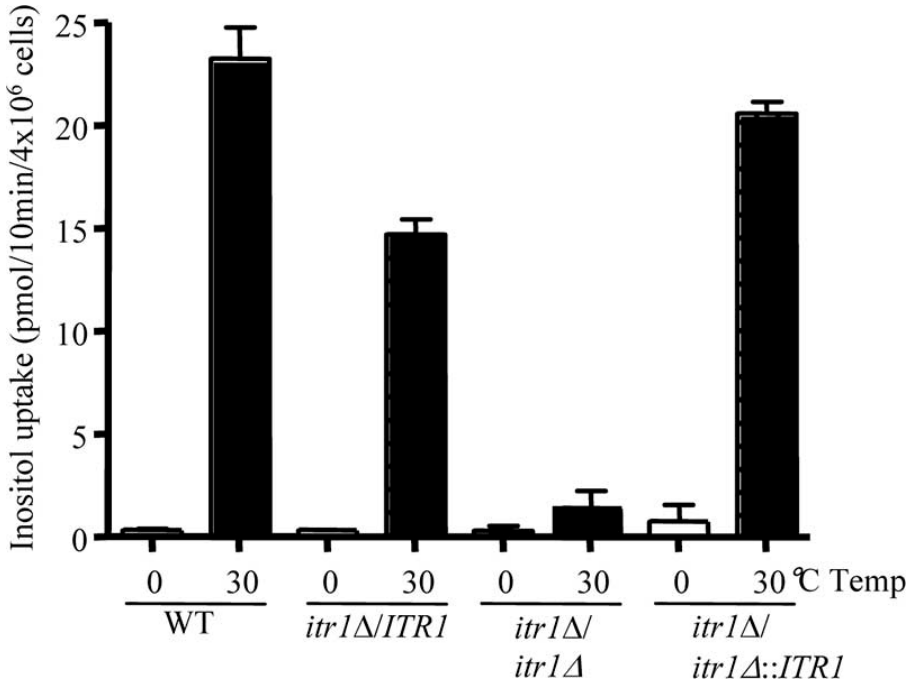


Fig 2- 3. The *ITR1* gene is required for inositol transport in *C. albicans.*

The inositol uptake assay revealed that the ability to import ^{3}H-myo-inositol was greatly reduced in the *itr1*Δ/*itr1*Δ mutant, whereas the wild-type, heterozygous, and reconstituted strains exhibited the ability to import inositol. Each strain was assayed at 0˚C (white bars) and 30˚C (black bars).

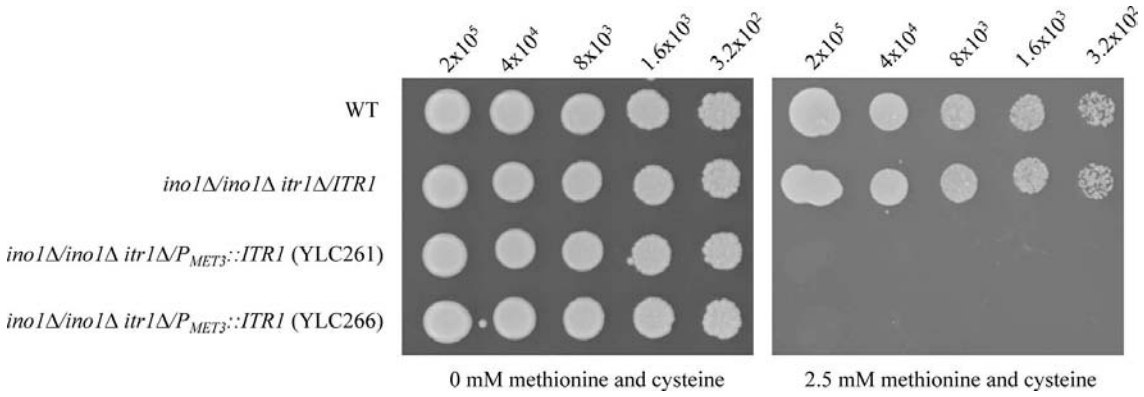
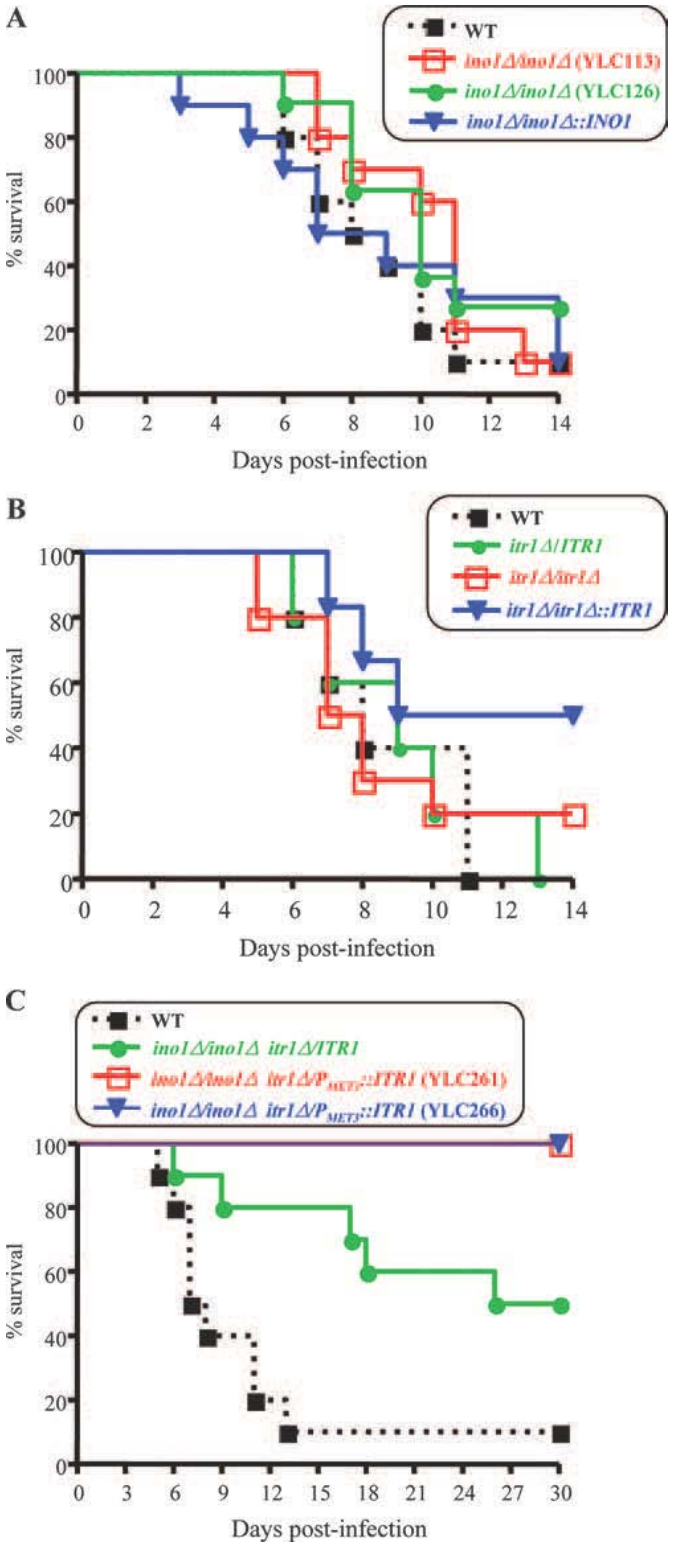


Fig 2- 4. The *INO1* and *ITR1* genes show synthetic defects in growth on agar plates.

The growth of wild-type, *ino1*Δ/Δ *itr1*Δ*/ITR1*, and two separately derived *ino1*Δ/Δ *itr1*Δ*/*P_{MET3}::*ITR1* strains were compared in 5-fold serial dilutions on minimal medium containing 75μM inositol ± 2.5mM methionine and cysteine.

Fig 2- 5. The *INO1* and *ITR1* genes show synthetic defects in virulence.

The survival of mice was monitored following intravenous challenge with 10^6 *C. albicans* blastospores. (A) Mice were injected with *INO1* mutant strains. Wild-type (n=10); *ino1*Δ/Δ (YLC113, n=10); *ino1*Δ/Δ (YLC126, n=11); *ino1*Δ/Δ::*INO1* (n=10). (B) Mice were injected with *ITR1* mutant strains. Wild-type (n=5); *itr1*Δ/*ITR1* (n=5); *itr1*Δ/Δ (n=10); *itr1*Δ/Δ::*ITR1* (n=6). (C) Mice were injected with *ino1*Δ/Δ *itr1*Δ/P_{MET3}::*ITR1* conditional double mutant strains. Wild-type (n=10); *ino1*Δ/Δ *itr1*Δ/*ITR1* (n=10); *ino1*Δ/Δ *itr1*Δ/P_{MET3}::*ITR1* (YLC261, n=10); *ino1*Δ/Δ *itr1*Δ/P_{MET3}::*ITR1* (YLC266, n=10). Strains for experiments A and B were pre-grown in YPD before the injection while strains for experiment C were pre-grown in minimal medium$^{-meth-cys}$ plus 75μM inositol.


A
WT
ino1Δ/ino1Δ (YLC113)
ino1Δ/ino1Δ (YLC126)
ino1Δ/ino1Δ::INO1
% survival
Days post-infection
B
WT
itr1Δ/ITR1
itr1Δ/itr1Δ
itr1Δ/itr1Δ::ITR1
% survival
Days post-infection
C
WT
ino1Δ/ino1Δ itr1Δ/ITR1
ino1Δ/ino1Δ itr1Δ/P$_{MET3}$::ITR1 (YLC261)
ino1Δ/ino1Δ itr1Δ/P$_{MET3}$::ITR1 (YLC266)
% survival
Days post-infection

Chapter 3: Phosphatidylserine synthase is essential for virulence and cell wall integrity in *Candida albicans*

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ABSTRACT

Candida albicans is one of the most commonly isolated human fungal pathogens, especially in immunocompromised patients. There are only a few effective antifungals available to treat fungal infections, creating an urgent need to identify new drug targets. The *S. cerevisiae* phosphatidylserine (PS) synthase, Cho1p, is required for PS synthesis, and is conserved in fungi, but not mammals. We have found that a *C. albicans cho1*Δ*/cho1*Δ mutant lacks detectable PS, and is completely avirulent in a mouse model of systemic candidiasis. The *cho1*Δ*/cho1*Δ mutant also exhibits defects in cell wall integrity, mitochondrial function, and filamentous growth, and it is an ethanolamine and choline auxotroph. However, its growth in choline supplemented media is extremely poor, with a doubling time of ~36 hours. PS is a precursor for the *de novo* synthesis of phosphatidylethanolamine (PE) via PS decarboxylase, and it was hypothesized that some of the *cho1*Δ*/cho1*Δ mutant's defects might be due to loss of *de novo* PE biosynthesis. A *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant, which lacks PS decarboxylase activity, was found to exhibit defects in cell wall integrity, mitochondrial function, filamentous growth, and virulence. However, for all of these phenotypes except filamentous growth, the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant was not as defective as the *cho1*Δ*/cho1*Δ mutant. In addition, the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant's growth in minimal media was rescued similarly with 1 mM ethanolamine or 10 μM choline. The severe virulence defect of the *cho1*Δ*/cho1*Δ mutant is likely due, in part, to a loss of *de novo* PE biosynthesis, but loss of PS itself contributes to this phenotype as well. We predict that *C. albicans* PS synthase could provide an excellent target for the development of a novel antifungal drug.

INTRODUCTION

The incidence of human systemic fungal infections has increased dramatically in the last thirty years. This is based, in part, on an increase in the number of immunocompromised patients due to conditions such as acquired immunodeficiency syndrome (AIDS), cancer-related chemotherapy, and leukemia (20, 65). *Candida* species are the leading cause of fungal infections in humans and can cause mucosal, cutaneous, and bloodstream infections. Bloodstream infections are of the greatest concern with a high associated mortality rate (31.8%) (116).

There are only five main types of antifungals used to combat *Candida* infections, and a number of these drugs are limited by emerging drug resistance or toxicity to the patient (43). Therefore, there is an urgent need to identify new targets for development of antifungal agents. The lipid ergosterol has served as a very potent drug target in fungi, and three of the major classes of antifungals work by targeting ergosterol or ergosterol biosynthesis (43). In particular, the azole and allylamine classes affect ergosterol biosynthesis (12, 53), and the polyenes target ergosterol in the membrane (46). The key to the effectiveness of these drugs is that their targets are not conserved in the host.

Phospholipid biosynthesis is an emerging area in which to look for novel drug targets in a number of pathogens. Although similar phospholipids are found in mammals, bacteria, protozoans, and fungi (81), the enzymes involved in synthesizing some phospholipids are not conserved between pathogens and mammals. Some of these nonconserved enzymes may serve as useful drug targets if they are required for growth or virulence in the host. In the fungal pathogen *Cryptococcus neoformans*, inositol-phosphoryl ceramide synthase 1 (*IPC1*) is required for sphingolipid metabolism. This enzyme is essential for virulence in a rabbit model of infection (102). In the bacterial pathogen *Brucella abortus*, the enzymes PcsA or PmtA, both required for phosphatidylcholine (PC) biosythesis, are necessary for virulence in BALB/c mice (30). Similarly, phosphatidylethanolamine (PE) biosynthesis in *B. abortus*, which is dependent on the phosphatidylserine (PS) synthase (PssA), is required for optimal virulence in a murine model (18). In the parasite *Plasmodium falciparum*, a potential novel anti-malarial drug target was identified: serine decarboxylase phosphoethanolamine methyltransferase (SDPM) encoded by *PfPMT* gene. This enzyme methylates phosphoethanolamine (Etn-P) to form phosphocholine (Cho-P), and is required for PC synthesis in *P. falciparum*. A *pfpmt*Δ mutant exhibits defects in parasite growth and viability, suggesting that this gene plays an important role in the pathogenesis of

intraerythrocytic *Plasmodium* parasites (182).

Little research has concentrated on the roles of phospholipid biosynthesis in the pathogenesis of *C. albicans*. It has been demonstrated that phospholipomannan, a fungal mannose inositol phosphate ceramide (MIPC) derived from phospholipids, is required for virulence in a mouse model of systemic infection (115). In addition, the sphingolipid biosynthetic gene *IPT1* is required for *C. albicans* to undergo hyphal formation, a virulence factor, in laboratory culture conditions, but its role in virulence in an animal model has not been examined (142).

A comparison of the basic pathways for phospholipid biosynthesis between the well-characterized yeast *S. cerevisiae* and mammals has revealed a potential target that is conserved in fungi (81). Although phospholipid biosynthetic pathways in mammals and yeast share many general characteristics in common, a major difference is found in the synthesis of PS (Fig 3-1). In yeast PS is synthesized from cytidyldiphosphate-diacylglycerol (CDP-DAG) and serine by the enzyme ScCho1p, with the associated release of cytidylmonophosphate (CMP) (92). In mammals, a very different pair of enzymes, encoded by *PSS1* and *PSS2*, generate PS by exchanging serine for the head groups of PC and PE, respectively (9, 160, 162). The Cho1p enzyme was among a number of enzymes on a comprehensive list of proteins that are conserved among fungi, but not found in mammals, and is, therefore, considered a potential drug target (16).

PS is normally asymmetrically distributed to the cytoplasmic side of the membrane bilayer in eukaryotic cells (170). PS is also considered both an end product and a biosynthetic intermediate, since it is found in eukaryotic cell membranes and serves as a precursor of PE (81)(Fig 3-1). The first description of PS synthesis mutants in eukaryotic cells (*S. cerevisiae*) was 30 years ago. Atkinson and colleagues demonstrated that *Sccho1* mutants were unable to synthesize PS and were auxotrophic for choline or ethanolamine (4, 5). The enzyme responsible for PS synthesis, and the gene encoding it, were later isolated (92). The PS synthesis mutant (*Sccho1*Δ) exhibited slow growth on nonfermentable carbon sources, mitochondrial abnormalities, and the formation of a high proportion of petite (respiratory-deficient) cells at lower temperatures (5). Interestingly, a mutant in the conserved PS synthase of fission yeast *Schizosaccharomyces pombe* (*pps1*Δ) was recently demonstrated to have divergent phenotypes including defects in cell morphology, cytokinesis, actin cytoskeleton, and cell wall integrity. In addition to those defects, the *pps1*Δ mutant is auxotrophic for ethanolamine, but cannot be supported by choline, even on fermentable carbon sources, unlike a *S. cerevisiae Sccho1*Δ mutant (112).

PS is clearly not necessary for viability in either of these fungi, however if its homolog is necessary for virulence or growth of *C. albicans* in the host, then CaCho1p may be a good drug target.

In this report we describe that the *cho1*Δ*/cho1*Δ mutant in *C. albicans* is avirulent in a mouse model of systemic candidiasis. In addition, the mutant exhibits overlapping, but distinct phenotypes with PS synthase mutants in *S. cerevisiae* and *S. pombe*. The *C. albicans cho1*Δ*/cho1*Δ mutant exhibits defects in cell wall integrity, mitochondrial function, and is very poorly rescued for growth by supplementation with choline. It is further demonstrated that many of the defects in the *cho1*Δ*/cho1*Δ mutant are attributable to a loss of *de novo* PE biosythesis, but that the loss of PS itself appears to exacerbate these problems. This study indicates that *C. albicans* PS synthase is absolutely necessary for growth in the host and therefore represents a new potential drug target.

MATERIAL AND METHODS

Strains, chemicals, and growth media

C. albicans strains used in this study are shown in Table S3-1 (see all tables and figures in the appendix B). The following media were used in this study: 1% yeast extract-2% peptone-2% glucose (YPD) liquid media and agar plates. YPD plates containing 250μg/ml nourseothricin was used to select for disruptants. Ethanolamine-free medium was made using the recipe for inositol-free minimal medium and then adding back 75μM inositol (163). The following supplements, Cyclosporin A (Alexis Biochem), FK506 (Tecoland), Paraquat (Sigma), Bovine calf serum (HyClone), Fluconazole (AB Biodisk), Amphotericin B (Fisher), Hygromycin B (Sigma), Nikkomycin Z (Sigma), Sodium dodecyl sulfate (SDS; Fisher), Caffeine (Acros Organics), Congo Red (ICN Biochemicals), Calcofluor white (Polysciences), Cyclophosphamide monohydrate (Sigma), or Papuamide B (gift from Todd Graham, Vanderbilt University, TN) were added to media at the concentrations described.

The phospholipid standards: phosphatidylserine (#830034), phosphatidylethanolamine (#850757), phosphatidylinositol (#840042), and phosphatidylcholine (#850457) were purchased from Avanti Polar Lipids Inc. or provided by Stephen Wright (Carson-Newman College, TN). Ethanolamine and choline chloride were purchased from Acros Organics Inc., and Fisher, respectively. Trypan blue & Eosin Y indicator plates for the mitochondrial dysfunction test included 0.15g KH_2PO_4, 0.15g $(NH_4)_2SO_4$, 0.1g $MgSO_4 \cdot 7H_2O$, 0.15g peptone, 0.15g yeast extract, 2g glucose, 1mg

eosin Y, 1mg trypan blue, and 1.5g agar in 100ml H_2O (62, 120).

Strain construction

The first allele of the *CHO1* gene was disrupted by using the *CaNAT1-FLP* cassette (154), whereas its second allele was disrupted by using the *SAT1* flipper (143). The *PSD1* and *PSD2* genes were disrupted by using the *SAT1* flipper.

For the *CHO1* disruption construct, an approximately 500-bp 5' upstream noncoding region (NCR) of *CHO1* (5' *CHO1*^{NCR}) was amplified with primers JCO43 and JCO44 (Table S3-3), which introduced KpnI and ApaI restriction sites, and was cloned into pJK863 (154), 5' of the *CaNAT1-FLP* cassette using the same enzymes (Fig 3-2A). An approximately 500-bp 3' *CHO1*^{NCR} was amplified with primers JCO45 and JCO46, which introduced SacII and SacI sites, and was cloned into pJK863 3' of the *CaNAT1-FLP* cassette using the same enzymes (Fig 3-2A). This procedure created the *CHO1* knock out construct plasmid pYLC130 (Table S3-2; Fig 3-2A), which was cut with KpnI and SacI to release the ~ 5.2kb disruption construct, and the wild type SC5314 strain was transformed with the disruption construct by electroporation (36). The *CaNAT1* construct could not be flipped out from *cho1*Δ/*cho1*Δ::*NAT1-FLP* strains due to the homozygote being unable to grow sufficiently in the Yeast Carbon Base-Bovine Serum Albumin (YCB-BSA) medium used to induce the *SAP2* promoter that drives the *FLP* recombinase on the *CaNAT1-FLP* cassette (154). We therefore used a similar cloning strategy as described above to clone 5'- and 3'- *CHO1*^{NCR} into the *SAT1* flipper (pSFS2A) (143), resulting in pYLC326 (Table S3-2; Fig 3-2A). See Table S3-3 for appropriate primers, restriction sites, etc. The disruption construct on pYLC326 was used to disrupt the second allele of *CHO1* in the *cho1*Δ*/CHO1* heterozygote, and it was flipped out easily because the homozygote could grow quite well in YPD induction medium (143). The *CHO1* reconstitution construct was made by amplifying a 1.3kb fragment containing the *CHO1* open reading frame (ORF) and the 5' NCR from SC5314 genomic DNA by using primers (JCO43 and JCO169) that introduced KpnI and HindIII sites. This fragment was ligated into the KpnI-HindIII digested pYLC326 which already contained the 3'*CHO1*^{NCR}. This procedure resulted in the *CHO1* reconstitution plasmid pYLC341 (Table S3-2; Fig 3-2B).

We used a similar approach to knock out *PSD1* and *PSD2* genes, but only the *SAT1* flipper was used. The primers used to generate each disruption construct are listed in Table S3-3. The *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ double mutant (YLC375) was made by knocking out the *PSD1* gene in the *psd2*Δ/*psd2*Δ mutant (YLC271) background.

Southern blot analysis

The cells were grown in liquid YPD at 30ºC overnight. The genomic DNA was extracted using the Winston-Hoffman method (70), and 20µg of genomic DNA was subjected to Southern blotting. The genomic DNA of *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ mutants were cut with XhoI, while the genomic DNA of the *psd2*Δ/*psd2*Δ mutant was cut with BamHI. PCR products containing the ~500-bp 3'*CHO1*^{NCR} (amplified with primers JCO45 and JCO46), the 3' *PSD1*^{NCR} (amplified with primers JCO143 and JCO144), and the 3' *PSD2*^{NCR} (amplified with primers JCO148 and JCO149) were used as probes. Hybridization conditions were described previously (25).

Phospholipid composition assay

The wild-type and phospholipid biosynthetic mutants were grown in shaking YPD liquid cultures overnight at 30ºC. Cells were washed twice in sterile water and diluted in 5ml YPD containing 5µCi/ml [^{32}P]-phosphoric acid (MP biomedicals) to an OD_{600} of 0.4 and grown for 3h at 37ºC at 250 rpm. Cells were harvested by centrifugation, washed with water, and transferred to screw-capped glass tubes (Corning, 16x100mm). The simple and efficient phospholipid extraction was adapted, in part, from the protocol of Hanson and Lester (64). In brief, 3ml of ethanol/H_2O (4:1) was added to the cell pellets and heated in a boiling water bath for 15 minutes. After centrifugation in a table top centrifuge (1000 x g), the supernatants containing radiolabeled phospholipids were transferred to fresh screw-capped glass tubes. The remaining cell pellets were extracted two additional times, using 1ml of ethanol/H_2O (4:1) for each extraction. All supernatants (~ 5ml) were pooled, and N_2 gas was used to dry down the phospholipids, which were then resuspended in 50µl of chloroform/methanol (2:1).

The individual phospholipids were resolved using one-dimensional silica gel thin-layer chromatography (TLC) plates (Whatman). The TLC plate was first incubated in the oven at 100ºC for 15 min, then prewashed (removing debris) thoroughly by chloroform/methanol (1:1) through migration on the TLC. The prewashed TLC plate was dried in the hood for 5 min and then baked in the oven at 100ºC for 15 min. Two µl of extracted radiolabeled phospholipids were spotted on the TLC plate and developed in cold chloroform/ethanol/water/triethylamine (30:35:7:35). The phospholipids were detected and quantitated with a Storm PhosphoImager.

The identity of phospholipids was determined by running the samples with phospholipid standards PS, PE, PC, and PI, and by mass spectrometry. The primuline spray [5mg primuline (Sigma) dissolved in 100ml of acetone/water (4:1)] was used to

visualize the lipid spots under UV light.

Mouse infection studies

Five- to 6-week-old male ICR mice (~ 20g) from Harlan Sprague Dawley were used in this study. Mice were housed in groups of five per cage. For infection, colonies from each *C. albicans* strain were inoculated into 5ml of YPD. Cultures were grown overnight and washed twice with 10ml of sterile water, and cells were counted by hemacytometer and resuspended in sterile water at 10^7 cells per ml. Dilutions of the cells were plated onto YPD containing 1M sorbitol and incubated at 37ºC for 48h to determine viability. Mice were injected via the lateral tail vein with 0.1ml of the cell suspension (10^6 cells), and the course of infection was monitored for up to 42 days. Survival was monitored twice daily, and moribund mice were euthanized. All experimental procedures were carried out according to the NIH guidelines for the ethical treatment of animals.

For the immunocompromised mouse model, five- to 6-week-old male ICR mice (~ 20g) from Harlan Sprague Dawley were intraperitoneally injected with 150mg/kg cyclophosphamide 5 days and 1 day prior to tail-vein injection with *C. albicans* blastospores. The *cho1*Δ/*cho1*Δ mutants were injected at 10^6 cells, while the wild type, *psd1*Δ/*psd1*Δ, and *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ double mutants were injected at 10^4 cells each (100-fold less than the *cho1*Δ/*cho1*Δ mutant). The 100mg/kg cyclophosphamide treatments were continued by injecting the mice every four days until the experiment was terminated.

For fungal burden analysis, the kidneys were dissected at day 5 in immunocompetent and at day 4 in immunocompromised mice in order to determine fungal burden. The kidneys were weighed, crushed, serial diluted in sterile water, and plated onto YPD containing 1M sorbitol. The plates were incubated at 37ºC for 48h to determine CFU per gram of kidney. For histopathological analysis, the kidneys were excised, halved longitudinally, fixed in 10% phosphate-buffered formalin (Fisher), embedded, sectioned with a Ridge microtome (Knoxville, TN), and stained with Gomori methenamine silver (GMS) or hematoxylin and eosin (H&E) by standard protocols.

Growth curve measurement

Overnight cultures were washed twice, diluted in YPD to 0.4 OD_{600}/ml and grown for 3h at 30 ºC. The log-phase cells were then collected, washed twice, and diluted into Etn/Cho-free medium supplemented with indicated concentrations of Etn or Cho at 0.1 OD_{600}/ml for growth at 37 ºC with shaking at 250 rpm. The media was buffered to pH 4.0 with 150 mM HEPES to ensure that *C. albicans* stayed in yeast form even when

higher concentrations of Etn were added since Etn is basic and otherwise induces hyphal growth. The cell concentration was measured with a spectrophotometer at OD_{600} at 0, 2, 4, 6, 8, and 24 hours. Afterwards, the concentrations were measured daily for up to 14 days.

Susceptibility to paraquat

For spot dilution tests overnight cultures were washed twice with water, five-fold diluted, and spotted onto YPD agar plates with or without 10mM paraquat, and grown at 37ºC for 48h. For the quantitative viability test, the cells in liquid culture were diluted and spotted at ~ 200 cells per plate onto YPD agar plates containing 0, 2, 4, 6, 8, or 10mM paraquat and cultured at 37ºC for 24 ~ 48h. The survival percentage was calculated by the formula: (CFU of YPD containing paraquat) / (CFU of YPD).

Transmission electron microscopy

Cells growing at log phase were harvested and fixed for electron microscopy using a modification of a protocol by Wright (183). After harvesting the cells they were suspended in the primary fixative consisting of 3% glutaraldehyde and 3% paraformaldehyde in 0.1M phosphate buffer. After five minutes the cells were centrifuged, then re-suspended in fresh fixative overnight at 4ºC. After primary fixation the cells were washed in 0.1M phosphate buffer for 10 minutes three times. Cells were washed in 1% $KMnO_4$ for 5 minutes, centrifuged, and re-suspended in fresh $KMnO_4$ for an additional 90 minutes. Cells were then washed in water for 10 minutes three times, and en-bloc stained in 1% aqueous uranyl acetate for 60 minutes. Cells were washed in water for 10 minutes three times, and dehydrated in a graded ethanol series at 15 minute intervals. Final dehydration was accomplished with two changes in fresh 100% ethanol. Cells were infiltrated with gradually increasing ratios of Spurr resin/ethanol over a 24 hour period. The final four hours of incubation were in 100% Spurr resin under vacuum. After changing to fresh Spurr resin, samples were placed in a 68 ºC oven for 24 hours. Thin sections were cut with a Reichert OMU3 ultramicrotome, mounted on copper grids, stained with lead citrate, and examined with a Hitachi H800 transmission electron microscope operating at 75KeV.

Identification of phosphatidylserine by electrospray mass spectrometry

The putative PS spot was scraped from the wild type lane on a TLC plate (Primuline spray and UV light were applied to visualize lipid spots). The scraped powder containing the putative PS spot was mixed with 4 ml of Chloroform:Methanol:Water (5:5:1) in the screw-capped glass tube, vortexed, and incubated on ice for 5 min. After centrifugation (1000 x g for 5 min), the supernatant containing putative PS was collected

in a fresh glass tube and dried under N_2 gas. Fifty µl of chloroform was used to resuspend the dried PS. The eluted putative PS was initially confirmed by running one dimensional TLC along with PS standard, then identified by electrospray mass spectrometry. Negative electrospray mass spectra were obtained from an ion-trap mass spectrometer (LCQ Deca XP Plus, ThermoElectronic Corp.) by direct infusion of PS samples into electrospray ionization (ESI) source at a flow rate of 5 µl/min. The capillary temperature was maintained at 150ºC. The optimized ESI source conditions listed as follow: -4 V for the capillary voltage, 4.5 kV for the spray voltage, and 30 V for the tube lens offset. Nitrogen was used as sheath gas at 5 arbitrary units.

Statistical analysis

This was done using Prism 4.0 software (GraphPad Software). For the mouse model of systemic infection, Kaplan-Meier survival curves were compared for significance by using the Mantel-Haenszel log rank test. The significance of differences in phospholipid composition, fungal burden, growth curve, paraquat susceptibility, and cell wall thickness between strains was determined using the two-tailed unpaired *t* test. Statistical significance was set at a *P* value of <0.05.

RESULTS

CHO1* is required for phosphatidylserine synthesis in *C. albicans

A *C. albicans* homolog of the *S. cerevisiae* phosphatidylserine (PS) synthase gene (*CaCHO1,* hereinafter referred to as *CHO1*) was identified by BLASTing the ScCho1p protein sequence against the *Candida* Genome Database (CGD, http://www.candidagenome.org). This search revealed that *C. albicans orf19.677* encodes the closest homolog of ScCho1p, and these proteins share 61% amino acid identity.

The first allele of the *CHO1* gene was disrupted by replacing the ORF with the nourseothricin resistance cassette, *CaNAT1-FLP* (154). We were unable to obtain a *cho1*Δ*/cho1*Δ homozygous mutant in which *CaNAT1-FLP* was excised from the second disrupted allele of the *cho1*Δ*/cho1*Δ::*NAT1-FLP* strain. This was because the homozygote could not grow in the nutrient poor medium (YCB-BSA) required to drive *FLP* recombinase expression from *SAP2* promoter on this cassette (154). Therefore, the second allele of the *CHO1* gene was disrupted using another nourseothricin resistance cassette (*SAT1* flipper) where *FLP* recombinase expression is driven by the leaky *CaMAL2* promoter (143) even in YPD medium. The *cho1*Δ disruption constructs are diagrammed in Fig 3-2A. The *CHO1* gene was reconstituted in the *cho1*Δ*/cho1*Δ strain

using the reconstitution construct shown in Fig 3-2B. Gene deletions and replacements were checked for accuracy using PCR (data not shown) and Southern blotting (Fig 3-2C).

In order to determine if the *CHO1* gene product serves as the *C. albicans* PS synthase, we tested to see if the *cho1*Δ*/cho1*Δ mutant failed to generate PS as is seen in a *S. cerevisiae Sccho1*Δ mutant (4). Phospholipids were *in vivo* labeled with ^{32}P-phosphoric acid, extracted using boiling ethanol:water (4:1) (64), separated by thin layer chromatography (TLC), and visualized by autoradiography with a phosphorimager (Fig 3-2D). A spot from the wild-type strain, that comigrates with the PS standard, was missing in the *cho1*Δ*/cho1*Δ mutant. This spot, which is found in both the wild-type and *cho1*Δ*/cho1*Δ::*CHO1* reconstituted strain was confirmed to represent PS by mass spectrometry. The spots representing the PS standard and PS from the wild-type strain were both eluted from the TLC plate and examined by mass spectrometry. Mass peaks were observed at 760.85 and 760.69 for the spots representing the PS standard and the putative wild-type PS, respectively, indicating that the spot in question did represent *C. albicans* PS (data not shown). Quantification of the phospholipid spots from TLC plates (representative shown in Fig 3-2D) revealed that the radioactivity associated with the PS spot in the *cho1*Δ*/cho1*Δ mutant was only 3% that found in the wild-type strain (Table 3-1). This is very similar to the reduction in PS production measured in *Sccho1*Δ mutants in *S. cerevisiae* (92) and is equal to background levels (Table 3-1). As a further test for the role of *CHO1* in making PS in *C. albicans*, the mutant and wild-type strains were tested for their resistance to Papuamide B. Papuamide B is a a cytotoxic lipopeptide that binds PS, and in *S. cerevisiae*, a genetic screen revealed that the *Sccho1*Δ mutant, which lacks PS, is resistant to Papuamide B (137). The *C. albicans cho1*Δ*/cho1*Δ mutant is resistant to Papuamide B, while the wild-type and *CHO1* reconstituted strains are sensitive (Fig 3-2E), further indicating that *CHO1* encodes the *C. albicans* PS synthase.

The *CHO1* gene is essential for virulence

The virulence of the *cho1*Δ*/cho1*Δ mutant was tested in a mouse model of systemic candidiasis. Outbred ICR mice (Harlan) were injected with 10^6 blastospores via the lateral tail vein and monitored for signs of infection. It was found that *cho1*Δ*/cho1*Δ mutants are avirulent, whereas the wild type, *cho1*Δ*/CHO1* (data not shown), and *cho1*Δ*/cho1*Δ::*CHO1* reconstituted strains exhibited full virulence (Fig 3-3A). The difference in virulence was reflected in mouse fungal burdens measured at day 5 by examination of the kidneys (Fig 3-3C). The fungal burden of *cho1*Δ*/cho1*Δ mutants was (7±3) x 10^3 CFU/g of kidney while the wild type proliferated ~15-fold better than the

mutant exhibiting (110±2) x 10^3 CFU/g of kidney (P<0.0001). Similar survival curves and fungal burdens were observed in mice that were immunocompromised with cyclophosphamide. In this case, the mice were injected with 100-fold more blastospores (10^6) of *cho1*Δ*/cho1*Δ mutants than wild type (10^4). The *cho1*Δ*/cho1*Δ mutant was avirulent under these conditions as well (Fig S3-1), and by day 14, the mice, despite being immunocompromised, had cleared the *cho1*Δ*/cho1*Δ mutant from the kidneys, as no CFUs could be detected (data not shown).

Histological examination of the kidneys revealed that *cho1*Δ*/cho1*Δ mutant cells or hyphae were not observable in the kidneys by Gomori Methenamine Silver (GMS) staining and there was no detectable damage to kidney tissue (H&E staining after 5d infection). In contrast, in mice infected with wild type *C. albicans*, hyphae were readily observed in the kidney and caused considerable necrosis at microscopic (Fig 3-4) and macroscopic levels (Fig S3-1C).

The *cho1*Δ*/cho1*Δ mutant exhibits ethanolamine auxotrophy and very poor growth in choline

The *S. cerevisiae Sccho1*Δ mutant is an ethanolamine (Etn) auxotroph because it lacks PS, which serves as a substrate for the *de novo* synthesis of phosphatidylethanolamine (PE). In the *de novo* synthesis pathway PS is decarboxylated to PE by two different PS decarboxylase enzymes named ScPsd1p and ScPsd2p (Fig 3-1). The *Sccho1*Δ mutant is only able to grow if Etn or Cho are exogenously supplied. The mutant can make PE via the Etn branch of the Kennedy pathway, and PC by methylating PE via the *de novo* pathway (Fig 3-1). Alternatively, if supplied with Cho, the *Sccho1*Δ mutant can make PC by the Cho branch of the Kennedy pathway, and PE by deriving phosphoethanolamine (Etn-P) from dihydrosphingosine (DHS) via the *DPL1* salvage pathway (Fig 3-1). However, the ability to use Cho alone is dependent on growth on a fermentable carbon source. Growth on a non-fermentable carbon source can only be supported with Etn in the media due to an increased requirement for mitochondrial respiration (13). Mitochondria are enriched in PE, and PE is important for full mitochondrial respiratory function (13, 56, 173).

The *cho1*Δ*/cho1*Δ mutant is an ethanolamine auxotroph, like the *Sccho1*Δ mutant (Fig 3-5A&B), and shows a reduction in its PE level (Table 3-1). Even in medium containing 1 mM Etn, the *cho1*Δ*/cho1*Δ mutant did not grow as well as the wild-type (Fig 3-5B), and the doubling time calculated for the wild-type and *cho1*Δ*/cho1*Δ strains was ~1.6 hours and ~3.3 hours, respectively (Table 3-2). In contrast, the *cho1*Δ*/cho1*Δ mutant

could not be rescued on plates by addition of Cho alone to the medium (Fig 3-5A), even on a fermentable carbon source (glucose). In liquid medium containing Cho alone, there was no rescue of growth in the first 8 hours of a growth curve, but by 24 hours, there was some modest growth that eventually became more substantial, but never approached that of wild-type (Fig 3-5C). The calculated doubling times for the wild-type and *cho1*Δ*/cho1*Δ strains in the Cho supplemented medium were ~1.5 hours and ~36 hours, respectively (Table 3-2). Thus, Cho supplementation allows very little growth in this mutant. Addition of 100 fold higher levels of Cho (1mM) rescued the mutants only slightly better than the 10μM shown (Fig 3-5C, and data not shown).

Mutations in PS decarboxylase genes synthesis lead to ethanolamine or choline auxotrophy

The above results suggested the possibility that the *cho1*Δ*/cho1*Δ mutant's virulence and growth defects were primarily due to a block in the *de novo* synthesis of PE by PS decarboxylase activity (Fig 3-1). In order to test this hypothesis, the homologs of the two *S. cerevisiae* PS decarboxylase genes *ScPSD1* and *ScPSD2* were identified by BLAST searches of their protein sequences against CGD. These homologs, *orf19.6045* (*CaPSD1*, hereinafter called *PSD1*) and *orf19.3954* (*CaPSD2*, hereinafter called *PSD2*) were disrupted using the *SAT1* flipper and their genotypes confirmed by PCR (data not shown) and Southern blot (Fig S3-2). Reconstituted strains were created for each of these genes as well.

The *psd1*Δ*/psd1*Δ was found to grow poorly without exogenous Etn (Fig 3-5A), and it showed a reduction in growth compared to wild-type even when supplemented with exogenous Etn, although it grew better than the *cho1*Δ*/cho1*Δ mutant in both liquid and solid media (Fig 3-5A&B and Table 3-2). The wild-type and *psd1*Δ*/psd1*Δ mutants had doubling times of ~1.6 hours and ~2.0 hours in Etn supplemented media, respectively (Table 3-2). PE production in the *psd1*Δ*/psd1*Δ mutant was reduced, as it produced only 77% as much as the wild-type strain (Table 3-1)(P=0.005). However, this is higher than in the *cho1*Δ*/cho1*Δ mutant, which produced only 60% as much PE as wild-type strain (Table 3-1). The *psd2*Δ*/psd2*Δ mutant, in contrast, behaved like wild-type for these phenotypes (Fig 3-5A and data not shown). The *psd1*Δ*/psd1*Δ mutant grew much better than the *cho1*Δ*/cho1*Δ mutant in media supplemented only with Cho, even in the earlier parts of the growth curve, although it again grew more slowly than wild-type (Fig 3-5A&C). The doubling time for the *psd1*Δ*/psd1*Δ mutant in this medium was ~2.0 hours compared to 1.5 hours for the wild-type (Table 3-2).

Although the *psd2*Δ*/psd2*Δ mutant did not show auxotrophic or growth phenotypes, the *PSD2* gene does appear to affect these phenotypes as a *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant was more defective for growth than the *psd1*Δ*/psd1*Δ mutant in the presence of Cho or Etn (Fig 3-5A-C). The doubling time of the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant was ~2.3 hours in both types of media (Table 3-2). This phenotype correlated with a decreased level of PE, as PE in the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant was 55% the level of wild-type as compared with 77% in the *psd1*Δ*/psd1*Δ single mutant (Table 3-1). The level observed in the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant is similar to that seen in the *cho1*Δ*/cho1*Δ mutant (Table 3-1). Interestingly, the PS level was increased ~two-fold in *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant as compared with that of wild-type (Table 3-1) (P=0.003). The behavior of the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant was somewhat complex compared to the *cho1*Δ*/cho1*Δ mutant when growing in medium supplemented with Etn or Cho. In liquid medium supplemented with Etn or Cho the double mutant had a faster doubling time than the *cho1*Δ*/cho1*Δ mutant (Fig 3-5B,C and Table 3-2). However, the double mutant exhibited similar growth defects to the *cho1*Δ*/cho1*Δ mutant on agar plates supplemented with Etn or Cho (Fig 3-5A).

PS decarboxylase genes *PSD1* and *PSD2* are synthetically required for virulence

Although the *psd1*Δ*/psd1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants differed in some respects from the *cho1*Δ*/cho1*Δ mutants, they exhibited a number of similarities which suggested that these mutants might affect virulence in the mouse model of systemic candidiasis. Therefore, mice were challenged with 10^6 blastospores of the *psd1*Δ/*psd1*Δ or *psd2*Δ*/psd2*Δ single mutants or the *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ double mutant as previously described. The *psd1*Δ/*psd1*Δ and *psd2*Δ*/psd2*Δ mutants were fully virulent and behaved like wild-type (Fig 3-3B). In contrast, the *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ double mutant exhibited very attenuated virulence (Fig 3-3B), although it was more virulent than the *cho1*Δ*/cho1*Δ mutant as a few mice infected with the double mutant did succumb to infection whereas no mice infected with the *cho1*Δ*/cho1*Δ mutant showed any symptoms of disease (Fig 3-3A). This difference in virulence was reflected in a two-fold larger fungal burden in the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant [(15± 0.5) x 10^3 CFU/g of kidney] compared to the *cho1*Δ*/cho1*Δ mutant [(7±3) x 10^3 CFU/g of kidney] (Fig 3-3C). This difference was not statistically significant, however, a similar difference was also seen in the cyclophosphamide treated mice (Fig S3-1). In addition, there was no evidence of *C. albicans* cells in histological sections of kidneys taken from

mice infected with the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant (Fig 3-4). There was also no sign of infection observed in the kidneys from immunocompromised mice when examined macroscopically (Fig S3-1C).

Although the *psd1*Δ*/psd1*Δ mutant exhibited no difference from wild-type in virulence as measured by the time it took for mice to succumb to the infection (Fig 3-3B), there was a statistically significant increase in the number of CFU/g of kidney of the *psd1*Δ*/psd1*Δ mutant compared to the wild-type strain ($P<0.0001$, Fig 3-3C).

Defects in PE and PS biosynthesis result in mitochondrial respiration defects

In *S. cerevisiae*, the loss of PS or PE biosynthesis leads to an increase in the formation of petite mutants, which are mutants in which the mitochondrial genome has been lost or the mutants exhibit mitochondrial respiration defects. The increase in petite formation appears to be due to the fact that mitochondria are enriched for PE, and the major site of *de novo* PE synthesis is the inner mitochondrial membrane where ScPsd1p converts PS to PE. PE is necessary for proper mitochondrial function, and a large decrease in PE levels correlates with mitochondrial defects and petite formation (5, 13, 28). It was suspected that the mitochondrial respiratory function in the *C. albicans cho1*Δ*/cho1*Δ mutant might be compromised. *C. albicans* is a non-petite forming yeast, meaning that it cannot survive without an intact mitochondrial genome (3), and defects in PE or PS biosynthesis may compromise mitochondrial respiration. In order to test this, the mutants were examined for sensitivity to paraquat. Paraquat is a herbicide that generates superoxide radicals and oxidative damage in the mitochondria based on its interactions with complex I and/or alternative NAD(P)H dehydrogenases in the electron transport chain (29). In *S. cerevisiae*, mutants that are defective for mitochondrial respiration show enhanced resistance to paraquat due to decreased electron transport chain activity (14). In addition, a *C. albicans* mutant that exhibits decoupling of oxidative phosphorylation is also more resistant to paraquat (26). Therefore, we examined the *cho1*Δ*/cho1*Δ*, psd1*Δ*/psd1*Δ*,* and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants for paraquat resistance. All three mutants were clearly more resistant to paraquat based on an agar dilution assay on plates containing 10 mM paraquat (Fig 3-6A). The *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants did not grow as well as the single mutants on paraquat plates, but this mutant exhibits poorer growth than that of the single mutants on regular YPD plates, and it grew better than the wild-type on the paraquat plate (Fig 3-6A). The mutants were also examined by a more quantitative assay in which cells were pregrown in liquid YPD medium and then plated at equal concentrations on YPD plates containing 0, 2, 4, 6, 8, or

10 mM paraquat. The number of colony forming units was then examined. It was found that the *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ mutants exhibited a quantitatively similar resistance to paraquat (Fig 3-6B). The *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant was not very resistant based on this assay, but that may be related to its poorer growth on YPD agar plates and not reflect a lowered resistance. The paraquat resistance exhibited by these mutants was not due to increased resistance to oxidative stress in general as they were not hyper-resistant to hydrogen peroxide (Y. -L. Chen and T. B. Reynolds, data not shown).

The *Sccho1*Δ mutant has been noted to exhibit a cold-sensitive increase in petite formation, such that the percentage of cells that loose their mitochondrial genome increases as the temperature decreases (5). As noted above, *C. albicans* is petite negative and presumably loss of the mitochondrial genome is lethal. The *cho1*Δ*/cho1*Δ mutant was found to be cold-sensitive for growth. The mutant exhibited decreased growth at 30˚C compared to 37˚C, but was completely unable to grow at 15˚C, even when the assay was extended out to 4 days (Fig 3-7A). The *psd1*Δ*/psd1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants also exhibited cold sensitive growth, although the *psd1*Δ*/psd1*Δ mutant was not as sensitive as the *cho1*Δ*/cho1*Δ mutant.

A common test for mitochondrial respiratory deficiency in yeast strains has been use of the indicator plates containing trypan blue and eosin Y. On these plates mutants lacking mitochondrial respiratory function take up the dye more readily than wild-type (62, 120). This assay has been shown to work in *C. albicans* as well (26, 62). This assay was performed on the *C. albicans* phospholipid biosynthetic mutants, and it was found that all of the mutants took up the dye at greater levels than the wild-type, but there was an increasing defect indicated in the mutants such that the *cho1*Δ*/cho1*Δ mutant appeared most defective, *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ was somewhat less defective, and the *psd1*Δ*/psd1*Δ mutant was the least defective, but was slightly more perturbed than the wild-type strain (Fig 3-7B).

Defects in PE and PS biosynthesis result in cell wall defects

The *cho1*Δ*/cho1*Δ*, psd1*Δ*/psd1*Δ*,* and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants all exhibit growth defects compared to the wild-type strain (Figs 3-5 and 3-8). A PS synthase mutant in *Schizosaccharomyes pombe* (*pps1*Δ) has been shown to exhibit slow growth due to cell wall defects (112). The *C. albicans* phospholipid biosynthetic mutants were therefore tested to determine if they too exhibited slow growth due to cell wall defects by growing them on plates containing osmotic stabilizers. All three mutants grew

like wild-type on plates containing 1M sorbitol, 0.8M NaCl, or 0.1M $CaCl_2$ (Fig 3-8A). This indicated that these mutants all exhibited some form of cell wall defect. The cold-sensitive growth described above (Fig 3-7A) was not rescued by growth in osmoremedial media (Y. -L. Chen and T. B. Reynolds, data not shown). In addition, osmotic stabilization did not rescue the auxotrophic defects reported above (data not shown).

The nature of the cell wall defect was further explored by examining different insults that perturb the cell wall. The *cho1*Δ*/cho1*Δ mutant and the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant both showed a strong sensitivity to 0.05% SDS, which is thought to measure cell wall permeability (167). The *cho1*Δ*/cho1*Δ mutant was more defective in this assay than the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant. The *psd1*Δ*/psd1*Δ mutant did not appear to be more sensitive than wild-type on YPD plates containing 0.05% SDS (Fig 3-8B). In contrast, all three phospholipid biosynthetic mutants exhibited increased resistance to caffeine compared to the wild-type strain (Fig 3-8C). None of the mutants were affected by calcofluor white in the media (data not shown). Addition of the chitin synthase inhibitor nikkomycin Z appeared to cause a very slight defect in growth of the *cho1*Δ*/cho1*Δ mutant, although it was hard to measure as the mutant exhibits a growth defect already compared to the wild-type, and the wild-type strain is also slightly inhibited by nikkomycin Z (see Appendix). The *cho1*Δ*/cho1*Δ mutant also exhibited a defect visualized by transmission electron microscopy (TEM) that was not seen in the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant or the *psd1*Δ*/psd1*Δ single mutant. TEM revealed that the *cho1*Δ*/cho1*Δ mutant had a higher percentage of cells that exhibited an altered distance between the plasma membrane and cell wall (Fig 3-8D), suggesting a defective plasma membrane or cell wall.

The *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants exhibit defects in filamentous growth

Filamentous growth is a well known virulence factor in *C. albicans*, and mutants that show severe defects in this phenotype also often exhibit altered virulence. Mutants in mitochondrial respiration enzymes such as Ndh51p have shown defects in filamentous growth (114). Therefore, filamentous growth was examined in the *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants. Strains were streaked onto plates and grown in conditions known to promote filamentous growth including medium 199 at pH 7.0 (M199-7), Spider medium, and YPD+10% serum. In comparison to the wild-type, the *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants were poorly filamentous (Fig 3-9A). However, if the media were osmotically stabilized by the addition of 1M sorbitol,

then the *cho1*Δ*/cho1*Δ mutant exhibited greater filamentous growth (Fig 3-9B). It was still less filamentous than wild-type on M199-7, but it was more filamentous than wild-type on spider and YPD+10% serum media. Filamentous growth in the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant, in contrast, was not rescued by osmotic stabilization (Fig 3-9B).

At very early time points, the *cho1*Δ*/cho1*Δ mutant was actually hyperfilamentous on YPD+10% serum medium compared to wild-type, even without osmotic stabilization (data not shown), but at later time points the colonies appeared less filamentous (Fig 3-9A). The *cho1*Δ*/cho1*Δ mutant also formed hyper-wrinkled colonies on YPD containing medium, both ± serum (data not shown). Neither of these effects were observed in the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant (data not shown).

The behavior of the mutants was also examined in liquid serum. In 100% bovine calf serum, it was found that the *cho1*Δ*/cho1*Δ mutant exhibited slower germ tube formation than the wild-type, and after a few hours growth was arrested altogether (Fig 3-9C). The addition of extra Etn rescued filamentous growth in the *cho1*Δ*/cho1*Δ mutant, but extra Cho had no effect (data not shown). The *psd1*Δ*/psd1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants, in contrast, exhibited nearly wild-type levels of filamentous growth (Fig 3-9C).

DISCUSSIONS

The *cho1*Δ*/cho1*Δ mutant in *C. albicans* is clearly avirulent in a mouse model of systemic candidiasis (Fig 3-3), and cannot even cause disease in a mouse immunocompromised with cyclophosphamide when infected with 100-fold more blastospores than the wild-type strain (Fig S3-1). Examination of the mouse kidneys quantitatively (Fig 3-3C, S3-1) and qualitatively (Figs 3-4 and S3-1) reveal that the *cho1*Δ*/cho1*Δ mutant is eventually completely cleared from mice and causes no apparent tissue damage. The loss of PS synthesis plays a very important role in the loss of virulence, but the downstream loss of *de novo* PE synthesis (Fig 3-1) also causes a loss in virulence. This conclusion is based on the fact that a *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ double mutant is extremely attenuated for virulence (Fig 3-3B). This double mutant lacks *de novo* PE biosynthesis, like the *cho1*Δ*/cho1*Δ mutant, and has a very similar drop in total cellular PE (Table 3-1). However, unlike *cho1*Δ*/cho1*Δ, the double mutant does not lack PS.

Based on our results, we propose that the *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants share a number of defects in cell wall integrity, mitochondrial

function, and filamentous growth that contribute to their loss of virulence. However, the *cho1*Δ*/cho1*Δ mutant has an additional phenotype, not shared with the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant, that makes it completely avirulent. This phenotype is its severe growth defect in the presence of Cho as the sole exogenous source for the Kennedy pathway (Fig 3-1).

We could not find the serum level of choline or ethanolamine in mice, but rat serum is estimated to contain ~10 μM Cho, and is higher in some other organisms (6, 185) . We found that the *cho1*Δ*/cho1*Δ mutant had a very slow doubling time of ~36 hours in media supplemented with 10 μM Cho (Table 3-2). Supplementing the media with 1 mM Cho (100 x increase) did not increase the growth rate in the early time points and had a modest effect only after several days (data not shown). Rat serum also contains an estimated 30 μM Etn (87), however, *C. albicans cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants require a minimum of ~1 mM Etn to fulfill their auxotrophic requirements, so the level of Etn in the serum is inadequate to support growth of the *cho1*Δ*/cho1*Δ mutant. This is reflected by the inability of the *cho1*Δ*/cho1*Δ mutant to progress beyond initial germ tube formation in 100% serum unless supplemented with 1 mM Etn (Fig 3-9C), and the inability to appreciably grow in liquid or solid media supplemented with 30 μM Etn (data not shown). We propose that the *cho1*Δ*/cho1*Δ mutant is extremely avirulent because it is unable to grow in the host environment due to insufficient Etn and Cho levels.

The behavior of the *C. albicans cho1*Δ*/cho1*Δ mutant in Etn vs Cho supplemented glucose media is very different from the *S. cerevisiae Sccho1*Δ mutant when it is grown on glucose media containing Etn or Cho, and is more akin to the *Sccho1*Δ mutant grown on a non-fermentable carbon source supplemented with Etn or Cho (13). On non-fermentable carbon sources, such as lactate, the *Sccho1*Δ mutant is also only able to grow on Etn. This is believed to be caused by the increased need for mitochondrial activity, which is an organelle rich in PE. The *DPL1* salvage pathway is not able to efficiently supply Etn for the increased demand for mitochondrial activity. In fact, overexpression of *DPL1* rescues growth of *Sccho1*Δ mutants on non-fermentable carbon sources (13).

Based on the work from *S. cerevisiae*, it was expected that the lack of responsiveness to Cho in the *C. albicans cho1*Δ*/cho1*Δ mutant was due to the lack of *de novo* PE formation and poor mitochondrial activity. If this were the case, then the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant should share this phenotype as it has a similar loss of *de novo* PE synthesis (Table 3-1, Fig 3-1). However, this is clearly not the case in *C.*

albicans (Fig 3-5). In fact, in this regard, *C. albicans* even differs from the PS synthase mutant *pps1*Δ in *S. pombe*, another petite negative yeast. The *pps1*Δ mutant is also an Etn auxotroph that cannot be supported by Cho even on fermentable carbon sources (112). However, further work with a mutant lacking all three *S. pombe* PS decarboxylase genes (*psd1-3*Δ) has revealed that a lack of PE was the cause of the inability to respond to Cho (103). Thus, inability to respond to Cho due to a loss of PS in particular is specific to *C. albicans*. The reason for this defect is unknown, but several possibilities present themselves. 1) In the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant there is a buildup of PS. Perhaps, PS can compensate for the lack of PE in the mitochondria. PS decarboxylase mutants in *S. pombe* and *S. cerevisiae* also experience a buildup of PS (13, 112). So if this explanation is correct, *C. albicans* is unusual. 2) The *DPL1* pathway in *C. albicans* may be compromised by a lack of PS, and this does not allow it to supply sufficient PE even on a fermentable carbon source. 3) PS or PE is required for the function of several transporters in *S. cerevisiae* (124, 131, 132, 146), and it is possible that the transporter for Cho in *C. albicans* is inhibited by a loss of PS in the membrane. This has not been observed for the Cho transporter, Hnm1p (96), in *S. cerevisiae*, but may be the case for the transporter in *C. albicans*. If this is the case, then cells growing on Cho alone would not be able to make sufficient PC via the Kennedy pathway to grow efficiently (Fig 3-1). Whatever the reason for the inability of Cho to supplement growth of the *cho1*Δ*/cho1*Δ mutant in the presence of Cho alone, we believe that is an important contributor to its loss of virulence.

Loss of *de novo* PE biosynthesis also contributes to a loss of virulence

The *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant is very attenuated for virulence. It suffers a growth defect compared to wild-type, but in comparison to the fully virulent *psd1*Δ*/psd1*Δ mutant, its growth is not seriously diminished (Fig 3-5, Table 3-2). This result suggests to us that the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant is defective in virulence for reasons other than a slightly diminished growth rate. This mutant, like the *cho1*Δ*/cho1*Δ mutant exhibits defects in cell wall biosynthesis, mitochondrial function, and hyphal growth. All of these factors might contribute to a loss of virulence and inability to grow in the host.

Three of the phospholipid mutants in this analysis, *psd1*Δ*/psd1*Δ*, cho1*Δ*/cho1*Δ*,* and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ, all exhibited some level of cell wall defects including osmoremedial growth and resistance to caffeine (Fig 3-8). However, only the two virulence-defective mutants, *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ, exhibited a strong sensitivity to SDS. SDS sensitivity can be interpreted to indicate an increased

permeability of the cell wall (167). The *cho1*Δ*/cho1*Δ mutant has a stronger defect than the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant in this regard, and also exhibits an increased distance between the cell wall and plasma membrane in the electron micrographs that is not seen in the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant (Fig 3-8D). The reason for this may be due to cell wall or plasma membrane defects, but is not known.

Both mutants suffer from a defect in *de novo* PE biosynthesis, which clearly impacts the cell wall, but loss of PS itself has an impact on this phenotype. The loss of PE may affect the cell wall by impacting glycosylphosphatidylinositol (GPI) anchored protein maturation as has been seen for the *Scpsd1*Δ *Scpsd2*Δ mutant in *S. cerevisiae*. In the *Scpsd1*Δ *Scpsd2*Δ mutant loss of *de novo* PE biosynthesis leads to poor maturation of ScGas1p even in the presence of exogenous Etn in the media. An attempt was made to compare the maturation of GPI-anchored proteins in *C. albicans cho1*Δ*/cho1*Δ and wild-type strains by Western blotting using an antibody specific to ScGas1p that has been shown to cross-react with similar *C. albicans* cell wall proteins. However, the results were inconclusive (data not shown), and we do not know if GPI-anchor synthesis is impacted in *C. albicans* in a similar manner as in *S. cerevisiae*. If so, then this may help to explain the cell wall defects seen in the *C. albicans psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant.

Alternatively, PE biosynthesis defects may impact virulence by affecting the mitochondria. Three of the phospholipid biosynthetic mutants, *psd1*Δ*/psd1*Δ, *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ, all exhibited resistance to paraquat on plates (Fig 3-6A) suggesting that they have defects that affect respiration. The mutants also exhibited very similar resistances to paraquat quantitatively, expect for the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant, but this may be due to its growth defect in non-osmoremedial media (Fig 3-6B). The paraquat resistance could be due to uncoupling of oxidative phosphorylation, as an undefined *C. albicans* mutant that is uncoupled for oxidative phosphorylation was also resistant to paraquat (26).

There was not an obvious difference between the virulent and non-virulent mutants by the paraquat resistance assay, however the trypan blue/eosin Y indicator plate assay revealed that the less virulent *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants gave a much stronger phenotype than the *psd1*Δ*/psd1*Δ mutant (Fig 3-7B). The basis for this assay is not understood, so the particular mitochondrial dysfunction that appears to be worse in the *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants compared to the *psd1*Δ*/psd1*Δ mutant is not known.

The cold-sensitive growth assay suggests that the *cho1*Δ*/cho1*Δ mutant has a

greater defect in mitochondrial genome stability. *S. cerevisiae Sccho1*Δ mutants show a cold-sensitive increase in petite mutant formation. The *psd1*Δ*/psd1*Δ, *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants all showed a cold-sensitive growth defect, but *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants showed a stronger defect than the *psd1*Δ*/psd1*Δ mutant (Fig 3-7A). PE has been shown to be important for maintenance of the mitochondrial genome, and these mutants' phenotype may reflect a general instability in mitochondrial proteins.

The defect observed in the mitochondria correlates with defects in the cell wall and it is possible that these phenotypes are connected. The function of PE in maintaining mitochondrial function overlaps with that of another mitochondrial lipid called cardiolipin (CL) (56). Mutants that lack CL due to a defect in the Pgs1p enzyme in *S. cerevisiae* have been shown to have cell wall defects due to dampened cell wall integrity MAP kinase (CWI-MAPK) signaling (188, 189). It is possible that the PE deficiencies caused by the *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants lead to defects in CWI-MAPK signaling.

The *cho1*Δ*/cho1*Δ mutant is more defective for cell wall biosynthesis and mitochondrial function than the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant. The reason for the increasing cell wall defect in the *cho1*Δ*/cho1*Δ mutant may be related to the function of PS in signal transduction. PS has been shown to stimulate ScPkc1p activity through ScRom2p in *S. cerevisiae* (79). ScPkc1p is at the head of the CWI-MAPK cascade and influences a number of aspects of cell wall biosynthesis (93). The loss of PS in *C. albicans* may hamper Pkc1p activity and cell wall biosynthesis in this yeast. In addition, PS has been shown to affect calcineurin activity as well (140). Calcineurin influences the cell wall in bakers' yeast and *C. albicans* (52, 181). Addition of 100mM $CaCl_2$ to the phospholipid mutants osmostabilizes their growth (Fig 3-8A), but addition of the calcineurin inhibitors cyclosporine A or FK506 (66) slightly inhibits the osmoremediated growth of the *cho1*Δ*/cho1*Δ mutant (Fig S3-3), suggesting that PS is required for full calcineurin activity in *C. albicans*.

The *S. pombe pps1*Δ PS synthase mutant also shows cell wall defects, however its defects are exclusively due to the loss of PS, as a mutant lacking all three PS decarboxylase genes (*Sppsd1-3*Δ) does not exhibit similar cell wall defects as a *pps1*Δ mutant (103). In addition, the nature of the cell wall defects may differ as well. The *C. albicans* mutants are not sensitive to calcofluor white and show only a very slight sensitivity to the chitin synthase inhibitor Nikkomycin Z (data not shown). The *pps1*Δ

mutant is very sensitive to calcofluour white which is associated with binding to chitin (112). Thus, the basic defects in the cell wall may be quite different in these two yeasts.

The cell wall defects in the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant may help to explain the defects in virulence as a number of cell wall mutants have been found that are reduced in virulence (7, 110, 115, 119, 129, 135). One explanation for the reduced virulence in some cell wall mutants is increased "unmasking" of ß1,3-glucan, and greater immune response from the host (177, 178). This may be the case for the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant, but this remains to be tested.

An interesting contrast to the strong virulence defect of the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant is the *psd1*Δ*/psd1*Δ mutant. In *S. cerevisiae*, the ScPsd1p enzyme is localized to the mitochondria and produces 90% of the *de novo* synthesized PE in the cell. The *C. albicans* Psd1p enzyme is likely similar since it is much more defective for growth in the absence of Etn supplementation. In fact, as in *S. cerevisiae*, the Kennedy pathway does not seem to be able to fully compensate in *C. albicans* in the absence of Psd1p or both enzymes (13). The *psd1*Δ*/psd1*Δ mutant is fully virulent even though it does have cell wall and mitochondrial defects, although milder than the double mutant. Surprisingly, the *psd1*Δ*/psd1*Δ mutant actually colonized the kidneys to a greater extent than the wild-type. The reason for this is not known, but it bears some resemblance to an undefined *C. albicans* oxidative phosphorylation decoupling mutant. This mutant also colonized the kidneys efficiently, but it was avirulent. This mutant was suggested by the authors to carry multiple undefined mutations, so its avirulence and heavy colonization of the kidneys may be unlinked (26). Thus, it is possible that the *psd1*Δ*/psd1*Δ mutation's mitochondrial defect may help lead to better immune evasion and colonizing of the kidneys. However, if this is the case, then even greater defects related to PE depletion (*i.e.* *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant) lead to a loss of virulence and colonization. These questions all remain to be resolved.

Filamentous growth in the absence of PE and PS

The *cho1*Δ*/cho1*Δ and *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutants exhibited defects in filamentous growth in response to several different environmental stimuli such as pH, glucose starvation, and serum (Fig 3-9). Filamentous growth has been associated with virulence in *C. albicans*, and this could be a contributor to the avirulence of these strains as well. The *cho1*Δ*/cho1*Δ mutant was arrested in germ tube formation in serum (Fig 3-9C), but this is likely related to its growth defect in medium supplemented with Cho (Fig 3-5), as addition of extra Cho did not improve its growth, but 1 mM Etn did allow a

recovery of filamentous growth (data not shown). The *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant did not exhibit this defect in 100% serum (Fig 3-9C). It is of note that it has been shown that disruption of the mitochondrial electron transport chain NADH dehydrogenase complex I gene *NDH51* leads to defects filamentous growth in basal salts medium (114). Thus, the filamentous growth defects in the phospholipid mutants may be related to a loss of mitochondrial capacity. Interestingly, the addition of 1 M sorbitol allowed a hyperfilamentous growth in the *cho1*Δ*/cho1*Δ mutant, but not the *psd1*Δ*/psd1*Δ *psd2*Δ*/psd2*Δ mutant (Fig 3-9). The reason for this recovery in the one mutant, but not the other is not clear.

Conclusions

The phospholipid biosynthetic mutants described in this work make it clear that loss of PS and *de novo* PE synthesis cause virulence defects. The pleiotropic nature of the mutants makes it difficult to determine precisely why the mutants are less virulent, but we believe the evidence supports the hypothesis that the loss PS leads to avirulence due to extremely poor growth without Etn. The loss of PE may lead to a loss of virulence more because of cell wall, mitochondrial, and/or filamentous growth defects. We suspect that all of these defects are related to mitochondrial dysfunction, but this remains to be determined.

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APPENDIX C: TABLES AND FIGURES

Table 3- 1. Phospholipid composition of wild type and phospholipid mutants in *C. albicans**

Strain	Phospholipid composition (% of total)				
	PS	PE	PI	PC	Others
WT	6.1 ± 0.8 [a,b,c]	14.5 ± 0.8 [d,e,f,g]	13.6 ± 0.9	53.6 ± 1.0 [h,i]	12.2 ± 1.7
*cho1*Δ/*cho1*Δ	0.2 ± 0.2 [a]	8.7 ± 1.5 [d]	15.6 ± 1.8	61.0 ± 3.4 [h]	14.5 ± 0.5
*cho1*Δ/*cho1*Δ::*CHO1*	2.8 ± 0.0 [b]	12.5 ± 0.3 [e]	15.3 ± 1.0	56.3 ± 0.9 [i]	13.2 ± 0.5
*psd1*Δ/*psd1*Δ	8.0 ± 2.0	11.1 ± 0.7 [f]	14.4 ± 1.5	55.2 ± 2.0	11.3 ± 1.4
*psd1*Δ/*psd1*Δ::*PSD1*	5.5 ± 0.2	13.8 ± 0.5	13.0 ± 0.8	56.1 ± 1.6	11.5 ± 0.6
*psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ	11.2 ± 1.0 [c]	8.0 ± 1.1 [g]	14.0 ± 4.8	56.4 ± 8.0	10.4 ± 1.2

*Cultures were grown to uniform labeling in YPD medium containing 5 µCi/ml of [^{32}P] phosphoric acid. Lipids were extracted and analyzed as described in Material and Methods. Data represent the mean ± standard deviation of one independent experiment and one duplicate experiment. The P values indicated by superscripted lower case were a (0.0003); b (0.002); c (0.003); d (0.004); e (0.02); f (0.005); g (0.001); h (0.02); i (0.02).

Table 3- 2. Doubling times of wild type and phospholipid mutants in Etn and Cho supplemented minimal media

Strain	Doubling time (hours)	
	Etn (1mM)	Cho (10μM)
Wild-type	1.6	1.5
cho1Δ/cho1Δ	3.3	36.0
psd1Δ/psd1Δ	2.0	2.0
psd1Δ/psd1Δ psd2Δ/psd2Δ	2.3	2.2

Table S3- 1. *C. albicans* strains

Strain	Parent	Genotype	Source of reference
SC5314 (wild-type)	Clinical isolate	Prototrophic wild type	(55)
YLC132	SC5314	*cho1*Δ::*NAT1-FLP*/*CHO1*	This study
YLC137	YLC132	*cho1*Δ/*CHO1*	This study
YLC142	YLC137	*cho1*Δ/*cho1*Δ::*NAT1-FLP*	This study
YLC334	YLC137	*cho1*Δ/*cho1*Δ::*SAT1-FLP*	This study
YLC337	YLC334	*cho1*Δ/*cho1*Δ	This study
YLC344	YLC337	*cho1*Δ/*cho1*Δ::*CHO1-SAT1*	This study
YLC335	YLC137	*cho1*Δ/*cho1*Δ::*SAT1-FLP*	This study
YLC339	YLC335	*cho1*Δ/*cho1*Δ	This study
YLC345[a]	YLC339	*cho1*Δ/*cho1*Δ::*CHO1-SAT1*	This study
YLC240	SC5314	*psd1*Δ::*SAT1-FLP*/*PSD1*	This study
YLC247	YLC240	*psd1*Δ/*PSD1*	This study
YLC254	YLC247	*psd1*Δ/*psd1*Δ::*SAT1-FLP*	This study
YLC280	YLC254	*psd1*Δ/*psd1*Δ	This study
YLC294	YLC280	*psd1*Δ/*psd1*Δ::*PSD1-SAT1*	This study
YLC243	YLC250	*psd2*Δ::*SAT1-FLP*/*PSD2*	This study
YLC250	YLC243	*psd2*Δ/*PSD2*	This study
YLC257	YLC250	*psd2*Δ/*psd2*Δ::*SAT1-FLP*	This study
YLC271	YLC257	*psd2*Δ/*psd2*Δ	This study
YLC290	YLC271	*psd2*Δ/*psd2*Δ::*PSD2-SAT1*	This study
YLC299	YLC271	*psd2*Δ/*psd2*Δ *psd1*Δ::*SAT1-FLP*/*PSD1*	This study
YLC300	YLC299	*psd2*Δ/*psd2*Δ *psd1*Δ/*PSD1*	This study
YLC331[b]	YLC300	*psd2*Δ/*psd2*Δ *psd1*Δ/*SAT1-*P_{MET3}*-PSD1*	This study
YLC373	YLC300	*psd2*Δ/*psd2*Δ *psd1*Δ/*psd1*Δ::*SAT1-FLP*	This study
YLC375	YLC373	*psd2*Δ/*psd2*Δ *psd1*Δ/*psd1*Δ	This study

[a] another separately derived *cho1*Δ/*cho1*Δ::*CHO1* reintegrant strain.

[b] conditional construct, second allele of *PSD1* was controlled by *MET3* promoter.

Table S3- 2. Plasmids

Plasmids	Characteristics	Source of reference
pJK863	*CaNAT1-FLP* cassette carrying nourseothricin resistance gene	(154)
pSFS2A	*SAT1* flipper carrying nourseothricin resistance gene	(143)
pYLC130	pJK863 flanked 5' and 3' *CHO1*^{NCR} for *CHO1* 1st allele knock out	This study
pYLC326	pSFS2A flanked 5' and 3' *CHO1*^{NCR} for *CHO1* 2nd allele knock out	This study
pYLC341	*CHO1* reconstitution construct	This study
pYLC231	pSFS2A flanked 5' and 3' *PSD1*^{NCR} for *PSD1* gene knock out	This study
pYLC286	*PSD1* reconstitution construct	This study
pYLC234	pSFS2A flanked 5' and 3' *PSD2*^{NCR} for *PSD2* gene knock out	This study
pYLC288	*PSD2* reconstitution construct	This study
pYLC314	*SAT1* marked *CaMET3* promoter construct	This study
pYLC324	*PSD1* conditional construct controlled by *CaMET3* promoter	This study

Table S3- 3. PCR primers

Primer	Use	Sequence (5' → 3')
JCO43	Disrupt *CHO1*	AAAAAAGGTACCCCCCCCACTGAACTTTAACCAA
JCO44	Disrupt *CHO1*	AAAAAAGGGCCCGAAAAGTGGAAGAAGAGCTGA
JCO45	Disrupt *CHO1*	AAAAAACCGCGGTTTTGGTATTATCAGACGCTG
JCO46	Disrupt *CHO1*	AAAAAAGAGCTCTGCTGTGAAGAGAGCAAATG
TRO369	Confirm c*ho1*Δ	GCACGTCAAGACTGTCAAGG
JCO96	Confirm c*ho1*Δ, *psd1*Δ, *psd2*Δ	TGAAGGGGGAGATTTTCACT
JCO99	Confirm c*ho1*Δ	AGATCATTCAAGCTACCCAG
JCO169	Restore *CHO1*	AAAAAAGCTTTCTCTATGGTTTAGGAATT
JCO141	Disrupt *PSD1*	AAAAAAGGGCCCTAAAGCAGCGTGGTATCGTGA
JCO142	Disrupt *PSD1*	AAAAAACTCGAGCAGTCAAACAAAGGAGTAACG
JCO143	Disrupt *PSD1*	AAAAAACCGCGGAGGAAGCTCAGCGTAATGGTT
JCO144	Disrupt *PSD1*	AAAAAAGAGCTCCAGTTGCAGATCTTGGTGTTG
JCO145	Confirm psd*1*Δ	TTCAACTTGTTGGTGACCCA
JCO157	Restore *PSD1*	AAAAAAGATATCCCTCTATACGAACCCACCTAA
JCO146	Disrupt *PSD2*	AAAAAAGGGCCCGAGTTGTAGCAAAAGTTACCG
JCO147	Disrupt *PSD2*	AAAAAACTCGAGTGCTGGGTTGAGTTGAATTCT
JCO148	Disrupt *PSD2*	AAAAAACCGCGGGTCGTACATTAAGCAAATGAA
JCO149	Disrupt *PSD2*	AAAAAAGAGCTCCCGATTACTTTCCTCATCGT
JCO150	Confirm *psd2*Δ	AGCGGGGAATCAAGTTACAA
JCO158	Restore *PSD2*	AAAAAAGATATCACTTTAATCTTCACTTTTGA
JCO165	*PSD1* conditional construct	AAAGAATTCCGTCAAAACTAGAGAATAATAAAG
JCO166	*PSD1* conditional construct	AAAGAATTCGTTTTCTGGGGAGGGTATTT
JCO167	*PSD1* conditional construct	AAACCGCGGATGCCACTCAAACCTATTTCG
JCO168	*PSD1* conditional construct	AAAGAGCTCCTATACGAACCCACCTAATGA

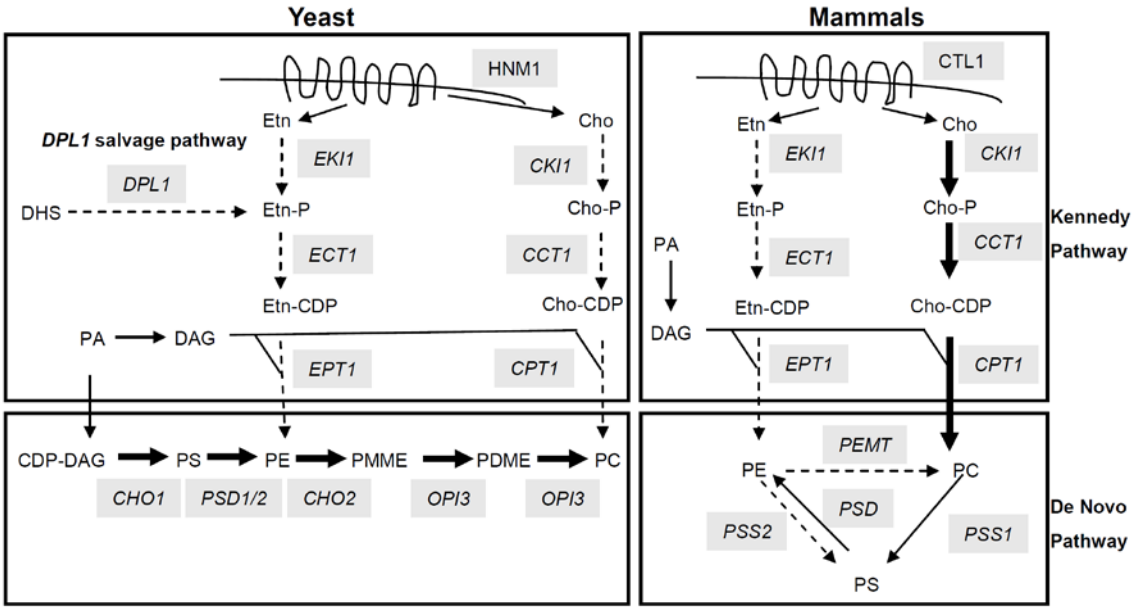
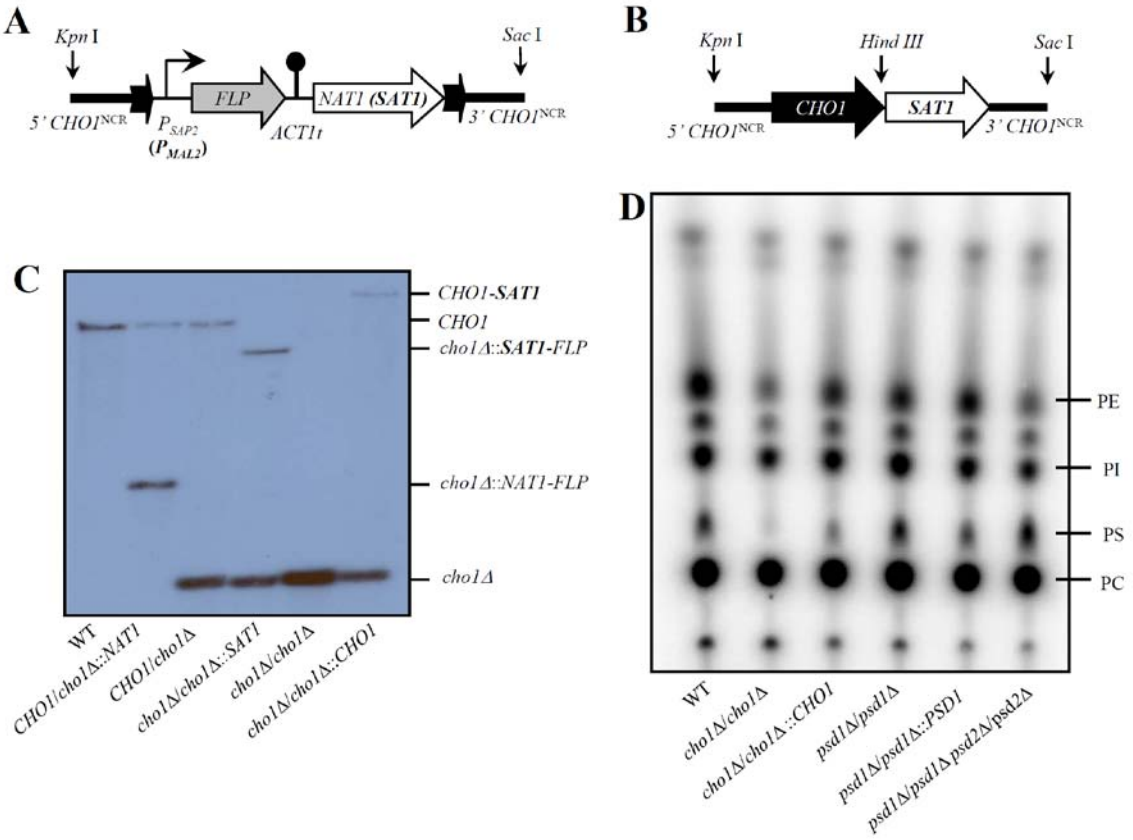


Fig 3- 1. The aminophospholipid biosynthetic pathways of both yeast and humans consist of two alternative pathways:

The de novo pathway and the Kennedy pathway, but there are significant differences in the de novo pathways of mammals and fungi. The Kennedy pathways of the two organisms are similar in structure, but the level to each is used to generate particular phospholipids is indicated by the thickness of the arrows. Dark bold arrow are used the most, dotted arrows the least, and thin solid arrows at intermediate levels. The de novo pathways differ between mammals and yeast. The yeast makes PS from CDP-DAG via the PS synthase (Cho1p) while mammals use Pss1 or Pss2 to make PS from PC or PE, respectively. Yeast carries two decarboxylases which convert PS to PE while mammals only carry one (PSD). Yeast convert PE to PC via three methylation reactions that are performed by two different sequential methylase enzymes whereas mammals only have one methylase (PEMT) that performs all three reactions. Etn: ethanolamine, Cho: choline, PS: phosphatidylserine, PE: phosphatidylethanolamine, PC: phosphatidylcholine, CDP-DAG: cytidyldiphosphate diacylglycerol, DAG: diacylglycerol, PMME: phosphatidylmonomethylethanolamine, PDME: phosphatidyldimethylethanolamine, Etn-P: phosphoethanolamine, Cho-P: phosphocholine, Etn-CDP: ethanolamine-cytididyldiphosphate, Cho-CDP: cholinecytidyldiphosphate, DHS: dihydrosphingosine.

Fig 3- 2. *CHO1* is required for phosphatidylserine synthesis in *C. albicans*.

(A) Structure of the *CHO1* disruption construct. Approximately 500 base pairs of non-coding DNA flanking the 5' and 3' ends of the *CHO1* gene (5'-and 3'-$CHO1^{NCR}$, respectively) were cloned onto either flank of the *CaNAT1-FLP* construct or *SAT1* flipper (143, 154). The SAT1 flipper regions are in parentheses. The thick dark arrows represent the FRT sites of the *FLP* recombinase. The ball and stick represents the *ACT1* terminator (*ACT1t*) and the thinner arrow on a raised line represents the *SAP2* promoter (P_{SAP2}, *CaNAT1-FLP*) or *MAL2* promoter (P_{MAL2}, *SAT1* flipper). (B) The *CHO1-SAT1* construct used to reintegrate *CHO1* into the *cho1*Δ/*cho1*Δ mutant. (C) Southern blotting was used to confirm the *CHO1* disruptions. (D) Separation of phospholipids from wild type and mutant strains. The four major phospholipids were examined in the wild-type and mutant strains. Positions of the different phospholipid spots were determined by comparing them with the migration of phospholipid standards, which are marked to the side of autoradiogram. The identity of the PS band from the wild type has also been confirmed by mass spectrometry (see text). (E) Five-fold spot dilution assays depicting the sensitivity and resistance of the wild type and *cho1*Δ/*cho1*Δ mutants to papuamide B. The strains were grown on YPD plates with or without papuamide B for 24h at 37°C.


A
Kpn I
Sac I
5' CHO1NCR
PSAP2
(PMAL2)
FLP
ACT1t
NAT1 (SAT1)
3' CHO1NCR
B
Kpn I
Hind III
Sac I
5' CHO1NCR
CHO1
SAT1
3' CHO1NCR
C
CHO1-SAT1
CHO1
cho1Δ::SAT1-FLP
cho1Δ::NAT1-FLP
cho1Δ
WT
CHO1/cho1Δ::NAT1
CHO1/cho1Δ
cho1Δ/cho1Δ::SAT1
cho1Δ/cho1Δ
cho1Δ/cho1Δ::CHO1
D
PE
PI
PS
PC
WT
cho1Δ/cho1Δ
cho1Δ/cho1Δ::CHO1
psd1Δ/psd1Δ
psd1Δ/psd1Δ::PSD1
psd1Δ/psd1Δpsd2Δ/psd2Δ
E
No Papuamide B
5 μg/ml Papuamide B
WT
cho1Δ/cho1Δ
cho1Δ/ cho1Δ::CHO1

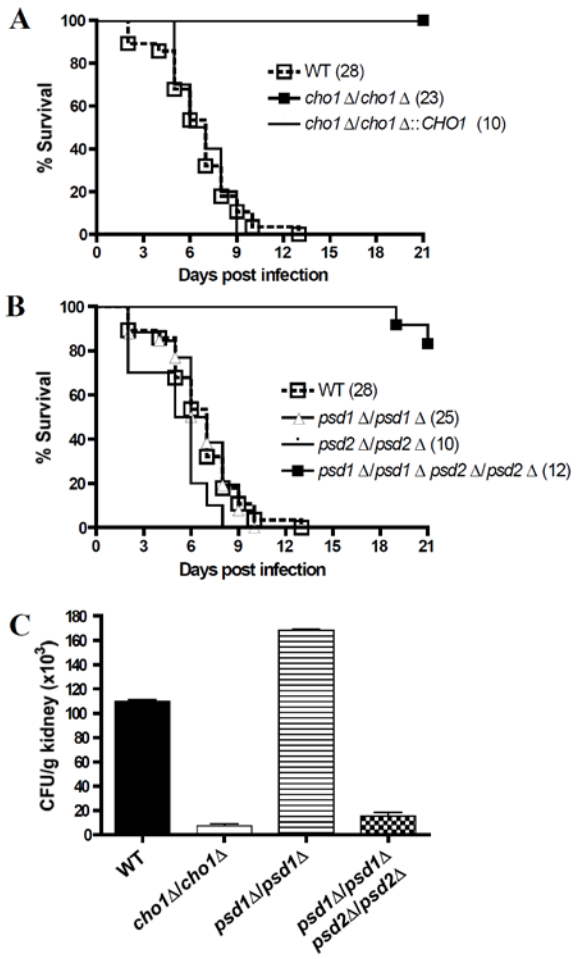


Fig 3- 3. The *cho1*Δ/*cho1*Δ and *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ double mutants are compromised for virulence.

The survival of mice following intravenous challenge with 10^6 *C. albicans* blastospores was monitored. The number of mice used in a specific strain is indicated in parentheses. (A) Mice injected with *cho1*Δ/*cho1*Δ mutants exhibited avirulence ($P<0.0001$ as compared with wild type). (B) Mice injected with *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ double mutants exhibited very attenuated virulence ($P<0.0001$ as compared with wild type). (C) The fungal burden in the kidneys was quantified at day 5 after infection. The error bar represents the mean ± standard deviation of three randomly selected mice infected with specific strains.

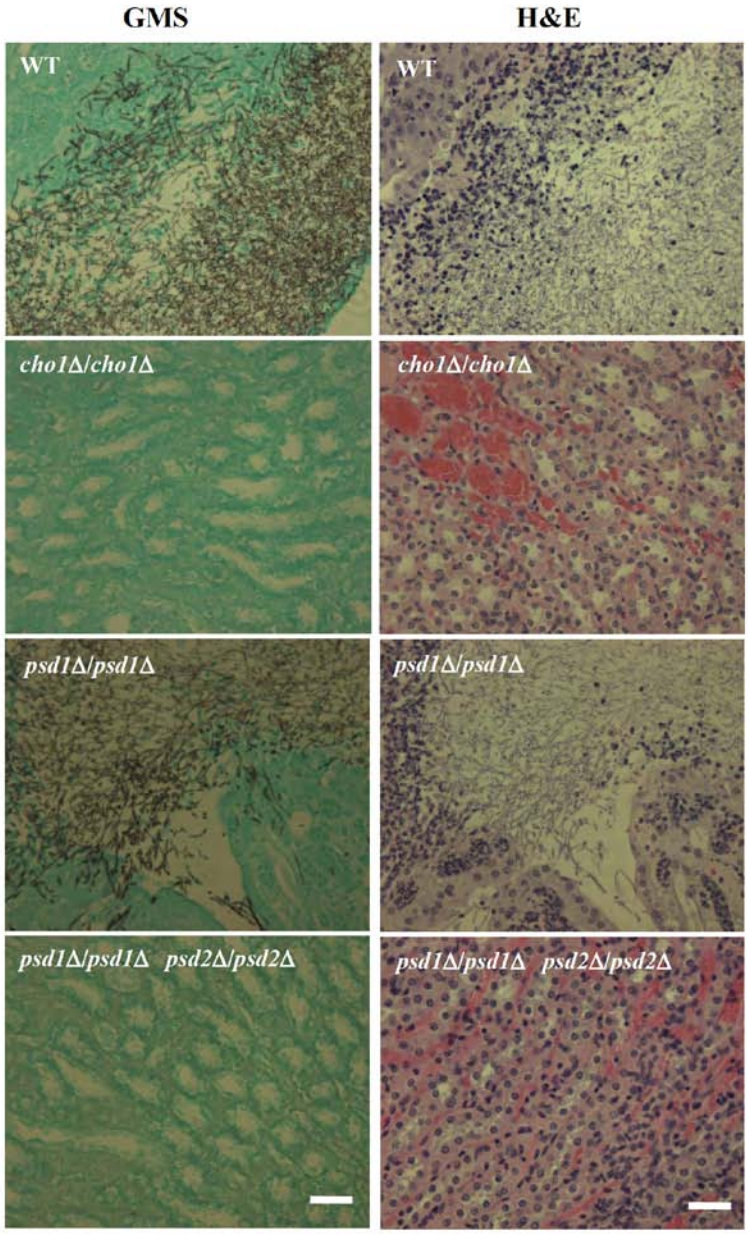


Fig 3- 4. Histological sections of kidneys dissected from mice infected with specific strains.

The mice were sacrificed at day 5 after infection. Gomori Methenamine Silver (GMS) and Hematoxylin and Eosin (H&E) staining were used to observe *C. albicans* colonization and tissue damage, respectively. Bar = 50μm.

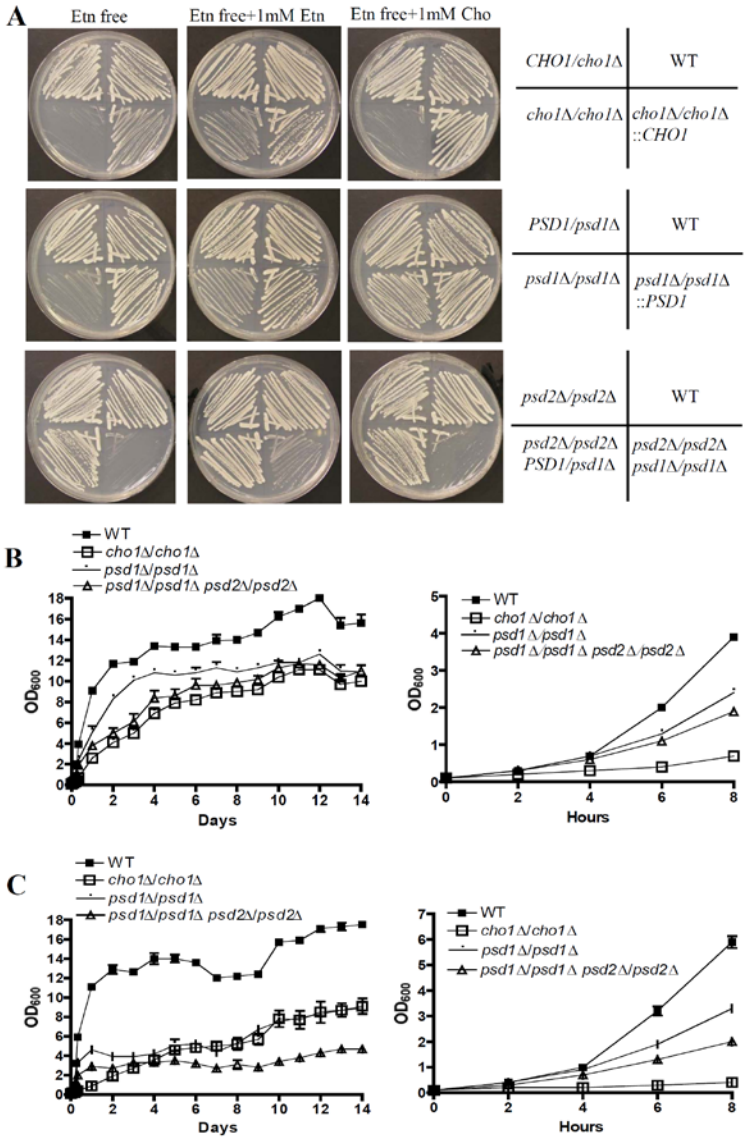


Fig 3- 5. The *cho1*Δ/*cho1*Δ and *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ mutants are ethanolamine auxotrophs.

(A) The cells were streaked onto Etn/Cho free medium supplemented with Etn , Cho, or neither, and grown for 2 days at 37°C. For the growth curves shown in B and C, log-phase cells were diluted into Etn free medium plus 1mM Etn (B) or 10 µM Cho (C) at a concentration of 0.1 OD_{600}/ml. The growth curves were measured every two hours for 8 hr (right hand graphs) and then once daily for 14 days (left hand graphs). The error bars represent mean ± standard deviation of a triplicate experiment. .

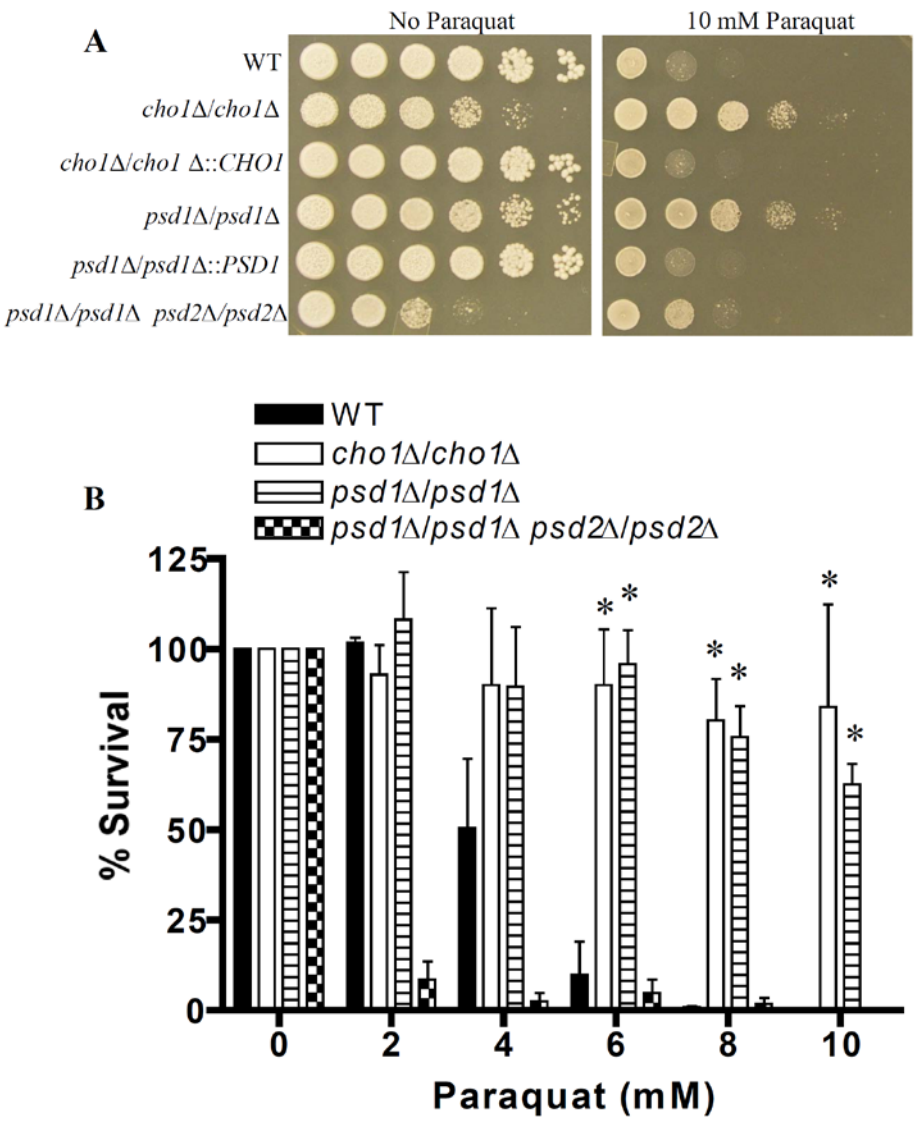


Fig 3- 6. The phospholipid biosynthetic mutants are resistant to paraquat.

(A) The overnight cultures were five-fold diluted and spotted onto YPD plates with or without 10mM paraquat. The plates were incubated for 24h at 37°C. (B) The number of colonies of the wild type and phospholipid biosynthetic mutant strains that could grow on medium containing various concentrations of paraquat (0-10mM) were counted for each strain and plotted. The colonies were counted after plates had been incubated at 37°C for 1 to 2 days. The asterisk represents a P value of <0.02 when comparing mutant's survival percentage to that of the wild type. The error bar represents the mean ± standard error of the mean of one independent and one duplicate experiment.

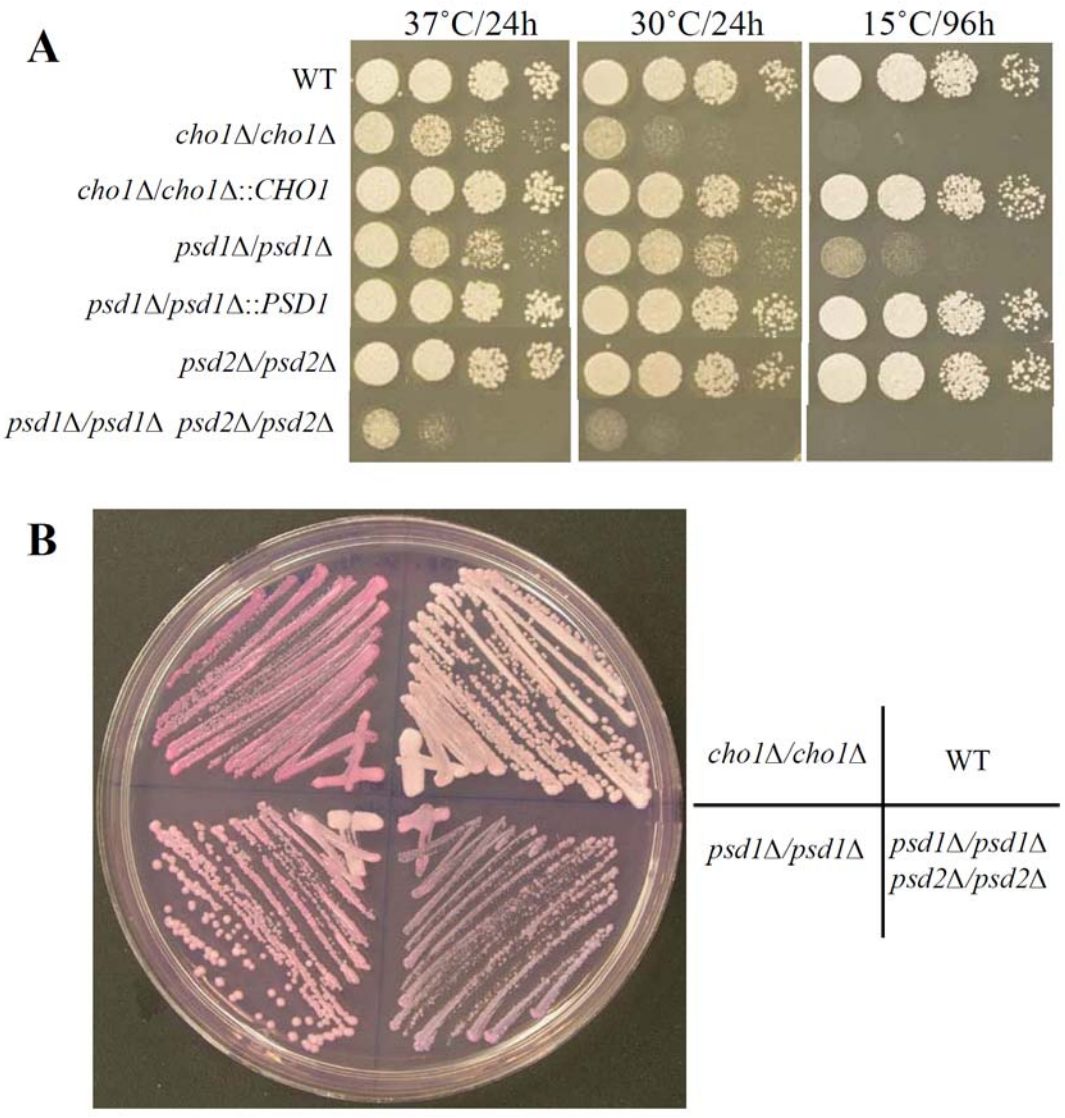


Fig 3- 7. Defects in PE and PS biosynthesis result in cold sensitivity and mitochondrial respiration defects.

(A) Five fold dilutions of overnight cultures were spotted onto YPD medium and grown at various temperatures for the indicated amounts of time. (B) A trypan blue & eosin Y indicator plate was used to test for mitochondrial dysfunction. The streaked cells were grown for 44 h at 37°C.

Fig 3- 8. Defects in PE and PS biosynthesis result in cell wall defects.

(A) The overnight culture cells were five-fold diluted, spotted onto YPD containing osmotic stabilizers $CaCl_2$, sorbitol, or NaCl, and grown for 24 h at 37°C. (B) & (C) The overnight culture cells were five-fold diluted and spotted onto YPD plates containing SDS or caffeine. (D) The overnight cultures cells were washed and diluted into YPD liquid medium at 0.4 OD/ml and grown at 37°C for 3h before collecting the cells for transmission electron microscopy observation. The cell wall thickness was quantitatively measured and plotted. The asterisk represents P value < 0.0001 as compared with wild-type.

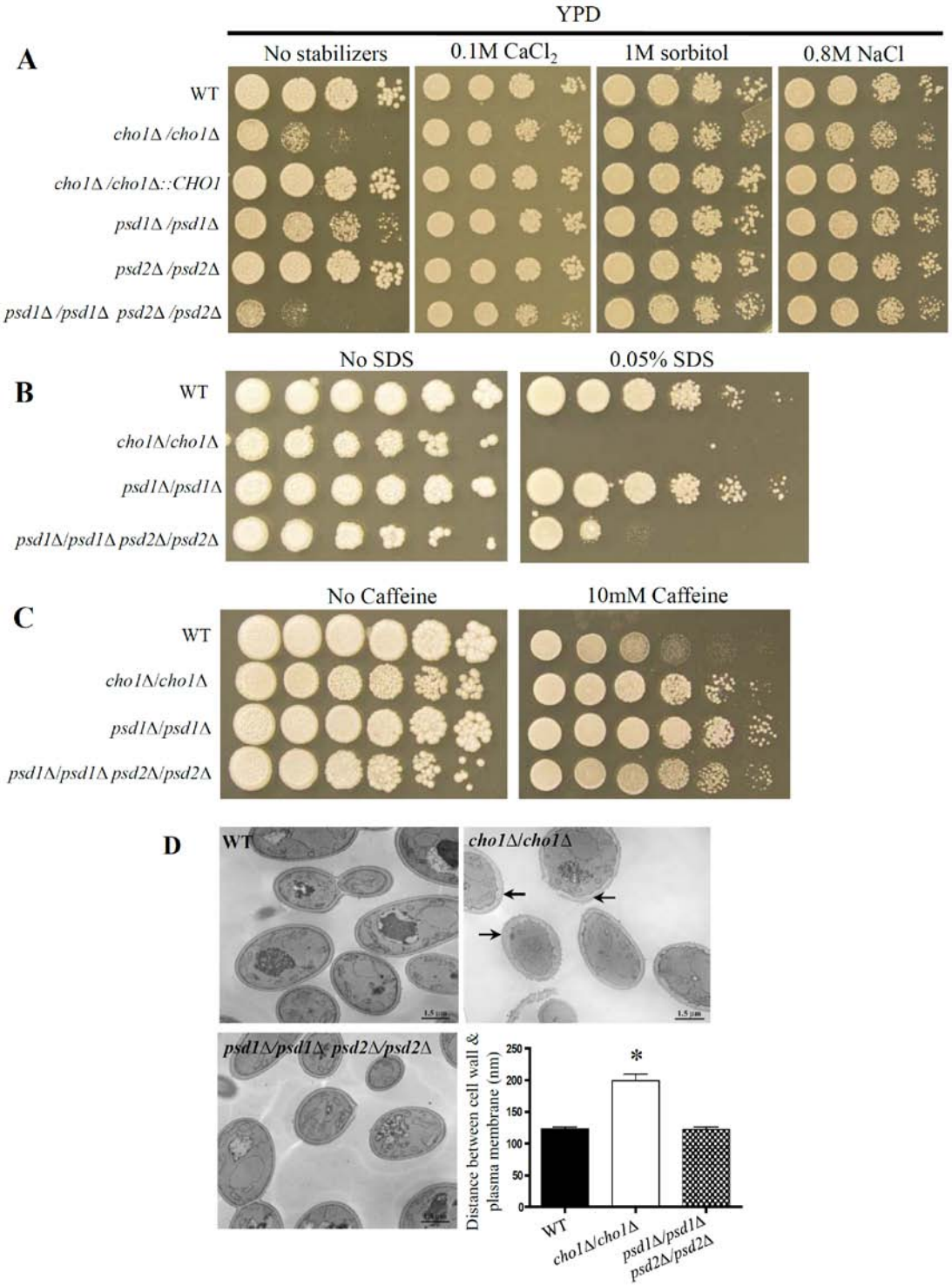

A
YPD
No stabilizers
0.1M CaCl2
1M sorbitol
0.8M NaCl
WT
cho1Δ /cho1Δ
cho1Δ /cho1Δ::CHO1
psd1Δ /psd1Δ
psd2Δ /psd2Δ
psd1Δ /psd1Δ psd2Δ /psd2Δ
B
No SDS
0.05% SDS
WT
cho1Δ/cho1Δ
psd1Δ/psd1Δ
psd1Δ/psd1Δ psd2Δ/psd2Δ
C
No Caffeine
10mM Caffeine
WT
cho1Δ/cho1Δ
psd1Δ/psd1Δ
psd1Δ/psd1Δ psd2Δ/psd2Δ
D
WT
cho1Δ/cho1Δ
psd1Δ/psd1Δ psd2Δ/psd2Δ
Distance between cell wall & plasma membrane (nm)
250
200
150
100
50
0
*
WT
cho1Δ/cho1Δ
psd1Δ/psd1Δ
psd2Δ/psd2Δ

Fig 3- 9. The *cho1*Δ/*cho1*Δ and *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ mutants exhibit filamentous growth defects.

Strains were streaked onto plates without (A) and with (B) 1M sorbitol and grown at 37°C for appropriate time. (C) Overnight cultures grown at 37°C with shaking were washed twice and diluted into 100% serum to a concentration of 0.01 OD/ml. Cells in 100% serum were transferred to polystyrene plastic well and incubate at 37°C for 4 hrs. Pictures were taken under microscope at 600X magnification.

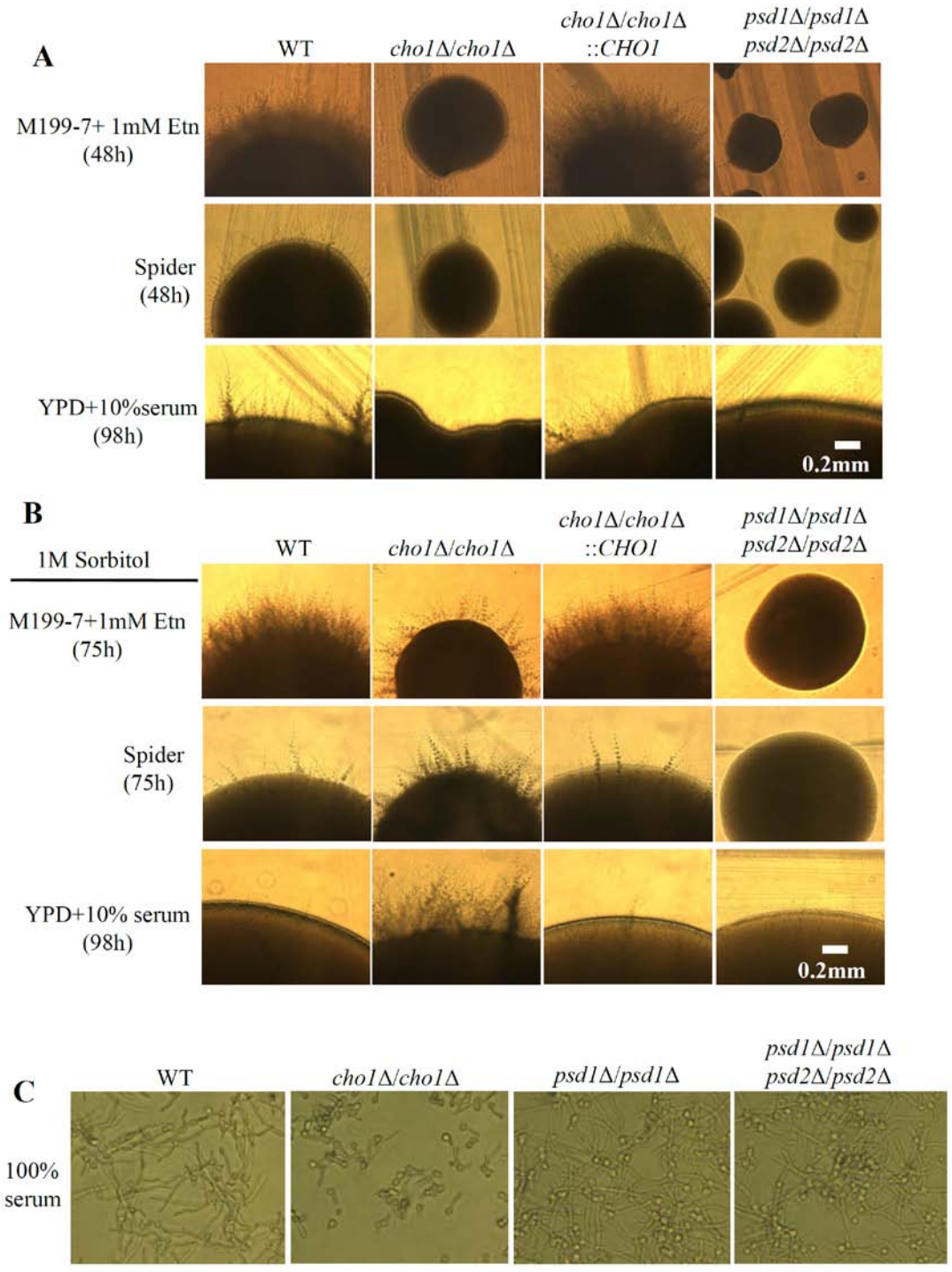
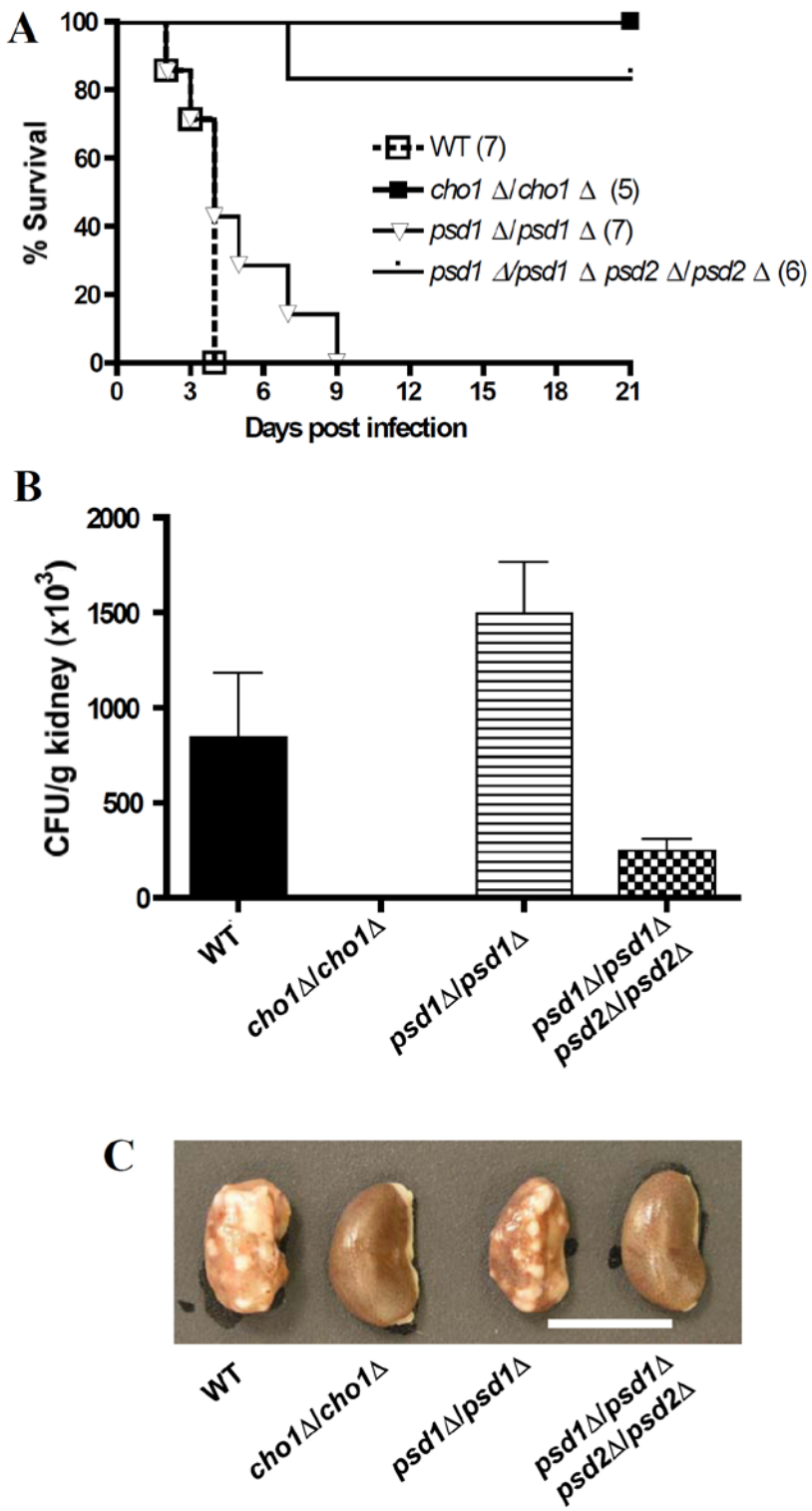

A
WT
cho1Δ/cho1Δ
cho1Δ/cho1Δ ::CHO1
psd1Δ/psd1Δ psd2Δ/psd2Δ
M199-7+ 1mM Etn (48h)
Spider (48h)
YPD+10%serum (98h)
0.2mm
B
1M Sorbitol
WT
cho1Δ/cho1Δ
cho1Δ/cho1Δ ::CHO1
psd1Δ/psd1Δ psd2Δ/psd2Δ
M199-7+1mM Etn (75h)
Spider (75h)
YPD+10% serum (98h)
0.2mm
C
WT
cho1Δ/cho1Δ
psd1Δ/psd1Δ
psd1Δ/psd1Δ psd2Δ/psd2Δ
100% serum

Fig S3- 1. The *cho1*Δ/*cho1*Δ and *psd1*Δ/psd1Δ *psd2*Δ/*psd2*Δ double mutants are compromised for virulence in immunocompromised mice.

Mice that were immunocompromised with cyclophosphamide were injected with 10^6 blastospores of *cho1/cho1*Δ mutants, whereas mice injected with the wild type, *psd1*Δ/*psd1*Δ, or *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ double mutant strains received 10^4 blastospores. Thus, mice received 100-fold more *cho1*Δ/*cho1*Δ blastopores than blastospores from other strains. The survival of mice following intravenous challenge was monitored twice a day. The number of mice used in a specific strain is indicated in parantheses. (A) Mice injected with *cho1*Δ/*cho1*Δ and *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ double mutants exhibited avirulence and attenuated virulence, respectively. However, *psd1*Δ/*psd1*Δ was as fully virulent as the wild-type. (B) The fungal burden in the kidneys was quantified from mice sacrificed at day 4. The error bars represents the mean ± standard deviation of three randomly selected mice infected with specific strains. (C) The kidney appearance of infected mice at day 4 was examined. Bar=1cm.


A
100
80
60
40
20
0
% Survival
0
3
6
9
12
15
18
21
Days post infection
WT (7)
cho1 Δ/cho1 Δ (5)
psd1 Δ/psd1 Δ (7)
psd1 Δ/psd1 Δ psd2 Δ/psd2 Δ (6)
B
2000
1500
1000
500
0
CFU/g kidney (x10^3)
WT
cho1Δ/cho1Δ
psd1Δ/psd1Δ
psd1Δ/psd1Δ
psd2Δ/psd2Δ
C
WT
cho1Δ/cho1Δ
psd1Δ/psd1Δ
psd1Δ/psd1Δ
psd2Δ/psd2Δ

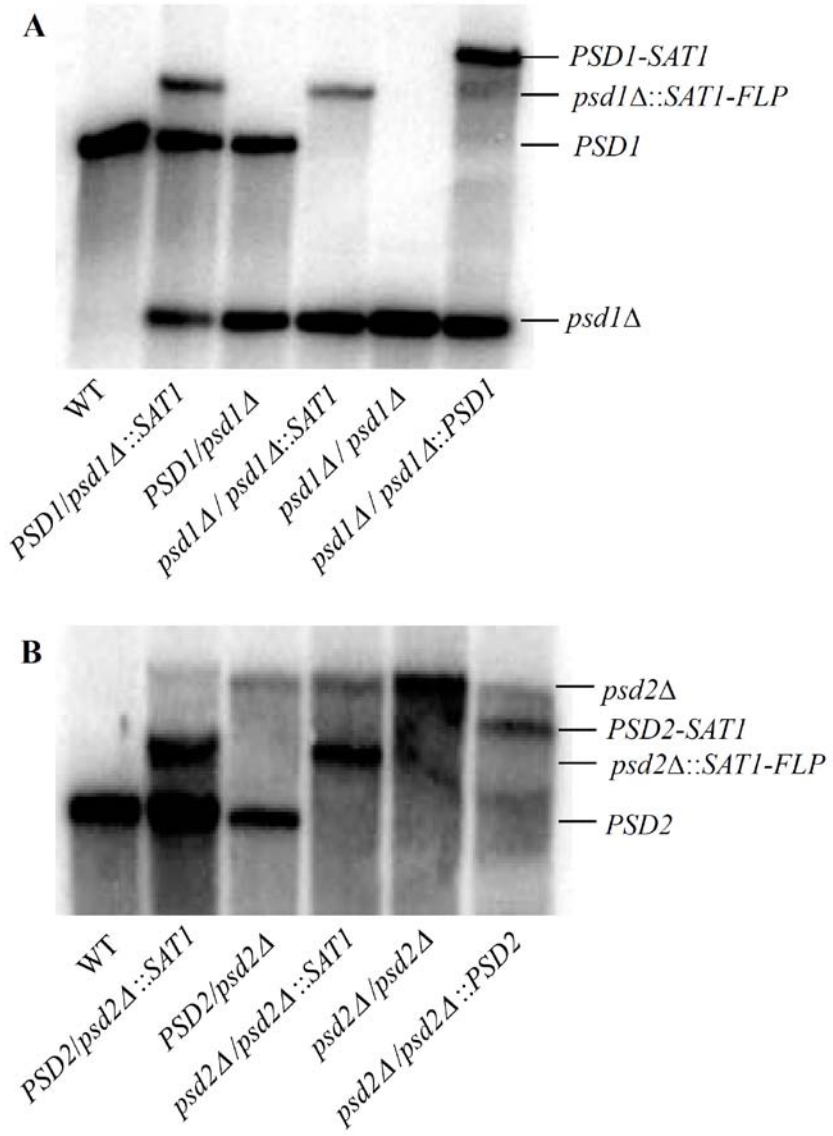


Fig S3- 2. Southern blotting confirmed the *PSD1* and *PSD2* disruptions.

The genomic DNAs were extracted from wild-type and mutants. (A) Twenty μg of DNA from wild type and *psd1*Δ/*psd1*Δ strains were digested by XhoI, and probed using a DNA fragment representing the 3' noncoding region of the *PSD1* gene. (B) Twenty μg of DNA from wild type and *psd2*Δ/*psd2*Δ strains were digested by BamHI, and probed using a DNA fragment representing the 3' noncoding region of the *PSD2* gene.

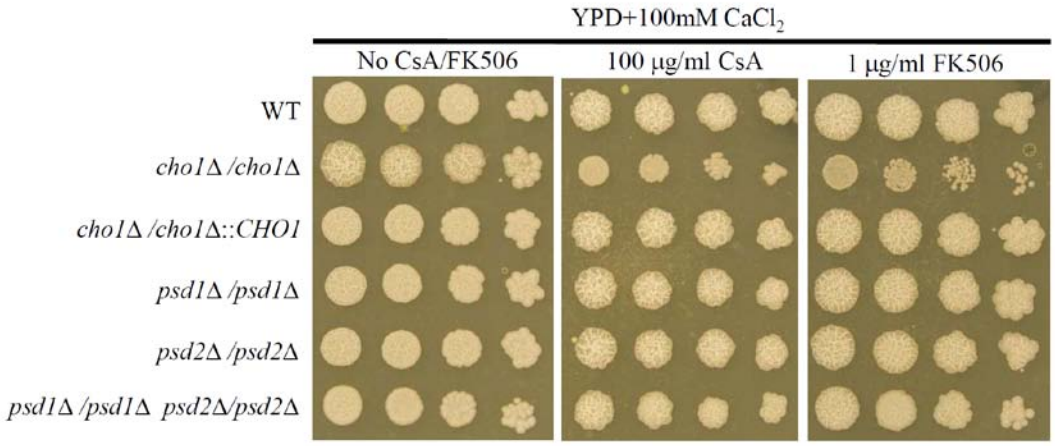


Fig S3- 3. The *cho1*Δ/*cho1*Δ cell wall defect appears to be in part due to perturbation of calcineurin.

The overnight cultures were five-fold diluted, spotted onto YPD containing 100mM $CaCl_2$with or without calcineurin inhibitor CsA or FK506, and grown for 45 h at 37°C.

Chapter 4: *CaOPI1* regulates morphogenesis and virulence, but not inositol biosynthesis

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ABSTRACT

The yeast *Candida albicans* is a very common cause of fungal infections in humans and serves as a model organism to study fungal virulence. *C. albicans* is a versatile pathogen that can cause life-threatening systemic infections in immunocompromised patients as well as painful mucosal infections of the oral and vaginal surfaces. Virulence factors exhibited by *C. albicans* include growth at 37°C, dimorphism, and production of secreted hydrolases such as proteases, lipases, and phospholipases. The pathways that regulate the transcription of secreted aspartyl protease (SAP) virulence factors in *C. albicans* are beginning to be understood, but much remains to be learned. Here we report the finding that the gene *OPI1* affects the virulence of *C. albicans*. Homozygous disruptants of *OPI1* (*opi1*Δ/*opi1*Δ) exhibits a significant reduction in virulence compared to wild-type in a rat model of vaginal infection. This phenotype is similar to that reported for the *sap2*Δ/*sap2*Δ mutant and suggests that *OPI1* may regulate *SAP2* during vaginal infections. Conversely, the *opi1*Δ/*opi1*Δ strain does not have a substantial effect on virulence in a mouse model of systemic infection. Thus, *OPI1* affects virulence in a host-site specific manner. The best characterized homologue of *OPI1* is the Saccharomyces cerevisiae gene *ScOPI1*, which encodes a negative regulator of inositol biosynthesis that acts by repressing transcription of the inositol biosynthetic gene *INO1*. We have found that in *C. albicans*, *OPI1* does not play a role in the regulation of *INO1*. Taken together, our results describe a novel regulatory protein is required for establishing infection in rat vagina and illustrate that the regulation of inositol biosynthesis in *C. albicans* and *S. cerevisiae* is different.

INTRODUCTION

Candida albicans is a commensal organism that lives as benign resident of the microflora of mammalian hosts. It can shift from a commensal to a pathogenic state in response to environmental stimuli that trigger developmental programs that induce the expression of virulence factors (65). Virulence factors exhibited by *C. albicans* include growth at 37°C, dimorphism, and production of secreted hydrolases such as proteases, lipases, and phospholipases. The pathways that regulate the transcription of secreted aspartyl protease (SAP) virulence factors in *C. albicans* are beginning to be understood, but much remains to be learned. SAPs are encoded by a gene family of 10 related genes (*SAP1* to *SAP10*) (123). Unlike *SAP*1 to *SAP8*, which encode secreted proteases, *SAP9* and *SAP10* encode GPI-anchored proteases, located at cell membrane or cell wall, and both are required for virulence (1). Among this family of genes, Sap2p is the most well-studied protease since it plays the major secreted protease during *in vitro* growth conditions, and also plays a role in pathogenesis.

The knowledge of SAP2 regulation is limited. *SAP2* is under the control of *STP1* transcription factor and *STP1*'s upstream GATA transcription factors *GLN3* and *GAT1* (34, 111). The importance of *SAP2* in pathogenesis has been discussed by several groups. For instance, De Bernardis et al. demonstrated that *SAP2* was the major virulence contributor in the rat vaginitis model (38). Schaller et al. showed that *SAP2* was required to cause tissue damage in an *in vitro* model of vaginal candidiasis (149). In addition, Hube et al. demonstrated that *SAP2* was required for virulence in a the rodent model of systemic infection (74). In contrast, Lermann and Morschhauser showed data contradictory to Schaller et al, 2003 that *SAP2* was not required to invade and damage in both oral and vaginal reconstituted human epithelium (91). Thus, there is controversy about the role of *SAP2* in pathogenesis.

S. cerevisiae OPI1 (*ScOPI1*) is a negative regulator of inositol biosynthesis which inhibits *ScINO1*'s transcriptional activator *ScINO2* (174, 179). In addition, it plays a role as a regulator of *ScFLO11*, an adhesion molecule, and is required for invasive growth and mat formation in *S. cerevisiae* (144). Inositol-3-phosphate synthase, encoded by *ScINO1*, catalyzes the conversion of glucose-6-phosphate to inositol-3-phosphate, which is then dephosphorylated by *INM1* or *INM2* to form inositol (24). The structural gene encoding *ScINO1* has been demonstrated to be conserved between *S. cerevisiae* and *C. albicans*, and shares similar function (25, 84). *C. albicans* and *S. cerevisiae INO1* homologs are similarly regulated in response to exogenously provided inositol (83). The *ScOPI1*

orthologue in *C. albicans* (*OPI1*) can complement *Scopi1*Δ for *INO1* regulation in *S. cerevisiae* (68). We therefore hypothesized that *OPI1* might function as an *INO1* negative regulator in *C. albicans*. However, a report from Hoppen et al. suggested that the regulation of the *INO1* expression in these two yeasts might not be conserved. The *C. albicans* heterodimeric transcription factors *INO2* and *INO4* (related to *ScINO2* and *ScINO4* from *S. cerevisiae*) did not regulate *INO1*, but instead activated ribosomal protein genes such as *RPL32* (71). The results indicated that inositol regulation might be transcriptional rewired between these two related eukaryotic organisms. In fact, Martchenko et al. has demonstrated that the *GAL4* transcription factor homologs appear rewired between these two organisms. In *C. albicans* the *GAL4* homolog activated the gluconeogenesis gene *LAT1* instead of galactose metabolism genes such as *GAL10*, and surprisingly *GAL10* was activated by another transcription factor *CPH1* (107). Therefore it is very interesting to investigate if *OPI1* shares similar inositol regulation as *ScOPI1*.

Here we report the finding that *OPI1* does not regulate inositol biosynthetic gene *INO1* but affects the virulence of *C. albicans*. In addition, *OPI1* affects morphogenesis at 30ºC, and establishing infection in a rat vaginitis model. These results illustrate that the regulation of inositol biosynthesis in *C. albicans* and *S. cerevisiae* is different.

MATERIALS AND METHODS

Strains and growth media

C. albicans strains used in this study are shown in Table 4-1 (see all tables and figures in the appendix C). Media used in this study include YPD (yeast extract-peptone-dextrose, 1% yeast extract, 2% peptone, 2% glucose), defined medium 199 (M199, Invitrogen, pH7.0 adjusted by 150mM HEPES), Spider (1% nutrient broth, 1% mannitol, 0.2% dipotassium phosphate, 1.35% agar), YPD containing 10% Bovine Fetal serum, YCB-BSA-YE (1.17% yeast carbon base, 0.2% BSA, 0.1% yeast extract, pH 5.0). Unless otherwise stated, agar plates were solidified with 2% agar (granulated, Fisher).

Strain construction

The *C. albicans OPI1* gene (*OPI1*) was disrupted by using the *CaNAT1-FLP* cassette (154). For the *OPI1* disruption construct, the 379 basepair (bp) 5' non-coding region (NCR) of *OPI1* was amplified with primers TRO522 and TRO526 (Table 4-3), and cloned as a *Kpn*I-*Apa*I digested 228bp fragment into pJK863 5' of the *CaNAT1-FLP* cassette (Fig 4-1A). The 448 bp 3' NCR of *OPI1* was amplified with primers TRO524 and TRO525 which introduced *Sac*II and *Sac*I sites, and was cloned into pJK863 3' of the

CaNAT1-FLP cassette (Fig 4-1A). This created the *OPI1* knock out construct plasmid pYLC36 (Table 4-2, Fig 4-1A), which was cut with *Kpn*I and *Sac*I to release the disruption construct which was transformed into the wild type SC5314 strain by electroporation as described previously (36, 143). The disruption construct was used to sequentially disrupt both alleles of *OPI1* as previously described (154). The *OPI1* reconstitution construct was made by amplifying a 1.7 Kb fragment containing the *OPI1* ORF and 5' NCR from SC5314 genomic DNA using primers (JCO12 and JCO14) that introduced *Kpn*I and *Sal*I sites. This fragment was ligated into the pRS316 vector along with another 1.7 Kb fragment containing the *NAT1*-3'*OPI1*^{NCR} amplified from plasmid pYLC36 using primers JCO50 and TRO525 which introduced *Sal*I and *Sac*I sites. This resulted in the *OPI1* reconstitution plasmid pYLC37 (Table 4-2, Fig 4-1B). The 3.4 Kb *Kpn*I-*Sac*I fragment from pYLC37 was transformed into the *opi1*Δ/*opi1*Δ mutant (YLC88) in order to create the reconstituted *opi11*Δ/*opi1*Δ::*OPI1* strain (YLC117).

The SAP2 constitutively expression construct was made by cloning dominant selectable marker *NAT1* with primers JCO129 and JCO130 to *Nde*I-digested pAU34 (168) and resulted in pYLC219. The *SAP2* ORF was then cloned to *Xma*I-digested pYLC219 with primers JCO131 and JCO132, and resulted in pYLC221 which can constitutively express *SAP2* under the control of the *ACT1* promoter. To transform this constitutive construct to wild type and *opi1*Δ/*opi1*Δ mutant, a *Ppu*MI-digested linear plasmid pYLC221 was used to integrate at URA3 site of *Candida* genome, and resulted in *OPI1*/*OPI1*::pACT1::*SAP2* (YLC223) and *opi1*Δ/*opi1*Δ:: pACT1::*SAP2* (YLC226).

Northern blot analysis

Northern blotting for *SAP2* and *INO1* expression was performed as described (144) with the following exceptions. Strains grown in YCB-BSA-YE at 37°C for 12 hrs (for *SAP2*) and in liquid medium 199 (pH 7.0) at 37°C for 2 hrs (for *INO1*) were collected for total RNA extraction by the hot phenol method (144). The PCR product containing bps 17-571 of the *SAP2* ORF (primers JCO35 and JCO36) and bps 76-581 of the *INO1* ORF (primers TRO562 and TRO563) were used as probes. Expression was normalized against *C. albicans ACT1* gene expression probed on the same membrane. The *ACT1* probe was generated with the primers JCO48 and JCO49.

Southern blot analysis

Hybridization conditions for the Southern blot analysis were similar to those for Northern blot analysis, except that the Techne Hybrigene oven was set to 60°C for the incubation step, and 42°C and 60°C for washing steps. The cells were grown in liquid

YPD at 30°C overnight. The genomic DNA was extracted using the Winston-Hoffman method (70) and 20μg of genomic DNA were subjected to Southern blotting. The genomic DNA of *opi1* mutants was cut by *Kpn*I and *Sph*I restriction enzymes. PCR products containing the ~500bp 3' NCR of *OPI1* (primers TRO524 and TRO525) was used as probes for Southern blot confirmation (Fig 4-1C).

Mouse infection studies

Five- to six-week-old male CD1 mice (18 to 20g) from Charles River Laboratories were used in this study. Mice were housed at five per cage. For infection, colonies from each *C. albicans* strain were inoculated into 20ml of YPD. Cultures were grown overnight, washed twice with 25ml of sterile water, counted by hemacytometer, and resuspended at 10^7 cells per ml in sterile water. The cells were then plated on YPD to determine the viability. Mice were injected via the tail vein with 0.1ml of the cell suspension (10^6 cells), and the course of infection was monitored for up to 14 days. At least two independent infections were performed for each strain. Survival was monitored twice daily, and moribund mice were euthanized. All experimental procedures were carried out according to the NIH guidelines for the ethical treatment of animals.

The statistical analysis was done using Prism 4.0 software (GraphPad Software). For the mouse model of systemic infection, Kaplan-Meier survival curves were compared for significance using the Mantel-Haenszel log rank test. Statistical significance was set at $P < 0.05$.

Rat vaginitis studies

The protocol of estrogen-dependent rat vaginal infection model adapted from De Bernardis F. et al (38) was used throughout this study. Briefly, oophorectomized female Wistar rats (80-100g; Charles River, Calco, Italy) were injected subcutaneously with 0.5mg of estradiol benzoate (Benzatrone; Samil, Rome). Six days after the first estradiol treatment, the animals were inoculated intravaginally with 10^7 yeast cells of each *C. albicans* strain tested in 0.1mL. The inoculum was dispensed into the vaginal cavity through a syringe equipped with a multipurpose calibrated tip (Combitip; PBI, Milan, Italy). The yeast cells had been previously grown in YPD broth at 28°C on a gyratory shaker (200rpm), harvested by centrifugation (1500g), washed, counted in a hemacytometer, and suspended to the required number in saline solution. At least two independent experiments were done, and in each experiment, each candidal strain was inoculated into at least 5 rats. Kinetics of *C. albicans* growth in and clearance from the vaginal cavity was measured by cfu enumeration after culturing vaginal samples in CSA

medium at 28°C for 48h. The samples were harvested by scraping the vaginal cavity of each animal in indicated days with a calibrated, plastic loop (1μL) (Disponoic; PBI).

RESULTS

OPI1*, unlike *ScOPI1*, does not regulate *INO1* in *C. albicans

Though *C. albicans* Opi1p has been demonstrated to repress *INO1* expression in *S. cerevisiae* (68), we show that it does not play a role in inositol regulation in *C. albicans*. Both copies of the *CaOPI1* gene were disrupted using the *CaNAT1-FLP* cassette as described in Fig 4-1 and the materials and methods. The wild type and *opi1*Δ/*opi1*Δ strains were both found to express similar amounts of *INO1* mRNA in Medium 199, pH 7.0, which contains only ~ 10 μM inositol (Fig 4-2). *INO1* mRNA expression was repressed in the presence of 75μM inositol in both strains suggesting that inositol biosynthesis is regulated by different transcription factors in *C. albicans*.

***opi1*Δ/*opi1*Δ mutant exhibits hyperfilamentous growth in three filament-inducing media at 30ºC**

From our previous work, we showed that *ScOPI1* regulates mat formation, invasive growth, and *ScFLO11* expression (144). We therefore hypothesized that *OPI1* would affect filamentous growth in *C. albicans*. Three filament-inducing media were used to test our hypothesis. We found that the *opi1*Δ/*opi1*Δ exhibited hyperfilamentous growth at 30°C on solid filament-inducing agar plates (Fig 4-3); however, these effects were not seen at 37°C. These phenotypes were also not seen in those filament-inducing liquid media at both 30°C and 37°C.

Since we found the *OPI1* affected filamentous growth on filament-inducing solid agar plates, we hypothesize that this gene might affect the expression of other hyphae-specific genes, such as *HWP1* and *ECE1*. However, we found *OPI1* is not involved in regulating expression of those genes (data not shown).

***OPI1* does not affect virulence in a mouse model of systemic infection**

A gene involved in regulating *C. albicans* morphogenesis might play as a virulence factor (180). We therefore used a mouse model of systemic infection to test the roles of *OPI1* in terms of virulence. However, we demonstrated that *OPI1* did not contribute to the virulence in this model since *opi1*Δ/*opi1*Δ exhibited a similar survival curve as the wild type (Fig 4-4).

***OPI1* is involved in establishing infection in the rat vaginitis model**

We then use different animal model to test the roles of *OPI1* in terms of virulence. We demonstrated that *OPI1* was involved in establishing rat vaginitis. We injected 10^7 cells into the rat vaginal tract, and then recovered and count cells at specific days. The *opi1*Δ/*opi1*Δ was very quickly cleared by the host compared to the wild type (Fig 4-5). These results suggest that *OPI1* plays a role in establishing infection in rat vaginal.

OPI1* affects rat vaginal establishment through regulating *SAP2

In order to assess whether *OPI1* affects rat vaginal establishment through regulating *SAP2,* we performed an epistatis experiment in which *SAP2* was constitutively expresed in the *opi1*Δ/*opi1*Δ mutant under the control of the *ACT1* promoter. The results showed that *opi1*Δ/*opi1*Δ::pACT1::*SAP2* mutant rescued rat vaginal establishment similar to wild type establishment (Fig 4-6).

DISCUSSIONS

Our reports show that *OPI1* affects virulence in the rat vaginitis model and may affect *SAP2* expression *in vivo* because *opi1*Δ/*opi1*Δ::pACT1::*SAP2* mutant can restore *opi1*Δ/*opi1*Δ mutant colonization. However, *OPI1* did not regulate *SAP2* in our *in vitro* experiment (i.e. YCB-BSA-YE medium). Interestingly, *OPI1* is not involved in a mouse model of systemic infection, suggesting *OPI1* exhibited a host-specific virulence.

The previous report has demonstrated that *OPI1* may regulate inositol biosynthesis and act as the negative regulator of *INO1* (68). Our results show that *OPI1* does not regulate inositol biosynthesis or *INO1*. This difference may be due to the fact that in the previous report the *C. albicans OPI1* was only studied when expressed heterologously in *S. cerevisiae*. The fact that it could complement the *Scopi1*Δ mutant's defect in repressing *ScINO1* (ICRE-regulated reporter gene) expression was taken as evidence it did the same thing in *C. albicans* (68).

CaOpi1p may work in *S. cerevisiae* because it can interact with the ScIno2p and ScIno4p heterodimer to repress *ScINO1* expression. *CaOPI1* did not regulate *INO1* in *C. albicans* indicating that different transcriptional regulators control *INO1* in this pathogen.

We screened *C. albicans* transcription factor mutant library (provided by Aaron Mitchell and Dominique Slangard) to isolate *INO1* regulator. However, we did not find any candidate in these mutant libraries. It will be interesting to identify what are the transcriptional activators of *INO1* in *C. albicans*.

OPI1 affects morphogenesis at 30ºC, but not at 37ºC (Fig 4-3). The *opi1*Δ/*opi1*Δ

mutant exhibited hyperfilamentous growth in three filament-inducing agar plates including medium 199, spider, and 10% serum at 30ºC. The result indicated that *OPI1* might be a temperature regulator of filamentous growth. Csank et al. demonstrated that *C. albicans CPP1*, a tyrosine phosphatase, is required for repressing the yeast to hyphal transition at 23ºC in contact with solid surfaces (32, 71). The *cpp1*Δ/*cpp1*Δ mutant exhibited hyperfilamentous and invasive growth on spider and wide variety of rich and defined solid media including Lee's medium, YPD, YPM, and 10% serum at 23ºC, but not at 37ºC. The germ tube formation of *cpp1*Δ/*cpp1*Δ mutant was not observed at liquid culture at 37ºC, an effect similar to *opi1*Δ/*opi1*Δ mutant. In contrary to *opi1*Δ/*opi1*Δ, the *cpp1*Δ/*cpp1*Δ mutant exhibited reduced virulence/pathogenecity in mouse systemic infection and mouse mastitis model (32, 61). It is possible that Cpp1p and Opi1p may interact in some way.

In the future, it will be tested if *OPI1* regulates rat vaginitis through affecting *SAP2* expression in vivo using qRT-PCR to assay mRNA from collected rat vaginal fluid infected with *C. albicans* wild-type and mutant strains. Taken together, our data suggested that *C. albicans* has a transcriptionally rewired regulator *OPI1* which does not regulate inositol biosynthesis but affects morphogenesis, and virulence in rat vaginitis model.

APPENDIX D: TABLES AND FIGURES

Table 4- 1. *C. albicans* strains

Strain	Parent	Genotype	Source of reference
SC5314 (wild-type)	Clinical isolate	Prototrophic wild type	(55)
YLC58	SC5314	*opi1*Δ::*NAT1-FLP*/*OPI1*	This study
YLC85	YLC58	*opi1*Δ/*OPI1*	This study
YLC86	YLC85	*opi1*Δ/*opi1*Δ::*NAT1-FLP*	This study
YLC88	YLC86	*opi1*Δ/*opi1*Δ	This study
YLC117	YLC88	*opi1*Δ/*opi1*Δ::*OPI1*	This study
SAP2MS4A	SC5314	*sap2*Δ/*sap2*Δ	(159)
YLC223	SC5314	*OPI1/OPI1*::P_{ACT1}::*SAP2*	This study
YLC226	YLC88	*opi1*Δ/*opi1*Δ::P_{ACT1}::*SAP2*	This study

Table 4- 2. Plasmids

Plasmids	Characteristics	Source of reference
pJK863	*CaNAT1-FLP* cassette carrying nourseothricin resistance gene	(154)
pYLC36	pJK863 flanked 5' and 3' $OPI1^{NCR}$ for *OPI1* gene knock out	This study
pYLC37	*OPI1* reconstitution construct	This study
pAU34	Constitutively expression construct under the control of ACT1 promoter	(168)
pYLC219	pAU34 contained *NAT1* selectable marker	This study
pYLC221	pYLC219 contained SAP2 ORF for constitutive expression	This study

Table 4- 3. PCR primers

Primer	Use	Sequence (5' → 3')
JCO522	Disrupt *OPI1*	AAAAAAGGGCCCTACACACACACACACTTACACACAT
JCO526	Disrupt *OPI1*	AAAAAAGGGCCCCGGTTTCCCCCTTTTTATATA
JCO524	Disrupt *OPI1*	AAAAAACCGCGGTGAGTGGTGGTTTCTTTTTGTT
JCO525	Disrupt *OPI1*	AAAAAAGAGCTCCAAGTTGGACTACAAATGGTCAAG
JCO12	Restore *OPI1*	GTGTGGTACCTATTTCCAAATTCA
JCO14	Restore *OPI1*	AAAAGTCGACCTACTACACACTACCATCTA
TRO369[a]	Confirm *OPI1* disruptions	GCACGTCAAGACTGTCAAGG
TRO532	Confirm *OPI1* disruptions	ACGGCATGCTAGGTATATACTGCT
JCO48	*ACT1* Northern blot probe	CCAGCTTTCTACGTTTCC
JCO49	*ACT1* Northern blot probe	CTGTAACCACGTTCAGAC
JCO35	*SAP2* Northern blot probe	TTCATTGCTCTTGCTATTGCT
JCO36	*SAP2* Northern blot probe	CACCGGCTTCATTGGTTTTA
TRO562	*INO1* Northern blot probe	GAAAACTCTGTTGTTGAAAAAGATG
TRO563	*INO1* Northern blot probe	TTGTTGGCACGTTCACTTTG
JCO129	*SAP2* constitutive expression	AAAAAACATATGCCCCGCGGGATATCAAGC
JCO130	*SAP2* constitutive expression	AAAAAACATATGTGGGTACCGAATTCGAGCT
JCO131	*SAP2* constitutive expression	AAAAAACCCGGGATGTTTTTAAAGAATATTTTC
JCO132	*SAP2* constitutive expression	AAAAAACCCGGGTTAGGTCAAGGCAGAAATAC
JCO133	*SAP2* constitutive expression	AGAGAGCAGAAACTCATGCCT
JCO134	*SAP2* constitutive expression	TAAGCATTCCAACCAGCATC
JCO122	*SAP2* constitutive expression	AAAAAAGGTACCCGTCAAAACTAGAGAATAATAAAG

[a] TRO369 hybridizes to the TEF promoter in the NAT1 cassettes and served as a reverse primer in combination with the confirmational forward primers listed to confirm the mutations.

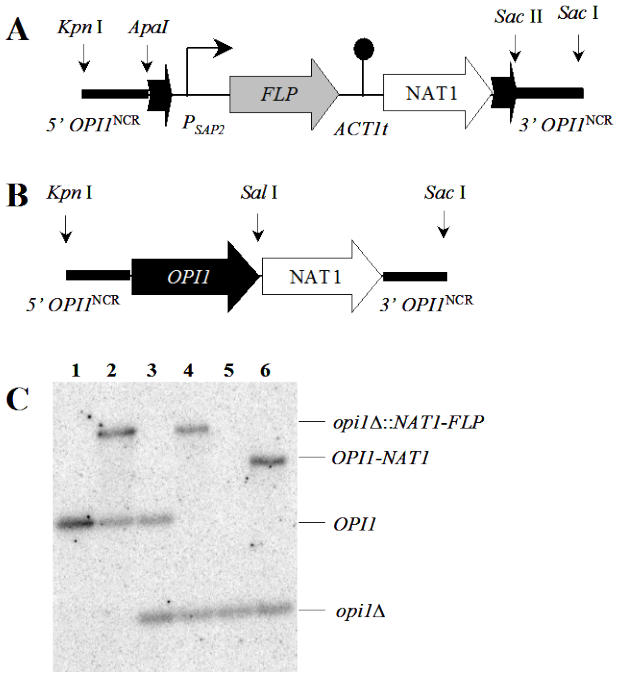

Fig 4- 1. The *OPI1* gene was disrupted in the strain SC5314 using the CaNAT1-FLP cassette .

(A) Structure of the *CaNAT1*-FLP-*OPI1* disruption construct. Approximately 500 base pairs of non-coding DNA flanking the 5' and 3' ends of the *OPI1* gene (5'- and 3'-NCR, respectively) were cloned onto either flank of the *CaNAT1*-FLP cassette. The thick dark arrows represent the FRT sites of the FLP recombinase. The ball-and-stick symbol represents the ACT1 terminator (ACT1t), and the thinner arrow on a raised line represents the SAP2 promoter (P_{SAP2}). (B) The *OPI1-NAT1* construct used to reintegrate *OPI1* into the *opi1*Δ/Δ mutant. (C) Southern blotting was used to confirm the *opi1*Δ/Δ disruptions. Lanes: 1, wild type; 2, *opi1*Δ ::NAT1-FLP/*OPI1* strain; 3, *opi1*Δ/*OPI1* strain; 4, *opi1*Δ/*opi1*Δ ::NAT1-FLP strain; 5, *opi1*Δ/*opi1*Δ strain; and 6, *opi1*Δ/*opi1*Δ::*OPI1* strain.

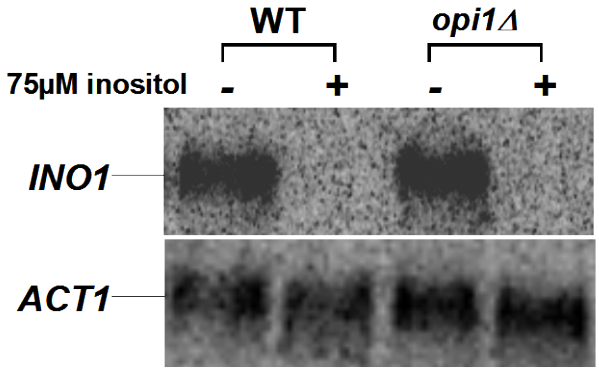

Fig 4- 2. The *opi1*Δ/*opi1*Δ mutant does not regulate *INO1* expression.

Strains were grown for 2 hrs in Medium 199, pH7.0 (± 75 μM inositol) at 37°C, collected, and subjected to Northern blotting against *INO1*. *ACT1* was reprobed on the same membrane as a loading control.

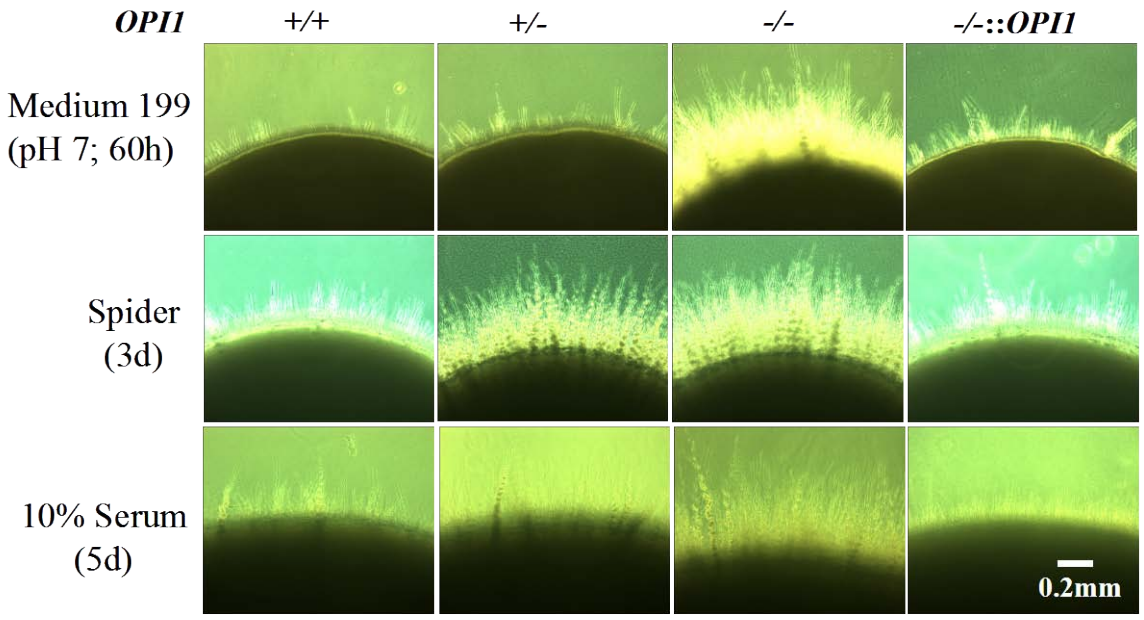

Fig 4- 3. *OPI1* -/- mutants exhibited hyperfilamentous growth in filament-inducing conditions on agar plates.

C. albicans strains from overnight cultures were diluted in sterile water and plated on the indicated medium and grown at 30°C for the indicated amount of time.

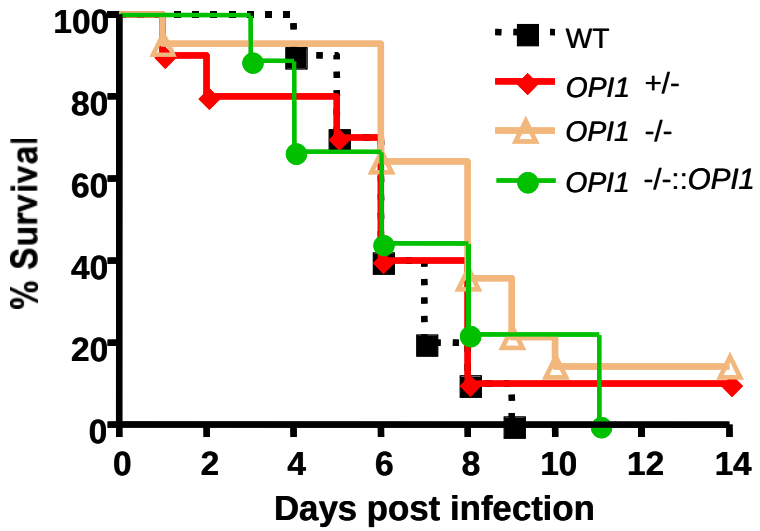

Fig 4- 4. *OPI1* is not required for virulence of C. albicans in a mouse model of systemic candidiasis.

Each strain was used to infect mice by injecting 10^6 *C. albicans* yeast-form cells into the tail-vein of each mouse. The mice were then assessed for viability over the course of 14 days.

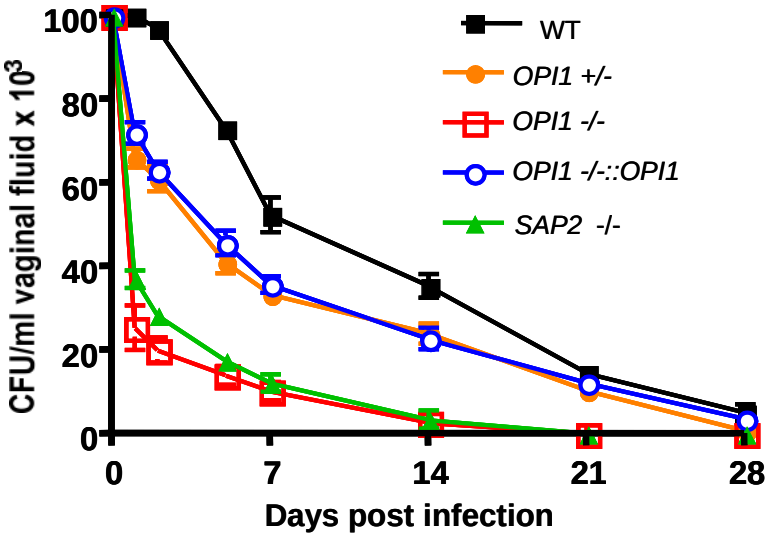

Fig 4- 5. *OPI1* is involved in establishing infection in the rat vaginitis model.

For each strain 5 rats were inoculated on day 0 with 10^7 blastospores, and vaginal cfu were counted by plating at the indicated time points.

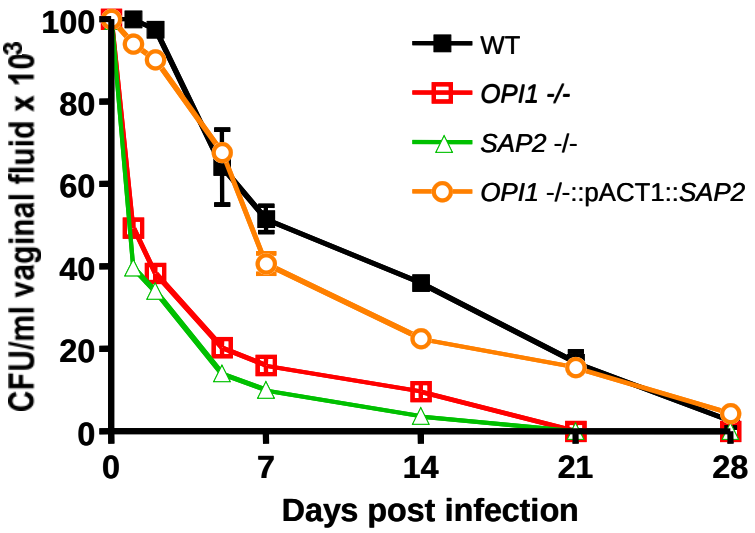

Fig 4- 6. *OPI1* affects rat vaginal establishment through regulating *SAP2*.

For each strain 5 rats were inoculated on day 0 with 10^7 blastospores, and vaginal cfu were counted by plating at the indicated time points

Chapter 5: Conclusions and Discussions

This thesis revolves around the roles of phospholipid biosynthetic pathways in *C. albicans* virulence. Currently, many studies have been concentrated on phospholipids because they play critical roles in growth, signal transduction, and virulence in addition to structure components. Our primary goal is to identify genes involved in these pathways that are required for virulence. The identification of genes involved in virulence may provide potential drug targets if there is no similar gene in mammalian cells. As mentioned in chapter 1, the five classes of antifungal drugs currently available have their own drawbacks such as kidney toxicity and fungal resistance. A new drug could offer an alternative new therapy to this opportunistic pathogen. Moreover, a new drug could be given in combination with the other drugs to treat systemic candidiasis even more efficiently (78).

Three related projects come together to investigate phospholipid metabolism genes and how they are involved in *C. albicans* virulence. 1) We have found that unlike some other pathogens, *C.albicans* is able to use *de novo* inositol biosynthesis (*INO1*) or import of exogenous inositol (*ITR1*) with equal efficiency to allow virulence. This is presumably because either mechanism allows effective synthesis of phosphatidylinositol. 2) The PS synthase gene *CHO1* is essential for virulence in mouse model of systemic candidiasis, as well as cell wall integrity, and mitochondrial function. 3) A putative regulator of phospholipid metabolism, *OPI1*, is required for morphogenesis and for colonization of the vaginal tract in a rat vaginitis model (see Chapter 4). Among the genes examined in this thesis, *CHO1* (phosphatidylserine synthase) is the most promising candidate for a drug target because there is no similar mammalian gene and this gene is conserved in opportunistic fungal pathogens including *Cryptococcus*, *Aspergillus*, and non-*albican Candida* species suggesting a drug with broad spectrum could be developed.

In addition to investigating the virulence, the bacterial pathogen *M. tuberculosis* and the parasitic pathogen *T. brucei*, which both must make inositol *de novo* in order to cause disease or even grow, respectively (25, 109, 118). The results showed that inositol biosynthesis and acquisition in *C. albicans* is conserved with *S. cerevisiae*. Interestingly, the putative inositol regulator *OPI1* gene is not conserved with *ScOPI1* because *OPI1* gene did not regulate expression of the inositol biosynthesis gene *INO1* in *C. albicans* suggesting inositol regulation between *S. cerevisiae* and *C. albicans* has been rewired (see Chapter 4). This phenomenon is also observed in *Yarrowia lipolytica*. Hirakawa et al. demonstrated that Yas3p, an opi1-family transcription factor does not regulate expression of *YlINO1*, instead it regulates the expression of *YlALK1*, an alkane-dependent gene (69).

Taken together, the structural *INO1* gene is conserved among fungi, and even other eukaryotes and prokaryotes, but the regulation of the *INO1* gene in fungal species has diverged.

Overall, the thesis suggests that Opi1p and Cho1p could be potential drug targets, but Cho1p seems to be more promising because *cho1*Δ/*cho1*Δ mutants are completely avirulent and eventually cleared by the host (see Chapter 3). Moreover, Cho1p represents a new class of targets (phospholipid) for antifungal agents. The more choices of drugs the more possible weapons to inhibit life-threaten pathogens.

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APPENDICES

APPENDIX E: SUPPLEMENTARY DATA

Table E-1. All *C. albicans* strains*

Ca genes	WT	Hetero-1	Hetero-2	Homo-1	Homo-2	Restored	Conditional	SAP2 Constitutive
OPI1	(+/+)	(+/-:NAT1)	(+/-)	(-/-:NAT1)	(-/-)	(-/-:OPI1)		223 (from 57)
	57	58	**85**	86	**88**	**117**		226 (from 88)
INO1	(+/+)	(+/-:NAT1)	(+/-)	(-/-:NAT1)	(-/-)	(-/-:INO1)		
	57	100	**105**	111	**113**	**120**		
	57	101	108	126				
CHO1	(+/+)	(+/-:NAT1)	(+/-)	(-/-:NAT1)		(-/-:CHO1, MPAr)		
	57	132	137	142		172 (partial)		
	57	131	135	140		168 (partial)		
	57	131	135	191				
				(-/-:SAT1)		**(-/-::CHO1-SAT1)**		
	57	132	**137**	334	**337**	**344**		
ITR2	(+/+)	(+/-:SAT1)	(+/-)	(-/-:SAT1)	(-/-)	(-/-:ITR2)		
	57	176	**185**	192	**196**	**211**		
	57	179	193	199	201			
INO1 -/- ITR2	*INO1-/- ITR2+/+*	*INO1 -/- ITR2 +/-:SAT1*	*INO1 -/- ITR2 +/-*				INO1-/- ITR2-/MET3::ITR2	
	113	180	**184**				**261**	
	113	181	187				**266**	
PSD1	(+/+)	(+/-:SAT1)	(+/-)	(-/-:SAT1)	(-/-)	(-/-:PSD1)		
	57	240	**247**	254	**280**	**294**		
PSD2	(+/+)	(+/-:SAT1)	(+/-)	(-/-:SAT1)	(-/-)	(-/-:PSD1)		
	57	243	**250**	257	**271**	**290**		
PSD2-/- PSD1	*PSD2 -/- PSD1 +/+*	*PSD2 -/- PSD1 +/-::SAT1*	*PSD2 -/- PSD1 +/-*	*PSD2-/- PSD1-/-::SAT1*	*PSD2-/- PSD1-/-*		PSD2 -/- PSD1 -/MET3::PSD1	
	271	299	**300**	373	**375**		**331**	
OPI3	(+/+)	(+/-:NAT1)	(+/-)	(-/-:NAT1)	(-/-)	(-/-:OPI3)		
	57	393	**400**	405	**406**	**412**		
CHO2	(+/+)	(+/-:NAT1)	(+/-)					
	57	394	**401**					

*Numbers represent YLC strains

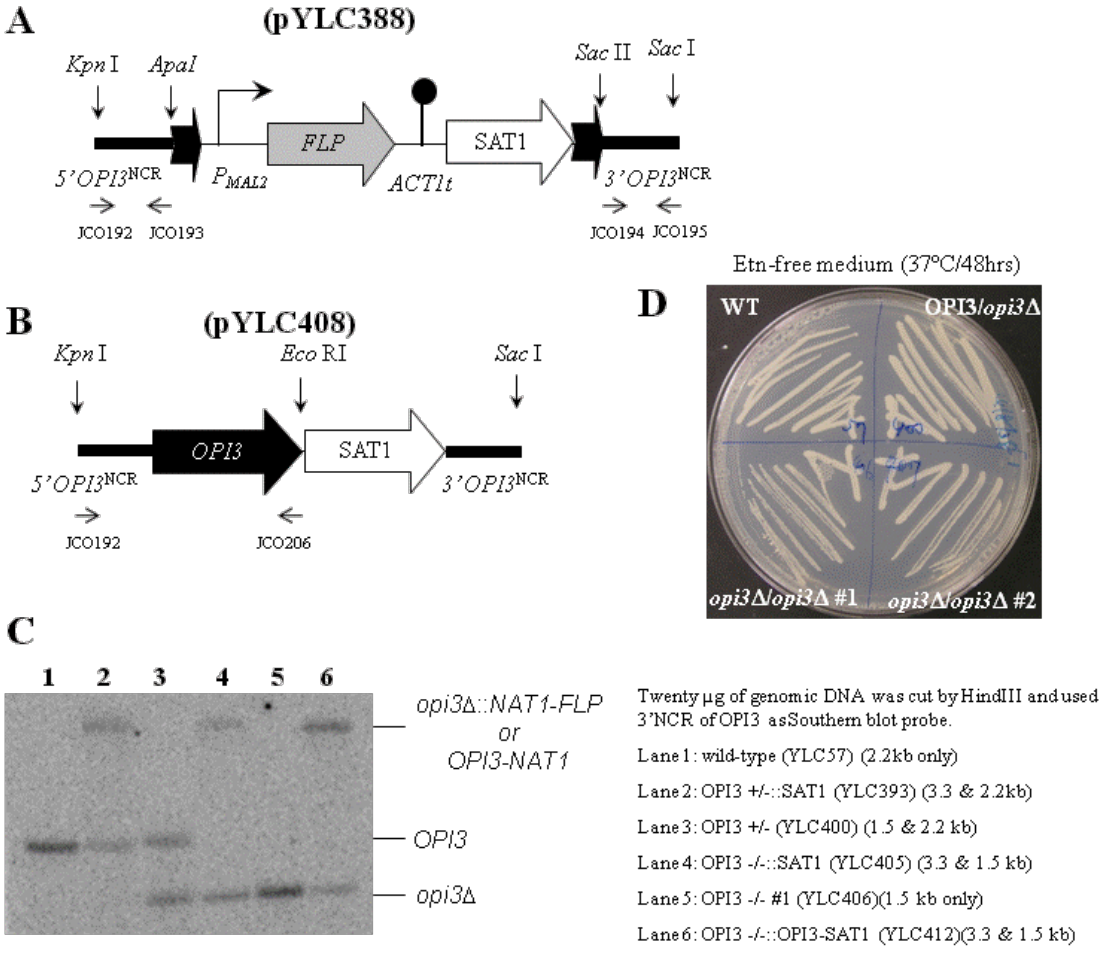


Fig E-1. The *OPI3* gene was disrupted in the strain SC5314 using the *SAT1* flipper.

(A) Structure of knock out construct. Approximately 500 base pairs of non-coding DNA flanking the 5' and 3' ends of the *OPI3* gene (5'-and 3'-NCR, respectively) were cloned onto either flank of *SAT1* flipper. The thick dark arrows represent the FRT sites of the FLP recombinase. The ball-and-stick symbol represents the ACT1 terminator (ACT1t), and the thinner arrow on a raised line represents the MAL2 promoter (P_{MAL2}). (B) The restored construct used to reintegrate *OPI3* into the *opi3Δ/opi3Δ* mutant. (C) Southern blotting was used to confirm the *opi3Δ/opi3Δ* mutant. (D) *opi3Δ/opi3Δ* mutant did not exhibit ethanolamine auxotrophy.

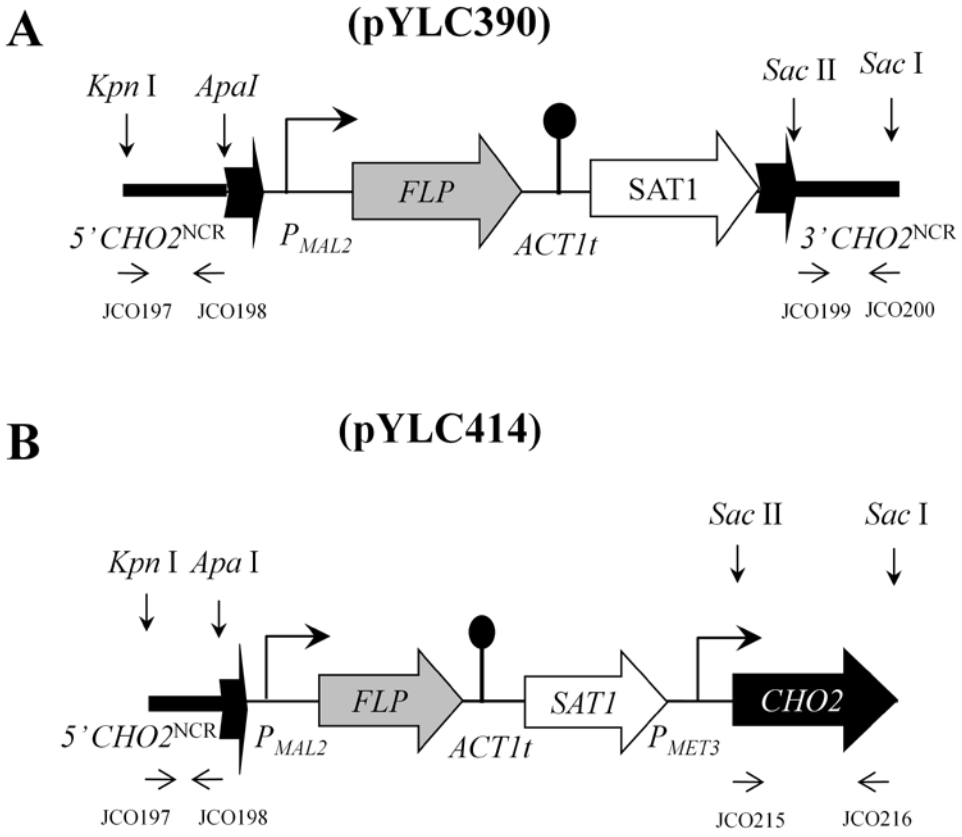


Fig E-2. The *CHO2* gene was disrupted in the strain SC5314 using the *SAT1* flipper.

(A) Structure of knock out construct. Approximately 500 base pairs of non-coding DNA flanking the 5' and 3' ends of the *CHO2* gene (5'-and 3'-NCR, respectively) were cloned onto either flank of *SAT1* flipper. The thick dark arrows represent the FRT sites of the FLP recombinase. The ball-and-stick symbol represents the ACT1 terminator (ACT1t), and the thinner arrow on a raised line represents the MAL2 promoter (P_{MAL2}). (B) The *CHO2* conditional expression construct which was used to replace second wild-type *CHO2* allele of CHO2/*cho2*Δ heterozygote due to inability to obtain *cho2*Δ/*cho2*Δ mutant.

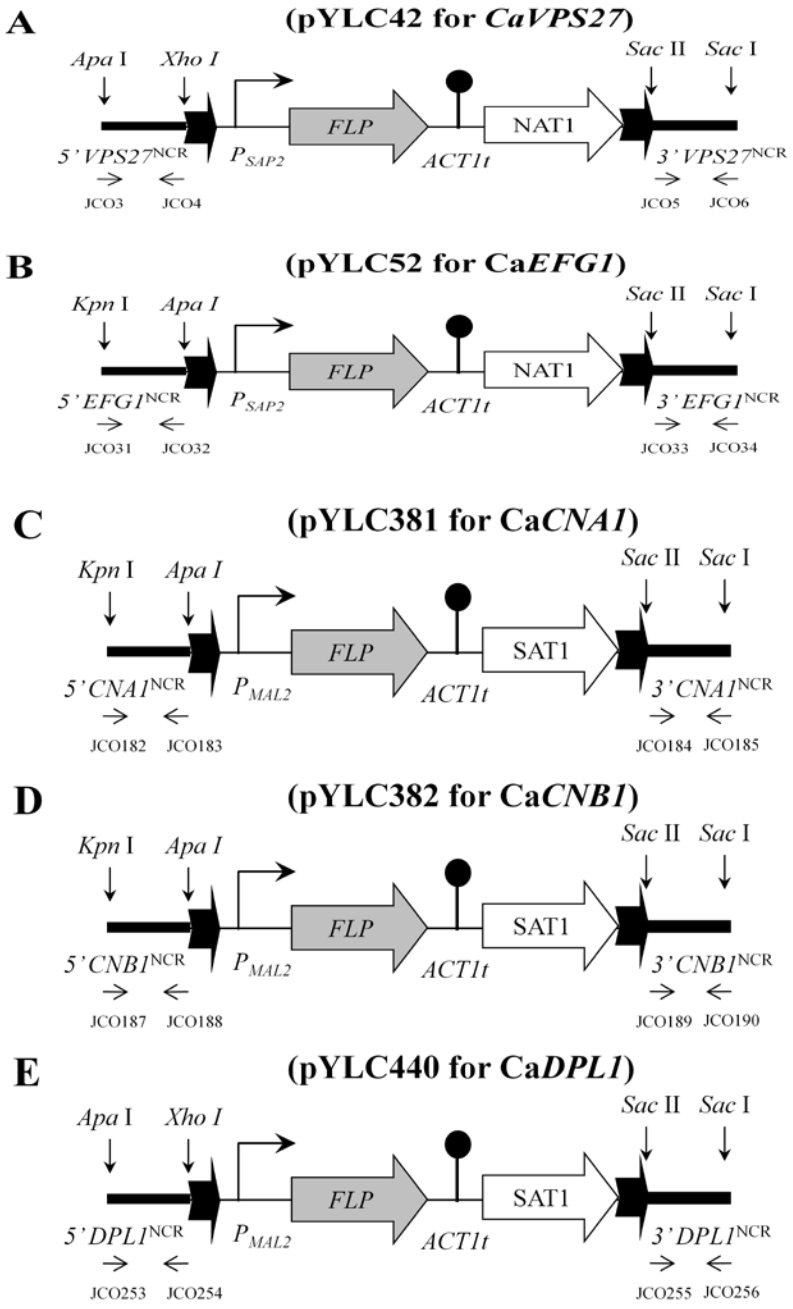


Fig E-3. The *VPS27*, *EFG1*, *CNA1*, *CNB1*, and *DPL1* knock out constructs. Refer A to E for more details.

SAT1 marked *MET3* conditional promoter construct (pYLC314 and pYLC315)

* ***SAT1*** included *CaACt1* promoter, *SAT1* ORF, and *CaURA3* terminator (~ 1.7kb) was amplified from pSFS2A (*SAT1* flipper).
* *CaMET3* promoter (P_{MET3}):

  1362 bp upstream of *CaMET3* gene was amplified from SC5314 genome.
* *SAT1* marked *MET3* promoter sequence was cloned into *Eco* RI site of pBluescript SK+
* A *Pst I* site located between *SAT1* and P_{MET3}

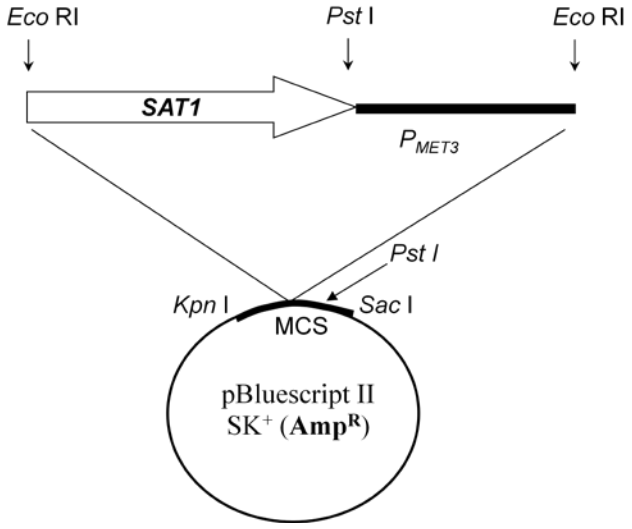


Fig E-4. The *SAT1*-marked MET3 conditional promoter construct was made to test essential gene.

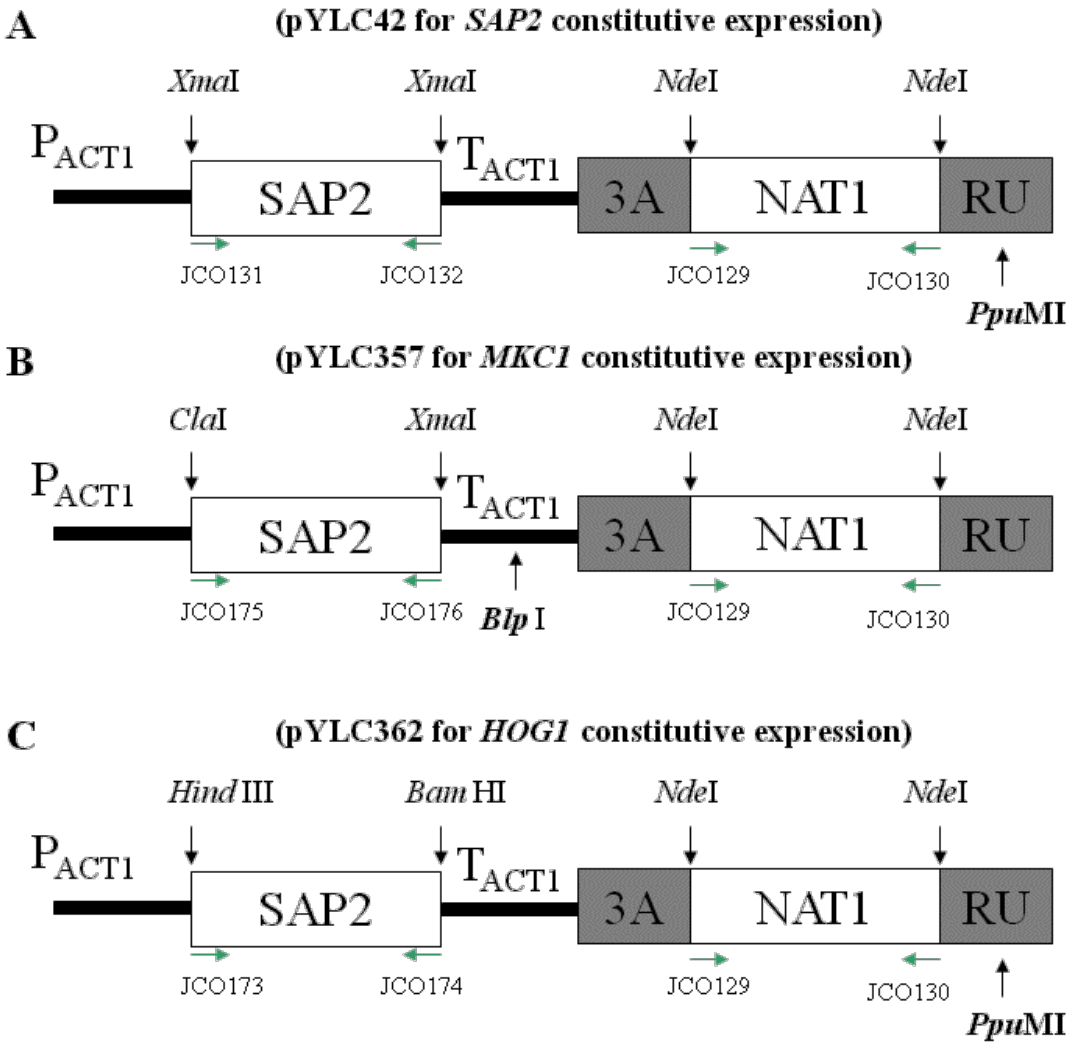


Fig E-5. The NAT1-marked construct of constitutive expression under the control of ACT1 promoter.

The linear plasmids cut by PpuMI or BlpI were transformed and integrated into *CaURA3* or *ACT1* terminator locus, respectively.

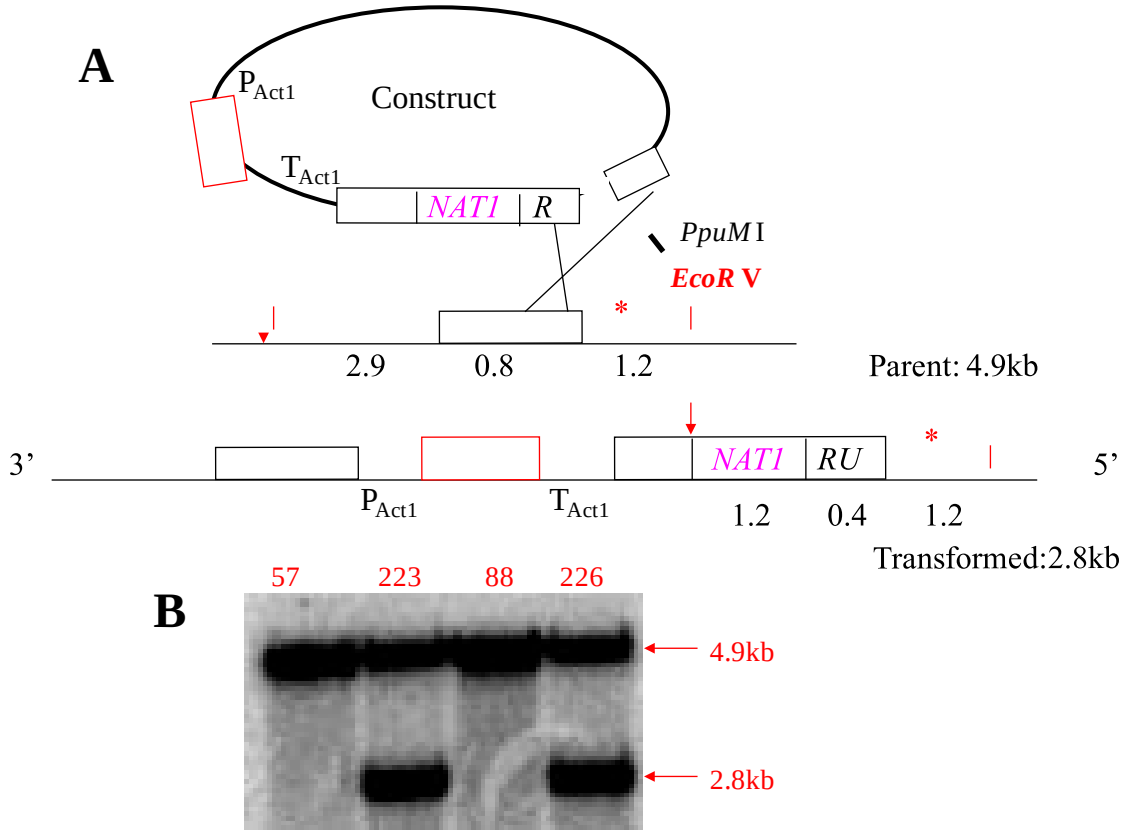


Fig E-6.The constitutively expression construct of *SAP2*.

(A) Diagram. (B) Southern blot. The genomic DNA was digested by EcoRV (arrow) and 5' noncoding region of URA3 was amplified as probe (asterisk). The strain number 57, 223, 88, and 226 represent *C. albicans* WT, WT::ACT1::*SAP2*, *opi1*Δ/*opi1*Δ, and *opi1*Δ/*opi1*Δ::ACT1::*SAP2*, respectively.

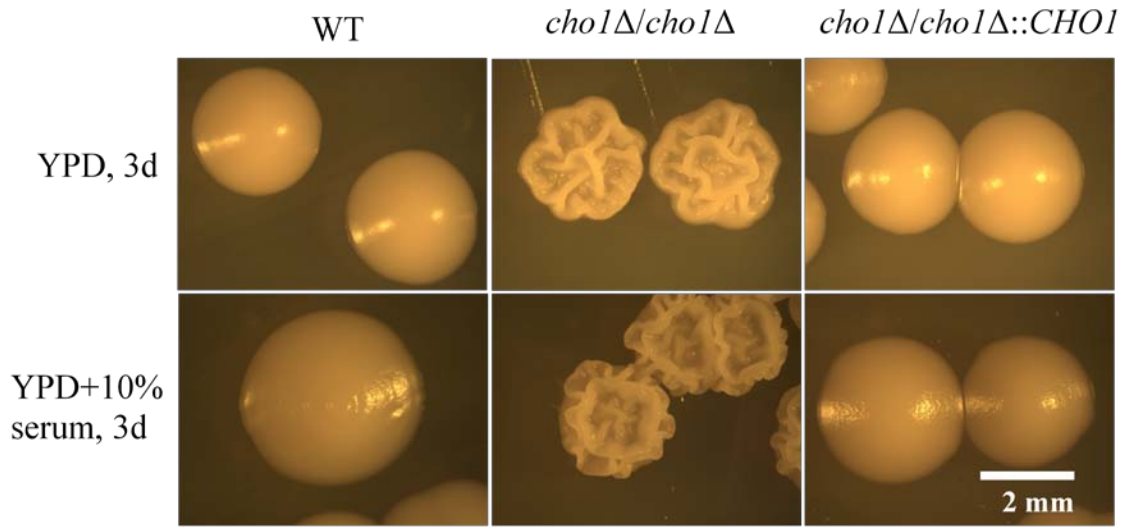


Fig E-7. The *cho1*Δ/*cho1*Δ mutant formed hyper-wrinkled colonies on YPD containing medium, both ± serum at 37ºC.

Neither of these effects were observed in the *psd1*Δ/*psd1*Δ and *psd1*Δ/*psd1*Δ *psd2*Δ/*psd2*Δ mutants (data not shown).

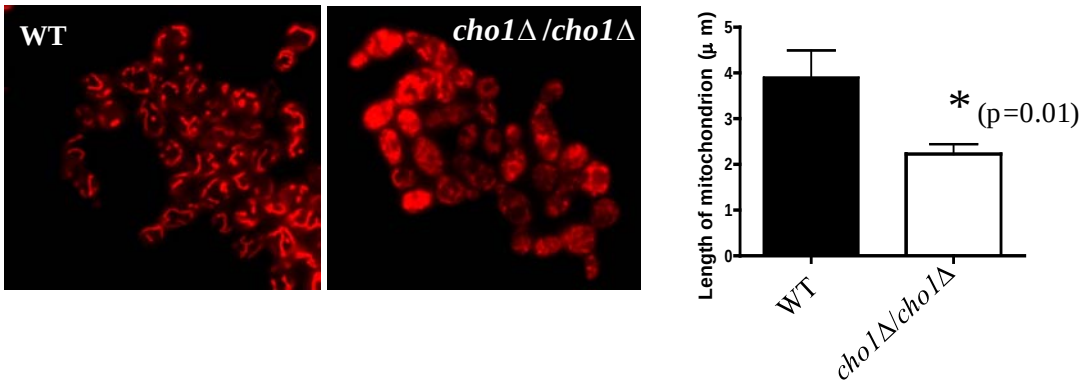


Fig E-8. The length of mitochondria in *cho1*Δ/*cho1*Δ mutant might be shorted.

Strains were stained with the vital dye Mitotracker Red CMXRos® after growth to log phase in YPD at 30ºC.

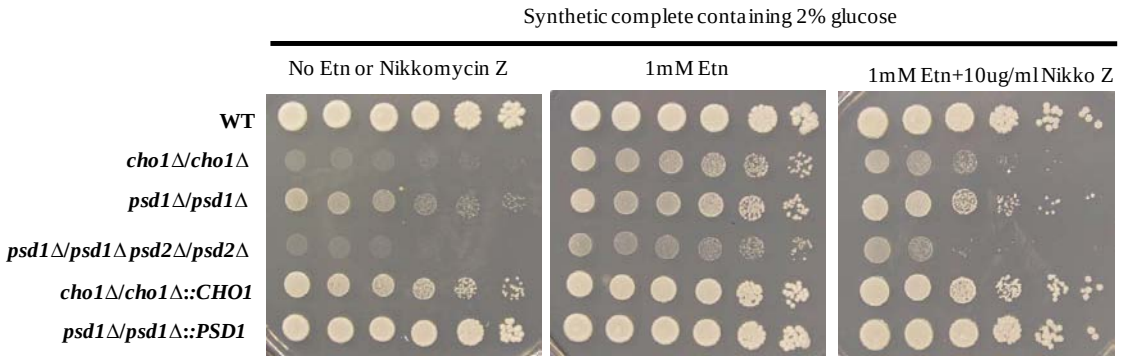


Fig E-9. The phospholipid mutants were slightly susceptable to chitin synthase inhibitor, nikkomycin Z.

Strains were grown for 48 hrs at 37ºC.

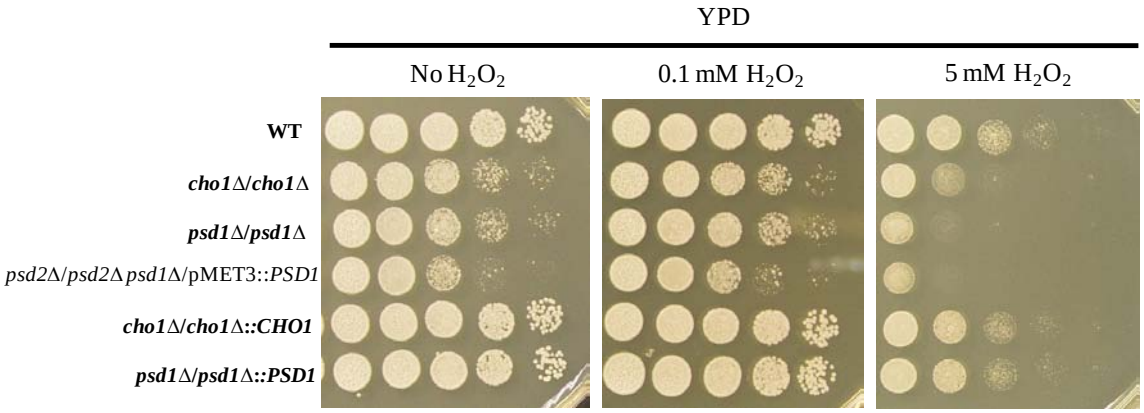


Fig E-10. Hydrogen peroxide has no observable effects on phospholipid mutants. Strains were grown for 24 hrs at 37ºC

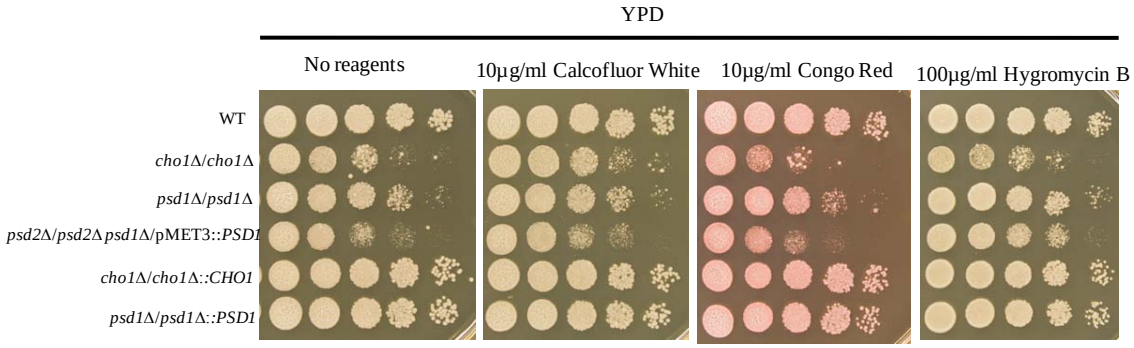


Fig E-11. Cell wall perturbating regagents Calcofluor white, CongoHydrogen, and drug Hygromycin B have no observable effects on the growth of phospholipid mutants.

Strains were grown for 24 hrs at 37ºC.

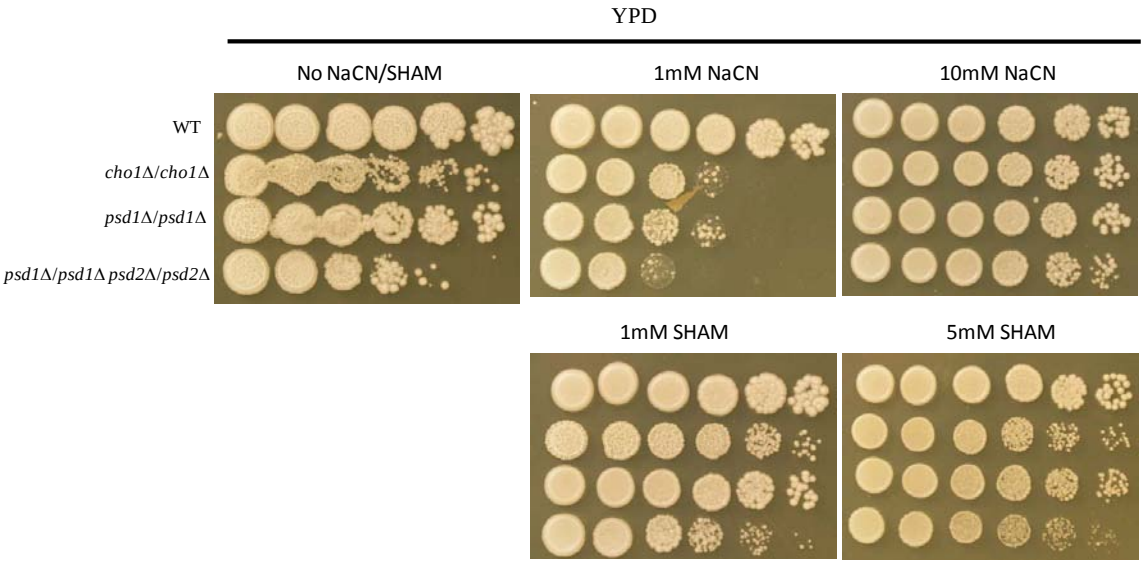


Fig E-12. 10mM NaCN (inhibiting cytochrome aa3) seems rescue the growth of phospholipid mutants.

However, SHAM (inhibiting alternative electron transport chain) has no observable effects on phospholipid mutants. Strains were grown for 42hrs at 37ºC.

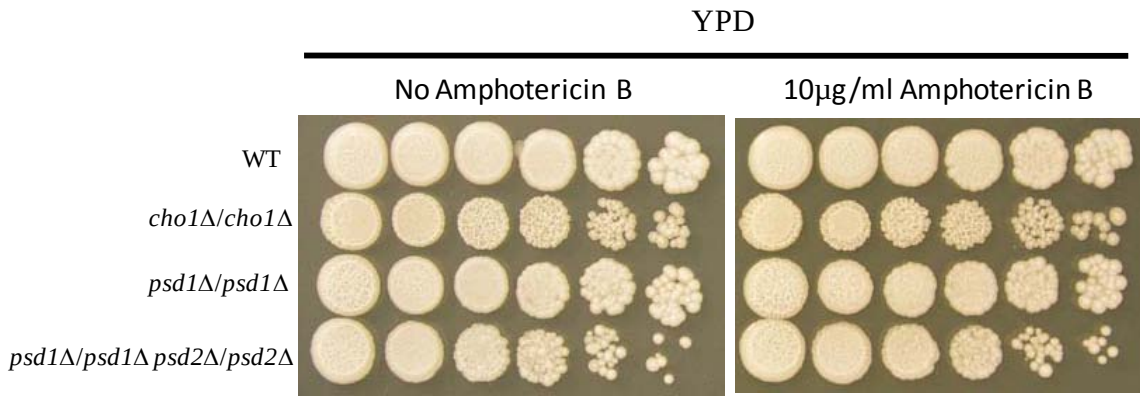


Fig E-13. Amphotericin B has no observable effects on the growth of phospholipid mutants.

Strains were grown for 48 hrs at 37ºC.

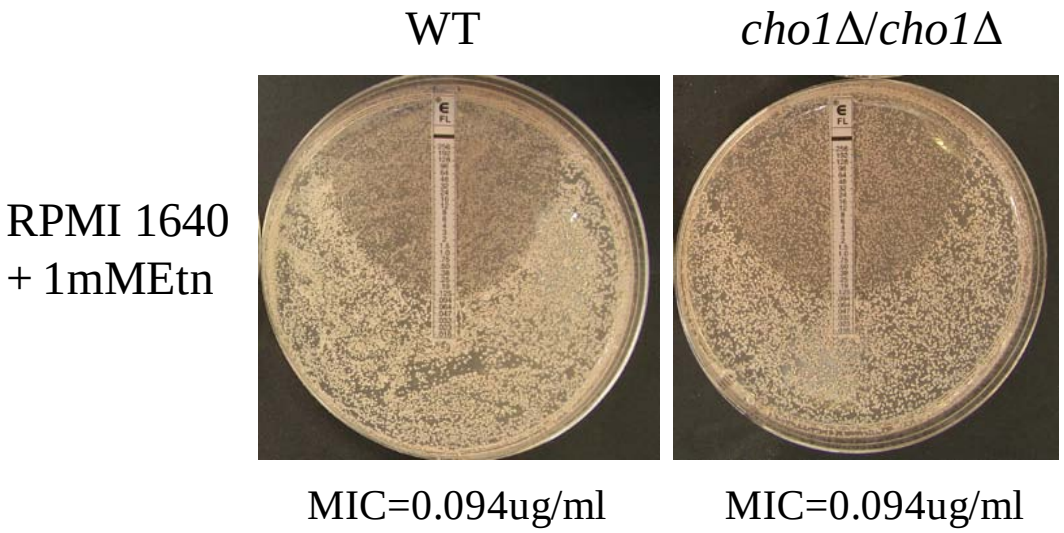


Fig E-14. Antifungal fluconazole has no effects on the growth of *cho1*Δ/*cho1*Δ mutant. Strains were grown for 36 hrs at 37ºC.

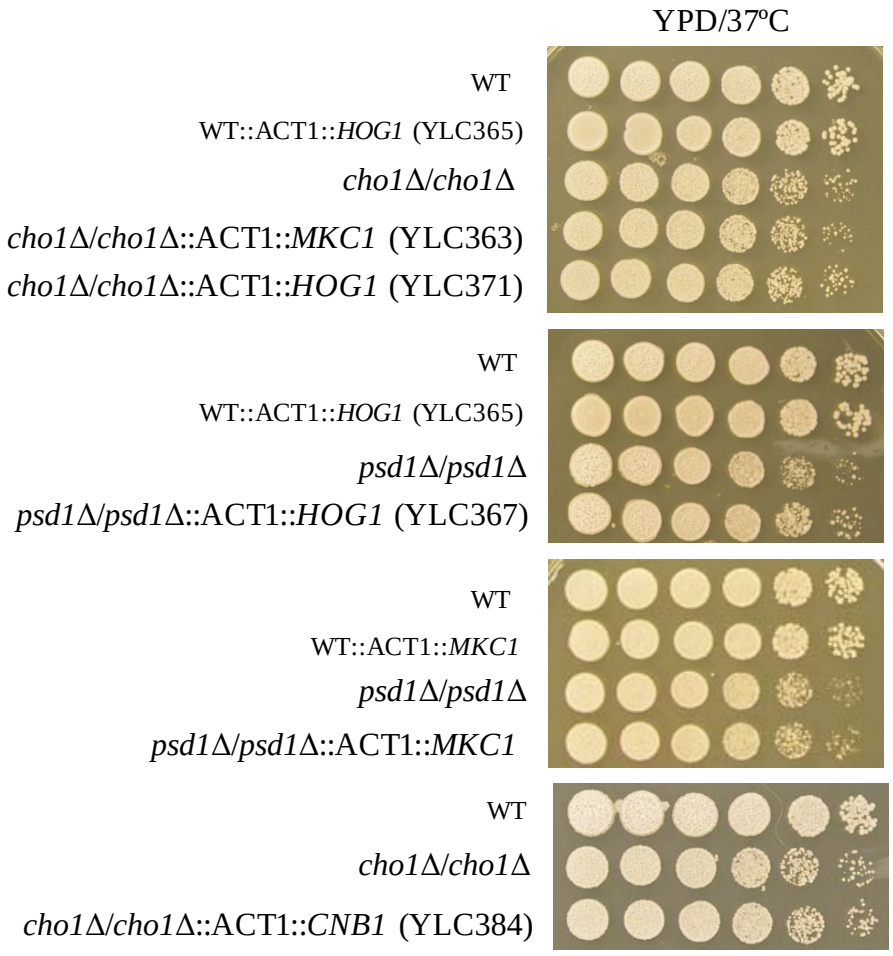


Fig E-15. The constitutively expression of *C. albicans MKC1, HOG1*, and *CNB1* signaling pathways.

These pathways cannot rescue the growth of mutants' growth on YPD or YPD containing sorbitol (data not shown). might not involve in regulating the growth of phospholipid mutants. Note that constitutively expressionof CNA1 has not obtained.

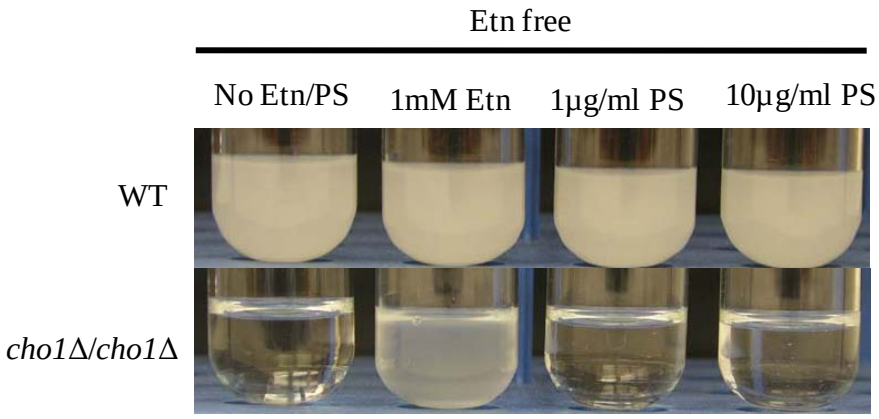
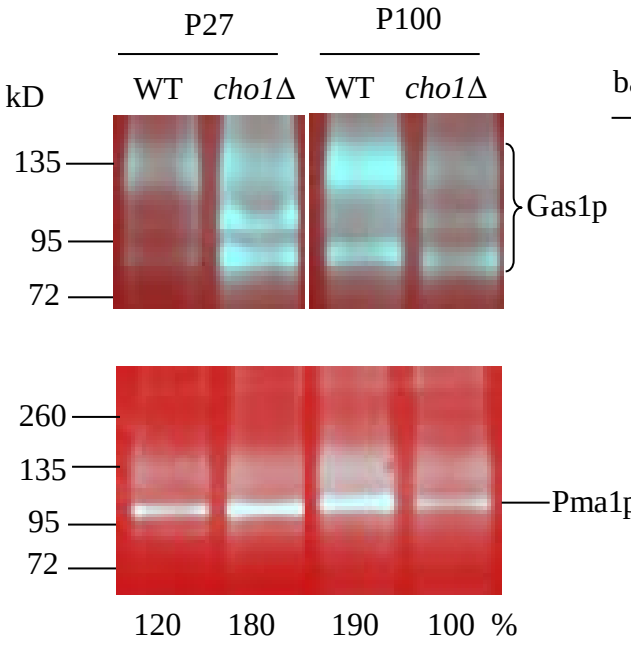


Fig E-16. Phosphatidylserine (PS) can not rescue the growth of *cho1*Δ/*cho1*Δ mutant in our tested condition.

Strains with same concentration were grown overnight in indicated concentration of Etn or PS at 30ºC.



	P27		P100	
band	WT	*cho1*Δ	WT	*cho1*Δ
Top	105	146	167	100 %
Mid	100	310	220	190 %
Bot	100	230	190	290 %

Fig E-17. *C. albicans* Gas1p might be affected in *cho1*Δ/*cho1*Δ mutant.

The intensity of Western blot results was normalized by Pma1p. Strains were grown to log phase at 37ºC and ultra centrifuged (27K & 100K x g) proteins were collected.

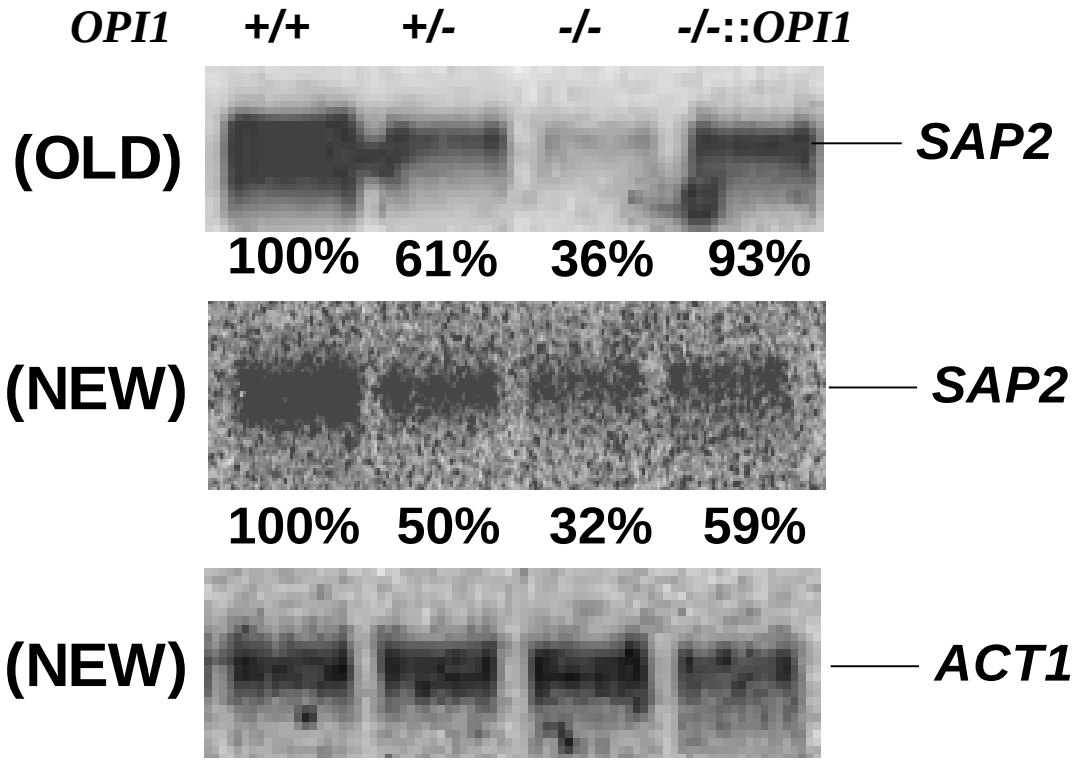


Fig E-18. The *OPI1* gene affects the transcription of the *SAP2* secreted aspartyl protease.

Cells were grown in yeast carbon base (YCB) medium where protein (bovine serum albumin, BSA) was the only nitrogen source (YCB-BSA) for 12 hours at 37˚C. Total RNA was isolated, Northern blotted, and probed for the *SAP2* gene. Expression levels were normalized to the *ACT1* gene. Note that he old northern blot included serial *opi1*Δ mutants obtained from the defective wild type.

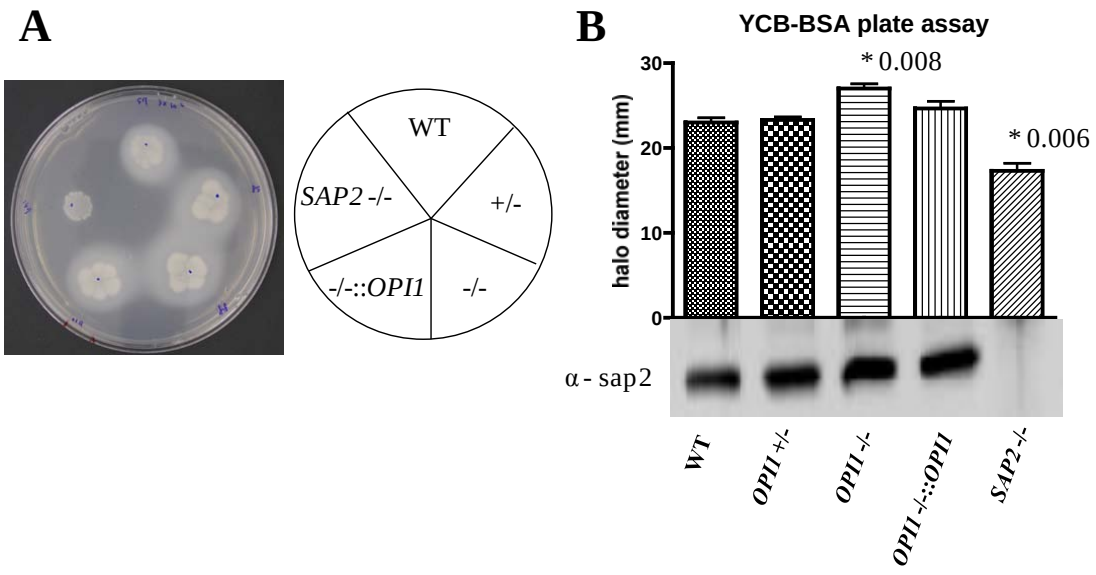


Fig E-19. *OPI1* -/- mutant exhibits hyper-protease activity in YCB-BSA plate.

(A) Plate assay. (B) quantitative measurement of zones, and western blot results in liquid YCB-BSA (collected supernatant with anti-sap2p). Asterisks indicate P value as compared to the wild type.

Lab solutions:

1. SC+2% glucose (dextrose)

10X YNB 25ml

(1) add 16.75g yeast nitrogen base w/o amino acids

(2) add water to 250ml, then filter sterilize and store at 4ºC

10X Yeast Synthetic drop-out medium supplement w/o uracil 25ml

(1) add 4.8g Yeast synthetic drop-out medium supplement w/o uracil

(2) add water to 250ml, then filter sterilize

10X uracil 25 ml (or uridine for *C. albicans*)

(1) add 50mg uracil

(2) add water to 250ml, then filter sterilize

40% glucose 12.5ml

Add water to 250ml

SC: Synthetic Complete (YNB + all amino acids)

SD: Synthetic Dextrose (YNB + Dextrose)

2. YCB-BSA-YE (Yeast Carbon Base-Bovine Serum Albumin-Yeast Extract)

2.9g YCB

0.5g BSA (avoid autoclave; add from filter sterilized 10% BSA stock)

0.25g YE

Add wáter to 200ml → adjust pH → bring water to 250ml.

3. YCB-BSA protease halo (plate) assay

5.8g YCB (Bacto)

10g Agar (Bacto)

Add water to 490ml → autoclave →cool down, then add 10ml of 50X BSA stock (10%)

4. RPMI 1640 for Fluconazole E test strips

5.2g RPMI 1640 (powder, Cat#50-020-PB,e Cell Gro, w/ L-Glutamine, Phenol Red, w/out $NaHCO_3$)

17.25g MOPS (0.165mole/L)

9.0g glucose (note that 1g has already in the powder)

7.5 g Agar (Bacto) (1.5%)

Add water to 450ml → adjust pH from ~5.6 to pH7.0 → add water to 500ml

5. M199 medium (pH7.0)

425ml original M199 (9.5g M199, GIBCO Cat#31100-027, Invitrogen,1 pack in 1L water)

75ml 1M HEPES (pH7.0) (150mM HEPES final)

Auto clave.

For making a solid plate, add 10g agar. Note that solid plate (pH4.0) cannot be solidified.

1M HEPES (MW=238.3): add 47.6g of HEPES into 200ml water → adjust pH

6. Spider (Haoping Liu, Science)

5g Nutrient broth

5g D-Mannitol

1g K_2HPO_4

6.75g Agar (if solid)

Add water to 500ml

7. YPD containing 15% glycerol (for stock in -80ºC)

1g Yeast Extract (1%)

2g Peptone (2%)

2g Dextrose (2%)

Add water to 70ml → add 30ml of 50% glycerol → autoclave

8. Calcofluor White stock (10mg/ml stock)

Add 100mg of Calcofluor White (M2R) to 10ml water

Add 3µl of 10M NaOH to dissolve the powder completely

Filter sterilize → cover with Aluminum Foil and store at 4ºC.

9. Amphotericin B (10mg/ml stock)

5mg Amphotericin B in 0.5ml DMSO

10. Frequent working concentration in testing *C. albicans*

Calcofluor White (CFW, 10µg/ml); Congo Red (10µg/ml); Nikkomycin Z (10µg/ml) Hygromycin B (100µg/ml); Amphotericin B (10µg/ml); Cyclosporin A (100µg/ml); FK506 (1µg/ml); Fluconazole (100µg/ml); Ketaconazole (50µg/ml); Caffeine (10mM); Hydrogen Peroxide (1~5mM).

VITA

Ying-Lien (Joseph) Chen received his Bachelor degree in Plant Pathology from National Ping-Tung Polytechnic College, Taiwan, in 1996. In 1998 he received a Master degree in Plant Pathology from National Taiwan University (NTU). He used to serve in Army, and work in Pediatrics department of NTU Hospital and Asia Hepto Gene Inc. before his arrival into United States. He was awarded *Eukaryotic Cell* outstanding young investigator and Science Alliance Awards in 2008. He was also granted Woods Hole scholarship for summer course 'Molecular Mycology' and awarded 'Excellence for in vivo Research' in 2008. He will receive his doctorate degree in Microbiology in May 2009. Following graduation, he plans to conduct fungal research as post-doctoral fellow at Duke University Medical Center, North Carolina.