

**The Identification and Characterization of Anti-Virulence Genes Within
Candida albicans Phospholipid Biosynthetic Pathways**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

**Robert Nielsen Tams
August 2019**

Copyright © 2019 by Robert Nielsen Tams
All rights reserved.

Dedication

This work is dedicated to my father, Dr. James F. Tams, who was always my best advocate, gave me the tools necessary to accomplish anything, instilled within me his competitive nature, and always pushed me to accomplish all tasks to the best of my ability.

Acknowledgements

First and foremost, I would like to thank my PhD advisor, Dr. Todd Reynolds, a.k.a. “The Toddfather,” for his guidance, patience, and understanding during my time in his Lab. I feel extremely lucky to have been a part of your group and will never forget the time spent here. You always said graduate school would be the “best ten years of my life!” and while it fortunately didn’t last that long, it was a great experience. Also, thank you for the multiple research assistantships that you funded, for funding so many trips to conferences, and for allowing me to branch out to chase pet projects of mine. Dr. Liz Fozo, thank you for acting as my undergraduate advisor (from Russia with love!) and as my backup PhD advisor on days when I couldn’t find Todd in his office, and also for being so frequently available for great discussions, scientific or otherwise, and for your continued advice on so many matters. I would like to thank the other members of my doctoral committee, Dr. Tim Sparer and Dr. Gladys Alexandre, for your direction and advice throughout my PhD work. I don’t think I could have asked for a better committee. Elizabeth McPherson, you have been such an excellent teaching mentor and friend. Thank you so much for your constant understanding, and I have always appreciated the great conversations we’ve had over the years.

Thanks to my lab-mates both past and present, you guys are amazing. Sarah Davis, thanks for taking me under your wing when I first joined the lab and for teaching me how to do mouse work. Chelsi “Chelsius” Cassilly, thank you for all the help with experiments, advice, and all the laughs. Tian Chen, I have always appreciated your support over the years, thanks for being a great friend. I would also like to thank Kyle Bonifer (I doubt you’ll ever read this, but I spelled your name correctly this time!) and Andy

Wagner for the help both in and outside the lab over the years. It takes a team for science to work, and our lab had a great group of friends that was always willing to help when needed. Karissa Cross, thanks for letting Ellis fry hot chicken, man that stuff is good. I would like to thank Joseph Jackson for help dissecting untold numbers of mice, and for teaching me flow cytometry. Thanks to Jia Wen and John Harp for the great scientific discussions. Also, Jia, I sincerely miss the constant reminders that “I have fat in my brain.” To the Wilhelm lab, thank you for your insights on transcriptomic analysis (Dr. Lauren Krausfeldt, Eric Gann, and Dr. Robbie Martin) and for the many discussions we’ve had over the years at the breweries of Knoxville.

Dr. Lauren Krausfeldt, thank you so much for your continued support over the years, I don’t think that I could have made it this far without you. Thank you for always pushing me to strive for excellence and for believing in me. Thanks for all the scientific discussions, the countless times you listened to or edited my presentations, and for being there for me when I needed it the most.

Finally, I would like to thank the entire Microbiology department, as there are so many people that have supported my efforts throughout this journey, and there are so many of you that I consider great friends that have not been mentioned here.

Abstract

Human fungal pathogens, which cause approximately two million life-threatening infections annually, represent a leading cause of morbidity and mortality for immunocompromised individuals. Numerous fungal pathogens with increased virulence, known as hypervirulence, have been identified, and the study of these organisms merits further investigation as this would lead to a greater understanding of host-pathogen interaction and regulation of virulence in fungi. We have identified two strains of hypervirulent *Candida albicans*, which is the most frequent cause of systemic fungal infections worldwide. These hypervirulent strains have mutations within the two major phospholipid biosynthetic routes in *C. albicans*, known as the cytidine-diphosphate diacylglycerol (CDP-DAG) and Kennedy pathways. Deletion of the Pem1 and Pem2 methyltransferases results in a buildup of the phospholipid phosphatidylethanolamine (PE) within the CDP-DAG pathway, and this correlates with hypervirulence. Similarly, overexpression of the Kennedy pathway, via constitutive expression of *EPT1*, results in hypervirulence that correlates with increased PE synthesis. Deletion of *EPT1* correlates with reduced PE and reduced virulence. Prior research indicates that decreasing CDP-DAG derived PE, by deletion of the Psd1 and Psd2 enzymes causes a decrease in virulence. Thus, modulation of PE synthesis acts as a rheostat for virulence in *C. albicans*. In this dissertation, we have determined that loss of PE synthesis by the CDP-DAG route results in avirulence due to an inability to obtain sufficient ethanolamine from the host. To determine how increased PE causes hypervirulence, transcriptomic analysis was performed for both high PE and low PE strains. Interestingly, while there were no transcriptional differences identified for the *EPT1* overexpressor, there were increases in

cell wall chitin and also hyphal growth, which suggests that an increase in PE modulates virulence via a post-transcriptional mechanism. While the mechanism has not been fully elucidated, hyphae are a major virulence factor for *C. albicans* that contribute to tissue damage, and it has been demonstrated that chitin modulates immune responses. Taken together, these phenotypes could explain why the *EPT1* overexpressor is hypervirulent. To our knowledge, the *EPT1* overexpressor and *PEM1 PEM2* null mutants are the first descriptions of fungal hypervirulence resulting from mutations in phospholipid biosynthetic pathways.

Table of Contents

Chapter 1 : Hypervirulent Fungal Pathogens	1
Introduction.....	2
Fungal Virulence Factors and Common Virulence-Moderating Genes.....	4
Hypervirulence Driven by Increased Invasion or Filamentation	7
Hypervirulence In Non-Hyphal Pathogens.....	9
Cell Wall Composition and Hypervirulence.....	11
Drug Resistant HVFPs.....	17
Mechanisms by Which Chitin May Contribute to Virulence.....	20
Novel Virulence Moderating Genes	23
References	26
Chapter 2 : Overproduction of Phospholipids by the Kennedy Pathway Leads to Hypervirulence in <i>Candida albicans</i>.....	32
Publication Note	33
Abstract	33
Introduction.....	34
Materials and Methods	38
Results.....	50
Discussion	68
References	73
Appendix B	76
Chapter 3 : Overexpression of <i>Candida albicans</i> <i>EPT1</i> Alters Cell Wall Composition and Hyphal Length	81
Publication Note	82
Abstract	82
Introduction.....	83
Materials and Methods	87
Results.....	94
Discussion	111
References	118
Chapter 4 : <i>Candida albicans</i> Cannot Acquire Sufficient Ethanolamine from the Host to Support Virulence in the Absence of <i>De Novo</i> Phosphatidylethanolamine Synthesis	122
Publication Note	123
Abstract	124
Introduction.....	125
Results.....	128
Discussion	140
Materials and Methods	144
References	151
Chapter 5 : Conclusions and Future Directions	154
Summary of Findings.....	155
Future Directions	159
Concluding Remarks	166
References	168

Vita.....	170
------------------	------------

List of Tables

Table 2.1. <i>C. albicans</i> strains used in chapter two.	39
Table 2.2. <i>E. coli</i> strains used in chapter two.	40
Table B.1. Primers used in chapter two.	77
Table 3.1. <i>C. albicans</i> strains used in chapter three.	88
Table 4.1. <i>C. albicans</i> strains used in chapter four.	145

List of Figures

Figure 1.1. Common fungal virulence factors.....	6
Figure 1.2. Alterations in chitin may modulate host immune response.	22
Figure 1.3. Yeast phospholipid biosynthesis.	24
Figure 2.1. Synthesis of yeast phospholipids.	36
Figure 2.2. The <i>pem1Δ/Δ pem1Δ/Δ</i> mutant is a choline auxotroph.....	51
Figure 2.3. Choline auxotrophy correlates to a loss of PC.	53
Figure 2.4. <i>BTA1</i> overexpression rescues choline auxotrophy.	56
Figure 2.5. Radiolabeling of <i>pem1Δ/Δ pem2Δ/Δ</i>	57
Figure 2.6. The <i>pem1Δ/Δ pem2Δ/Δ</i> mutant is hypervirulent.	59
Figure 2.7. <i>C. albicans</i> dual-purpose ethanolamine/choline phosphotransferase.	61
Figure 2.8. Synthesis of both PC and PE via the Kennedy pathway requires <i>EPT1</i>	64
Figure 2.9. Overexpression of <i>EPT1</i> causes hypervirulence.....	66
Figure 3.1. PCA analysis and housekeeping gene expression profiles.	96
Figure 3.2. Differential expression analysis.	98
Figure 3.3. Heat map of co-expressed genes.	99
Figure 3.4. Differentially expressed pathways in <i>psd1Δ/Δ psd2Δ/Δ</i>	103
Figure 3.5. Overexpression of <i>EPT1</i> reduces β(1,3)-glucan exposure.....	106
Figure 3.6. Caspofungin induced β(1,3)-glucan unmasking.	108
Figure 3.7. Overexpression of <i>EPT1</i> increases total chitin.....	109
Figure 3.8. Overexpression of <i>EPT1</i> results in increased hyphal length.	112
Figure 4.1. Pathways for synthesizing aminophospholipids in <i>C. albicans</i>	127
Figure 4.2. The <i>cho1Δ/Δ</i> and <i>psd1Δ/Δ psd2Δ/Δ</i> mutants are attenuated for virulence during <i>in vivo</i> and <i>in vitro</i> models of OPC.	129
Figure 4.3. 1mM ethanolamine is required to support growth of the <i>cho1Δ/Δ</i> and <i>psd1Δ/Δ psd2Δ/Δ</i> mutants in minimal medium.	131
Figure 4.4. Expression of <i>AtSDC</i> restores ethanolamine prototrophy in <i>cho1Δ/Δ</i> and <i>psd1Δ/Δ psd2Δ/Δ</i> mutants.	133
Figure 4.5. Expression of <i>AtSDC</i> restores virulence in <i>cho1Δ/Δ</i> and <i>psd1Δ/Δ psd2Δ/Δ</i> in a mouse model of systemic candidiasis. A & B.....	135
Figure 4.6. Expression of <i>AtSDC</i> restores virulence to <i>cho1Δ/Δ</i> or <i>psd1Δ/Δ psd2Δ/Δ</i> for <i>in vivo</i> and <i>in vitro</i> models of OPC.	137
Figure 4.7. Expression of <i>AtSDC</i> suppresses <i>cho1Δ/Δ</i> β(1,3)-glucan unmasking in <i>cho1Δ/Δ</i>	138
Figure 4.8. Expression of <i>AtSDC</i> in the <i>cho1Δ/Δ</i> mutant leads to PS production.....	141

Chapter 1 : Hypervirulent Fungal Pathogens

Introduction

Fungal infections are a major cause of co-morbidity and mortality in immunocompromised patients (Perlroth et al., 2007). A recent study performed by the Centers for Disease Control and Prevention estimated that there were approximately 75,000 hospitalizations and almost 9 million outpatient visits for fungal infections in the United States in 2017, which came at an estimated cost of \$7.2 billion (Benedict et al., 2018). Of the fungi that cause disseminated infection, some of the most prevalent infections in 2017 were caused by *Candida*, *Aspergillus*, *Histoplasma*, *Cryptococcus*, and *Pneumocystis* spp. (Benedict et al., 2018). Worldwide, there are over 2 million reported cases of life-threatening infection and ~1.7 billion cases of superficial mycoses each year as a result of these five fungi (Brown et al., 2012). The world-wide health crisis resulting from fungal infections is worsened by an increasing prevalence of antifungal resistance, and multi-drug resistant fungal infections (Revie et al., 2018). In addition, the increased frequency at which systemic fungal infections are observed can be partially attributed to medical advances that increase the longevity of immunosuppressed individuals (Perlroth et al., 2007). Compounding these issues, novel fungal pathogens have emerged (e.g. *Candida auris*), and there is a common yet understudied phenomenon of hypervirulence in human fungal pathogens (Brown et al., 2016; de Cassia Orlandi Sardi et al., 2018). As a combined result of these factors, there will likely be a rise in the incidence of life-threatening fungal infections and fungal associated mortality for immunocompromised individuals unless there is continued development of novel antifungals, which will require continued advances in our understanding of fungal virulence and host-pathogen interactions.

One method we can use to develop a better understanding of fungal virulence and

regulation of virulence, is to investigate the phenomenon of “enhanced virulence,” which has been widely observed in fungi and is commonly referred to “hypervirulence.” While hypervirulence can encompass a variety of phenotypes, such as lower infectious dose or increased damage against tissue culture cells, the term typically refers to occasions in which an organism obtains a higher fungal burden within the host and can kill the host more rapidly than its wild-type counterpart. Hypervirulent fungi have been isolated from the environment, from patients in a clinical setting, and have also been created in the laboratory (D'Souza et al., 2001; Wheeler et al., 2003; Kamran et al., 2004; Ma and May, 2010; Yu et al., 2018; Tams et al., 2019). The study of these pathogens has led to the identification of specific genes that have a negative impact on virulence, which have been described as “anti-virulence” or “virulence moderating genes,” as disruption of these genes leads to a hypervirulent phenotype (Foreman-Wykert and Miller, 2003; Kamran et al., 2004).

Since mutations that arise in virulence-moderating genes increase the virulence of fungal pathogens, it is important to identify and characterize mutants with this phenotype as the appearance of clinically important hypervirulent fungal pathogens (HVFPs) could increase mortality in susceptible patients, allow for more frequent incidences of infection in immunocompetent individuals, and potentially result in more severe superficial and mucosal mycoses. Furthermore, several studies have identified drug resistant HVFPs (Angiolella et al., 2008). Although antifungal resistance usually results in a fitness cost, there are several examples, notably in *C. glabrata*, in which drug resistance correlates with the development of hypervirulence (Vale-Silva and Sanglard, 2015). If drug resistance is a selective pressure for hypervirulence, this could create dual-threat fungal

pathogens as they would not only become more difficult to treat due to reduced anti-fungal efficacy, but because they also have an enhanced virulence phenotype (Angiolella et al., 2008; Ferrari et al., 2009; Vale-Silva and Sanglard, 2015). Given the already problematic rise in anti-fungal drug resistance, it is important to more thoroughly investigate any relationship between drug resistance and hypervirulence.

Besides potential threats posed by HVFPs, the study of these organisms may help answer questions regarding the regulation of virulence factors in fungi and may allow for the identification of novel virulence factors or anti-fungal drug targets. The traditional approach to search for novel drug targets has been to identify mutations which confer a fitness disadvantage or avirulence. Therefore, there has been less focus on those mutations for which an advantage is conferred. However, given the frequency at which hypervirulence has been observed, and because regulatory virulence moderating genes (e.g. transcription factors) are often identified, the identification and evaluation of HVFPs can serve as a tool to develop a more thorough understanding of how fungal virulence is regulated and thus is important for the development of novel antifungal agents.

Fungal Virulence Factors and Common Virulence-Moderating Genes

Saprophytic fungi such as *Aspergillus* spp. typically cause disease only in immunocompromised individuals, however there are some saprophytic pathogens such as *Histoplasma* and *Cryptococcus* spp. that can cause disease in otherwise healthy individuals (Wheat, 2006; Kwon-Chung et al., 2014; van de Veerdonk et al., 2017). Other fungi such as *Candida glabrata* and *Candida albicans* are commensal organisms that can be found in the vaginal, oral, and gut microbiomes, and therefore must maintain the ability to survive host defenses and not damage the host during normal growth (Nobile and

Johnson, 2015). This is a delicate balancing act that can be disrupted if the immune system does not effectively stop fungal overgrowth or cannot respond to fungal mediated damage as a result of immunosuppression (Badiie and Hashemizadeh, 2014). As is the case for other infectious organisms, the ability of these fungal pathogens to cause disease is reliant upon virulence factors that allow for growth in the host, manipulation of host defenses, and damage to the host. The major virulence factors in fungi vary depending on species, but can include growth at 37°C, temperature dependent dimorphism, formation of capsule, invasive growth, evasion from immunosurveillance or escape from immune cells, and the production of lipases, proteases, adhesins, invasins, and toxic peptides (Ghannoum, 2000; Naglik et al., 2003; Yang, 2003; O'Meara and Alspaugh, 2012; Mayer et al., 2013; Badiie and Hashemizadeh, 2014) (Figure 1.1, A-D).

Mutations in virulence moderating genes are frequently identified in HVFPs which cause them to more easily cross the boundary from commensalism to pathogenesis. Such mutations may cause increased expression of virulence factors or an increased readiness to enter a pathogenic state. The most common mutations that are associated with hypervirulence occur within transcription factors, and given that transcription factors regulate numerous genes, it is often difficult to determine which gene(s) the transcription factor impacts that are causative of hypervirulence (Kamran et al., 2004; Ferrari et al., 2009; O'Meara et al., 2010; Kakade et al., 2019). In many cases, hypervirulence caused by mutation in a transcription factor results in several phenotypes that, either independently or together, may drive hypervirulence.

Hypervirulence can be induced not only by alterations in virulence factors presented by the fungus, but also by changes that impact cell wall architecture (Bahnan

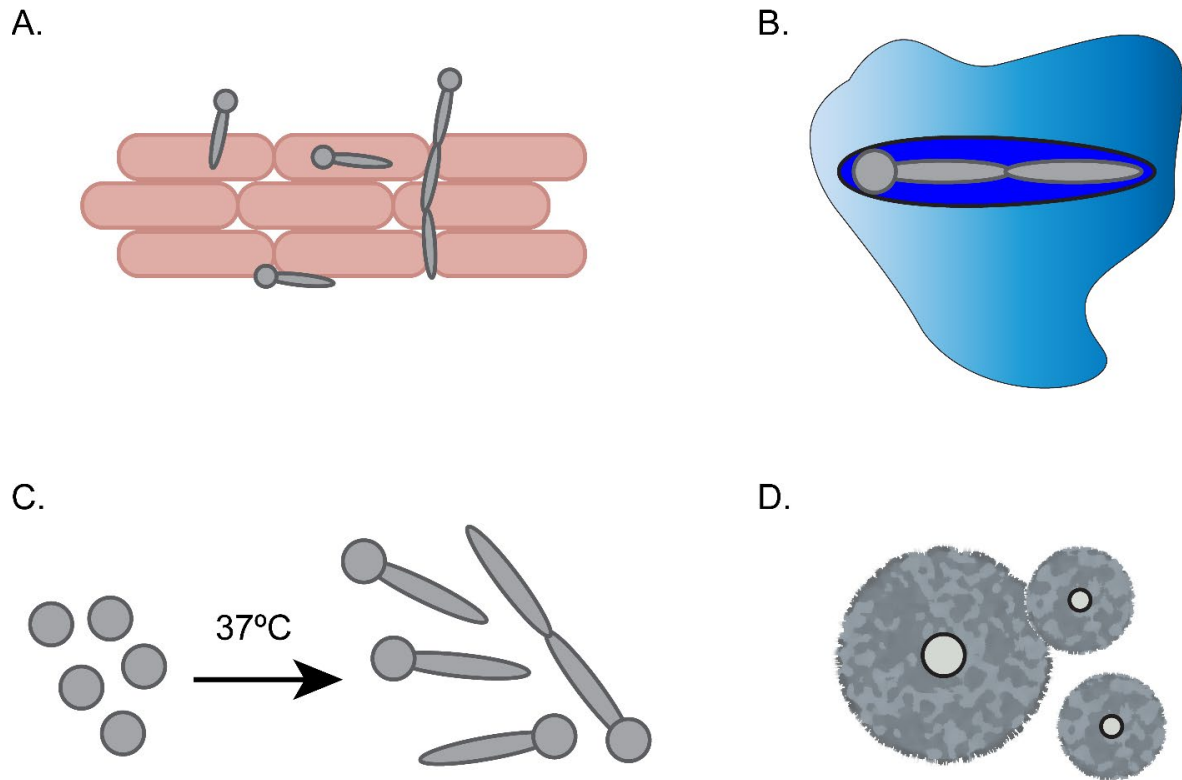


Figure 1.1. Common fungal virulence factors.

(A) Some fungi can form filaments that aid in dissemination across epithelial and endothelial barriers. For example, *C. albicans* can produce hyphae that actively penetrate these barriers or induce their own endocytosis. **(B)** Filamentation can aid in escape from the phagolysosome of immune cells and thus aid in immune evasion. **(C)** Dimorphism, the switch from yeast to pseudohyphae or to hyphae, and in some cases from filamentous to yeast form cells, is an important virulence factor. **(D)** *C. neoformans* can form a polysaccharide capsule that aids in immune evasion.

et al., 2012; Duvenage et al., 2019). It is conceivable that alterations in the cell wall which lead to hypervirulence may occur due to exposure of antigenic cell wall components and subsequent inflammation with immunopathology, or alternatively as a result of decreased fungal antigen recognition by the immune system and immune evasion. In this chapter, several commonly observed phenotypes associated with hypervirulence will be discussed including (i) increased invasion or filamentation, (ii) changes to cell wall composition and subsequent modification of host immune response, and (iii) drug resistance.

Hypervirulence Driven by Increased Invasion or Filamentation

Filamentation is used by some fungi, such as *Candida* and *Aspergillus* spp., to form biofilms or to physically rupture cells in host tissues (Figure 1.1, A) (Mayer et al., 2013; van de Veerdonk et al., 2017). In addition to directly damaging cells, *C. albicans* hyphae can induce their own phagocytosis by endothelial cells to aid in migration across endothelial cell barriers, and hyphae can aid in immune evasion as they mediate escape from the phagolysosome of granulocytes (Figure 1.1, A-B). (Wachtler et al., 2012; Mayer et al., 2013). The formation of hyphae is arguably one of the most important virulence factors for *C. albicans*, as locking it in the yeast form results in avirulence in the mouse systemic model of infection, and also because there are numerous virulence factors for which expression is dependent upon formation of hyphae (Figure 1.1, C). (Lo et al., 1997; Wilson et al., 2016). Avirulence of yeast-locked cells is most likely due to a combined loss of the ability to actively penetrate cells and to induce endocytosis, as well as down-regulation of hyphal associated virulence factors (Wilson et al., 2016). Interestingly, locking *C. albicans* in the filamentous form also results in avirulence, presumably due to an inability to disseminate effectively (Braun and Johnson, 1997; Murad et al., 2001).

Therefore, it is important to be able to switch between the yeast and hyphal forms for the organism to be virulent. Several studies have shown that hypervirulence of *C. albicans* is correlated with an increased filamentation phenotype (Angiolella et al., 2008; Carlisle et al., 2009; Bahnan et al., 2012; Duvenage et al., 2019). This suggests that pushing *C. albicans* towards increased filamentation causes increased virulence, however *C. albicans* must still be able to form yeast cells or virulence is lost, as is the case for *C. albicans* locked completely in the filamentous state (Braun and Johnson, 1997; Murad et al., 2001).

To determine how the degree of filamentation impacts virulence for *C. albicans*, one study employed the use of a doxycycline repressible system to overexpress *UME6*, which is a transcriptional activator of hyphal formation and other hyphal associated genes (Carlisle et al., 2009). The result of *UME6* overexpression *in vitro* was increased hyphal production and increased agar invasion, and this correlated to hypervirulence in the mouse model of disseminated candidiasis (Carlisle et al., 2009). Importantly, in this mouse model, mice were infected with yeast form cells, and expression of *UME6* was induced within the mouse by the absence of doxycycline in the drinking water. This allowed for yeast form cells to disseminate throughout the mouse, and then for strong formation of hyphae to occur (Carlisle et al., 2009). One caveat to this approach is that a transcription factor was overexpressed, and other hyphal associated genes were induced. Hypervirulence in this mutant is therefore likely due to a pleiotropic effect of increased hyphal formation as well as increased expression of other virulence factors. For example, it was observed that transcription of candidalysin, a small pore forming toxic peptide, was also increased in the *UME6* overexpression strain *in vitro* (Carlisle et al., 2009).

A deletion of the *C. albicans* *PIR32* gene, which encodes a cell wall protein that is homologous to *S. cerevisiae* Pir proteins that are important for cell wall integrity, also results in increased hyphal formation at 37°C in potato dextrose broth (Bahnan et al., 2012). Despite increased hyphal formation, this mutant exhibits a decreased capability to form biofilms, a decreased capacity for adhesion to plastic, and a slightly delayed ability to adhere to epithelial cells (Bahnan et al., 2012). Regardless, the mutant has a stark increase in virulence in the mouse model of systemic infection as compared to the wild-type strain (Bahnan et al., 2012). One possible explanation is that this strain has a drastic increase in the amount of chitin in the cell, which is known to be a stress response to compensate for the loss of cell wall proteins (Bahnan et al., 2012). The authors hypothesize that the increase in filamentation causes increased damage to organs, and that the increased chitin, which is a structural component of the cell wall important for rigidity, may increase resistance to host immune attacks (Bahnan et al., 2012).

Hypervirulence In Non-Hyphal Pathogens

C. glabrata and *C. neoformans* may invade tissue without filamentation, while others are required to transition from mycelium into a yeast state to be able to cause disease, such as *H. capsulatum* (Medoff et al., 1987; Chang et al., 2004; Brunke and Hube, 2013). It is thought that in the case of *C. glabrata*, invasion occurs via induced endocytosis rather than active penetration, however, there have been several observed cases in which *C. glabrata* is able to form pseudohyphae, which could contribute to active penetration (Csank and Haynes, 2000; Brunke et al., 2014).

In fact, a 2014 study demonstrated that the formation of pseudohyphae correlates with a hypervirulent phenotype in *C. glabrata* (Brunke et al., 2014). A microevolution

experiment was performed in which *C. glabrata* was co-incubated with macrophages for a period of six months, and this resulted in the formation of an “EVO” strain that displayed pseudohyphal growth, wrinkled colonies, and a hypervirulent phenotype (Brunke et al., 2014). Hypervirulence of this strain is evidenced by increased macrophage damage at an early timepoint, better escape from macrophages, and an increased ability to kill chicken embryos. In mice, this mutant also exhibited increased brain fungal burden that correlates with an increased immune response, and more rapid weight loss of infected mice as compared to those infected with wild-type (Brunke et al., 2014). However, as with many other hypervirulent phenotypes, filamentation was not the only observation that could result in hypervirulence, as the EVO strain also displayed an increase in cell wall thickness and a separation defect, which has been observed in other hypervirulent mutants (Wheeler et al., 2003; Kamran et al., 2004; Brunke et al., 2014).

There are multiple reasons that shifting the fungal cell population towards increased filamentation may increase virulence. These may include (i) an increased ability to penetrate and damage cells due to mechanical stress (Mayer et al., 2013; Brunke et al., 2014), (ii) an increased ability to escape immune cell phagolysosomes or to be phagocytosed due to large particle size (Wachtler et al., 2012), (iii) expression of other virulence factors can be tied to hyphal formation (Wilson et al., 2016), and (iv) increased ability to translocate across epithelial and endothelial cell barriers as a response to increased induced phagocytosis may aid in dissemination (Wachtler et al., 2012).

Cell Wall Composition and Hypervirulence

The fungal cell wall is the primary interface by which fungi interact with their environment, and a site where numerous virulence factors are presented for interaction with the host (Munro, 2010). The composition of fungal cell walls varies widely between species, but common components may include polymers of glucose like $\alpha(1,3)$ -glucan, $\beta(1,3)$ -glucan, $\beta(1,6)$ -glucan, and mixed $\beta(1,3)/\beta(1,4)$ -glucan. Additional polysaccharide components may include mannan, chitin, and chitosan. Mannan consists of heavily glycosylated cell wall proteins that are typically attached to the cell wall via a remnant of a glycosylphosphoinositol (GPI) anchor or covalent linkage to cell wall glucans (Free, 2013). Chitin is a polymer of $\beta(1,4)$ -N-acetyl glucosamine to which cell wall mannans, glucans, and ultimately cell wall proteins are linked (Munro, 2010). Chitin may be deacetylated into chitosan, which is a more soluble form of the polymer (Free, 2013). Some species also contain the non-polysaccharide molecule melanin in their cell walls, which is formed from the cross linking of phenolic compounds and serves to protect fungi from UV irradiation as well as oxidative damage (Free, 2013). One species that produces melanin, *C. neoformans*, also produces a polysaccharide capsule that decreases fungal immune recognition, modulates phagocytosis, protects from damage caused by host reactive oxygen species, and is considered to be an important virulence factor (Figure 1.1, D.) (McClelland et al., 2006; O'Meara and Alspaugh, 2012). This capsule is mostly comprised of a galactose backbone decorated with mannose, xylose, and glucuronic acid side chains, which has collectively been referred to as glucuronoxylomannogalactan (GXMGal), and while it is evident that capsule is a critical virulence factor for *C. neoformans*, there are conflicting reports in which both hyper-capsulated or hypo-

capsulated mutants strains are avirulent (McClelland et al., 2006; Doering, 2009; Casadevall et al., 2018).

For all fungal pathogens however, maintaining the integrity and composition of the cell wall is important, as mutations that result in cell wall damage or in the exposure of pathogen associated molecular patterns (PAMPs) can result in increased immune recognition, clearance, and virulence attenuation (Brown and Gordon, 2005; Plato et al., 2015). For example, some *C. albicans* strains with increased $\beta(1,3)$ -glucan exposure are avirulent in the mouse model of disseminated candidiasis, and $\beta(1,3)$ -glucan exposure is correlated to increased tumor necrosis factor alpha (TNF- α) release from macrophages *in vitro* (Chen et al., 2010; Davis et al., 2014). While the ability of $\beta(1,3)$ -glucan to induce an immune response has been extensively studied, the role of chitin responsible maintaining cell rigidity is less clear.

Purified fungal chitin may act as a pro-inflammatory or anti-inflammatory molecule depending on particle size (Da Silva et al., 2009). Induction of chitin synthesis occurs in a cell cycle dependent manner, is regulated mostly in a post-transcriptional manner, and expression of chitin synthases is increased upon the dimorphic switch to hyphal morphology in *C. albicans* (Munro et al., 1998; Orlan, 2012). Cell wall stress, such as that induced by the drug caspofungin or by the deletion of important cell wall proteins, may also induce the production of chitin (Wheeler and Fink, 2006). The consequences of altered chitin levels on host-pathogen interactions remain somewhat unclear, however there are several instances in which hypervirulent *S. cerevisiae*, *C. albicans*, and *C. glabrata* strains with increased chitin content have been identified, and this area of study therefore merits further research.

In 2004, Kamran *et al.* identified a virulence moderating gene in *C. glabrata* by screening a transposon library for mutants with an increased ability to persist *in vivo* (Kamran et al., 2004). One mutant was represented by a gene that has homology to the transcription factor *ACE2* in *S. cerevisiae* that is a positive transcriptional activator of the chitinase *CTS1* (Dohrmann et al., 1992). The *C. glabrata ace2Δ* mutant exhibited a clumping phenotype that correlated to a loss of *CTS1* mRNA, and this strain was hypervirulent in a neutropenic mouse model of disseminated candidiasis (Kamran et al., 2004). To test if hypervirulence was due to vascular occlusion, chitinase treatment was performed to stop clumping, and *ace2Δ* cells were still hypervirulent as compared to wild-type. Furthermore, the infectious dose of *ace2Δ* is 200-fold lower than wild-type. Inactivation of *ACE2* also lead to increases in TNF- α and Interleukin 6 (IL-6), both of which are pro-inflammatory cytokines (Kamran et al., 2004).

In a follow up study, a proteomic analysis was performed which determined that there were decreases in several proteins involved in cell wall biogenesis and remodeling in *ace2Δ*, and that hypervirulence was therefore likely a result of changes in host-pathogen interactions, especially given the increase in host immune response (Kamran et al., 2004; Stead et al., 2005). It was further confirmed that *ACE2* regulates *CTS1* in *C. glabrata* in a proteomic analysis of the secretome of the *ace2Δ* mutant (Stead et al., 2010). There was also an observed decrease in a putative endoglucanase, *ETF2*, and there were increases in intracellular proteins on the cell surface (Stead et al., 2010). The authors postulate that the increased leakiness of these intracellular proteins, as well as changes in recognition of antigenic cell wall components may result in the hypervirulence of this strain (Stead et al., 2010). It was not determined if the loss of *CTS1* leads to a

build-up of chitin, and this would be interesting as there are reports that suggest that chitin plays a major role in hypervirulence. As a complement to this, it would also be interesting to determine if *C. glabrata* or *S. cerevisiae* *CTS1* mutants have a hypervirulent phenotype, or if the further changes in the transcriptional profile conferred by loss of *C. glabrata* *ACE2* are required for this hypervirulent phenotype.

Kelly *et al.* investigated *ACE2* in *C. albicans* (Kelly *et al.*, 2004). In *S. cerevisiae* and *C. glabrata* there is a paralog to *ACE2* known as *SWI5* that is not present in *C. albicans*, thus *ACE2* may perform the function of both transcription factors in *C. albicans* (Kelly *et al.*, 2004). In addition, for *C. albicans* there are three chitinase genes (*CHT1*, *CHT2*, and *CHT3*), whereas *C. glabrata* and *S. cerevisiae* have a single chitinase (*CTS1*) (Kelly *et al.*, 2004). In the *C. albicans* *ace2Δ/Δ* mutant, there was an increase in the formation of pseudohyphae, an increase in agar invasion, and hyphae were longer (Kelly *et al.*, 2004). However, this mutant was avirulent in the mouse model of disseminated candidiasis likely due to an inability of the cells to disseminate (Kelly *et al.*, 2004).

As previously discussed, *C. albicans* must be able to transition between both yeast and hyphal forms for virulence. Also, for *C. albicans* there may be a different gene set that is regulated by *ACE2* since there are two paralogs in *S. cerevisiae* and *C. glabrata*. qRT-PCR showed that a loss of *ACE2* caused a decrease in *CHT3* but not *CHT2* (*CHT3* is a functional homolog to *cgCTS1*) (Dunkler *et al.*, 2005). Therefore, it would be interesting to determine if *C. albicans* null mutants of the *CHT1-3* genes would result in avirulence or hypervirulence. In addition, it would be interesting to determine if repression of *C. albicans* *ACE2* via a doxycycline repressible system would be sufficient to induce hypervirulence by driving strong hyphal formation after dissemination, as was performed

for overexpression of *UME6*.

A final study was undertaken with *ACE2* mutants from *C. albicans*, *C. glabrata*, and *S. cerevisiae* to compare the effects of knocking out *ACE2* on virulence for each strain in the same controlled environment and the same mouse model. In this model there was a minimal increase in mean survival time for the *C. albicans ace2Δ* mutant and the *C. glabrata ace2Δ* strain was confirmed as hypervirulent, even using a ten-fold lower inoculum than wild-type. (MacCallum et al., 2006). Hypervirulence correlated to a higher fungal burden in kidney brain and lung (MacCallum et al., 2006). For a virulent strain of *S. cerevisiae* there was a decrease in survival of mice infected with the *ace2Δ* knockout, however there was no difference in fungal burden (MacCallum et al., 2006). Importantly, infection in immunocompetent mice did not result in hypervirulence, which further indicates that the hypervirulence is not due to clumping and vascular occlusion (MacCallum et al., 2006).

Changes to chitin may also influence the hypervirulent phenotype for the previously discussed EVO strain of *C. glabrata*, as it was found that in addition to the formation of pseudohyphae, there was a point mutation in the *CHS2* gene, and that the cells have a separation defect (Brunke et al., 2014). The authors note that a separate clinical isolate that also exhibited pseudohyphal formation, but had no mutation in *CHS2*, also had increased macrophage damage. The authors concluded that since the genetic basis for the pseudohyphal phenotype is not due to a *CHS2* mutation, then the formation of pseudohyphae is driving hypervirulence. This is possible, however since a chitin synthesis defect can drive pseudohyphal growth, it is possible that hypervirulence is related more directly chitin levels. Mutants that show hypervirulence and altered chitin

levels include *C. glabrata ada2Δ*, *S. cerevisiae ssd1Δ*, and the previously discussed *C. glabrata ace2Δ* mutant, and *C. albicans pir32Δ* (Wheeler et al., 2003; Kamran et al., 2004; Bahnan et al., 2012; Yu et al., 2018).

In *C. albicans*, *ADA2* is a component of the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex, which is important for drug tolerance (Yu et al., 2018). In multiple fungi, a loss of this gene confers growth defects and sensitivity to cell wall stressors (Yu et al., 2018). Loss of *ADA2* in *C. glabrata* also increases sensitivity to antibiotics and cell wall stressors, however, the strain was hypervirulent in a mouse model of disseminated infection and infected animals had higher fungal burden in the kidneys, spleen, and brain (Yu et al., 2018). In addition, the strain had increased filamentation, which was also observed for the *C. glabrata ace2Δ* mutant, and there was an increase in TNF- α response in infected mice (Kamran et al., 2004; Yu et al., 2018). Transcriptional profiling determined that 26 adhesin genes were upregulated, which suggests that hypervirulence could be related to greater adhesion to the host and is again attributed to multiple factors (Yu et al., 2018).

Although *S. cerevisiae* is typically not considered pathogenic, there have been occurrences of infection in immunocompromised individuals, and DBA/2 mice are susceptible to infection with some *S. cerevisiae* isolates (Wheeler et al., 2003). In a 2002 study, a strain with a mutation in *SSD1*, which is important for high temperature growth, was demonstrated to be hypervirulent in a systemic mouse model (Wheeler et al., 2003). Interestingly, the *ssd1Δ* strain exhibits an increase in both cell wall $\beta(1,3)$ -glucan and chitin content and has a clumping phenotype similar to the *ace2Δ* mutants (Wheeler et al., 2003; Kamran et al., 2004). The cell wall changes correlate to increased TNF- α , IL-6

and IL1- β *in vitro*, and therefore immune modulation may play a role in the increased virulence (Wheeler et al., 2003). Importantly, Balb/c mice, which are not susceptible to *S. cerevisiae* infection, do not succumb when infected with this mutant, indicating that the death of the DBA/2 mice was not due to vascular occlusion secondary to the *SSD1* clumping phenotype, but rather changes in the cell wall of this mutant (Wheeler et al., 2003).

Drug Resistant HVFPs

There is evidence that drug resistance in fungi may also drive hypervirulence, which is especially concerning given the current and problematic rise in drug resistance (Vale-Silva and Sanglard, 2015). While the correlation between HVFPs and drug resistance is somewhat unclear, it is possible that the mechanisms for development of drug resistance have pleiotropic effects which also allow for greater damage to the host or resistance to the immune response. For example, biofilms are a major virulence factor in fungi that allow them to colonize medical implants and intravenous catheters and are also important for the invasion of host tissues and drug resistance (Nett et al., 2008; Fanning and Mitchell, 2012). A study performed with clinical isolates of *Candida albicans* demonstrated that when serially passaged in the presence of either fluconazole or miconazole, not only did drug resistance emerge, but also hypervirulence (Angiolella et al., 2008). This phenotype was associated with increased hyphal formation and branching, increased agar invasion, increased biofilm formation, and an increased capacity to adhere to polystyrene (Angiolella et al., 2008). These drug resistant isolates had a stark increase in virulence, which was demonstrated in a disseminated mouse model of infection in which 57% and 70% mortality was observed for fluconazole and miconazole resistant strains,

respectively, whereas there was only 10% mortality observed in mice infected with the wild-type *C. albicans* (Angiolella et al., 2008). Impacts observed for these strains that may explain the drug resistance include mutations in *MDR1* and *FKS1* (Angiolella et al., 2008). Given that biofilms confer a fitness advantage for organisms under the pressure of antifungal treatment, and that increased filamentation is correlated with hypervirulence, it is a logical conclusion that the use of antibiotics may, in some cases, drive hypervirulence.

Another example in which drug resistance correlated with hypervirulence was observed in 2009 with *C. glabrata*. It was demonstrated that increased resistance to fluconazole via a gain of function (GOF) mutation in the *PDR1* transcription factor, which regulates the drug efflux pumps *CDR1*, *CDR2*, and *SNQ2*, resulted in hypervirulence even in the absence of fluconazole (Ferrari et al., 2009). The azole resistant isolate had increased kidney fungal burden and caused increased mortality rate in a disseminated model of either immunosuppressed or immunocompetent mice (Ferrari et al., 2009). This was independent of the strain background, as introduction of the hyperactive allele was performed in both drug sensitive and resistant *pdr1Δ* backgrounds, with the same result (Ferrari et al., 2009). Further, four different *PDR1* gain of function alleles (L280F, T588A, E1083Q, and P822L) with single amino acid substitutions, which conferred differential transcriptional increases of *CDR1*, *CDR2*, or *SNQ2*, resulted in hypervirulence and increased kidney burden, indicating that overactivation of *PDR1* by several different mutations can confer hypervirulence (Ferrari et al., 2009). The authors hypothesized that hypervirulence was due differential expression of genes involved in stress response, resistance to DNA damage, and cell wall structure. This was later confirmed with a microarray analysis (Vermitsky et al., 2006).

In the same study, three azole resistant clinical isolates with mutations which did not map to *PDR1* were identified, and it was observed that these mutants had a petite phenotype and mitochondrial dysfunction (Ferrari et al., 2009). However, a fourth hypervirulent azole resistant *C. glabrata* isolate (BP41) with mitochondrial defects has been isolated, and this strain did have increased expression of *PDR1* (Ferrari et al., 2011). Therefore, it is difficult to determine if mitochondrial defects alone may contribute to hypervirulence, or if *PDR1* plays an important role (Ferrari et al., 2011). Regardless, this study demonstrated that a clinical isolate which had acquired fluconazole resistance (BP41) was hypervirulent in the rat vaginal model as well as a mouse model of disseminated candidiasis as compared to its azole susceptible parental strain. In the disseminated model mice infected with this strain had higher fungal burden in both the spleen and kidneys (Ferrari et al., 2011). In addition to increased expression of *PDR1*, BP41 demonstrated an increase in expression of numerous cell wall remodeling genes, as well as GPI anchored aspartyl proteases, which are known virulence factors (Ferrari et al., 2011). Another mutant strain with mitochondrial defects (DSY4339) was created *in vitro* by ethidium bromide mutagenesis in this study, however the virulence phenotype differed in that it had decreased virulence and it did not display increased expression of *PDR1* (Ferrari et al., 2011). The authors did not determine the susceptibility to fluconazole for DSY4339, so it is not clear if there is a correlation of drug resistance to virulence. For *C. albicans*, inhibition of classical respiration processes by treatment with both sodium nitroprusside and salicylhydroxamic acid not only induces increased hyphal formation, but also induces cell wall remodeling, increased expression of virulence factors, and hypervirulence (Duvenage et al., 2019). Therefore, between the two organisms the

relationship between mitochondrial dysfunction and hypervirulence remains unclear, although for *C. glabrata*, *PDR1* overexpression does seem to drive hypervirulence.

Since drug resistance can in some cases result in hypervirulence, then the problems caused by antifungal resistance may be two-fold. The over/misuse of drugs can select for drug resistant organisms that are harder to treat and may aid in the evolution of more aggressive HVFPs, further exacerbating this issue. While the correlation between hypervirulence and drug resistance has been observed numerous times in bacteria, especially in the organism *Klebsiella pneumonia*, there have been less reports of this phenomenon in fungi, and this is therefore an area of research that merits greater investigation (Lee et al., 2017).

Mechanisms by Which Chitin May Contribute to Virulence

Although chitin typically only makes up 3-4% of the fungal cell wall, this component is important for rigidity of the cell and virulence (Gow et al., 2017). It has been clearly demonstrated that chitin impacts the immune response in several studies. For example, when added to peritoneal macrophages, purified fungal particles of intermediate chitin (IC) size (40-70 μm) can induce an inflammatory TNF- α response, whereas small chitin (SC) particles of less than 40 μm induce the strong production of anti-inflammatory interleukin 10 (IL-10), and also the production of TNF- α albeit to a lesser extent than that of IC (Da Silva et al., 2009). It was demonstrated that induction of a TNF- α response for IC was dependent on toll like receptor 2 (*TLR2*) and to some extent the C-type signaling lectin, Dectin-1 (Da Silva et al., 2009). For smaller chitin particles, IL-10 induction from macrophages was completely dependent on Dectin-1, but *TLR2* was dispensable (Da Silva et al., 2009). Other studies have demonstrated that chitin can induce host arginases,

which reduce arginine, a substrate used for the synthesis of reactive nitrogen species (RNS). This causes macrophages to decrease RNS production that would otherwise damage the fungus (Wagener et al., 2017).

As chitin can have an impact on immune response, there are several mechanisms by which changes to cell wall chitin may induce hypervirulence that act oppositely of one another with regard to immune response (Figure 1.2, A-C). Normally, chitin is at the basal layer of the cell wall, and other cell wall components are bound to it, including immunogenic components such as $\beta(1,3)$ -glucan (Figure 1.2, A). Increases in chitin could damage/alter the cell wall and internal components could leak out of the cell, or this may cause exposure of immune epitopes within the cell wall, such as $\beta(1,3)$ -glucan, that increase immune response and therefore produce an increase in immunopathology (Figure 1.2, B). This is supported in several cases of hypervirulence by the observation of increased TNF- α release. Alternatively, overproduction of chitin could result in interaction of chitin with immune cells (Figure 1.2, C). Since chitin could act in an immunosuppressive capacity, this could allow the fungus to avoid the host immune response and therefore continue to grow and damage the host. Besides these hypotheses, increased chitin production or a decrease in chitinase activity may decrease the ability of cells to separate properly, which could result in a clumping phenotype. Clumping of cells may prevent granulocytes from phagocytosing larger groups of cells, and for those cells that have been phagocytosed, clumping may aid in physical pressure that results in escape from the phagolysosome. Most studies investigating the effects of chitin on the immune response have been performed with purified chitin, either from

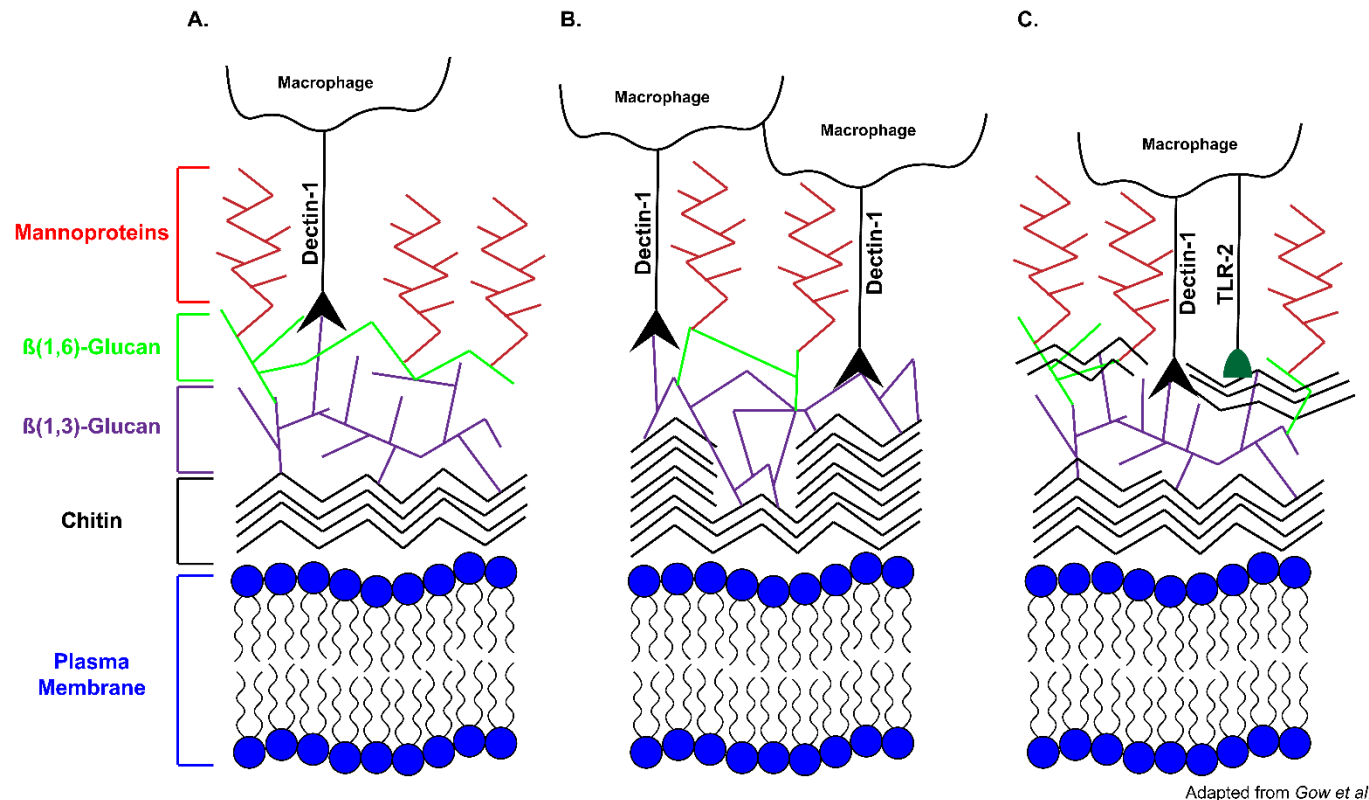


Figure 1.2. Alterations in chitin may modulate host immune response.

The *C. albicans* cell wall is used as a model. The wall contains a chitin base linked to $\beta(1,3)$ -glucan. To $\beta(1,3)$ -glucan, $\beta(1,6)$ -glucans are linked. Heavily mannosylated GPI anchored proteins decorate the outer wall. **(A)** Exposed $\beta(1,3)$ -glucan is an immunogenic epitope, and exposure can occur via host induced damage. Dectin-1 is a C-type signaling lectin that can detect $\beta(1,3)$ -glucan thus inducing a pro-inflammatory response. **(B)** Overproduction of chitin could cause increased exposure of $\beta(1,3)$ -glucan, or other immunogenic cell wall components, resulting in increased immunopathology and hypervirulence. **(C)** Alternatively, increased chitin could be recognized by Dectin-1 and PAMPs such as toll like receptor 2 (TLR2), which could result in immune suppression.

fungal or invertebrate sources, and these chitin particles are typically exceptionally large compared to the size of a fungal cell. Thus, more work should be done with live mutants and strains to determine if varying levels of chitin in the context of live cells impact immune responses.

Novel Virulence Moderating Genes

To our knowledge, no mutations have been identified in fungal phospholipid biosynthetic pathways that result in hypervirulence, until recently. In this dissertation we describe evidence for the first mutations in two separate phospholipid biosynthetic routes for *C. albicans* that, surprisingly, result in hypervirulence. Phospholipid biosynthetic pathways are a requirement for the growth of fungal pathogens, as phospholipids are the major structural components of all cell membranes, and numerous studies have demonstrated that they are not only essential for growth but are also required for virulence (Chen et al., 2008; Chen et al., 2010; Davis et al., 2018). The mutations conferring hypervirulence in *C. albicans* that we have identified lie within two major phospholipid biosynthetic routes known as the cytidine-diphosphate diacylglycerol (CDP-DAG) pathway, and the Kennedy pathway (Figure 1.3). Within the CDP-DAG pathway, CDP-DAG is converted to either phosphatidylinositol (PI) or alternatively to phosphatidylserine (PS) (Figure 1.3, red arrows) (Paulus and Kennedy, 1960; Atkinson et al., 1980; Letts et al., 1983). PS can be decarboxylated to form phosphatidylethanolamine (PE) which can then be methylated to form phosphatidylcholine (PC) (Figure 1.3, red arrows) (Kodaki and Yamashita, 1987; Clancey et al., 1993; Trotter and Voelker, 1995). PC and PE, which are the major phospholipids in yeast, can also be formed via the Kennedy pathway (Figure 1.3, green arrows) (Gibellini and Smith, 2010; Cassilly et al., 2017; Tams et al., 2019). Exogenous

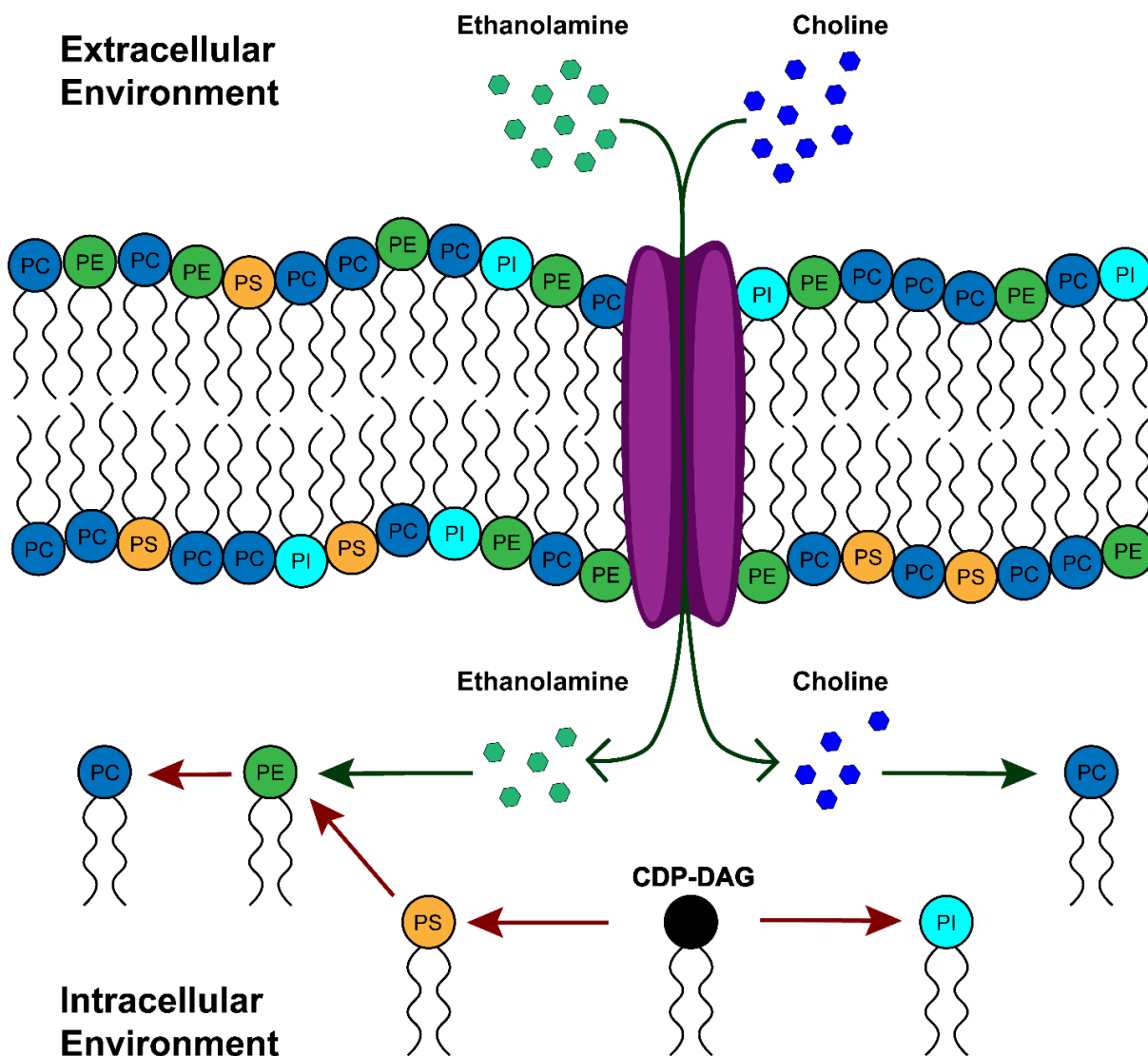


Figure 1.3. Yeast phospholipid biosynthesis.

There are two major routes for phospholipid synthesis in yeast: the cytidine diphosphate diacylglycerol (CDP-DAG) pathway (red arrows), and the Kennedy pathway (green arrows). In the CDP-DAG pathway, CDP-DAG is converted to either phosphatidylinositol (PI) or phosphatidylserine (PS). PS can be decarboxylated to form phosphatidylethanolamine (PE) which can be further methylated to form phosphatidylcholine (PC). Alternatively, exogenous ethanolamine (green hexagons) or choline (blue hexagons) can be imported into the cell and converted into PE and PC, respectively, via the Kennedy pathway.

ethanolamine and choline can be imported into the cell, and once internalized can be converted into PE and PC, respectively, through a three-step enzymatic process (Gibellini and Smith, 2010). Interestingly, overexpression of Ept1, which catalyzes the final step of the Kennedy pathway to form PE and PC, results in hypervirulence (Figure 1.3) (Tams et al., 2019). In addition, disruption of Pem1 and Pem2, which catalyze the methylation of PE to PC in the CDP-DAG pathway, results in hypervirulence that correlates with a buildup of PE (Tams et al., 2019). While the mechanism of hypervirulence is not yet fully understood for these mutants, several phenotypes we have observed are common to other hypervirulent mutants discussed in this chapter, including increased cell wall chitin levels and increased hyphal length.

References

- Angiolella, L., Stringaro, A.R., De Bernardis, F., Posteraro, B., Bonito, M., Toccaceli, L., et al. (2008). Increase of virulence and its phenotypic traits in drug-resistant strains of *Candida albicans*. *Antimicrob Agents Chemother* 52(3), 927-936. doi: 10.1128/AAC.01223-07.
- Atkinson, K.D., Jensen, B., Kolat, A.I., Storm, E.M., Henry, S.A., and Fogel, S. (1980). Yeast mutants auxotrophic for choline or ethanolamine. *J Bacteriol* 141(2), 558-564.
- Badiee, P., and Hashemizadeh, Z. (2014). Opportunistic invasive fungal infections: diagnosis & clinical management. *Indian J Med Res* 139(2), 195-204.
- Bahnan, W., Koussa, J., Younes, S., Abi Rizk, M., Khalil, B., El Sitt, S., et al. (2012). Deletion of the *Candida albicans* PIR32 results in increased virulence, stress response, and upregulation of cell wall chitin deposition. *Mycopathologia* 174(2), 107-119. doi: 10.1007/s11046-012-9533-z.
- Benedict, K., Jackson, B.R., Chiller, T., and Beer, K.D. (2018). Estimation of direct healthcare costs of fungal diseases in the United States. *Clin Infect Dis* 68(11), 1791-1797. doi: 10.1093/cid/ciy776.
- Braun, B.R., and Johnson, A.D. (1997). Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1. *Science* 277(5322), 105-109. doi: 10.1126/science.277.5322.105.
- Brown, G.D., Denning, D.W., Gow, N.A., Levitz, S.M., Netea, M.G., and White, T.C. (2012). Hidden killers: human fungal infections. *Sci Transl Med* 4(165), 165rv113. doi: 10.1126/scitranslmed.3004404.
- Brown, G.D., and Gordon, S. (2005). Immune recognition of fungal beta-glucans. *Cell Microbiol* 7(4), 471-479. doi: 10.1111/j.1462-5822.2005.00505.x.
- Brown, N.A., Urban, M., and Hammond-Kosack, K.E. (2016). The trans-kingdom identification of negative regulators of pathogen hypervirulence. *FEMS Microbiol Rev* 40(1), 19-40. doi: 10.1093/femsre/fuv042.
- Brunke, S., and Hube, B. (2013). Two unlike cousins: *Candida albicans* and *C. glabrata* infection strategies. *Cell Microbiol* 15(5), 701-708. doi: 10.1111/cmi.12091.
- Brunke, S., Seider, K., Fischer, D., Jacobsen, I.D., Kasper, L., Jablonowski, N., et al. (2014). One small step for a yeast--microevolution within macrophages renders *Candida glabrata* hypervirulent due to a single point mutation. *PLoS Pathog* 10(10), e1004478. doi: 10.1371/journal.ppat.1004478.
- Carlisle, P.L., Banerjee, M., Lazzell, A., Monteagudo, C., Lopez-Ribot, J.L., and Kadosh, D. (2009). Expression levels of a filament-specific transcriptional regulator are sufficient to determine *Candida albicans* morphology and virulence. *Proc Natl Acad Sci U S A* 106(2), 599-604. doi: 10.1073/pnas.0804061106.
- Casadevall, A., Coelho, C., Cordero, R.J.B., Dragotakes, Q., Jung, E., Vij, R., et al. (2018). The capsule of *Cryptococcus neoformans*. *Virulence*, 1-10. doi: 10.1080/21505594.2018.1431087.
- Cassilly, C.D., Farmer, A.T., Montedonico, A.E., Smith, T.K., Campagna, S.R., and Reynolds, T.B. (2017). Role of phosphatidylserine synthase in shaping the phospholipidome of *Candida albicans*. *FEMS Yeast Res* 17(2), (2): fox007. doi: 10.1093/femsyr/fox007.

- Chang, Y.C., Stins, M.F., McCaffery, M.J., Miller, G.F., Pare, D.R., Dam, T., et al. (2004). Cryptococcal yeast cells invade the central nervous system via transcellular penetration of the blood-brain barrier. *Infect Immun* 72(9), 4985-4995. doi: 10.1128/IAI.72.9.4985-4995.2004.
- Chen, Y.L., Kauffman, S., and Reynolds, T.B. (2008). *Candida albicans* uses multiple mechanisms to acquire the essential metabolite inositol during infection. *Infect Immun* 76(6), 2793-2801. doi: 10.1128/IAI.01514-07.
- Chen, Y.L., Montedonico, A.E., Kauffman, S., Dunlap, J.R., Menn, F.M., and Reynolds, T.B. (2010). Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence in *Candida albicans*. *Mol Microbiol* 75(5), 1112-1132. doi: 10.1111/j.1365-2958.2009.07018.x.
- Clancey, C.J., Chang, S.C., and Dowhan, W. (1993). Cloning of a gene (PSD1) encoding phosphatidylserine decarboxylase from *Saccharomyces cerevisiae* by complementation of an *Escherichia coli* mutant. *J Biol Chem* 268(33), 24580-24590.
- Csank, C., and Haynes, K. (2000). *Candida glabrata* displays pseudohyphal growth. *FEMS Microbiol Lett* 189(1), 115-120. doi: 10.1111/j.1574-6968.2000.tb09216.x.
- D'Souza, C.A., Alspaugh, J.A., Yue, C., Harashima, T., Cox, G.M., Perfect, J.R., et al. (2001). Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. *Mol Cell Biol* 21(9), 3179-3191. doi: 10.1128/MCB.21.9.3179-3191.2001.
- Da Silva, C.A., Chalouni, C., Williams, A., Hartl, D., Lee, C.G., and Elias, J.A. (2009). Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J Immunol* 182(6), 3573-3582. doi: 10.4049/jimmunol.0802113.
- Davis, S.E., Hopke, A., Minkin, S.C., Jr., Montedonico, A.E., Wheeler, R.T., and Reynolds, T.B. (2014). Masking of beta(1-3)-glucan in the cell wall of *Candida albicans* from detection by innate immune cells depends on phosphatidylserine. *Infect Immun* 82(10), 4405-4413. doi: 10.1128/IAI.01612-14.
- Davis, S.E., Tams, R.N., Solis, N., Wagner, A.S., Chen, T., Jackson, J.W., et al. (2018). *Candida albicans* cannot acquire sufficient ethanolamine from the host to support virulence in the absence of de novo phosphatidylethanolamine synthesis. *Infect Immun* 86(8), e00815-00817. doi: 10.1128/IAI.00815-17.
- de Cassia Orlandi Sardi, J., Silva, D.R., Soares Mendes-Giannini, M.J., and Rosalen, P.L. (2018). *Candida auris*: Epidemiology, risk factors, virulence, resistance, and therapeutic options. *Microb Pathog* 125, 116-121. doi: 10.1016/j.micpath.2018.09.014.
- Doering, T.L. (2009). How sweet it is! Cell wall biogenesis and polysaccharide capsule formation in *Cryptococcus neoformans*. *Annu Rev Microbiol* 63, 223-247. doi: 10.1146/annurev.micro.62.081307.162753.
- Dohrmann, P.R., Butler, G., Tamai, K., Dorland, S., Greene, J.R., Thiele, D.J., et al. (1992). Parallel pathways of gene regulation: homologous regulators SWI5 and ACE2 differentially control transcription of HO and chitinase. *Genes Dev* 6(1), 93-104.
- Dunkler, A., Walther, A., Specht, C.A., and Wendland, J. (2005). *Candida albicans* CHT3 encodes the functional homolog of the Cts1 chitinase of *Saccharomyces cerevisiae*. *Fungal Genet Biol* 42(11), 935-947. doi: 10.1016/j.fgb.2005.08.001.

- Duvenage, L., Walker, L.A., Bojarczuk, A., Johnston, S.A., MacCallum, D.M., Munro, C.A., et al. (2019). Inhibition of Classical and Alternative Modes of Respiration in *Candida albicans* Leads to Cell Wall Remodeling and Increased Macrophage Recognition. *MBio* 10(1), e02535-02518. doi: 10.1128/mBio.02535-18.
- Fanning, S., and Mitchell, A.P. (2012). Fungal biofilms. *PLoS Pathog* 8(4), e1002585. doi: 10.1371/journal.ppat.1002585.
- Ferrari, S., Ischer, F., Calabrese, D., Posteraro, B., Sanguinetti, M., Fadda, G., et al. (2009). Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. *PLoS Pathog* 5(1), e1000268. doi: 10.1371/journal.ppat.1000268.
- Ferrari, S., Sanguinetti, M., De Bernardis, F., Torelli, R., Posteraro, B., Vandeputte, P., et al. (2011). Loss of mitochondrial functions associated with azole resistance in *Candida glabrata* results in enhanced virulence in mice. *Antimicrob Agents Chemother* 55(5), 1852-1860. doi: 10.1128/AAC.01271-10.
- Foreman-Wykert, A.K., and Miller, J.F. (2003). Hypervirulence and pathogen fitness. *Trends Microbiol* 11(3), 105-108.
- Free, S.J. (2013). Fungal cell wall organization and biosynthesis. *Adv Genet* 81, 33-82. doi: 10.1016/B978-0-12-407677-8.00002-6.
- Ghannoum, M.A. (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* 13(1), 122-143, table of contents. doi: 10.1128/cmr.13.1.122-143.2000.
- Gibellini, F., and Smith, T.K. (2010). The Kennedy pathway--De novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life* 62(6), 414-428. doi: 10.1002/iub.337.
- Gow, N.A.R., Latge, J.P., and Munro, C.A. (2017). The Fungal Cell Wall: Structure, Biosynthesis, and Function. *Microbiol Spectr* 5(3), (3):FUNK-0035-2016. doi: 10.1128/microbiolspec.FUNK-0035-2016.
- Kakade, P., Mahadik, K., Balaji, K.N., Sanyal, K., and Nagaraja, V. (2019). Two negative regulators of biofilm development exhibit functional divergence in conferring virulence potential to *Candida albicans*. *FEMS Yeast Res* 19(2), 5057869. doi: 10.1093/femsyr/foy078.
- Kamran, M., Calcagno, A.M., Findon, H., Bignell, E., Jones, M.D., Warn, P., et al. (2004). Inactivation of transcription factor gene ACE2 in the fungal pathogen *Candida glabrata* results in hypervirulence. *Eukaryot Cell* 3(2), 546-552.
- Kelly, M.T., MacCallum, D.M., Clancy, S.D., Odds, F.C., Brown, A.J., and Butler, G. (2004). The *Candida albicans* CaACE2 gene affects morphogenesis, adherence and virulence. *Mol Microbiol* 53(3), 969-983. doi: 10.1111/j.1365-2958.2004.04185.x.
- Kodaki, T., and Yamashita, S. (1987). Yeast phosphatidylethanolamine methylation pathway. Cloning and characterization of two distinct methyltransferase genes. *J Biol Chem* 262(32), 15428-15435.
- Kwon-Chung, K.J., Fraser, J.A., Doering, T.L., Wang, Z., Janbon, G., Idnurm, A., et al. (2014). *Cryptococcus neoformans* and *Cryptococcus gattii*, the etiologic agents of cryptococcosis. *Cold Spring Harb Perspect Med* 4(7), a019760. doi: 10.1101/cshperspect.a019760.
- Lee, C.R., Lee, J.H., Park, K.S., Jeon, J.H., Kim, Y.B., Cha, C.J., et al. (2017).

- Antimicrobial Resistance of Hypervirulent *Klebsiella pneumoniae*: Epidemiology, Hypervirulence-Associated Determinants, and Resistance Mechanisms. *Front Cell Infect Microbiol* 7, 483. doi: 10.3389/fcimb.2017.00483.
- Letts, V.A., Klig, L.S., Bae-Lee, M., Carman, G.M., and Henry, S.A. (1983). Isolation of the yeast structural gene for the membrane-associated enzyme phosphatidylserine synthase. *Proc Natl Acad Sci U S A* 80(23), 7279-7283.
- Lo, H.J., Kohler, J.R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., and Fink, G.R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 90(5), 939-949.
- Ma, H., and May, R.C. (2010). Mitochondria and the regulation of hypervirulence in the fatal fungal outbreak on Vancouver Island. *Virulence* 1(3), 197-201. doi: 10.4161/viru.1.3.11053.
- MacCallum, D.M., Findon, H., Kenny, C.C., Butler, G., Haynes, K., and Odds, F.C. (2006). Different consequences of ACE2 and SWI5 gene disruptions for virulence of pathogenic and nonpathogenic yeasts. *Infect Immun* 74(9), 5244-5248. doi: 10.1128/IAI.00817-06.
- Mayer, F.L., Wilson, D., and Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence* 4(2), 119-128. doi: 10.4161/viru.22913.
- McClelland, E.E., Bernhardt, P., and Casadevall, A. (2006). Estimating the relative contributions of virulence factors for pathogenic microbes. *Infect Immun* 74(3), 1500-1504. doi: 10.1128/IAI.74.3.1500-1504.2006.
- Medoff, G., Kobayashi, G.S., Painter, A., and Travis, S. (1987). Morphogenesis and pathogenicity of *Histoplasma capsulatum*. *Infect Immun* 55(6), 1355-1358.
- Munro, C. (2010). "Candida albicans Cell Wall Mediated Virulence," in *Pathogenic Yeasts*, eds. R. Ashbee & E.M. Bignell. (Berlin, Heidelberg: Springer Berlin Heidelberg), 69-95.
- Munro, C.A., Schofield, D.A., Gooday, G.W., and Gow, N.A. (1998). Regulation of chitin synthesis during dimorphic growth of *Candida albicans*. *Microbiology* 144 (Pt 2), 391-401. doi: 10.1099/00221287-144-2-391.
- Murad, A.M., Leng, P., Straffon, M., Wishart, J., Macaskill, S., MacCallum, D., et al. (2001). NRG1 represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. *EMBO J* 20(17), 4742-4752. doi: 10.1093/emboj/20.17.4742.
- Naglik, J.R., Challacombe, S.J., and Hube, B. (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* 67(3), 400-428, table of contents. doi: 10.1128/mmbr.67.3.400-428.2003.
- Nett, J.E., Guite, K.M., Ringeisen, A., Holoyda, K.A., and Andes, D.R. (2008). Reduced biocide susceptibility in *Candida albicans* biofilms. *Antimicrob Agents Chemother* 52(9), 3411-3413. doi: 10.1128/AAC.01656-07.
- Nobile, C.J., and Johnson, A.D. (2015). *Candida albicans* Biofilms and Human Disease. *Annu Rev Microbiol* 69, 71-92. doi: 10.1146/annurev-micro-091014-104330.
- O'Meara, T.R., and Alspaugh, J.A. (2012). The *Cryptococcus neoformans* capsule: a sword and a shield. *Clin Microbiol Rev* 25(3), 387-408. doi: 10.1128/CMR.00001-12.
- O'Meara, T.R., Norton, D., Price, M.S., Hay, C., Clements, M.F., Nichols, C.B., et al. (2010). Interaction of *Cryptococcus neoformans* Rim101 and protein kinase A regulates capsule. *PLoS Pathog* 6(2), e1000776. doi:

- 10.1371/journal.ppat.1000776.
- Orlean, P. (2012). Architecture and biosynthesis of the *Saccharomyces cerevisiae* cell wall. *Genetics* 192(3), 775-818. doi: 10.1534/genetics.112.144485.
- Paulus, H., and Kennedy, E.P. (1960). The enzymatic synthesis of inositol monophosphatide. *J Biol Chem* 235, 1303-1311.
- Perlroth, J., Choi, B., and Spellberg, B. (2007). Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med Mycol* 45(4), 321-346. doi: 10.1080/13693780701218689.
- Plato, A., Hardison, S.E., and Brown, G.D. (2015). Pattern recognition receptors in antifungal immunity. *Semin Immunopathol* 37(2), 97-106. doi: 10.1007/s00281-014-0462-4.
- Revie, N.M., Iyer, K.R., Robbins, N., and Cowen, L.E. (2018). Antifungal drug resistance: evolution, mechanisms and impact. *Curr Opin Microbiol* 45, 70-76. doi: 10.1016/j.mib.2018.02.005.
- Stead, D., Findon, H., Yin, Z., Walker, J., Selway, L., Cash, P., et al. (2005). Proteomic changes associated with inactivation of the *Candida glabrata* ACE2 virulence-moderating gene. *Proteomics* 5(7), 1838-1848. doi: 10.1002/pmic.200401064.
- Stead, D.A., Walker, J., Holcombe, L., Gibbs, S.R., Yin, Z., Selway, L., et al. (2010). Impact of the transcriptional regulator, Ace2, on the *Candida glabrata* secretome. *Proteomics* 10(2), 212-223. doi: 10.1002/pmic.200800706.
- Tams, R.N., Cassilly, C.D., Anaokar, S., Brewer, W.T., Dinsmore, J.T., Chen, Y.-L., et al. (2019). Overproduction of Phospholipids by the Kennedy Pathway Leads to Hypervirulence in *Candida albicans*. *Frontiers in Microbiology* 10, 86. doi: 10.3389/fmicb.2019.00086.
- Trotter, P.J., and Voelker, D.R. (1995). Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (PSD2) in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 270(11), 6062-6070. doi: 10.1074/jbc.270.11.6062.
- Vale-Silva, L.A., and Sanglard, D. (2015). Tipping the balance both ways: drug resistance and virulence in *Candida glabrata*. *FEMS Yeast Res* 15(4), fov025. doi: 10.1093/femsyr/fov025.
- van de Veerdonk, F.L., Gresnigt, M.S., Romani, L., Netea, M.G., and Latge, J.P. (2017). *Aspergillus fumigatus* morphology and dynamic host interactions. *Nat Rev Microbiol* 15(11), 661-674. doi: 10.1038/nrmicro.2017.90.
- Vermitsky, J.P., Earhart, K.D., Smith, W.L., Homayouni, R., Edlind, T.D., and Rogers, P.D. (2006). Pdr1 regulates multidrug resistance in *Candida glabrata*: gene disruption and genome-wide expression studies. *Mol Microbiol* 61(3), 704-722. doi: 10.1111/j.1365-2958.2006.05235.x.
- Wachtler, B., Citiulo, F., Jablonowski, N., Forster, S., Dalle, F., Schaller, M., et al. (2012). *Candida albicans*-epithelial interactions: dissecting the roles of active penetration, induced endocytosis and host factors on the infection process. *PLoS One* 7(5), e36952. doi: 10.1371/journal.pone.0036952.
- Wagener, J., MacCallum, D.M., Brown, G.D., and Gow, N.A. (2017). *Candida albicans* Chitin Increases Arginase-1 Activity in Human Macrophages, with an Impact on Macrophage Antimicrobial Functions. *MBio* 8(1), e01820-01816. doi: 10.1128/mBio.01820-16.
- Wheat, L.J. (2006). Histoplasmosis: a review for clinicians from non-endemic areas.

- Mycoses* 49(4), 274-282. doi: 10.1111/j.1439-0507.2006.01253.x.
- Wheeler, R.T., and Fink, G.R. (2006). A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog* 2(4), e35. doi: 10.1371/journal.ppat.0020035.
- Wheeler, R.T., Kupiec, M., Magnelli, P., Abeijon, C., and Fink, G.R. (2003). A *Saccharomyces cerevisiae* mutant with increased virulence. *Proc Natl Acad Sci U S A* 100(5), 2766-2770. doi: 10.1073/pnas.0437995100.
- Wilson, D., Naglik, J.R., and Hube, B. (2016). The Missing Link between *Candida albicans* Hyphal Morphogenesis and Host Cell Damage. *PLoS Pathog* 12(10), e1005867. doi: 10.1371/journal.ppat.1005867.
- Yang, Y.L. (2003). Virulence factors of *Candida* species. *J Microbiol Immunol Infect* 36(4), 223-228.
- Yu, S.J., Chang, Y.L., and Chen, Y.L. (2018). Deletion of ADA2 Increases Antifungal Drug Susceptibility and Virulence in *Candida glabrata*. *Antimicrob Agents Chemother* 62(3), e01924-01917. doi: 10.1128/AAC.01924-17.

Chapter 2 : Overproduction of Phospholipids by the Kennedy Pathway Leads to Hypervirulence in *Candida albicans*

Publication Note

A version of this chapter has been previously published by Robert N. Tams, Chelsi D. Cassilly, Sanket Anaokar, William T. Brewer, Justin T. Dinsmore, Ying-Lien Chen, Jana Patton-Vogt, and Todd B. Reynolds:

Tams RN, Cassilly CD, Anaokar S, Brewer WT, Dinsmore JT, Chen Y-L, Patton-Vogt J, Reynolds TB. 2019. *Overproduction of Phospholipids by the Kennedy Pathway Leads to Hypervirulence in Candida albicans*. *Frontiers in Microbiology* 10.

The article was revised in the following ways for inclusion in this dissertation: Methods and results regarding experiments involving the *BTA1* gene were incorporated. The author contributions are as follows: RT, TR, and Y-LC participated in plasmid design and constructions, and *C. albicans* strain construction. RT performed mouse infection models with the assistance of WB or CC. RT and JD performed growth curves. SA and CC performed radiolabeling assays with direction from JP-V. RT performed all other work. TR directed the project and edited the manuscript. JP-V edited the manuscript as well.

Abstract

Candida albicans is an opportunistic human fungal pathogen that causes life-threatening systemic infections, as well as oral mucosal infections. Phospholipids are crucial for pathogenesis in *C. albicans*, as disruption of phosphatidylserine (PS) and phosphatidylethanolamine (PE) biosynthesis within the cytidine diphosphate diacylglycerol (CDP-DAG) pathway causes avirulence in a mouse model of systemic infection. The synthesis of PE by this pathway plays a crucial role in virulence, but it was unknown if downstream conversion of PE to phosphatidylcholine (PC) is required for pathogenicity. Therefore, the enzymes responsible for methylating PE to PC, Pem1 and

Pem2, were disrupted. The resulting *pem1* Δ/Δ *pem2* Δ/Δ mutant was not less virulent in mice, but rather hypervirulent. Since the *pem1* Δ/Δ *pem2* Δ/Δ mutant accumulated PE, this led to the hypothesis that increased PE synthesis increases virulence. To test this, the alternative Kennedy pathway for PE/PC synthesis was exploited. This pathway makes PE and PC from exogenous ethanolamine and choline, respectively, using three enzymatic steps. In contrast to *Saccharomyces cerevisiae*, *C. albicans* was found to use one enzyme, Ept1, for the final enzymatic step (ethanolamine/cholinephosphotransferase) that generates both PE and PC. *EPT1* was overexpressed, which resulted in increases in both PE and PC synthesis. Moreover, the *EPT1* overexpressor strain is hypervirulent in mice and causes them to succumb to systemic infection more rapidly than wild-type. In contrast, disruption of *EPT1* causes loss of PE and PC synthesis by the Kennedy pathway, and decreased kidney fungal burden during the mouse systemic infection model, indicating a mild loss of virulence. In addition, the *ept1* Δ/Δ mutant exhibits decreased cytotoxicity against oral epithelial cells *in vitro*, whereas the *EPT1* overexpression strain exhibits increased cytotoxicity. Taken altogether, our data indicate that mutations that result in increased PE synthesis cause greater virulence and mutations that decrease PE synthesis attenuate virulence.

Introduction

Candida albicans is a fungus that typically resides as a commensal in the gastrointestinal tract of up to 70% of healthy individuals, as well as within the oral mucosa (Bouza and Munoz, 2008). However, *C. albicans* can cause vaginal infections as well as opportunistic oral and systemic infections, which are more commonly seen in immunocompromised or immunosuppressed individuals (Kabir et al., 2012). Systemic blood stream infections

(BSIs) are of particular concern as they have mortality rates of approximately 30-50% (Williams and Lewis, 2011), and *Candida* spp. are the fourth most common causes of BSIs in the United States (Kabir et al., 2012). Treatment of systemic infections has encountered some limitations as a result of poor oral availability and high drug toxicity of some drugs (Letscher-Bru and Herbrecht, 2003; Mukherjee et al., 2005). In addition, resistance to standard antifungal treatments such as fluconazole, by *Candida* spp., is an emerging issue (Sanguinetti et al., 2015). It is therefore imperative that novel drug targets are discovered.

Phospholipid biosynthetic pathways are an attractive area to search for drug targets, as phospholipids are the major structural lipids that form cellular membranes (de Kroon et al., 2013). Cells must therefore synthesize them from precursors acquired from their environment to support growth during infection. In addition to having a structural role within the cell, intermediate phospholipid metabolites can act as second messengers, and may therefore serve important regulatory functions (Exton, 1994; de Kroon et al., 2013). As such, they are required for the growth and pathogenesis of *C. albicans*.

The most abundant phospholipids in *C. albicans* are phosphatidylglycerol (PG), cardiolipin (CL) phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI) (Singh et al., 2010; Cassilly et al., 2017). The major biosynthetic pathway for the phospholipids PS, PE, and PC in *C. albicans* is known as the cytidine-diphosphate diacylglycerol (CDP-DAG) pathway (Figure 2.1) (Henry et al., 2012). Within this pathway, the common phospholipid precursor CDP-DAG is condensed with serine to form PS by the PS synthase Cho1 (Bae-Lee and Carman, 1984; Henry et al., 2012). PS is then decarboxylated by one of two PS

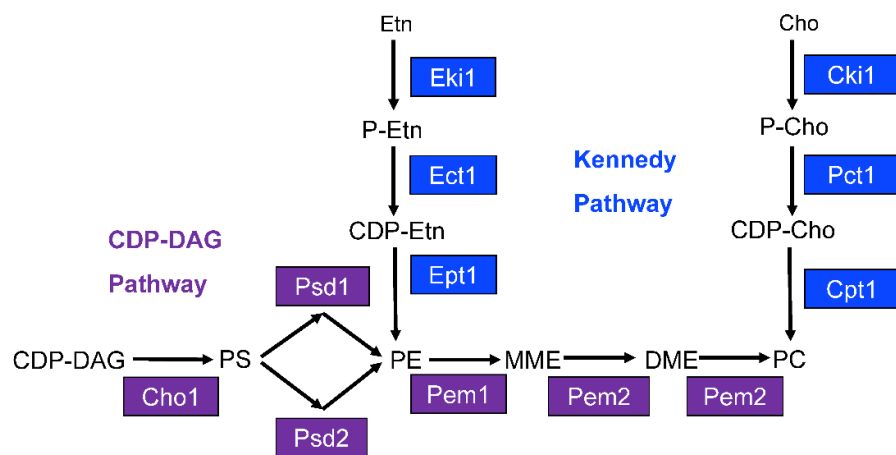


Figure 2.1. Synthesis of yeast phospholipids.

Model for yeast phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) biosynthesis. Proteins involved in CDP-DAG pathway mediated phospholipid biosynthesis are shown in purple, and those involved in Kennedy pathway mediated phospholipid biosynthesis are shown in blue. Abbreviations: CDP-DAG, cytidine diphosphate diacylglycerol; MME, monomethylphosphatidylethanolamine; DME, dimethylphosphatidylethanolamine; Etn, ethanolamine; Cho, choline; P-Etn, phosphoethanolamine; P-Cho, phosphocholine; CDP-Etn, cytidinediphosphoethanolamine; CDP-Cho, cytidinediphosphocholine.

decarboxylases, Psd1 or Psd2, to form PE (Henry et al., 2012). Based on work from *S. cerevisiae*, PE is then sequentially methylated by the PE methyltransferases Pem1 and Pem2 to form PC (Henry et al., 2012). Previously, our lab has demonstrated that the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants are avirulent in the mouse model of systemic infection (Chen et al., 2010). Interestingly, these mutants are avirulent despite having the ability to utilize an alternative phospholipid biosynthetic pathway known as the Kennedy pathway. This pathway uses exogenous ethanolamine or choline to synthesize PE or PC, respectively (Figure 2.1) (Gibellini and Smith, 2010). We have recently reported that the Kennedy pathway alone cannot support virulence because the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants cannot import sufficient ethanolamine from the host to support PE synthesis (Davis et al., 2018). Growth of either mutant is restored *in vitro* in medium containing >100 μM ethanolamine, which is higher than the estimated concentration of ~30 μM within the host (Houweling et al., 1992; Davis et al., 2018). In addition, virulence is restored if PE synthesis is supported by heterologous expression of the *Arabidopsis thaliana* serine decarboxylase (*AtSDC*) (Davis et al., 2018). This enzyme allows for decarboxylation of cytoplasmic serine to yield ethanolamine, which can be used to synthesize PE via the Kennedy pathway. Thus *AtSDC* bypasses deficiencies due to importing low levels of exogenous ethanolamine (Davis et al., 2018). Therefore, wild-type fungus requires the CDP-DAG pathway to generate enough PE to cause disease since it cannot import enough ethanolamine to compensate for PE loss using the Kennedy pathway. Loss of PE synthesis should also impact downstream PC synthesis, and the impact of PC on virulence has been largely unstudied in this organism. In this study, we sought to determine if the loss of PC biosynthesis from the CDP-DAG pathway would

attenuate virulence, or if the Kennedy pathway could compensate for this loss. In addition, we directly determined if Kennedy pathway mediated production of PE and/or PC is also important for virulence in *C. albicans* when the CDP-DAG pathway is still functional.

Materials and Methods

Strains and growth media

Candida albicans strains used in this study were derived from the SC5314 background (Gillum et al., 1984) (Table 2.1). Standard medium used for culturing *C. albicans* was YPD (1% yeast extract, 2% dextrose, and 2% peptone) and cultures were maintained aerobically at 30°C in a shaking incubator at 225 RPM (Styles, 2002). DH5- α *Escherichia coli* (NEB, C2987I) were used for plasmid construction (Table 2.2), and were cultured in LB broth (1% tryptone, 1% NaCl, 0.5% yeast extract) in a rotating incubator at 37°C (Guthrie C., 2002).

Plasmid construction

Primers described in this section are shown in appendix B. The *PEM1* knockout vector was created using the *SAT1* flipper plasmid pSFS2A (Cat^R) (Reuss et al., 2004). A 589 bp region of the *PEM1* (*CHO2/CR_02540W_A/orf19.169*) 5' non-coding region (NCR) was amplified using the primers JCO197 and JCO198 which introduced *KpnI* and *ApaI* sites, respectively. This fragment was cloned into the *KpnI* and *ApaI* sites of pSFS2A. In addition, a 589 bp 3' NCR of *PEM1* was amplified using JCO199 and JCO200 and was subsequently cloned into the *SacII* and *SacI* sites of pSFS2A to create the pYLC390 *PEM1* knockout vector. In addition to the *PEM1* knockout vector, a *P_{MET3}* repressible

Table 2.1. *C. albicans* strains used in chapter two.

Strain	Parent Strain	Genotype	Source
SC5314	Clinical isolate	Prototrophic Wild Type	Gillum <i>et al.</i> , 1984
YLC394	SC5314	<i>PEM1/pem1Δ-SAT1</i>	This Study
YLC401	YLC394	<i>PEM1/pem1Δ-FRT</i>	This Study
YLC417	YLC401	<i>pem1Δ/pem1::P_{MET3}-PEM1-SAT1</i>	This Study
YLC393	SC5314	<i>PEM2/pem2Δ-SAT1</i>	This Study
YLC400	YLC393	<i>PEM2/pem2Δ-FRT</i>	This Study
YLC405	YLC400	<i>pem2Δ/Δ-SAT1</i>	This Study
YLC406	YLC405	<i>pem2Δ/Δ-FRT</i>	This Study
BTY38	YLC406	<i>PEM1/pem1Δ pem2Δ/Δ-SAT1</i>	This Study
BTY66	BTY38	<i>PEM1/pem1Δ pem2Δ/Δ-FRT</i>	This Study
BTY72	BTY66	<i>pem1Δ/Δ pem2Δ/Δ-SAT1</i>	This Study
BTY77	BTY72	<i>pem1Δ/Δ pem2Δ/Δ-FRT</i>	This Study
BTY138	BTY77	<i>pem1Δ/Δ pem2Δ/pem2Δ::PEM2-SAT1</i>	This Study
BTY147	BTY138	<i>pem1Δ/Δ pem2Δ/pem2Δ::PEM2 FRT</i>	This Study
BTY167	BTY147	<i>pem1Δ/Δ::PEM1 pem2Δ/pem2Δ::PEM2-SAT1</i>	This Study
BTY169	BTY167	<i>pem1Δ/Δ::PEM1 pem2Δ/pem2Δ::PEM2-FRT</i>	This Study
BTY88	SC5314	<i>P_{ENO1}::P_{ENO1}-EPT1</i>	This Study
BTY97	SC5314	<i>EPT1/ept1Δ-SAT1</i>	This Study
BTY101	BTY97	<i>EPT1/ept1Δ-FRT</i>	This Study
BTY104	BTY101	<i>ept1Δ/Δ-SAT1</i>	This Study
BTY108	BTY104	<i>ept1Δ/Δ-FRT</i>	This Study
BTY130	BTY108	<i>ept1Δ/Δ P_{ENO1}::P_{ENO1}-EPT1-SAT1</i>	This Study
BTY192	BTY108	<i>ept1Δ/Δ::EPT1-SAT1</i>	This Study
BTY200	BTY192	<i>ept1Δ/Δ::EPT1-FRT</i>	This Study
BTY81	BTY77	<i>pem1Δ/Δ pem2Δ/Δ P_{ENO1}::P_{ENO1}-BTA1-SAT1</i>	This Study

Table 2.2. *E. coli* strains used in chapter two.

Plasmid	Description	Parent Vector
pSFS2A	<i>SAT1</i> flipper cassette; (CAT ^R)	Reuss <i>et al.</i> , 2004
pYLC390	<i>PEM1</i> knockout construct; 5' NCR (<i>KpnI</i> - <i>ApaI</i>) + 3' NCR (<i>SacII</i> - <i>I</i>) of <i>PEM1</i>	pSFS2A
pYLC388	<i>PEM2</i> knockout construct; 5' NCR (<i>KpnI</i> - <i>ApaI</i>) + 3' NCR (<i>SacII</i> - <i>I</i>) of <i>PEM2</i>	pSFS2A
pYLC314	Methionine repressible expression cassette; <i>SAT1-P_{MET3}</i> (<i>EcoRI</i> - <i>EcoRI</i>); (AMP ^R)	pBluescript SK+
pYLC315	Methionine repressible expression cassette; <i>SAT1-P_{MET3}</i> (<i>EcoRI</i> - <i>EcoRI</i>); (AMP ^R)	pBluescript SK+
pYLC410	<i>PEM1</i> repressible expression vector; 5' NCR of <i>PEM1</i> (<i>KpnI</i> - <i>ApaI</i>) + <i>SAT1-P_{MET3}</i> (<i>EcoRI</i> - <i>EcoRI</i>)	pYLC315
pYLC414	<i>PEM1</i> repressible expression vector; 5' NCR of <i>PEM1</i> (<i>KpnI</i> - <i>ApaI</i>) + <i>SAT1-P_{MET3}</i> (<i>EcoRI</i> - <i>EcoRI</i>) + <i>PEM1</i> ORF (<i>SacII</i> - <i>SacI</i>)	pYLC410
pBT33	<i>PEM2</i> reintegration construct; 5' NCR and <i>PEM2</i> ORF (<i>KpnI</i> - <i>ApaI</i>) + 3' NCR (<i>SacII</i> - <i>SacI</i>) of <i>PEM2</i>	pYLC388
pBT47	<i>PEM1</i> reintegration construct; 5' NCR + <i>PEM1</i> ORF (<i>ApaI</i> - <i>XhoI</i>)	pSFS2A
pBT49	<i>PEM1</i> reintegration construct; 5' NCR and <i>PEM1</i> ORF (<i>ApaI</i> - <i>XhoI</i>) + 3' NCR (<i>NotI</i> - <i>SacI</i>) of <i>PEM1</i>	pBT47
pBT1	Overexpression Vector; <i>P_{ENO1}</i> (<i>BamHI</i> - <i>NotI</i>)	pYLC314
pBT11	<i>EPT1</i> overexpression cassette; <i>P_{ENO1}</i> - <i>EPT1</i> (<i>NotI</i> - <i>SacI</i>)	pBT1
pBT16	<i>EPT1</i> knockout construct; 5' NCR and <i>EPT1</i> ORF (<i>KpnI</i> , <i>XhoI</i>) + 3' NCR (<i>NotI</i> , <i>SacI</i>) of <i>EPT1</i> (CAT ^R)	pSFS2A
pBT51	<i>EPT1</i> reintegration construct; 5' NCR + <i>EPT1</i> ORF (<i>KpnI</i> - <i>XhoI</i>)	pSFS2A
pBT53	<i>EPT1</i> reintegration construct; 5' NCR + <i>EPT1</i> ORF (<i>KpnI</i> - <i>XhoI</i>) + 3' NCR (<i>SacII</i> - <i>SacI</i>)	pBT51
pBT5	<i>BTA1</i> overexpression construct; <i>P_{ENO1}</i> - <i>BTA1</i> (<i>NotI</i> - <i>SacI</i>)	pBT1

PEM1 expression vector was also created. To create this vector JCO165 and JCO166 were used to amplify an *EcoRI*-flanked *SAT1-P_{MET3}* fragment from pYLC229 (Chen et al., 2008), which was subsequently cloned into the *EcoRI* site of a pBluescript SK+ plasmid to yield two isogenic clones: pYLC314 and pYLC315. The 5' NCR of *PEM1* was amplified from genomic *C. albicans* DNA with JCO199 and JCO200 as a *KpnI-ApaI* fragment and cloned into pYLC315 (*KpnI-ApaI*) upstream of the *SAT1* marked *P_{MET3}* promoter yielding pYLC410. To complete the construct, the *PEM1* ORF was amplified along with the 3' NCR with JCO215 and JCO216 and this *SacII-SacI* fragment was cloned into pYLC410 downstream of the *P_{MET3}* promoter (*SacII-SacI*) to yield the *PEM1* conditional expression vector pYLC414.

The *SAT1* flipper vector pSFS2A was used to create a *PEM2* knockout vector, pYLC388 (Reuss et al., 2004). A 592 bp 5' NCR to *PEM2* (*OPI3/C3_06570C_A/orf19.7446*) was amplified using JCO192 and JCO193 and cloned in the *KpnI* and *ApaI* sites of pSFS2A. A 587 bp NCR 3' to the *PEM2* ORF was amplified using JCO194 and JCO195 and cloned into the *SacII* and *SacI* sites, resulting in pYLC388.

pYLC388 was used to create a reintegration construct for *PEM2*. The 5' NCR was released from pYLC388 by restriction with *ApaI* and *KpnI*. A 1297 bp fragment including the *PEM2* ORF (*OPI3/C3_06570C_A/orf19.7446*) and approximately 500 bp of NCR 5' to the ORF was amplified with BTO67 and JCO192. This fragment was subcloned into pYLC388 at the *ApaI-KpnI* sites to create pBT33.

The *PEM1* reintegration construct was created using pSFS2A (Reuss et al., 2004). pSFS2A was linearized using *ApaI* and *XhoI*. A 3451 bp fragment including approximately

500 bp of the 5' NCR of *PEM1*, the *PEM1* ORF (CHO2/CR_02540W_A/orf19.169), and approximately 300 bp of the 3' NCR of *PEM1* was amplified using BTO52 and BTO53 to introduce *Apal-XhoI* sites, and subsequently was cloned into the linearized vector to create pBT47. pBT47 was linearized using *NotI* and *SacI*. A 622 bp NCR 3' to the *PEM1* ORF (-356 to -978) was amplified using BTO54 and BTO55 to introduce *NotI-SacI* sites, and subsequently cloned into the linearized pBT47 vector to create the *PEM1* reintegration vector, pBT49.

For the construction of an overexpression vector, pYLC314 was used. The *P_{MET3}* promoter was removed from pYLC314 by restriction with *PstI*, which flanks both sides of the insert. The constitutively active *P_{ENO1}* promoter, +928 to +1 5' of the *ENO1* ORF (C1_08500C_A/orf19.395), was amplified from wild type *C. albicans* genomic DNA using the primers BTO30 and BTO31 to introduce *BamHI* and *NotI* sites, and was cloned into the *BamHI* and *NotI* sites of pYLC314 to create pBT1.

To overexpress *EPT1*, a 1,509 bp fragment including the *EPT1* ORF (C7_02690C_A/orf19.3695) and approximately 300 bp of NCR 3' to the ORF was amplified from *C. albicans* genomic DNA using BTO35 and BTO36, and was cloned into the *NotI* and *SacI* sites of pBT1 to create the *EPT1* overexpression vector, pBT11.

To create an *EPT1* knockout vector, the 5' and 3' NCRs of the *EPT1* ORF (C7_02690C_A/orf19.3695) were cloned into pSFS2A plasmid flanking the *SAT1* flipper construct (Reuss et al., 2004). First, a 471 bp fragment of the 5' NCR of the *EPT1* ORF was amplified from *C. albicans* genomic DNA using BTO44 and BTO45 and subsequently cloned into the *KpnI* and *XhoI* sites of pSFS2A. In addition, a 494 bp fragment of the 3' NCR of *EPT1* was amplified with BTO46 and BTO47 and cloned into the *NotI* and *SacI*

sites to create pBT16.

To create an *EPT1* reintegration construct a 2312 bp fragment including the *EPT1* ORF, 903 bp 5' NCR of the *EPT1* ORF, and 200 bp of the 3' NCR was amplified from genomic *C. albicans* DNA using TRO1044 and TRO1045. This PCR product was cloned into pSFS2A at the *KpnI* and *XhoI* sites to create pBT51. A 596 bp fragment of the 3' NCR (+202 to +798) was amplified from genomic DNA using TRO1046 and TRO1047 and cloned into the *SacI* and *SacII* sites of pBT51 to create the finalized *EPT1* reintegration construct, pBT53.

To create a *BTA1* (C1_11490C_A/orf19.1171) overexpression construct, a 2570 bp fragment including the *BTA1* ORF and approximately 300 bp of the 3' NCR was amplified with BTO28 and BTO29 and was subsequently cloned into the *NotI* and *SacI* sites of pBT1 to create pBT5.

***Candida albicans* strain construction**

Plasmids were cut at the indicated restriction site(s) and linearized fragments were purified using the QIAquick PCR Purification Kit (QIAGEN, 28106) for single restriction digests, or the QIAquick Gel Extraction Kit (QIAGEN, 28704) if restriction digests resulted in multiple fragments. All *Candida albicans* transformations were performed via electroporation as described previously (Hasim et al., 2014) and plates containing 200 µg/mL nourseothricin (GoldBio, N-500-1) were used for selection of *C. albicans* transformants (Table 2.1). Following transformation, mutations were confirmed via PCR using the indicated primers (Appendix B).

To create the *pem2*Δ/Δ strain, pYLC388 was linearized (*KpnI*, *SacI*) and

transformed into wild type *C. albicans* (SC5314). The *SAT1* marker was removed by *FLP*-mediated recombination from YLC393 using the *SAT1* flipper method to create YLC400 (*PEM2/pem2Δ*) (Reuss et al., 2004). Linearized pYLC388 was again used to transform YLC400, which resulted in the *pem2Δ/Δ-SAT1* strain (YLC405). The *SAT1* marker was removed by recombination from YLC405 to create YLC406 (*pem2Δ/Δ*).

YLC406 was transformed with a linearized pYLC390 fragment (*KpnI*, *SacI*) to create the *PEM1/pem1Δ-SAT1 pem2Δ/Δ* mutant BTY38. The *SAT1* marker was removed by recombination to create BTY66 (*PEM1/pem1Δ pem2Δ/Δ*). BTY66 was again transformed with linearized pYLC390 (*KpnI*, *SacI*) to create a *pem1Δ/Δ-SAT1 pem2Δ/Δ* mutant (BTY72), and the *SAT1* marker was removed by recombination to create BTY77 (*pem1Δ/Δ pem2Δ/Δ*). To overexpress *BTA1* in the *pem1Δ/Δ pem2Δ/Δ* background, pBT5 was linearized with *MscI* and transformed into BTY77 to create a *pem1Δ/Δ pem2Δ/Δ P_{ENO1}::P_{ENO1}-BTA1* strain, BTY81.

To reintegrate *PEM1* and *PEM2* into the homozygous double mutant, pBT33 was linearized (*KpnI*, *SacI*) and transformed into BTY77 to create a *pem1Δ/Δ pem2Δ/pem2Δ::PEM2-SAT1* strain, BTY138. The *SAT1* marker was removed by recombination from BTY138, resulting in BTY147 (*pem1Δ/Δ pem2Δ/pem2Δ::PEM2-SAT1*). BTY147 was transformed with linearized pBT49 (*Apal*, *SacI*) to create BTY167 (*pem1Δ/Δ::PEM1 pem2Δ/pem2Δ::PEM2-SAT1*). The *SAT1* marker was removed by recombination from BTY167 to create the *pem1Δ/Δ::PEM1 pem2Δ/pem2Δ::PEM2* mutant (BTY169).

To create a repressible *PEM1* mutant, SC5314 was transformed with linearized pYLC390 (*KpnI-SacI*) to yield a *PEM1/pem1Δ-SAT1* strain (YLC394). The *SAT1* marker

was removed by recombination from YLC394 to yield a *PEM1/pem1Δ* strain (YLC401). YLC401 was then transformed with the *PEM1* conditional expression vector pYLC414 (*KpnI*) to yield a *pem1Δ/pem1Δ::P_{MET3}-PEM1-SAT1* strain (YLC417).

To overexpress *EPT1*, SC5314 was transformed using linearized pBT11 (*MscI*) to create BTY88 (*P_{ENO1}::P_{ENO1}-EPT1*). To knock out *EPT1*, pBT16 was linearized (*KpnI*, *SacI*) and transformed into SC5314 resulting in an *EPT1/ept1Δ-SAT1* strain (BTY97). The *SAT1* marker was removed by recombination from BTY97, resulting in BTY101 (*EPT1/ept1Δ*). BTY101 was transformed with linearized pBT16 again to create the *ept1Δ/Δ-SAT1* strain, BTY104. The *SAT1* marker was removed by recombination from BTY104 to create BTY108 (*ept1Δ/Δ*), so that a reintegrant strain could be produced. Two distinct types of reintegrant were produced: one in which the *EPT1* gene was constitutively expressed, and another in which one allele of *EPT1* was reintegrated into the *EPT1* locus under its native promoter. For the first reintegrant, the overexpression cassette pBT11 was linearized (*MscI*) and transformed into BTY108, resulting in the *ept1Δ/Δ P_{ENO1}::P_{ENO1}-EPT1* strain (BTY130). To reintegrate the gene under its native promoter and at the *EPT1* locus, pBT53 was linearized using *KpnI* and *SacI* and subsequently transformed into BTY108 to yield BTY192 (*ept1Δ/Δ::EPT1-SAT1*). The *SAT1* marker was then removed by recombination to yield BTY200 (*ept1Δ/Δ::EPT1*).

Growth curves

To determine choline auxotrophy, strains were cultured overnight in liquid YNB medium (0.67% yeast nitrogen base containing ammonium sulfate (BD Difco, 291940), 2% dextrose), and then diluted to OD_{600nm} = 0.1 in triplicate in fresh YNB (Styles, 2002).

Growth was measured at OD_{600nm} every two hours for 12 hours or at other time points as indicated. To rescue choline auxotrophy YNB medium was supplemented with choline chloride (Fisher Scientific, O1972-250), glycerophosphocholine (Sigma-Aldrich, G5291-10MG), or lysophosphatidylcholine (Avanti Polar Lipids INC., 845875P).

Lipid extraction and TLC

C. albicans strains were cultured overnight in 5mL YNB medium supplemented with 0.25 μ M L-methionine/L-cysteine (represses *PEM1* in pYLC417), and then diluted to an OD_{600nm} = 0.4 in 25 mL of YNB with 0.25 μ M L-methionine/L-cysteine or YNB supplemented with 1 mM choline chloride and 0.25 μ M L-methionine/L-cysteine. Cultures were maintained at 30°C for 12 hours at 225 RPM and then transferred to 50 mL conical tubes and pelleted at 3000 RPM for 10 minutes. Pellets were lyophilized and weighed to normalize for the amount of cells present. Following lyophilization, pellets were washed with 25 mL water and resuspended in 3 mL ethanol:water (4:1). Suspensions were heated in boiling water for 15 minutes and pelleted at 3,000 RPM for 10 minutes. The supernatant was transferred to new tubes and back extractions were completed twice using 1 mL of ethanol:water (4:1) following the above procedure. Lipids were dried under nitrogen gas and resuspended in chloroform:methanol (2:1). TLC plates were washed once with chloroform:methanol (2:1) and dried at 100°C for 15 minutes. Lipid extracts were spot inoculated on TLC plates (Millipore, HX377581) and chloroform:ethanol:water:triethylamine (35:30:7:35) was used as a solvent system for separation (Chen et al., 2010). Phospholipids were visualized under UV following treatment with primuline (Fisher Scientific, 8064-60-6).

Mouse models

Outbred male ICR mice at 5 to 6 weeks of age were obtained from Envigo for use in this study. *C. albicans* strains were cultured overnight in 50 mL YPD at 30°C and 225 RPM. After approximately 16 hours they were transferred to 50 mL Falcon tubes and centrifuged at 3,500 RPM for 5 minutes. The pellets were subsequently washed twice with 25 mL of water. Cells were counted via hemocytometer and diluted to 5×10^6 cells/mL unless otherwise noted. Mice were injected via the lateral tail vein with 0.1 mL of the *C. albicans* suspension. *C. albicans* suspensions were plated on YPD and incubated overnight at 30°C to determine cell viability. Following infection, mice were monitored for signs of illness for 21 days and were sacrificed after succumbing to infection.

For experiments in which fungal burden was measured, mice were sacrificed 5 days post infection and kidneys were harvested. Kidneys were placed in 1 mL water within pre-weighed whirl-pack bags and homogenized. Serial dilutions (10^{-1} , 10^{-2} , and 10^{-3}) were prepared in water, and 1 mL of each dilution was added to 1.1% Noble agar pours (3.5 mL) at 55°C and poured onto YPD plates in duplicate for each dilution set. Plates were incubated at 30°C overnight and colony forming unit (CFUs) were counted after 24 hours.

Ethics statement

All mouse model experiments in this study were performed under an animal protocol (0016-0714) that was approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC) and followed the ethical guidelines set forth by the National Institute of Health (NIH) for the ethical treatment of animals.

In vitro cytotoxicity assays

The FaDu oral epithelial cell line was used in this study (Martinez-Lopez et al., 2006). Cells were routinely cultured in EBSS medium (Fisher Scientific, SH30024.01) with 10% fetal bovine serum (Fisher Scientific, BP1600-100) and 1% penicillin/streptomycin (Invitrogen, 15140-122) at 37°C and 5% CO₂. Prior to the cell killing assay, 5x10⁵ cells/mL of FaDu cells were plated in a 24 well plate. For each *C. albicans* strain, 1 mL of FaDu cells was plated in triplicate. *C. albicans* strains were cultured overnight in 5 mL YPD, and then washed twice with sterile water. Cells were resuspended in 10 mL H₂O and counted via hemocytometer. Solutions containing 2.5x10⁶ cells/mL of each strain (MOI=5) were made in EBSS supplemented with 2% Human Serum (MP Biomedicals, ICN2930149) and 1% penicillin/streptomycin.

Wells containing FaDu cells were aspirated and 1 mL of *C. albicans* cell suspensions were added to FaDu cells for co-incubation. In addition, the following control wells were included in triplicate: *C. albicans* alone, FaDu cells alone, medium alone, FaDu cells with lysis solution, and lysis solution with medium. Plates were centrifuged at 250xg for 5 minutes and incubated for 4 hours at 37°C at 5% CO₂. Following incubation, plates were spun at 250xg for 5 minutes, and 50 µL of each supernatant was assayed for LDH release using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, G1780) using the manufacturer's protocol. Cytotoxicity was expressed as a relative percentage of average wild type cytotoxicity and three biological replicates were performed (each with technical replicates in triplicate).

PC synthesis assay

This procedure was done as previously described for a phosphatidylserine synthase assay with some changes (Bae-Lee and Carman, 1984; Cassilly et al., 2017). The optimal PC synthesis assay mixture contained 50 mM Tris-HCl pH 7.5, 0.1% Triton X-100, 0.5 mM MnCl_2 and 0.1 mM DAG (Avanti Polar Lipids INC.) added as a suspension in 1% Triton X-100 and 0.5 mg protein in a total volume of 0.1 mL. The assay was performed by monitoring the incorporation of 0.5 mM cytidine diphosphocholine (Sigma-Aldrich) spiked with 10% by volume ^{14}C -methyl cytidine diphosphocholine ($\sim 0.2 \mu\text{Ci}$) into the chloroform-soluble product at 37°C for a predetermined amount of time. The reaction was terminated by the addition of 1 mL chloroform:methanol (2:1). Following this the reaction was extracted as previously described. Samples were fully dried in scintillation vials, 2.5 mL scintillation fluid was added to each vial, and they were measured with a liquid scintillation counter (Tri-Carb 2900TR).

In vivo PE synthesis assay

Cultures were grown to logarithmic phase at 30°C in YNB in media supplemented with $100 \mu\text{M}$ choline chloride and $0.2 \mu\text{Ci/mL}$ of ^{14}C ethanolamine. Phospholipids were isolated as described in Surlow *et al.* (Surlow et al., 2014). Lipid extracts representing equivalent amounts of optical density units (ODUs) were spotted onto silica gel TLC plates and plates were developed in chloroform:ethanol:water:triethylamine (30:35:7:35). A Typhoon 8600 phosphorimager was used for visualization and quantitation of PC and PE regions of intensity (ROI).

Statistical analyses and BLAST parameters

All statistical analyses were carried out using GraphPad Prism (version 7.04). For mouse survival curves the log-rank (Mantel-Cox) test was performed and for all other analyses unpaired t-tests between the indicated strains were used to determine statistical significance. For all analyses, $p < 0.05$ was used as a cutoff to determine statistical significance. To align protein sequences from *S. cerevisiae* to *C. albicans*, Basic Local Alignment Search Tool Protein-Protein (BLASTP) was used (NCBI, CGD). Sequences were obtained for *S. cerevisiae* S288C from the Saccharomyces Genome Database (SGD) and BLASTP was performed via the Candida Genome Database (CGD). The default settings were used to query assembly 22 of *C. albicans* SC5314 for hits. These settings included allowgaps = yes, gapext = 1, gapopen = 11, matrix = BLOSUM62.

Results

Disruption of PEM1 and PEM2 causes choline auxotrophy in *C. albicans*

To determine if CDP-DAG mediated PC biosynthesis is required for virulence in *C. albicans*, methylation of PE to PC was disrupted by constructing a *pem1* Δ/Δ *pem2* Δ/Δ knockout mutant using the *SAT1* flipper method (Reuss et al., 2004). The *pem1* Δ/Δ *pem2* Δ/Δ mutant is expected to be a choline auxotroph because it can only make PC from imported choline via the Kennedy pathway (Figure 2.1). Therefore, growth of the *pem1* Δ/Δ *pem2* Δ/Δ mutant was observed for 48 hours in minimal media (YNB) with or without 100 μ M choline supplementation (Figure 2.2, A). Little growth occurred in minimal medium lacking choline, indicating that the *pem1* Δ/Δ *pem2* Δ/Δ mutant cannot synthesize PC efficiently due to disruption of the CDP-DAG pathway. Residual growth of the *pem1* Δ/Δ *pem2* Δ/Δ may be due to the ability to store PC or choline within the cell. Addition of 100

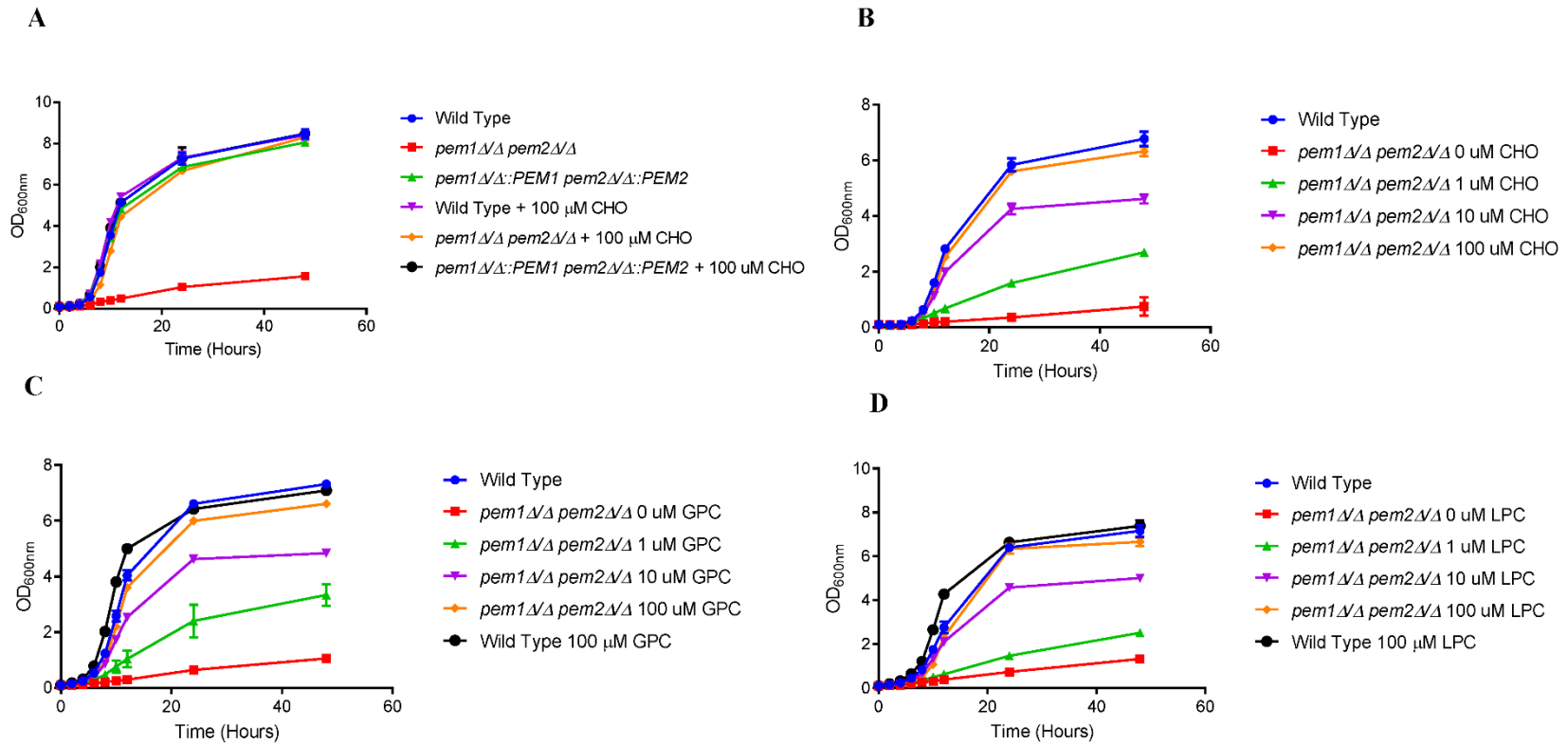


Figure 2.2. The *pem1ΔΔ pem2ΔΔ* mutant is a choline auxotroph.

(A) Wild type, *pem1ΔΔ pem2ΔΔ*, and *pem1ΔΔ::PEM1 pem2ΔΔ::PEM2* strains were grown overnight in minimal media, diluted to an OD_{600nm} = 0.1, and then cultured for 48 hours in minimal media, with or without 100 μM choline (CHO). Wild type and *pem1ΔΔ pem2ΔΔ* were also cultured for 48 hours in minimal media, with or without varying concentrations of (B) choline (CHO), (C) glycerophosphocholine (GPC), or (D) lysophosphatidylcholine (LPC). These assays were performed with three biological replicates.

μ M choline or reintegration of the methyltransferase genes (*pem1 Δ/Δ ::PEM1 pem2 Δ/Δ ::PEM2* strain) restored growth to wild-type levels, which indicates that exogenous choline can be used to synthesize PC via the Kennedy pathway. In fact, choline levels as low as 10 μ M also restored growth to nearly wild-type levels (Figure 2.2, B). Choline is found at $\sim$ 11 μ M in rodent serum (Klein et al., 1993).

To further investigate the ability of *C. albicans* to acquire choline from other exogenous sources we tested several choline-containing compounds that are commonly found in human and rodent tissues/serum, including lysophosphatidylcholine (LPC) and glycerophosphocholine (GPC), (Croset et al., 2000; Ilcol et al., 2005) to determine if they could also restore growth. LPC is as high as 450 μ M in rat serum (Suarez-Garcia et al., 2017) and GPC is as high as 4.5 μ M, but GPC can be over 1 mM in homogenized brain tissue and approximately 270 μ M/g protein in homogenized kidney tissue (Bauernschmitt and Kinne, 1993; Klein et al., 1993). The growth of the *pem1 Δ/Δ pem2 Δ/Δ* mutant was restored to the wild type level in minimal media when supplemented with either 100 μ M GPC or LPC and was restored to nearly wild-type levels even at 10 μ M (Figure 2.2, C-D).

To confirm that the *pem1 Δ/Δ pem2 Δ/Δ* mutant's lack of growth in choline-free media correlates with loss of PC synthesis, total lipids were extracted from cells growing in minimal medium lacking a source of choline and analyzed by thin layer chromatography (TLC) (Figure 2.3). While wild type *C. albicans* was able to produce PC regardless of supplementation, the *pem1 Δ/Δ pem2 Δ/Δ* mutant failed to produce PC unless supplemented with choline. Single mutants for both *PEM1* and *PEM2* were also tested for PC synthesis. For *PEM1*, a *pem1 Δ /PEM1::P_{MET3}-PEM1* conditional knockout was used, and for *PEM2* a *pem2 Δ/Δ* conventional knockout was made. Both were cultured

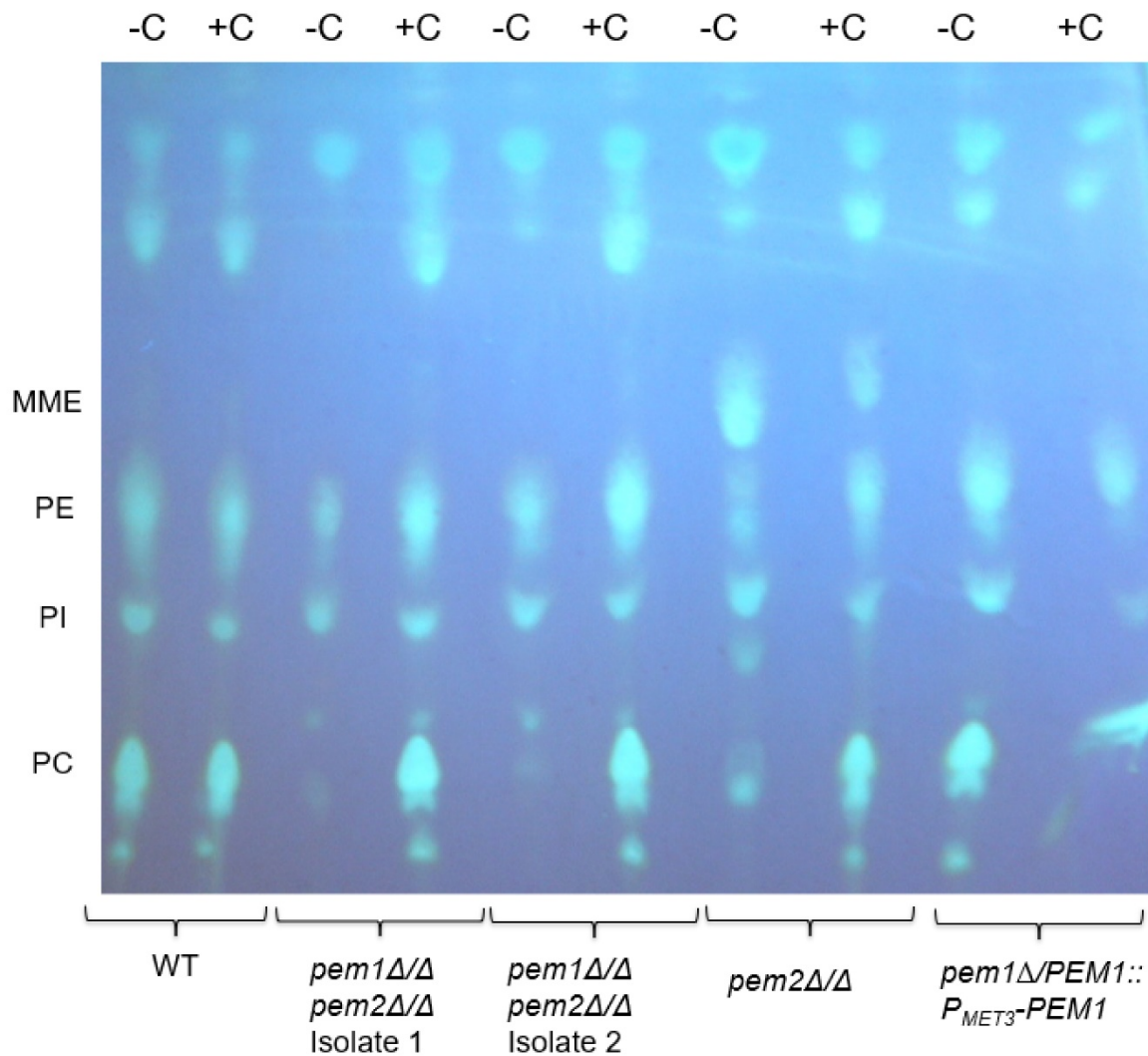


Figure 2.3. Choline auxotrophy correlates to a loss of PC.

Wild-type, *pem1*Δ/Δ *pem2*Δ/Δ, *pem2*Δ/Δ, and *pem1*Δ/*PEM1*::*P*_{MET3}-*PEM1* mutants were cultured in the presence of 0.25 μM L-methionine in minimal media (YNB) with (“+C”) or without (“-C”) 1 mM choline and total lipids were extracted. Phospholipids were separated with thin layer chromatography (TLC) and visualized using primulin. The *pem1*Δ/Δ *pem2*Δ/Δ mutant only produces PC when supplemented with choline.

with or without 1 mM choline (Figure 2.3). In addition, the YNB media included YNB 0.25 μ M cysteine/methionine to shut off expression of *PEM1* in the *pem1 Δ /PEM1::P_{MET3}-PEM1* strain. The *pem1 Δ /PEM1::P_{MET3}-PEM1* strain was fully capable of PC production even in the absence of choline when methionine/cysteine were present, but the *pem2 Δ/Δ* mutant failed to produce PC unless supplemented with choline and there was an increase in the intermediate product monomethylethanolamine. This resembles *S. cerevisiae*, in which *PEM2* has overlapping substrate specificity and can methylate PE (Preitschopf et al., 1993; Henry et al., 2012). This also indicates that Pem1 cannot efficiently methylate the intermediate products monomethylethanolamine or dimethylethanolamine to PC on its own. To further investigate this phenomenon, growth curves were performed for the *pem1 Δ /PEM1::P_{MET3}-PEM1* and *pem2 Δ/Δ* strains in minimal medium containing 0.25 μ M cysteine/methionine. It was found that loss of *PEM1* confers a slight growth defect, whereas loss of *PEM2* results in a severe growth defect, however, this defect is not as severe as that observed for the *pem1 Δ/Δ pem2 Δ/Δ* mutant (data not shown).

In some fungi, such as *Neurospora crassa*, there is an alternative for PC called diacylglycerol-N,N,N-trimethylhomoserine (DGTS) that can act as a functional analog to PC, but lacks phosphate, and is typically expressed under conditions of phosphate starvation (Riekhof et al., 2014). *Candida albicans* contains a putative *BTA1* ORF (C1_11490_A/orf19.8764), however the function of this gene has not been demonstrated experimentally. Given that the *pem1 Δ/Δ pem2 Δ/Δ* mutant is auxotrophic for PC, we hypothesized that overexpression of *BTA1* would restore growth for this strain in the absence of choline. A *pem1 Δ/Δ pem2 Δ/Δ P_{ENO1}-BTA1* strain was created and cultured in minimal media (YNB) lacking choline, and it was evident that overexpression of *BTA1*

restored growth (Figure 2.4). Thus, *C. albicans* contains a functional copy of *BTA1*, and overexpression of this DGTS synthase is able to compensate for the loss of PC in this organism. The fact that *C. albicans pem1Δ/Δ pem2Δ/Δ* fails to grow in the absence of choline indicates that *C. albicans* is not expressing *BTA1* as a means to compensate for loss of PE methylation.

PE from the CDP-DAG pathway accumulates in the pem1Δ/Δ pem2Δ/Δ mutant

If PE methylation to PC is blocked, it is expected that PE will accumulate in the cell and PC from this pathway will decrease. To test this, [¹⁴C]ethanolamine radiolabeling assays of whole cells were performed using the *pem1Δ/Δ pem2Δ/Δ* mutant. This experiment measures the accumulation of PE that is derived from the Kennedy pathway. There was an approximate 1.44-fold increase in radiolabeled PE in *pem1Δ/Δ pem2Δ/Δ* mutants as compared to the wild type (**p<0.001) (Figure 2.5) and little or no PC was detected. Thus, blockage of PE methylation results in the expected depletion of labeled PC and a buildup of labeled PE. When the methyltransferases are reintegrated into the mutant, statistically significant decreases in PE radiolabel accumulation and concurrent increases in PC radiolabel accumulation are measured relative to the mutant (*p<0.05). These data do not measure the total PE and PC in the cells, as the phospholipid pools produced by the CDP-DAG pathway for PE or the Kennedy pathway for PC are unlabeled (Figures 2.1, 2.5), but the data do reveal that blockage of the CDP-DAG pathway at this point increases PE and decreases PC specifically generated from imported ethanolamine.

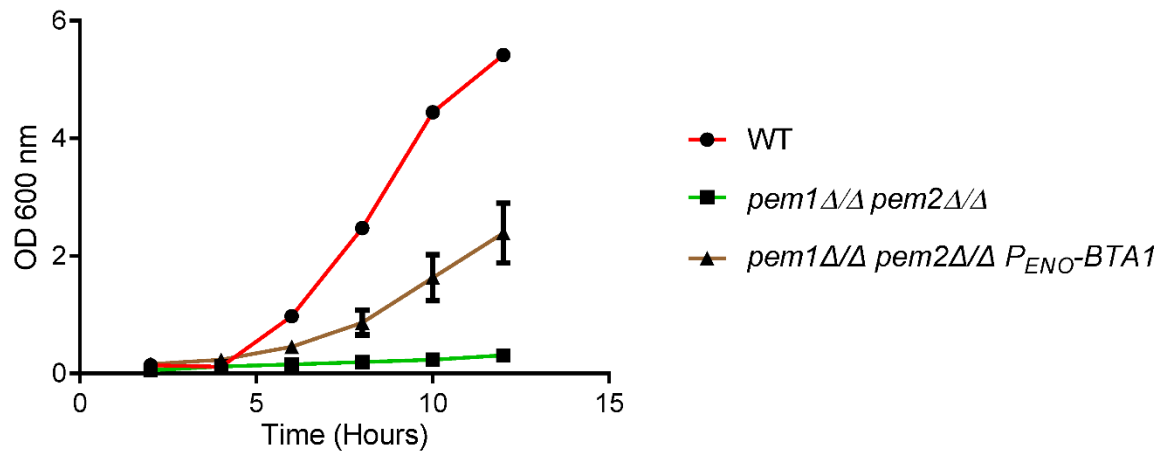


Figure 2.4. *BTA1* overexpression rescues choline auxotrophy.

Constitutive expression of the DGTS synthase, Bta1, suppresses growth defects caused by a loss of *de novo* PC synthesis in wild type *C. albicans* cultured in minimal media (YNB).

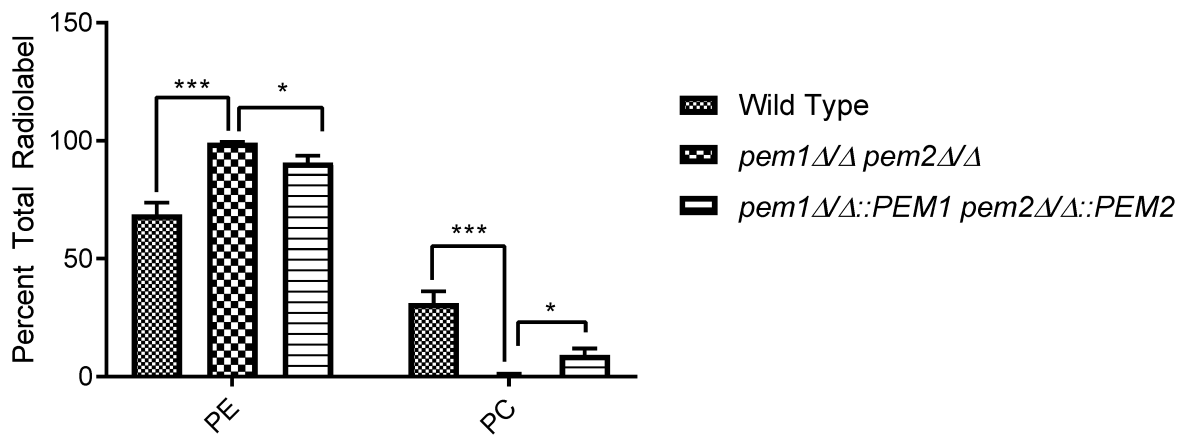


Figure 2.5. Radiolabeling of *pem1Δ/Δ pem2Δ/Δ*.

Strains were cultured in the presence of [¹⁴C]ethanolamine and incorporation of the radiolabel into PE and PC was quantified using thin-layer chromatography (TLC) of extracted lipids (***p<0.001, *p<0.05; unpaired *t*-test compared to wild-type). This assay was performed with biological replicates in triplicate

Blockage of PC synthesis by the PE methyltransferase mutant $pem1\Delta/\Delta$ $pem2\Delta/\Delta$ results in hypervirulence

Choline, GPC, and LPC can fully support growth of $pem1\Delta/\Delta$ $pem2\Delta/\Delta$ mutants *in vitro* (Figure 2.2). These compounds are all found in the host, and therefore can be used to make PC by pathways that are alternatives to the CDP-DAG pathway, such as the Kennedy pathway (Figure 2.1). However, it was still possible that loss of *PEM1* and *PEM2* would affect virulence, so the mutants were tested in a mouse model of systemic infection. Outbred ICR mice were infected with 3×10^5 cells each of either wild type (n=35), $pem1\Delta/\Delta$ $pem2\Delta/\Delta$ (n=25), or $pem1\Delta/\Delta::PEM1$ $pem2\Delta/\Delta::PEM2$ (n=10) via the tail vein. The $pem1\Delta/\Delta$ $pem2\Delta/\Delta$ strain is fully virulent (Figure 2.6), indicating that PC biosynthesis by the CDP-DAG pathway is not required for full virulence, as the Kennedy pathway can synthesize enough PC *in vivo* to support virulence using choline obtained from the host. Moreover, mice infected with the $pem1\Delta/\Delta$ $pem2\Delta/\Delta$ mutant succumb to infection more rapidly than those infected with wild type (**p<0.01) (Figure 2.6). This was unexpected, and we hypothesized that the $pem1\Delta/\Delta$ $pem2\Delta/\Delta$ mutant might be more virulent because the blockage of PC biosynthesis by the CDP-DAG pathway leads to increased PE, as seen *in vitro* (Figure 2.5). This could be because a block of the CDP-DAG pathway upregulates an alternative PE synthesis pathway, such as the Kennedy pathway. Alternatively, there may just be a buildup of PE as it cannot be methylated. In either case, this may have a downstream effect on virulence. If so, then overexpression of the Kennedy pathway (overexpression of PE synthesis enzymes by an alternative means), should increase virulence in a similar manner.

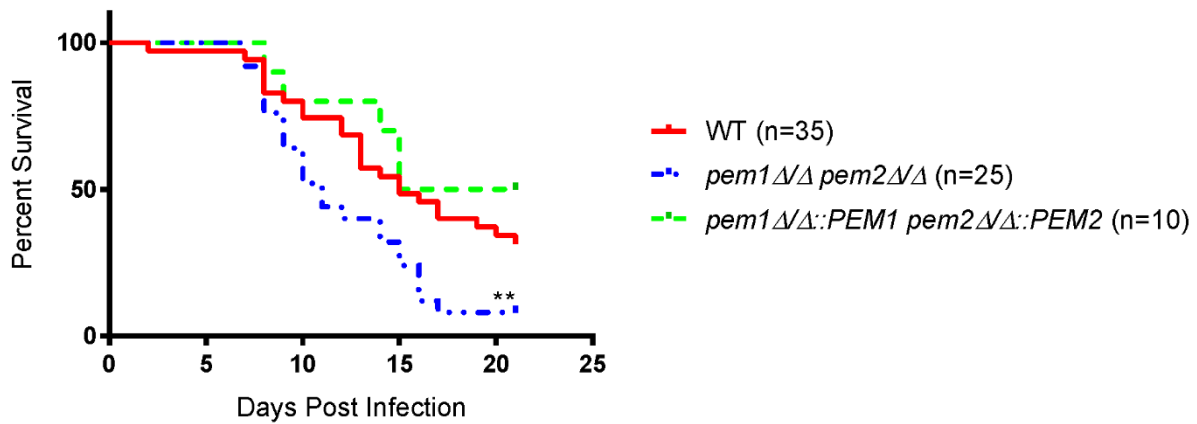


Figure 2.6. The *pem1*Δ/Δ *pem2*Δ/Δ mutant is hypervirulent.

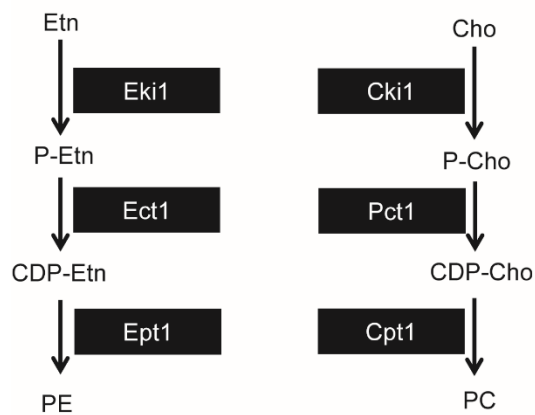
Mice were infected via the lateral tail vein with 3×10^5 wild type (WT), *pem1*Δ/Δ *pem2*Δ/Δ, or *pem1*Δ/Δ::PEM1 *pem2*Δ/Δ::PEM2 cells. Mice infected with the *pem1*Δ/Δ *pem2*Δ/Δ mutant succumb to infection more rapidly than those infected with wild type *C. albicans* (**p<0.01 compared to wild-type; log-rank (Mantel-Cox) test). The number of mice representing each strain is shown in parentheses beside each strain.

In contrast to S. cerevisiae, in C. albicans one enzyme, Ept1, catalyzes the final step for both PE and PC biosynthesis in the Kennedy Pathway.

The Kennedy pathway can use exogenous choline and ethanolamine from the environment to form PC and PE, respectively, and it also makes PC from internal stores of choline that have been released by phospholipase activity (Dowd et al., 2001; Fernandez-Murray et al., 2009). The current model for the *C. albicans* Kennedy pathway is inferred from that characterized in baker's yeast (Figure 2.1). In *S. cerevisiae* there are two branches of the Kennedy pathway; the CDP-ethanolamine and CDP-choline branches (Gibellini and Smith, 2010) (Figure 2.7, A). Each branch catalyzes analogous reactions to convert ethanolamine or choline into PE or PC, respectively. To begin synthesis of either PE or PC, ethanolamine or choline are imported into the cell by the ethanolamine/choline importer Hnm1 (Nikawa et al., 1986; Nikawa et al., 1990). These precursors are then phosphorylated by either ethanolamine (Eki1) or choline (Cki1) kinase to form phosphoethanolamine (P-Etn) or phosphocholine (P-Cho), respectively (Gibellini and Smith, 2010). P-Etn and P-Cho are used to form the high-energy intermediates CDP-ethanolamine and CDP-choline by the ethanolamine and choline cytidyltransferases, Ect1 and Pct1, respectively (Gibellini and Smith, 2010). Finally, diacylglycerol is condensed with CDP-ethanolamine by Ept1 to form PE, or CDP-choline by Cpt1 to form PC, with the release of CMP in both cases (Gibellini and Smith, 2010).

The two *S. cerevisiae* enzymes that represent each step of the Kennedy pathway (one from each branch, Figure 2.7, A) share high sequence identity. For example, when searching the *C. albicans* protein database in the Candida Genome Database (CGD) with the Basic Local Alignment Search Tool (BLAST) using the *S. cerevisiae* Eki1 sequence

A. *Saccharomyces cerevisiae*



B. *Candida albicans*

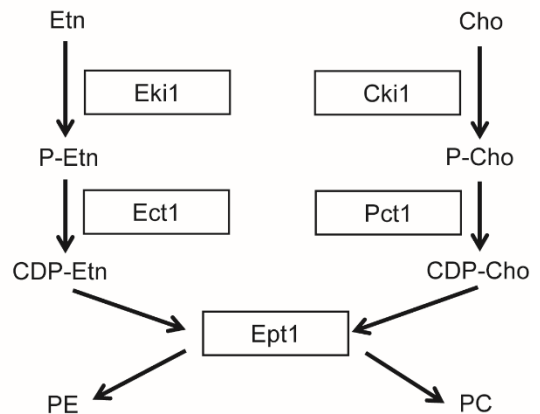


Figure 2.7. *C. albicans* dual-purpose ethanolamine/choline phosphotransferase. (A) Model for the *S. cerevisiae* Kennedy pathway and (B) proposed model for *C. albicans* Kennedy pathway. Abbreviations: Etn, ethanolamine; Cho, choline; P-Etn, phosphoethanolamine; P-Cho, phosphocholine; CDP-Etn, cytidinediphosphoethanolamine; CDP-Cho, cytidinediphosphocholine.

as a query, hits are given for both the *C. albicans* Eki1 (C7_01320W/orf19.6912) and Cki1 (C3_05300C/orf19.6966) sequences (e-values of $6e-7$ and $8e-87$, respectively). The same is true for *S. cerevisiae* Cki1. Thus, this holds true for the enzymes in the first two steps of the Kennedy pathway. However, we recovered only one protein sequence when we used BLAST to search CGD with the *S. cerevisiae* Ept1 or Cpt1 protein sequences. For either *S. cerevisiae* protein, a single *C. albicans* protein is revealed that is designated as *Ept1* (C7_02690C/orf19.3695; e-value of $3e-114$ for Ept1 and $7e-111$ for Cpt1). We hypothesized that *C. albicans* differs from *S. cerevisiae* in that it uses a single phosphotransferase (*C. albicans* Ept1) to catalyze the addition of both CDP-ethanolamine and CDP-choline to DAG to make PE and PC, respectively (Figure 2.7, B). If so, blocking this step of the pathway would impede Kennedy pathway mediated synthesis of both PE and PC. Alternatively, overexpressing it may increase synthesis of both PE and PC as well.

In order to determine if Ept1 alone catalyzes the final step of both branches of the Kennedy pathway in *C. albicans*, an *ept1* Δ/Δ knockout strain was generated using the SAT1 flipper method (Reuss et al., 2004). In addition, *EPT1* was cloned into an overexpression vector under the control of the strong, constitutive *ENO1* promoter in order to create a *P_{ENO1}-EPT1* overexpression plasmid. This *P_{ENO1}-EPT1* plasmid was integrated into wild-type *C. albicans*. In addition, reintegrants were made for *ept1* Δ/Δ in which one allele of *EPT1* was expressed at its native locus and under control of its native promoter or the *P_{ENO1}-EPT1* plasmid was used to reintegrate the gene.

To determine if these alterations in *EPT1* affected PC synthesis from its precursors CDP-choline and diacylglycerol (DAG), as predicted, a CDP-cholinephosphotransferase

assay was performed on membranes isolated from each strain. Membranes were isolated from the strains and incubated with [¹⁴C]CDP-choline and DAG, and the level of [¹⁴C]PC synthesized was measured. Disruption of *EPT1* resulted in a total loss of PC synthesis, while overexpression of *EPT1* in wild-type or *ept1Δ/Δ* resulted in twice the level of [¹⁴C]PC as found in wild-type (Figure 2.8, A, **p<0.01). Thus, Ept1 is solely responsible for catalyzing PC synthesis from CDP-choline and DAG.

Due to difficulty in obtaining radiolabeled [¹⁴C]CDP-ethanolamine, the role for *C. albicans* Ept1 in PE synthesis was tested by measuring synthesis of [¹⁴C]PE from [¹⁴C]ethanolamine in whole cells. Each strain was cultured in minimal (YNB) media supplemented with [¹⁴C]ethanolamine, and lipids were extracted (Surlow et al., 2014), separated by TLC, and quantified to measure incorporation of the radiolabel into PE (Figure 2.8, B, *p<0.05). Confirming our hypothesis, the *ept1Δ/Δ* strain does not incorporate a detectable amount of radiolabel into PE. In contrast, the *P_{ENO1}-EPT1* strain incorporates increased radiolabel into PE. Finally, the *ept1Δ/Δ* mutant transformed with *P_{ENO1}-EPT1* or *EPT1* on its native promoter has a restored ability to incorporate the radiolabels into both phospholipids (Figure 2.8, A-B). Thus, *EPT1* solely catalyzes the final step of both the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway in *C. albicans* (Figure 2.7, B).

Overexpression of EPT1 results in hypervirulence in the mouse systemic infection model

Given that overexpression of *EPT1* increases PE/PC synthesis by the Kennedy pathway, we wanted to determine if this would also cause hypervirulence as observed for

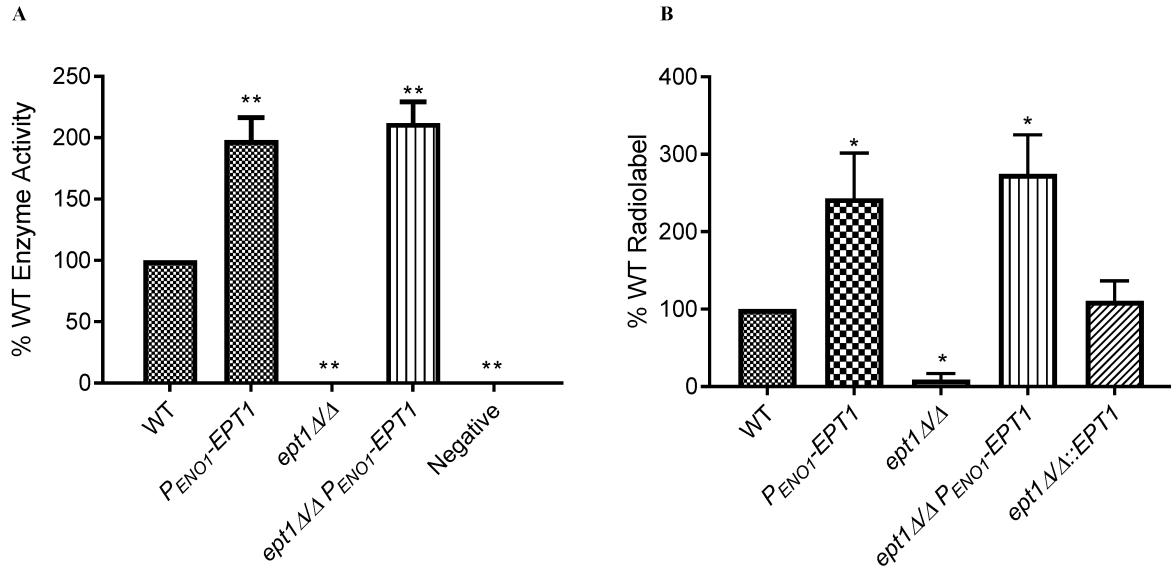


Figure 2.8. Synthesis of both PC and PE via the Kennedy pathway requires *EPT1*.

(A) Membranes were isolated from all strains and used in an *in vitro* PC synthesis assay, where [^{14}C]CDP-choline incorporation into PC was measured. Enzyme activity was measured as counts per minute per milligram of protein, and is expressed as a percentage of wild-type (** $p < 0.01$; unpaired *t*-test compared to wild-type, WT). **(B)** Each strain was cultured in the presence of [^{14}C]ethanolamine and incorporation of the radiolabel into PE was quantified by phosphorimager from thin-layer chromatography (* $p < 0.05$; compared to wild-type, WT by the unpaired *t*-test). Each of these assays was performed with three biological replicates.

pem1 Δ/Δ *pem2* Δ/Δ , which also accumulates PE (Figure 2.5). Therefore, wild-type and *P_{ENO1}-EPT1* strains were tested in the mouse model of systemic candidiasis. Mice infected with *P_{ENO1}-EPT1* *C. albicans* succumbed to infection more rapidly than those infected with wild type (***p*<0.01) (Figure 2.9, A). Thus, the *P_{ENO1}-EPT1* and *pem1* Δ/Δ *pem2* Δ/Δ mutants both accumulate PE and exhibit hypervirulence. We wanted to determine if this hypervirulence would correlate with increases in kidney fungal burden. Outbred ICR mice (Envigo) were infected with 5x10⁵ cells of the wild-type and *P_{ENO1}-EPT1* strains (n=10 for each strain). Five days post infection, the mice were necropsied, and kidneys were harvested to determine fungal burden. Mice infected with the *P_{ENO1}-EPT1* overexpression strain exhibited an average increase in fungal burden of 38.1% when compared to mice infected with wild type *C. albicans*, however this difference was not statistically significant (Figure 2.9, B, **p*=0.1885).

Disruption of EPT1 results in a decrease in kidney fungal burden in mice

As *EPT1* is necessary for PE and PC synthesis through the Kennedy pathway, and its overexpression increases virulence, we tested an *ept1* Δ/Δ mutant in this model to determine if it exhibits decreased virulence. For survival curves in the systemic infection model there was not a significant decrease in virulence for the *ept1* Δ/Δ strain compared to wild type (Figure 2.9, A). However, it should be noted that mice infected with the *ept1* Δ/Δ strain began to succumb to infection at 11 days post infection, which is slightly delayed compared to those infected with the wild-type (which begin to succumb to infection at 7 days post infection), which may indicate a modest, although not statistically significant, decrease in virulence. Although there was not a statistically significant

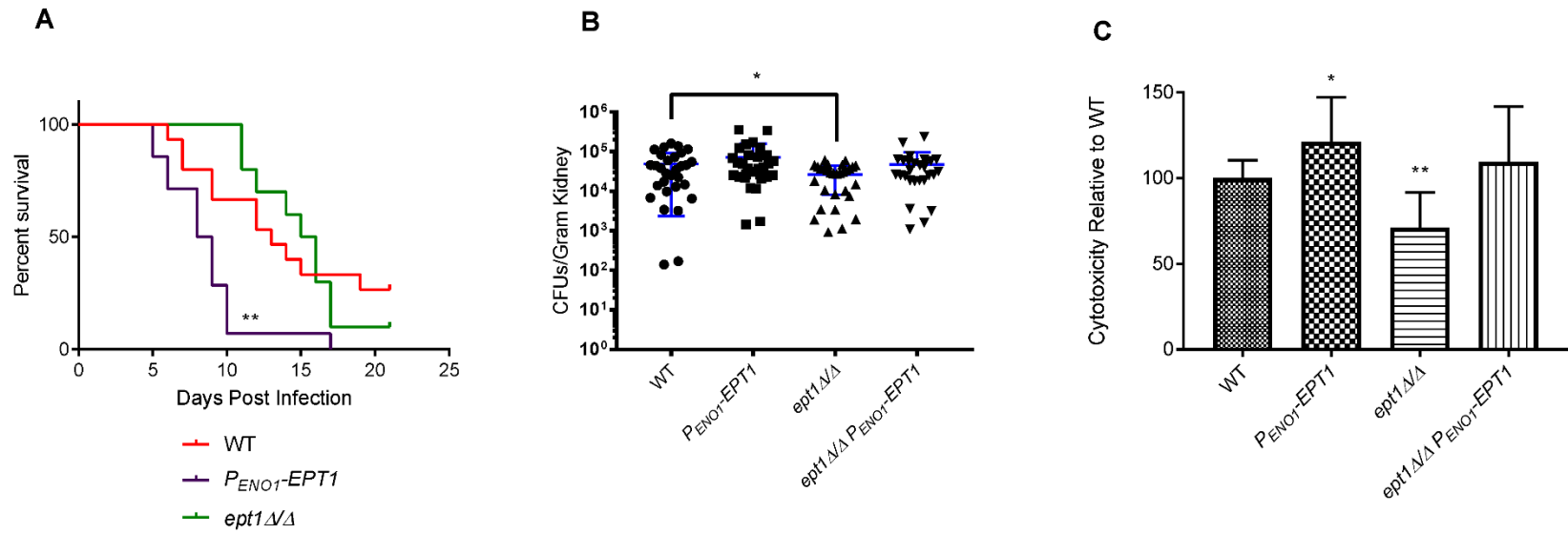


Figure 2.9. Overexpression of *EPT1* causes hypervirulence.

(A) Mice infected with P_{ENO1} -*EPT1* overexpression strain (n=14) succumb to infection more rapidly than the wild type (WT, n=15) (**p<.01; compared to WT, log-rank (Mantel-Cox) test). Mice infected with either one of two isogenic $ept1\Delta\Delta$ mutants do not differ significantly from those infected with wild type *C. albicans* (n=10 each). The n values for wild-type and P_{ENO1} -*EPT1* were derived from three separate experiments, and those for $ept1\Delta\Delta$ were derived from two separate experiments.

(B) Mice infected with $ept1\Delta\Delta$ have a decreased kidney fungal burden five days post infection compared to those infected with the wild type (*p<0.05; unpaired *t*-test, n=15 mice per strain). Mice infected with the $ept1\Delta\Delta$ P_{ENO1} -*EPT1* reintegrant resemble those infected with the wild type. Experiments were performed three times using five mice per strain and kidney homogenates were plated with two technical replicates for reproducibility.

(C) Expression level of *C. albicans EPT1* affects cytotoxicity in co-culture with human epithelial cells *in vitro*. The P_{ENO1} -*EPT1* overexpression strain has increased cytotoxicity (*p<0.05; unpaired *t*-test) compared to wild type, whereas $ept1\Delta\Delta$ has decreased cytotoxicity (**p<0.01). The $ept1\Delta\Delta$ P_{ENO1} -*EPT1* reintegrant resembles wild type for cytotoxicity. Biological replicates were performed in triplicate and technical replicates were also performed in triplicate (n=9 total replicates per strain).

difference in survival curves, mice infected with the *ept1* Δ/Δ mutant had a significant average decrease in kidney fungal burden of 44.97% as compared to the wild type (* $p < 0.05$) (Figure 2.9, B). This decrease in fungal burden may correlate with the slight delay seen in survival curves for mice infected with the *ept1* Δ/Δ strain. Conversely, mice infected with the reintegrand *ept1* $\Delta/ept1\Delta::P_{ENO1}$ -*EPT1* strain did not exhibit any significant differences in fungal burden compared to mice infected with wild type *C. albicans*.

***EPT1* expression affects cytotoxicity of *C. albicans* against epithelial cells**

The mechanism by which *EPT1* impacts virulence is unclear, as there are no clear impacts on growth rate in growth curves (data not shown). Thus, we examined another virulence factor, which is the ability of *C. albicans* to damage epithelial cells. This can be assessed by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) from host cells. In order to measure cytotoxicity of each mutant compared to wild-type, 2.5×10^6 *C. albicans* cells were co-cultured with human oral epithelial cells (FaDu cell line). A CytoTox 96[®] cytotoxicity assay kit (Promega) was used to quantify the release of LDH after four hours. There was a statistically significant decrease in cytotoxicity of the *ept1* Δ/Δ mutant as compared to the wild type (** $p < 0.01$) (Figure 2.9, C). Conversely, the *P_{ENO1}*-*EPT1* overexpression mutant has increased cytotoxicity compared to the wild type (* $p < 0.05$) (Figure 2.9, C). The *ept1* Δ/Δ *P_{ENO1}*-*EPT1* reintegrand does have a modest increase in cytotoxicity versus the wild type, but it is not statistically significant (Figure 2.9, C).

Discussion

In a previous communication we demonstrated that the synthesis of phospholipids via the CDP-DAG pathway is absolutely required for *Candida albicans* to be able to cause disease in the mouse model of systemic infection (Chen et al., 2010). The phospholipids produced in this pathway specifically are PS, PE, and PC (Figure 2.1). Blocking synthesis of PS or PE by disrupting the PS synthase (*cho1Δ/Δ* mutant) or both PS decarboxylases (*psd1Δ/Δ psd2Δ/Δ* mutant) results in avirulence (Chen et al., 2010). Although the full mechanism is not clear (Davis et al., 2018), PS and PE synthesis by the CDP-DAG pathway clearly play a crucial role in virulence. However, at the outset of this study the role of PE's downstream product, PC, was less clear. In addition, no prior studies have directly evaluated the role of the Kennedy pathway with regard to virulence in *C. albicans*.

Increased PE in two different pathways correlates with hypervirulence

To determine if PC was important for the ability to cause disease, we blocked CDP-DAG mediated PC biosynthesis by the *pem1Δ/Δ pem2Δ/Δ* mutation. Systemic infections carried out in mice revealed that this mutation did not block virulence, but instead mice infected with the *pem1Δ/Δ pem2Δ/Δ* mutant succumb to infection more rapidly than those infected with wild-type (Figure 2.6).

The *pem1Δ/Δ pem2Δ/Δ* mutation causes a loss of PC synthesis by methylation of PE, but simultaneously leads to a build-up of PE (Figure 2.5). Synthesis of PE has been shown to be required for virulence (Chen et al., 2010; Davis et al., 2018), which suggests the possibility that increased PE might lead to greater virulence. Therefore, we increased PE synthesis through the Kennedy pathway by overexpressing *EPT1* (Figures 2.7, 2.8),

and this led to hypervirulence as well (Figure 2.9, A). This suggests that increased PE synthesis causes hypervirulence, however it should be noted that overexpression of *EPT1* also causes increased PC synthesis.

We favor increased synthesis of PE rather than PC as an explanation of hypervirulence since PE generated by Ept1 builds up, but PC goes down in the hypervirulent *pem1* Δ/Δ *pem2* Δ/Δ mutant (Figure 2.5). Thus, the common phospholipid to increase in both *pem1* Δ/Δ *pem2* Δ/Δ and *P_{ENO1}-EPT1* (Figures 2.5 and 2.8) is PE. Furthermore, PE synthesized from either the CDP-DAG or Kennedy pathways can support virulence (Chen et al., 2010; Davis et al., 2018). Thus, taken altogether, these data suggest PE is the primary phospholipid influencing virulence.

However, the mechanism by which elevated PE causes virulence is not clear at this point. We do note that increased *EPT1* correlates with increased damage of epithelial cells, and although modest, this assay is measuring only short term (4 hours) damage, so increased damage over time (~10 days) may accumulate leading to more rapid terminal infection. However, the full mechanism by which increased virulence is mediated is unknown.

EPT1 is required for full virulence during systemic infections

Overexpression of *EPT1* leads to hypervirulence, but we also wanted to determine whether the Kennedy pathway is required for full virulence in *C. albicans*, as this pathway is present in a variety of eukaryotic pathogens and is required for virulence in some (Gibellini et al., 2009). Furthermore, it has been demonstrated that blocking the ability of *C. albicans* to import glycerophosphocholine (GPC), an intermediate metabolite that may

be shunted into the Kennedy pathway, attenuates virulence in the mouse model of systemic infection (Bishop et al., 2013). Until now, no virulence studies have been performed on the Kennedy pathway in *C. albicans*, and so it has also remained unknown whether this pathway is redundant in *C. albicans*, or if it is specifically required for growth in the host.

We found that disruption of *EPT1* caused a modest decrease in kidney fungal burden (Figure 2.9, B), but not a significant increase in mouse survival (Figure 2.9, A). This modest decrease in fungal burden might help explain the modest increase in the time in which mice begin to succumb to infection in the *ept1* Δ/Δ mutant (11 days) compared to wild-type (7 days) (Figure 2.9, A), however, the overall difference in curves was not statistically significant, so the modest decrease in fungal burden does not appear to have a large impact on overall virulence. The decrease in kidney fungal burden also correlated with a modest decrease in damage to epithelial cells (Figure 2.9, C). Thus, it is possible that the *ept1* Δ/Δ mutant is unable to damage host cells as well as wild-type *C. albicans*, and this could help explain the loss of fungal burden. Also, it is not entirely clear whether the effect is related to PE or PC, as Ept1 synthesizes both in *C. albicans*.

C. albicans Ept1 synthesizes both PE and PC in the Kennedy pathway

Prior to this study the model for the Kennedy pathway in *C. albicans* was based entirely on that elucidated for *S. cerevisiae*. However, we found that while all the enzymes involved in the first two steps of either PE or PC biosynthesis had homologs in *C. albicans*, only one enzyme for the last step of synthesis was found in *C. albicans*: Ept1. Given the results of the homology search and the fact that there is some overlap in substrate

specificity between *EPT1* and *PCT1* in *S. cerevisiae* (Gibellini et al., 2009), we hypothesized that this enzyme was responsible for both PE and PC biosynthesis in *C. albicans*. This was tested using [¹⁴C] radiolabeling. Our findings are that the knockout cannot incorporate radiolabeled precursors into either PE or PC, and that when the gene is overexpressed an increased amount of the radiolabeled precursors are incorporated into PE and PC. This indicates that Ept1 is the only enzyme that catalyzes this reaction in *C. albicans*, and we can therefore block the Kennedy pathway by knocking out *EPT1* and overexpress the pathway by overexpressing *EPT1*. We do not yet fully understand how the Kennedy pathway influences virulence, but the ability to modulate virulence in either direction using *EPT1* expression levels indicates that this is an important pathway for controlling the pathogenicity of this fungus.

Funding

This work was funded in part by National Institutes of Health grants NIH 1R01AL105690 (TBR), NIH 1R21AI130895 (TBR), and NIH R15 GM104876 (JP-V). The funders had no role in the design or execution of the project or the decision to publish.

Acknowledgements

We thank Dr. Joachim Morschhäuser for the *SAT1* flipper plasmid, pSFS2A. We also thank Eric Gann, Joseph Jackson, Andrew Wagner, Tian Chen, Kyle Bonifer, and Dr. Tim Sparer for their helpful comments and discussions of the manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Bae-Lee, M.S., and Carman, G.M. (1984). Phosphatidylserine synthesis in *Saccharomyces cerevisiae*. Purification and characterization of membrane-associated phosphatidylserine synthase. *J Biol Chem* 259(17), 10857-10862.
- Bauernschmitt, H.G., and Kinne, R.K. (1993). Metabolism of the 'organic osmolyte' glycerophosphorylcholine in isolated rat inner medullary collecting duct cells. II. Regulation by extracellular osmolality. *Biochim Biophys Acta* 1150(1), 25-34.
- Bishop, A.C., Ganguly, S., Solis, N.V., Cooley, B.M., Jensen-Seaman, M.I., Filler, S.G., et al. (2013). Glycerophosphocholine utilization by *Candida albicans*: role of the Git3 transporter in virulence. *J Biol Chem* 288(47), 33939-33952. doi: 10.1074/jbc.M113.505735.
- Bouza, E., and Munoz, P. (2008). Epidemiology of candidemia in intensive care units. *Int J Antimicrob Agents* 32 Suppl 2, S87-91. doi: 10.1016/S0924-8579(08)70006-2.
- Cassilly, C.D., Farmer, A.T., Montedonico, A.E., Smith, T.K., Campagna, S.R., and Reynolds, T.B. (2017). Role of phosphatidylserine synthase in shaping the phospholipidome of *Candida albicans*. *FEMS Yeast Res* 17(2), (2): fox007. doi: 10.1093/femsyr/fox007.
- Chen, Y.L., Kauffman, S., and Reynolds, T.B. (2008). *Candida albicans* uses multiple mechanisms to acquire the essential metabolite inositol during infection. *Infect Immun* 76(6), 2793-2801. doi: 10.1128/IAI.01514-07.
- Chen, Y.L., Montedonico, A.E., Kauffman, S., Dunlap, J.R., Menn, F.M., and Reynolds, T.B. (2010). Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence in *Candida albicans*. *Mol Microbiol* 75(5), 1112-1132. doi: 10.1111/j.1365-2958.2009.07018.x.
- Croset, M., Brossard, N., Polette, A., and Lagarde, M. (2000). Characterization of plasma unsaturated lysophosphatidylcholines in human and rat. *Biochem J* 345 Pt 1, 61-67.
- Davis, S.E., Tams, R.N., Solis, N., Wagner, A.S., Chen, T., Jackson, J.W., et al. (2018). *Candida albicans* cannot acquire sufficient ethanolamine from the host to support virulence in the absence of de novo phosphatidylethanolamine synthesis. *Infect Immun* 86(8), e00815-00817. doi: 10.1128/IAI.00815-17.
- de Kroon, A.I., Rijken, P.J., and De Smet, C.H. (2013). Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. *Prog Lipid Res* 52(4), 374-394. doi: 10.1016/j.plipres.2013.04.006.
- Dowd, S.R., Bier, M.E., and Patton-Vogt, J.L. (2001). Turnover of phosphatidylcholine in *Saccharomyces cerevisiae*. The role of the CDP-choline pathway. *J Biol Chem* 276(6), 3756-3763. doi: 10.1074/jbc.M003694200.
- Exton, J.H. (1994). Phosphatidylcholine breakdown and signal transduction. *Biochim Biophys Acta* 1212(1), 26-42.
- Fernandez-Murray, J.P., Gaspard, G.J., Jesch, S.A., and McMaster, C.R. (2009). NTE1-encoded phosphatidylcholine phospholipase b regulates transcription of phospholipid biosynthetic genes. *J Biol Chem* 284(52), 36034-36046. doi: 10.1074/jbc.M109.063958.
- Gibellini, F., Hunter, W.N., and Smith, T.K. (2009). The ethanolamine branch of the Kennedy pathway is essential in the bloodstream form of *Trypanosoma brucei*. *Mol*

- Microbiol* 73(5), 826-843. doi: 10.1111/j.1365-2958.2009.06764.x.
- Gibellini, F., and Smith, T.K. (2010). The Kennedy pathway--De novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life* 62(6), 414-428. doi: 10.1002/iub.337.
- Gillum, A.M., Tsay, E.Y., and Kirsch, D.R. (1984). Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3* and *E. coli* *pyrF* mutations. *Mol Gen Genet* 198(2), 179-182.
- Guthrie C., F.G.R. (2002). *Methods in Enzymology*. Academic Press.
- Hasim, S., Hussin, N.A., Alomar, F., Bidasee, K.R., Nickerson, K.W., and Wilson, M.A. (2014). A glutathione-independent glyoxalase of the DJ-1 superfamily plays an important role in managing metabolically generated methylglyoxal in *Candida albicans*. *J Biol Chem* 289(3), 1662-1674. doi: 10.1074/jbc.M113.505784.
- Henry, S.A., Kohlwein, S.D., and Carman, G.M. (2012). Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. *Genetics* 190(2), 317-349. doi: 10.1534/genetics.111.130286.
- Houweling, M., Tijburg, L.B., Vaartjes, W.J., and van Golde, L.M. (1992). Phosphatidylethanolamine metabolism in rat liver after partial hepatectomy. Control of biosynthesis of phosphatidylethanolamine by the availability of ethanolamine. *Biochem J* 283 (Pt 1), 55-61.
- Ilcol, Y.O., Ozbek, R., Hamurtekin, E., and Ulus, I.H. (2005). Choline status in newborns, infants, children, breast-feeding women, breast-fed infants and human breast milk. *J Nutr Biochem* 16(8), 489-499. doi: 10.1016/j.jnutbio.2005.01.011.
- Kabir, M.A., Hussain, M.A., and Ahmad, Z. (2012). *Candida albicans*: A Model Organism for Studying Fungal Pathogens. *ISRN Microbiol* 2012, 538694. doi: 10.5402/2012/538694.
- Klein, J., Gonzalez, R., Koppen, A., and Loffelholz, K. (1993). Free choline and choline metabolites in rat brain and body fluids: sensitive determination and implications for choline supply to the brain. *Neurochem Int* 22(3), 293-300.
- Letscher-Bru, V., and Herbrecht, R. (2003). Caspofungin: the first representative of a new antifungal class. *J Antimicrob Chemother* 51(3), 513-521.
- Martinez-Lopez, R., Park, H., Myers, C.L., Gil, C., and Filler, S.G. (2006). *Candida albicans* Ecm33p is important for normal cell wall architecture and interactions with host cells. *Eukaryot Cell* 5(1), 140-147. doi: 10.1128/EC.5.1.140-147.2006.
- Mukherjee, P.K., Sheehan, D.J., Hitchcock, C.A., and Ghannoum, M.A. (2005). Combination treatment of invasive fungal infections. *Clin Microbiol Rev* 18(1), 163-194. doi: 10.1128/CMR.18.1.163-194.2005.
- Nikawa, J., Hosaka, K., Tsukagoshi, Y., and Yamashita, S. (1990). Primary structure of the yeast choline transport gene and regulation of its expression. *J Biol Chem* 265(26), 15996-16003.
- Nikawa, J., Tsukagoshi, Y., and Yamashita, S. (1986). Cloning of a gene encoding choline transport in *Saccharomyces cerevisiae*. *J Bacteriol* 166(1), 328-330.
- Preitschopf, W., Luckl, H., Summers, E., Henry, S.A., Paltauf, F., and Kohlwein, S.D. (1993). Molecular cloning of the yeast OPI3 gene as a high copy number suppressor of the *cho2* mutation. *Curr Genet* 23(2), 95-101.
- Reuss, O., Vik, A., Kolter, R., and Morschhauser, J. (2004). The SAT1 flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene* 341, 119-127. doi:

- 10.1016/j.gene.2004.06.021.
- Riekhof, W.R., Naik, S., Bertrand, H., Benning, C., and Voelker, D.R. (2014). Phosphate starvation in fungi induces the replacement of phosphatidylcholine with the phosphorus-free betaine lipid diacylglycerol-N,N,N-trimethylhomoserine. *Eukaryot Cell* 13(6), 749-757. doi: 10.1128/EC.00004-14.
- Sanguinetti, M., Posteraro, B., and Lass-Flörl, C. (2015). Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. *Mycoses* 58 Suppl 2, 2-13. doi: 10.1111/myc.12330.
- Singh, A., Prasad, T., Kapoor, K., Mandal, A., Roth, M., Welti, R., et al. (2010). Phospholipidome of *Candida*: each species of *Candida* has distinctive phospholipid molecular species. *OMICS* 14(6), 665-677. doi: 10.1089/omi.2010.0041.
- Styles, C. (2002). How to set up a yeast laboratory. *Methods in Enzymology* 350, 42-71. doi: 10.1016/S0076-6879(02)50955-1.
- Suarez-Garcia, S., Arola, L., Pascual-Serrano, A., Arola-Arnal, A., Aragones, G., Blade, C., et al. (2017). Development and validation of a UHPLC-ESI-MS/MS method for the simultaneous quantification of mammal lysophosphatidylcholines and lysophosphatidylethanolamines in serum. *J Chromatogr B Analyt Technol Biomed Life Sci* 1055-1056, 86-97. doi: 10.1016/j.jchromb.2017.04.028.
- Surlow, B.A., Cooley, B.M., Needham, P.G., Brodsky, J.L., and Patton-Vogt, J. (2014). Loss of Ypk1, the yeast homolog to the human serum- and glucocorticoid-induced protein kinase, accelerates phospholipase B1-mediated phosphatidylcholine deacylation. *J Biol Chem* 289(45), 31591-31604. doi: 10.1074/jbc.M114.581157.
- Williams, D., and Lewis, M. (2011). Pathogenesis and treatment of oral candidosis. *J Oral Microbiol* 3. doi: 10.3402/jom.v3i0.5771.

Appendix B

Genetic modifications

Short oligonucleotides (primers) were used for the creation of plasmids in *E. coli*, and to knock out, overexpress, or reintegrate genes in *C. albicans* (Table B.1). In addition, primers were used to verify the integration or absence of genes from specific loci in the *C. albicans* genome, as well as within plasmid constructs in *E. coli*, and to sequence genes upon integration to identify any potential mutations (Table B.1).

Table B.1. Primers used in chapter two.

Primer	Description/Function	Sequence
JCO95	<i>P_{MAL2}</i> reverse primer to confirm presence/absence of <i>SAT1</i> flipper vectors	5'-GATTATTAGTTAAACCACTGC-3'
JCO96	<i>URA3</i> terminator forward primer to confirm presence/absence of <i>SAT1</i> flipper vectors	5'-TGAAGGGGGAGATTTTCACT-3'
JCO165	<i>EcoRI</i> linked forward primer to amplify the <i>SAT1</i> marker for conditional <i>PEM1</i> knockout	5-AAAGAATTCCGTCAAACCTAGAGAATAATAAAG-3'
JCO166	<i>EcoRI</i> linked reverse primer to amplify 5' NCR of <i>P_{MET3}</i> for conditional <i>PEM1</i> knockout	5'-AAAGAATTCGTTTTCTGGGGAGGGTATTT-3'
JCO192	<i>KpnI</i> linked forward primer to amplify 5' NCR of <i>PEM2</i>	5'-AAAGGTACCCAGTTTCCATACCAGTCTCA-3'
JCO193	<i>Apal</i> linked reverse primer to amplify 5' NCR of <i>PEM2</i>	5'-AAAGGGCCCGACAGGAGTAATGAATGTAT-3'
JCO194	<i>SacII</i> linked forward primer to amplify 3' NCR of <i>PEM2</i>	5'-AAACCGCGGTCTTGTGGTATTGATTCGGGG-3'
JCO195	<i>SacI</i> linked reverse primer to amplify 3' NCR of <i>PEM2</i>	5'-AAAGAGCTCACCCGTTTTTTTTTTCATTGC-3'
JCO196	<i>PEM2</i> 3' NCR reverse primer to confirm <i>PEM2</i> knockout	5'-TTGTCCACTTCTTCATCAGCA-3'
JCO197	<i>KpnI</i> linked forward primer to amplify 5' NCR of <i>PEM1</i>	5'-AAAGGTACCTTTCATTGATCTTTATTGAAA-3'
JCO198	<i>Apal</i> linked reverse primer to amplify 5' NCR of <i>PEM1</i>	5'-AAAGGGCCCTGTTCAAAAAAAAAAAAAA-3'
JCO199	<i>SacII</i> linked forward primer to amplify 3' NCR of <i>PEM1</i>	5'-AAACCGCGGGGAGTTTTGTATTAATTTT-3'
JCO200	<i>SacI</i> linked reverse primer to amplify 3' NCR of <i>PEM1</i>	5'-AAAGAGCTCATTGAACATAAATTGAGTT-3'
JCO201	<i>PEM1</i> 3' NCR reverse primer to confirm <i>PEM1</i> knockout	5'-ATTTGGTCCACGTCCAATCA-3'
JCO203	<i>PEM2</i> ORF reverse primer to confirm <i>PEM2</i> knockout	5'-GGTTCTTCAAACAATAATGCG-3'
JCO215	<i>SacII</i> linked forward primer to amplify <i>PEM1</i> ORF for conditional knockout	5'-AAACCGCGGATGACTTTCACAATCAATAAT-3'
JCO216	<i>SacI</i> linked reverse primer to amplify <i>PEM1</i> ORF for conditional knockout	5'-AAAGAGCTCTTATACATCTTTTTTATCAGT-3'
JCO217	Forward primer to amplify 5' NCR of <i>PEM1</i> to confirm conditional knockout	5'-TCCCCCTTTTCTCTAAAAG-3'

Table B.1. (Continued)

Primer	Description/Function	Sequence
JCO220	Reverse primer to amplify <i>SAT1</i> ORF to confirm conditional knockout	5'-CTTTATTATTCTCTAGTTTTGACG-3'
BTO24	<i>PEM1</i> 5' NCR forward primer to confirm <i>PEM1</i> knockout	5'-CACGAGTACCCTTTGATTG-3'
BTO25	<i>PEM1</i> 5' NCR forward primer to confirm <i>PEM1</i> knockout	5'-GATAGACAATCATTCTTATACAGT-3'
BTO26	<i>PEM1</i> 3' NCR reverse primer to confirm <i>PEM1</i> knockout	5'-GTAGTGGACAAGTTAGACTTACA-3'
BTO27	<i>PEM1</i> ORF reverse primer to confirm <i>PEM1</i> knockout	5'-GTATTGAATTCCACTGGCATGGA-3'
BTO28	<i>NotI</i> linked forward primer to amplify <i>BTA1</i> ORF	5'- AAAAGCGGCCGCGAATGTCAGAATTCATAGTTG- 3'
BTO29	<i>SacI</i> linked forward primer to amplify <i>BTA1</i> ORF	5'-AAAAGAGCTCCACATAAAGAGGGGATATTC-3'
BTO30	<i>BamHI</i> linked forward primer to amplify 5' NCR of <i>ENO1</i>	5'-AAAAGGATCCAGGTCTGTCATATTTCTATC-3'
BTO31	<i>NotI</i> linked reverse primer to amplify 5' NCR of <i>ENO1</i>	5'- AAAAGCGGCCGCGTTGTAATATTCCTGAATTATC- 3'
BTO33	<i>P_{ENO1}</i> colony PCR forward to confirm overexpression inserts	5'-GACTAACGATTTCTATAAGG-3'
BTO34	<i>BTA1</i> colony PCR reverse	5'-GAAACAAATCAACTATGAATT C-3'
BTO35	<i>NotI</i> linked forward primer to amplify <i>EPT1</i> ORF	5'-AAAAGCGGCCGCATGGGACTTTTCATTCCAAC- 3'
BTO36	<i>SacI</i> linked reverse primer to amplify <i>EPT1</i> ORF	5'-AAAAGAGCTCCAAGATGTTTTAGCTCCTG-3'
BTO37	<i>NotI</i> linked forward primer to amplify <i>PCT1</i> ORF	5'- AAAAGCGGCCGCAATATGGCTAGACTTACTAG-3'
BTO38	<i>SacI</i> linked reverse primer to amplify <i>PCT1</i> ORF	5'-AAAAGAGCTCCAATTAAATCCTGTGACATTG-3'
BTO42	<i>PCT1</i> ORF reverse primer to confirm <i>PCT1</i> overexpression	5'-CTCTTTCTAGTAAGTCTAGC-3'
BTO43	<i>EPT1</i> ORF reverse primer to confirm <i>EPT1</i> overexpression	5'-GTTAGTTGGAATGAAAAGTC-3'
BTO44	<i>KpnI</i> linked forward primer to amplify 5' NCR of <i>EPT1</i>	5'-AAAAGGTACCCTTTTGTTAGGAGCTGTAG-3'
BTO45	<i>XhoI</i> linked reverse primer to amplify 5' NCR of <i>EPT1</i>	5'-AAAACGAGGTGTTCTTGGTGTGATTG-3'

Table B.1. (Continued)

Primer	Description/Function	Sequence
BTO46	<i>NotI</i> linked forward primer to amplify 3' NCR of <i>EPT1</i>	5'-AAAAGCGGCCGCGATACGTTTTGTTTGGTTAC-3'
BTO47	<i>SacI</i> linked reverse primer to amplify 3' NCR of <i>EPT1</i>	5'-AAAAGAGCTCCACCAGAACAAAGAGAATC-3'
BTO52	<i>ApaI</i> linked forward primer to amplify <i>PEM1</i> ORF for reintegration	5'-AAAAGGGCCCCCTGTTGAATACAATCTCTTG-3'
BTO53	<i>XhoI</i> linked reverse primer to amplify <i>PEM1</i> ORF for reintegration	5'-AAAAC TCGAGCTGACTGAAGATGGAATTG-3'
BTO54	<i>NotI</i> linked forward primer to amplify 3' NCR of <i>PEM1</i> for reintegration	5'-AAAAGCGGCCGCGTAAGTCTAACTTGTCCAC-3'
BTO55	<i>SacI</i> linked reverse primer to amplify 3' NCR of <i>PEM1</i> for reintegration	5'-AAAAGAGCTCCAATGAGTGATAGAAGACG-3'
BTO56	<i>EPT1</i> 5' NCR forward primer to confirm <i>EPT1</i> knockout	5'-GTGATGATGATGGTTCTTTC-3'
BTO57	<i>EPT1</i> ORF reverse primer to confirm <i>EPT1</i> knockout	5'-GAGTTTCATTTAAATAAGGATC-3'
BTO58	<i>EPT1</i> 3' NCR reverse primer to confirm <i>EPT1</i> knockout	5'-CACATTATCGATGAAGCTAG-3'
BTO59	<i>EPT1</i> ORF forward primer to confirm <i>EPT1</i> knockout	5'-GCTTATCGAGGATTATATCC-3'
BTO67	<i>ApaI</i> linked reverse primer to amplify <i>PEM2</i> ORF for reintegration	5'-AAAAGGGCCCCCCCCGAATCAATACCACAAGA-3'
BTO87	<i>PEM2</i> ORF reverse primer to confirm <i>PEM2</i> reintegration	5'-CTTGAGCAATTGATGACATG-3'
BTO88	<i>PEM2</i> 5' NCR forward primer to confirm <i>PEM2</i> reintegration	5'-GCAATGACATTTGATAGCTG-3'
BTO89	<i>NotI</i> linked forward primer to amplify <i>ECT1</i> ORF	5'-AAAAGCGGCCGCGATGTACGAAAGGCCAGAAG-3'
BTO90	<i>SacI</i> linked reverse primer to amplify <i>ECT1</i> ORF	5'-AAAAGAGCTCCTAGAACTAACAAATGGACTG-3'
BTO91	<i>NotI</i> linked forward primer to amplify <i>PSD2</i> ORF	5'-AAAAGCGGCCGCGATGAGATCTTCTGGCTATTTAA-3'
BTO92	<i>SacI</i> linked reverse primer to amplify <i>PSD2</i> ORF	5'-AAAAGAGCTCCATAATCCATATTCACGAATG-3'
BTO95	<i>ECT1</i> ORF reverse primer to confirm <i>ECT1</i> overexpression	5'-CTTCTGGCCTTTCGTACAT-3'
BTO96	<i>PSD2</i> ORF reverse primer to confirm <i>PSD2</i> overexpression	5'-TTAAATAGCCAGAAGATCTC-3'

Table B.1. (Continued)

Primer	Description/Function	Sequence
BTO105	<i>EPT1</i> 3' NCR reverse primer to confirm <i>EPT1</i> reintegration	5'-GATCAAGAAATTGCTCTTAGAG-3'
BTO106	<i>EPT1</i> 5' NCR forward primer to confirm <i>EPT1</i> reintegration	5'-CTGGATTTATATTCAGAATCAT-3'
TRO1044	<i>KpnI</i> linked forward primer to amplify <i>EPT1</i> for reintegration	5'-AAAAGGTACCCCCCCTCTGAACAAAAATTG-3'
TRO1045	<i>XhoI</i> linked reverse primer to amplify <i>EPT1</i> for reintegration	5'-AAAACGCGAGCCAAAAGCAAAGTAAAAGCAA-3'
TRO1046	<i>SacII</i> linked forward primer to amplify 3' NCR of <i>EPT1</i> for reintegration	5'-AAAACCGCGGTGGATTGGATTGGATTGGATC-3'
TRO1047	<i>SacI</i> linked reverse primer to amplify 3' NCR of <i>EPT1</i> for reintegration	5'-AAAAGAGCTCTCAACTTTTCCTCCAACCTACAAA-3'

Chapter 3 : Overexpression of *Candida albicans* *EPT1* Alters Cell Wall Composition and Hyphal Length

Publication Note

A version of this chapter has been submitted for peer review and publication. If accepted for publication, the authors and title for this publication will be as follows:

Robert N. Tams, Andrew S. Wagner, Joseph W. Jackson, Eric R. Gann, Timothy E. Sparer, and Todd B. Reynolds. *Overexpression of Candida albicans EPT1 Alters Cell Wall Composition and Hyphal Length.*

The author contributions are as follows: RT and TBR designed and created plasmids and *C. albicans* strains. RT performed RNA extractions and sequencing. Bioinformatic analysis was performed by RT and EG. AW performed chitin and $\beta(1,3)$ -glucan staining and subsequent flow cytometry. JJ and RT performed staining and flow cytometry of macrophages. RT performed all other assays and wrote the manuscript. TBR directed the project and edited the manuscript.

Abstract

Candida albicans is a leading cause of systemic bloodstream infections in immunocompromised patients and phospholipid biosynthesis is an important requirement for this organism to grow and cause disease. We have previously demonstrated that the *psd1* Δ/Δ *psd2* Δ/Δ mutant, which cannot synthesize the phospholipid phosphatidylethanolamine (PE) by the CDP-DAG pathway, is avirulent in the mouse model of systemic candidiasis and has decreased cytotoxicity against oral epithelial cells *in vitro*. Similarly, an *ept1* Δ/Δ mutant, which cannot produce PE by the Kennedy pathway, exhibits decreased kidney fungal burden in systemically infected mice and has decreased epithelial cell cytotoxicity *in vitro*. Conversely, overexpression of *EPT1* results in a hypervirulent phenotype both *in vivo* and *in vitro*. Thus, mutations that increase PE

synthesis increase virulence, and mutations that decrease PE synthesis decrease virulence. However, the mechanism by which virulence is regulated by these PE regulatory enzymes is only partially understood. In the present study RNA sequencing was performed to elucidate this mechanism by identifying transcriptional dysregulation that occurs in strains with either deficient or excessive PE biosynthesis. It was found that decreased PE synthesis by either loss of *EPT1* or *PSD1* and *PSD2* leads to down-regulation of genes involved in the TCA cycle, glycolysis, amino acid biosynthesis, and fatty acid oxidation, which implicates impacts on mitochondrial function. Loss of *PSD1* and *PSD2*, but not *EPT1*, causes significant increases in transcription of glycosylation machinery genes, which may reflect the substantial cell wall defects in this mutant. These accumulated defects could contribute to the defects in virulence experienced by mutants with deficient PE synthesis. In contrast to mutants with decreased PE synthesis, there were no transcriptional differences between the *EPT1* overexpression strain and the wild-type besides an increase in *EPT1*, indicating that the hypervirulent phenotype is a consequence of post-transcriptional changes. Upon further analysis it was found that overexpression of *EPT1* causes increased chitin content, decreased $\beta(1,3)$ -glucan exposure, and increased hyphal length. These phenotypes may explain the previously observed hypervirulence in the *EPT1* overexpressor and appear to be a result of post-transcriptional regulation.

Introduction

The opportunistic human fungal pathogen *Candida albicans* causes oral and vaginal mucosal infections as well as dangerous systemic infections. Both mucosal and systemic candidiasis are common in immunocompromised individuals, and it remains problematic

that there are only three major classes of drugs that can be used to treat systemic infections (Cowen et al., 2015). Drugs within these classes target some component of the cell membrane or cell wall (Cowen et al., 2015). However, there are currently no antifungal drugs for the treatment of systemic candidiasis that target phospholipid biosynthetic pathways. These pathways are not entirely conserved amongst mammals and fungi and are required for growth and pathogenesis of *C. albicans* (Chen et al., 2008; Chen et al., 2010). Therefore, these are good areas in which to identify novel drug targets. Since many phospholipid biosynthetic processes in *C. albicans* have been inferred from the model yeast *Saccharomyces cerevisiae*, it is imperative to gain a better understanding of phospholipid biosynthetic processes and to evaluate their impact on virulence in *C. albicans*.

The major phospholipids of *C. albicans* are phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC) (Singh et al., 2010; Cassilly et al., 2017). The *de novo* route for the synthesis of PS, PE, and PC is the cytidine-diphosphate diacylglycerol (CDP-DAG) pathway (Carman and Han, 2011). In this pathway, PS is synthesized by the Cho1 enzyme from serine and CDP-DAG (Atkinson et al., 1980; Letts et al., 1983). PS is then decarboxylated by Psd1 and Psd2 to form PE, which can then be methylated by Pem1 and Pem2 to form PC (Kodaki and Yamashita, 1987; Clancey et al., 1993; Trotter and Voelker, 1995). We have previously reported that PE synthesis by the CDP-DAG pathway is a requirement for virulence in the mouse systemic model of infection as a *psd1Δ/Δ* *psd2Δ/Δ* mutant has a heavy attenuation of virulence and the *cho1Δ/Δ* mutant is completely avirulent (Chen et al., 2010).

In addition to the CDP-DAG pathway, *C. albicans* can synthesize PE and PC by an alternative route known as the Kennedy pathway (Gibellini and Smith, 2010). We have recently reported that the ethanolamine/choline phosphotransferase gene *EPT1* is required for the synthesis of both PE and PC by the Kennedy pathway, which is different from the model organism *S. cerevisiae* in which there is one phosphotransferase enzyme for the synthesis of each lipid (Gibellini and Smith, 2010; Tams et al., 2019). Furthermore, a modest decrease in fungal burden is observed in the kidneys of *ept1Δ/Δ* infected mice, and *ept1Δ/Δ* exhibits decreased cytotoxicity against oral epithelial cells *in vitro* compared to wild-type cells (Tams et al., 2019). Interestingly, we found that when synthesis of PE is increased by overexpressing the *EPT1* gene, the fungus becomes hypervirulent in the mouse systemic model of infection and has increased cytotoxicity against oral epithelial cells (Tams et al., 2019). Another mutant which cannot methylate PE to PC, *pem1Δ/Δ pem2Δ/Δ*, has increased PE, and this strain is also hypervirulent in the mouse systemic infection model (Tams et al., 2019). Thus, the synthesis level of PE in the cell correlates with virulence and may act as a dimmer switch for virulence. However, the mechanism by which the level of Ept1/PE regulates virulence remains unknown.

To elucidate this mechanism, a transcriptomic analysis of the wild-type, *psd1Δ/Δ psd2Δ/Δ*, *ept1Δ/Δ*, and *EPT1* overexpression strains was performed. Since we have previously determined that these strains have a gradient of cytotoxicity against epithelial cells, and that increased damage correlated to increased PE, we hypothesized that there would be a gradient of gene expression for impacted genes (Tams et al., 2019). For example, there may be a gene that is overexpressed in the *EPT1* overexpressor that has decreased expression in the wild-type and even lower expression in PE deficient mutants

(or vice versa). Therefore, strains were cultured under similar conditions in which cytotoxicity defects were observed (i.e. hyphal growth in tissue culture), and transcriptional profiling was used to elucidate how PE levels impact virulence.

In addition to this approach, and because it has been demonstrated that changes in phospholipid biosynthesis can impact cell wall structure (Davis et al., 2014), our alternative hypothesis was that there were changes in cell wall structure, which could lead to changes in host-pathogen interactions. Therefore, we examined two components of the cell wall that have been shown to have immunomodulatory functions. Of these components, $\beta(1,3)$ -glucan is an extensively studied cell wall carbohydrate polymer that impacts immune recognition in the host. For example, it has been demonstrated that immune recognition of $\beta(1,3)$ -glucan is a key factor in the ability of tissue resident macrophages, which are primary mediators of immunosurveillance, to recognize and clear fungal infections (Davis et al., 2014; Xu and Shinohara, 2017). *C. albicans* strains with significant exposure or “unmasking” of $\beta(1,3)$ -glucan are less virulent than those that mask these residues, and mice deficient in the Dectin-1 receptor, which is a C-type signaling lectin involved in recognition of $\beta(1,3)$ -glucan, are more susceptible to *C. albicans* infections (Herre et al., 2004; Taylor et al., 2007; Chen et al., 2010; Davis et al., 2014). A loss of phosphatidylserine biosynthesis increases exposure of $\beta(1,3)$ -glucan, and thus it is possible that overexpression of phospholipid biosynthesis may also impact exposure of $\beta(1,3)$ -glucan (Davis et al., 2014).

Another cell wall component that can modulate host-pathogen interactions is chitin, which is a $\beta(1,4)$ -linked N-acetylglucosamine polymer common in fungal cell walls and numerous invertebrate organisms (Lenardon et al., 2010). Purified fungal chitin can

modulate the immune system by inducing inflammatory tumor necrosis factor alpha (TNF- α) mediated response, or alternatively anti-inflammatory interleukin 10 (IL-10) mediated response in macrophages in a particle size-dependent manner (Da Silva et al., 2009). Further, fungal chitin can drive decreased production of reactive nitrogen species in macrophages (Wagener et al., 2017). Therefore, in hypervirulent strains it is possible that there are changes to the cell wall that allow the fungus to modulate the immune system in order to better evade immunosurveillance. Since $\beta(1,3)$ -glucan masking is important for immune evasion and because chitin can modulate the host immune response, the second goal of this study was to identify differences in these components between the wild-type and hypervirulent *EPT1* overexpression strain.

Materials and Methods

Culture conditions and growth media

Candida albicans strains from the SC5314 background were routinely cultured in YPD (1% yeast extract, 2% dextrose, 2% peptone) in a shaking incubator at 225 RPM and 30°C (Gillum et al., 1984) (Table 1). The RAW 264.7 macrophage cell line was routinely cultured in Dulbecco's Modified Eagle Medium with L-Glutamine (DMEM) (Gibco, 11965118) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, 16000044) and 1% penicillin/streptomycin at 37°C and 5% CO₂ (Raschke et al., 1978). *Escherichia coli* strains (DH5- α ; NEB, C29871) were cultured in LB broth (1% tryptone, 1% NaCl, 0.5% yeast extract) in a rotating incubator at 37°C.

Table 3.1. *C. albicans* strains used in chapter three.

Strain	Parent Strain	Genotype	Source
SC5314	Clinical isolate	Prototrophic Wild Type	Gillum <i>et al.</i>
YLC339	YLC335	<i>cho1</i> Δ/Δ	Chen <i>et al.</i>
YLC375	YLC373	<i>psd1</i> Δ/Δ <i>psd2</i> Δ/Δ	Chen <i>et al.</i>
BTY88	SC5314	<i>P_{ENO1}::P_{ENO1}-EPT1-NAT1</i>	Tams <i>et al.</i>
BTY104	BTY101	<i>ept1</i> Δ/Δ - <i>SAT1</i>	Tams <i>et al.</i>
BTY204	YLC339	<i>cho1</i> Δ/Δ <i>P_{ENO1}::P_{ENO1}-EPT1-NAT1</i>	This Study
pBT11 (<i>E. coli</i>)	pBT1 (<i>E. coli</i>)	<i>EPT1</i> Overexpression Vector	Tams <i>et al.</i>

Strain construction

Candida albicans strains (Table 1) were created with the use of the *SAT1* flipper method (Reuss et al., 2004). To overexpress *EPT1* in the *cho1Δ/Δ* background, a *cho1Δ/Δ P_{ENO1}-EPT1* strain was constructed. The *EPT1* overexpression construct, pBT11, was linearized (*MscI*) and transformed into YLC339 (*cho1Δ/Δ*) via electroporation as described previously (Hasim et al., 2014; Tams et al., 2019). Transformants were selected with the use of YPD supplemented with 200 µg/mL nourseothricin (Gold Bio, N-500-1), and successful transformants were confirmed using PCR with the primers BTO33 (5'-GACTAACGATTTCTATAAGG-3') and BTO43 (5'-GTTAGTTGGAATGAAAAG TC-3').

RNA Isolation, purification, and sequencing

The wild-type, *EPT1* overexpressor, *ept1Δ/Δ*, and *psd1Δ/Δ psd2Δ/Δ* strains were cultured for approximately 16 hours in 5 mL YPD shaking at 225 RPM and 30°C. Following incubation, each strain was washed twice with 35 mL sterile ddH₂O and resuspended in 5 mL ddH₂O. Each strain was diluted, in duplicate, to OD_{600nm} = 0.5 in 25 mL of Earl's balanced salt solution (EBSS) (GE Life Sciences, SH30024.01) supplemented with 1% penicillin/streptomycin (Gibco, 15140122) and 2% human serum (MP Biomedicals, 92930149) that was pre-warmed to 37°C in T125 tissue culture flasks. Flasks were incubated at 37°C and 5% CO₂ for 4 hours, as was performed in prior cytotoxicity assays (Tams et al., 2019). Following incubation, hyphae were removed with a sterile rubber cell scraper and transferred to 50 mL conical tubes. Samples were washed twice with sterile PBS and were resuspended in 750 µL acid-phenol (pH = 4.5), vortexed, and transferred to 2 mL screw cap tubes that contained 750 µL TES buffer (10 mM Tris pH 7.5, 10 mM

EDTA pH 8.0, 0.5% SDS) and 0.5 grams Zirconia beads. Each tube was filled completely with TES so that no air remained and was placed on ice for 2 minutes. Samples were homogenized in a bead beater for one minute and allowed to rest on ice for 2 minutes. Four total cycles of homogenization were performed, and samples were subsequently incubated at 65°C for 30 minutes with vigorous vortexing at 10-minute intervals. After incubation, samples were centrifuged for 5 minutes at 13,000 RPM at room temperature and the aqueous layer was transferred to a fresh tube containing 700 µL acid-phenol/chloroform, vortexed, and centrifuged at 13,000 RPM for 5 minutes. This process was repeated using 600 µL phenol/chloroform (pH=6.7) until the interface was clear. The supernatant was transferred to a fresh tube and nucleic acids were precipitated with DEPC treated 3M NaOAc and 100% ethanol. Samples were stored at -80°C for approximately 16 hours, thawed, and the RNA pellet was centrifuged, washed with 70% ice-cold ethanol, air dried, resuspended in 50 µl RNase Free H₂O, and quantified using a spectrophotometer. Following quantification, DNase treatment was performed with a Turbo DNase-Free kit (Ambion, AM1907) according to the manufacturer's protocol.

To determine final RNA concentration and quality, samples were analyzed with the 2100 series bioanalyzer (Agilent Technologies, CA, USA) at the University of Tennessee Genomics Core. Following quality control analysis, a cDNA library was prepared with the Nextera XT library kit (Illumina Inc., CA, USA) by following the manufacturer's protocol, and the library was validated via bioanalyzer (Agilent Technologies, CA, USA). The cDNA library was pooled and sequenced in-house using an Illumina MiSeq V2 flow cell (Illumina Inc, CA, USA).

Transcriptomic analysis

The *C. albicans* reference genome (version A22-s07-m01-r71) and corresponding annotations were downloaded from the Candida Genome Database (Skrzypek et al., 2017). Samples were analyzed using the University of Tennessee Advanced Computing Facility (ACF) resources. The quality of each sample was analyzed using FastQC (version 0.11.5) and trimming was performed using Trimmomatic (version 0.36). Read quality was again confirmed following trimming with FastQC (version 0.11.5). The reference genome and corresponding annotations were converted to a haploid reference and annotation set by removing the B allele for each gene using in-house scripts. This was also repeated the A allele. Read mapping and annotation was performed with STAR (version 2.5.3a) (Dobin et al., 2013). Mapped read counts were obtained with htseq (version 0.10.0) (Anders et al., 2015) and DEseq2 (version 1.18.1) (Anders and Huber, 2010) was used within the R-Studio Environment (R version 3.4.3) to perform differential expression analysis. In-house Python scripts were used to generate a list of genes that were co-upregulated and co-downregulated in both the *ept1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* strains as compared to the wild-type. Specifically, this list included genes that had greater or less than a 0.6 log₂-fold (~1.5-fold) change in both mutants compared to wild-type. The genes in this list also meet the requirement of having a Benjamini-Hochberg adjusted p-value of <0.05. GraphPad Prism (version 7.04) was used to generate heat maps, and the *Candida* Genome Database (CGD) GOslim mapper feature was used to determine functional groupings of differentially expressed genes (Skrzypek et al., 2017). In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis tool was used to determine which pathways had multiple genes that were differentially expressed (Kanehisa and Goto,

2000). To validate RNA sequencing data, transcripts per kilobase million (TPM) was calculated using CLC Genomics Workbench (version 10.1.1) and the proportion of transcripts mapping to housekeeping genes for each sample set was determined. Briefly, Illumina reads were imported into CLC and trimmed with limit = 0.01, maximum number of ambiguities = 2 and automatic read-through adapter trimming = yes. Reads were mapped with mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, and similarity fraction = 0.8.

β(1,3)-glucan and chitin detection assays

Chitin staining and surface β(1,3)-glucan exposure of cells at either stationary or log phase of growth was quantified with the use of flow cytometry. To stain cells at stationary phase, 5 mL cultures of *C. albicans* in YPD were grown shaking for approximately 16 hours at 30°C and 225 RPM, and then diluted to OD_{600nm} = 0.5 for β(1,3)-glucan staining, or OD_{600nm} = 1.5 for chitin staining. To stain log-phase cells, stationary cultures were diluted to OD_{600nm} = 0.1 in 5 mL YPD and incubated for 3.5 hours at 30°C and 225 RPM. Staining of β(1,3)-glucan was performed as described previously with three biological replicates for each strain (Chen et al., 2019).

Chitin staining was achieved using calcofluor white (PolySciences Inc., 4359). Stationary phase cultures were diluted to OD_{600nm} = 1.5, washed three times with 500 μL PBS, and resuspended in 500 μL of 0.01 mg/mL calcofluor white. Cells were then rocked in the dark for 5 minutes at room temperature, washed 3 times with 500 μL water, and resuspended in 500 μL FACS buffer for flow cytometry analysis. All samples were analyzed with an LSRII flow cytometer (Becton Dickinson Biosciences) and data for each

sample was collected for 100,000 gated events. This experiment was performed with three biological replicates. Data was analyzed with FlowJo (version 10.11) and statistical significance was determined by performing an unpaired *t*-test with GraphPad Prism (version 7.04).

Surface exposed $\beta(1,3)$ -glucan was further assessed when cells were put under physical or chemical stress. Heat was used to induce physical stress. Stationary phase *C. albicans* was diluted to an $OD_{600nm} = 0.5$ in 1 mL of PBS and incubated at 90°C for 20 minutes (Wheeler and Fink, 2006). After incubation, tubes were removed and $\beta(1,3)$ -glucan staining was performed as described above. Alternatively, chemical stress was induced by the addition of sub-minimum inhibitory concentrations (MIC) of caspofungin (Merck, NJ, USA). Stationary cultures of *C. albicans* were diluted to $OD_{600nm} = 0.5$ in 5 mL of YPD containing either 15.6 ng/mL (1/8th M.I.C.), 31.3 ng/mL (1/4th M.I.C.), or 46.9 ng/mL (3/8th M.I.C.) caspofungin. Cultures were incubated at 30°C and 225 RPM for 30 minutes and 1 mL of each sample was removed for analysis of either $\beta(1,3)$ -glucan exposure or chitin as previously described. Chitin analysis after caspofungin treatment was performed in biological and technical triplicate. $\beta(1,3)$ -glucan analysis after caspofungin treatment was performed with a minimum of two biological replicates per strain and per condition, each in technical triplicate.

Yeast growth and hyphal length measurements

For yeast form cells, each strain was cultured to stationary phase for approximately 16 hours in YPD shaking at 225 RPM and 30°C and then diluted to $OD_{600nm} = 0.1$ in fresh YPD in triplicate. Cells were again cultured by shaking at 225 RPM and 30°C, and

OD600_{nm} was measured every two hours for 12 hours.

For hyphal length measurements, stationary phase cells were washed twice with 30 mL sterile PBS. Following washes, cells were resuspended in 5 mL PBS. Cultures were diluted to OD600_{nm} = 1.0 in 1 mL of RPMI + 20% human serum that was pre-warmed to 37°C. Serial dilutions were performed and 100 µL of each culture was plated in triplicate in a 96 well plate at a final OD600_{nm} of 1.0x10⁻⁴. Cells were then incubated for 3 hours at 37°C to induce the formation of hyphae. Following incubation, six images per well were obtained on an inverted microscope. For each experiment the length of 200 hyphae, from the base of the cell to the hyphal tip, was measured for each strain using ImageJ software (version 1.52a) and was expressed as percent average wild-type hyphal length. This experiment was performed three times (n=600/strain total) (Schindelin et al., 2015).

Results

Loss of PE synthesis causes global changes to the *C. albicans* transcriptome

The *EPT1* overexpression strain has increased cytotoxicity against oral epithelial cells that correlates with increased PE synthesis by the Kennedy pathway (Tams et al., 2019). Conversely, loss of PE synthesis by either the CDP-DAG pathway or the Kennedy pathway results in decreased cytotoxicity (Davis et al., 2018; Tams et al., 2019). To determine the mechanism by which PE level impacts virulence, a transcriptional profile of these strains was obtained after growth under the same conditions in which differences in cytotoxicity were previously observed. Each strain was cultured in duplicate in tissue culture flasks (37°C, 5% CO₂, EBSS + 2% Human Serum, 1% pen/strep) for four hours and RNA was extracted and sequenced with an Illumina MiSeq. RNA integrity number (RIN) values ranged from 8.6-9.8 and nearly 38.5 million 150 bp paired-end reads were

obtained for the sample set after sequencing. FastQC was used to remove index or adapter contamination from reads, and to remove leading and trailing reads with PHRED scores <30. Sequence quality was determined both before and after trimming. The diploid reference genome and annotations (version A22-s07-m01-r71) were obtained from the *Candida* Genome Database (CGD) (Skrzypek et al., 2017) and were converted to haploid versions using in-house scripts that removed the B chromosomes and corresponding annotations. This was performed to circumvent issues with proper mapping, as most reads were thrown out or assigned poor mapping quality scores when a diploid reference was used. STAR was used to map reads, and approximately 90% of reads mapped uniquely to the genome for all samples (Anders and Huber, 2010). Mapped reads were counted using htseq and differential expression analysis was performed using DEseq2. To ensure that there was no bias caused by removal of the B alleles, this analysis was also performed by mapping to reference genomes in which the A alleles had been removed, and it was found that the difference between the two mapping scenarios was negligible. Transcripts per kilobase million (TPM) was obtained using CLC Genomics Workbench.

A principal component plot (PCA) was created using R Studio to determine similarity of samples and replicates (Figure 3.1, A). Between all replicates, there was tight clustering, indicating little technical and biological variability. The wild-type and *EPT1* overexpressor strains also clustered tightly, indicating similar gene expression profiles. More significant differences were observed for the mutants deficient for PE production, with the *psd1Δ/Δ psd2Δ/Δ* strain displaying the greatest variance from wild-type (Figure 3.1, A). To verify that gene expression estimates derived from this dataset are valid, the

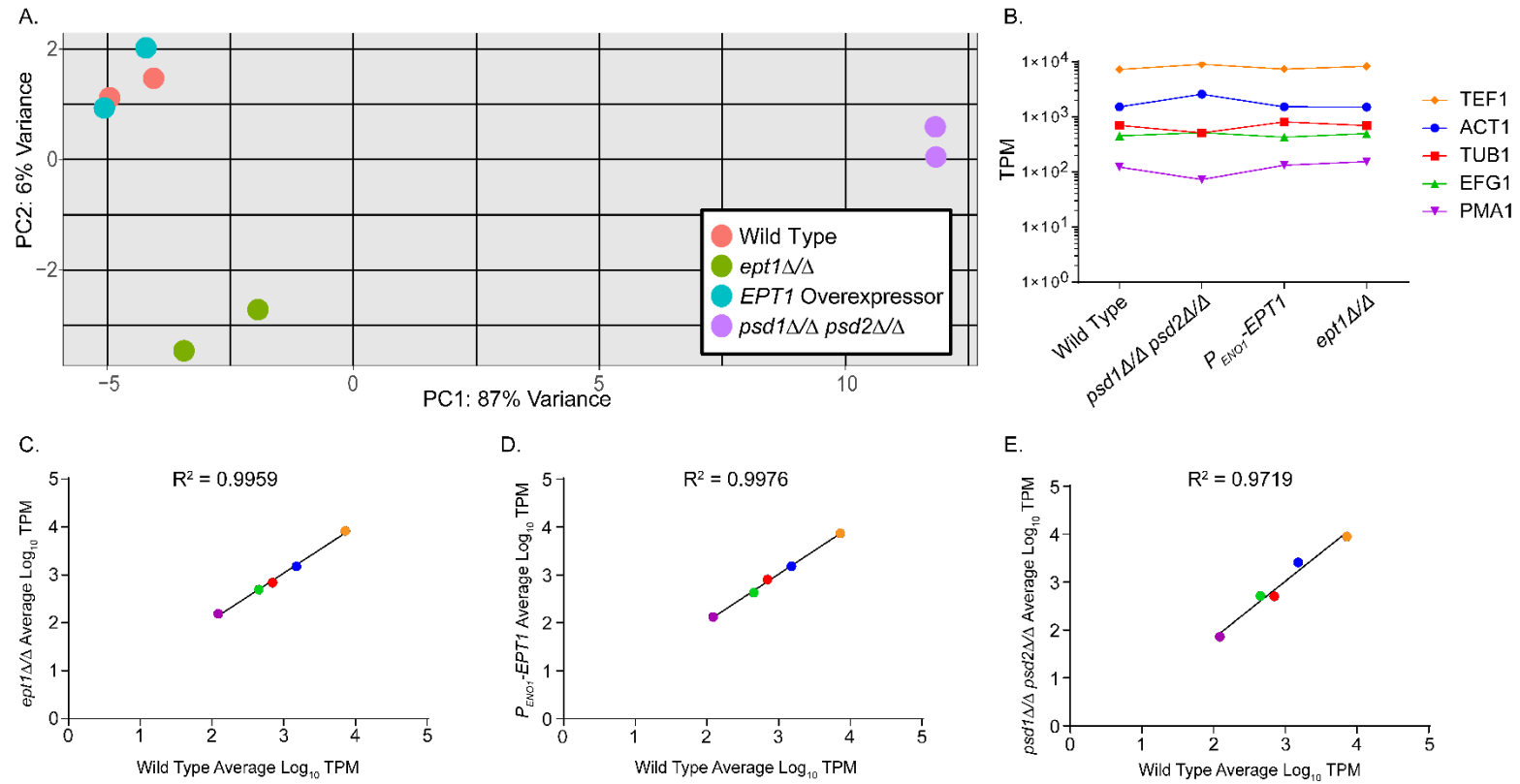


Figure 3.1. PCA analysis and housekeeping gene expression profiles.

(A) Principal component plot for transcriptomic analysis reveals that replicates have little variation. The *EPT1* strain is similar to wild-type, and the PE deficient strains have the greatest differences versus wild-type. **(B)** TPM for housekeeping genes are similar among all strains. **(C-D)** Log transformed TPM for housekeeping genes (*TEF1*, *ACT1*, *TUB1*, *EFG1*, and *PMA1*) is plotted versus wild-type for **(C)** *ept1* Δ/Δ , **(D)** *EPT1* overexpressor, and **(E)** *psd1* Δ/Δ *psd2* Δ/Δ ; The color for each gene corresponds to the figure legend in panel B.

proportion of reads mapping to five common housekeeping genes (*TEF1*, *ACT1*, *TUB1*, *EFG1*, and *PMA1*) were obtained, and the average TPM of replicates for each sample set was plotted as was performed by Martin *et al.* (Figure 3.1, B) (Martin *et al.*, 2017). Housekeeping gene expression was similar among all sample sets, even for *PMA1* which had expression as low as $\sim 10^2$ TPM (Figure 3.1, B). To further demonstrate that expression of housekeeping genes was similar for each sample set as compared to the wild type, the average TPM of each sample set was log transformed and plotted against the corresponding wild type values (Figures 3.1, C-E). Linear regression analysis was performed, and the R-squared values were >0.97 for each sample set, indicating a strong correlation.

For this analysis, genes that had greater than a $\log_2$ fold-change of 0.6 (approximately 1.5-fold) compared to wild-type were considered differentially expressed, and the cut-off for statistical significance used a Benjamini-Hochberg adjusted $*p < 0.05$. There were vast changes to the transcriptome of the *psd1 Δ/Δ psd2 Δ/Δ* mutant, and to a lesser extent the *ept1 Δ/Δ* mutant (Figure 3.2, B-C). In total, there were 618 statistically significant up-regulated genes and 599 down-regulated genes in the *psd1 Δ/Δ psd2 Δ/Δ* mutant as compared to the wild type. For the *ept1 Δ/Δ* knockout, there were 21 statistically significant up-regulated genes and 48 down-regulated genes when compared to wild-type. To determine how loss of PE by either pathway affected gene expression, genes that were co-upregulated or co-downregulated in both mutants were compiled. There were 6 genes that were co-upregulated in both the *ept1 Δ/Δ* and *psd1 Δ/Δ psd2 Δ/Δ* mutants, and 35 genes co-downregulated in both mutants compared to wild-type (Figure 3.3, A). The set of co-regulated genes was used as an input for Kyoto Encyclopedia of

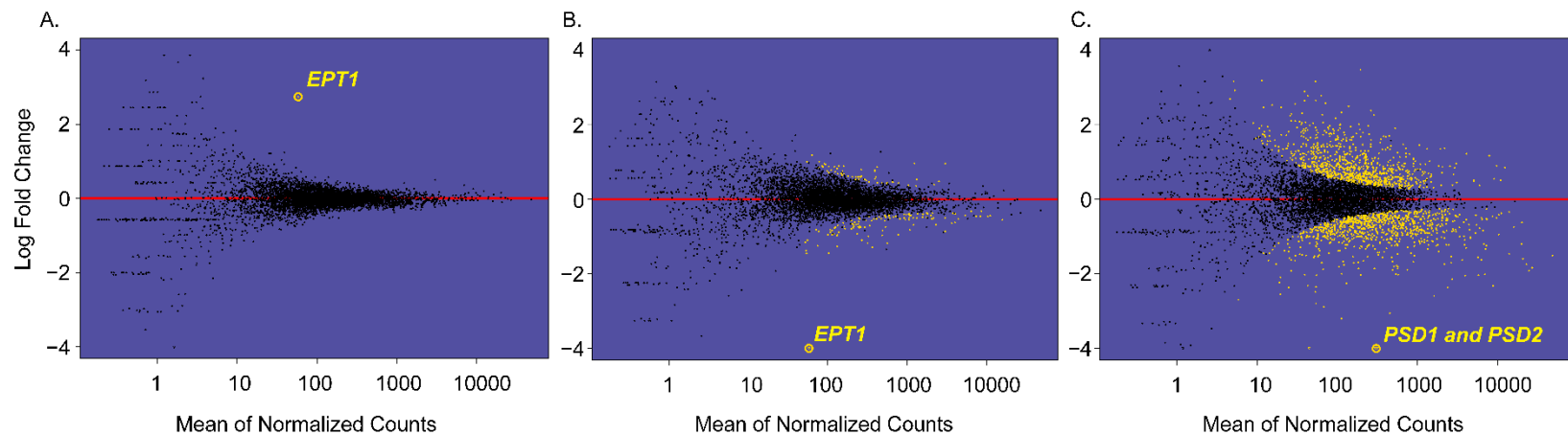
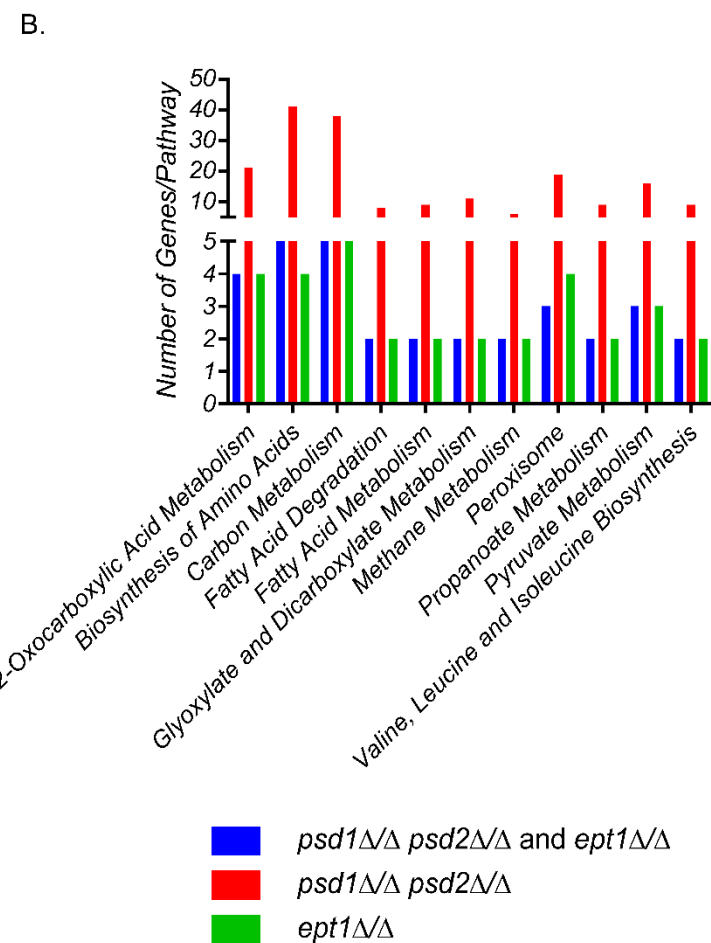
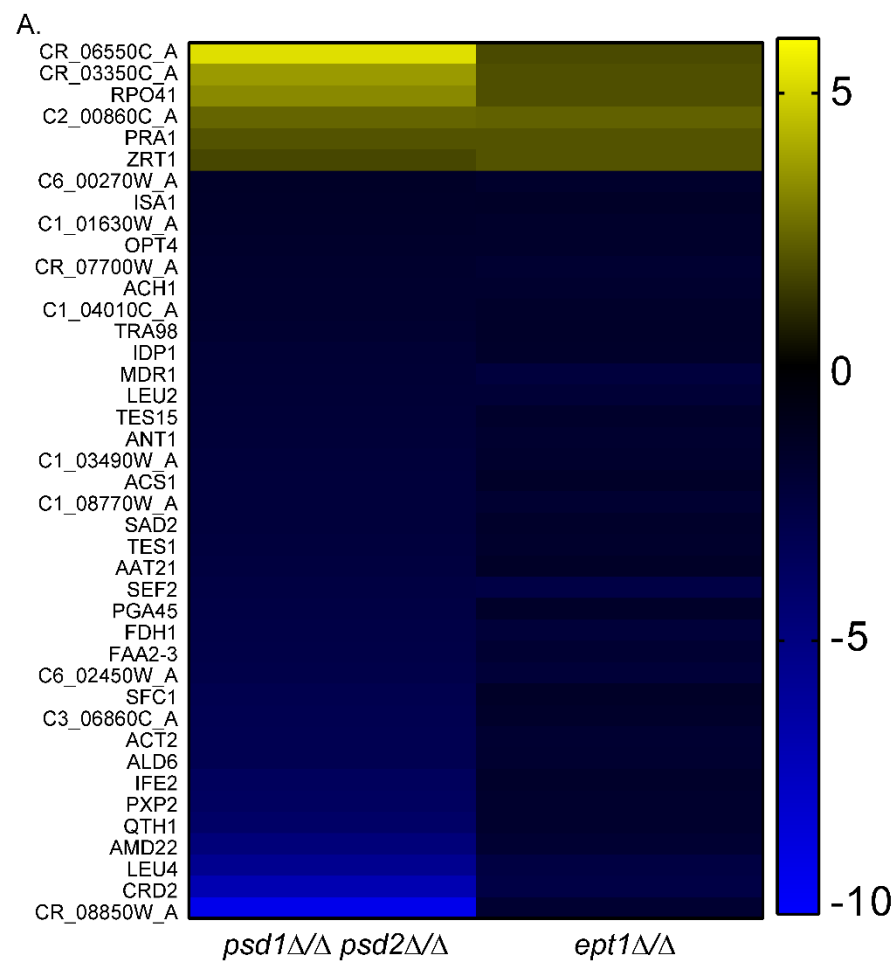


Figure 3.2. Differential expression analysis.

MA plots were used to visualize log fold-change of (A) *EPT1* overexpressor, (B) *ept1Δ/Δ* and (C) *psd1Δ/Δ psd2Δ/Δ* mutants versus wild-type. Gold points represent genes that have Benjamini-Hochberg adjusted p-values < 0.05 and black points are those with p-values > 0.05. Genes above the red line have positive expression compared to wild type, and those that are below the line have negative expression values. The only statistically significant differentially expressed gene in the *EPT1* overexpressor strain is *EPT1* ($\log_2$ fold-change = 2.74, $*p < 0.05$). For the *ept1Δ/Δ* strain, there are 69 statistically significant differentially expressed genes (greater or less than $\log_2$ -fold change of 0.6), and for the *psd1Δ/Δ psd2Δ/Δ* mutant there are 1,217 statistically significant differentially expressed genes.

Figure 3.3. Heat map of co-expressed genes.

(A) Heat map of genes that have >0.6 $\log_2$ -fold change versus wild-type with a Benjamini-Hochberg adjusted p-value of <0.05 in both the *psd1* Δ/Δ *psd2* Δ/Δ and *ept1* Δ/Δ mutants. Genes that are co-down-regulated are blue and genes that are co-up-regulated are yellow. **(B)** Pathways that are impacted by the loss of PE were determined using KEGG pathway analysis. Pathways with more than one gene differentially expressed in both the *psd1* Δ/Δ *psd2* Δ/Δ and *ept1* Δ/Δ mutants are displayed for each mutant.



Genes and Genomes (KEGG) pathway analysis to determine which pathways had multiple genes that were differentially expressed when PE was depleted by either the CDP-DAG or Kennedy pathway. A bar chart identifying pathways containing more than one gene with differential expression was compiled (Figure 3.3, B). Each mutant was individually examined for differential expression of genes involved in these specific pathways using KEGG (Figure 3.3, B).

Overall, eleven pathways emerged that had more than two genes with differential expression present for both mutants. When the *psd1Δ/Δ psd2Δ/Δ* mutant is examined alone the number of genes differentially expressed within these pathways greatly increases (Figure 3.3, B). There were multiple commonalities between these pathways. First, 2-oxocarboxylic acid metabolism and pyruvate metabolism are tied to the citric acid (TCA) cycle. Second, the synthesis of valine, leucine, and isoleucine are potential end products from the TCA cycle. Thirdly, the peroxisome is involved in the β -oxidation of fatty acids to form acetyl-CoA for the TCA cycle. There are also changes to fatty acid degradation and metabolism, which is not unexpected as we have disrupted major phospholipid biosynthetic routes.

In addition to the eleven identified pathways with genes that are differentially expressed in both mutants, there were several pathways identified in the *psd1Δ/Δ psd2Δ/Δ* mutant that had genes with differential expression and for which the *ept1Δ/Δ* mutant had fewer than two genes differentially expressed. These included genes directly involved in the TCA cycle, protein processing at the ER, glycolysis, oxidative phosphorylation, tRNA biosynthesis, and N and O glycan biosynthesis. Overall, it was found that the genes involved in amino acid biosynthesis, the TCA cycle, the peroxisome,

oxidative respiration, and glycolysis were down-regulated (Figure 3.4). Several of these functions are again part of the core carbon metabolism in the cell, and down-regulation of the TCA cycle and oxidative phosphorylation may indicate that there are mitochondrial defects in the *psd1Δ/Δ psd2Δ/Δ* mutant. In fact, this aligns with previous research that demonstrated mitochondrial defects in the *psd1Δ/Δ psd2Δ/Δ* mutant via increased growth in the presence of paraquat and hypersensitivity to farnesol (Chen et al., 2010; Hasim et al., 2018).

Pathways that involve genes that were up-regulated in the *psd1Δ/Δ psd2Δ/Δ* mutant included N and O glycan biosynthesis, tRNA synthesis, and protein processing at the endoplasmic reticulum (ER) (Figure 3.4). Glycosylation of cell wall proteins occurs within the ER and Golgi, and this function is important for proper folding and export of glycoproteins (Orlean, 2012). The increased expression of genes involved in these processes may be in response to cell wall stress incurred by a loss of PE. The *psd1Δ/Δ psd2Δ/Δ* mutant is known to have cell wall stability issues which can be suppressed by addition of an osmotic stabilizer to growth medium (Chen et al., 2010).

Overexpression of EPT1 does not impact the transcriptome, but does reduce $\beta(1,3)$ -glucan exposure

Surprisingly, it was found that in the PE overexpressor (*P_{ENO1}-EPT1*), there was only one gene with statistically significant differential expression compared to the wild-type. This gene was *EPT1* (log₂-fold change = 2.74, *p< 0.05), which is constitutively expressed in this strain (Figure 3.2, A; Table 1). While we have previously used radiolabeling assays to confirm that the *EPT1* overexpressor has increased Ept1 activity, this data further

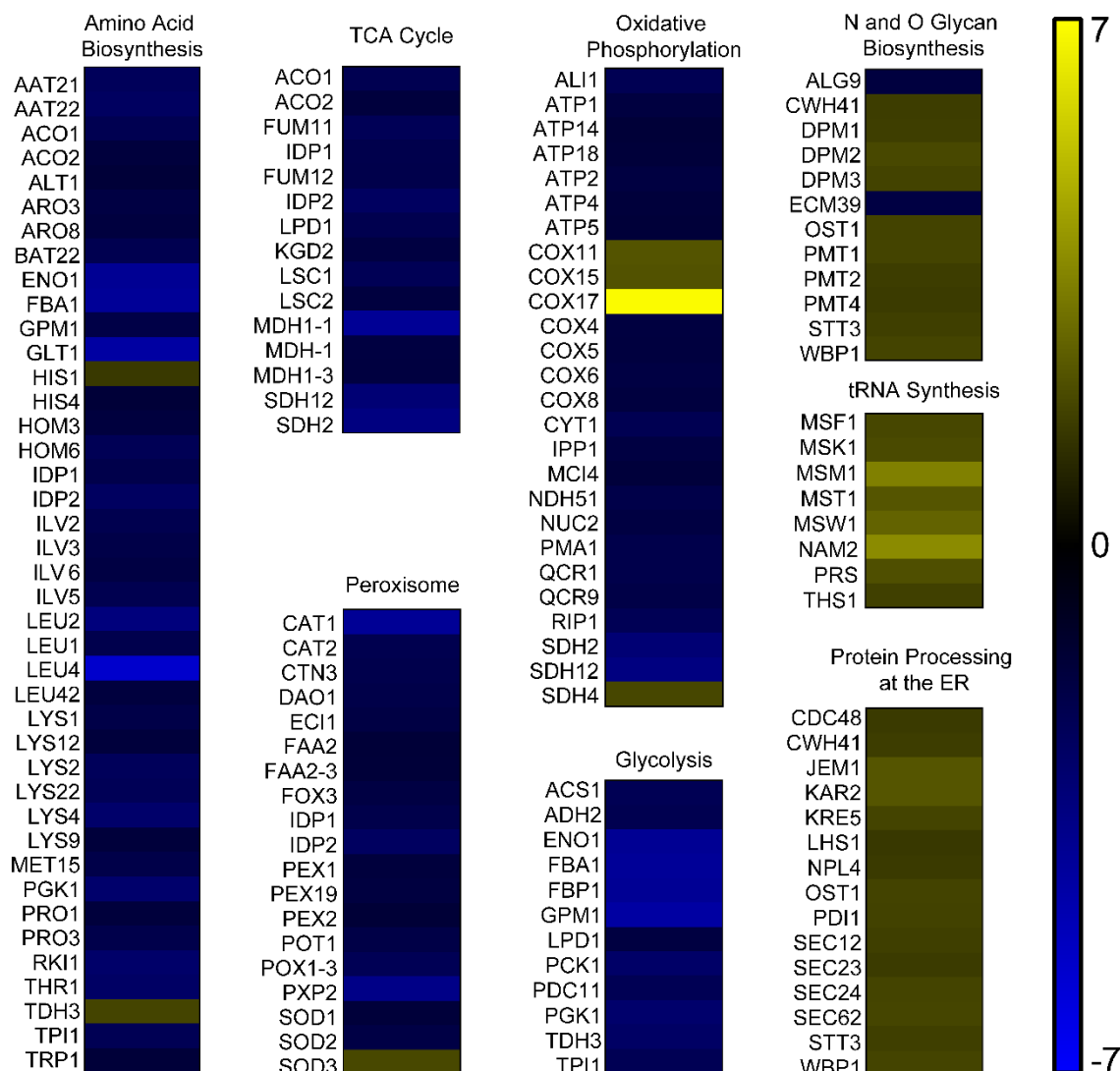


Figure 3.4. Differentially expressed pathways in *psd1Δ/Δ psd2Δ/Δ*.

Several pathways have multiple differentially expressed genes in both the *psd1Δ/Δ psd2Δ/Δ* and *ept1Δ/Δ* mutants. These pathways, along with other significantly different genes/pathways were further investigated for the *psd1Δ/Δ psd2Δ/Δ* mutant. Down-regulated pathways included those involved in glycolysis, oxidative phosphorylation, the TCA cycle, peroxisomal functions, and amino acid biosynthesis. Several pathways were found to be modestly up-regulated in the *psd1Δ/Δ psd2Δ/Δ* mutant, including N- and O- glycan biosynthesis, tRNA synthesis, and protein processing at the ER. Yellow values are up-regulated and blue values are down-regulated. All genes shown have greater or less than a log₂-fold change of 0.6 and Benjamini-Hochberg adjusted *p<0.05.

validates that the overexpression construct functions as expected (Tams et al., 2019). However, since there are no other differentially expressed genes, we hypothesized that the hypervirulent phenotype is due to post-transcriptional changes in the cell that either alter the virulence properties of the fungus or host immune recognition.

Alterations that affect virulence often do so by affecting the cell wall. The cell wall is the interface whereby *C. albicans* interacts with the host, and because the cell must be able to mask immunogenic components of the wall to effectively avoid the host immune response, we investigated cell wall components in the *EPT1* overexpressor that influence host-pathogen interactions.

The fungal cell wall is largely composed of chitin, $\beta(1,3)$ glucan, $\beta(1,6)$ glucan, and mannosylated cell wall glycoproteins (mannan) (Gow et al., 2017). The mannan of the cell wall allows the fungus to hide or mask $\beta(1,3)$ -glucan during infection, which is an important function as $\beta(1,3)$ -glucan is an immunogenic polysaccharide that interacts with, among other receptors, host Dectin-1 on resident macrophages to initiate a proinflammatory response (Gow et al., 2007; Gow et al., 2017). We have previously found that there is an increase in exposure of $\beta(1,3)$ -glucan in the *cho1 Δ/Δ* mutant due to a loss of CDP-DAG generated PS (Davis et al., 2018). We hypothesized that a loss of Kennedy pathway mediated PE biosynthesis would result in increased $\beta(1,3)$ -glucan exposure and that overexpression of *EPT1*, which overproduces PE, could have an impact on cell wall architecture and perhaps mask $\beta(1,3)$ -glucan (Tams et al., 2019).

Immunofluorescent staining and flow cytometry were performed to determine the amount of exposure for wild-type, *EPT1* overexpressor, and *ept1 Δ/Δ* strains. In addition, *cho1 Δ/Δ* was used as a positive control for $\beta(1,3)$ -glucan exposure based on our previous

observations (Davis et al., 2018). A *cho1Δ/Δ P_{ENO1}-EPT1* strain was also constructed to determine if overexpression of the Kennedy pathway in the *cho1Δ/Δ* background would decrease β-1,3 glucan exposure in this mutant. Each strain was cultured in YPD and stained with a mouse anti-β(1,3)-glucan primary antibody and a phycoerythrin conjugated goat anti-mouse IgG secondary antibody. Samples were then measured by flow cytometry. In stationary phase cells, no difference was observed for β(1,3)-glucan exposure (Figure 3.5, A). However, in log phase cells there is a modest but statistically significant decrease in β(1,3)-glucan exposure in the *EPT1* overexpressor compared to wild-type (*p<0.05) (Figure 3.5, B). The *cho1Δ/Δ* strain, as expected, had increased β(1,3)-glucan exposure when compared to the wild-type (Figure 3.5, B; ***p<0.0001). Overexpression of *EPT1* in the *cho1Δ/Δ* background resulted in a statistically significant decrease in β(1,3)-glucan exposure compared to the parental *cho1Δ/Δ* strain (Figure 3.5, B; ^{aa}p<0.01). These data suggest that overexpression of *EPT1*, which causes an increase in Kennedy pathway mediated PE synthesis, can cause decreased β(1,3)-glucan exposure, even in highly exposed cells. Therefore, we chose to further investigate this phenomenon.

Within the host during infection, *Candida* cells exhibit β(1,3)-glucan exposure over time (Hopke et al., 2016) or in response to treatment with drugs like caspofungin (Wheeler and Fink, 2006). Because *EPT1* overexpression could cause decreased β(1,3)-glucan exposure in *cho1Δ/Δ* and wild-type cells, we wanted to test the impact of *EPT1* overexpression on β(1,3)-glucan exposure in response to stresses that induce unmasking. Two cell wall stressors that cause unmasking were applied to stationary phase cells: (1) a sub-minimum inhibitory concentration (MIC) of caspofungin and (2) heat

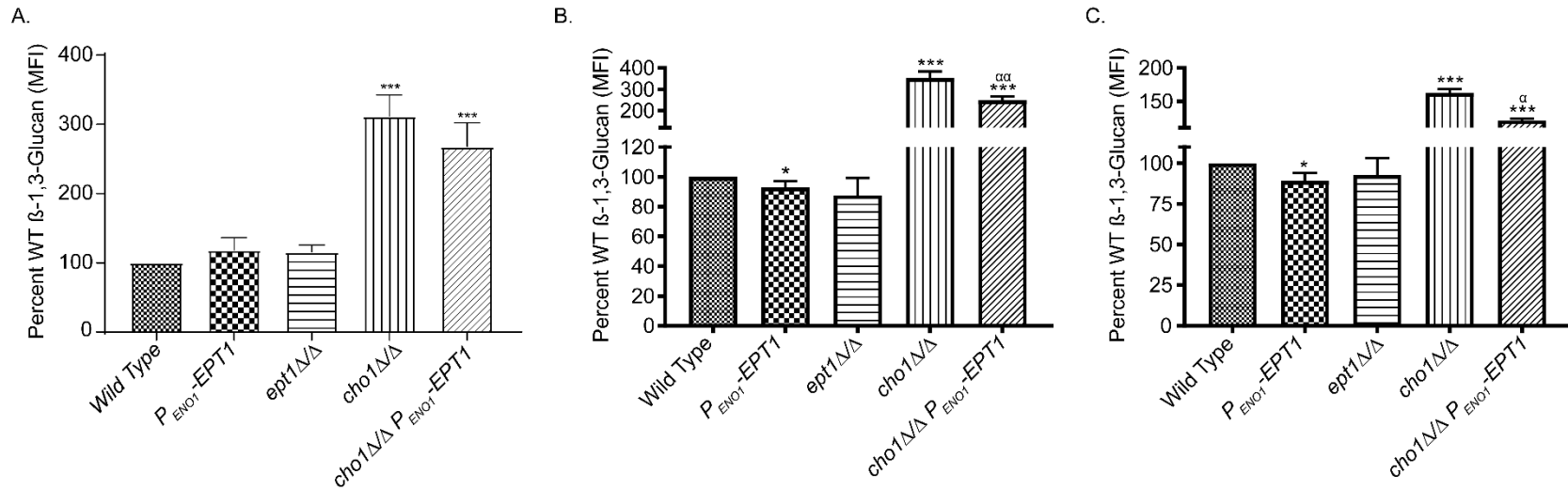


Figure 3.5. Overexpression of *EPT1* reduces $\beta(1,3)$ -glucan exposure.

(A) There is a statistically significant increase in $\beta(1,3)$ -glucan exposure for the $cho1\Delta/\Delta$ for stationary phase cells, and this exposure is decreased upon overexpression of *EPT1*. There was no statistically significant difference when *EPT1* is overexpressed in the wild-type background (** $p < 0.0001$ vs. wild-type, unpaired *t*-test). **(B)** There is a decrease in mean fluorescent intensity (MFI) of $\beta(1,3)$ -glucan exposure when *EPT1* is overexpressed in the $cho1\Delta/\Delta$ background, and a modest decrease in MFI of the *EPT1* overexpressor as compared to wild-type in log phase cells (** $p < 0.0001$ vs. WT, * $p < 0.05$ vs. WT, $\alpha\alpha p < 0.01$ vs. $cho1\Delta/\Delta$; $n = 3$, unpaired *t*-test). **(C)** This difference is also observed for stationary phase cells overexpressing *EPT1* that have been heat shocked at 90°C for 20 minutes (** $p < 0.0001$ vs. WT, * $p < 0.05$ vs. WT, $\alpha p < 0.05$ vs. $cho1\Delta/\Delta$; $n = 3$, unpaired *t*-test).

shock at 90°C. The cells were subsequently analyzed for $\beta(1,3)$ -glucan exposure with flow cytometry. A decrease in $\beta(1,3)$ -glucan exposure was observed for stationary phase cells overexpressing *EPT1* in either the wild-type or *cho1 Δ/Δ* backgrounds in response to heat shock (Figure 3.5, C). Treatment of stationary phase cells with a sublethal concentration of caspofungin (47 ng/mL) induced $\beta(1,3)$ -glucan exposure for both the wild-type and *EPT1* overexpressor as compared to their respective untreated controls (Figure 3.6, A; *** $p < 0.0001$). However, overexpression of *EPT1* diminished caspofungin induced $\beta(1,3)$ -glucan exposure at 15.6 ng/mL (1/8th MIC), 31.3 ng/mL (1/4th MIC), and 47 ng/mL (3/8th MIC) caspofungin (Figure 3.6, B; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$). These data suggest that overexpression of *EPT1* confers a general resistance to $\beta(1,3)$ -glucan exposure in response to cell wall stress, and this protection could potentially aid in evasion from immunosurveillance during stresses encountered *in vivo*.

Overexpression of *EPT1* increases cell wall chitin

Chitin levels correlate with changes in $\beta(1,3)$ -glucan exposure as well as alterations in virulence; thus, it was important to determine if overexpression of *EPT1* altered chitin level (Marakalala et al., 2013; Hasim et al., 2017; Wagener et al., 2017). Purified fungal chitin less than 40 μ M in size can cause increased production of IL-10 *in vivo*, which drives an anti-inflammatory response (Da Silva et al., 2009). Alternatively, purified chitin can cause a pro-inflammatory TNF- α response *in vivo* if particle size is between 40-70 μ M (Da Silva et al., 2009). Therefore, chitin levels in the wild-type, *EPT1* overexpressor, *cho1 Δ/Δ* , and *cho1 Δ/Δ EPT1* overexpressor strains were assessed by staining with calcofluor white (CFW) and measuring by flow cytometry (Figures 3.7, A-B). The *cho1 Δ/Δ*

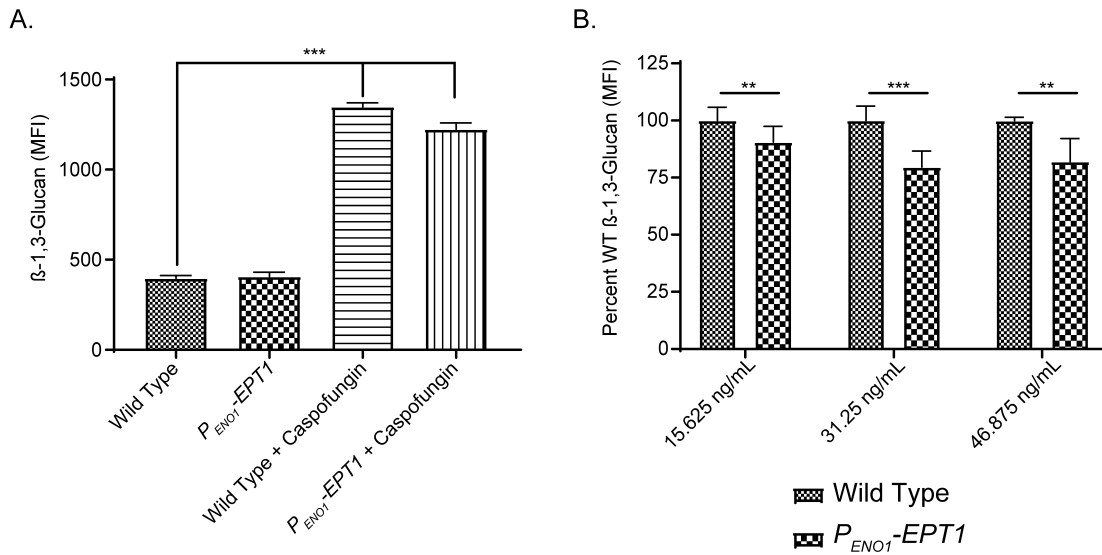


Figure 3.6. Caspofungin induced β(1,3)-glucan unmasking.

(A) Treatment with caspofungin (46.875 ng/mL) induces β(1,3)-glucan unmasking in both the wild-type (3.39-fold) and *EPT1* overexpression (3.00-fold) strains compared to untreated controls ($n = 3$, $***p < 0.0001$; unpaired *t*-test). **(B)** The wild-type and *EPT1* overexpressor were incubated with the indicated sublethal dose of caspofungin and immunofluorescent staining of β(1,3)-glucan was analyzed with flow cytometry. Overexpression of *EPT1* provides protection from caspofungin induced β(1,3)-glucan unmasking ($n = 6-9$ /strain, $***p < 0.0001$, $**p < 0.01$; unpaired *t*-test).

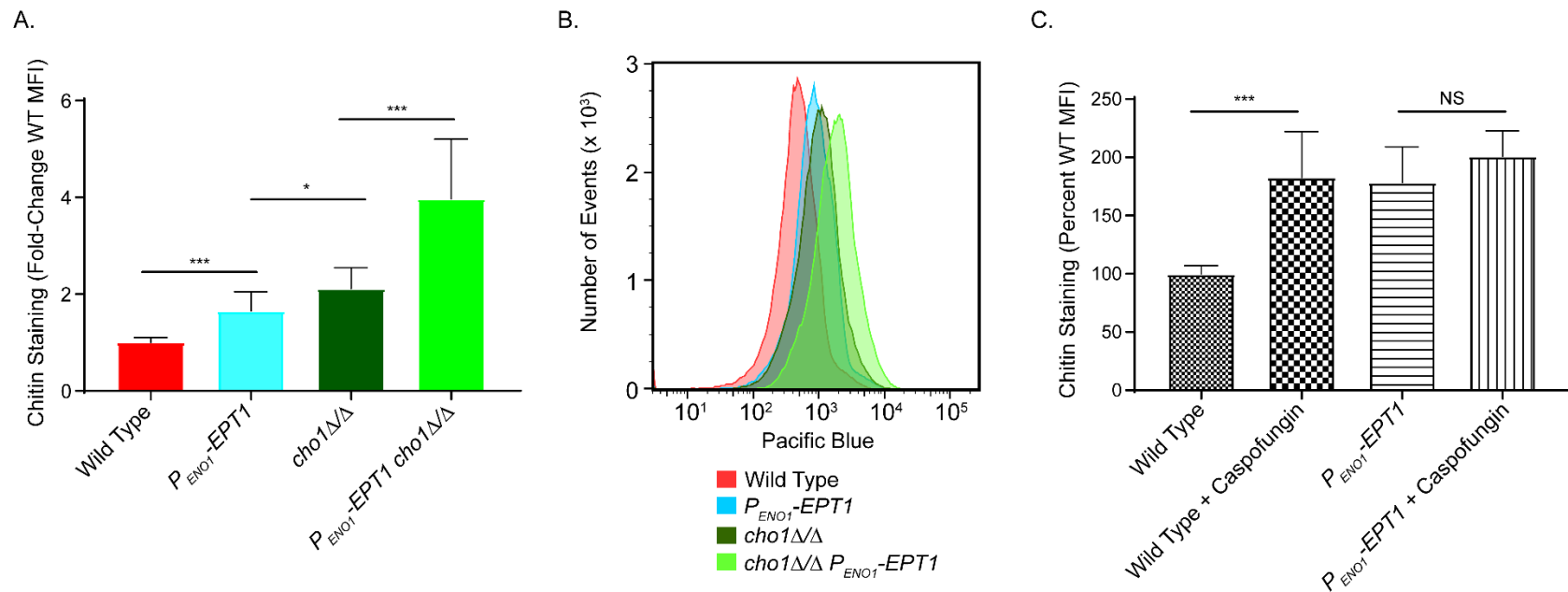


Figure 3.7. Overexpression of *EPT1* increases total chitin.

(A) Flow cytometry data of calcofluor white stained cells normalized to the wild-type mean fluorescent intensity (MFI) ($n = 9$ /strain, *** $p < 0.0001$, * $p < 0.05$ unpaired; t -test). **(B)** A representative histogram for flow cytometry of calcofluor white stained cells ($n = 100,000$ events/strain). An average increase of 1.65-fold MFI was observed for the *EPT1* overexpressor compared to wild-type, and overexpression of *EPT1* in the $cho1\Delta/\Delta$ background resulted in a 1.88-fold increase compared to $cho1\Delta/\Delta$, or a 3.96-fold increase compared to wild-type (*** $p < 0.0001$, unpaired t -test). **(C)** Cells were treated with 46.875 ng/mL caspofungin for 30 minutes and flow cytometry was performed to determine chitin content. There is a 1.83-fold increase in MFI for wild-type cells exposed to caspofungin, but the *EPT1* overexpression strain already exhibits increased chitin and does not increase further upon treatment ($n = 6$ /strain, *** $p < 0.0001$, NS = not significant; unpaired t -test).

strain was used as a positive control as we have previously demonstrated an increase in chitin for this strain (Hasim et al., 2017). In the *EPT1* overexpression strain, a 1.65-fold increase in chitin staining was observed when compared to the wild-type strain (Figure 3.7, A; *** $p < 0.0001$). The *cho1* Δ/Δ mutant has a more intense increase in chitin staining of 2.11-fold change as compared to wild-type (Figure 3.7, A). This effect is compounded when the *EPT1* gene is overexpressed in the *cho1* Δ/Δ background to a 1.88-fold increase over *cho1* Δ/Δ (*** $p < 0.0001$, Figure 3.7, A) or 3.96-fold increase over wild-type. Treatment with echinocandins such as caspofungin causes an increase in chitin content (Walker et al., 2008). Therefore, wild-type and *EPT1* overexpressor were stressed for 30 minutes with 47 ng/mL caspofungin and then stained with CFW and measured with flow cytometry. Interestingly, there was a statistically significant increase in chitin staining for the wild-type in the presence of caspofungin of 1.83-fold, but not the *EPT1* overexpressor, which already has an increase in chitin (*** $p < 0.0001$, Figure 3.7, C). However, when we tested the *EPT1* overexpressor for changes in TNF- α release from macrophages, we observed no significant differences from wild-type (data not shown).

Overexpression of EPT1 increases hyphal length

In addition to defensive virulence factors like $\beta(1,3)$ -glucan exposure, *Candida* has a variety of offensive virulence factors. The most well studied of these is hyphal growth. Hyphal formation is a major virulence factor in *C. albicans* that allows for escape from macrophages, damage to mammalian cells, and aids in biofilm formation (Angiolella et al., 2008; Carlisle et al., 2009; Mayer et al., 2013; Duvenage et al., 2019). There have been several instances in which hypervirulence in *C. albicans* was associated with an

increased ability to form hyphae. One such study explored the phenotype of a *PIR32* deletion strain and found that not only was this strain hyper-filamentous and hypervirulent, but that it also had an increase in chitin content (Bahnan et al., 2012). Since we observed hypervirulence and an increase in chitin content in the *EPT1* overexpressor, we hypothesized that this strain may also have an increased ability to form filaments. When induced to form hyphae in RPMI with 20% human serum at 37°C, differences were seen in hyphal length after 3 hours. A 9.5% increase in length was observed on average for the *EPT1* overexpressor strain compared to wild-type (Figure 3.8, A; $p < 0.0001$). The *cho1Δ/Δ* mutant had a 32.1% decrease in hyphae length compared to wild-type, but overexpression of *EPT1* in the *cho1Δ/Δ* background resulted in a 16% increase compared to the *cho1Δ/Δ* mutant ($*p < 0.0001$). These data indicate that overexpression of *EPT1* does increase filamentation. However, in yeast form cells there was no difference in growth curves between either the *EPT1* overexpressor, the *ept1Δ/Δ* mutant, or the wild-type strains (Figure 3.8, B). Growth was also tested via spot dilutions on YPD plates and there were no differences in growth rate at 30°C (data not shown). Therefore, the increase in filamentation seems to be hyphal specific.

Discussion

The amount of PE synthesized in the cell has a direct impact on *C. albicans* virulence. Cells that cannot produce PE by either of the two major pathways are less virulent in the mouse model of systemic candidiasis and less cytotoxic against epithelial cells *in vitro* (Chen et al., 2010; Davis et al., 2018; Tams et al., 2019). Synthesis of PE via the CDP-DAG pathway is more important for virulence than is Kennedy pathway mediated PE synthesis, as loss of PE synthesis by the CDP-DAG pathway results in a greater virulence

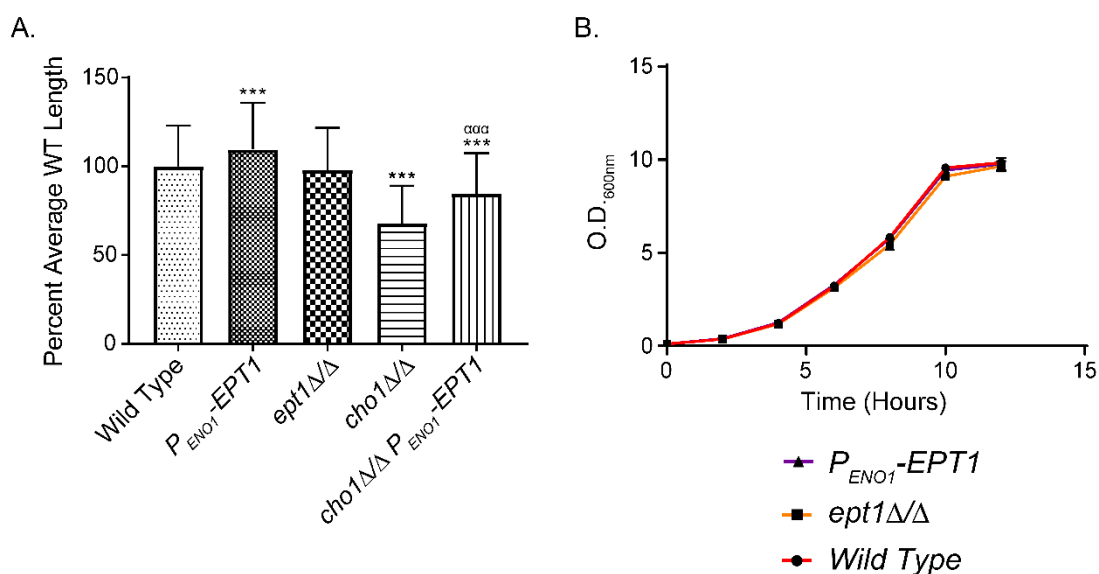


Figure 3.8. Overexpression of *EPT1* results in increased hyphal length.

(A) Stationary cultures were diluted to O.D._{600nm} = 1.0×10^{-4} in RPMI + 20% human serum and were incubated for 3 hours at 37°C in technical triplicate. Following incubation, six images per well were obtained on an inverted microscope at 200x total magnification. The length of hyphae was obtained using ImageJ software, and was expressed as percent average wild-type hyphae length. This experiment was performed three times (n=600 hyphae/strain, ***p<0.0001 vs. WT, $\alpha\alpha\alpha$ p<0.0001 vs. $cho1\Delta/\Delta$, unpaired *t*-test). **(B)** Stationary cultures were diluted to O.D._{600nm} = 0.1 in YPD and incubated at 225 RPM and 30°C. Growth was measured by obtaining the O.D._{600nm} at two-hour time points for 12 hours. Strains were cultured in triplicate.

defect in the mouse model and a larger decrease in cytotoxicity than seen in *ept1Δ/Δ* mutants (Davis et al., 2018; Tams et al., 2019). Conversely, overexpression of *EPT1* results in increased PE synthesis and hypervirulence (Tams et al., 2019). To determine why increased PE would lead to increased virulence, a transcriptomic approach has been taken. However, we have found that there are no transcriptional differences in the *EPT1* overexpressor as compared to the wild-type under similar conditions in which we see differing levels of cytotoxicity *in vitro* (Figure 3.2, A). Therefore, the changes in cytotoxicity must be due to post-transcriptional changes within the cell.

In contrast, loss of PE greatly impacts the transcriptomic profile of the cell (Figure 3.2, B-C). This impact is greater for the *psd1Δ/Δ psd2Δ/Δ* mutant, which is expected given its known growth defects, cell wall issues, and strongly attenuated virulence in the mouse model (Chen et al., 2010). Interestingly, we have found that the *psd1Δ/Δ psd2Δ/Δ* mutant has decreased expression of genes in several pathways involved in energy production and core carbon metabolism of the cell; specifically, the TCA cycle, glycolysis, and oxidative respiration (Figure 3.4). PE is an important component of the mitochondrial membrane as disruption of PE synthesis decreases inner membrane potential (Bottinger et al., 2012), which explains our findings of decreased expression of cellular respiration genes. The *psd1Δ/Δ psd2Δ/Δ* mutant also has increased expression of genes involved in protein processing and glycosylation (Figure 3.4). These are important for secretion of properly manufactured glycoproteins on the cell surface, and we believe that the increased regulation of these genes is a compensatory mechanism for cell wall stress (Orlean, 2012). We have previously found that this mutant has a growth defect that can be restored when an osmotic stabilizer such as sorbitol is added to the growth medium

(Chen et al., 2010). However, the main goal of transcriptomic analysis was to determine why overexpression of *EPT1* increased virulence, and we found no differences in the transcriptional profile for this mutant, which indicates that there must be post-transcriptional changes in the cell that occur with increased PE that cause virulence to increase. Therefore, we tested an alternative hypothesis that the cell wall architecture is altered, which could have an immunomodulatory effect or aid in evasion of immunosurveillance.

The two primary changes observed in the cell wall of the *EPT1* overexpressor were decreased $\beta(1,3)$ -glucan exposure when exposed to cell wall stressors, and an increase in chitin (Figures 3.5, C; 3.6, B; 3.7, A). $\beta(1,3)$ -glucan exposure causes a Dectin-1 mediated TNF- α response in macrophages and it has been shown that neutrophils can cause unmasking of cells *in vitro* (Taylor et al., 2007; Davis et al., 2014). To cause unmasking of cells *in vitro*, our cells were stressed with a sub-MIC dose of caspofungin or heat shock (Figures 3.5, C; 3.6, B). We have determined that cells overexpressing *EPT1* have protection from $\beta(1,3)$ -glucan exposure when in log phase or alternatively in stationary phase when stressed with caspofungin or heat stress (Figures 3.5, C; 3.6, B). If the *EPT1* overexpressor can more effectively resist $\beta(1,3)$ -glucan exposure *in vivo*, then immune evasion could partially explain the hypervirulent phenotype. It has been demonstrated that small particles of purified fungal chitin can increase production of IL-10 *in vivo*, and that large chitin particles can induce a TNF- α response (Da Silva et al., 2009). While there was a 1.65-fold increase of chitin for the *EPT1* overexpressor as compared to the wild-type (Figure 3.7, A), we were not able to elucidate any differences in TNF- α response from RAW 264.7 macrophages (data not shown). Most of the chitin

synthesis in *C. albicans* is performed by Chitin Synthase 3 (*CHS3*), which is transcriptionally controlled based on cell cycle (Lenardon et al., 2007). However, localization of *CHS3* to the site of polarized growth is the major regulatory mechanism, and this is controlled by post-transcriptional events (Lenardon et al., 2007). Therefore, it is reasonable that there are no transcriptional changes of chitin synthase genes even though there is an increase in chitin content in the *EPT1* overexpression mutant.

Besides changes in cell wall architecture that may contribute to immune evasion as a defensive mechanism, we have demonstrated that overexpression of *EPT1* also causes increased filamentation (Figure 3.8, A). The ability to form hyphae is a major virulence determinant in *C. albicans*, as they are used to escape the phagosome, lyse epithelial and endothelial cells, and help to form biofilms (Mayer et al., 2013; Westman et al., 2018). To our knowledge there have been at least four other instances in which hypervirulence in *C. albicans* correlated with increased hyphae length or production, and in one case this correlated with an increased level of chitin (Angiolella et al., 2008; Carlisle et al., 2009; Bahnan et al., 2012; Duvenage et al., 2019). It is currently unclear as to why the *EPT1* overexpressing cells form longer hyphae at an early timepoint, but one possible explanation is that increased phospholipid synthesis in the cell increases availability for this structural component of cell membrane synthesis. However, yeast form cells overexpressing *EPT1* do not replicate at an increased rate, so this may be a hyphal specific difference (Figure 3.8, B).

Since hyphae are a major virulence factor of *C. albicans* that contribute to the ability to lyse mammalian cells and contribute to tissue invasion, this is one possible explanation for hypervirulence of the *EPT1* overexpression strain (Mayer et al., 2013). It

is important for *C. albicans* to switch between yeast and hyphae forms, as locking it in either form results in avirulence, and this is presumably due to an inability to disseminate for hyphae locked cells, or an inability to damage tissue for yeast locked cells (Saville et al., 2003). However, it has been shown that driving cells into hyphal formation (by overexpression of the *UME6* transcription factor, for example) increases virulence in the mouse model (Carlisle et al., 2009). Since hyphae are a major determinant in the ability to form deep-seated infections, it is possible that increased hyphal development in the *EPT1* overexpressor, in conjunction with changes in cell wall architecture that could impact immune evasion, may contribute to the previously observed increase in cytotoxicity and virulence (Tams et al., 2019). Given that the increase in chitin observed for the *EPT1* overexpressor does not cause a clear change in TNF- α release from macrophages, the increase in hyphal growth is the most likely explanation for the increased virulence.

Author contributions

RT and TBR designed and created plasmids and *C. albicans* strains. RT performed RNA extractions and sequencing. Bioinformatic analysis was performed by RT and EG. AW performed chitin and $\beta(1,3)$ -glucan staining and subsequent flow cytometry. JJ assisted with flow cytometry. RT performed all other assays and wrote the manuscript. TBR directed the project. TBR and TS edited the manuscript.

Funding

This work was funded by the National Institutes of Health grants NIH 1R21AI130895 (TBR), NIH 1R01AL105690 (TBR), and NIH R01DK079984 (TS). The funders had no role in the design or execution of the project or the decision to publish.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

We thank Veronica Brown and the UT Genomics Core for assistance in cDNA library preparation and sequencing, and Dr. Lauren Krausfeldt for her comments and suggestions regarding transcriptomic analysis. We also thank Steven Wilhelm, Robbie Martin, Tian Chen and Kyle Bonifer for helpful discussions and other contributions.

References

- Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol* 11(10), R106. doi: 10.1186/gb-2010-11-10-r106.
- Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31(2), 166-169. doi: 10.1093/bioinformatics/btu638.
- Angiolella, L., Stringaro, A.R., De Bernardis, F., Posteraro, B., Bonito, M., Toccaceli, L., et al. (2008). Increase of virulence and its phenotypic traits in drug-resistant strains of *Candida albicans*. *Antimicrob Agents Chemother* 52(3), 927-936. doi: 10.1128/AAC.01223-07.
- Atkinson, K.D., Jensen, B., Kolat, A.I., Storm, E.M., Henry, S.A., and Fogel, S. (1980). Yeast mutants auxotrophic for choline or ethanolamine. *J Bacteriol* 141(2), 558-564.
- Bahnan, W., Koussa, J., Younes, S., Abi Rizk, M., Khalil, B., El Sitt, S., et al. (2012). Deletion of the *Candida albicans* PIR32 results in increased virulence, stress response, and upregulation of cell wall chitin deposition. *Mycopathologia* 174(2), 107-119. doi: 10.1007/s11046-012-9533-z.
- Bottinger, L., Horvath, S.E., Kleinschroth, T., Hunte, C., Daum, G., Pfanner, N., et al. (2012). Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes. *J Mol Biol* 423(5), 677-686. doi: 10.1016/j.jmb.2012.09.001.
- Carlisle, P.L., Banerjee, M., Lazzell, A., Monteagudo, C., Lopez-Ribot, J.L., and Kadosh, D. (2009). Expression levels of a filament-specific transcriptional regulator are sufficient to determine *Candida albicans* morphology and virulence. *Proc Natl Acad Sci U S A* 106(2), 599-604. doi: 10.1073/pnas.0804061106.
- Carman, G.M., and Han, G.S. (2011). Regulation of phospholipid synthesis in the yeast *Saccharomyces cerevisiae*. *Annu Rev Biochem* 80, 859-883. doi: 10.1146/annurev-biochem-060409-092229.
- Cassilly, C.D., Farmer, A.T., Montedonico, A.E., Smith, T.K., Campagna, S.R., and Reynolds, T.B. (2017). Role of phosphatidylserine synthase in shaping the phospholipidome of *Candida albicans*. *FEMS Yeast Res* 17(2), (2): fox007. doi: 10.1093/femsyr/fox007.
- Chen, T., Jackson, J.W., Tams, R.N., Davis, S.E., Sparer, T.E., and Reynolds, T.B. (2019). Exposure of *Candida albicans* beta (1,3)-glucan is promoted by activation of the Cek1 pathway. *PLoS Genet* 15(1), e1007892. doi: 10.1371/journal.pgen.1007892.
- Chen, Y.L., Kauffman, S., and Reynolds, T.B. (2008). *Candida albicans* uses multiple mechanisms to acquire the essential metabolite inositol during infection. *Infect Immun* 76(6), 2793-2801. doi: 10.1128/IAI.01514-07.
- Chen, Y.L., Montedonico, A.E., Kauffman, S., Dunlap, J.R., Menn, F.M., and Reynolds, T.B. (2010). Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence in *Candida albicans*. *Mol Microbiol* 75(5), 1112-1132. doi: 10.1111/j.1365-2958.2009.07018.x.
- Clancey, C.J., Chang, S.C., and Dowhan, W. (1993). Cloning of a gene (PSD1) encoding phosphatidylserine decarboxylase from *Saccharomyces cerevisiae* by

- complementation of an *Escherichia coli* mutant. *J Biol Chem* 268(33), 24580-24590.
- Cowen, L.E., Sanglard, D., Howard, S.J., Rogers, P.D., and Perlin, D.S. (2015). Mechanisms of Antifungal Drug Resistance. *Cold Spring Harb Perspect Med* 5(7), a019752. doi: 10.1101/cshperspect.a019752.
- Da Silva, C.A., Chalouni, C., Williams, A., Hartl, D., Lee, C.G., and Elias, J.A. (2009). Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J Immunol* 182(6), 3573-3582. doi: 10.4049/jimmunol.0802113.
- Davis, S.E., Hopke, A., Minkin, S.C., Jr., Montedonico, A.E., Wheeler, R.T., and Reynolds, T.B. (2014). Masking of beta(1-3)-glucan in the cell wall of *Candida albicans* from detection by innate immune cells depends on phosphatidylserine. *Infect Immun* 82(10), 4405-4413. doi: 10.1128/IAI.01612-14.
- Davis, S.E., Tams, R.N., Solis, N., Wagner, A.S., Chen, T., Jackson, J.W., et al. (2018). *Candida albicans* cannot acquire sufficient ethanolamine from the host to support virulence in the absence of de novo phosphatidylethanolamine synthesis. *Infect Immun* 86(8), e00815-00817. doi: 10.1128/IAI.00815-17.
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29(1), 15-21. doi: 10.1093/bioinformatics/bts635.
- Duvenage, L., Walker, L.A., Bojarczuk, A., Johnston, S.A., MacCallum, D.M., Munro, C.A., et al. (2019). Inhibition of Classical and Alternative Modes of Respiration in *Candida albicans* Leads to Cell Wall Remodeling and Increased Macrophage Recognition. *MBio* 10(1), e02535-02518. doi: 10.1128/mBio.02535-18.
- Gibellini, F., and Smith, T.K. (2010). The Kennedy pathway--De novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life* 62(6), 414-428. doi: 10.1002/iub.337.
- Gillum, A.M., Tsay, E.Y., and Kirsch, D.R. (1984). Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3* and *E. coli* *pyrF* mutations. *Mol Gen Genet* 198(2), 179-182.
- Gow, N.A., Netea, M.G., Munro, C.A., Ferwerda, G., Bates, S., Mora-Montes, H.M., et al. (2007). Immune recognition of *Candida albicans* beta-glucan by dectin-1. *J Infect Dis* 196(10), 1565-1571. doi: 10.1086/523110.
- Gow, N.A.R., Latge, J.P., and Munro, C.A. (2017). The Fungal Cell Wall: Structure, Biosynthesis, and Function. *Microbiol Spectr* 5(3), (3):FUNK-0035-2016. doi: 10.1128/microbiolspec.FUNK-0035-2016.
- Hasim, S., Allison, D.P., Retterer, S.T., Hopke, A., Wheeler, R.T., Doktycz, M.J., et al. (2017). beta-(1,3)-Glucan Unmasking in Some *Candida albicans* Mutants Correlates with Increases in Cell Wall Surface Roughness and Decreases in Cell Wall Elasticity. *Infect Immun* 85(1), e00601-00616. doi: 10.1128/IAI.00601-16.
- Hasim, S., Hussin, N.A., Alomar, F., Bidasee, K.R., Nickerson, K.W., and Wilson, M.A. (2014). A glutathione-independent glyoxalase of the DJ-1 superfamily plays an important role in managing metabolically generated methylglyoxal in *Candida albicans*. *J Biol Chem* 289(3), 1662-1674. doi: 10.1074/jbc.M113.505784.
- Hasim, S., Vaughn, E.N., Donohoe, D., Gordon, D.M., Pfiffner, S., and Reynolds, T.B. (2018). Influence of phosphatidylserine and phosphatidylethanolamine on farnesol tolerance in *Candida albicans*. *Yeast* 35(4), 343-351. doi: 10.1002/yea.3297.

- Herre, J., Marshall, A.S., Caron, E., Edwards, A.D., Williams, D.L., Schweighoffer, E., et al. (2004). Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* 104(13), 4038-4045. doi: 10.1182/blood-2004-03-1140.
- Hopke, A., Nicke, N., Hidu, E.E., Degani, G., Popolo, L., and Wheeler, R.T. (2016). Neutrophil Attack Triggers Extracellular Trap-Dependent Candida Cell Wall Remodeling and Altered Immune Recognition. *PLoS Pathog* 12(5), e1005644. doi: 10.1371/journal.ppat.1005644.
- Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28(1), 27-30.
- Kodaki, T., and Yamashita, S. (1987). Yeast phosphatidylethanolamine methylation pathway. Cloning and characterization of two distinct methyltransferase genes. *J Biol Chem* 262(32), 15428-15435.
- Lenardon, M.D., Munro, C.A., and Gow, N.A. (2010). Chitin synthesis and fungal pathogenesis. *Curr Opin Microbiol* 13(4), 416-423. doi: 10.1016/j.mib.2010.05.002.
- Lenardon, M.D., Whitton, R.K., Munro, C.A., Marshall, D., and Gow, N.A. (2007). Individual chitin synthase enzymes synthesize microfibrils of differing structure at specific locations in the Candida albicans cell wall. *Mol Microbiol* 66(5), 1164-1173. doi: 10.1111/j.1365-2958.2007.05990.x.
- Letts, V.A., Klig, L.S., Bae-Lee, M., Carman, G.M., and Henry, S.A. (1983). Isolation of the yeast structural gene for the membrane-associated enzyme phosphatidylserine synthase. *Proc Natl Acad Sci U S A* 80(23), 7279-7283.
- Marakalala, M.J., Vautier, S., Potrykus, J., Walker, L.A., Shepardson, K.M., Hopke, A., et al. (2013). Differential adaptation of Candida albicans in vivo modulates immune recognition by dectin-1. *PLoS Pathog* 9(4), e1003315. doi: 10.1371/journal.ppat.1003315.
- Martin, R.M., Dearth, S.P., LeClerc, G.R., Campagna, S.R., Fozo, E.M., Zinser, E.R., et al. (2017). Microcystin-LR does not induce alterations to transcriptomic or metabolomic profiles of a model heterotrophic bacterium. *PLoS One* 12(12), e0189608. doi: 10.1371/journal.pone.0189608.
- Mayer, F.L., Wilson, D., and Hube, B. (2013). Candida albicans pathogenicity mechanisms. *Virulence* 4(2), 119-128. doi: 10.4161/viru.22913.
- Orlean, P. (2012). Architecture and biosynthesis of the Saccharomyces cerevisiae cell wall. *Genetics* 192(3), 775-818. doi: 10.1534/genetics.112.144485.
- Raschke, W.C., Baird, S., Ralph, P., and Nakoinz, I. (1978). Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell* 15(1), 261-267.
- Reuss, O., Vik, A., Kolter, R., and Morschhauser, J. (2004). The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. *Gene* 341, 119-127. doi: 10.1016/j.gene.2004.06.021.
- Saville, S.P., Lazzell, A.L., Monteagudo, C., and Lopez-Ribot, J.L. (2003). Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection. *Eukaryot Cell* 2(5), 1053-1060.
- Schindelin, J., Rueden, C.T., Hiner, M.C., and Eliceiri, K.W. (2015). The ImageJ ecosystem: An open platform for biomedical image analysis. *Mol Reprod Dev* 82(7-8), 518-529. doi: 10.1002/mrd.22489.

- Singh, A., Prasad, T., Kapoor, K., Mandal, A., Roth, M., Welti, R., et al. (2010). Phospholipidome of *Candida*: each species of *Candida* has distinctive phospholipid molecular species. *OMICS* 14(6), 665-677. doi: 10.1089/omi.2010.0041.
- Skrzypek, M.S., Binkley, J., Binkley, G., Miyasato, S.R., Simison, M., and Sherlock, G. (2017). The *Candida* Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and visualization of high throughput sequencing data. *Nucleic Acids Res* 45(D1), D592-D596. doi: 10.1093/nar/gkw924.
- Tams, R.N., Cassilly, C.D., Anaokar, S., Brewer, W.T., Dinsmore, J.T., Chen, Y.-L., et al. (2019). Overproduction of Phospholipids by the Kennedy Pathway Leads to Hypervirulence in *Candida albicans*. *Frontiers in Microbiology* 10, 86. doi: 10.3389/fmicb.2019.00086.
- Taylor, P.R., Tsoni, S.V., Willment, J.A., Dennehy, K.M., Rosas, M., Findon, H., et al. (2007). Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol* 8(1), 31-38. doi: 10.1038/ni1408.
- Trotter, P.J., and Voelker, D.R. (1995). Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (PSD2) in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 270(11), 6062-6070. doi: 10.1074/jbc.270.11.6062.
- Wagener, J., MacCallum, D.M., Brown, G.D., and Gow, N.A. (2017). *Candida albicans* Chitin Increases Arginase-1 Activity in Human Macrophages, with an Impact on Macrophage Antimicrobial Functions. *MBio* 8(1), e01820-01816. doi: 10.1128/mBio.01820-16.
- Walker, L.A., Munro, C.A., de Bruijn, I., Lenardon, M.D., McKinnon, A., and Gow, N.A. (2008). Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. *PLoS Pathog* 4(4), e1000040. doi: 10.1371/journal.ppat.1000040.
- Westman, J., Moran, G., Mogavero, S., Hube, B., and Grinstein, S. (2018). *Candida albicans* Hyphal Expansion Causes Phagosomal Membrane Damage and Luminal Alkalinization. *MBio* 9(5), e01226-01218. doi: 10.1128/mBio.01226-18.
- Wheeler, R.T., and Fink, G.R. (2006). A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog* 2(4), e35. doi: 10.1371/journal.ppat.0020035.
- Xu, S., and Shinohara, M.L. (2017). Tissue-Resident Macrophages in Fungal Infections. *Front Immunol* 8, 1798. doi: 10.3389/fimmu.2017.01798.

**Chapter 4 : *Candida albicans* Cannot Acquire Sufficient Ethanolamine
from the Host to Support Virulence in the Absence of *De Novo*
Phosphatidylethanolamine Synthesis**

Publication Note

A version of this chapter has been previously published by Sarah E. Davis, Robert N. Tams, Norma V. Solis, Andrew S. Wagner, Tian Chen, Joseph W. Jackson, Sahar Hasim, Anthony E. Montedonico, Justin Dinsmore, Timothy E. Sparer, Scott G. Filler, and Todd B. Reynolds:

Davis, S.E., Tams, R.N., Solis, N., Wagner, A.S., Chen, T., Jackson, J.W., et al. 2018. *Candida albicans cannot acquire sufficient ethanolamine from the host to support virulence in the absence of de novo phosphatidylethanolamine synthesis*. Infection and Immunity. 86(8).

It should be noted that this article has two primary authors, Sarah E. Davis, and Robert N. Tams. The author contributions are as follows: SED initially wrote the manuscript, which was edited by NVS, SH, AM, RNT, SGF and TBR. Several re-submissions were made, which required extensive revisions. These revisions were performed primarily by RNT, and by ASW, TC, and JWJ.

SED, SH, and RNT performed mouse intravenous models. SH created and utilized the *AtSDC* construct. SED and RNT performed mouse oral model studies, and cytotoxicity assays. RNT and SED performed growth curves. SED and TC performed immunofluorescence studies. ASW and JWJ performed flow cytometry (data not included). RNT performed lipid extractions, TLC, and papaumide-A kill assays. SED, TBR, and RNT created and edited figures. Further editing of the manuscript was performed by RNT and TBR, as well as other authors, and the project was directed by TBR. TBR, SGF, and SED provided funding for these experiments.

Abstract

Candida albicans mutants for phosphatidylserine (PS) synthase (*cho1Δ/Δ*) and PS decarboxylase (*psd1Δ/Δ psd2Δ/Δ*) are compromised for virulence in mouse models of systemic infection and oropharyngeal Candidiasis (OPC). Both of these enzymes are necessary to synthesize phosphatidylethanolamine (PE) by the *de novo* pathway, but these mutants are still capable of growth in culture media as they can import ethanolamine from media to synthesize PE through the Kennedy pathway. Given that the host has ethanolamine in its serum, the exact mechanism by which virulence is lost in these mutants is not clear. There are two competing hypotheses to explain their loss of virulence. 1) PE from the Kennedy pathway cannot substitute for *de novo* synthesized PE. 2) The mutants cannot acquire sufficient ethanolamine from the host to support adequate PE synthesis. These hypotheses can be simultaneously tested if ethanolamine availability is increased for *Candida* while it is inside the host. We accomplish this by transcomplementation of *C. albicans* with the *Arabidopsis thaliana* serine decarboxylase gene (*AtSDC*), which converts cytoplasmic serine to ethanolamine. Expression of *AtSDC* in either mutant restores PE synthesis even in the absence of exogenous ethanolamine. *AtSDC* also restores virulence to *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* in systemic and OPC infections. Thus, in the absence of *de novo* PE synthesis, *C. albicans* cannot acquire sufficient ethanolamine from the host to support virulence. In addition, expression of *AtSDC* restores PS synthesis in the *cho1Δ/Δ* mutant, which may be due to causing PS decarboxylase to run backwards and convert PE to PS.

Introduction

Candida spp. are human commensal fungi that colonize the gastrointestinal tract, as well as vaginal and oral mucosa. They reside as oral flora in approximately 30-60% of healthy individuals and usually do not cause disease (Pankhurst, 2009). However, in immunocompromised patients *Candida spp.* are able to cause a common oral infection known as oropharyngeal Candidiasis (OPC) or thrush (Pankhurst, 2009; Thompson et al., 2010). OPC is one of the first indicators of suppression of adaptive immunity and is seen in up to 95% of acquired immune deficiency syndrome (AIDS) patients at some time during their infection (Rabeneck et al., 1993). Other risk factors for OPC include use of steroid inhalers or dentures, and it is becoming a common infection in recipients of solid organ transplants (Lynch, 1994; Hancock et al., 2003; Soysa et al., 2008; Dongari-Bagtzoglou et al., 2009). These infections are detrimental to the comfort of patients, causing difficulty in chewing and swallowing, and in severe cases will result in reduced food intake and weight loss (Pankhurst, 2009). It is important to note that these oral infections can be refractory to medications and exhibit drug resistance, making treatment challenging (Revankar et al., 1998; Thompson et al., 2010). Out of all *Candida* species, *C. albicans* is the species most commonly isolated from oral lesions (Vazquez and Sobel, 2002).

C. albicans and related species are also capable of causing more serious systemic infections, when it enters the bloodstream and deep organs, and these infections have a mortality rate of ~30% (Eggimann et al., 2003; Bustamante, 2005; Morrell et al., 2005). Systemic infections tend to occur in neutropenic patients and are associated with intravascular catheters. Previously, we discovered that mutations that block the *de novo*

pathway for synthesizing the phospholipids phosphatidylserine (PS) and/or phosphatidylethanolamine (PE) compromise the virulence of *C. albicans* in a mouse model of systemic candidiasis and could potentially serve as good drug targets (Chen et al., 2010). The *cho1* Δ/Δ mutation disrupts PS synthase, the first step of the *de novo* pathway (Figure 4.1, enzymes shown in green), which generates PS from cytidyldiphosphate-diacylglycerol (CDP-DAG) and serine (Atkinson et al., 1980; Yamashita and Nikawa, 1997). A second pair of mutations (*psd1* Δ/Δ *psd2* Δ/Δ) blocks the second step of the pathway, whereby a pool of PS is decarboxylated to PE by Psd1p or Psd2p (Clancey et al., 1993; Trotter and Voelker, 1995; Birner et al., 2001). The *cho1* Δ/Δ mutant has a complete loss of PS and cannot make PE by this pathway, whereas the *psd1* Δ/Δ *psd2* Δ/Δ mutant cannot synthesize PE from PS, and therefore accumulates PS (Chen et al., 2010; Cassilly et al., 2017). In both mutants, PE can be synthesized via an alternative pathway called the Kennedy pathway (Figure 4.1, enzymes shown in blue), where diacylglycerol (DAG) and ethanolamine are used to make PE (Henry et al., 2012) (Figure 4.1). Thus, the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants are ethanolamine auxotrophs (Chen et al., 2010).

The *cho1* Δ/Δ mutant is avirulent and *psd1* Δ/Δ *psd2* Δ/Δ is highly attenuated in a mouse model for systemic Candidiasis (Chen et al., 2010). The *cho1* Δ/Δ mutant exhibits more severe cell wall defects than the *psd1* Δ/Δ *psd2* Δ/Δ mutant, and this is manifested in increased exposure of cell wall β (1-3)-glucan (unmasking) to the immune system, as well as an altered cell wall structure as seen by transmission electron microscopy (TEM) and increased chitin levels (Wheeler et al., 2008; Chen et al., 2010; Davis et al., 2014). The avirulence of the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants is somewhat surprising given

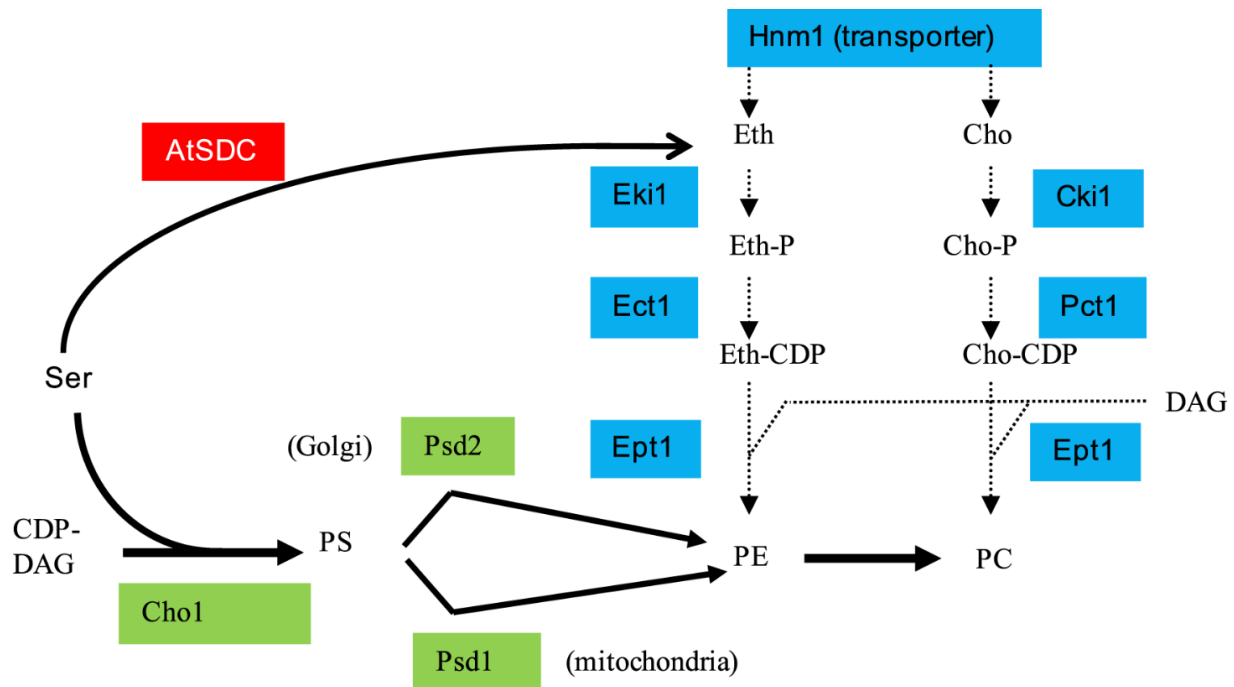


Figure 4.1. Pathways for synthesizing aminophospholipids in *C. albicans*.

Enzymes are named after their confirmed or predicted homologs in *S. cerevisiae*. The Kennedy pathway enzymes are shown in blue, and the *de novo* pathway proteins are shown in green. *Arabidopsis thaliana* Serine Decarboxylase (*AtSDC*) is shown in red. Ser: serine, 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, DAG:

that both mutants can grow in the presence of exogenous ethanolamine, and host serum contains ~30 μ M ethanolamine (Houweling et al., 1992). Therefore, in this communication we specifically address the molecular mechanism by which these mutants are compromised for virulence *in vivo* using both systemic and OPC infection models.

Results

To determine if *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ have defects in oral infections by *C. albicans*, we compared these mutants to wild-type and reintegrant strains in the mouse OPC model. It was discovered that there was a decrease in pathogenicity for both *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ in OPC infections (Figure 4.2, 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 units (CFUs).

In addition to the mouse OPC model, a tissue culture model has been used to study the damage of oral epithelial cells (FaDu) by *C. albicans*. This can be measured based on the release of lactate dehydrogenase (LDH), a cytoplasmic enzyme, as a proxy for FaDu cell damage (Sun et al., 2010). When *C. albicans* strains are co-incubated with FaDu cells, the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants show reduced damage of the epithelial cells (Figure 4.2, C).

Loss of de novo PE synthesis compromises virulence because mutants cannot acquire sufficient ethanolamine in the host

The *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ phospholipid biosynthesis mutants show decreased virulence in three models of virulence: murine systemic infection (Chen et al.,

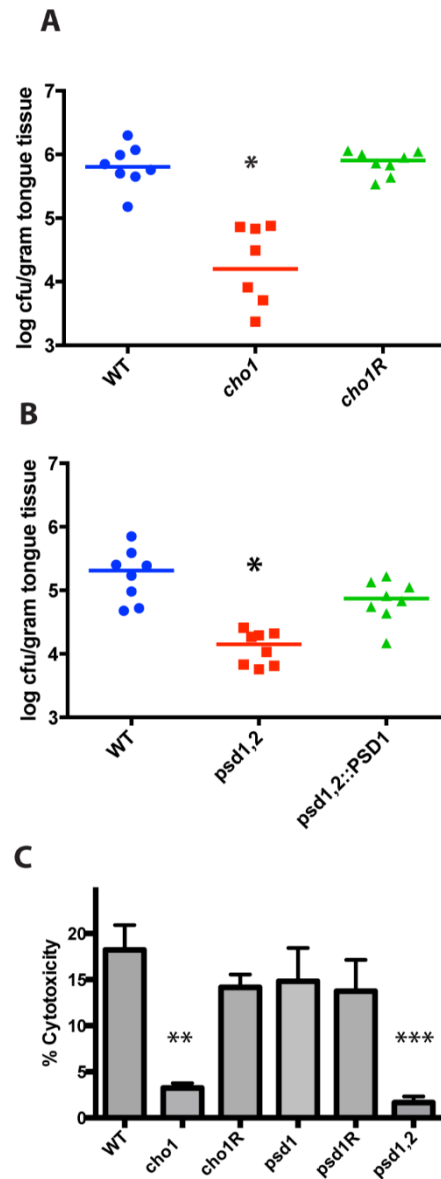


Figure 4.2. The *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants are attenuated for virulence during *in vivo* and *in vitro* models of OPC.

Mice were inoculated orally with **A.** wild-type (WT), *cho1* Δ/Δ (*cho1*) and *cho1* $\Delta/\Delta::CHO1$ (*cho1R*) reintegant strains or **B.** WT, *psd1* Δ/Δ *psd2* Δ/Δ (*psd1,2*), or *psd1* Δ/Δ *psd2* $\Delta/\Delta::PSD1$ (*psd1,2R*) reintegant strains. After five days their tongues were harvested, and the cfus/gram of tissue were measured. *p-value<0.0002, compared to WT. **C.** The strains were co-incubated with FaDu cells, and the level of lactate dehydrogenase (LDH) released into the media was measured as a proxy for FaDu cell damage. **p-value 0.0007, *** p-value 0.0005, all compared to wild-type.

2010), murine OPC (Figure 4.2, A-B), and FaDu epithelial damage (Figure 4.2, C). The main feature shared by both mutants is the inability to synthesize PE from PS. However, it is not clear why loss of PE synthesis through the *de novo* pathway compromises virulence given the existence of a functional Kennedy pathway. One hypothesis is that PE synthesized by Psd1 or Psd2 is fundamentally different in composition or localization than PE synthesized through the Kennedy pathway, and therefore Kennedy pathway PE cannot compensate for *de novo* pathway PE. A competing hypothesis is that PE from either pathway is sufficient, but *C. albicans* cannot acquire enough ethanolamine from the host to satisfy its PE requirement via the Kennedy pathway.

The way to test these competing hypotheses is to increase PE synthesis through the Kennedy pathway while *C. albicans* is in the host during an infection. This requires that we provide sufficient ethanolamine intracellularly to *C. albicans*, as it is possible that the amount of ethanolamine in the host is not sufficient to support growth or virulence of these mutants. The level of ethanolamine in mouse serum is estimated to be ~30µM (Houweling et al., 1992). However, we found that the level of ethanolamine required to support growth of these *Candida* mutants in culture media at 37°C is greater than 100µM (Figure 4.3). Therefore, the mutants may not be able to import sufficient ethanolamine from the host to support virulence.

In order to provide ethanolamine intracellularly, a codon optimized serine decarboxylase enzyme derived from *Arabidopsis thaliana* (*AtSDC*) was expressed in our *C. albicans* strains (Rontein et al., 2001). Serine decarboxylase activity is found in plants, but not in mammals or fungi. *AtSDC* decarboxylates serine to ethanolamine within the

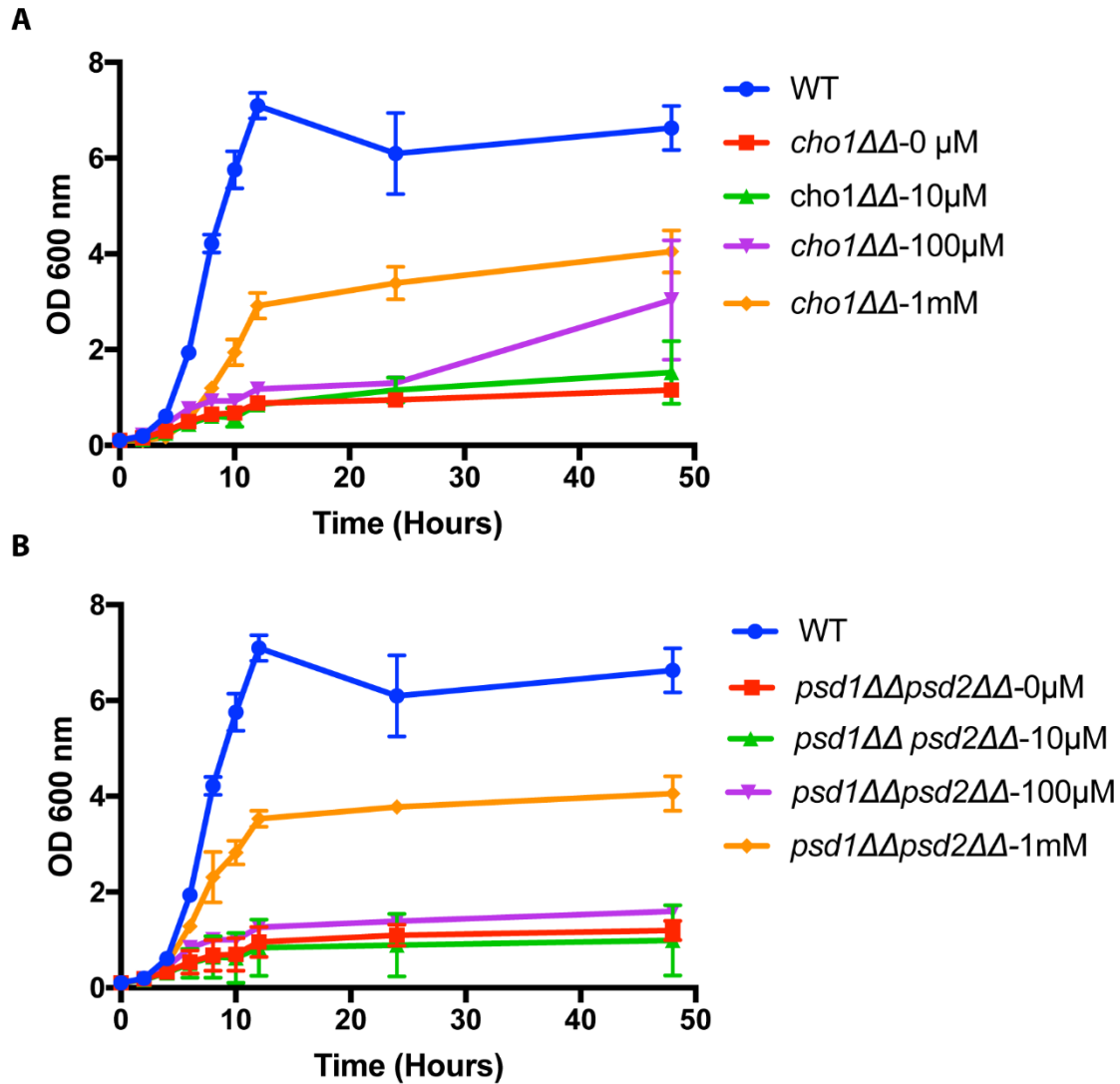


Figure 4.3. 1mM ethanolamine is required to support growth of the *cho1ΔΔ* and *psd1ΔΔ psd2ΔΔ* mutants in minimal medium.

A. The *cho1ΔΔ* and **B.** *psd1ΔΔ psd2ΔΔ* strains were grown overnight in YPD, and then diluted to an OD⁶⁰⁰ of 0.1 and grown in minimal media + various levels of ethanolamine (Eth) at 37°C with shaking, and their growth was measured for up to 48 hours. Growth is compared to wild-type (WT) with 0μM ethanolamine.

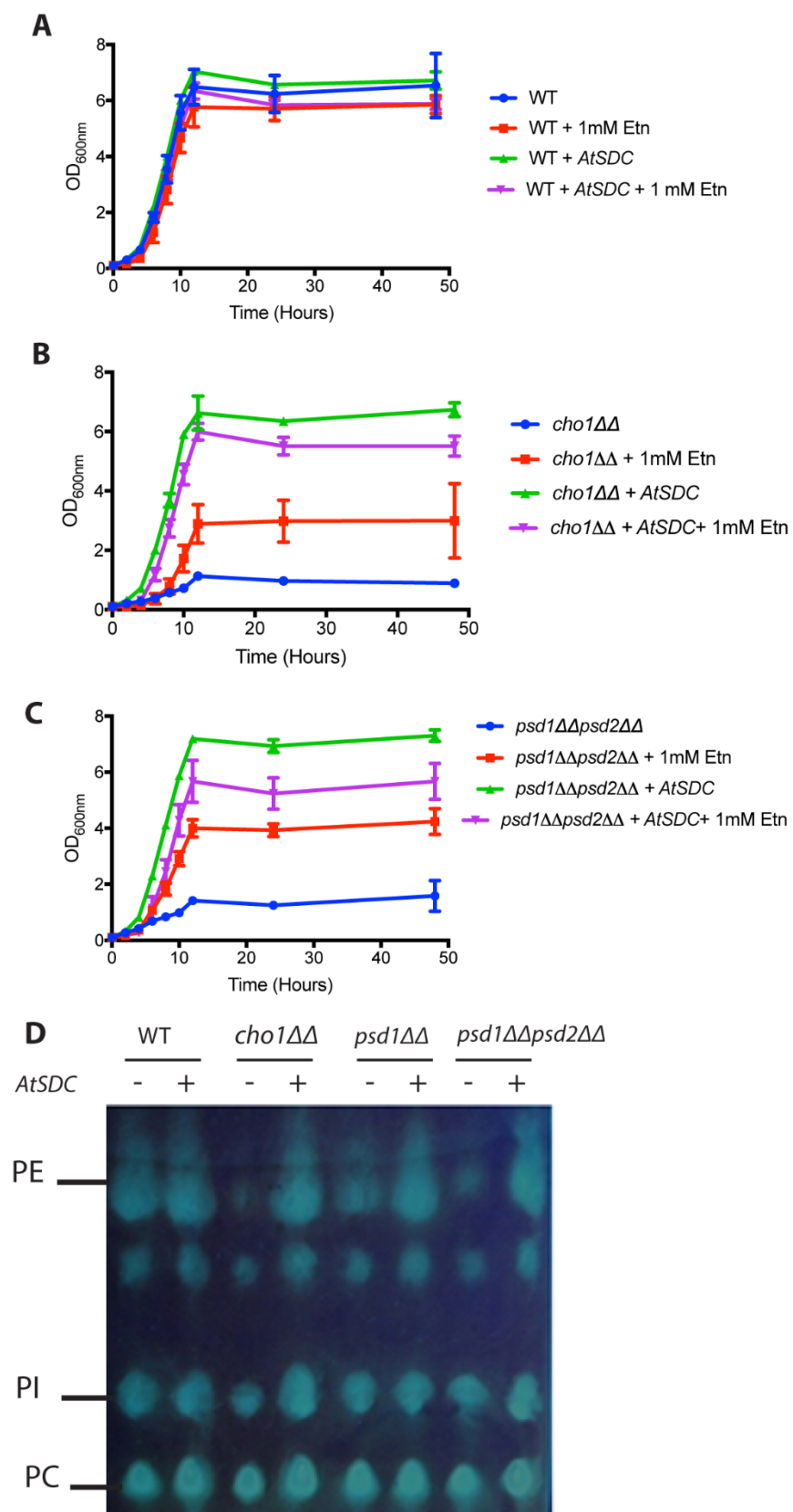
cytoplasm, and the ethanolamine can be used by the Kennedy pathway to synthesize PE (Figure 4.1). If the loss of virulence in both mutants is due to an inability to acquire sufficient ethanolamine from the host, then addition of *AtSDC* should restore ethanolamine prototrophy and virulence in mouse models of Candidiasis. If, alternatively, PE must be made by the *de novo* pathway to support virulence because Kennedy pathway derived PE is insufficient, then *AtSDC* will not rescue the virulence defect for either mutant.

The ability of *AtSDC* to support PE synthesis by the Kennedy pathway was tested in two ways. First, it was found to restore ethanolamine prototrophy in the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* strains by supporting growth in minimal media lacking ethanolamine (Figure 4.4, A-C). In fact, when grown in media lacking ethanolamine, mutants expressing *AtSDC* grew at rates similar to wild-type and exhibited better growth than the same mutant strain supplemented with ethanolamine (Figure 4.4, A-C). Second, thin layer chromatography (TLC) confirmed that PE levels were restored in the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants growing in the absence of ethanolamine when *AtSDC* is expressed (Figure 4.4, D).

We then examined if addition of *AtSDC* would restore virulence for the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants in a mouse model of systemic infection (Figure 4.5, A-B). Indeed, the addition of this enzyme fully restored virulence to both the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants in the systemic infection model. The differences seen in the survival curves were reflected in fungal burden measurements (Figure 4.5, C). It is interesting to note that although both the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants had

Figure 4.4. Expression of *AtSDC* restores ethanolamine prototrophy in *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants.

A, B, & C Mutant and wild-type (WT) strains $\pm$ *AtSDC* were grown in minimal media $\pm$ ethanolamine (Eth) at 37°C and a growth curve was plotted. **D** Phospholipids were isolated from each strain grown in minimal media lacking ethanolamine, and then separated by TLC. Lipid spots were visualized with primuline stain under ultraviolet light. Strains included *cho1* Δ/Δ (*cho1*), *psd1* Δ/Δ (*psd1*), and *psd1* Δ/Δ *psd2* Δ/Δ (*psd1 psd2*), all $\pm$ *AtSDC*.



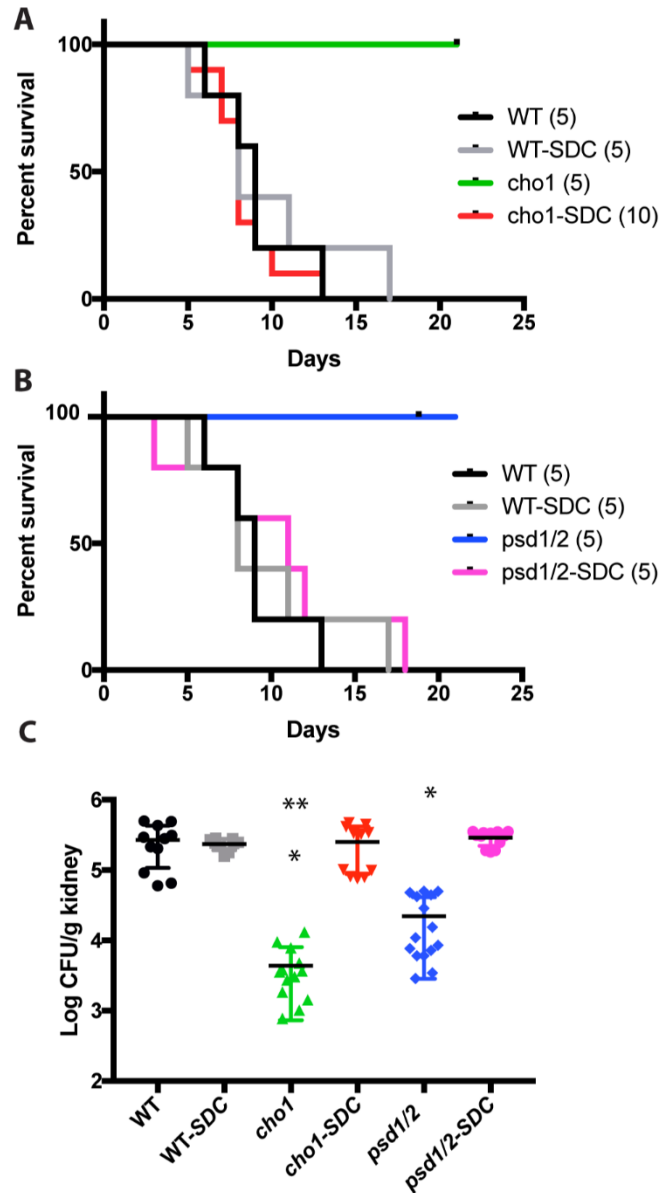


Figure 4.5. Expression of *AtSDC* restores virulence in *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ in a mouse model of systemic candidiasis. A & B

Mice were infected by tail-vein with 5×10^5 cells from each strain and survival was graphed over time. The number of mice per strain are shown beside the strain name in parenthesis. The *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ survival curves were significantly different than wild-type (*P value = 0.0004). **C.** Mice were infected with 5×10^5 cells by tail-vein and after five days, they were euthanized, and kidneys were removed, and colony forming units (cfus)/gram of kidney were measured by plating. * p-value < 0.0001, compared to WT (wild-type), and ** p-value = 0.0031 compared to *cho1* Δ/Δ . Mutant strains: *cho1* (*cho1* Δ/Δ), *psd1* (*psd1* Δ/Δ), *psd1,2* (*psd1* Δ/Δ *psd2* Δ/Δ), *SDC* (*AtSDC*).

much lower fungal burden than wild-type, the *cho1* Δ/Δ mutant exhibited a significantly lower fungal burden than *psd1* Δ/Δ *psd2* Δ/Δ (Figure 4.5, C).

We further determined if the *AtSDC* expressing mutants would be restored for virulence in the OPC model. Indeed, when these strains were tested in this model, they revealed that the mutants expressing *AtSDC* had virulence levels similar to wild-type (Figure 4.6, A). In the previous experiments shown in Figure 4.2, *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ were tested separately, and with outbred (ICR) and inbred (Balb/c) mice, respectively. However, when compared in the same experiment with outbred mice (CD-1), the *psd1* Δ/Δ *psd2* Δ/Δ mutant appears to be more virulent than the *cho1* Δ/Δ mutant. Finally, we determined if the addition of *AtSDC* would restore the ability of the mutants to damage epithelial cells. This was found to be the case for both mutants (Figure 4.6, B). In both cases, the mutants with *AtSDC* caused as much damage as wild-type.

Expression of the AtSDC restores $\beta(1,3)$ -glucan unmasking in *cho1* Δ/Δ by restoring PS synthesis

Previous work from Davis *et al* (Davis et al., 2014) revealed that *cho1* Δ/Δ , but not *psd1* Δ/Δ *psd2* Δ/Δ , exhibits increased exposure of $\beta(1,3)$ -glucan (unmasking). This data showed that PS, but not PE, regulates unmasking. Therefore, complete restoration of virulence in *cho1* Δ/Δ by *AtSDC* was unexpected as *AtSDC* synthesizes ethanolamine specifically. Therefore, we measured whether $\beta(1,3)$ -glucan unmasking in the *cho1* Δ/Δ mutant would be affected by PE synthesis through the Kennedy pathway due to expression of *AtSDC*. The addition of *AtSDC* led to full restoration of $\beta(1,3)$ -glucan unmasking in the *cho1* Δ/Δ mutant similar to wild-type (Figure 4.7). Thus, production of PE from the Kennedy

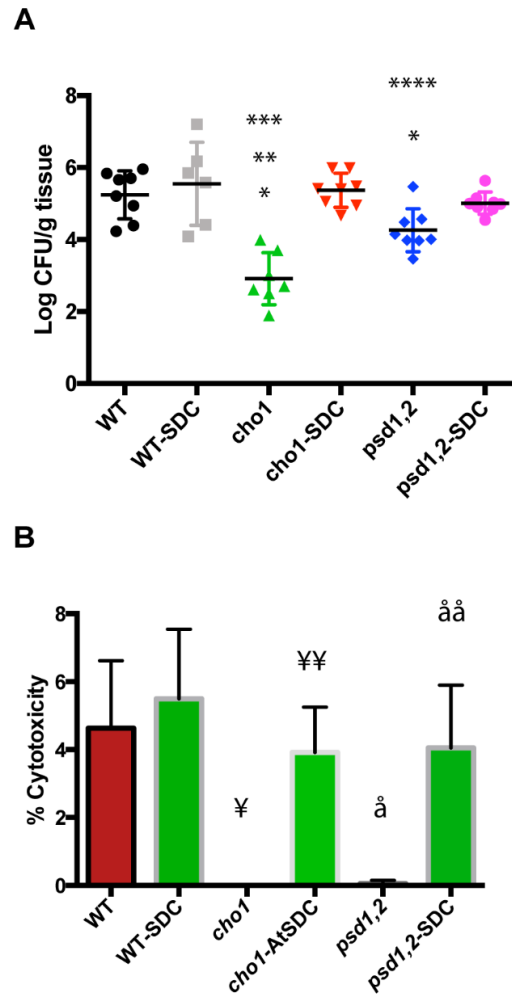
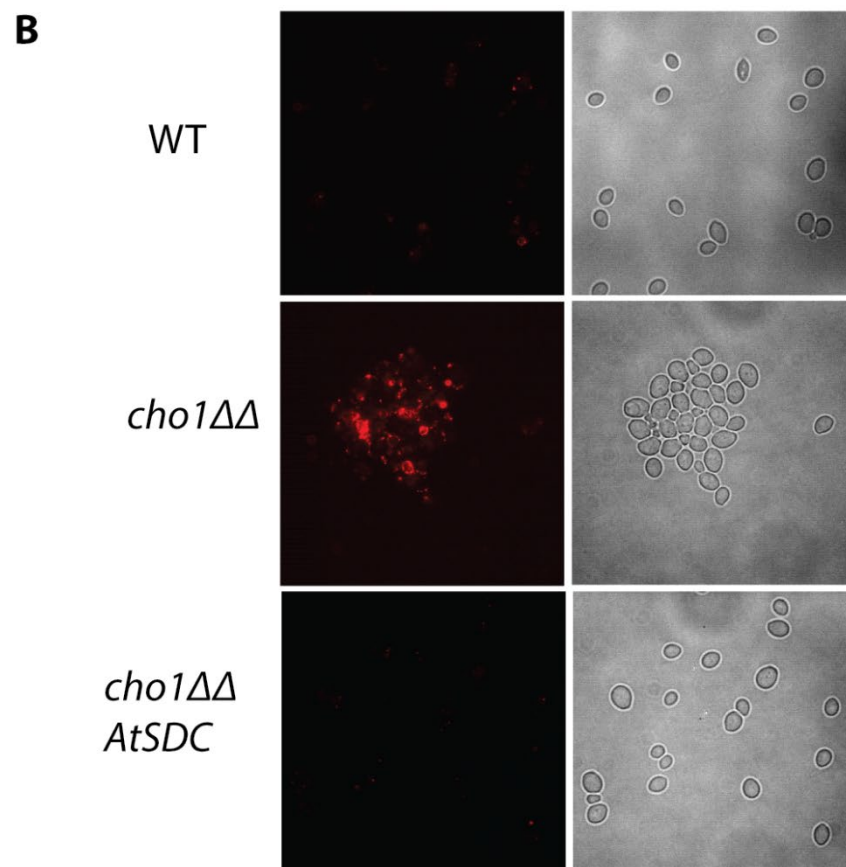
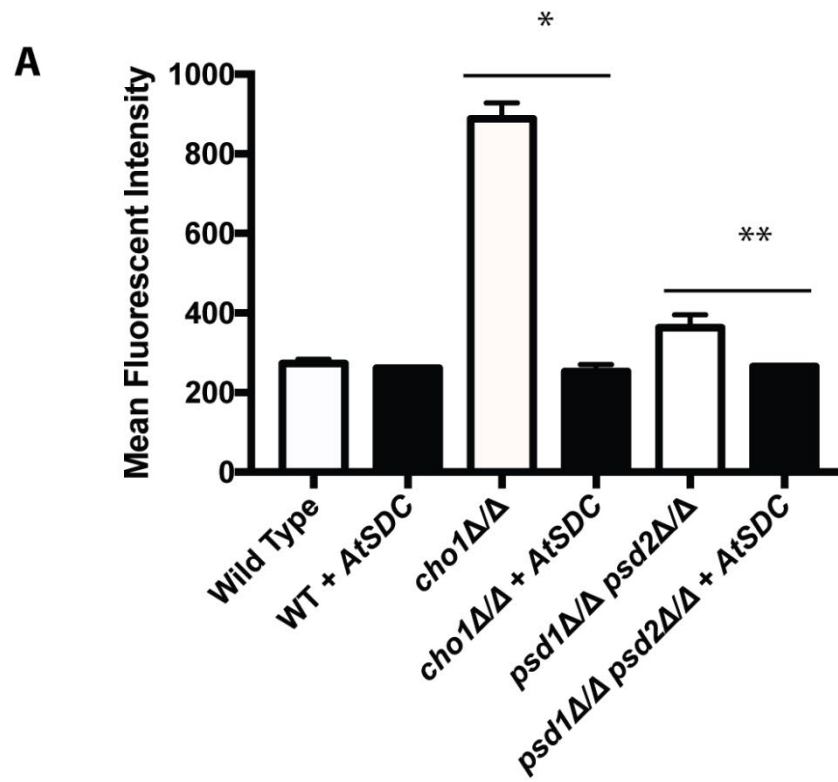


Figure 4.6. Expression of *AtSDC* restores virulence to *cho1*Δ/Δ or *psd1*Δ/Δ *psd2*Δ/Δ for *in vivo* and *in vitro* models of OPC.

A. An OPC infection model was performed with mice and after five days, they were sacrificed, tongues were removed, and CFUs/gram tongue were measured. * P-value<0.01, compared to wild-type (WT). ** P-value<0.01, compared to *cho1*Δ/Δ-*AtSDC*. ***P-value<.01, compared to *psd1*Δ/Δ *psd2*ΔΔ. **** P-value<0.01, compared to *psd1*Δ/Δ *psd2*ΔΔ-*AtSDC*. **B.** The wild-type (WT), *cho1*Δ/Δ and *psd1*Δ/Δ *psd2*Δ/Δ mutants were compared to their respective *AtSDC* transformants, for their abilities to damage FaDu cells based on release of lactate dehydrogenase (LDH). ¥ p-value=0.0157, å p-value=0.0163, both compared to WT, ¥¥ p-value=0.0071 compared to *cho1*Δ/Δ, åå p-value=0.0201 compared to *psd1*Δ/Δ *psd2*ΔΔ. Strains: *cho1* (*cho1*Δ/Δ), *psd1* (*psd1*Δ/Δ), *psd1,2* (*psd1*Δ/Δ *psd2*ΔΔ), *SDC* (*AtSDC*)

Figure 4.7. Expression of *AtSDC* suppresses *cho1* Δ/Δ β (1,3)-glucan unmasking in *cho1* Δ/Δ .

A Cells were stained with anti- β (1,3)-glucan primary and phycoerythrin-conjugated secondary antibodies, and 100,000 cells were measured by flow cytometry for each replicate sample. Experiments were performed with three replicates per strain. **B** Cells were stained with anti- β (1,3)-glucan primary and Cy3-conjugated secondary antibodies and viewed by immunofluorescent microscopy. *P-value<0.0001, **P-value=0.0067.



pathway can bypass the *cho1* Δ/Δ mutation regarding $\beta(1,3)$ -glucan exposure.

This left the possibility that PE production by *AtSDC* was allowing the regeneration of PS. There is no other PS synthase in *C. albicans*. However, if sufficient product is present, an enzyme can be made to run backward, and the *cho1* Δ/Δ mutant possess the two PS decarboxylase enzymes Psd1 and Psd2. Thus, we hypothesized that PS synthesis is restored in the *cho1* Δ/Δ mutant when *AtSDC* is expressed. Therefore, we ran thin-layer chromatography to determine if the *cho1* Δ/Δ *AtSDC* mutant synthesizes PS, and we found that it did (Figure 4.8, A). Furthermore, strains that contain PS are susceptible to the PS-binding toxin papuamide A (PapA) at 10 and 5 μ g/ml PapA, while the *cho1* Δ/Δ mutant (lacking PS) is resistant, as reported previously (Cassilly et al., 2016). In contrast to *cho1* Δ/Δ , the *cho1* Δ/Δ *AtSDC* strain is susceptible, indicating that it contains PS (Figure 4.8, B). This would explain why *AtSDC* restores $\beta(1,3)$ -glucan masking to the *cho1* Δ/Δ mutant.

Discussion

The data presented in this communication address the main question of whether the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants' losses in virulence are due to a shared loss of PE synthesis from one specific pathway (*de novo*) or if it was due to an overall loss of PE synthesis. Based on the results with *AtSDC* strains (Figures 4.5 and 4.6), PE synthesized by the Kennedy pathway can compensate for loss of *de novo* synthesized PE. However, the Kennedy pathway does not compensate for loss of virulence in the *cho1* Δ/Δ or *psd1* Δ/Δ *psd2* Δ/Δ mutants lacking *AtSDC*, and this is likely because the mutants are unable to import sufficient ethanolamine to support virulence in the host. In culture, these mutants require >100 μ M (Figure 4.3), and serum ethanolamine is estimated to be 30 μ M

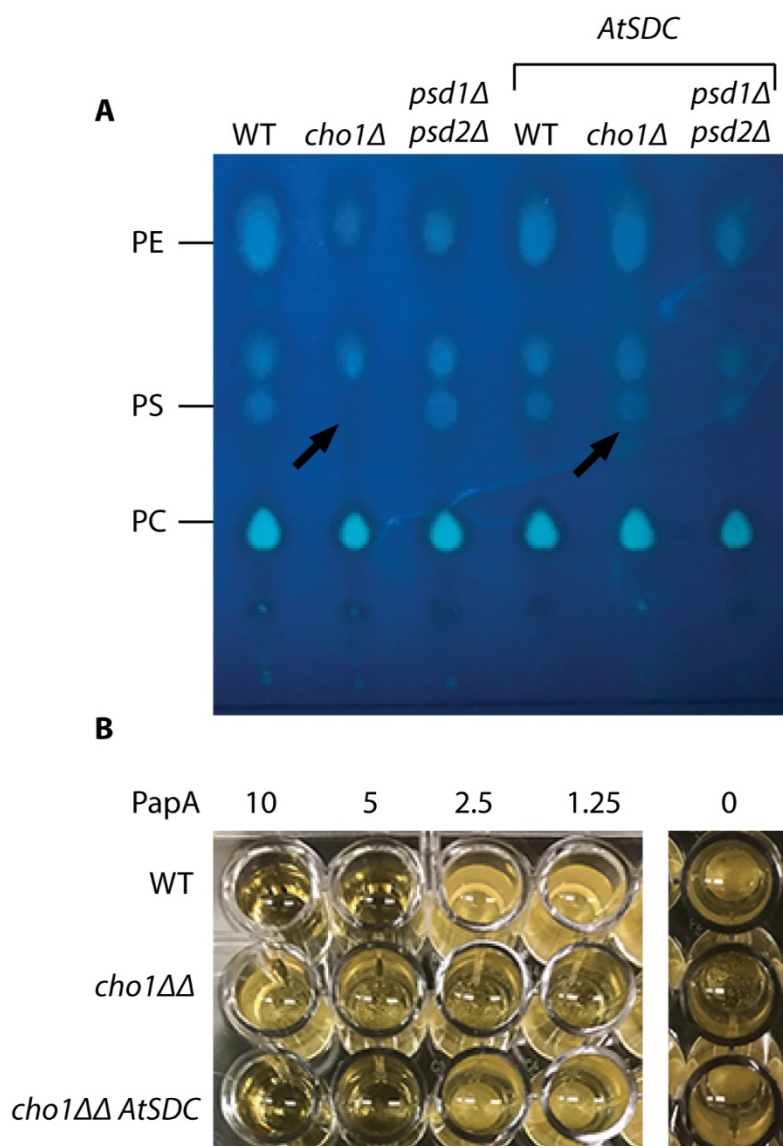


Figure 4.8. Expression of *AtSDC* in the *cho1ΔΔ* mutant leads to PS production.

A Strains were grown overnight in YPD and phospholipids were extracted and run on TLC to determine if PS was being synthesized. Lipid spots were compared to purified standards, which are marked on the TLC. The PS spot is marked with a black arrow in the *cho1ΔΔ* and *cho1ΔΔ AtSDC* lanes. **B** Cells were grown overnight in YPD, diluted to 10^5 /ml in fresh YPD, and 100μl were added to wells containing YPD + PapA or the methanol/water solvent control at the concentrations shown and then incubated overnight without shaking at 30°C.

(Houweling et al., 1992). In addition, even with 1 mM ethanolamine, neither the *cho1* Δ/Δ nor *psd1* Δ/Δ *psd2* Δ/Δ mutants recover wild-type levels of growth, but they do so with *AtSDC* (Figures 4.3, 4.4), thus one possible explanation is that *C. albicans* is unable to transport ethanolamine into the cell at an efficiency that allows it to restore virulence.

The inability of ethanolamine auxotrophs like *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ to cause disease is interesting given that auxotrophic mutants in *Candida spp.* are only sometimes associated with avirulence. For instance, an auxotroph for inositol (*ino1* Δ/Δ), a metabolite used to synthesize the phospholipid phosphatidylinositol is not compromised for virulence (Chen et al., 2008). In addition, auxotrophs for the amino acids lysine, histidine, leucine, or arginine are fully virulent (Noble and Johnson, 2005; Gabriel et al., 2014). In addition, the related fungal pathogen *Candida glabrata*, is a natural niacin auxotroph (Domergue et al., 2005) and *C. albicans* is a biotin auxotroph (Odds, 1988; Ahmad Hussin et al., 2016), yet they are both virulent. In contrast, mutants that are auxotrophs for the nucleotides uridine or adenine are avirulent (Lay et al., 1998; Donovan et al., 2001), as is a mutant for fatty acid synthesis (*fas2* Δ/Δ) (Zhao et al., 1997). Thus, it is not a forgone conclusion that auxotrophic mutants are going to be comprised for virulence, but this is clearly the case with the ethanolamine auxotrophs in this study.

This study also has an unusual innovation in that we tested for auxotrophy *in vivo* during infection, which is rarely done because of technical difficulties. Often auxotrophs are assumed to be avirulent due to insufficient nutrient acquisition in the host based on correlations with *in vitro* phenotypes on plates or in culture media. Thus, auxotrophies often cannot be easily tested *in vivo* during infection. However, in this study, with the *AtSDC* gene we were able to demonstrate that provision of ethanolamine from a source

other than the host restored virulence *in vivo*. This confirms that ethanolamine auxotrophy is indeed a major contributor to the loss of virulence in the *cho1* Δ/Δ and *psd1* Δ/Δ mutants.

One potential related explanation for the virulence loss of *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ besides auxotrophy is that they grow more slowly compared to wild-type even with ethanolamine supplementation (Figure 4.4). In contrast, expression of *AtSDC* restores both mutants to wild-type growth. However, as reported in Chen *et al.* (Chen *et al.*, 2010) the *cho1* Δ/Δ and *psd1* Δ/Δ *psd2* Δ/Δ mutants' growth in media supplemented with ethanolamine was similar to that of the single mutant *psd1* Δ/Δ which is fully virulent in the tail-vein infection model. Thus, there is not necessarily a strong correlation between *in vitro* growth in culture and virulence, an observation that was also made by Noble, *et al* in their study of the virulence of ~700 homozygous *Candida albicans* mutants (Noble *et al.*, 2010).

Finally, it was noted, based on fungal burden levels, that the *psd1* Δ/Δ *psd2* Δ/Δ mutant had a modest, but significantly, higher fungal burden in both the systemic model of infection and the OPC model. These differences in fungal burdens do correlate with the *cho1* Δ/Δ mutant's β (1-3)-glucan unmasking compared to that of *psd1* Δ/Δ *psd2* Δ/Δ (Davis *et al.*, 2014), however there may be other explanations as these mutants are both pleiotropic.

The role of Kennedy pathway PE in β -glucan exposure

Regarding unmasking, another observation made in this study is that supplying sufficient PE via the *AtSDC* can restore masking in the *cho1* Δ/Δ mutant. Although, initially we thought it might be acting to restore unmasking in the absence of PS, we have found that *AtSDC* can cause PS synthesis in the absence of PS synthase gene. The most likely

explanation is that the excess PE synthesized by the Kennedy Pathway in the *cho1Δ/Δ* strain expressing *AtSDC* causes the Psd1 and/or Psd2 PS decarboxylase enzymes to run in reverse and restores PS and therefore $\beta(1,3)$ -glucan masking. This is a surprising result and is the first time, to our knowledge, that this has been suggested *in vivo* for a PS decarboxylase enzyme.

Materials and Methods

Strains and media

The *C. albicans* strains used in this study are in Table 4.1. The media used for growth and maintenance of the strains was YPD (yeast extract, peptone, dextrose) broth and plates (Styles, 2002). For growth curves, we used minimal media (yeast nitrogen base, dextrose) $\pm$ ethanolamine (Styles, 2002). The *AtSDC* strains were generated as follows: First, the *AtSDC* gene was codon optimized by site-directed mutagenesis to change its single CTG codon at amino acid position 447 to TTG in the yeast expression vector pVT103-U (Rontein et al., 2001), and was then PCR amplified with SDO17 (5'-AAAACCATGGATGGTTGGATCTTTGGAATCTGA-3') and SDO18 (5'-AAAAAGATCTCTAGACCATGCTCCTACCTAGA-3') which introduced *NcoI* and *BglII* sites that were used to replace the *dTomato* ORF found between these sites in the *P_{ENO1}-dTomato-NATr* plasmid (Brothers et al., 2013), creating the plasmid *P_{ENO1}-AtSDC*. This plasmid was linearized with *EcoRV*, and then transformed by electroporation as previously described (Chen et al., 2010), and selected on YPD plates containing 200 μ g/ml nourseothricin.

Table 4.1. *C. albicans* strains used in chapter four.

Strain	Parent Strain	Genotype	Source
SC5314	Clinical Isolate	Prototrophic wild type	Gillum et al., 1984
YLC337	SC5314	<i>cho1</i> Δ/Δ	Chen et al., 2010
YLC344	YLC337	<i>cho1</i> Δ/Δ:: <i>CHO1-SAT1</i>	Chen et al., 2010
YLC280	SC5314	<i>psd1</i> Δ/Δ	Chen et al., 2010
YLC294	YLC280	<i>psd1</i> Δ/Δ:: <i>PSD1-SAT1</i>	Chen et al., 2010
YLC375	SC5314	<i>psd2</i> Δ/Δ <i>psd1</i> Δ/Δ	Chen et al., 2010
SED029	YLC375	<i>psd1</i> Δ/Δ <i>psd2</i> Δ/Δ:: <i>PSD1-SAT1</i>	This study
SED030	SC5314	<i>P_{ENO1}-AtSDC-SAT1</i>	This study
SED031	YLC337	<i>cho1</i> Δ/Δ <i>P_{ENO1}-AtSDC-SAT1</i>	This study
SED032	YLC280	<i>psd1</i> Δ/Δ <i>P_{ENO1}-AtSDC-SAT1</i>	This study
SED033	YLC375	<i>psd1</i> Δ/Δ <i>psd2</i> Δ/Δ <i>P_{ENO1}-AtSDC-SAT1</i>	This study

Papuamide A toxicity assay

The PapA assay was performed as described in (Cassilly et al., 2016).

LDH release assay from FaDu cells during *C. albicans* infection in tissue culture

For measurement of LDH, cytotoxicity assays were performed using the protocol and materials found in the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega, G1780). Briefly, 5×10^5 FaDu cells/mL were plated in EBSS media with 10% FBS in a 24 well plate with a total volume of 1 mL/well. *C. albicans* strains were grown overnight in 5 mL YPD. The following day *C. albicans* cultures were washed with water and resuspended in EBSS with 2% human serum. Media was aspirated from FaDu cells, and 1mL/well *C. albicans* was added at a multiplicity of infection of 5 and centrifuged at 200xg for 5 minutes. Wells containing FaDu or *C. albicans* cells were included as controls and all samples were tested in triplicate by incubating for 4 hours at 37°C and 5% CO₂. Following incubation, the plate was again centrifuged at 200xg for 5 minutes and 50µL of each supernatant was transferred to a 96 well plate. An equal volume of reconstituted substrate mix was added to each well and incubated for 30 minutes protected from light. 50µL of stop solution was added to each well and absorbance was measured at 490nm. Percent cytotoxicity was calculated as described in the manufacturer's protocol by comparing samples to epithelial cells treated with lysis solution (Sun et al., 2010).

Hot ethanol extraction of total phospholipids and thin layer chromatography (TLC)

Sample extraction and TLC for Figure 4.8 was performed as previously described (Chen et al., 2010) using plates from GE Healthcare that can separate PS very well, but are no

longer available for sale, and are therefore a limited resource. Due to this limitation, for the TLC shown in Figure 4.4, which focused on PE, the following exceptions were made to conserve the GE plates. 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 (13). Lipids were visualized on the dried plate by spraying with Primuline dye (5mg of Primuline, cat# 206865, Sigma) in 100ml of acetone, and illuminating with UV light.

Microscopy and flow cytometry

For flow cytometry, cell staining was performed with an anti- β -(1,3)-glucan primary antibody (Biosupplies Australia), and a goat anti-mouse-phycoerythrin (PE) secondary antibody (Jackson ImmunoResearch). Three biological replicates for all strains were used in each experiment, and all were analyzed on the same day. In brief, 5ml cultures of *C. albicans* strains were grown shaking overnight at 30°C and diluted the following morning to an OD₆₀₀ of 0.5 in 1ml of 1x PBS. Cells were subsequently washed twice with PBS and blocked for 1 hour in PBS plus 3% BSA. Following blocking, cells were rocked at 4°C for 90 minutes in a 1:800 dilution of anti- β -(1,3)-glucan primary antibody in PBS plus 3% BSA. Cells were then washed four times with PBS and rocked once more at 4°C for 20 minutes in a 1:300 dilution of goat anti-mouse-PE secondary antibody in PBS plus 3% BSA. Cells were washed 4 additional times with PBS and finally resuspended in 500ul of FACS buffer for flow cytometry analysis. Samples were run on a FACSCalibur LSRII flow

cytometer (Becton Dickinson) and data for each sample was collected for 100,000 gated events. Data was analyzed with the use of FlowJo 10.11 (FlowJo LLC, OR, USA) and all statistics were run using a Student *t* test.

For immunofluorescence, this procedure was done as described in (Davis et al., 2014). *C. albicans* cells were grown overnight in YPD medium at 30°C. An anti- β (1-3)-glucan antibody (Biosupplies Australia Pty Ltd., Australia) at a 1:800 dilution was used as the primary antibody, and a goat anti-mouse antibody conjugated to Cy3 (Jackson ImmunoResearch Inc., USA) at 1:300 dilution was used as secondary antibody. For imaging, *Candida* cells were resuspended in 100 μ L of PBS and visualized with LEICA DM5500B epi-fluorescent microscope with Hamamatsu Orca-ER CCD digital camera. The pictures were taken through Leica Application Suite AF (Advanced Fluorescence) software.

Oropharyngeal candidiasis model

This experiment was performed as previously described (Kamai et al., 2001; Solis and Filler, 2012). Briefly, eight mice per strain of *C. albicans* were immunosuppressed with cortisone acetate, which administered subcutaneously at 225 mg/kg on days -1, 1 and 3 relative to infection. To induce infection, the mice were anesthetized with ketamine and xylazine, after which a calcium alginate swab saturated with 10^6 *C. albicans* cells was placed sublingually for 75 min. After recovery from anesthesia, the mice were provided with food and water *ad libitum*. They were sacrificed after 5 days of infection, and then their tongues were harvested and divided lengthwise. One half was weighed, homogenized and quantitatively cultured.

Mouse systemic infection model

To prepare strains for injection, 1.0 OD^{600} of cells from overnight test tube cultures were washed 5 times with 1 mL ddH₂O, resuspended, and counted by hemocytometer. These washed cultures were then diluted to 5×10^6 cells/ml and 100 μl of cell suspension was injected into ICR (Harlan) mice for a total of 5×10^5 yeast/mouse. N=5 mice for each *Candida* strain/experiment. 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 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.

Ethics statement

The mouse studies carried out in this report followed ethical guidelines set forth by the National Institutes of Health for the ethical treatment of animals. For the systemic infection model, these experiments were carried out at the University of Tennessee under the UT-Animal Care and Use Committee (UT-ACUC) approved protocol #0016-0714. The OPC model was carried out under the approved animal welfare assurance #A3330-01 from the Institutional Animal Care and Use Committee (IACUC) of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center.

Acknowledgements

We thank Dr. Steven Minkin for helpful comments on the manuscript. We appreciate Dr. Andrew Hanson for his gift of a plasmid expressing the *Arabidopsis thaliana* SDC gene. This work was funded in part by grants NIH R01AL105690 (T.B.R.), R01DE017088 (S.F.), and NIH F31DE022512 (S.E.D.). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

References

- Ahmad Hussin, N., Pathirana, R.U., Hasim, S., Tati, S., Scheib-Owens, J.A., and Nickerson, K.W. (2016). Biotin Auxotrophy and Biotin Enhanced Germ Tube Formation in *Candida albicans*. *Microorganisms* 4(3). doi: 10.3390/microorganisms4030037.
- Atkinson, K., Fogel, S., and Henry, S.A. (1980). Yeast mutant defective in phosphatidylserine synthesis. *J Biol Chem* 255(14), 6653-6661.
- Birner, R., Burgermeister, M., Schneider, R., and Daum, G. (2001). Roles of phosphatidylethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*. *Mol Biol Cell* 12(4), 997-1007.
- Brothers, K.M., Gratacap, R.L., Barker, S.E., Newman, Z.R., Norum, A., and Wheeler, R.T. (2013). NADPH oxidase-driven phagocyte recruitment controls *Candida albicans* filamentous growth and prevents mortality. *PLoS Pathog* 9(10), e1003634. doi: 10.1371/journal.ppat.1003634
- Bustamante, C.I. (2005). Treatment of *Candida* infection: a view from the trenches! *Curr Opin Infect Dis* 18(6), 490-495.
- Cassilly, C.D., Farmer, A.T., Montedonico, A.E., Smith, T.K., Campagna, S.R., and Reynolds, T.B. (2017). Role of phosphatidylserine synthase in shaping the phospholipidome of *Candida albicans*. *FEMS Yeast Res.* doi: 10.1093/femsyr/fox007.
- Cassilly, C.D., Maddox, M.M., Cherian, P.T., Bowling, J.J., Hamann, M.T., Lee, R.E., et al. (2016). SB-224289 Antagonizes the Antifungal Mechanism of the Marine Depsipeptide Papuamide A. *PLoS One* 11(5), e0154932. doi: 10.1371/journal.pone.0154932.
- Chen, Y.L., Kauffman, S., and Reynolds, T.B. (2008). *Candida albicans* uses multiple mechanisms to acquire the essential metabolite inositol during infection. *Infect Immun* 76(6), 2793-2801. doi: 10.1128/IAI.01514-07.
- Chen, Y.L., Montedonico, A.E., Kauffman, S., Dunlap, J.R., Menn, F.M., and Reynolds, T.B. (2010). Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence in *Candida albicans*. *Mol Microbiol* 75(5), 1112-1132. doi: 10.1111/j.1365-2958.2009.07018.x.
- Clancey, C.J., Chang, S.C., and Dowhan, W. (1993). Cloning of a gene (PSD1) encoding phosphatidylserine decarboxylase from *Saccharomyces cerevisiae* by complementation of an *Escherichia coli* mutant. *J Biol Chem* 268(33), 24580-24590.
- Davis, S.E., Hopke, A., Minkin, S.C., Jr., Montedonico, A.E., Wheeler, R.T., and Reynolds, T.B. (2014). Masking of beta(1-3)-glucan in the cell wall of *Candida albicans* from detection by innate immune cells depends on phosphatidylserine. *Infect Immun* 82(10), 4405-4413. doi: 10.1128/IAI.01612-14.
- Domergue, R., Castano, I., De Las Penas, A., Zupancic, M., Lockett, V., Hebel, J.R., et al. (2005). Nicotinic acid limitation regulates silencing of *Candida* adhesins during UTI. *Science* 308(5723), 866-870. doi: 10.1126/science.1108640.
- Dongari-Bagtzoglou, A., Dwivedi, P., Ioannidou, E., Shaqman, M., Hull, D., and Burleson, J. (2009). Oral *Candida* infection and colonization in solid organ transplant recipients. *Oral Microbiol Immunol* 24(3), 249-254. doi: 10.1111/j.1399-

302X.2009.00505.x.

- Donovan, M., Schumuke, J.J., Fonzi, W.A., Bonar, S.L., Gheesling-Mullis, K., Jacob, G.S., et al. (2001). Virulence of a phosphoribosylaminoimidazole carboxylase-deficient *Candida albicans* strain in an immunosuppressed murine model of systemic candidiasis. *Infect Immun* 69(4), 2542-2548. doi: 10.1128/IAI.69.4.2542-2548.2001.
- Eggimann, P., Garbino, J., and Pittet, D. (2003). Management of *Candida* species infections in critically ill patients. *Lancet Infect Dis* 3(12), 772-785.
- Gabriel, I., Kur, K., Laforce-Nesbitt, S.S., Pulickal, A.S., Bliss, J.M., and Milewski, S. (2014). Phenotypic consequences of LYS4 gene disruption in *Candida albicans*. *Yeast* 31(8), 299-308. doi: 10.1002/yea.3021.
- Gillum, A.M., Tsay, E.Y., and Kirsch, D.R. (1984). Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3* and *E. coli* *pyrF* mutations. *Mol Gen Genet* 198(2), 179-182.
- Hancock, P.J., Epstein, J.B., and Sadler, G.R. (2003). Oral and dental management related to radiation therapy for head and neck cancer. *J Can Dent Assoc* 69(9), 585-590.
- Henry, S.A., Kohlwein, S.D., and Carman, G.M. (2012). Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. *Genetics* 190(2), 317-349. doi: 10.1534/genetics.111.130286.
- Houweling, M., Tijburg, L.B., Vaartjes, W.J., and van Golde, L.M. (1992). Phosphatidylethanolamine metabolism in rat liver after partial hepatectomy. Control of biosynthesis of phosphatidylethanolamine by the availability of ethanolamine. *Biochem J* 283 (Pt 1), 55-61.
- Kamai, Y., Kubota, M., Kamai, Y., Hosokawa, T., Fukuoka, T., and Filler, S.G. (2001). New model of oropharyngeal candidiasis in mice. *Antimicrob Agents Chemother* 45(11), 3195-3197. doi: 10.1128/AAC.45.11.3195-3197.2001.
- Lay, J., Henry, L.K., Clifford, J., Koltin, Y., Bulawa, C.E., and Becker, J.M. (1998). Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. *Infect Immun* 66(11), 5301-5306.
- Lynch, D.P. (1994). Oral candidiasis. History, classification, and clinical presentation. *Oral Surg Oral Med Oral Pathol* 78(2), 189-193.
- Morrell, M., Fraser, V.J., and Kollef, M.H. (2005). Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. *Antimicrob Agents Chemother* 49(9), 3640-3645. doi: 10.1128/AAC.49.9.3640-3645.2005.
- Noble, S.M., French, S., Kohn, L.A., Chen, V., and Johnson, A.D. (2010). Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. *Nat Genet* 42(7), 590-598. doi: 10.1038/ng.605.
- Noble, S.M., and Johnson, A.D. (2005). Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot Cell* 4(2), 298-309. doi: 10.1128/EC.4.2.298-309.2005.
- Odds, F.C. (1988). *Candida* and candidosis: A review and bibliography. *J. Basic Microb.* 30, 382-383.
- Pankhurst, C.L. (2009). Candidiasis (oropharyngeal). *Clin Evid (Online)* 2009.

- Rabeneck, L., Crane, M.M., Risser, J.M., Lacke, C.E., and Wray, N.P. (1993). A simple clinical staging system that predicts progression to AIDS using CD4 count, oral thrush, and night sweats. *J Gen Intern Med* 8(1), 5-9.
- Revankar, S.G., Kirkpatrick, W.R., McAtee, R.K., Dib, O.P., Fothergill, A.W., Redding, S.W., et al. (1998). A randomized trial of continuous or intermittent therapy with fluconazole for oropharyngeal candidiasis in HIV-infected patients: clinical outcomes and development of fluconazole resistance. *Am J Med* 105(1), 7-11.
- Rontein, D., Nishida, I., Tashiro, G., Yoshioka, K., Wu, W.I., Voelker, D.R., et al. (2001). Plants synthesize ethanolamine by direct decarboxylation of serine using a pyridoxal phosphate enzyme. *J Biol Chem* 276(38), 35523-35529. doi: 10.1074/jbc.M106038200.
- Solis, N.V., and Filler, S.G. (2012). Mouse model of oropharyngeal candidiasis. *Nat Protoc* 7(4), 637-642. doi: 10.1038/nprot.2012.011.
- Soysa, N.S., Samaranayake, L.P., and Ellepola, A.N. (2008). Antimicrobials as a contributory factor in oral candidosis--a brief overview. *Oral Dis* 14(2), 138-143. doi: 10.1111/j.1601-0825.2006.01357.x.
- Styles, C. (2002). How to set up a yeast laboratory. *Methods Enzymol* 350, 42-71.
- Sun, J.N., Solis, N.V., Phan, Q.T., Bajwa, J.S., Kashleva, H., Thompson, A., et al. (2010). Host cell invasion and virulence mediated by *Candida albicans* Ssa1. *PLoS Pathog* 6(11), e1001181. doi: 10.1371/journal.ppat.1001181.
- Thompson, G.R., 3rd, Patel, P.K., Kirkpatrick, W.R., Westbrook, S.D., Berg, D., Erlandsen, J., et al. (2010). Oropharyngeal candidiasis in the era of antiretroviral therapy. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 109(4), 488-495. doi: 10.1016/j.tripleo.2009.11.026.
- Trotter, P.J., and Voelker, D.R. (1995). Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (PSD2) in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 270(11), 6062-6070.
- Vazquez, J.A., and Sobel, J.D. (2002). Mucosal candidiasis. *Infect Dis Clin North Am* 16(4), 793-820, v.
- Wheeler, R.T., Kombe, D., Agarwala, S.D., and Fink, G.R. (2008). Dynamic, morphotype-specific *Candida albicans* beta-glucan exposure during infection and drug treatment. *PLoS Pathog* 4(12), e1000227. doi: 10.1371/journal.ppat.1000227.
- Yamashita, S., and Nikawa, J. (1997). Phosphatidylserine synthase from yeast. *Biochim Biophys Acta* 1348(1-2), 228-235.
- Zhao, X.J., McElhaney-Feser, G.E., Sheridan, M.J., Broedel, S.E., Jr., and Cihlar, R.L. (1997). Avirulence of *Candida albicans* FAS2 mutants in a mouse model of systemic candidiasis. *Infect Immun* 65(2), 829-832.

Chapter 5 : Conclusions and Future Directions

Summary of Findings

Prior to the initiation of these studies it was known that mutations within phospholipid biosynthetic pathways can have a negative impact on the virulence of *Candida albicans*. Mutations blocking synthesis of PS and PE via the CDP-DAG pathway result in avirulence (*cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ*) and blockage of inositol biosynthesis and import together (*ino1Δ/Δ itr2Δ/Δ*), which is required for phosphatidylinositol (PI) synthesis, results in avirulence (Chen et al., 2008; Chen et al., 2010). However, it was unknown whether CDP-DAG mediated biosynthesis of PC was required for virulence or if the alternative route for PE and PC synthesis, known as the Kennedy pathway, could compensate for this loss. In addition, it was completely unknown whether the Kennedy pathway was required for the growth or virulence of this organism, or alternatively if this pathway is redundant with the CDP-DAG pathway. Thus, this is the first study that addressed the requirement of the Kennedy pathway for virulence in *C. albicans*.

While auxotrophic mutants are not always avirulent, it is not necessarily surprising that mutations in major phospholipid biosynthetic routes can confer avirulence, as these mutations cause the organism to have a decreased capacity for synthesis of key components of cellular membranes. This could have detrimental effects on numerous cellular processes including signaling, protein localization or secretion, mitochondrial membrane potential and function, altered membrane stability/fluidity, and changes to cell wall architecture secondary to stress incurred by the plasma membrane. Contrary to many auxotrophic mutants for phospholipid synthesis, in this work we found that *pem1Δ/Δ pem1Δ/Δ*, which cannot produce PC via the CDP-DAG pathway, is hypervirulent (Tams et al., 2019). To our knowledge, this is the first hypervirulent fungal pathogen (HVFP) for

which hypervirulence is caused by a phospholipid biosynthetic null mutation.

Since *pem1Δ/Δ pem1Δ/Δ* is hypervirulent, the Kennedy pathway must therefore have the capacity to obtain sufficient choline from the mouse to produce PC and support growth and pathogenesis, regardless of the fact that *pem1Δ/Δ pem1Δ/Δ* is a choline auxotroph (Tams et al., 2019) (Chapter 2). This is in contrast to the *psd1Δ/Δ psd2Δ/Δ* mutant, for which ethanolamine auxotrophy does result in avirulence, likely because there is not enough ethanolamine in murine serum to support virulence for *psd1Δ/Δ psd2Δ/Δ* (Chen et al., 2010; Davis et al., 2018) (Chapter 4). While we have not yet been able to identify why the *pem1Δ/Δ pem2Δ/Δ* strain is hypervirulent, preliminary histology does indicate increased tissue necrosis and neutrophil invasion in the kidney tissue of *pem1Δ/Δ pem2Δ/Δ* infected mice as compared to wild-type (data not shown). Besides hypervirulence, the only other major phenotype that we have been able to elucidate for this mutant is a build-up of PE synthesized from the Kennedy pathway (Tams et al., 2019). Since blocking PC synthesis via the CDP-DAG pathway resulted in hypervirulence, we hypothesized that the cell wall was compensating for the loss of PC by increasing flux of phospholipid synthesis through the Kennedy pathway, and that this possibly resulted in hypervirulence. Therefore, in an attempt to re-capitulate the hypervirulent phenotype, we overexpressed the Kennedy pathway via constitutive expression of *EPT1* and found that this mutant was also hypervirulent in the mouse model of disseminated candidiasis, and additionally, that it has an increased cytotoxicity against oral epithelial cells *in vitro* (Tams et al., 2019) (Chapter 2). Other phenotypes observed for this mutant are a buildup of PE and PC (correlative to the increased PE observed in the hypervirulent *pem1Δ/Δ pem2Δ/Δ* mutant), increased chitin content, protection from stress-induced $\beta(1,3)$ -glucan

unmasking, and increased hyphal growth. Further, it was found that none of these phenotypes were the result of transcriptional changes, as evidenced by a transcriptional profile analysis.

In addition to overexpression, *EPT1* was knocked out to determine if the Kennedy pathway was required for the virulence of this organism, as it is in some other species (Tams et al., 2019) (Chapter 2). It was observed that deletion of *EPT1* (*ept1Δ/Δ*) blocked activity of the Kennedy pathway, and upon infection with the *ept1Δ/Δ* strain, there was not a heavy attenuation of virulence in mice (Tams et al., 2019). However, it was observed that there was a modest decrease in kidney fungal burden for infected mice, and also that there was a decrease in cytotoxicity against oral epithelial cells *in vitro* (Tams et al., 2019). Regardless, while the Kennedy pathway can compensate for a loss of CDP-DAG mediated PC synthesis, this pathway alone is dispensable for virulence and growth so long as PC can be produced via the *de novo* (CDP-DAG) route.

This brings up an interesting evolutionary question as to why the Kennedy pathway is present if it is not necessary in wild type *C. albicans*. It has been demonstrated that lipids produced by this pathway have different acyl chain substituents in *S. cerevisiae*, so it is possible that PE and PC produced by this pathway enter a separate “pool” of phospholipids produced by the CDP-DAG pathway (de Kroon et al., 2013). PE and PC can be broken down via de-acylation and subsequent hydrolysis to create ethanolamine or choline, respectively, which can be shunted back into the Kennedy pathway and thus “re-modeled” when necessary, such as upon oxidative damage (Dowd et al., 2001). This pathway is up-regulated at 37°C in *S. cerevisiae*, and if the same occurs for *C. albicans*, up-regulation of the Kennedy pathway could act as a pre-emptive attempt to correct future

membrane damage signaled by changes to environmental stimulus (Dowd et al., 2001). However, there are also different energy requirements for the synthesis of phospholipids by each pathway (ATP vs. SAM), so the cell may simply alter usage of pathways based on metabolic needs (Henry et al., 2012).

It is evident from these data that the production of PE is important for growth and virulence. However, given that synthesis of PE by the CDP-DAG pathway but not the Kennedy pathway is required for virulence, a final study was undertaken to further investigate the cellular requirements of PE *in vivo*. *In vitro*, *psd1Δ/Δ psd2Δ/Δ* requires ~1 mM ethanolamine to restore growth in minimal medium, which is much higher than the estimated available amount of ethanolamine in mouse serum (~30 μM) (Houweling et al., 1992; Davis et al., 2018). To determine if ethanolamine limitation was the cause of avirulence of the *cho1Δ/Δ* and *psd1Δ/Δ psd2Δ/Δ* mutants, a serine decarboxylase was introduced (*atSDC*), which converts intracellular serine to ethanolamine, and it was found that this fully restored virulence for both mutants (Davis et al., 2018). Thus, the reason the *psd1Δ/Δ psd2Δ/Δ* mutant is avirulent is because there is not enough ethanolamine in mouse sera, or because the capability of *C. albicans* to import ethanolamine is too low. Interestingly, *AtSDC* introduction also resulted in a restoration of β(1,3)-glucan unmasking for the *cho1Δ/Δ* mutant, and we hypothesized that this was due to a restoration of PE (Davis et al., 2018). However, further examination revealed that PS was being produced in the *cho1Δ/Δ atSDC* mutant, presumably by reverse action of the *PSD1* and *PSD2* enzymes (Davis et al., 2018).

While these studies have expanded upon our knowledge of the requirements of PE synthesis for *C. albicans*, and the impact that PE has on virulence, there are several

areas that merit a continuation of study. There are numerous directions this project could take but the primary considerations for future studies should be (1) to more fully elucidate the mechanism for hypervirulence of the *EPT1* overexpression strain with a strong emphasis on chitin and cell wall changes, and (2) to independently evaluate the impact of chitin on virulence and immune response.

Future Directions

Let's talk about chitin

As we have noted, increases in cell wall chitin can be induced for *C. albicans* via cell wall stress, such as that induced by mutation of cell wall proteins or treatment with caspofungin (Wheeler and Fink, 2006; Bahnan et al., 2012). Since mutations in phospholipid biosynthetic pathways can also cause cell wall stress, it is possible that overproduction of phospholipids, by overexpression of *EPT1*, causes disequilibrium in the cell membrane that results in a compensatory activation of chitin production. Although there are no transcriptional differences in chitin synthase expression for the *EPT1* overexpressor as compared with wild-type, chitin synthesis is typically controlled via post-transcriptional events, and therefore any differences in activity of this enzyme may have been missed in our experimental approach (Lenardon et al., 2007).

Given the increase in chitin for the *EPT1* overexpressor and the potential for chitin to impact virulence, evaluation of the mechanism for increased chitin level in this strain is one of the most important considerations for continuation of this project. Chitin synthase 3 (*CHS3*) is the major chitin synthase in *C. albicans*, and this gene should be labeled with green fluorescent protein (GFP) in the *EPT1* overexpression strain to determine if there are any changes in localization in our hypervirulent mutants. Thus far, a plasmid has been

created (pBT71) that includes a hygromycin resistance cassette suitable for use with the *EPT1* overexpressor strain. As an aside, it should also be noted that this plasmid can be used as an important cloning tool for the lab when using strains for which a *NAT1* cassette has been used and there is a need for a secondary drug marker. In the case of pBT71, Hyg^R was inserted within the *NotI* site of the commercially available pBlueScript KS+ plasmid, and it has been confirmed that pBT71 confers resistance to hygromycin. A second plasmid with a GFP label has been created (pBT75) from pBT71, and primers BTO142 and BTO143 can be used to amplify a construct from pBT75 to tag *CHS3* with GFP. This has already been attempted, and although colony PCR positive clones have been obtained with this construct (~75), there have been no colonies for which fluorescence was obtained. Regardless, this should be attempted again with either this plasmid or with another construct.

We have determined that there is an increase in hyphal length of the *EPT1* overexpression strain at an early timepoint, and that this increase in length also occurs when *EPT1* is overexpressed in the *cho1Δ/Δ* background. It is unclear as to why overexpression of *EPT1* increases hyphal length, but it is possible that this correlates to increased chitin production. While confocal microscopy with video could be used to quantify the exact rate at which hyphae form for comparison to wild-type, a *P_{ENO1}-EPT1* *CHS3*-GFP strain should be examined for differences in intensity or localization of the protein. *CHS3* typically localizes to the site of polarized growth on hyphae, and it would be interesting to determine if this localization occurs at an earlier time point (or differentially) in the *EPT1* overexpresser than it does for the wild-type (Lenardon et al., 2007). Alternatively, hyphae could be stained with calcofluor white, which stains total

chitin, and the amount of chitin specifically within hyphal tips could be measured and quantified with ImageJ software.

Another study that should be performed is to directly evaluate the impact of an increase in chitin on virulence and immune regulation. There are several strains of wild type *C. albicans* that have varying levels of chitin, and the amount of chitin for each strain was shown to correspond to virulence, however, there were two reports with conflicting results (Marakalala et al., 2013; Wagener et al., 2017). Given that our experiments demonstrate an association of increased chitin with increased virulence, this should be studied with greater detail. If possible, one should obtain the strains with low chitin levels from the Aberdeen Fungal Group and overexpress *EPT1* in these strains. It should then be determined if overexpression of *EPT1* increases chitin level of low chitin strains, and if the increased chitin level correlates to increased virulence. Since SC5314, which was used in this study, is at baseline already a highly virulent strain, overexpression of *EPT1* in less virulent wild-type strains may induce a greater degree of hypervirulence as compared to the respective parent strain.

Little is understood about how chitin levels in the cell wall and in its native form can impact virulence and alter immune responses in live animals. The impact of chitin on virulence and immune response should be measured directly by modulation of *CHS3* with a dose responsive regulatable system (such as the TetOff system). With this system, the expression of the *CHS3* gene could be regulated by the use of a gradient of doxycycline concentration. Chitin content of cells treated with differing doxycycline concentrations could be evaluated with flow cytometry. Once confirmed that chitin levels are altered in a dose dependent manner, mouse experiments could be performed when doxycycline is

added to the drinking water of mice. Multiple parameters could be monitored including immunohistochemistry (noting not only necrosis and immune infiltration, but also again confirming staining of chitin correlative of doxycycline dose *in vivo*) of biopsied kidneys, immune infiltration via flow cytometry, and immune response (TNF- α and IL-10). This would be extremely interesting to perform because most studies investigating the ability of chitin to impact the immune response do so with tissue culture cells and purified chitin particles. Alternative to a regulatable system, *CHS3* could be overexpressed in the *EPT1* overexpression strain, BTY88 (using the pBT71 plasmid as a starting point). If, however, there were issues with the use of these strains *in vivo*, one could use them to perform *in vitro* studies to examine immune response to varying chitin level in *C. albicans* in its native state and in a uniform wild-type strain (SC5314), which has also not been done. If experiments were to be performed *in vitro*, it would be also worthwhile to examine capacity for reactive nitrogen species production from naïve bone marrow derived macrophages as was performed by Wagener *et al.*, as purified chitin has demonstrated the potential decreased production of RNS (Wagener *et al.*, 2017). This could be performed with the use of an arginase detection kit as described in the same study.

Alternative cell wall components

GPI anchors are an important cell wall component required for the addition of the heavily mannosylated proteins bound to the cell wall, and mutation that cause a disruption in GPI anchor synthesis can result in non-viable yeast strains and issues with cell wall integrity (Orlean, 2012). These anchors are also important for virulence as many virulence factors including proteases and adhesins are covalently attached to the cell wall via a GPI anchor

remnant (Kapteyn et al., 2000; Orlean and Menon, 2007). A requirement for the proper construction of GPI anchors is the addition of phosphoethanolamine groups, which are obtained from PE (Orlean and Menon, 2007). Based on this, we hypothesize that increases in PE level of the cell may increase the amount of this substrate and thus more GPI anchor may readily be formed. This could result in an abundance of GPI anchored proteins on the cell surface and increase mannosylation, which could help in immune evasion. Alternatively, increasing GPI anchored proteins on the cell surface could increase the abundance of GPI anchored virulence factors, resulting in more cellular damage. Either way, it would be interesting to determine if there are differences in GPI anchors for the *EPT1* overexpression and *pem1Δ/Δ pem1Δ/Δ* strains. We have tried several methods of doing so but have not been able to effectively quantify GPI anchored protein levels. We have attempted to use an Alexa Fluor conjugate of proaerolysin (FLAER), a compound commonly used for mammalian GPI anchor detection in a clinical setting, however this approach was unsuccessful. In addition, several common GPI anchored cell wall proteins were fractionated and examined using Western blotting techniques (*GAS1*, *HWP1*, *ALS3*) but we could not detect differences. Some of these antibodies are not available commercially and were dated, so if one could obtain new antibodies and probe the cells using immunofluorescence this may help to gain insight into changes of GPI anchored proteins.

Another possibility for the investigation of GPI anchored cell wall components would be to radiolabel the GPI anchor precursors, such as either [¹³C] ethanolamine or [¹³C] inositol, to then obtain cell wall protein fractions, and attempt to quantify differences with mass spectrometry. While this could be performed in-house, it may be worthwhile to

collaborate with a group that specializes in cell wall structure for further evaluation. Other, and more simple methods that could be used to identify changes in cell wall protein mannosylation are to quantify intensity of concanavalin A stained cells (a fluorescent compound which binds to mannose residues) with flow cytometry. These studies could be performed for both the *pem1Δ/Δ pem1Δ/Δ* and *EPT1* overexpression strains.

An alternative approach for the evaluation of GPI anchored proteins, which we have also attempted previously, is to label an extremely common GPI anchored protein, such as *PGA59* or *PGA62*, with GFP. Fluorescence could then be quantified using flow cytometry, or cell wall protein fractions could be quantified using Western blotting with an α-GFP antibody as a proxy for changes in GPI anchored proteins. If this were to be performed, several different common GPI anchored proteins should be examined to ensure the differences are not only in the one tagged protein of interest. This experiment would now be easier to perform since we have a second functional antibiotic marker (HygR, pBT71), whereas there were issues with this previously.

A final cell wall experiment that should be performed with the *pem1Δ/Δ pem1Δ/Δ* or *EPT1* overexpressor is to examine the cell wall with TEM or atomic force microscopy (AFM). While we have examined the cells with SEM and have not identified differences, TEM would be a better method to identify any differences in cell wall thickness, and as several HVFPs display both increased chitin and cell wall thickness, this would be a pertinent experiment.

Other unanswered questions

While the *pem1Δ/Δ pem1Δ/Δ* mutant cannot produce PC via the CDP-DAG pathway, it can still obtain PC from the Kennedy pathway, whereas the *EPT1* overexpressor increases both PC and PE synthesis (Tams et al., 2019) (Chapter 2). Therefore, an important question that should be asked regarding these mutants is whether changes to PE or PC are causative of hypervirulence, and this should be addressed. We favor increased PE synthesis as the reason for hypervirulence as increased PE is a commonality to both *pem1Δ/Δ pem1Δ/Δ* and the *EPT1* overexpressor, and because PC synthesis via methylation is decreased in the *pem1Δ/Δ pem1Δ/Δ* mutant, yet it is still hypervirulent. It would therefore be interesting to determine the exact phospholipid composition of the *pem1Δ/Δ pem1Δ/Δ*, *EPT1* overexpressor, and wild-type strains with lipidomic analysis (mass spectrometry) to determine if there are any correlations between the hypervirulent strains. An examination of these strains with mass spectrometry may also help elucidate differences in acyl chain composition of phospholipids produced via the Kennedy pathway as compared to those produced by the CDP-DAG pathway, which has not been done in *C. albicans*, and this would determine if separate pools of PC/PE are being produced by each pathway.

A final matter that has not been resolved is whether it is PS or PE that is required for a restoration of $\beta(1,3)$ -glucan unmasking in the *cho1Δ/Δ* strain, as there was an unexpected occurrence in which PS was produced in the *cho1Δ/Δ atSDC* strain, presumably by reverse action of the phosphatidylserine decarboxylase enzymes (Davis et al., 2018). While it is most likely a combination of a loss of both phospholipids, one way to definitively answer this question would be to knock out *cho1Δ/Δ* in the *psd1Δ/Δ*

psd2Δ/Δ AtSDC triple mutant background. This transformation would be technically challenging because of underlying cell wall issues, but there would be no way for this quadruple mutant strain to create PS, as all enzymes with potential for its production would be absent. Immunofluorescent imaging of $\beta(1,3)$ -glucan in this strain would parse apart whether it is really PS or PE that is responsible for restoration of $\beta(1,3)$ -glucan masking (or if it is a combination of the two).

Concluding Remarks

A major confounding problem with the study of phospholipids is that many of these pathways are intertwined, and if one pathway is disrupted there are often compensatory changes in expression or activity of alternate pathways to ameliorate issues with cell function or viability. While we do have a good general outline of phospholipid biosynthetic pathways for yeast, we do not have a full understanding of phospholipid pathways in *C. albicans* because our common basis for our understanding of them is often elucidated from *S. cerevisiae* and other *Candida* species. Another difficulty in the study of phospholipid biosynthetic pathways is that proteins can localize to different regions of phospholipid enrichment, and it is difficult to discern local changes to the profiles of lipids in a given portion of a membrane. In addition, mutations in phospholipid synthesis are often pleiotropic: proteins may not be secreted properly, transmembrane (and other) proteins may localize improperly, and the balance of phospholipid intermediates which often serve as second messengers can be disrupted. Beyond these difficulties, there are “redundant” pathways that produce the same phospholipids. For instance, the Kennedy pathway produces both PE and PC as does the CDP-DAG pathway, and one is required for virulence while the other is not, as we have demonstrated in this dissertation. There

are also redundancies for production of the same phospholipid within a single pathway (Psd1 and Psd2 for example), for which the enzymes may localize to different locations yet perform the same function, and this adds another layer of complexity to the gallimaufry of phospholipid biosynthesis in *C. albicans*. Regardless, in this work we have ascertained important insights into the function of the two most abundant phospholipids in *C. albicans* with regard to virulence, and we have identified potential mechanisms by which changes in the synthesis of PE and PC induce hypervirulence.

References

- Bahnan, W., Koussa, J., Younes, S., Abi Rizk, M., Khalil, B., El Sitt, S., et al. (2012). Deletion of the *Candida albicans* PIR32 results in increased virulence, stress response, and upregulation of cell wall chitin deposition. *Mycopathologia* 174(2), 107-119. doi: 10.1007/s11046-012-9533-z.
- Chen, Y.L., Kauffman, S., and Reynolds, T.B. (2008). *Candida albicans* uses multiple mechanisms to acquire the essential metabolite inositol during infection. *Infect Immun* 76(6), 2793-2801. doi: 10.1128/IAI.01514-07.
- Chen, Y.L., Montedonico, A.E., Kauffman, S., Dunlap, J.R., Menn, F.M., and Reynolds, T.B. (2010). Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence in *Candida albicans*. *Mol Microbiol* 75(5), 1112-1132. doi: 10.1111/j.1365-2958.2009.07018.x.
- Davis, S.E., Tams, R.N., Solis, N., Wagner, A.S., Chen, T., Jackson, J.W., et al. (2018). *Candida albicans* cannot acquire sufficient ethanolamine from the host to support virulence in the absence of de novo phosphatidylethanolamine synthesis. *Infect Immun*. doi: 10.1128/IAI.00815-17.
- de Kroon, A.I., Rijken, P.J., and De Smet, C.H. (2013). Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. *Prog Lipid Res* 52(4), 374-394. doi: 10.1016/j.plipres.2013.04.006.
- Dowd, S.R., Bier, M.E., and Patton-Vogt, J.L. (2001). Turnover of phosphatidylcholine in *Saccharomyces cerevisiae*. The role of the CDP-choline pathway. *J Biol Chem* 276(6), 3756-3763. doi: 10.1074/jbc.M003694200.
- Henry, S.A., Kohlwein, S.D., and Carman, G.M. (2012). Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. *Genetics* 190(2), 317-349. doi: 10.1534/genetics.111.130286.
- Houweling, M., Tijburg, L.B., Vaartjes, W.J., and van Golde, L.M. (1992). Phosphatidylethanolamine metabolism in rat liver after partial hepatectomy. Control of biosynthesis of phosphatidylethanolamine by the availability of ethanolamine. *Biochem J* 283 (Pt 1), 55-61.
- Kapteyn, J.C., Hoyer, L.L., Hecht, J.E., Muller, W.H., Andel, A., Verkleij, A.J., et al. (2000). The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. *Mol Microbiol* 35(3), 601-611.
- Lenardon, M.D., Whitton, R.K., Munro, C.A., Marshall, D., and Gow, N.A. (2007). Individual chitin synthase enzymes synthesize microfibrils of differing structure at specific locations in the *Candida albicans* cell wall. *Mol Microbiol* 66(5), 1164-1173. doi: 10.1111/j.1365-2958.2007.05990.x.
- Marakalala, M.J., Vautier, S., Potrykus, J., Walker, L.A., Shepardson, K.M., Hopke, A., et al. (2013). Differential adaptation of *Candida albicans* in vivo modulates immune recognition by dectin-1. *PLoS Pathog* 9(4), e1003315. doi: 10.1371/journal.ppat.1003315.
- Orlean, P. (2012). Architecture and biosynthesis of the *Saccharomyces cerevisiae* cell wall. *Genetics* 192(3), 775-818. doi: 10.1534/genetics.112.144485.
- Orlean, P., and Menon, A.K. (2007). Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we

- learned to stop worrying and love glycerophospholipids. *J Lipid Res* 48(5), 993-1011. doi: 10.1194/jlr.R700002-JLR200.
- Tams, R.N., Cassilly, C.D., Anaokar, S., Brewer, W.T., Dinsmore, J.T., Chen, Y.-L., et al. (2019). Overproduction of Phospholipids by the Kennedy Pathway Leads to Hypervirulence in *Candida albicans*. *Frontiers in Microbiology* 10(86). doi: 10.3389/fmicb.2019.00086.
- Wagener, J., MacCallum, D.M., Brown, G.D., and Gow, N.A. (2017). *Candida albicans* Chitin Increases Arginase-1 Activity in Human Macrophages, with an Impact on Macrophage Antimicrobial Functions. *MBio* 8(1). doi: 10.1128/mBio.01820-16.
- Wheeler, R.T., and Fink, G.R. (2006). A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog* 2(4), e35. doi: 10.1371/journal.ppat.0020035.

Vita

Robert Nielsen Tams (Bob) was born in Gallatin, TN on 11 December 1989 to James and Sherry Tams. He attended high school at Pope John Paul II in Hendersonville, TN and graduated in 2008. After high school, he moved to Knoxville and attended The University of Tennessee, where he completed the degree of Bachelor of Science with a major in microbiology. Following graduation, he briefly worked as a laboratory technician until he enrolled in graduate school to continue research at the University of Tennessee with guidance from Dr. Todd Reynolds. During graduate school he enjoyed cooking, gardening, watching Tennessee football, hiking in the Grand Teton and Smoky mountains, brewing and (occasionally) drinking beer with friends, and travelling with his girlfriend Lauren whenever possible. Just before graduation, he considered moving to Florida with Lauren, their three legged cat (Rocky), and two Labrador retrievers (Moose and Theodore) to open “Dr. Bob’s Crab Shack” where he could sell over-priced T-shirts (“We put the science in the flavor!”) and two for one domestic beers, but that was probably a pipe dream. He completed his Doctor of Philosophy degree in microbiology in August 2019.