

The role of Phosphatidylserine and Phosphatidylethanolamine
in *Candida albicans* virulence

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

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August 2015

Dedication

Thank you Mom and Dad for believing in me and the never ending support. Thanks to my colleagues in lab for the laughs and conversation, and sharing in the stress of graduate school. Last not but not least, thank you to all the wonderful teachers I have had through the years that have encouraged my love for science and have been a constant inspiration for me to fulfill my dreams.

Acknowledgements

I'd like to thank all of my committee members, Dr. Becker, Dr. Sparer, Dr. Alexandre, Dr. Su and Dr. Reynolds for your advice over the years. I would like to especially thank my PI Dr. Reynolds, who was incredibly patient with my unorthodox lab hours and supported me through it all. Thank you Dr. Minkin and Dr. Dunlap for assisting with microscopy and all the quantification it required. I would like to thank Dr. Melinda Hauser and Dr. Elizabeth Fozo for their critical review of my prelim, and also constantly being available for advice of both a professional and personal nature. Thank you Elizabeth McPherson, you helped me become a better teacher and thank you for your endless patience and support. Thanks to Dr. Becker and Dr. Sparer for allowing me to use their lab space and reagents often.

Before graduate school, I was a technician at Virginia Tech under the guidance of Dr. Liwu Li. Dr. Li, thank you for the opportunity to become a better scientist and encouraging me to go to graduate school; without your help I don't know if I would have made it this far. Thank you to my first academic mentor Dr. Harry Kurtz Jr., my undergraduate PI at Clemson University. Conducting undergraduate research in your lab gave me a passion for scientific research, and ultimately propelled me into my professional career.

Abstract

In hospitalized patients with neutropenia, *Candida albicans* is the fourth leading cause of systemic bloodstream infections, which have a mortality rate of approximately 30 %. The phosphatidylserine synthase enzyme of *C. albicans*, Cho1p, appears to be a good drug target as a mutant lacking this enzyme (the *cho1Δ/Δ* [null mutant]) is avirulent in animal models of *Candida* infections and this enzyme is not conserved in humans. We discovered that the loss of phosphatidylserine (PS) synthesis affects *C. albicans*' expression of the Als3p adhesin, a virulence protein, and loss of PS synthesis also compromises the cell wall, causing increased exposure of the polysaccharide β [beta]-(1,3) glucan that ultimately leads to increased recognition by the immune system.

Downstream of phosphatidylserine synthase are the decarboxylase enzymes Psd1p and Psd2p, which are essential for *de novo* synthesis of phosphatidylethanolamine from phosphatidylserine. When these enzymes are lost, the only option the yeast has to acquiring phosphatidylethanolamine is by synthesizing it by the Kennedy Pathway utilizing ethanolamine acquired from the environment. The final chapter of my dissertation explores how ethanolamine is difficult to acquire from the host *in vivo*, and is ultimately a bottleneck for *C. albicans* to cause virulence in the mouse model of disseminated candidiasis.

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Chapter I: Background and Introduction

An Overview of *Candida albicans* Virulence

Candida spp. are human commensal fungi that colonize the gastrointestinal tract, as well as vaginal and oral mucosa (1). They occur as oral flora for approximately 30-60% of healthy individuals and usually do not cause disease (2, 3). However, *C. albicans* and related species are capable of causing more serious systemic infections, where it is present in the bloodstream and deep organs, and are of great concern as they have a high associated mortality rate once fungal septicemia is established (31.8%) (4). Systemic infections are a common problem in intensive care units (ICUs), and are associated with risk factors such as major abdominal surgery, catheterization and prolonged stays in the ICU (5). Patients with neutropenia are also more susceptible to systemic fungal infections, making patients with leukemia or undergoing vigorous chemotherapy treatments generally more susceptible to systemic infections (6, 7). In addition, *C. albicans* is the leading blood-borne fungi to colonize patients' prosthetic heart valves (8). This affliction, which is known as fungal endocarditis, is extremely life threatening with a mortality rate of up to 40%, and is difficult to treat with antifungals once established (9, 10).

Currently there are only five classes of antifungal drugs available to treat fungal infections, and toxicity and resistance limit their effectiveness. Only 3 of the 5 can be used for systemic infections, further limiting options for treatment of fungal bloodstream infections (11-13). The polyenes bind ergosterol in the cell membrane, disrupting cell integrity and resulting in cell death. An example is amphotericin B, which is the only polyene that is an effective treatment for systemic infections, but must be given intravenously and has the ability to cause kidney damage (14, 15). As ergosterol is a lipid that is unique to fungi, it has also been targeted by the azole drug family, which inhibits the enzyme lanosterol 14 α -demethylase which converts lanosterol to ergosterol. Ultimately inhibition of this pathway results in a lack of ergosterol in the fungal cell membrane, and a fungistatic control of infection (16). Apart from targeting of ergosterol, cell wall components that are unique to fungi can be targeted, in particular the

β -(1,3) glucan saccharide that is important for cell wall infrastructure. Drugs that belong to the echinocandins family (such as caspofungin) are able to inhibit the enzyme β -(1,3) glucan synthase, thus causing the cell wall to eventually lose integrity (16). The remaining drugs are hydrophobic and can only be used topically for fungal skin or nail infections. For example, allylamines are hydrophobic and are not amenable for administration orally or intravenously (17).

In immunocompromised patients *Candida* spp. are able to cause an extremely common oral infection known as oropharyngeal Candidiasis (OPC) (2). OPC is one of the first indicators of suppression of adaptive immunity, and is seen in up to 95% of AIDS patients at some time during their infection (18). Other risk factors for OPC include use of steroid inhalers and the use of dentures, and is becoming a common infection in recipients of solid organ transplants (19-22). These infections are deleterious to the comfort of the patient causing difficulty chewing and swallowing, and in severe cases will result in reduced eating and weight loss (2). It is important to note that these oral infections are refractory to medications and often exhibit drug resistance, making treatment challenging (23). Out of all *Candida* species, *C. albicans* is the species most commonly isolated from oral lesions (24).

***C. albicans* morphology and its role in virulence**

Morphology is also known to be involved in *C. albicans* virulence. Morphology differences are dependent on the phenomenon known as "phenotypic switching", which is the ability of *C. albicans* to switch between its 3 forms: yeast, pseudohyphae, and hyphae (Fig. 1.1). In regards to virulence, the yeast form is regarded as being important for dispersion and travel within the bloodstream, and the hyphal form important for tissue invasion and damage (Fig 1.1) (25-28). This is at least partially due to differential gene regulation in the yeast or hyphal forms, with the hyphal form expressing specific adhesins as well as secreted aspartyl proteases that altogether enable it to become an effective bloodstream pathogen (29-31). Hyphal cells also contain up to three times more chitin in comparison to yeast form, which may play a role in

immune resistance and robustness in surviving *in vivo* as it lends strength to the overall structure of the cell (32). For instance, *C. albicans* strains that are upregulated in chitin synthesis by upregulation of Pir32 are more virulent than wild type (33). Pir32 is also upregulated when *C. albicans* is phagocytosed by macrophages, which may aid in *C. albicans*' survival and escape of the macrophage phagosome (34).

The importance of hyphal formation of *C. albicans* for virulence is also demonstrated in mutants that are unable to form hyphae. The *efg1Δ/Δ cph1Δ/Δ* double mutant is permanently fixed in yeast-form and is less virulent, and is reduced in the ability to invade host endothelial cells in the mouse oropharyngeal candidiasis model (25). However, there is evidence suggesting that hyphal formation is not the end-all and be-all of *C. albicans* virulence. When the hyper-filamentous *C. albicans* mutant *tup1Δ/Δ* is generated, it is reduced in virulence and dissemination (25). Altogether these findings suggest that both yeast and hyphal forms work together in concert to disseminate in the bloodstream as well as invade host tissue.

A Phospholipid biosynthesis overview for fungi and humans

Apart from the morphology of *Candida albicans*, there are a variety of other components that can contribute to virulence, such as secreted aspartyl proteases (SAPs), adhesin molecules, and phospholipases (35). Inversely, many factors can contribute to a lack of virulence for *C. albicans* including auxotrophy of essential nutrients such as amino acids (36, 37). As this dissertation is focused on how a loss of phospholipids can result in a loss of virulence, and how yeast phospholipid synthesis enzymes could be unique drug targets, it is vital to understand how yeast and mammals differ in the steps that it takes to generate these various phospholipids. Yeast including *C. albicans* have two main means of synthesizing phospholipids, either by the *de novo* process or via the Kennedy pathway (Fig. 1.2). The *de novo* pathway is the sole means by which *C. albicans* can synthesize phosphatidylserine. It does so from cytidine diphosphate diacylglycerol (CDP-DAG) and serine by the Cho1p enzyme (38). Phosphatidylserine (PS) can then be decarboxylated into phosphatidylethanolamine (PE)

by the phosphatidylserine decarboxylases Psd1p (located in the mitochondria) or Psd2p (located in the Golgi/vacuole) (39-42). Lastly, phosphatidylethanolamine can then be methylated by the two methylases Opi3 and Cho2 to generate phosphatidylcholine (PC), the most abundant phospholipid in yeast genera.

Apart from the *de novo* pathway, phosphatidylethanolamine and phosphatidylcholine can be generated from the Kennedy pathway, which directly incorporates ethanolamine and choline into diacylglycerol (DAG) to make their respective phospholipids (Fig.1.2) (41, 43). Synthesis of PE or PC via the Kennedy pathway consists of a total of 3 enzymatic steps. First, ethanolamine is phosphorylated in an ATP-dependent manner by an ethanolamine kinase. After phosphorylation, the enzyme cytidine tri-phosphate (CTP) phosphoethanolamine cytidyltransferase uses the CTP as an energy source to generate (cytidine di-phosphate) CDP-ethanolamine. Lastly, CDP-ethanolamine 1,2-diacylglycerol ethanolamine phosphotransferase completes the final step of PE synthesis, using CDP-ethanolamine and DAG to form PE. Synthesis of PC via the Kennedy pathway requires the same steps, but uses choline as the head group substrate instead of ethanolamine. It has been found however that generation of phosphatidylethanolamine via this pathway is limited by the supply of ethanolamine in the media, and growth does not become as robust as wild type until exogenous ethanolamine is supplied at 1 mM ethanolamine in minimal media (44).

Phospholipid synthesis and its role in virulence in organisms

Auxotrophy is known as the loss of an organism's ability to synthesize an organic compound required for growth, such as essential amino acids, fatty acids, or vitamins (45). *URA3* (synthesizes uracil), *HIS3* (synthesizes histidine), and *LYS4* (synthesizes lysine) are three examples in which when knocked out in *C. albicans*, the yeast fails to grow in the absence of these amino acids; as these amino acids are not readily available in the host, losses of these genes also result in a reduction in virulence in an immune-competent mouse model of systemic candidiasis (36, 46, 47). Auxotrophy in *C. albicans* for the synthesis of oleic acid also makes it

more susceptible to the antifungal drug amphotericin B, an important antifungal administered intravenously (48). These fatty acids are important for the organism as they are essential for synthesizing a variety of compounds within the cell, including phospholipids. Other fungal pathogens may also be affected in virulence by auxotrophy. *Aspergillus fumigatus*, when it is auxotrophic for uridine and uracil, is reduced in virulence in a mouse model of invasive aspergillosis (37).

Phospholipid synthesis is an essential function for all living organisms, and when it is disrupted there are repercussions, some which can be fatal, and others which have severe side effects. For *C. albicans*, we have discovered that the loss of phosphatidylserine synthase in the *cho1Δ/Δ* mutant results in growth defects in minimal media, ethanolamine auxotrophy, avirulence and cell wall defects (44). This mutant is also diminished in phosphatidylethanolamine (PE) synthesis (a downstream product in the *de novo* pathway, Fig 1.2). In addition, the *psd1Δ/Δ psd2Δ/Δ* double mutant is comparably diminished in PE levels (though not in PS, as the *cho1Δ/Δ* mutant). We have discovered that for both of these mutants, the loss of PE is responsible for both of them losing virulence in a mouse model of candidiasis, as well as a diminished ability to colonize the oropharynxes.

Interestingly, a role for phospholipids in virulence is not unique to *C. albicans* and defects can occur in other organisms when phospholipid synthesis is compromised. One example of phospholipid synthesis being important for virulence is exemplified by the bacterial pathogen *Brucella abortus*, which is a facultatively intracellular bacterium that is responsible for causing spontaneous abortions in cattle (a condition known as *brucellosis*) (49). When PE as well as PS synthesis are diminished in a phosphatidylserine synthase mutant (*pssAΔ*), there is an increased sensitivity to detergents which indicates altered cell membrane properties (50). This mutant was unable to successfully colonize a mouse and had difficulty surviving intracellularly in an intro assay, suggesting that PE is essential for virulence (50). In addition, *Mycobacterium tuberculosis*, which is a significant pathogen affecting hundreds of thousands

per year with lung infections (51) is diminished in virulence when it is no longer able to synthesize inositol, a precursor for phosphatidylinositol (PI) synthesis (52). A loss of this essential substrate resulted in a diminished ability to colonize macrophages as well as reduced overall lethality in a pulmonary mouse model. PI is an important constituent for the virulence factor lipoarabinomannan, which may explain how a loss of PI causes a reduction in virulence (52, 53).

It has also been discovered that phospholipid synthesis is vital for virulence in a variety of eukaryotes, including *Plasmodium falciparum*, a protozoan parasite that causes malaria in humans and is one of the most significant pathogens even to this day (54, 55). It was revealed that when phosphatidylcholine (PC) synthesis is inhibited in *P. falciparum*, it is unable to survive *in vitro* in culture suggesting that it is vital for survival (56). This finding was determined by the administration of the PC analog miltefosine, and then measuring the organism's proliferation rate in a blood culture (56). Yet another eukaryote that is influenced by the loss of lipid synthesis is *Toxoplasma gondii*, another obligate intracellular protozoan that is responsible for causing toxoplasmosis, a disease that affects an incredibly wide array of warm-blooded animals (57, 58). The *T. gondii* fatty acid synthesis II (FAS II) pathway is essential for lipoylation of the apicoplast organelle. When this is abolished in the null mutant *AcpΔ*, it is unable to survive in a tissue culture plaque assays, and eliminating this enzyme from the pathogen eliminates all mortality when injected in the mouse model (59), ultimately suggesting that synthesis of this important lipid molecule is vital for the survival of this organism (59).

Ultimately the synthesis of phospholipids is vital for avirulence in several microbes. Interestingly, despite studies in bacteria and parasites similar studies in pathogenic fungi are largely lacking.

Importance of adhesins in virulence for *C. albicans*

The Als family of adhesins expressed in *C. albicans* consists of 8 different proteins, that are all GPI-anchored cell wall proteins that all generally play a role in adherence to surfaces as well as inducing endocytosis by mammalian cells (60). Some of these Als proteins have been implicated in virulence: for instance, the Als1p protein has been shown to greatly increase adhesion to epithelial cells when artificially expressed in *Saccharomyces cerevisiae* (61). Comparatively, addition of the Als3p protein to *S. cerevisiae* also increased adhesion to epithelial cells to a statistically significant degree (61), and *C. albicans* that had Als3p removed from the genome had a drastically lower level of adherence to epithelial cells (62).

The cell wall protein Als3p is a virulence factor that acts as an adhesin, invasin, and binds host ferritin (31, 63-66), and it is specifically regulated by the transcription factor Rim101p which mediates the pH mediated response (67, 68). There is also evidence that *ALS1*, *ALS3*, and *HWP1* are regulated by the Rlm1p transcription factor (69). The Als3p protein is produced exclusively on the hyphal form of *C. albicans* (*C. albicans* morphology illustrated in Fig. 1.1)(70). From an *in vivo* perspective, this pathway is implicated in virulence as it is activated when *C. albicans* detects the pH neutral bloodstream as well as the presence of serum (67). While Als3p does not appear to contribute to virulence in a systemic mouse model of candidiasis, it does affect virulence in an tissue culture model of oropharyngeal candidiasis (OPC) using a FaDu cell line, as well as a modest role in virulence in the mouse OPC model (31, 71).

The immune response to *C. albicans*

C. albicans inhabits the gastrointestinal (GI) tract primarily as a yeast-form and as a commensal does not typically cause disease (72). The intestines are protected from invasion of normal flora by the intestinal mucosal barrier, which consists of a physical barrier as well as an immunological barrier (part of the initial immunological recognition is mediated by Peyer's patches, which play a part in sensing overgrowth in the GI tract and prevent invasion into the

host tissue) (73). This is the first line of defense the host has against the fungus, but can become compromised in those who are immunocompromised. If a breach in the physical barriers of the host occur, the immune system plays an important role in elimination of *C. albicans* and is critical for the survival of the host.

The main purpose of the yeast cell wall is to protect it from environmental stress such as osmolarity and detect its surroundings, but can also serve an important role in concealing it from the immune system (74-76). In systemic bloodstream infections of *C. albicans* (known as candidiasis), the cell wall surface helps conceal *C. albicans* from recognition from the innate immune system by concealing its main pathogen-associated molecular pattern (PAMP) β -(1,3) glucan, which enables the pathogen to disseminate with minimal detection by the innate immune system (77, 78). The surface of the cell wall largely consists of a thick layer of heavily mannosylated proteins, which conceals/masks the β -(1,3) glucan underneath (79-81). When the innate immune system detects structural β -(1,3) glucan sugars they elicit a pro-inflammatory response, promoting elimination of the infection (82, 83).

While the innate immune system predominantly plays a role in systemic infections, the adaptive immune response can play a role in mucosal infections, resulting in a pro-inflammatory Th1 response (84). Examples of these infections are oropharyngeal candidiasis as well as over colonization of the vaginal tract, both which can lead to discomfort to those whom that are over-colonized.

Phagocytic effector cells of the innate immune system like neutrophils and macrophages are the first line of defense against systemic fungal infections (such as candidiasis). These effector cells detect fungi by binding β -(1,3) glucan in the fungal cell wall with a C-type lectin signaling protein called Dectin-1 that is found on a number of innate immune effector cells, and induces production of pro-inflammatory cytokines such as TNF α as well as yeast phagocytosis (82, 85, 86). Apart from Dectin-1, there is Dectin-2 which can detect mannan (82, 85). These Dectin receptors are C-type lectin transmembrane receptors (87). Dectin-1 can mediate

signaling by itself with a tyrosine-based action motif (ITAM); in contrast, the Dectin-2 receptor requires an associated adaptor molecule for ITAM-based activation (88). Once binding of their appropriate ligand occurs, these Dectin receptors will be phosphorylated on a tyrosine residue by the spleen tyrosine kinase (syk), a protein tyrosine kinase (89). Once phosphorylated, the Dectin-1 receptor initiates a signaling cascade that promotes phagocytosis, release of reactive oxygen species (ROS) and cytokine release (Fig. 1.3) (89)

Separately from these fungal-specific receptors, toll like receptors (TLRs) 2 (which recognizes phospholipomannan) and 4 (which recognize O-bound mannans) play an important role in the innate immune response. While these receptors are able to bind fungal components, TLR2 is also able to bind and recognize peptidoglycan, a predominant cell wall component of gram + bacteria, and TLR4 is a classical LPS receptor, which makes them versatile signaling receptors in the innate immune system (90, 91). Once activated, these receptors associate with MyD88 to activate the transcription factor NF κ B and results in pro-inflammatory macrophage proliferation (92). In addition, some receptors have synergy with one another (such as TLR2 and Dectin-1) which together promote phagocytosis by macrophages and a TNF α response (87, 93). Lastly, there is the mannose receptor (MR) that recognizes oligosaccharides that terminate in mannose and N-acetyl glucosamine (94, 95).

Apart from effector cells, there are bloodstream components such as defensins and histatins that can destroy *C. albicans* outright based on the surface charge of the microbe (96), which may be mediated by either changes in the cell wall, cell membrane, or both. Complement, another non-cellular component of the immune system, in particular the C3 component, has been implicated in *C. albicans* colonization of mucosa and may play a role in controlling colonization (97). C3b and iC3b in particular can bind to *C. albicans*, and result in opsonization (78). These complement components cannot kill the yeast outright due to their protective thick cell wall structure but opsonization mediates recruitment of inflammatory cells (78). In addition to C3b, C3d is known to directly bind to β -(1,6) glucan, an important structural component of the

cell wall, and binding of these C3 components ultimately results in the recruitment of neutrophils to the site of colonization (98). In conclusion, neutrophils are the major component of the innate immune system that deals with *C. albicans* bloodstream infections, with complement and macrophages playing a minor role in cytokine production and labeling the *C. albicans*' cell wall.

Due to the fact that our *cho1* Δ/Δ mutant has severe cell wall defects, we were curious as to whether this would result in exposure of cell wall components that could be recognized by the immune system (44). In Chapter 2, I will discuss how a loss of phosphatidylserine synthesis by the *cho1* Δ/Δ mutant results in an increased exposure of β -(1,3) glucan and an increased recognition by macrophages.

Appendix. Chapter I Figures

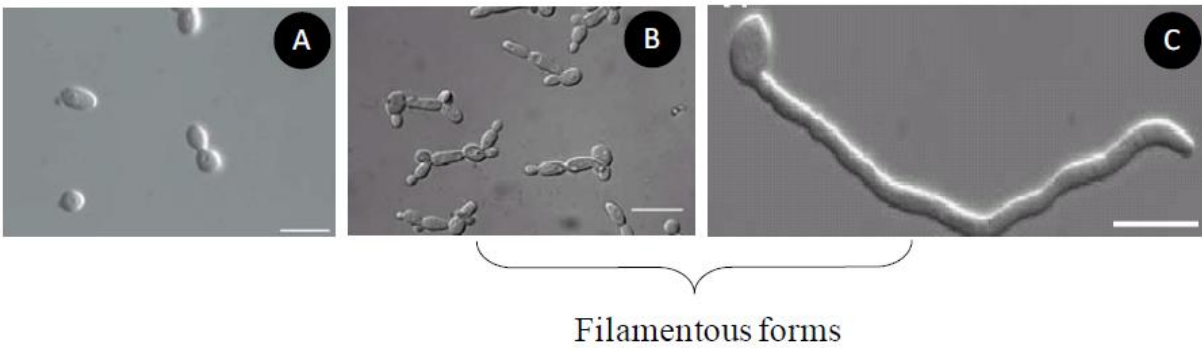


Figure 1.1. Representation of the various morphologies that *C. albicans* can exhibit. **A** yeast form, **B** pseudo-hyphae form and **C** Hyphae. Figure from Joseph Chen's dissertation.

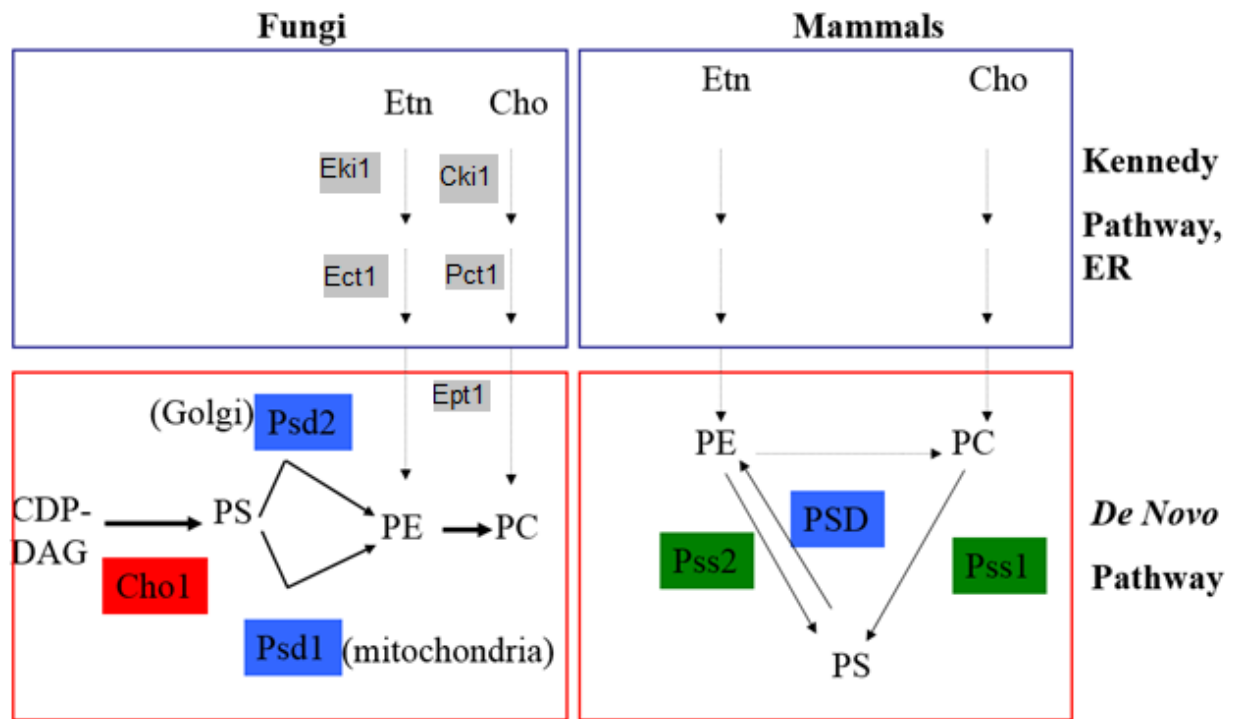


Figure 1.2. There are significant differences between humans and *C. albicans* in the enzyme involved in the *de novo* synthesis of phosphatidylserine (PS). Cho1p does not have a homolog in mammals.

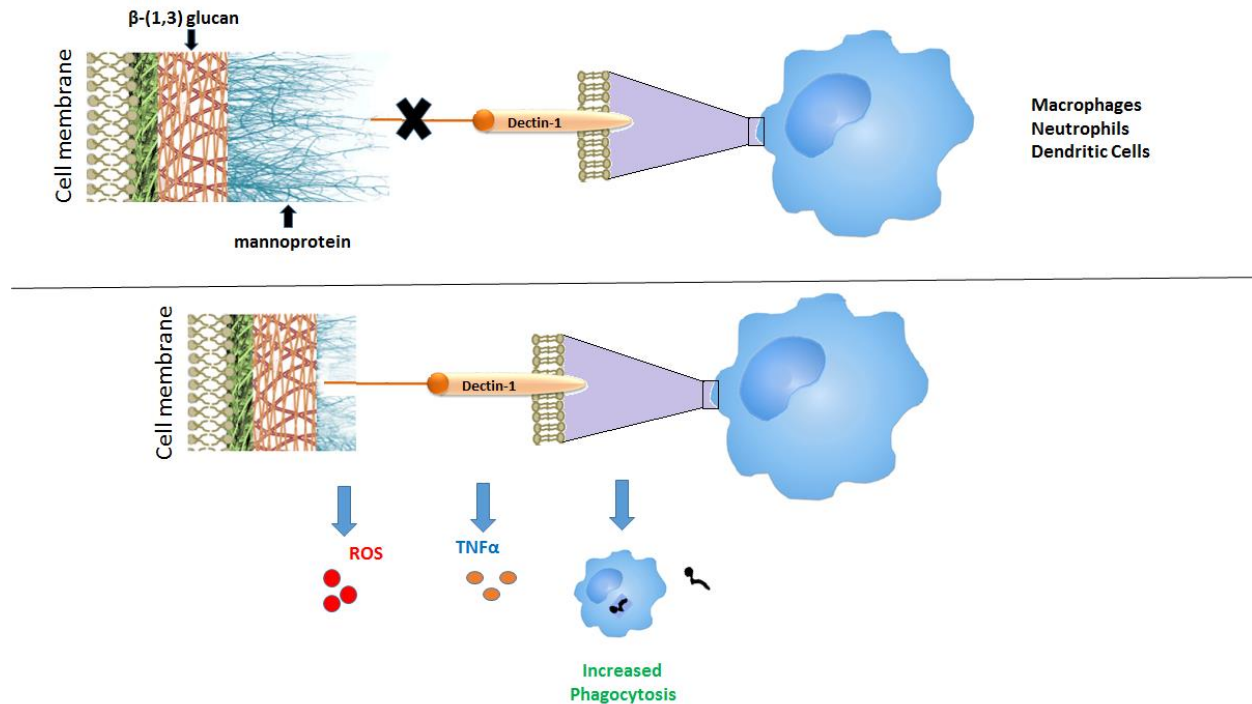


Figure 1.3. The Dectin-1 receptor is expressed on the surface of macrophages, dendritic cells and neutrophils. The Dectin-1 receptor mediates binding to β -(1,3) glucan. Normally, *Candida albicans* has a cell consisting of chitin at the base, then β -(1,3) glucan and β -(1,6) glucan in the center and the surface is coated with mannoproteins. These mannoproteins conceal the glucan underneath, preventing Dectin-1 binding (top part of figure). However with certain cell wall mutants, there is a loss of the mannoprotein coat resulting in exposure of glucan resulting in binding of the Dectin-1 receptor. This binding induces phagocytosis of the bound target, as well as production of the cytokine TNF α as well as reactive oxygen species (ROS) (bottom part of figure). Figure adapted from a previous publication (99).

Chapter II: β -(1,3) glucan is unmasked in a phosphatidylserine synthase mutant

(*cho1* Δ/Δ), which leads to an innate immune response mediated by the Dectin-1 receptor

Abstract

The virulence of *Candida albicans* in a mouse model of invasive candidiasis is dependent on the phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE). Disruption of the PS synthase gene *CHO1* (i.e., *cho1Δ/Δ*) eliminates PS and blocks the *de novo* pathway for PE biosynthesis. In addition, the *cho1Δ/Δ* mutant's ability to cause invasive disease is severely compromised.

The *cho1Δ/Δ* mutant also exhibits cell wall defects, and in this study, it was determined that loss of PS results in decreased masking of cell wall β -(1,3) glucan from the immune system. In wild-type *C. albicans*, the outer mannan layer of the wall masks the inner layer of β -(1,3) glucan from exposure and detection by innate immune effector molecules like the C-type signaling lectin Dectin-1, which is found on macrophages, neutrophils, and dendritic cells. The *cho1Δ/Δ* mutant exhibits increases in exposure of β -(1,3) glucan, which leads to greater binding by Dectin-1 in both yeast and hyphal forms. The unmasking of β -(1,3) glucan also results in increased elicitation of TNF- α from macrophages in a Dectin-1-dependent manner. The role of phospholipids in fungal pathogenesis is an emerging field, and this is the first study showing that loss of PS in *C. albicans* results in decreased masking of β -(1,3) glucan, which may contribute to our understanding of fungus-host interactions.

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Alex Hopke and Dr. Robert Wheeler performed the flow cytometry, as well as immunofluorescence of sDectin-1 staining in Figure 2.3 and generation of the Fc-Dectin-1 receptor used in these experiments. Dr. Anthony Montedonico assisted in mouse experiments Mentioned in Figure 2.2 B & C. Dr. Steven Minkin assisted in Figure 2.2 A, where he operated the equipment and performed data analysis on hyphal length. The rest of the experiments were performed by Dr. Sarah Davis. Most of these data are also reported in publication “Masking of β -(1,3) glucan in the cell wall of *Candida albicans* from detection by innate immune cells depends on phosphatidylserine” listed in references.

Introduction

Both the innate and adaptive immunological responses of the host play a role in controlling and eliminating fungal infections, but the innate response is the early responder and is crucial for controlling bloodstream infections (100-102). The innate response is mediated largely by phagocytic cells such as macrophages and neutrophils, which recognize, phagocytose, and kill the pathogen (103). Recognition is dependent, in part, on pathogen-associated molecular patterns (PAMPs) that are present on the surface of the pathogen, which can then be recognized by phagocytic pattern recognition receptors (PRRs) (104-107). Fungal cell walls contain the PAMP β -(1,3) glucan . The fungal wall is composed of two relatively indistinct layers; mannosylated proteins (mannan) are enriched on the outer surface layer, and

β -(1,3) glucan and chitin are enriched in the layer beneath (Fig. 2.2) (108, 109). β -(1,3) glucan is a known PAMP that is recognized by Dectin-1, a C-type lectin PRR that is expressed by macrophages, neutrophils, and dendritic cells (104). Several cell wall mutants with increased exposure of β -(1,3) glucan also exhibit increased recognition by phagocytic cells, resulting in increased phagocytosis by macrophages and production of cytokines, inducing a strong response by the innate immune system that may play a role in the faster elimination of these mutants (110, 111). In this communication, we report that the loss of PS in the *C. albicans* *cho1 Δ Δ* mutant results in changes in cell wall structure, notably, an increase in β -(1,3) glucan exposure on the cell surface. The full mechanism of how phospholipids affect the cell wall is not known, but it may relate to the manner in which phospholipids like PS modulate signaling pathways like calcineurin, protein kinase C, Hog1p, or Cek1p, which regulate the cell wall (112, 113). Increased β -(1,3) glucan exposure induces increased Dectin-1 binding, mediating macrophage release of the cytokine tumor necrosis factor alpha (TNF α). The TNF α response is important in protecting against systemic candidiasis, and when the TNF α response is ablated by either knocking out or neutralizing the TNF α response with an anti-TNF α antibody, mice are more susceptible to systemic candidemia (114, 115). Interestingly, we find that the *psd1 Δ Δ* *psd2 Δ Δ* double mutant does not exhibit these phenotypes as strongly, suggesting that PS is specifically required for β -(1,3) glucan “masking” from immune recognition.

Results

Previous work has shown that the *cho1 Δ Δ* and *psd1 Δ Δ* *psd2 Δ Δ* mutants show poor hyphal formation on plates in response to a neutral pH or Spider medium, a medium that is poor in nutrients and that promote hyphal formation. However, the *cho1 Δ Δ* and *psd1 Δ Δ* *psd2 Δ Δ* mutants could both form hyphae in response to mouse serum (44). These previous data were only qualitative, and it was not clear whether the mutants grew as hyphae at a rate similar to that of the wild type in response to serum. Consequently, the hyphal extension rates of the

cho1 Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants were compared to those of the wild type and *psd1* Δ/Δ mutants in human serum by time-lapse microscopy. Through this assay, it was found that all of the strains except the *cho1* Δ/Δ mutant exhibited similar hyphal extension rates, with the *cho1* Δ/Δ mutant exhibiting a slightly shorter hyphal growth; however the difference was not statistically significant (Fig. 2.2 A, Videos A1-4).

In order to determine if these mutants could also form hyphae in response to more *in vivo* stimuli, such as host tissue, the strains were compared for growth after they infected mice and were localized to the kidneys. Each strain was transformed with a construct that would express cytoplasmic GFP from the constitutive *ENO1* promoter (116). Then yeast-form cells of each strain were stained on the exterior with Alexa Fluor 555 dye and administered to mice via tail vein injection. Kidney homogenates were analyzed by microscopy 6 h after infection to determine if hyphal growth occurred in the kidney. The Alexa Fluor stain allows the identification of injected yeast-form cells, and GFP-expressing hyphae that lack Alexa Fluor staining indicate new growth after infection of the kidney. Analysis of kidney homogenates revealed that all four strains were able to survive and grow similarly within the mouse (Figs. 2.2 B & C). Thus, when grown under host-simulating conditions, the mutants appear to form hyphae at a rate similar to that of the wild type, at least for several hours.

Loss of PS results in increased exposure of β -(1,3) glucan on the *C. albicans* yeast-form cell surface. In a previous report, the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants' cell wall defects were studied in the context of yeast-form cells grown at 30°C in rich medium (YPD). In these studies, the *cho1* Δ/Δ mutant exhibited stronger cell wall defects than the *psd1* Δ/Δ *psd2* Δ/Δ mutant, including a much greater increase in chitin levels than in wild-type cells and a thicker, more irregular cell wall, as shown by transmission electron microscopy (44). It was hypothesized that these cell wall defects would correlate with phenotypes that impact virulence, such as increased exposure of β -(1,3) glucan, which affects host-pathogen interactions. We examined the effects of these mutations on β -(1,3) glucan exposure by immunofluorescence

with an anti- β -(1,3) glucan antibody (110). β -(1,3) glucan is noticeably more exposed in the log and stationary phases of *cho1 Δ/Δ* mutant cells than in wild-type, *cho1 Δ/Δ ::CHO1*, *psd1 Δ/Δ* , or even *psd1 Δ/Δ psd2 Δ/Δ* mutant cells (Fig. 2.3 A & B). β -(1,3) glucan on the surface of *C. albicans* is bound and detected by Dectin-1, a C-type lectin that is expressed on innate immune effector cells such as macrophages, neutrophils, and dendritic cells (110, 117). This binding and recognition aids macrophages and neutrophils in the phagocytosis of yeast and results in the secretion of cytokines like TNF α (110, 118). Fluorescent staining with sDectin-1–Fc reveals that *cho1 Δ/Δ* mutant yeast-form cells exhibit greater binding by Dectin-1 than the other strains (Fig. 2.4 A). Flow cytometry to quantify the binding of sDectin-1–Fc to the surface of yeast-form *C. albicans* supports the microscopy results and indicates that the *cho1 Δ/Δ* mutant has significantly greater binding by Dectin-1 than the other strains and that the *psd1 Δ/Δ psd2 Δ/Δ* double mutant (solid pink line) does not differ from the wild type (black) (Fig 2.4 B). The *cho1 Δ/Δ* cells exhibit an average increase in binding by Dectin-1 over wild type of approximately 58% (Fig 2.4 C). These results support those showing increased anti- β -(1,3) glucan staining exposure on the *cho1 Δ/Δ* mutant cell surface (Fig. 2.4). β -(1,3) glucan is bound by Dectin-1, but there are data that suggest that anti- β -(1,3) glucan antibody and sDectin-1–Fc may bind to somewhat distinct epitopes (110). If so, then it should be possible to see co-localization of staining with both probes. To assess this, yeast-form cells were double stained with an anti β -(1,3) glucan antibody and sDectin-1–Fc and secondary antibodies with differential fluorophores. It is clear that the signals co-localized and are very prominent for the *cho1 Δ/Δ* mutant (see Fig. A1). Phospholipid mutants generate hyphae when growing in human serum. Since the *cho1 Δ/Δ* but not the *psd1 Δ/Δ psd2 Δ/Δ* mutant cells exhibit increased exposure of β -(1,3) glucan in the yeast form, the effects of these mutations on the exposure of β -(1,3) glucan in hyphae needed to be addressed to determine if there were any cell wall differences that are dependent on morphology.

Loss of PS increases exposure of β -(1,3) glucan in hyphae

The *cho1 Δ/Δ* mutant exhibits a greater exposure of β -(1,3) glucan than the wild-type, *psd1 Δ/Δ psd2 Δ/Δ* double mutant, and other strains in yeast form (Figs. 2.3 A & B, Fig. A1). In order to determine if this held true for hyphae as well, cells were grown in human serum at 37°C to induce hyphal growth and then stained for β -(1,3) glucan exposure with an anti- β -(1,3) glucan antibody. When staining the *C. albicans*' hyphae with sDectin-1–Fc or an anti- β -(1,3) glucan antibody was performed, only the *cho1 Δ/Δ* mutant exhibited strong staining with either probe, and the staining, like that in yeast-form *cho1 Δ/Δ* mutant cells, was found in overlapping patterns (Fig 2.5 A & B, Fig. A2). In addition, a number of the hyphae of the *cho1 Δ/Δ* and *psd1 Δ/Δ psd2 Δ/Δ* mutants were also malformed compared to those of the wild-type and *psd1 Δ/Δ* strains. They were enlarged at the tips in comparison and in many cases were somewhat curved near the tip (Fig. 2.5 A, enlargement indicated by arrows). Approximately 48% of the *cho1 Δ/Δ* mutant hyphae and 52% of the *psd1 Δ/Δ psd2 Δ/Δ* mutant hyphae showed this phenotype, whereas none of the other strains exhibited this phenotype (Table 3). The frequency of co-localization of anti- β -(1,3) glucan antibody and Dectin-1 staining was quantified for all of the strains, revealing that they bind to unfixed hyphal cells similarly for the most part (Fig. A3). The *psd1 Δ/Δ* and *psd1 Δ/Δ psd2 Δ/Δ* mutants did have stronger staining than the wild type, but it was generally much weaker than that of the *cho1 Δ/Δ* mutant strain. Interestingly, for many of the mutants staining with sDectin-1–Fc tended to be stronger for the mother yeast cells when they were grown in serum (Fig. A2).

Phospholipid mutants are not more susceptible to killing by innate immune responses.

As our *cho1 Δ/Δ* mutant has increased β -(1,3) glucan exposure and increased binding by sDectin-1–Fc, it was hypothesized that increased binding of Dectin-1 would result in increased susceptibility to killing by macrophages. However, when *in vitro* killing assays were performed with macrophages (RAW264.7 or Raw BLUE), it was found that there was no difference in killing for any of the strains (Fig. 2.6 A). Neutrophils are typically more efficient at killing *C.*

albicans than macrophages, so the susceptibility of the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants to neutrophils was also tested. However, the mutants were not more readily killed by neutrophils either (Fig. 2.6 B). In addition to cellular innate immune responses, it is also possible that the mutants might be more susceptible to non-cellular innate immune response elements like cationic peptides, as membranes of our *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants are defective (44). We tested the susceptibility of our mutants to the human cationic peptide β -defensin, which is known to act against the negatively charged membranes of invading pathogens and can play a role in mucosal defense against *C. albicans* (96, 119-121). Surprisingly, the *cho1Δ/Δ* mutant appeared to be more resistant than the wild type to β -defensin at a concentration of 500 nM, but these values were not statistically significant. The *psd1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants showed no differences (Fig. 2.6 C) from the wild type. In addition, the mutants showed no difference in binding by complement C (data not shown).

Loss of PS in *C. albicans* causes increased elicitation of cytokines from macrophages.

Increased exposure of β -(1,3) glucan in *C. albicans* cell wall mutants has been shown to cause an increase in TNF α release from macrophages (110). The differences in β -(1,3) glucan exposure reported in the *cho1Δ/Δ* mutant was hypothesized to cause an increase in TNF α production. A TNF α -specific ELISA revealed that the *cho1Δ/Δ* mutant elicits greater TNF α production from RAW-BLUE macrophages than the wild type strain (Fig. 2.7 A). This is not the case for the reintegrand *psd1Δ/Δ*, or *psd1Δ/Δ psd2Δ/Δ* mutant strain. The Dectin-1 receptor expressed on immune effector cells binds to β -(1,3) glucan on the *C. albicans* cell surface, and this has been reported to trigger TNF α release (117). Thus, Dectin-1 was presumed to be responsible for the increase in TNF α release. In order to rule out other non-Dectin-1 receptors as mediators of increased TNF α production by macrophages in response to the *cho1Δ/Δ* mutant, an anti-Dectin-1 antibody was administered to the macrophages prior to their exposure to *cho1Δ/Δ* mutant cells in order to block Dectin-1 activation during co-incubation with yeast cells. The results in Fig. 2.7 A show that blocking of Dectin-1 reduces TNF α production to nearly

wild-type levels, revealing that Dectin-1 is responsible for cytokine release. When the RAW 264.7 macrophage cell line was used, a similar elicitation of TNF α in a Dectin-1-dependent manner was observed as well (see Fig. A4). In addition to these data, it was determined that there was an statistically significant increase in phagocytosis of the *cho1 Δ/Δ* mutant by RAW-BLUE macrophages after an 8 hour incubation, which is a typical reaction of macrophages to yeast in the bloodstream (Fig. 2.7 B) (122).

Discussion

Loss of the PS synthase gene *CHO1* results in changes in immune recognition, most notably, increased exposure of β -(1,3) glucan in the cell wall. The increased exposure of β (1-3)-glucan can be seen from the increased binding of anti- β -(1,3) glucan antibodies (Fig. 2.3 A & B). On the basis of current models, this may be due to glycosylated cell surface proteins on the *cho1 Δ/Δ* mutant cell wall not adequately concealing the layer of β -(1,3) glucan underneath, causing “chinks” in the masking of this important yeast cell wall PAMP. These differences in β -(1,3) glucan exposure in the *cho1 Δ/Δ* mutant are also supported by Dectin-1 receptor binding to both yeast-form (Fig. 2.4 A) and hyphal cells (Fig. 2.5 B) and elicitation of TNF α (Fig. 2.7 A, Fig. A4). These data reveal that the immune response to the *cho1 Δ/Δ* mutant is stronger than that to all of the other strains tested. The more severe defects in immune evasion in the *cho1 Δ/Δ* mutant (lacks PS and has deficient PE) than the *psd1 Δ/Δ* *psd2 Δ/Δ* double mutant, which has deficient PE but abundant PS, reflect the stronger cell wall deficiencies of the *cho1 Δ/Δ* mutant (44). The loss of PS for the *cho1 Δ/Δ* mutant may also affect the membrane charge of the cell, which would reduce the negative surface charge that defensins are known to interact with. This may explain why the *cho1 Δ/Δ* mutant appears to be more resistant to human β -defensin 2, as it has a different surface charge than any of the other strains we tested (Fig. 2.6 C).

The correlation between increased β -(1,3) glucan exposure and increased TNF α production in the *cho1 Δ/Δ* mutant is notable, as Wheeler and Fink (110) found some mutants of *Saccharomyces cerevisiae* that exhibit increased binding by anti- β -(1,3) glucan antibody but

did not necessarily bind better to Dectin-1 or hyper-elicite TNF α . Thus, while increased staining by anti- β -(1,3) glucan antibody does not always correlate with increased immune recognition, the increase that occurs in the *cho1 Δ/Δ* mutant does yield a more productive immune response as well as increased phagocytosis (Fig. 2.7 A & B, Fig. A4). These data suggest that the loss of PS alone is responsible for unmasking in *C. albicans*, and this conclusion is drawn from the following observations. (i) Loss of *CHO1* results in a complete loss of PS and a 40% drop in PE levels, since this population of PE molecules is derived from PS by decarboxylation via Psd1p or Psd2p (44). (ii) Loss of *PSD1* and *PSD2* simultaneously (*psd1 Δ/Δ psd2 Δ/Δ*) causes a 40% drop in PE similar to that observed in the *cho1 Δ/Δ* mutant, but there is an increase in PS, since it is not converted to PE by decarboxylation. (iii) Although the *cho1 Δ/Δ* and *psd1 Δ/Δ psd2 Δ/Δ* mutants have similar drops in PE, only the *cho1 Δ/Δ* mutant, with its lack of PS, shows a strong increase in β -(1,3) glucan exposure by staining with anti- β (1-3)-glucan antibody or sDectin-1–Fc (Fig. 2.3 A & B, Fig. 2.4 A, Fig. 2.5 A & B, Figs. A1 & A2) and increased elicitation of TNF α from macrophages (Fig. 2.7 A, Fig. A4). Thus, it appears that the loss of PS is responsible for the majority of the increased exposure of β -(1,3) glucan . There is a subtle contrast between the hyphal form and the yeast form in regard to β -(1,3) glucan exposure. In particular, although *cho1 Δ/Δ* mutant hyphae have the strongest exposure of β -(1,3) glucan , the *psd1 Δ/Δ* and *psd1 Δ/Δ psd2 Δ/Δ* mutants also exhibit greater exposure of β -(1,3) glucan and binding of sDectin-1–Fc than the wild type, but exposure is lower than that of the *cho1 Δ/Δ* mutant (Fig. 2.5 A & B).

Thus, for hyphae, the defect in masking is affected primarily by PS but is impacted by PE as well. We did not test to see if this difference in the hyphal form leads to greater TNF α release in macrophages because of technical difficulties when working with hyphae in this assay, as they were susceptible to aggregations and hence difficult to distribute evenly to macrophages for stimulation to elicit TNF α . Alternatively, these data may indicate that β -(1,3) glucan is more readily unmasked in the *psd1 Δ/Δ psd2 Δ/Δ* mutant along with the *cho1 Δ/Δ*

mutant, than in the *psd1Δ/Δ* mutant. This could be of interest, as the unmasking that occurs under these conditions correlates with these strains' defects in virulence.

The precise defect that leads to increased exposure of β -(1,3) glucan in yeast-form cells of the PS synthase mutant alone is unclear. This increase does not appear to resemble that of the *kre5Δ/Δ* mutant, which has increased exposure all over the cell surface (110). Instead, the increased exposure we observe is localized to foci at the periphery of yeast-form cells providing a punctate appearance to the exposure (Fig. 2.3 A & B, Fig. A1). These increases also do not correlate with bud scars (data not shown). Transmission electron microscopy images of *cho1Δ/Δ* mutant cells reveal irregularities in the membrane and cell wall that look like invaginations (44). The increased exposure of β -(1,3) glucan in the cell wall of the *cho1Δ/Δ* mutant may correspond to these irregularities, although that remains to be determined. The mechanism by which PS controls β (1-3)-glucan masking could be related to effects of PS on cell wall signal transduction pathways, general endocytic trafficking, or alterations of the plasma membrane (123, 124). A number of cell wall signaling pathways are directly affected by PS, including the protein kinase C (Pkc1p) and calcineurin pathways (125-129). In *S. cerevisiae*, PS is a cofactor for ScPkc1p activity *in vitro*. In addition, calcineurin is recruited to membranes by PS *in vitro*. Therefore, these pathways could potentially affect the cell wall because of PS loss. The role that β -(1,3) glucan exposure in the *cho1Δ/Δ* mutant plays in compromising virulence in the host is not clear. The yeast form of the *cho1Δ/Δ* mutant elicits a greater release of cytokines from macrophages than the *psd1Δ/Δ psd2Δ/Δ* mutant and has more exposure of β -(1,3) glucan as yeast cells or hyphae, suggesting a stronger immune response. However, these mutants are pleiotropic, so there are likely other mechanisms at work in addition to unmasking to affect virulence. For example, both mutants are ethanolamine auxotrophs (44). Even in YPD, which contains ethanolamine, the *cho1Δ/Δ* mutant has a 2.5-fold lower growth rate than the wild type, which suggests that the growth rate could play a role in the *cho1Δ/Δ* mutant's ability to be pathogenic; however, it can clearly grow for several hours in serum or in the host (Fig. 2.2 A, B

& C), but this may not translate into growth over several days, which is required to overwhelm the host during sepsis. Thus, the mutants may not be getting enough ethanolamine to make sufficient PE, which is essential (44), as it is in other yeasts like *S. cerevisiae* and *Schizosaccharomyces pombe* (130). Alternatively, auxotrophy may play a role, but the increased exposure of β -(1,3) glucan may result in more rapid clearance of the mutant. PS clearly affects the cell wall and masking independently of PE, but PE appears to at least subtly affect the cell wall in hyphae. These defects compromise an important defensive virulence factor of fungi, “masking” of the cell wall PAMP β -(1,3) glucan. This report sets the stage for a better understanding of how PS and PE regulate pathways that ultimately affect virulence traits and cell wall physiology in this pathogen.

Materials and Methods

Purification of sDectin-1–Fc.

HEK293T cells were transfected with the soluble Dectin-1–Fc (sDectin-1–Fc) construct previously described (131). After selection for stable transfection with Zeocin, the cells were seeded into a CELLline AD1000 bioreactor (Wheaton) and cell supernatants were harvested twice weekly. Supernatants were purified over Hi-Trap protein A columns (GE Healthcare) and dialyzed overnight with phosphate-buffered saline (PBS) in Slide-A-Lyzer Dialysis cassettes (Thermo Scientific). Dialyzed supernatants were concentrated with Amicon Ultra centrifugal filters (Millipore). Elution from the column directly into 1 M Tris (pH 9) neutralization buffer was performed under low-pH conditions with 100mM glycine (pH 2.8). The concentration of sDectin- –Fc was determined with the Bradford assay.

sDectin-1–Fc staining and flow cytometry.

C. albicans strains were streaked onto YPD agar plates and left at 37°C overnight. A single colony per strain was picked and transferred into 5 ml YPD liquid, which was put into a rotator wheel and left overnight at 37°C. A sample of culture was centrifuged and washed three times with PBS. Samples were blocked in PBS plus 2% bovine serum albumin for 1 hour at

room temperature. After blocking, samples were stained with sDectin-1–Fc at 16.5 µg/ml for 1.5 h on ice. Samples were washed with PBS five times and then stained with donkey anti-human IgG DyLight 488 antibody (Jackson ImmunoResearch) at 0.83 µg/ml for 20 min on ice. Samples were washed five times with PBS and then resuspended in 500 µl PBS for flow cytometry. Flow cytometry data were obtained for 10,000 gated events per strain, and statistics were calculated with the paired Student *t* test. For imaging, *Candida* cells were prepared as outlined above but were resuspended in 50 µl PBS and visualized with a Zeiss AxioVision Vivotome microscope (Carl Zeiss Microscopy, LLC). This experiment was repeated twice.

In other immunofluorescence experiments with sDectin-1–Fc staining, donkey anti-human IgG-Cy3 or rabbit-anti-human IgG-Cy2 (both from Jackson ImmunoResearch) was used as the secondary antibody.

Immunofluorescence of β -(1,3) glucan and complement.

Strains were grown overnight in YPD at 30°C. For yeast-form staining, 0.1 optical density (O.D) unit of each strain was washed three times with PBS. Blocking and primary and secondary antibody incubations occurred on ice in PBS plus 3% bovine serum albumin. Blocking was for 1 hour, primary-antibody incubation was for 1.5 hour, and secondary-antibody incubation was for 20 minutes with five washes between antibodies. An anti- β -(1,3) glucan antibody (Biosupplies Australia) at a 1:800 dilution was used as the primary antibody, and a goat anti-mouse antibody conjugated to Cy3 (Jackson ImmunoResearch) at 1:600 was used as the secondary antibody. For hyphal form staining (see Fig. 2.5, Fig. A2), overnight cultures grown at 30°C were washed three times with PBS, added to 20% human serum in RPMI in Lab-Tek glass chamber slides (Thermo Scientific), and incubated at 37°C for 3 hours to ensure adequate filamentation. Antibody staining was performed in the same way as yeast-form staining. A formaldehyde fixation step with 37% formaldehyde in PBS was used for yeast and hyphae in some cases before staining began, and this is noted in the figure legends if that was the case.

Complement staining with the primary antibody against human C1q-c complement (Santa Cruz catalog no. sc-365301) (132) and a Cy3- conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) was performed with overnight cultures grown at 30°C that were washed three times with PBS. The yeast cells were then incubated with 50% human serum for 30 minutes and then stained according to the yeast-form immunofluorescence protocol.

For all immunofluorescence studies, experiments were repeated in triplicate. In each experiment, $n=100$ for yeast cells and 20 for hyphae.

Quantification of immunofluorescence signal of hyphae for β -(1,3) glucan and Dectin-1 immunofluorescence.

The fluorescence intensities of labeled hyphae were assessed with NIS-Elements AR 3.2 image analysis software (Nikon Instruments Inc., Melville, NY). Hyphae were viewed by using a rainbow display scale of pixel intensity and assigned a score based on fluorescence intensity (red=high [3], yellow=medium [2], green=low [1]).

Strains and growth media

All of the strains used for these experiments are described in Table 1 or derived from these strains. Strains were cultured in YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose) or on YPD agar plates (133). The RAW264.7 mouse-derived macrophage cell line (134) and human neutrophils were both cultured in RPMI plus 10% fetal bovine serum and 1X penicillin-streptomycin (Gibco). The RAW-BLUE cells that expressed elevated levels of Dectin-1 were cultured in the same medium as RAW264.7 macrophages, only with the addition of 200 $\mu\text{g/ml}$ Zeocin (Research Products International Corporation). For the generation of strains that express cytoplasmic green fluorescent protein (GFP) constitutively under the control of the promoter of the gene for enolase (*ENO1*), we used the plasmid pENO1-yEGFP3-NAT (116) and transformation methods that have been previously described (135). To generate the *cho1 Δ : Δ ::CHO1* reintegrant strain that expresses GFP, the

SAT1 marker was flipped out of the CHO1 reintegration construct (pCHO1-SAT1-flipper) (strain SED022) and then transformed with the above-described GFP plasmid using nourseothricin selection at a concentration of 200 µg/ml (Werner BioAgents).

Killing assays

Killing assays were performed with macrophages and neutrophils. Neutrophil isolation was done by a method adapted from a previously published protocol (136). Neutrophils were obtained from whole blood from a human donor. The blood was diluted with an equal volume of PBS, Lymphocyte Separation medium (BioWhittaker) was underlaid, and the cells were centrifuged to pellet neutrophils and erythrocytes. The pellet was subjected to 3% dextran sedimentation to isolate neutrophils and further treated with 0.2% sodium chloride to lyse any residual erythrocytes. Yeast-form cells (from an overnight culture grown in YPD) and phagocytes were co-incubated at a 1:40 ratio for 2 hours at 37°C and 5%CO₂, and afterward, yeast cells were plated on YPD plates for total colony form units (CFU) counting. The experiment was performed three separate times, and for each strain in an individual experiment, n=5, for a total of n=15 for each strain.

ELISA of TNFα

RAW264.7 macrophages were plated the day prior at 5*10⁵/well in a 24-well plate. Macrophages and UV-killed yeast-form *Candida* cells were co-incubated at a 1:10 ratio for 8 h at 37°C and 5%CO₂. The enzyme-linked immune-sorbent assay (ELISA) kit instructions of the manufacturer (R&D Systems) were followed. To determine if the release of TNFα was due to Dectin-1 binding, a commercial anti-Dectin-1 neutralizing antibody (InvivoGen MAb-mDect) was administered to the macrophages for 1 h prior to co-incubation at a concentration of 100 ng/ml. Three individual experiments were performed. For each of these experiments, three individual wells were stimulated for 8 hours with the RAW 264.7 macrophage cell line or for 4 hours when RAW-BLUE macrophages were used (For RAW-BLUE the setup for the experiment was the same). From each well, the sample was taken and measured in triplicate, for a total of nine data

points per group. Statistical analysis of the data was performed by the unpaired-sample *t* test with two-tailed *P* values. The RAW 264.7 macrophages were a gift from Dr. Chunlei Su at the University of Tennessee, and the RAW-BLUE were a gift from Dr. Robert Wheeler at the University of Maine.

β-Defensin Assay

By using a protocol adapted from reference (137), strains were grown overnight in YPD at 30°C, washed with 10 mM sodium phosphate buffer (NaPB), and counted with a hemacytometer and 10⁵ cells were incubated with various concentrations of human β-defensin 3 (AnaSpec catalog no. 60741) for 1 hour in 10mMNaPB in a shaking incubator at 37°C. After incubation, cells were washed with 10 mM NaPB, diluted 1:100, and plated for CFU counting. The assay was performed three separate times. For each strain in each assay, *n* = 4. Statistical analysis of the data was performed by the unpaired-sample *t* test with two-tailed p-values.

Time-lapse microscopy of hyphal formation

A 2% agarose gel was overlaid on the surface of a Delta T dish (Bioprotechs, Inc.) with wells formed in the gel to contain medium and yeast. Strains were grown overnight in YPD at 30°C and washed with PBS, and cells were counted with a hemacytometer. RPMI plus 25% human serum was added to the wells, and 5 *10³ yeast cells were added to each well. The Delta T dish was placed on a heated stage set to 37°C and imaged automatically to determine hyphal length over a 3 hour period. Hyphal length was determined by software analysis (NIS-Elements version 3.1; Nikon Instruments). Quantification was performed by monitoring 20 cells of each strain.

In vivo hyphal formation

GFP-expressing strains were washed with PBS and stained with Alexa Fluor 555 (Invitrogen) in 100 mM NaCO₃ buffer (pH 10) for 30 minutes at room temperature. After cells were washed three times in PBS, 10⁷ cells were injected per mouse via the tail vein. After 6 hours, the mice were sacrificed and their kidneys were removed and immediately homogenized

with a Dounce tissue homogenizer and visualized by microscopy. This experiment was performed in triplicate with two or three mice per genotype, so there were eight or nine mice per strain. For each experiment, both kidneys of each mouse were homogenized together and visualized to look for yeast cells. A minimum of 3 cells from each pair of kidneys were imaged, for a minimum of 24 to 27 cells of each strain.

Appendix. Chapter II Figures

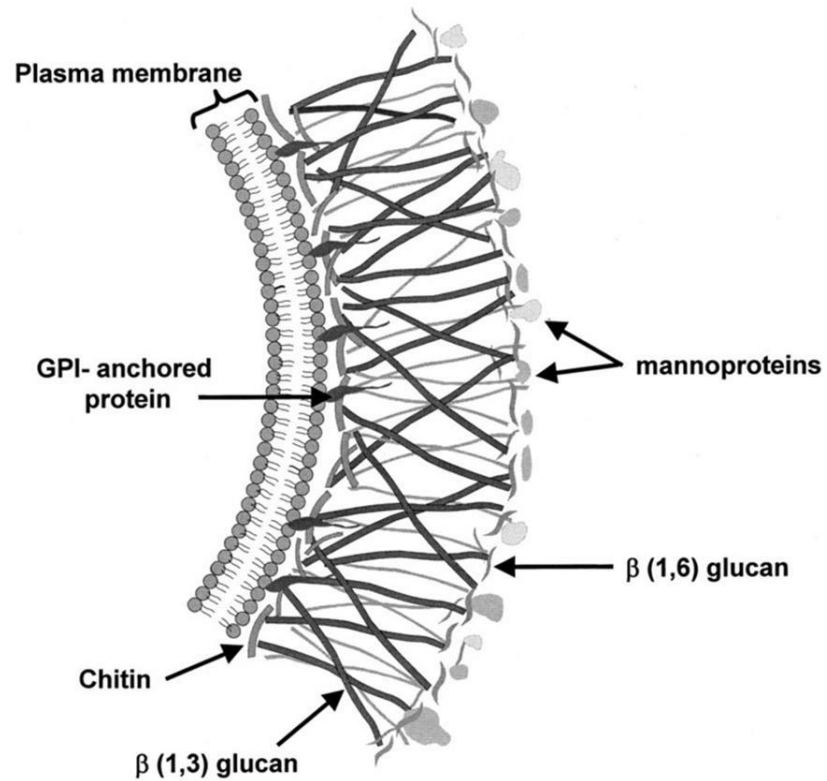


Figure 2.1. The structure of the fungal cell wall with the different polysaccharide and protein components depicted. (Image adopted from science.kennesaw.edu/fungalcellwall) (adapted by Anthony Montedonico, in dissertation at UTK).

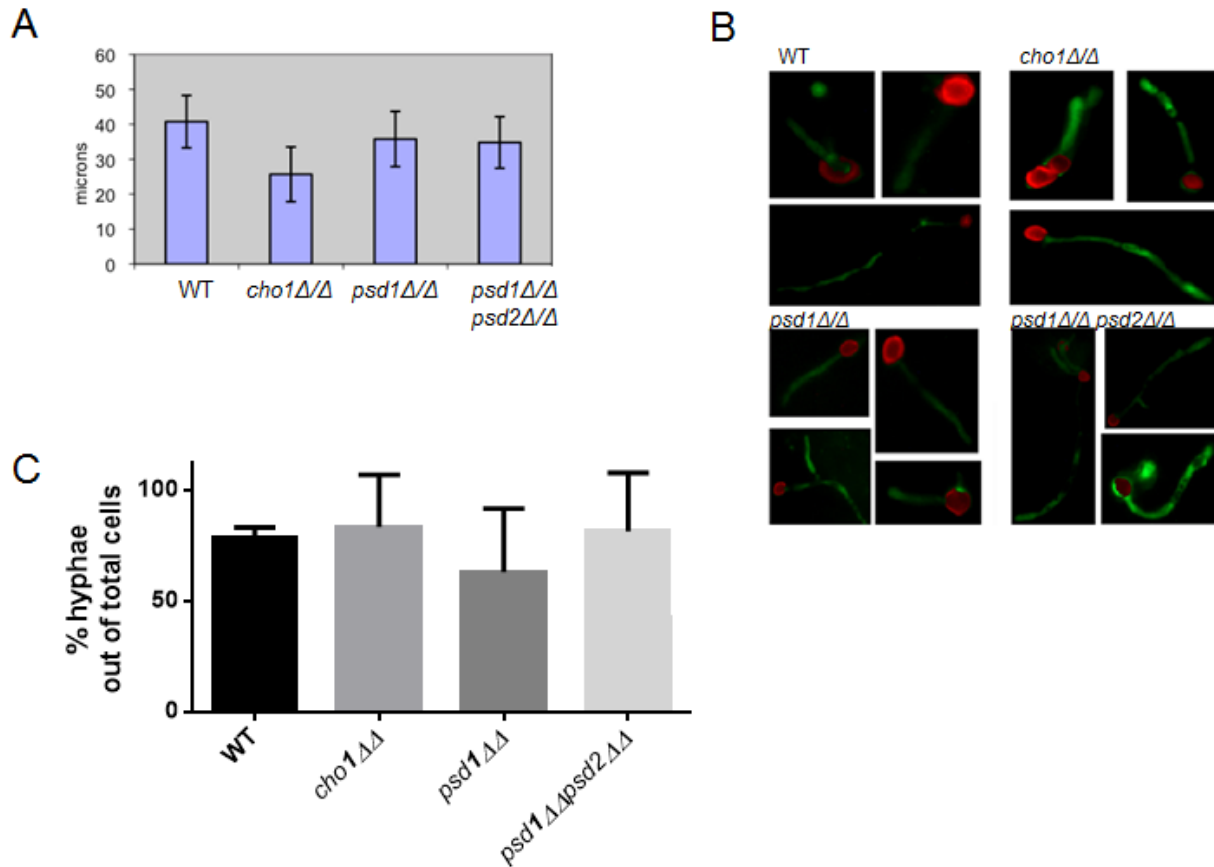


Figure 2.2. Hyphal formation of the *cho1Δ/Δ* mutant is not significantly impaired in serum or host tissue. **A** *C. albicans* strains were incubated in 100% human serum and were observed by time lapse microscopy on thermally regulated glass slides. After 3 hours the length of the hyphae was measured as an indicator of growth over time. **B** *C. albicans* strains expressing GFP were stained with Alexa Fluor dye during yeast form growth. Stained yeast cells were then injected into mice via the tail-vein and after 6 hours mice were sacrificed, kidneys were removed, and then homogenized and viewed by microscopy. Hyphae expressing GFP indicate new growth that occurred within the mouse tissue. **C** Percentage of yeast inoculated *in vivo* that generated hyphae in the kidneys.

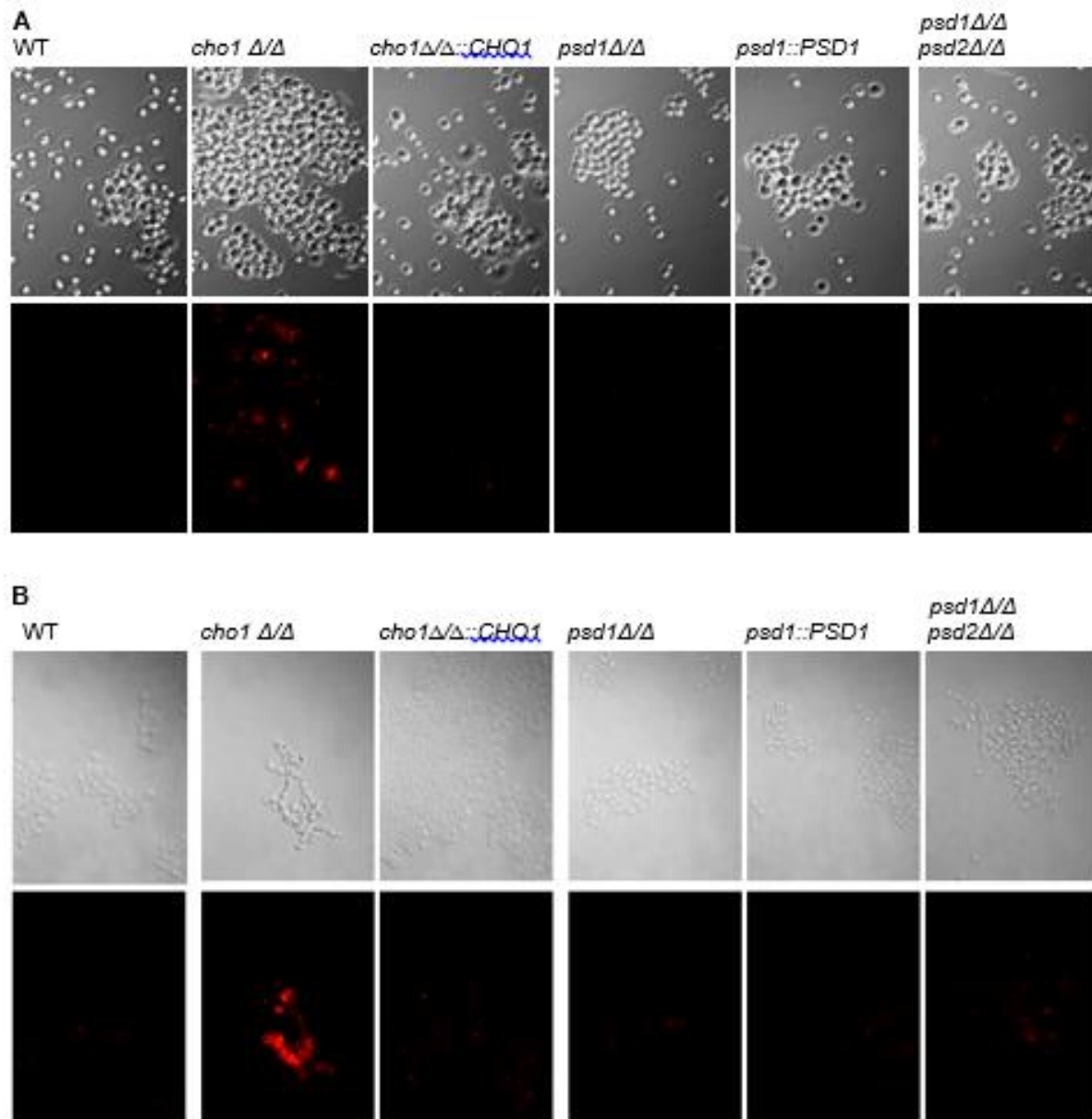


Figure 2.3. A The stationary-phase and log-phase yeast form of the *cho1* Δ/Δ mutant exhibits increased exposure of β -(1,3) glucan . **A** Immunofluorescence using an anti- β 1,3-glucan primary antibody reveals that the *cho1* Δ/Δ mutant has increased β -(1,3) glucan exposure in comparison to the other strains in both stationary phase (**A**) and log-phase (**B**).

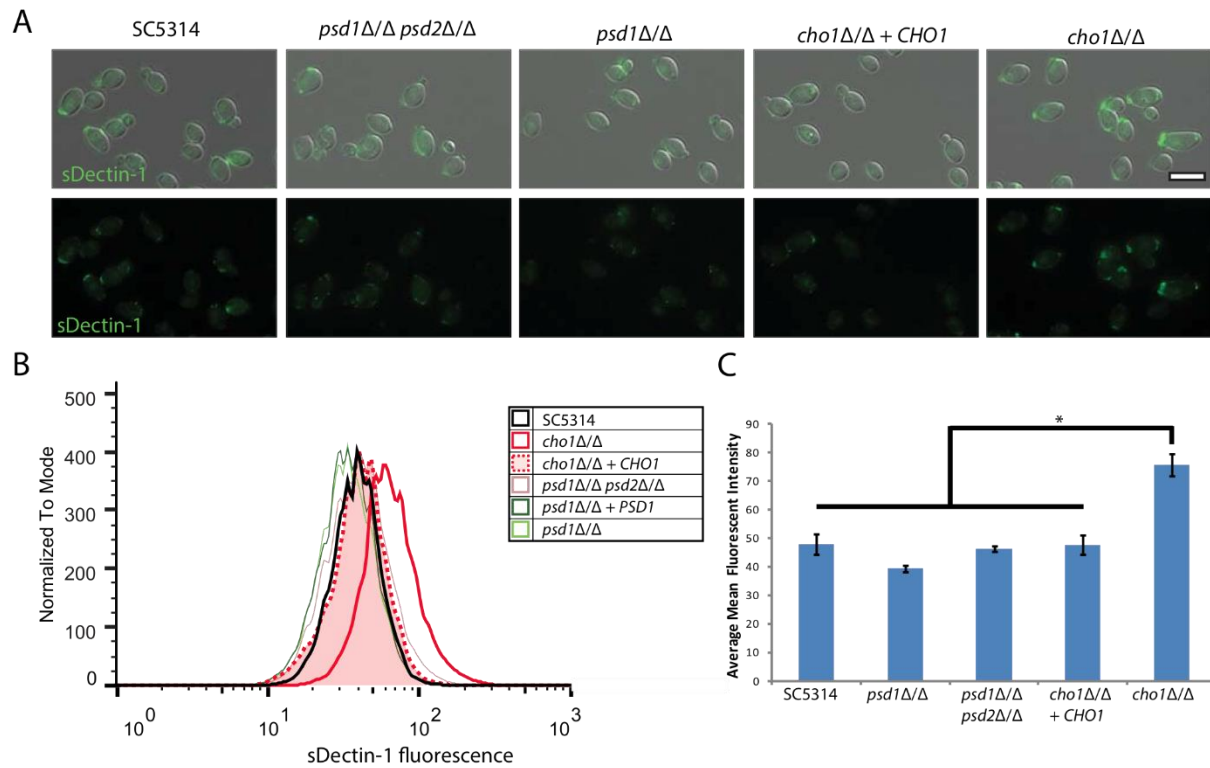


Figure 2.4. The *cho1Δ/Δ* mutant in yeast form has increased binding to the Dectin-1 receptor. **A** *C. albicans* strains grown overnight in YPD were stained with sDectin-1-Fc and fluorescently labeled secondary antibody, showing that the *cho1Δ/Δ* mutant exhibits greater staining than all other strains. **B** Flow cytometry reveals that the *cho1Δ/Δ* mutant has greater binding to the Dectin-1 receptor in comparison to other strains. **C** A graph of average mean fluorescent intensity from each sample reveals that the *cho1Δ/Δ* mutant exhibits significantly greater staining with sDectin-1-Fc than the other strains. * p-value < 0.05.

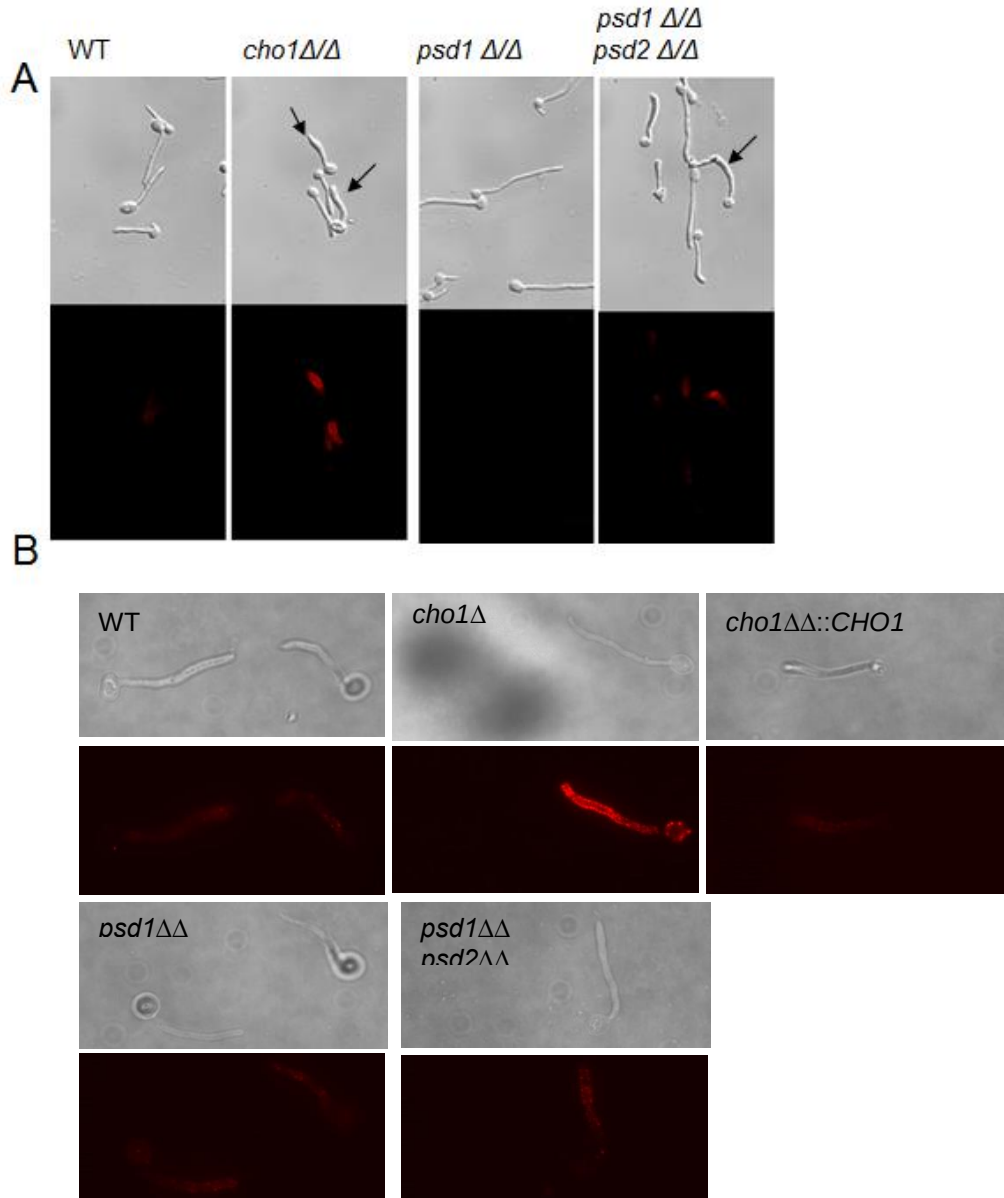


Figure 2.5. The hyphal form of the *cho1* Δ/Δ mutant has increased exposure of β -(1,3) glucan and binding by the Dectin-1 receptor. A Immunofluorescence of β -(1,3) glucan exposure using a anti- β 1,3-glucan antibody reveals that the *cho1* Δ/Δ mutant and the *psd1* Δ/Δ *psd2* Δ/Δ double mutant have increased β -(1,3) glucan exposure in comparison to the wild type and *psd1* Δ/Δ mutants. This exposure appears to localize at malformed tips of the hyphae (indicated by arrows). **B** Immunofluorescence using sDectin-1-Fc and secondary antibody shows that the *cho1* Δ/Δ mutant exhibits greater binding to Dectin-1 than the other strains.

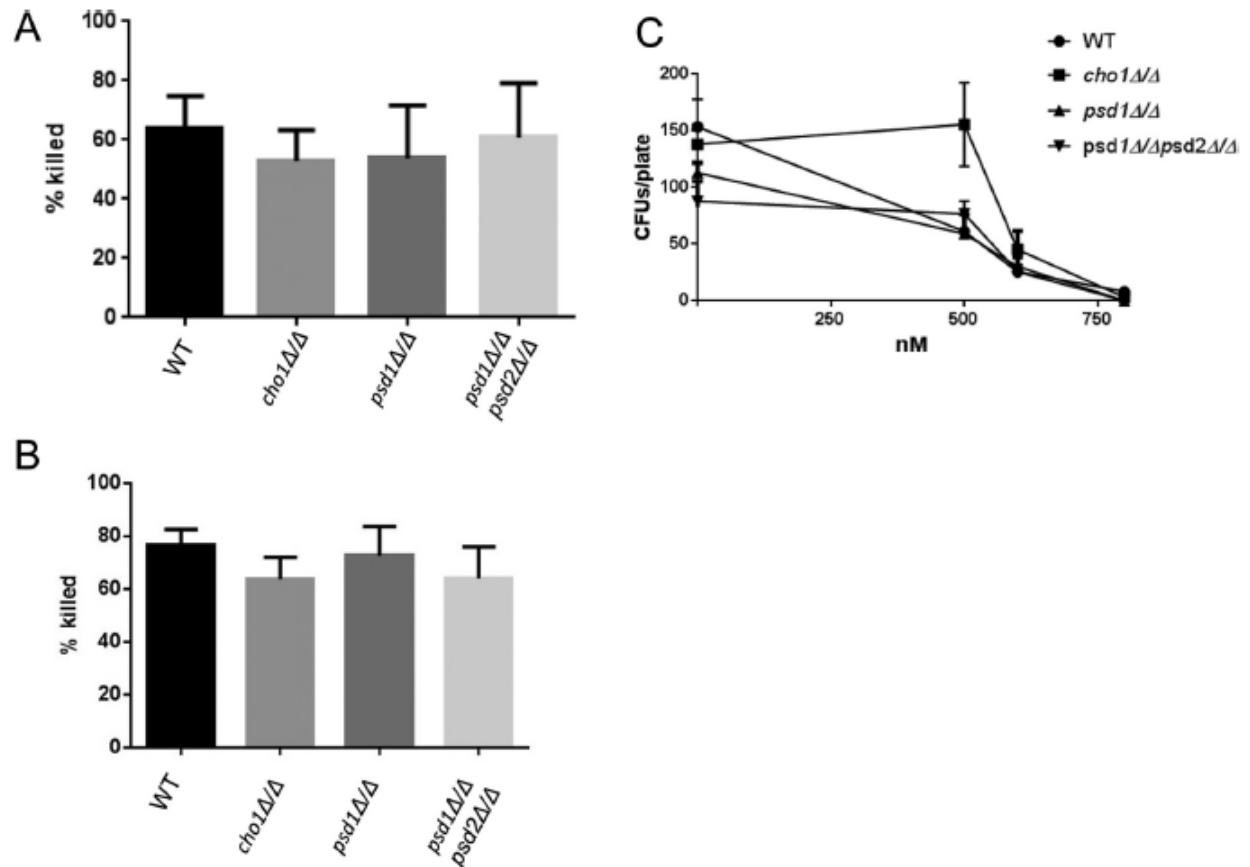


Figure 2.6. There are no differences in killing by phagocytes or defensins. **A** RAW 264.7 macrophages and *C. albicans* cells were co-incubated for 8 hours and *C. albicans* plated to measure viable CFUs. *C. albicans* strains mock challenged with macrophages were used to calculate the percentage of *C. albicans* killed. **B** Neutrophils were measured for their ability to kill the different strains using the same approach as in A, except that they were co-incubated for only two hours. **C** The *cho1Δ/Δ* mutant was incubated with β -defensin or a mock challenge and then viable CFUs were measured. No values were statistically significant from one another.

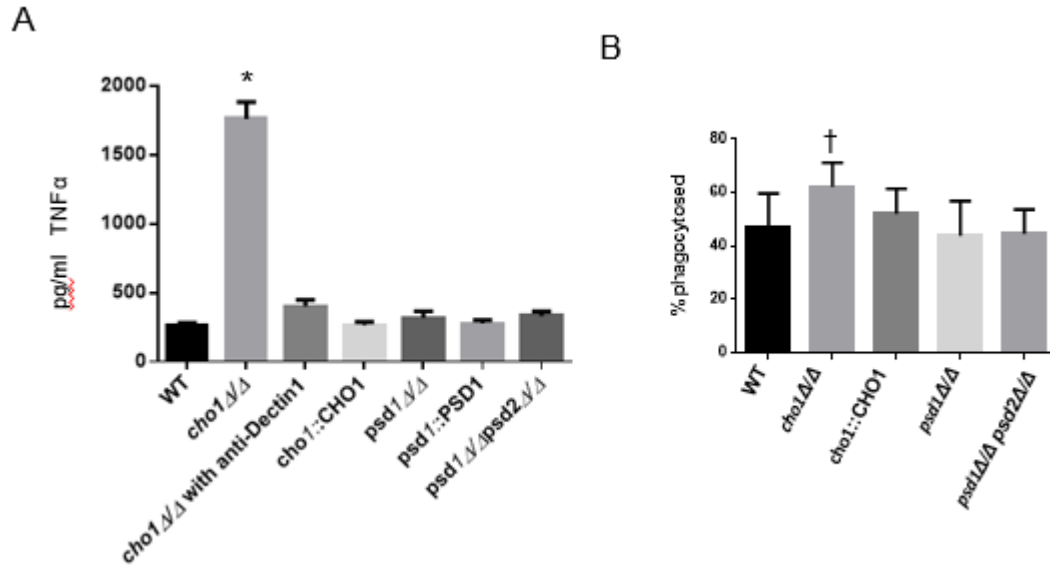


Figure 2.7. The *cho1Δ/Δ* mutant elicits TNFα from macrophages in a Dectin-1-dependent manner. **A** The TNFα-specific ELISA was used to measure TNFα elicited from RAW-BLUE macrophages (Invitrogen) in response to *C. albicans* challenge after 4 hours. Pre-treatment with anti-Dectin-1 neutralizing antibody reveals that the TNFα response is Dectin-1 mediated. **B** The *cho1Δ/Δ* mutant was more efficiently phagocytosed by RAW-BLUE macrophages in comparison to wild type and other strains.

* p-value < 0.0001

† p-value < 0.05

Chapter III: The loss of *de novo* phosphatidylserine and phosphatidylethanolamine results in reduction of the adhesin Als3p and downstream virulence factors

Abstract

The *Candida albicans* mutants *cho1* Δ/Δ (phosphatidylserine synthase) and *psd1* Δ/Δ *psd2* Δ/Δ (phosphatidylserine decarboxylase) are avirulent in a mouse model of systemic candidiasis. In this chapter, we find that the mutants are also avirulent in a mouse model of oropharyngeal candidiasis (OPC). Given that the *cho1* Δ/Δ mutant has cell wall defects and β (1-3)-glucan unmasking (explored in Chapter II) but the *psd1* Δ/Δ *psd2* Δ/Δ mutant did not indicated that there may be other phenotypes more associated with a loss of offensive virulence factors.

There a variety of cell wall proteins expressed by *C. albicans* that are important for virulence in the oropharyngeal candidiasis (OPC) model. One of these proteins is Als3p, an adhesin that binds to cadherins, promotes attachment to epithelial and endothelial cells and induces endocytosis and tissue damage. The mutants were examined for defects in Als3p and it was found that both mutants poorly expressed Als3p on the cell surface. This correlated with defects in adhesion to epithelial cells as well as endocytosis by, and damage to, epithelial cells. Interestingly, the mutants did not show defects in other adhesins like Als1p or Hwp1p, suggesting a specific regulation of the adhesin Als3p.

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The mouse model of oropharyngeal candidiasis, which includes the mouse model and histology were performed by Norma Solis in Dr. Scott Filler's lab. Dr. Filler also provided us with FaDu cells. Quantification of immunofluorescence LUTs were performed by Dr. Steven Minkin. The rest of the experiments were performed by Dr. Sarah Davis. Adhesin antibodies Als1, Als3, Als4, and Hwp1 were a donation from Dr. Lois Hoyer.

Introduction

Als3p is an adhesin that belongs to the Als adhesin family of *C. albicans* and is expressed exclusively on hyphal *C. albicans* (1, 60, 138-140). Adhesins play a key role in epithelial cell adhesion, which is a key feature in the establishment and progression of Oropharyngeal Candidiasis (OPC) (141-143). Als3p mediates adhesion by binding cadherins on the surface of epithelial and endothelial cells (31, 66). This binding induces endocytosis, enabling the hyphae to enter the host tissue and cause damage. When *ALS3* is knocked out *C. albicans* has a reduced ability to attach to oral epithelial cell lines and cause tissue damage in mice (31, 62).

The *de novo* pathway for synthesizing the phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) is required for the virulence of *C. albicans* in a mouse model of systemic Candidiasis (44). The first step of the *de novo* pathway (Fig 1.2), carried out by the PS synthase Cho1p, which generates PS from cytidyldiphosphate-diacylglycerol (CDP-DAG) and serine, is responsible for synthesizing all PS within the cell. PS is also a substrate for PE,

where the PS is decarboxylated by the PS decarboxylase enzymes Psd1p and Psd2p. When Cho1p or both Psd1p and Psd2p are knocked out, there is a comparable loss of PE to about half of wild type levels (44). The remaining PE is synthesized via the Kennedy pathway from diacylglycerol (DAG) and ethanolamine that is primarily imported from the environment (Fig. 1.2). In fact, the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants are ethanolamine auxotrophs that cannot make sufficient PE via the Kennedy pathway without importing ethanolamine.

Here we describe how loss of PE synthesis through PS synthase (*cho1* Δ/Δ) or PS decarboxylase (*psd1* Δ/Δ *psd2* Δ/Δ) mutations results in a reduction in Als3p and Als3p-associated virulence phenotypes such as epithelial adhesion and damage.

Results

The *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants exhibit cell wall defects (44). As PE is the donor for ethanolamine-phosphate moieties required for glycosylphosphatidylinositol (GPI) anchor synthesis (82, 144), we hypothesized that this may lead to poor expression of GPI-anchored proteins at the cell surface of hyphae. Many cell wall adhesins are GPI-anchored proteins, so we assessed whether GPI-anchored adhesins associated with hyphae were compromised in the mutants compared to wild-type using monoclonal antibodies. Analysis of the Als3p expression on the cell surface was assessed by immunofluorescence, revealing that the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants exhibit lower levels of Als3p on hyphae, with the lowest levels of fluorescence associated with the *cho1* Δ/Δ mutant (Fig. 3.1 A). In addition, there were fewer *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ cells expressing detectable Als3p compared to wild-type (Fig. 3.1 B). These original findings support our hypothesis that a loss of PE would ultimately result in a loss of GPI anchored proteins. The loss of Als3p suggested the possibility that other GPI-anchored adhesins were also diminished in hyphae, which may help explain decreased virulence and unmasking reported in chapter II. However, examination of Als1p, Als4p, and Hwp1p using immunofluorescence revealed no differences between strains (Fig. 3.2

A & B & data not shown). This suggested that the Als3p expression defect is not due to a general defect in GPI-anchored protein processing or hyphal protein distribution, but a consequence of reduced Als3p expression.

As Als3p expression is associated with adhesion to mammalian cell lines, we tested if the loss of Als3p expression would correlate with a loss in adhesion to the Chinese hamster ovary (CHO) cell line, which is used as an *in vitro* model for epithelial cell adhesion. It was found that the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants both exhibit reduced adhesion to CHO cells (Fig 3.3), which is correlated with the decrease they exhibit in Als3p expression (Fig. 3.1 A & B).

Als3p acts as an invasin that induces endocytosis of *C. albicans* hyphae into cell lines that express cadherins, such as FaDu oral epithelial lines, and mutants that lack *ALS3* have decreased endocytosis (31). When *C. albicans* strains are co-incubated with the FaDu cells, the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants show reduced endocytosis (Fig 3.4 A). *C. albicans* cells that have been endocytosed can subsequently damage epithelial cells which can be measured by the level of lactate dehydrogenase (LDH) released into the media. As predicted, the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants have lower LDH released into the media (Fig 3.4 B).

Als3p is not required for virulence in a systemic model of infection (71), however it has been shown to play a minor role in OPC (145). In order to investigate if the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants exhibit a loss of virulence in oropharyngeal infection, a mouse model of OPC was performed (146). There was a decrease in pathogenicity for both the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants in OPC infections (Fig. 3.5 A & B). The *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants both showed decreased colonization of the tongues of mice based on recovered colony forming unit counts (CFUs). The mutants also had decreased levels of colonization on the epithelium as can be seen by histology (Fig 3.5 C).

Discussion

ALS3 expression is controlled by phospholipid levels

The *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants have both a qualitative and quantitative decrease in the adhesin Als3p (Fig. 3.1 A & B), which is a virulence factor in the mouse model of OPC (145). Loss of Als3p results in a decrease in adhesion to CHO and FaDu cell lines, which correlates with a loss in damage to, and endocytosis by, epithelial cells (Fig. 3.4 A & B). It is important to note that this decrease is specific to Als3p, and there are no deficiencies in the immunofluorescence of other adhesins such as Als1p and Als4p (Fig. 3.2 A & B). This led us to believe that decreased PE results in a reduction of Als3p immunofluorescence, and this reduction is more prominent when PS as well as PE are reduced in the *cho1* Δ/Δ mutant. We hypothesize that the loss of these phospholipids affects signal transduction cascades that control the regulation of Als3p. We currently do not know which pathways are involved. One pathway known to be important in the specific Als3p regulation is the pH sensing pathway which is mediated by the transcription factor Rim101. This is dependent on proteolytic cleavage and activation of Rim101 in response to neutral pH in a cascade that depends on endosomal trafficking components (147-152). However, Western blots comparing our mutants to wild type showed that there are no differences in Rim101p processing in these strains (Fig. 3.6). It is important to note that there may be other pathways dependent on PS and/or PE that are regulating ALS3 expression. For example, Protein kinase C (Pkc1p) in the cell wall integrity MAP kinase pathway depends on PS in *S. cerevisiae* (66).

Materials & Methods

Strains and media

The *C. albicans* strains used in this study are in Table 1. The media used for most experiments for growth and maintenance of the strains was YPD (yeast extract, peptone, and dextrose) liquid media and plates. For growth curves, we used minimal media (yeast nitrogen

base, dextrose) $\pm$ ethanolamine. The *AtSDC* strains were generated as follows: First, the *AtSDC* gene was codon optimized by site-directed mutagenesis in the yeast expression vector pVT103-U (153), and was then PCR amplified with SDO17 and SDO18 which introduced *NcoI* and *BglII* sites that were used to replace the dTomato gene found between these sites in the pENO-dTomato-NATr plasmid (154), creating the plasmid pENO1-*AtSDC*. This plasmid was linearized with *EcoRV*, and then transformed by electroporation as previously described (44).

Generation of the Rim101-V5 strains

Rim101p was tagged with V5 in our strains using the pYLC314.1-RIM101-V5 plasmid, which was created as follows. A vector carrying the *SAT1* marker, pYLC314, was created first. pYLC314 was created from pBluescript II SK+ by cutting it with *EcoRI* and ligating into it a 1.7kb fragment containing the *SAT1* marker followed by the *C.albicans MET3* promoter. This fragment was generated by PCR from pYLC229 (155) using primers JCO165 and JCO166 that introduced *EcoRI* sites on both flanks (see Table 2). The *MET3* promoter was removed by cutting pYLC314 with *PstI*, which cut on either side of the *MET3* promoter and then re-ligating to create pYLC314.1. The *C. albicans RIM101* gene, plus 1000 base pairs (bps) of upstream DNA and 240 bps downstream, was amplified with primers TRO892 and TRO893, which introduced *Apal* and *XhoI* sites respectively, and was cloned into plasmid pYLC314.1 to create pYLC314.1-RIM101; for primer sequences see Table 2. A V5 epitope tag was added to *RIM101* at the internal *AgeI* site (147) by digesting the plasmid with *AgeI* and *BstBI* to remove a segment of *RIM101*, which was replaced by a fragment that was identical except that a V5 tag was added just after the *AgeI* site. This was done by amplifying the *RIM101* sequence with primers SD22, which introduced the V5 tag, and SDO20. pYLC314-RIM101-V5 was linearized with *BstBI* and it was transformed into *C. albicans* strains by electroporation (135).

Endocytosis of *Candida albicans* into epithelial cells

Endocytosis was measured as previously described (31).

LDH Release

The day prior to the experiment, FaDu cells were plated at 5×10^5 cells/well in a 24 well plate in HBSS media + 10% FBS. The next day, media was replaced with fresh HBSS + 2% human serum. *C. albicans* cells were added to wells at an M.O.I. of 5 and co-incubated for 6 hours at 37°C + 5% CO₂. At this time supernatant was harvested and LDH release was quantified using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Cat# G1782).

Adhesion Assay to epithelial cells

The day prior to the experiment, 4×10^5 Chinese hamster ovary (CHO) cells/well were plated in a 6 well plate with F12 media + 10% FBS + penicillin/streptomycin. The next day, 6×10^5 *C. albicans* cells from an overnight culture grown in YPD were washed 3X with 1mL of PBS and resuspended in 9mL complete F12 media in a glass test tube. These were incubated at 37°C for 1 hour in the shaking incubator. After 1 hour, media was removed from the CHO cells and 3 mL of the F12 yeast mixture were added to each well. These plates were returned to 37°C and incubated for 2 hours (no shaking). After incubation, media was removed from the wells which were washed 5X with 3 mL PBS. Once washing was complete, 3mL of water was added to each well in order to lyse the CHO cells. After 10 minutes, the bottom of the plates were scraped with a cell scraper to release remaining *Candida*. This cell mixture was then diluted 1:10, then 100 µl of diluent was plated onto YPD + 1M sorbitol plates and incubated overnight at 37°C for 24 hours. Colony forming units were then counted to determine CFU/ml.

Western Blot for Rim101p-V5 detection

Overnight cultures were diluted to 0.1 OD in 50 mL YPD and incubated for 3 hours in 30°C shaking incubator. After 3 hours, cells were stimulated with M199 media at a pH of 8 for 30 minutes in 30°C shaking incubator. After stimulation cells were pelleted by centrifugation at 3300rpm for 3 minutes then the pellet was resuspended in 250 µl PBS with protease inhibitors

(Roche cat# 4693124001) and bead beaten 3 times for 30 seconds each. Samples were spun down at 2000rpm and the supernatant was measured by Bradford and resuspended in Laemmli loading buffer. 20µg protein was run on an 8% acrylamide gel and transferred to PVDF overnight. Blocking was 1 hour in TBST + 5% milk, primary was 1:2000 dilution of V5 antibody conjugated to HRP (Abcam cat# ab1325) overnight. After primary, the blot was washed 3X quickly with TBST then 2X at 5 minutes each with TBST. Western blot was developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific cat # 34080).

Oropharyngeal Candidiasis Model

This experiment was adapted from a previous publication (145, 146).

Appendix. Chapter III Figures

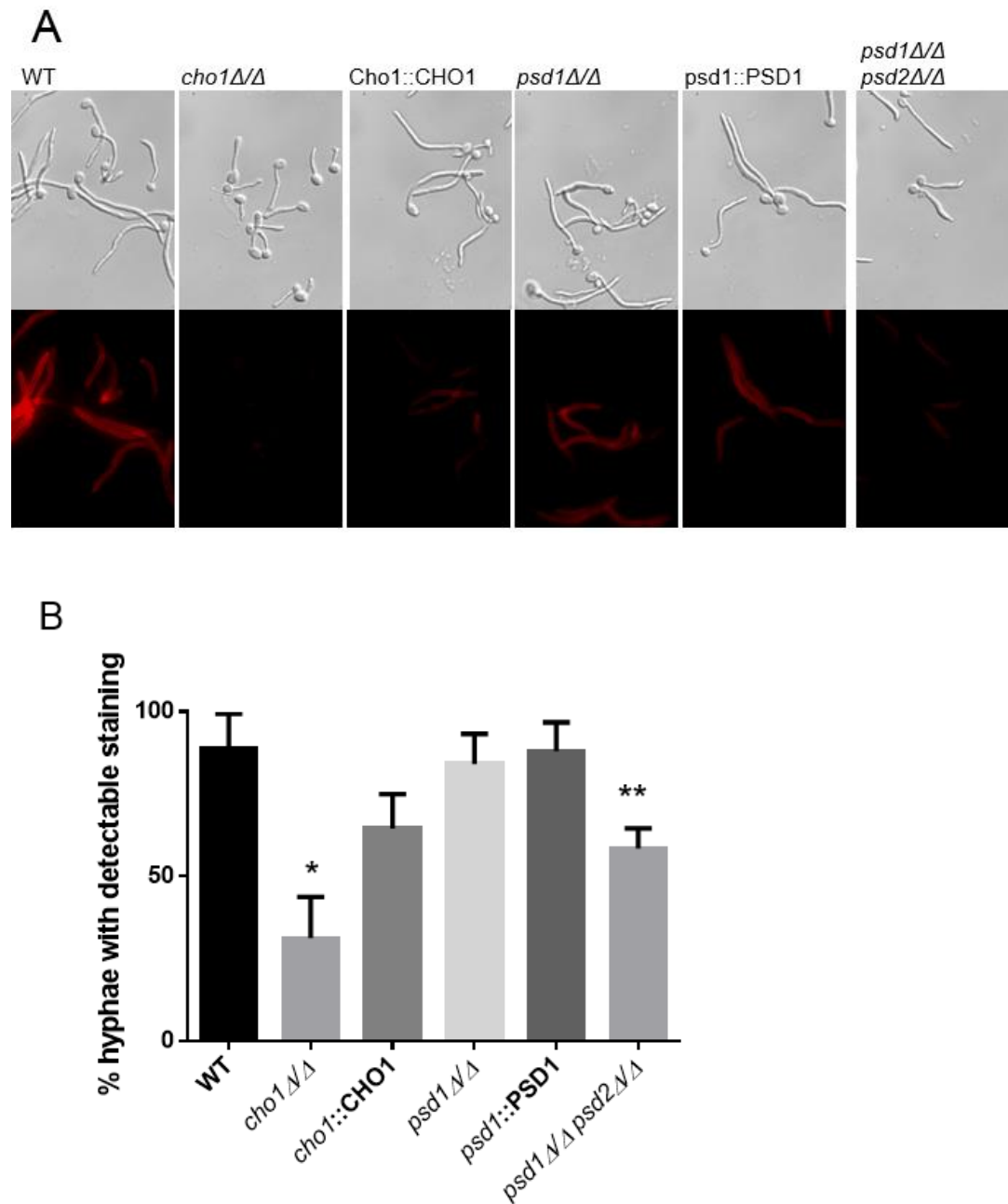


Figure 3.1. Mutants reduced in virulence are diminished in Als3p. A The *cho1*Δ/Δ and *psd1*Δ/Δ *psd2*Δ/Δ mutants are reduced in Als3p measured by immunofluorescence. **B**

Quantification of the number of cells in the population with detectable Als3p. * Comparing WT to *cho1*Δ/Δ, p-value < 0.0005 ** Comparing WT to *psd1*Δ/Δ *psd2*Δ/Δ, p-value < 0.005

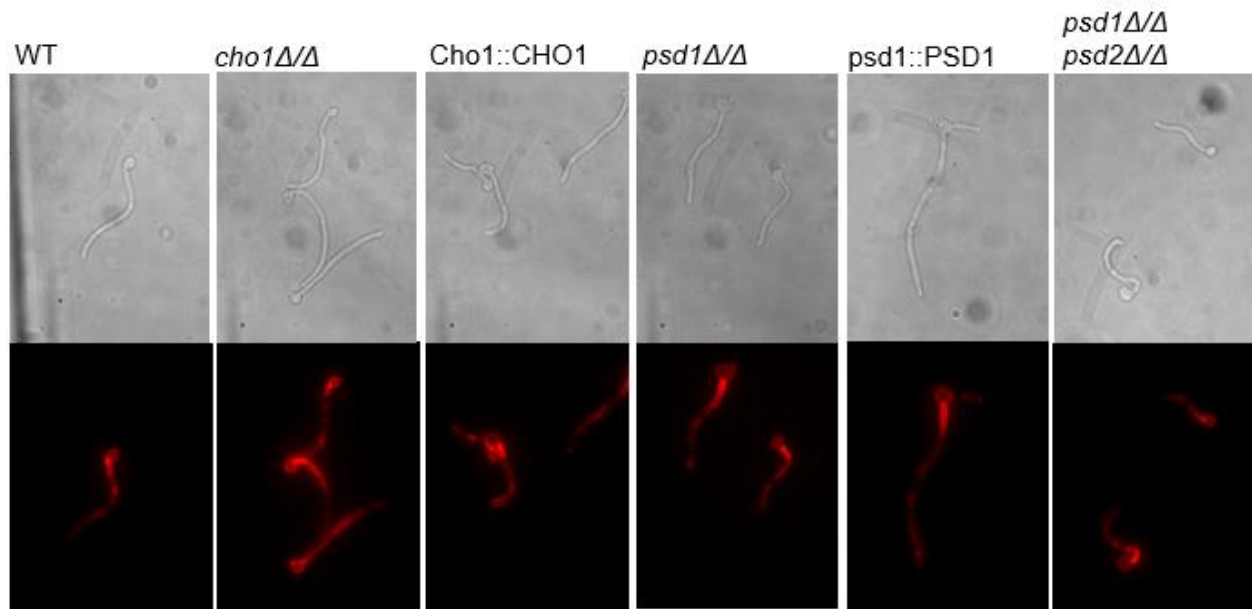
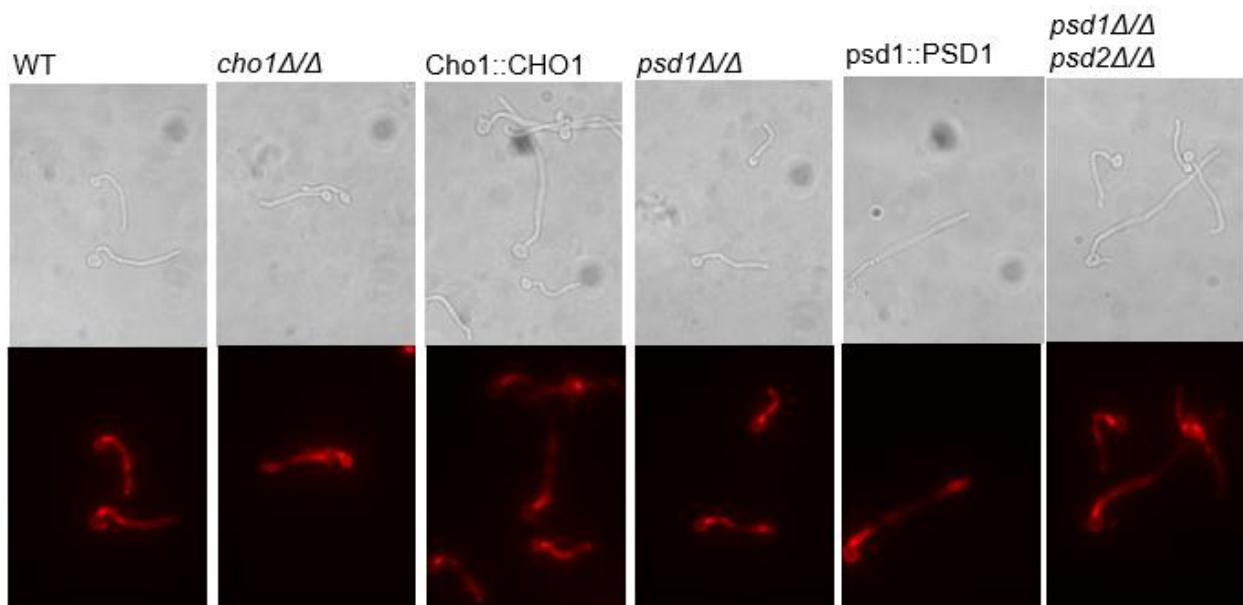
A**B**

Figure 3.2. The Als1p (A) and Als4p (B) adhesins are expressed equally among the phospholipid mutants and wild type.

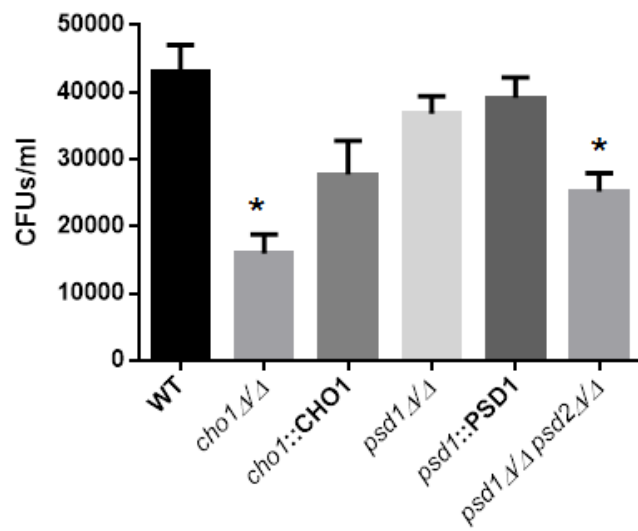


Figure 3.3. Loss of Als3p correlates with a loss of adhesion to the CHO cell line. The *cho1*Δ/Δ and *psd1*Δ/Δ *psd2*Δ/Δ mutants are both reduced in the ability to attach to mammalian epithelial cells. * p-value < 0.0001 compared to WT.

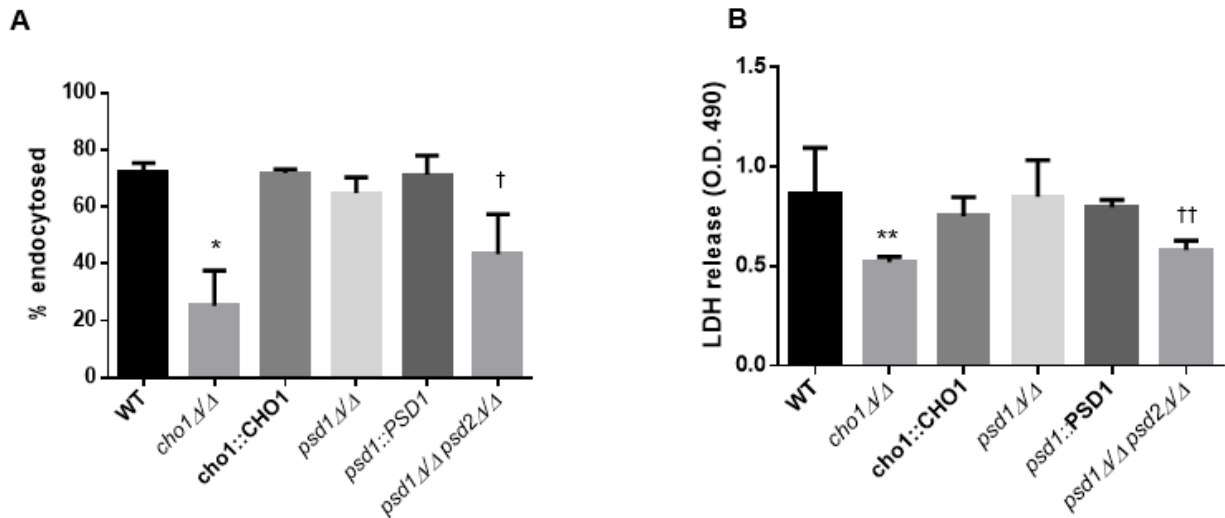


Figure 3.4. Mutants reduced in Als3p are also reduced in ability to induce endocytosis and tissue damage. **A** After co-incubation of *C. albicans* with FaDu cells and then counterstaining to visualize endocytosis, it was shown that for the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants there is a reduction in endocytosis by the epithelial cell line. **B** The *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants are both reduced in their ability to cause damage to the FaDu cells, indicated by the reduction in lactase dehydrogenase (LDH) release. * p-value < 0.005, † p-value < 0.05, ** p-value < 0.0005 †† p-value < 0.001, all compared to WT.

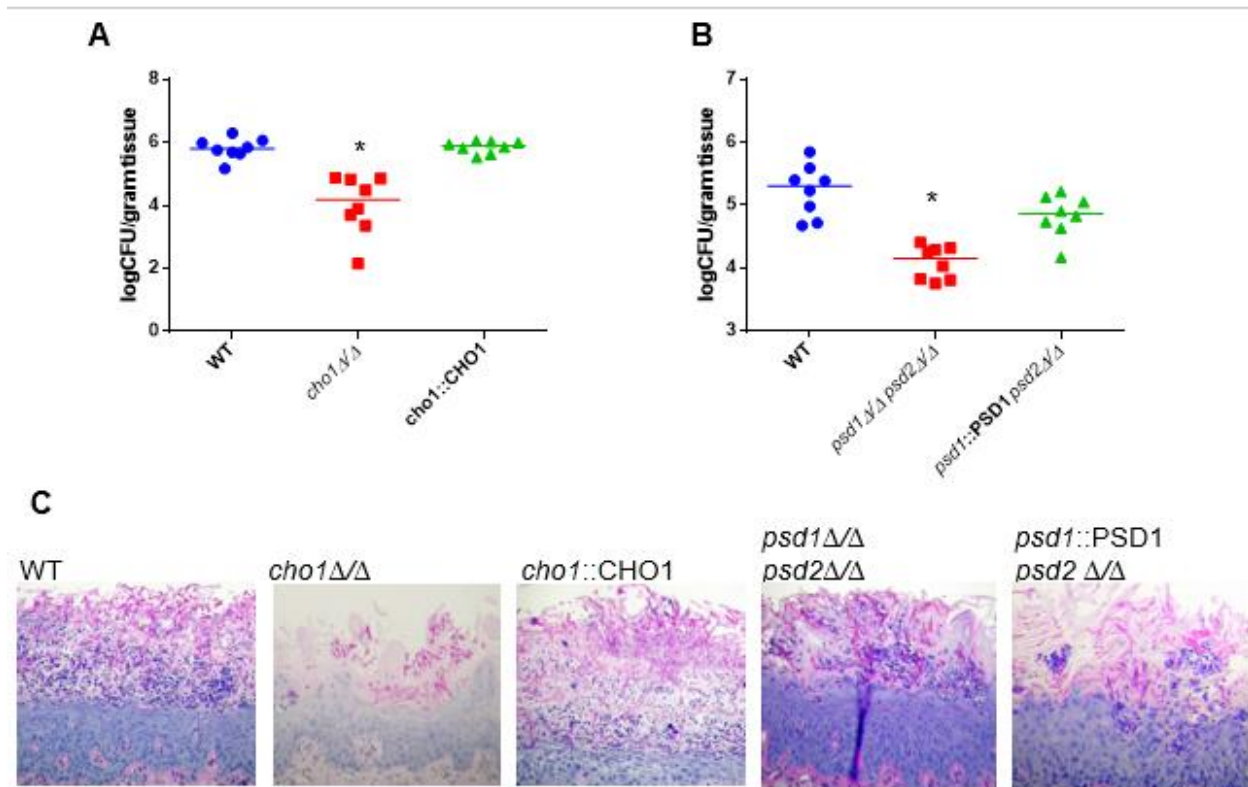


Figure 3.5. The *cho1*Δ/Δ and *psd1*Δ/Δ *psd2*Δ/Δ mutants are attenuated for virulence in OPC in mice. **A, B.** Mice were inoculated orally with *C. albicans* strains, and after five days, their tongues were harvested, and the CFUs/gram of tissue were measured. **C.** PAS (periodic acid-Schiff) staining of histological samples from a clipping of tissue from tongues in A and B is shown for each strain. * = P-value < 0.0002, Compared to WT.

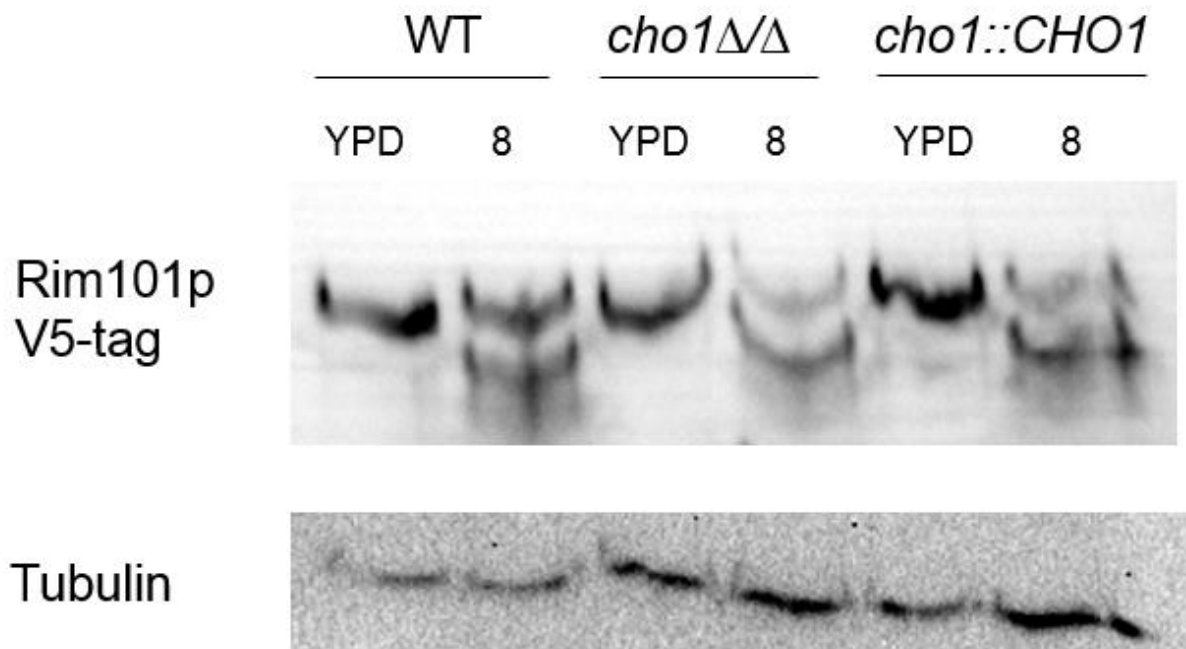


Figure 3.6. There are no differences in the proteolytic processing of the Rim101p transcription factor. Activation of the Rim101p transcription factor is the same for all strains, indicating that Als3p regulation is mediated by another means. YPD is a neutral pH of 6.7 and does not activate the pathway. “8” in the figure denotes M199 media adjusted to the pH 8 which activates Rim101p proteolytic cleavage.

Chapter IV: Ethanolamine auxotrophy is responsible for avirulence in the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants

Abstract

Candida albicans null mutants in the *de novo* synthesis of phosphatidylserine (*cho1* Δ/Δ mutant) and phosphatidylethanolamine (*psd1* Δ/Δ *psd2* Δ/Δ mutant) are greatly reduced in virulence in both a systemic infection model and an oropharyngeal model of Candidiasis. The *cho1* Δ/Δ mutant is unable to synthesize phosphatidylserine (PS) and is reduced in phosphatidylethanolamine (PE), whereas the *psd1* Δ/Δ *psd2* Δ/Δ mutant is only diminished in PE and has elevated levels of PS. Due to the loss of the *de novo* pathways, both mutants are ethanolamine auxotrophs. It was unclear if ethanolamine auxotrophy was the major contributor to this loss of virulence, or whether the attenuation in virulence was due to cell wall defects or cell signaling.

In order to delve into what factors were contributing to avirulence, we tested the hypothesis that the main defect in virulence was because of an inability to grow in the host due to ethanolamine auxotrophy. These mutants require 1mM ethanolamine in culture media for growth and host ethanolamine is estimated at ~30 μ M. This hypothesis was tested by supplying ethanolamine to the mutants through expression of the *Arabidopsis thaliana* serine decarboxylase (*AtSDC*), which converts cytoplasmic serine to ethanolamine for PE synthesis by the Kennedy pathway. The *AtSDC* completely restored ethanolamine prototrophy and virulence in the mice, indicating that ethanolamine auxotrophy is the main cause of avirulence in *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants.

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Dr. Sahar Hasim assisted with transformation of the *Arabidopsis thaliana* Serine decarboxylase gene into our strains, as well as performed growth curves with them and assisted me in the mouse model of systemic candidiasis. Dr. Anthony Montedonico assisted with sequencing the *Arabidopsis thaliana* Serine decarboxylase gene and performing site directed mutagenesis to make it codon-optimized for *Candida albicans*. Dr. Todd Reynolds cloned the codon-optimized *AtSDC* gene into the *PENO1* expression plasmid. All other experiments were performed by Dr. Sarah Davis.

Introduction

The *de novo* pathway for synthesizing the phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) is required for the virulence of *C. albicans* in a mouse model of systemic Candidiasis (44). The first step of the *de novo* pathway, carried out by the PS synthase Cho1p, is responsible for synthesizing all PS within the cell. This PS is also a substrate for PE in the *de novo* pathway, where the PS is decarboxylated by the PS decarboxylase enzymes Psd1p and Psd2p (Fig. 1.2). When Cho1p is knocked out (the *cho1Δ/Δ* mutant) or both Psd1p and Psd2p are knocked out (the *psd1Δ/Δ psd2Δ/Δ* mutant), there is a comparable loss of PE by about half of wild type levels (44).

Both the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants are ethanolamine auxotrophs, and cannot grow in minimal media without ethanolamine (44). It was unclear whether this auxotrophy is the major contributor to the loss of virulence, or whether the loss of PS and/or PE resulted in other problems (i.e. cell signaling, cell wall perturbations) that would be the major contributor to virulence. These were of concern due to the fact that the *cho1Δ/Δ* mutant exhibits

cell wall defects that results in β -(1,3) glucan exposure and elicitation of TNF α from macrophages, which could at least play a part in immune activation and elimination *in vivo* (83).

Due to the fact that the *psd1* Δ/Δ *psd2* Δ/Δ mutant does not have cell wall defects, but has reduced PE levels comparable to the *cho1* Δ/Δ mutant and is avirulent, we suspected that an inability to acquire sufficient ethanolamine *in vivo* to synthesize PE via the Kennedy Pathway could be a major contributor to the inability to survive in the host. In order to assess survival, we performed short-term assays an *in vitro* measure of hyphal length generation in human serum, as well as an *in vivo* mouse experiment (Fig 2.2). These assays revealed that at least for a short period of time the mutants were able to generate hyphae. However, we later speculated that hyphal generation could be due to the cell using ethanolamine that had been stored in the cell and did not reflect what would occur during a long-term infection. Therefore our goal was to determine whether the mouse could provide enough ethanolamine to the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants to support growth.

In order to investigate this question, we increased levels of endogenous ethanolamine by addition of the *Arabidopsis thaliana* serine decarboxylase gene, which decarboxylates serine to ethanolamine (Fig. 1.2). This ethanolamine can then be used by the Kennedy pathway to generate PE (Fig. 1.2). With this new means of making PE, the virulence of *Candida albicans* was restored for both the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants in a mouse model of systemic infection. This suggests that our attenuated mutants are unable to acquire sufficient levels of ethanolamine inside the host during infection, resulting in an inability to proliferate and cause disseminated disease. Altogether our findings indicate that PE is a virulence factor of *C. albicans* that can be targeted potentially by targeting activity of the Cho1p (phosphatidylserine synthase) enzyme, reducing virulence and making Cho1p an excellent drug target.

Results

Loss of *de novo* PE synthesis compromises virulence through ethanolamine auxotrophy

The *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants share a comparable loss in PE and are both ethanolamine auxotrophs (44), so we hypothesized that the reason for the loss of virulence is due to an inability to acquire sufficient amounts of ethanolamine for PE synthesis by the Kennedy pathway within the host. The level of ethanolamine in the host is estimated to be ~30 μ M, but the level required to support growth in culture media is greater than 100 μ M (44, 156). A full understanding of how the loss of PS and *de novo* PE contribute to avirulence and interfere with host-pathogen interactions will aid in the development of assays to search for future inhibitors that can be used in drug development, or will reveal other alternative drug targets which may also be exploited.

In order to test this hypothesis, a serine decarboxylase enzyme derived originally from *Arabidopsis thaliana* (*AtSDC*) was expressed in our *C. albicans* strains after codon optimization (153, 157). *AtSDC* decarboxylates serine to ethanolamine within the cytoplasm, and the ethanolamine can then be used by the Kennedy pathway to synthesize PE (Fig. 4.1). If ethanolamine auxotrophy is mediating the loss of virulence, addition of this enzyme should restore ethanolamine prototrophy and virulence in a mouse model of systemic Candidiasis.

The ability of *AtSDC* to restore ethanolamine prototrophy in the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ strains was tested by measuring growth in minimal media +/- ethanolamine (Fig. 4.2 A & B). As expected, the mutants that exhibited ethanolamine auxotrophy in the past were unable to grow in the absence of ethanolamine. When grown in media lacking ethanolamine, the transformed mutants expressing *AtSDC* grew at rates similar to the respective parental mutant strains in the presence of ethanolamine (Fig. 4.2 A & B). In addition, thin layer chromatography

(TLC) confirmed that PE levels were restored in the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants when *AtSDC* is expressed, even when grown in the absence of ethanolamine (Fig. 4.2 C).

We then examined if addition of *AtSDC* would restore virulence for the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants in a mouse model of systemic infection (Fig. 4.3 A & B). Indeed, the addition of this enzyme fully restored virulence to both the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants in the systemic infection model. The differences seen for the survival curve were reflected in fungal burden measurements (Fig. 4.3 C). Although it not clear what is directly responsible for this, it is interesting to note that although both the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants had much lower fungal burden than wild-type, the *cho1* Δ/Δ mutant is diminished more than the *psd1* Δ/Δ *psd2* Δ/Δ mutant (Fig. 4.3 C). This may be attributable to the diminished Als3p expression, increased unmasking, or both.

The Role of Kennedy pathway PE in regulating Als3p and cell wall unmasking

As Als3p is diminished for both the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants, it seemed likely that PE levels were important for this regulation. Therefore, Als3p levels were analyzed by immunofluorescence for the wild-type and mutant strains expressing *AtSDC*. The addition of the *AtSDC* increases Als3p for the *psd1* Δ/Δ *psd2* Δ/Δ mutant, but not the *cho1* Δ/Δ mutant (Fig. 4.4 A). Relative mean intensity reveals that for Als3p staining, the *cho1* Δ/Δ and *cho1* Δ/Δ -*AtSDC* mutants exhibit comparable intensity, while *psd1* Δ/Δ *psd2* Δ/Δ -*AtSDC* is more similar to wild type in levels (Fig. 4.4 E). However, the adhesion to CHO cells seems to be restored for both the *cho1* Δ/Δ -*AtSDC* and *psd1* Δ/Δ *psd2* Δ/Δ -*AtSDC* (Fig. 4.4 B). The *cho1* Δ/Δ mutant exposes more β -(1,3) glucan than wild-type in both yeast and hyphal forms, which leads to increased recognition by innate immune cells (83). This effect appears to be mediated solely by the loss of PS, as we did not see rescue of β -(1,3) glucan unmasking in the *cho1* Δ/Δ mutant by addition of *AtSDC* for either the yeast or the hyphae (Fig. 4.4 C & D). As Als3p is a known virulence factor for the development of oropharyngeal candidiasis (OPC), we tested the in vivo mouse model to

determine if addition of the AtSDC gene would affect virulence (31, 63). When the *cho1* Δ/Δ -AtSDC and the *psd1* Δ/Δ *psd2* Δ/Δ -AtSDC mutants were tested in this model, virulence was restored and there were no statistical differences between these strains and wild type (Fig. 4.5).

Discussion

Virulence defects in the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants are primarily the products of ethanolamine auxotrophy

The *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants exhibit a number of phenotypes, but the *cho1* Δ/Δ mutant has more substantial cell wall defects than the *psd1* Δ/Δ *psd2* Δ/Δ mutants, including greater exposure of β -(1,3) glucan, and loss of Als3p than *psd1* Δ/Δ *psd2* Δ/Δ (83). This has repeatedly raised the question of whether the losses of virulence observed for the *cho1* Δ/Δ or *psd1* Δ/Δ *psd2* Δ/Δ mutants are due more to deficiencies in synthesis of PS and PE, or PE alone. The PE synthesis defect is the primary culprit, as both mutants share this in common, and the mutants require a minimum of 1mM ethanolamine for growth in culture media and the level of ethanolamine in the host is only $\sim 30 \mu\text{M}$ (44, 156). The observation that AtSDC can suppress Als3p expression defects in *psd1* Δ/Δ *psd2* Δ/Δ , but not *cho1* Δ/Δ suggests that both PE and PS independently regulate Als3p, a surprising and interesting finding.

In order to address whether an inability to acquire PE is the result of avirulence for *C. albicans* mutants in the mouse model of disseminated candidiasis, we first heterologously expressed in *C. albicans* the AtSDC, which decarboxylates serine into ethanolamine in the cell's cytoplasm (153). This ethanolamine can then be used by the Kennedy pathway to create PE (Fig. 1.1 & 4.1). If virulence is restored for both mutants expressing AtSDC, then this shows that insufficient PE synthesis in the host environment due to an inability to acquire enough ethanolamine substrate is the main contributor to avirulence. Synthesis of ethanolamine by AtSDC supported growth of the mutants in culture media to levels similar to when they are supplemented with 1mM ethanolamine (Fig. 4.2 A & B) and also restored PE levels (Fig. 4.2 C).

Finally *AtSDC* restored virulence in the systemic mouse model based on survival curves and fungal burden, and also restored virulence in the OPC model (Fig. 4.3 & 4.5).

Materials and Methods

Immunofluorescent Staining of hyphae

This protocol was modified from a previous publication (83). It is important to note that hyphae were induced for *AtSDC* strains with mouse serum rather than human serum.

Quantification of Immunofluorescence

Quantification of LUT's were performed as previously described (83).

Immunofluorescence staining for β -(1,3) glucan

The method from Davis *et al* (83) was used, and stationary cultures were stained.

Hot ethanol extraction and TLC

Sample extraction and TLC protocols were performed as previously described (44) with the following exceptions. Before running with samples, TLC silica gel plates (Fisher cat# M1057290001) were prepared by baking them in a drying oven at 100°C for 15 minutes. After baking, these plates were run with 50mM EDTA. Plates were then run with a chloroform: methanol (1:1) migration solvent to remove EDTA, dried, and baked in the oven again for 15 minutes. The samples were then loaded and run as described. Lipids were visualized on the dried plate by spraying with Primuline dye (5mg of Primuline, cat# 206865, Sigma, in 100ml of acetone: and visualizing with UV light.

Mouse systemic infection model

To prepare strains for injection, 1 O.D. from overnight test tube cultures were washed 5 times with 1 mL ddH₂O then counted by hemacytometer. These washed cultures were then diluted to 5×10^6 cells/ml and 100 μ l of cell solution was injected into ICR (Harlan) mice for a total of 5×10^5 yeast/mouse. N=5 mice for each *Candida* strain. An animal protocol on file with the UT

IACUC ensures humane treatment of the animals. For fungal burdens, cultures were prepared and injected the same way as they were for the mouse mortality assay described above. 5 days after injection, mice were humanely euthanized, and then both kidneys were removed, one for histology and one for CFU counts. The kidney for histology was stored in 10% formaldehyde and later sections and stained with H&E by the University of Tennessee Veterinary School. The other kidney was weighed and homogenized with a dounce homogenizer in 5 ml ddH₂O. This homogenate was diluted 1:4 and 200 µl was plated on YPD + 1M sorbitol plates for CFU counts.

Appendix. Chapter IV Figures

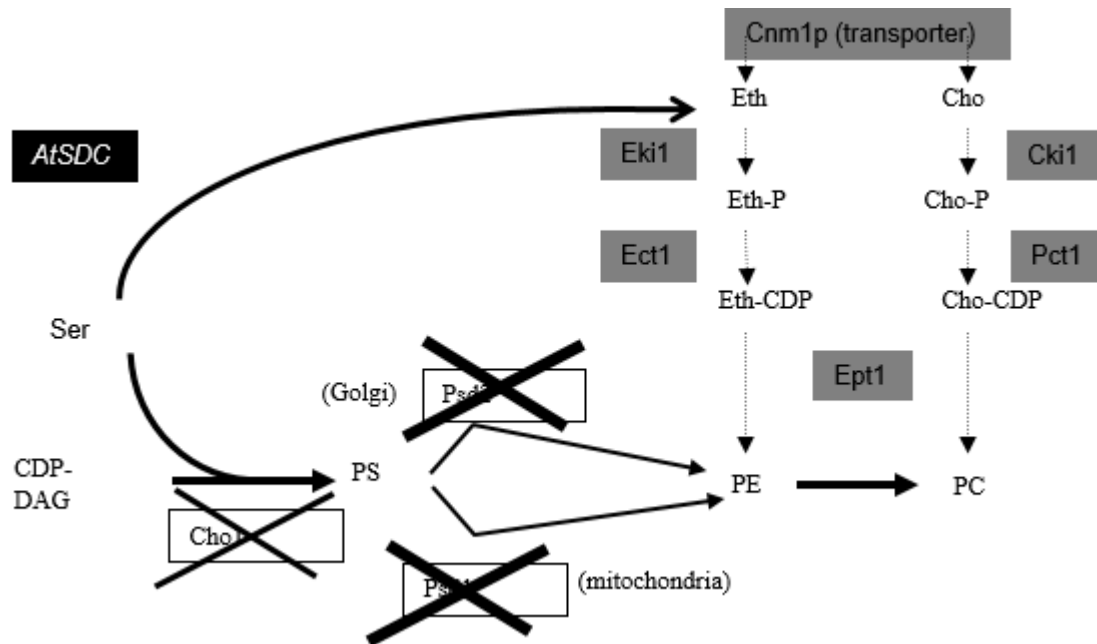


Figure 4.1. Pathways for synthesizing phosphatidylethanolamine in *C. albicans*. The Kennedy pathway genes are shown in gray, and are named after their predicted homologs in *S. cerevisiae*, and the *de novo* pathway proteins are shown in white. *Arabidopsis thaliana* Serine Decarboxylase (*AtSDC*) is shown in black. Eth: ethanolamine, Cho: choline, Eth-P: phosphoethanolamine, Cho-P: phosphocholine, Etn-CDP: cytidyldiphosphate-ethanolamine, Cho-CDP: cytidyldiphosphate-choline, PS: phosphatidylserine, PE: phosphatidylethanolamine, PC: phosphatidylcholine, CDP-DAG: cytidyldiphosphate-diacylglycerol, Ser: serine.

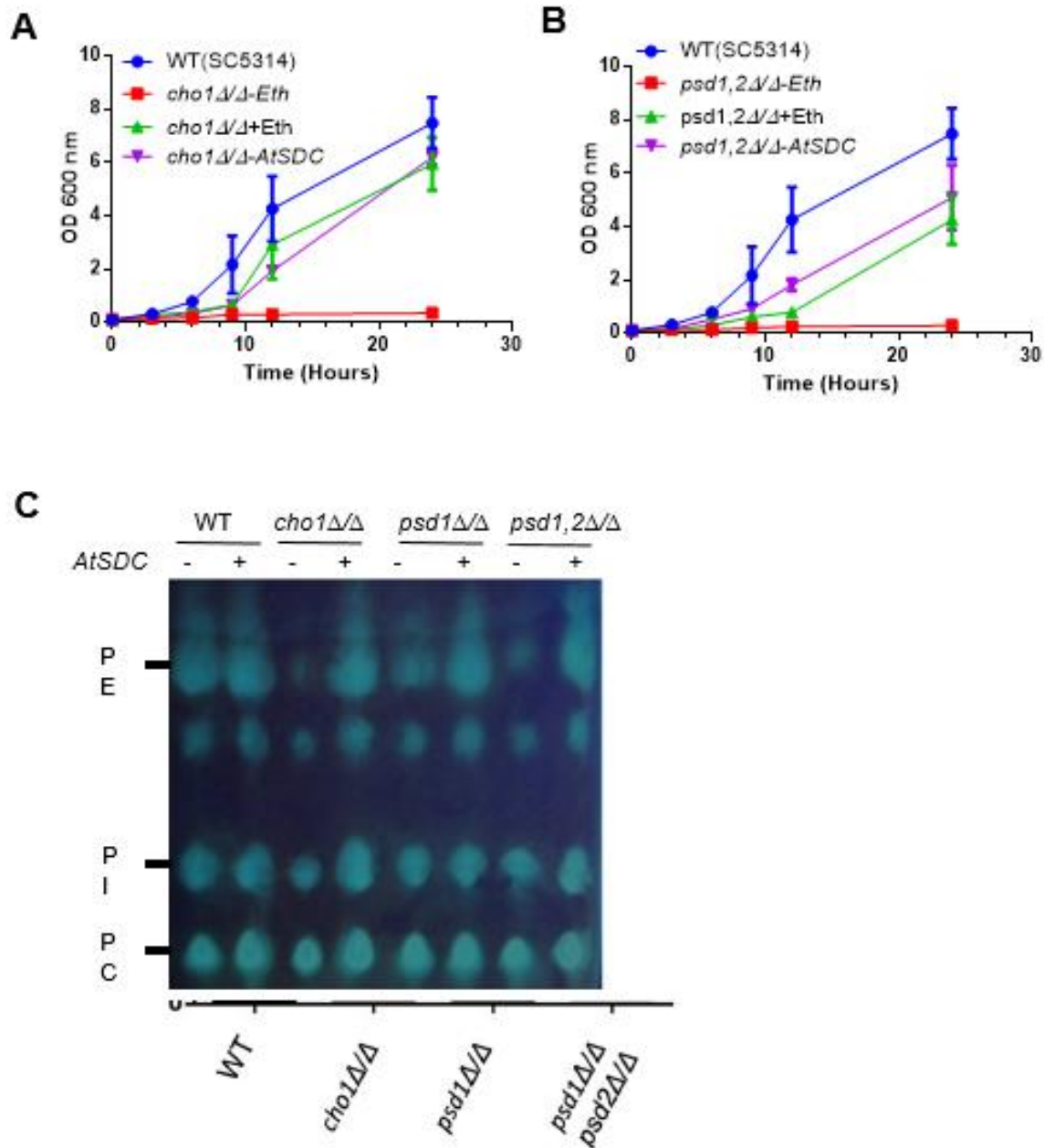


Figure 4.2. Expression of *AtSDC* restores ethanolamine prototrophy and virulence in *cho1Δ/Δ* or *psd1Δ/Δ psd2Δ/Δ* in a mouse model of disseminated *candidiasis*. **A,B** Mutant and wild-type (WT) strains $\pm$ *AtSDC* were grown in minimal media $\pm$ ethanolamine (Eth) and a growth curve was plotted using OD600 measurements. **C** Phospholipids were isolated from each strain grown in minimal media lacking ethanolamine, and then separated by TLC. They were visualized with primuline stain under ultraviolet light.

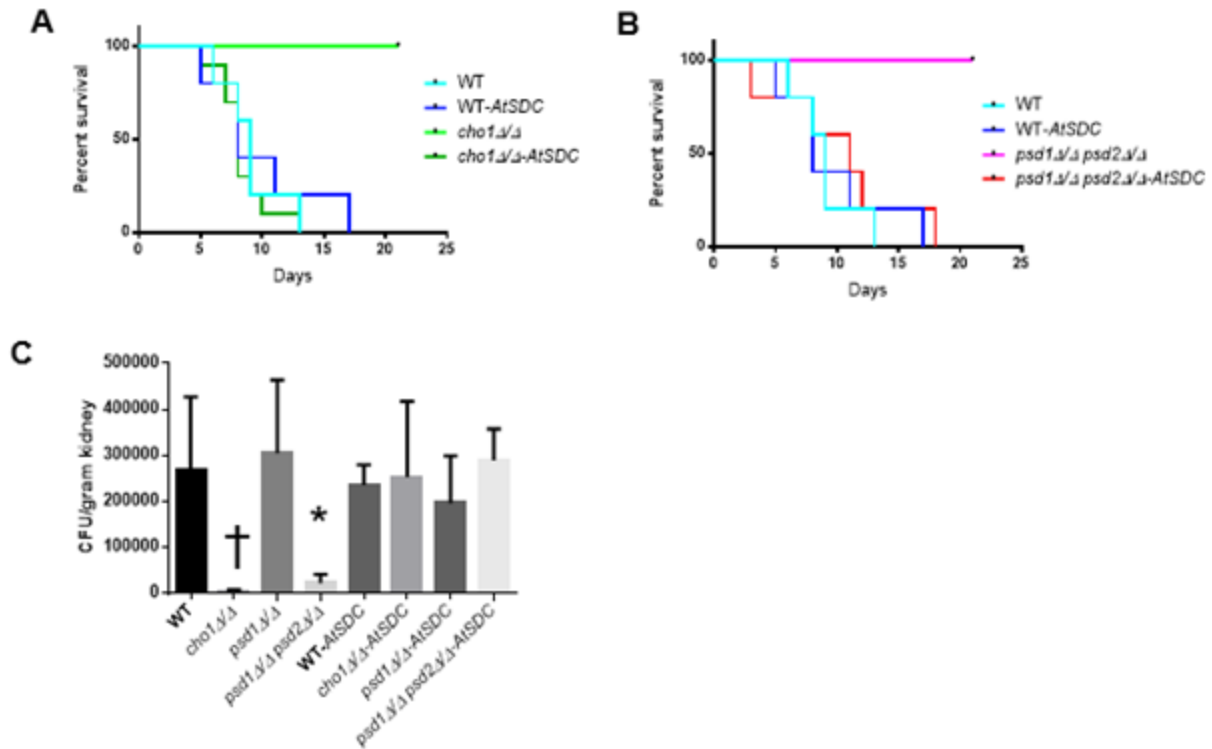
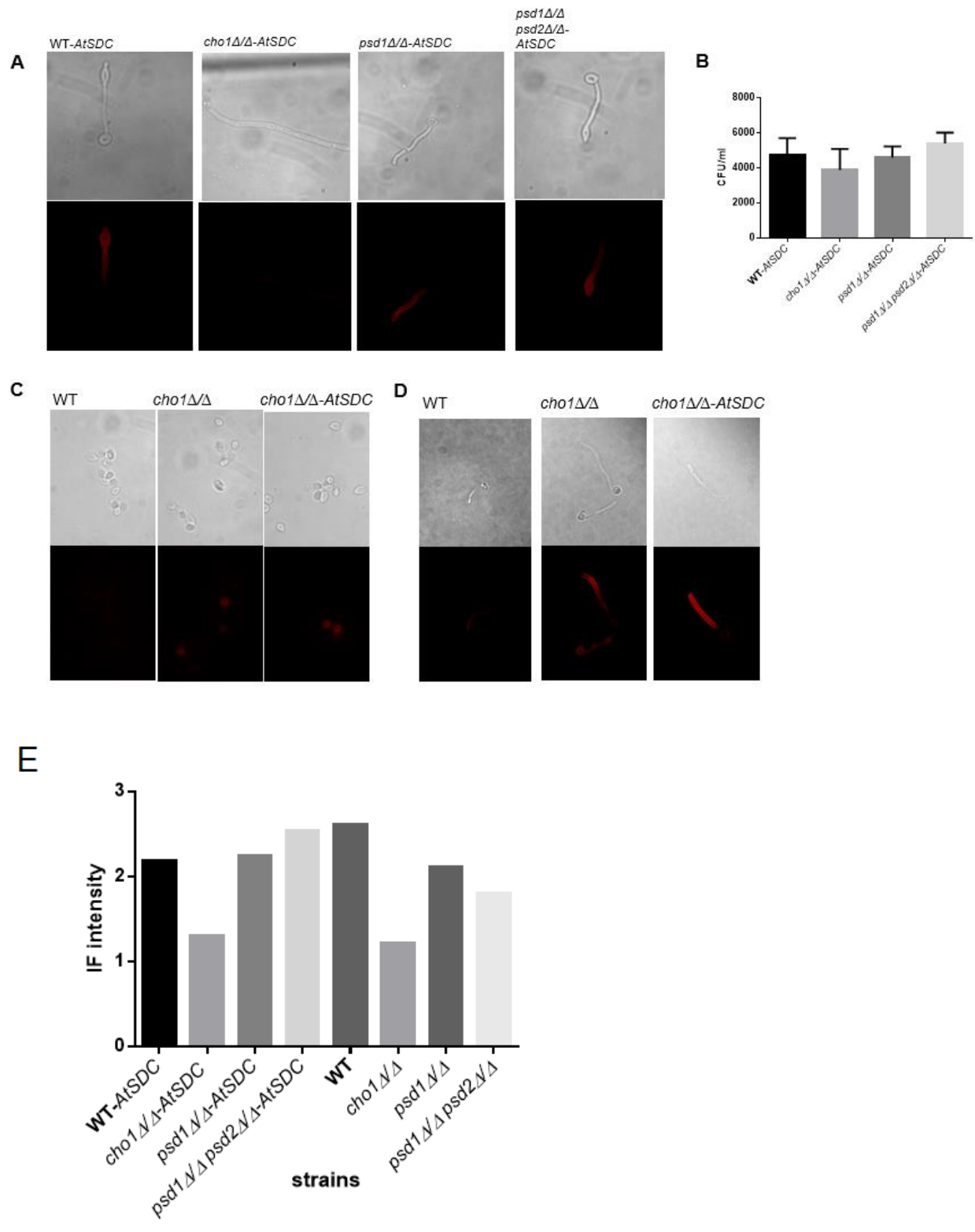


Figure 4.3. Expression of *AtSDC* restores virulence in *cho1*Δ/Δ or *psd1*Δ/Δ *psd2*Δ/Δ in a mouse model of disseminated candidiasis. A, B.. Mice were infected by tail-vein with 5×10^5 cells from each strain and survival was charted over time. **C** Mice were infected with 5×10^5 cells by tail-vein and after five days, they were sacrificed, and kidneys were removed, and CFUs/gram kidney were counted. * P-value < 0.05, Compared to WT. † P-value < .05, Compared to *psd1*Δ/Δ *psd2*Δ/Δ.

Figure 4.4. *AtSDC* restores phenotypes in the *psd1* Δ/Δ *psd2* Δ/Δ mutant, but not *cho1* Δ/Δ .

A The mutant and control strains were induced to form hyphae by serum, and Als3p was examined by secondary immunofluorescence using a monoclonal antibody to Als3p as the primary. **B** The *AtDSC* expressing strains were tested for adhesion to CHO cells in tissue culture. **C** The *cho1* Δ/Δ mutant and *cho1* Δ/Δ -*AtSDC* mutant was stained for β -(1,3) glucan exposure with the anti- β (1,3)-glucan antibody on yeast form. **D** The *cho1* Δ/Δ mutant and *cho1* Δ/Δ -*AtSDC* mutant was stained for β -(1,3) glucan exposure with the anti- β (1,3)-glucan antibody on hyphal form. **E** Quantification of Als3p by measuring relative intensity shows Als3p levels restored for the *psd1* Δ/Δ *psd2* Δ/Δ -*AtSDC* mutant but not the *cho1* Δ/Δ -*AtSDC* mutant.



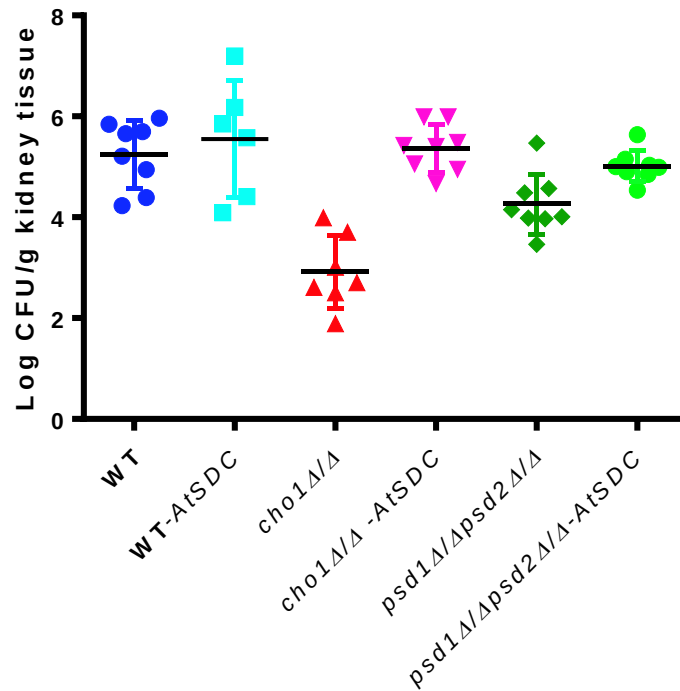


Figure 4.5. Addition of the *AtSDC* gene restores virulence in the *cho1*Δ/Δ and *psd1*Δ/Δ *psd2*Δ/Δ mutants expressing the serine decarboxylase. There are no statistical differences between wild type (WT), *cho1*Δ/Δ-*AtSDC* or *psd1*Δ/Δ *psd2*Δ/Δ-*AtSDC*.

Chapter V: Conclusions and Future Directions

Conclusions of Dissertation

Altogether, the findings in this dissertation illustrate how a loss of phosphatidylserine (PS) for *Candida albicans* via the avirulent *cho1* Δ/Δ mutant results in perturbations in the cell wall, ultimately resulting in disturbances in the surface which enables β -(1,3) glucan to become exposed on the surface. This polysaccharide can then be recognized by effector cells such as macrophages and neutrophils in the host, eliciting an immune response. This is a novel phenomenon showing a link between phospholipid synthesis and cell wall construction and/or maintenance.

In addition to a loss of PS, the *cho1* Δ/Δ mutant is reduced in phosphatidylethanolamine (PE) levels, a relatively abundant phospholipid. Ultimately the question was to determine whether a loss of virulence for the *cho1* Δ/Δ mutant was due to a loss of PS or PE. In order to address this, there was a double mutant generated that could not synthesize de novo PE, which was the *psd1* Δ/Δ *psd2* Δ/Δ mutant. This mutant was also avirulent, suggesting that PE was important for virulence. Since the *cho1* Δ/Δ mutant was shown to have cell wall defects, we speculated that this would lead to differences in proteins on the cell surface that may play a role in virulence. However studies of these proteins revealed that this may not be the case, although there is a difference in the Als3p adhesin protein and a reduction in the oropharyngeal candidiasis (OPC) model for both the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants.

Although the *psd1* Δ/Δ *psd2* Δ/Δ mutant was avirulent, it was unclear if it was due to difficulty with acquiring ethanolamine in the host or if it's Kennedy Pathway was simply insufficient to incorporate cellular ethanolamine into PE. By adding the Serine Decarboxylase to the strains we were able to reveal that the Kennedy Pathway is more than sufficient to support PE synthesis and thus restoring virulence for both the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants, showing us that the mechanism behind the loss of virulence in these phospholipid synthesis mutants is ultimately due to lack of ethanolamine inside the host.

Future Directions

Chen *et al.* discovered that there are significant cell wall aberrations in the *cho1Δ/Δ* mutant, but it is unclear how a loss of phospholipids results in cell wall perturbation. It is also interesting to note that this phenomenon only seems to occur with the *cho1Δ/Δ* mutant but not as much with the *psd1Δ/Δ psd2Δ/Δ* mutant, yet both are avirulent and have phospholipid deficiencies, suggesting that the loss of phosphatidylserine is specifically responsible for most cell wall issues. Our current hypothesis is that the loss of phosphatidylserine affects cell signaling beginning at cell surface receptors, ultimately resulting in downstream transcriptional regulation that regulate cell wall synthesis.

There is ample evidence suggesting that the problems that the *cho1Δ/Δ* mutant has with cell wall integrity as well as temperature and osmolarity sensitivity (44) are mediated by complications of signaling pathways. Data that supports this hypothesis is a western blot showing the high osmolarity pathway (HOG pathway) being constitutively activated in the *cho1Δ/Δ* mutant but becomes reduced when supplemented with sorbitol, which helps to stabilize osmolarity outside of the cell (Fig. A5) (44). This finding correlates with data previously generated in our lab, with the *cho1Δ/Δ* mutant having poor growth in regular YPD media but is restored when sorbitol is added to the media (44). The HOG pathway as well as the cell wall integrity (PCK) pathway are also implicated in cell wall stress, which can be activated by osmolarity stress (158). Interestingly, our western blots reveal that the Pkc1p-mediated pathway is constitutively activated for the *cho1Δ/Δ* mutant and does not seem to be rescued with any addition of sorbitol, which may indicate other factors are at play (Fig. A5). Additionally, the PKC pathway can be activated by caspofungin which induces cell wall stress. When the strains are stimulated, we see once again that the *cho1Δ/Δ* mutant is constitutively activated but no other strains are (Fig. A6). Additionally, under caspofungin stimulation the Hog1p seems to phosphorylate less for the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants than the wild type, suggesting

that there may be some inverse relationship between activation of the PKC1 and Hog1 pathways (Fig. A6). Other pathways that may be involved include the CEK pathway, which is also known to regulate cell wall reconstruction by regulating the switch from yeast to filamentous growth (113, 159). The Cek1p pathway regulates this filamentation by regulation of the GTPase Cdc42p, which when active is polarized to the hyphal tip to promote elongation. (113). In *Saccharomyces cerevisiae*, the *cho1Δ/Δ* mutant has a deficiency in Cdc42p polarization, leading to delayed bud formation (160). Due to the fact that the *cho1Δ/Δ* mutant has a complete lack of PS, the Cdc42p may have difficulties localizing to the proper location on the membrane. We have some preliminary evidence that the *cho1Δ/Δ* mutant does not have Cdc42p polarized to the hyphae, which also coincides with the *cho1Δ/Δ* mutant developing hyphae slower than our other strains in certain growth conditions (44).

Cdc42p has a sequence of 4 lysine residues near the C-terminus, lending it a general positive charge on its surface (161). This positive charge may have interactions with the PS located on the inner leaflet of the cell membrane, assisting in co-localization between the PS in the membrane of the emerging hyphae and Cdc42p. In order to determine whether Cdc42p mis-localization is influencing the cell wall architecture of the *cho1Δ/Δ* mutant, a Cdc42p mutant can be designed with targeted mutations in the important Lysine residues, changing them to a less positively charged amino acid (such as aspartic acid). This would potentially mimic the absence of PS in an otherwise wild-type cell. Alternatively, a hyperactive allele of Cdc42p, when expressed in *C. albicans* may restore localization in the *cho1Δ/Δ* mutant. If localization is restored, the cell wall phenotype of the constitutive mutant will be observed by electron microscopy, β -(1,3) glucan staining and SDS and caspofungin treatment. If Cdc42p also plays a role in any cell signaling, Mkc1p activation and Hog1p activation can be tested by western blot.

However, it is possible that mutations in the Lysine-containing motif do not affect Cdc42p localization and its downstream effects. If this is the case, an alternative approach would be to

transform a hyper-active Ste11 into the *cho1* Δ/Δ mutant (162). Ste11 is a Mitogen Activated Protein Kinase Kinase Kinase (MAP-KKK) that is activated downstream of Cdc42p, and if active can circumvent the issues with Cdc42p localization (163). If addition of this hyper-active Ste11p allele by transformation into the *cho1* Δ/Δ mutant reduces cell wall perturbations and sensitivity, it would indicate that the Cek1p pathway is involved.

As the *cho1* Δ/Δ mutant is also the only mutant that we have discovered to have increased exposure of β -(1,3) glucan, future directions will be conducting a large scale screening of mutants in various signaling pathways to determine if there are other mutants that have this exposure of β -(1,3) glucan, which would provide stronger evidence that there are signaling pathways at play.

Table 1. *Candida albicans* strains

Strain	Parent	Genotype	Source of Ref.
SC5314	Clinical isolate	Prototrophic Wild Type	(164)
YLC337		<i>cho1Δ/ cho1Δ</i>	(44)
YLC344		<i>cho1Δ/cho1Δ::CHO1-SAT1</i>	(44)
YLC280		<i>psd1Δ/ psd1Δ</i>	(44)
YLC294		<i>psd1Δ/ psd1Δ::PSD1-SAT1</i>	(44)
YLC375		<i>psd2Δ/psd2Δ psd1Δ/psd1Δ</i>	(44)
SED010	SC5314	Wild Type- <i>P_{ENO1}-GFP-SAT1</i>	This study
SED013	YLC337	<i>cho1Δ/ cho1Δ -P_{ENO1}-GFP-SAT1</i>	This study
SED016	YLC280	<i>psd1Δ/ psd1Δ -P_{ENO1}-GFP-SAT1</i>	This study
SED019	YLC375	<i>psd2Δ/psd2Δ psd1Δ/psd1Δ</i> <i>- P_{ENO1}-GFP-SAT1</i>	This study
SED022	YLC344	<i>cho1Δ/Δ::CHO1</i> with SAT1 flipped out	This study
SED024	SED022	<i>cho1Δ/ cho1Δ::CHO1 -P_{ENO1}-GFP-SAT1</i>	This study
SED025	SC5314	WT:: <i>Rim101-V5-SAT1</i>	This study
SED026	YLC337	<i>cho1Δ/cho1Δ::Rim101-V5-SAT1</i>	This study
SED027	YLC280	<i>psd1Δ/psd1Δ::Rim101-V5-SAT1</i>	This study
SED028	YLC375	<i>psd2Δ/psd2Δ psd1Δ/psd1Δ::</i> <i>Rim101-V5-SAT1</i>	This study
SED029	YLC375	<i>psd2Δ/psd2Δ psd1Δ/psd1Δ::PSD1-SAT1</i>	This study
SED030	SC5314	SC5314:: <i>AtSDC::SAT1</i>	This study
SED031	YLC337	<i>cho1Δ/cho1Δ::AtSDC::SAT1</i>	This study
SED032	YLC280	<i>psd1Δ/psd1Δ::AtSDC::SAT1</i>	This study
SED033	YLC375	<i>psd2Δ/psd2Δ psd1Δ/psd1Δ</i> <i>::AtSDC::SAT1</i>	This study

Table 2. Primers used in this study

Primer	Sequence	Use
SDO17	aaaaccatggATGGTTGGATCTTTGGAATCTGA	AtSDC plasmid
SDO18	aaaaagatctCTAGACCATGCTCCTACCTAGA	AtSDC plasmid
JCO165	aaagaattcCGTCAAAACTAGAGAATAATAAAG	Rim101p-V5 plasmid
JCO166	aaagaattcGTTTTCTGGGGAGGGTATTT	Rim101p-V5 plasmid
TRO892	AAAAGGGCCCTTTTGCATCCATAAACAGAGTACA	Rim101p-V5 plasmid
TRO893	AAAACCTCGAGAGGTCAAGCTGGTGTGTTGTA	Rim101p-V5 plasmid
SDO20	GTCGTCGTAGTTCAATCC	Rim101p-V5 plasmid
SDO22	aaaaaaACCGGTGGTAAGCCTATCCCTAACCTCTCC	
	TCGGTCTCGATTCTACGGCAAGTGAGAGTACTGC	Rim101p-V5 plasmid

Table 3. Quantification of the Occurrence of Abnormal Hyphae in Mutants

Strain	Cells with Deformity	Cells Total	Percentage (%)
<i>SC5314</i>	0	37	0.00%
<i>cho1Δ/Δ</i>	11	23	47.83%
<i>cho1::CHO1</i>	0	13	0.00%
<i>psd1Δ/Δ</i>	0	29	0.00%
<i>psd1::PSD1</i>	0	19	0.00%
<i>psd1Δ/Δ psd2Δ/Δ</i>	11	21	52.38%

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Appendix. Additional Figures

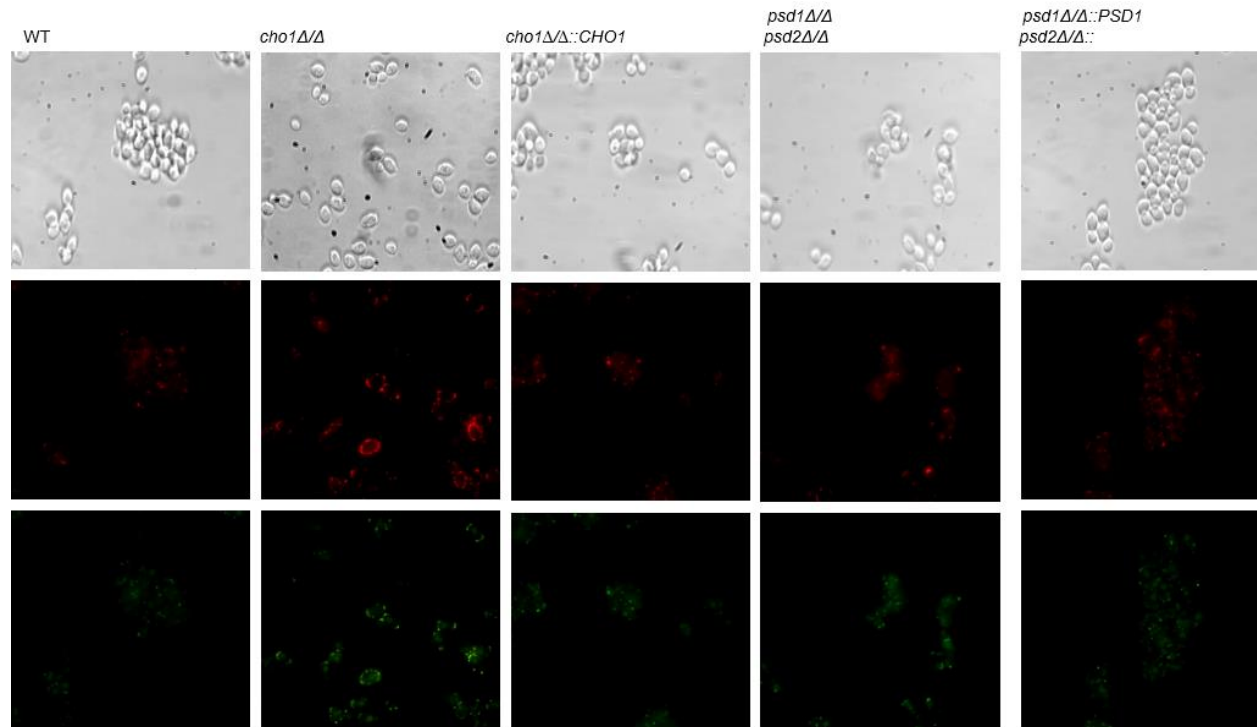


Figure A1. β -(1,3) glucan and Dectin-1 co-staining immunofluorescence of yeast. Top row panels: Brightfield optics. Middle row of panels: Staining with anti- β -(1,3) glucan antibody and C3 (red) conjugated secondary antibody. Bottom row of panels: Staining with sDectin-1-Fc and C2 (green) conjugated to secondary antibody.

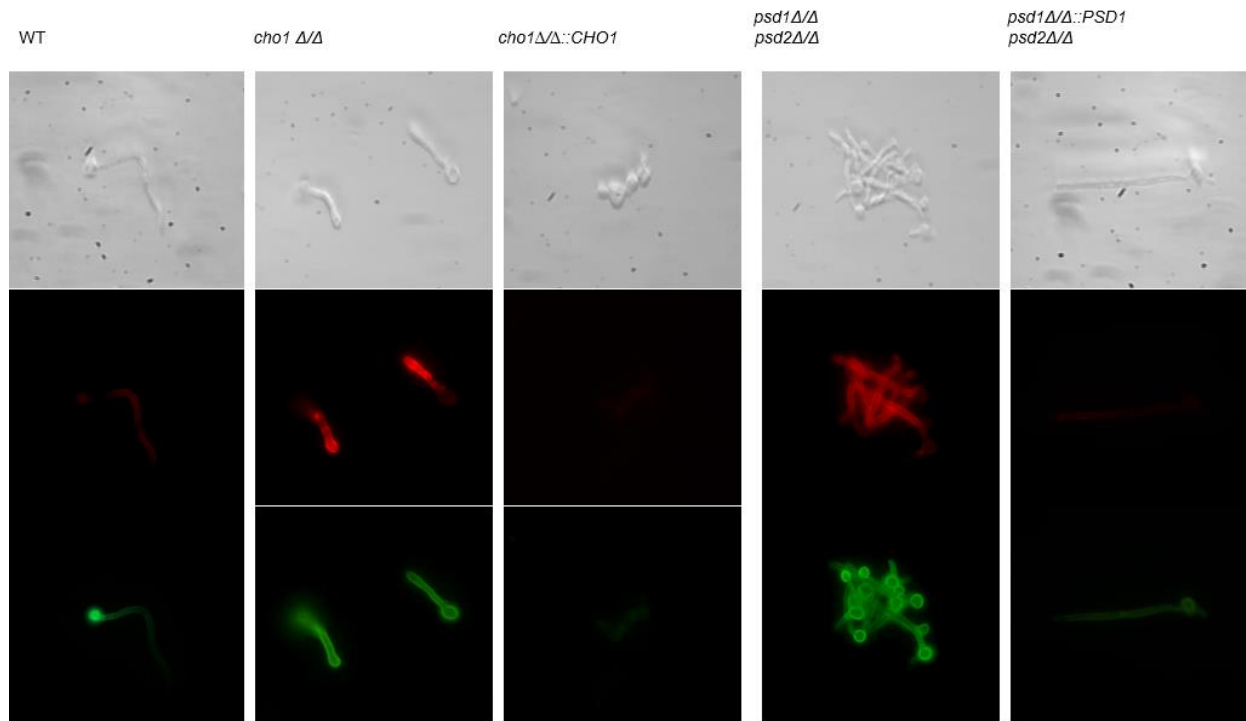


Figure A2. β -(1,3) glucan and Dectin-1 co-staining immunofluorescence of hyphae.

Staining illustrates the co-localization of β -(1,3) glucan staining and binding of the Dectin-1 receptor. Red = β -(1,3) glucan. Green = Dectin-1.

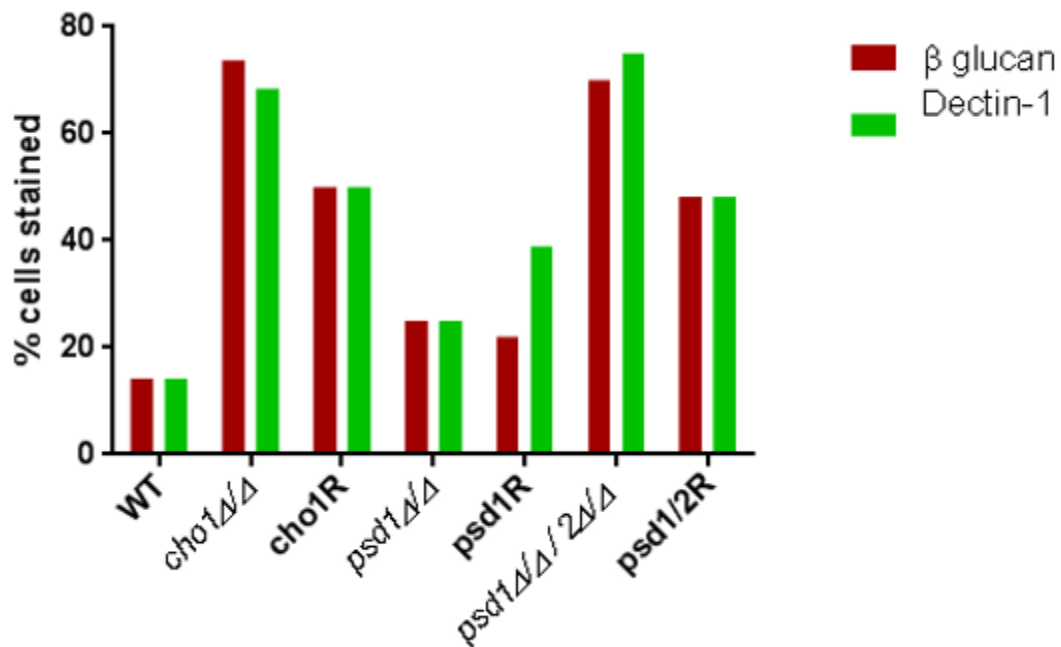


Figure A3. Hyphae co-staining is comparable between the β -(1,3) glucan antibody and sDectin-1-Fc. The number of hyphae for a given field of view that stained with β -(1,3) glucan and Dectin-1-Fc were independently divided by the total number of hyphae apparent by bright field optics to calculate the percent stained for each fluor. N=20 per strain. “R” after strain name denotes reintegration.

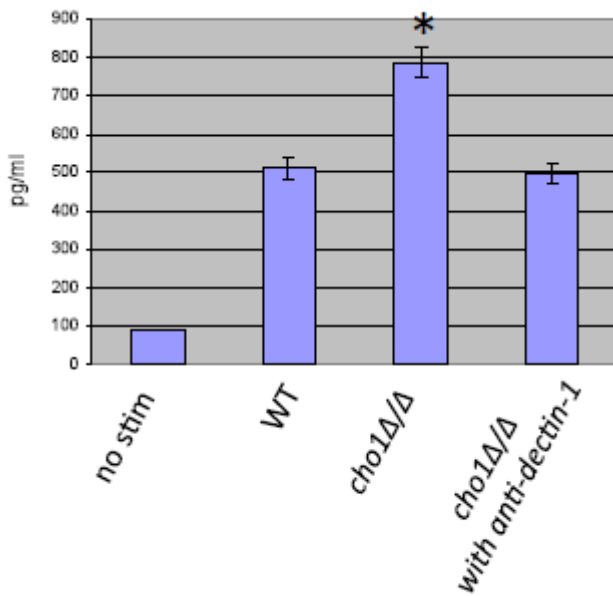


Figure A4. ELISA of TNF α elicitation of RAW264.7 macrophages in response to strains reveals that only the *cho1* Δ/Δ mutant elicits production, and is mediated by the Dectin-1 receptor. The *cho1* Δ/Δ mutant is elevated in TNF α elicitation after an 8 hour incubation, and this response is reduced to wild type levels when pre-treated with a neutralizing anti-Dectin-1 antibody.

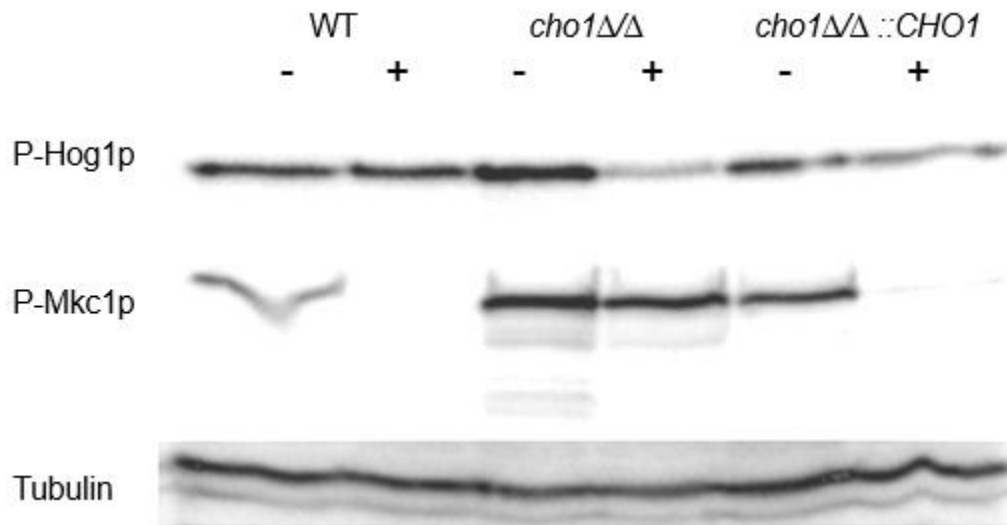


Figure A5. Stimulation of the High Osmolarity Pathway (HOG) with sorbitol reveals the *cho1Δ/Δ* mutant is activated differently than wild type. Stimulation reveals that sorbitol reduces activation of the HOG pathway via a decrease in Hog1p activation, but does not have an effect on the cell wall integrity pathway (mediated by Mkc1p). Also, this western reveals that the cell wall integrity pathway is constitutively activated in the *cho1Δ/Δ* mutant. Stimulation was 20 minutes with 1M sorbitol added to YPD.

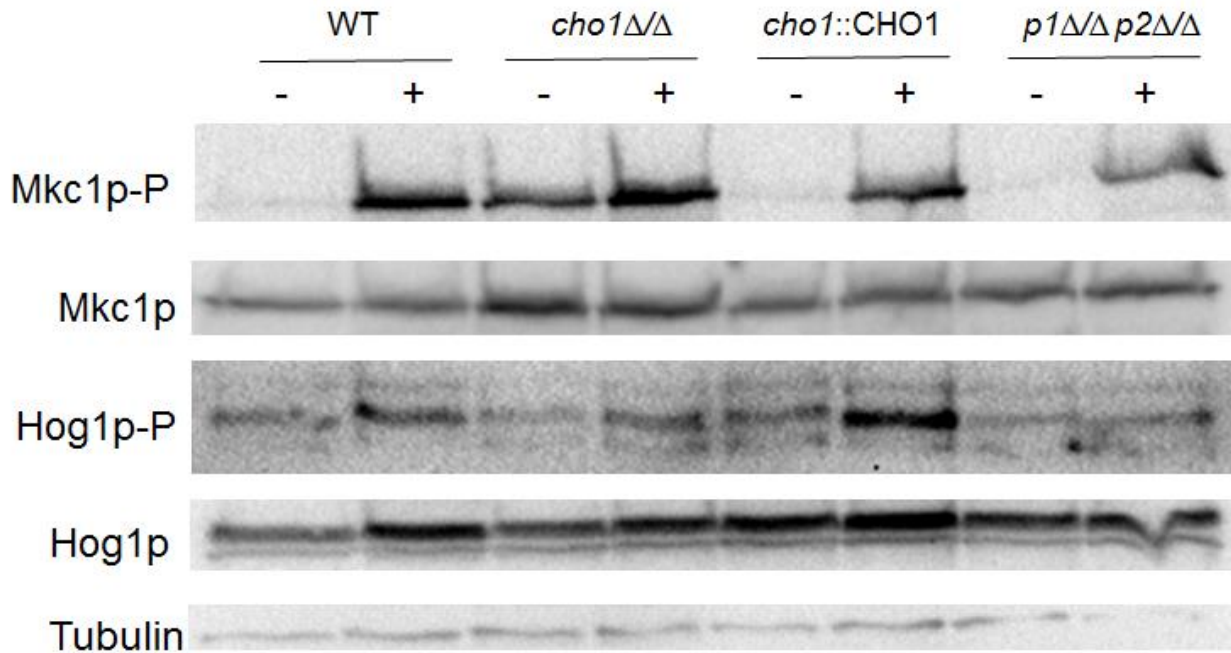


Figure A6. Stimulation of the cell wall integrity pathway and Hog1 pathway with caspofungin. When this pathway was stimulated, it was revealed that the *cho1Δ/Δ* mutant's Mkc1p phosphorylation (Mkc2p-P) is constitutively active with or without stimulation, but the wild-type, *cho1Δ/Δ* reintegant (*cho1::CHO1*) or the *psd1Δ/Δpsd2Δ/Δ* mutants (*p1Δ/Δp2Δ/Δ*) do not. Stimulation was 15 minutes with 125 ng caspofungin in YPD media.

Materials and Methods

Protocols pertaining to Appendix Figures A1, A2, A3 and A4 are described in the materials method section of Chapter 2.

Western Blot

For both the Hog1p and Mkc1p westerns, the experimental setup were the same. The day prior to stimulation, 5 mL of YPD were inoculated from plated strains and incubated overnight at 30°C. The next day, these strains were diluted into flasks with 40 mL of fresh YPD and grown for 3 hours at 30°C in a shaking incubator.

For the Hog1p western, the cultures were transferred to conicals and pelleted at 3300 rpm for 5 minutes. The pellets were then washed in 15 mL ddH₂O and pelleted again. After this, the pellets were resuspended in 40 mL YPD containing 1mL sorbitol and transferred to new flasks. These flasks were then incubated in a 30°C shaking incubator for 20 minutes and then pelleted after stimulation. The pellet was then resuspended in protease and phosphatase inhibitors (Roche 4906845001) and bead beat and extracted in the same manner as the Rim101 western mentioned in Chapter 3. SDS-PAGE was run with 20 ug protein, and transferred overnight at 10V. Primary antibodies were anti-P-Mkc1p (Cell Signaling Cat #4370), anti-P-Hog1p (Cell Signaling Cat# 9216), and anti-tubulin (AbD Serotec, cat# MCA78G). Primary incubations were performed with antibodies diluted 1:2000 in 5% BSA in TBST and incubated overnight at 4°C. Membranes were washed 3X quickly with TBST, then another 2X for 5 minutes with TBST. Secondary antibodies conjugated to horseradish peroxidase (HRP) were then incubated at 1:5000 in 5% with TBST, with anti-rat (for tubulin) (Jackson Immuresearch Cat#112-035-175), anti-mouse (for P-Hog1p) (Jackson Immunoresearch Cat# 315-035-008), and anti-rabbit (for P-Mkc1p) (Jackson Immunoresearch Cat# 211-032-171) for 20 minutes at room temperature. 3 quick washes then 2X 5 minute washes were once again performed in TBST. Western blot was developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific cat # 34080).

For the Mkc1p western, cells were grown to stationary phase overnight in 5mL YPD. The next day, strains were diluted to a concentration of 0.1 O.D./mL in 40 mL YPD, and incubated at 30°C in a shaking incubator for 3 hours. After this time, cells were stimulated with 125 ng/mL caspofungin for 10 minutes in a shaking incubator at 30°C (Mitchell 2006). Cells were centrifuged at 3300 rpm for 5 minutes and pellets were washed in 10 mL PBS. After washing and re-pelleting, cells were resuspended in 100µL of PBS with 1X protease inhibitor (Roche 04-693-124-001) and 1X phosphatase inhibitor (Roche 04-906-001) and transferred to a screwcap microcentrifuge tube. The cell slurry was then bead beat 3X for 1 minute each time, and then pelleted at 13000 rpm. Supernatant was then transferred to a new microcentrifuge tube. Bradford protein assay was performed to ascertain the total protein, then 20 µg of samples suspended in Laemmli buffer were boiled and then separated on 10% acrylamide gels for 90 minutes at 100 Volts at 4°C, then transferred overnight at 10 Volts at 4°C onto PVDF membrane. The next day, membranes were blocked for 1 hour in 5% BSA in TBST. Primary antibodies were anti-P-Mkc1p (Cell Signaling Cat #4370), anti-Mkc1p (Cell Signaling Cat# 9107), anti-P-Hog1p (Cell Signaling Cat# 9216), anti-Hog1p (Santa Cruz Cat# sc-9079), and anti-tubulin (AbD Serotec, cat# MCA78G). Primary incubations were performed with antibodies diluted 1:2000 in 5% BSA in TBST and incubated for 90 minutes at 4°C. The membranes were then quickly washed 3X with TBST, then another 2X for 5 minutes with TBST. Secondary antibodies conjugated to horseradish peroxidase (HRP) were then incubated at 1:5000 in 5% with TBST, with anti-rat (for tubulin) (Jackson Immuresearch Cat#112-035-175), anti-mouse (for P-Hog1p, Hog1p and Mkc1p) (Jackson Immunoresearch Cat# 315-035-008), and anti-rabbit (for P-Mkc1p) (Jackson Immunoresearch Cat# 211-032-171) for 20 minutes at room temperature. 3 quick washes then 2 5 minute washes were once again performed in TBST. Western blot was developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific cat # 34080).

Vita

Sarah Elizabeth Davis was born in Florence, S.C. on September 16, 1983. She graduated from West Florence High School in 2002, and pursued a B.S. in Microbiology at Clemson University which she completed in December 2006. After graduation she worked at Virginia Tech under the guidance of Dr. Liwu Li where she researched the role of the innate immune system's Interleukin-1 Associated Receptor (IRAK-1) in the role of diabetes and cardiovascular disease. After completing almost 2 years of research and helping colleagues to complete 2 research papers, she joined the University of Tennessee in August 2009.