Removing the Mask:
Regulation of Cell Wall β (1,3)-Glucan Unmasking in *Candida albicans*

A Dissertation Presented for

the Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Tian Chen

August 2019

Copyright © 2019 by Tian Chen
All rights reserved.

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my advisor Dr. Todd Reynolds for continuous support of my Ph.D. studies. I struggled at the beginning of my research and almost thought I wouldn't make it through. Fortunately, Dr. Reynolds guided me through this tough but rewarding journey. His patience, enthusiasm, perseverance, and immense knowledge inspired me all the way, and continues to motivate me to be a scientist like him. I could not have imagined having a better advisor and mentor for my Ph.D. studies.

I am especially indebted to my dissertation committee members: Drs. Jeffrey Becker, Elizabeth Fozo, Maitreyi Das, and Timothy Sparer, for their hard questions, insightful comments, and support. They encouraged my progress and pushed me to think critically about my research. I have learned tremendously from each committee/individual meeting with them. Without them, my scientific knowledge, research skill, and communication ability would not have gotten this far. Drs. Fozo and Das also inspire me as women scientists who maintain a successful career and family balance.

I have had great pleasure working with my fellows in the Reynolds lab: Bob Tams, Andy Wagner, Eric Gann, Kyle Bonifer, Elise Phillips, Aeric Zhou, and early members Drs. Chelsi Cassilly, Sahar Hasim, and Sarah Davis. Thank you for the help, advice and friendship. They really made lab life so much more enjoyable! Having them as my close friends is part of the best experience in my Ph.D. life, and I will miss them dearly. I also thank the Fozo lab and the Goodrich-Blair lab for their generosity of allowing me to use reagents and equipment. They always had what I was looking for, whether it was a protocol, or certain chemicals. I had great fortune of becoming close friends with Daren Ginete, Audra Hinson, Jonelle Basso, Bikash Bogati, and Mang Chang. They all provided support and helped me through my bumpy path.

I also thank my husband Xu for his support with love and understanding. He always stands by me and believes that I am a great scientist. His love and confidence in me are what have gotten me through those ups and downs. Thank you also to my little boy Ryan. It was hard to balance studying, being a teaching

assistant, and raising a child in graduate school, but Ryan has made me stronger and better than I could have ever imagined. Also, I could not have made it without help from my mother-in-law Zhaoli! I cherish her kindness and will always remember her help with Ryan when I needed it the most. And last, I am especially grateful that I have my family as a stable force and constant support for me. I owe everything to them!

ABSTRACT

Candida albicans is an important cause of systemic infections in the immune compromised population. However, drug resistance and toxicity have put limits on antifungals efficacy. The *C. albicans* cell wall is considered a good therapeutic target due to its role in fungal pathogenicity. Thus, a potential method for improving antifungal drugs could be to enhance the detection of fungal cell wall antigens by host immune cells. Detection of *C. albicans* largely occurs through the receptor Dectin-1 that can recognize β [beta] (1,3)-glucan, an important component of fungal cell walls. However, a layer of glycosylated proteins masks the β (1,3)-glucan, hiding it from immune detection. In order to better understand possible mechanisms of unmasking β (1,3)-glucan, we must develop a deeper comprehension of the mechanism behind unmasking.

The phosphatidylserine (PS) synthase enzyme (Cho1) was reported to control β (1,3)-glucan exposure. In this dissertation, I utilized classical genetics and biochemical methods to identify the potential protein(s) involved in causing this phenotype. Several fungal cell wall associated signaling pathways are overly activated when *CHO1* is disrupted, including GTPase Cdc42, a central regulator of cellular polarity and morphological development, and its regulated protein kinase Cek1. When Cek1 is activated independent of *CHO1*, it leads to β (1,3)-glucan exposure. To understand thoroughly how Cek1 and its associated pathway govern β (1,3)-glucan exposure, I further screened the upstream signaling protein(s) causing Cek1 over-activation. A novel signaling cascade was identified where the predicted GTPase activating protein (GAP) Lrg1 represses Cek1 activity by downregulating the GTPase Cdc42 and its downstream MAPKKK, Ste11. The consequences to virulence for upregulation of Cek1 are that pathogenicity is diminished in the mouse model of systemic infection, and this correlates with increased cytokine responses from macrophages. Data from RNA-sequencing demonstrates that a number of cell wall associated genes are significantly up-regulated transcriptionally when Cek1 is hyper-activated, which might be responsible for the cell wall exposure. Thus, we propose a model that Cek1 hyperactivation causes β (1,3)-glucan exposure by upregulating cell wall proteins

and leads to a more robust immune detection *in vivo*, promoting more effective clearance.

TABLE OF CONTENTS

CHAPTER I: Introduction 1

Construction of the bi-layered *C. albicans* cell wall 2

Immune-recognition of individual cell wall components 9

β (1,3)-glucan unmasking can be induced by manipulation of cell wall signaling pathways 15

Summary 26

Reference 27

CHAPTER II: Unmasking of *Candida albicans* β (1,3)-glucan Is Promoted by Activation of the Cek1 Pathway 36

Abstract 38

Introduction 39

Methods 42

Results 49

Discussion 63

Conclusions 69

References 70

Appendix 76

CHAPTER III: Hyperactivation of Cek1 Attenuates Virulence in *Candida albicans* 87

Abstract 89

Introduction 90

Methods 92

Results 98

Discussion 112

Reference 116
Appendix 122
CHAPTER IV: Genetic Analysis of Genes Expressed during β (1,3)-glucan Exposure Induced by Cek1 Hyperactivation 131
Abstract 133
Introduction 134
Methods 135
Results 138
Discussion 148
Reference 152
Appendix 155
CHAPTER V: Conclusion and Future Direction 165
Conclusion 166
Future direction 167
Reference 174
VITA 176

LIST OF TABLES

Table S.2. 1. *C. albicans* strains used in this study....76
Table S.2. 2. Plasmids used in this study 77
Table S.2. 3. Primers used in this study....78
Table S.3. 1. *C. albicans* strains used in this study....122
Table S.3. 2. Plasmids used in this study 123
Table S.3. 3. Primers used in this study....124
Table 4. 1: Commonly regulated genes induced by *STE11$^{\Delta N467}$*–doxycycline vs. Wild-type± and *STE11$^{\Delta N46}$* +doxycycline....147
Table S.4. 1. *C. albicans* strains used in this study....155
Table S.4. 2. Genes that were differentially regulated in *STE11$^{\Delta N467}$*–doxycycline 156

LIST OF FIGURES

Figure 1. 1. The structure of *C. albicans* cell wall. 4
Figure 1. 2. A model of *N*- and *O*-glycosylation of *C. albicans* cell wall proteins biosynthsis. 8
Figure 1. 3. *C. albicans* β (1,3)-glucan is an important PAMP for detecting fungi. 12
Figure 1. 4. The MAPKs pathways in *C. albicans* that are involved in cell wall construction. 17
Figure 2. 1. The Cek1 and Mkc1 MAP kinase signaling cascades in *C. albicans* are involved in cell wall biogenesis. 41
Figure 2. 2. Cek1 and Mkc1 MAPKs exhibit increased activation in *cho1Δ/Δ* yeast cells compared to wild-type. 51
Figure 2. 3. Hyperactive Ste11 ($STE11^{\Delta N467}$) causes significant increases in β (1,3)-glucan exposure and TNF-α secretion. 54
Figure 2. 4. Cdc42 activity is upregulated in *cho1Δ/Δ* compared to wild-type. 57
Figure 2. 5. Cho1 is essential for GFP-Cdc42 polarization at the plasma membrane. 60
Figure 2. 6 Deletion of *MKC1* in *cho1Δ/Δ* does not diminish β (1,3)-glucan exposure. 62
Figure 2. 7. Active GTP-Rho1 is de-localized in *cho1Δ/Δ*. 64
Figure 2. 8. Hyper-activated Rho1 causes β (1,3)-glucan exposure. 65
Figure S.2. 1. $STE11^{\Delta N467}$ exhibited significantly increased β (1,3)-glucan exposure compared to wild-type cells. 81
Figure S.2. 2. Growth curves were measured to determine the growth rate of strains in YPD vs YPM. 82
Figure S.2. 3. The exposed β (1,3)-glucan in $STE11^{\Delta N467}$ YPM cells was not restricted to bud scars. 83
Figure S.2. 4. $CDC42^{G12V}$ increases β (1,3)-glucan exposure, but also reduces the viable cell population. 84
Figure S.2. 5. *MKC1* was knocked out in *C. albicans* via CRISPR-Cas9. 85

Figure S.2. 6. Deleting one *PKC1* allele in *cho1Δ/Δ* did not rescue β (1,3)-glucan exposure. 86
Figure 3. 1. Lrg1 represses β (1,3)-glucan unmasking in *C. albicans*. 99
Figure 3. 2 .The Cek1p MAPK is hyper-phosphorylated in *lrg1Δ/Δ* cells compared to wild-type 101
Figure 3. 3. Lrg1 inhibits the activity of GTPases Cdc42 and Ras1, but not Rho1. 103
Figure 3. 4. Disruption of *LRG1* causes Cek1 over-activation through Ste11 MAPKKK 106
Figure 3. 5. *LRG1* disruption significantly induces TNF-α secretion. 109
Figure 3. 6. Activated Cek1 leads to attenuated fungal virulence in the mouse model of systemic infection. 110
Figure 3. 7. Ste11 hyper-activation causes decreased kidney fungal burden in the mouse model of systemic infection. 111
Figure 3. 8. A model shows that Lrg1 acts as a molecular switch regulating Cek1 activity negatively via the GTPase Cdc42. 114
Figure S.3. 1. Loss of Lrg1 decreases GTPase Rac1 activity. 126
Figure S.3. 2. The hyperactive *Ras1*^{G13V} does not display Cek1 phosphorylation. 127
Figure S.3. 3. Cek1 MAPK is hyper-phosphorylated when the *P*$_{tetOFF}$-*STE11*$^{\Delta N467}$ construct is induced. 128
Figure S.3. 4. An overnight culture of *P*$_{tetOFF}$-*STE11*$^{\Delta N467}$ exhibits increased β (1,3)-glucan exposure when induced. 129
Figure S.3. 5. The growth rate of different *Candida* strains was measured to determine if hyperactivation of Cek1 MAPK affects cellular growth. 130
Figure 4. 1. The *P*$_{tetOFF}$ -*STE11*$^{\Delta N467}$ strain exhibits β (1,3)-glucan exposure in a time-dependent manner in the absence of doxycycline. 140
Figure 4. 2. PCA plot displays the clustering of samples within each group. 141
Figure 4. 3. Housekeeping genes expression was similar among different *Candida* strains. 144

Figure 4. 4. Differentially expressed genes in *STE11*$^{\Delta N467}$-inducing condition are enriched for cell wall repair genes.....145
Figure 4. 5. Caspofungin treatment over-activates the Cek1 MAPK. 149

CHAPTER I: Introduction

Candida species are the most common human fungal pathogens and are also ranked as the fourth-greatest cause of hospital-acquired bloodstream infections, with up to 40% mortality in epidemiological studies [1, 2]. *Candida* species colonize the human gastrointestinal tract and skin asymptomatically in immuno-competent individuals. Under certain condition, however, *Candida* species can cause mucosal and systemic infections. Risk factors include central venous catheter implants, major surgeries such as organ transplants, neutropenia, cancer therapy, and HIV infection. Current anti-fungal drugs include three major classes –azoles, polyenes, and echinocandins, and each of them has its specific drug target [3, 4]. However, drug toxicity and drug resistance have limited the efficacy of current anti-fungal medicine.

The majority of life-threatening fungal infections are opportunistic in nature, and a novel approach that may be necessary to complement current antifungals will be to simultaneously improve host immune efficacy. This can include cytokine therapy and other adjunctive therapy approaches [5]. A related strategy to improve adjunctive therapy will be to make the fungi more recognizable to the host immune effector cells. A better understanding of the interaction of *Candida* species with their cognate host receptors, particularly of how different cell wall components are recognized by their receptors on immune cells, will provide new insights that will facilitate such an approach. It will also improve our understanding of the process of fungal pathogenesis.

The immune recognition of fungal pathogens is the first step in determining the host response to fungal infection, which also plays an important role in shaping fungal colonization and the subsequent induction of disease. Cell wall composition and architecture are critical in modulating host immune responses. This review will summarize various conditions leading to the modification of cell wall structure, and their immune-modulatory significance.

Construction of the bi-layered *C. albicans* cell wall

The *C. albicans* cell wall is a firm, but dynamic structure which is essential for fungal viability since it serves as a tough, but malleable barrier that sustains cell

shape and prevents osmotic lysis. It is also important for fungal virulence because it is the interface between the fungus and the host, and the architecture of its antigenic polysaccharides, lipids, and proteins presents virulence factors and dampens host responses. As a result, some cell wall impaired mutants (*e.g. cho1Δ/Δ*, *sun41Δ/Δ, kre5Δ/Δ*, *phr1Δ/Δ)* exhibit avirulence in the mouse systemic infection model [6-9]. When exposed to the immune system, cell wall components modulate host immune recognition in a variety of ways [10]. For instance, Dectin-1, a C-type signaling lectin, located at the surface of myeloid-derivative immune cells, specifically recognizes the immunogenic polysaccharide β (1,3)-glucan and initiates pro-inflammatory antifungal responses including TNF-α secretion [11]. Chitin, a type of polysaccharide composed of *N*-acetylglucosamine subunit, is shown to have immune-inhibitory effects in a concentration- and size- dependent manner [10]. However, the cell wall is a multi-layered structure that minimizes the exposure of some of these components to the immune system. The *C. albicans* cell wall is composed of an outer layer of *N*- and *O*-linked glycosylated proteins with 80%-90% mannose content [12], as well as an inner layer of the core polysaccharides β (1,3)-glucan, β (1,6)-glucan and chitin. Each layer serves different roles in fungal physiology and virulence and have different components (Figure 1. 1).

Inner layers

β-glucans

β-glucans are the main cell wall components in *C. albicans* and account for about 60% by weight of the cell wall [8]. β (1,3)-glucan is present in the cell walls of plants, bacteria and fungi. It is a crucial cell wall structural polysaccharide residing in the inner layer of the cell wall and represents about 40% of cell wall weight [13]. This polysaccharide is synthesized by a plasma membrane-bound glucan synthase complex and uses UDP-glucose as a substrate to create a linear polymer of glucose covalently linked at 1-, 3- position through glycosidic bonds. The resultant β (1,3)-glucan is extruded into the periplasmic space where it is released from membrane and covalently attached to other cell wall components by various

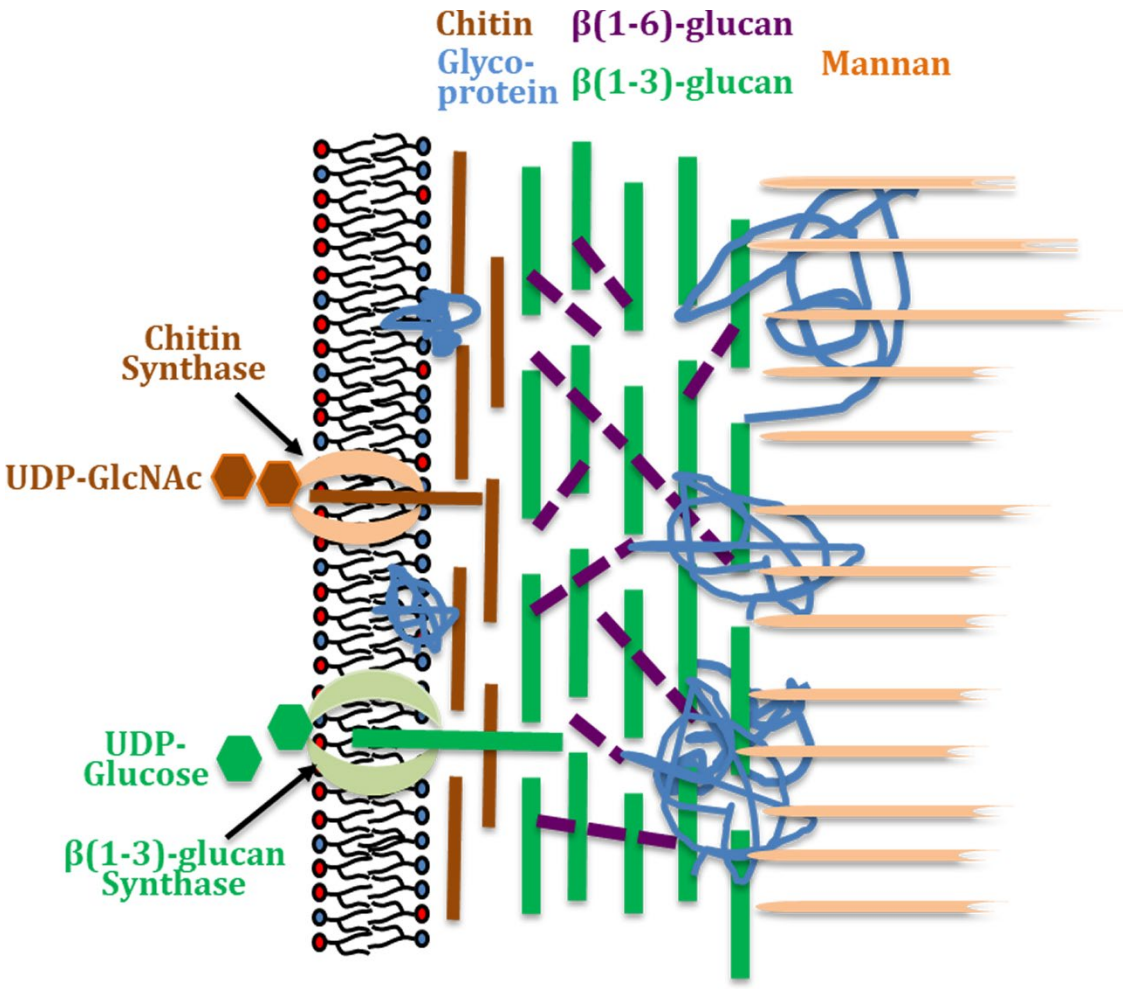


Figure 1. 1. The structure of *C. albicans* cell wall.

C. albicans has layered cell wall structure, where the inner layer is comprised of chitin and β-glucan, and the outer layer is made of glycosylated proteins. Chitin is synthesized by chitin synthases using substrate UDP-GlcNAc, and β (1,3)-glucan is synthesized by its synthase and substrate UDP-Glucose. Glycosylated proteins (glyco-proteins) are modified by *N*- and *O*-mannans with 80%-90% mannose content

trans-glycosidase enzymes in the periplasmic space and/or cell wall [14] (Figure 1.1). The *C. albicans* glucan synthase complex include two subunits: a catalytic subunit Fks1 and a regulatory subunit Rho1 [15]. Rho1 and Fks1 are essential in *C. albicans* [16], and this enzyme complex is the target of the echinocandin anti-fungal drug class, which acts by inhibiting β (1,3)-glucan synthesis. Fks1 encodes a 210-kDa transmembrane protein, and is important for echinocandin resistance, as the mechanism involves point mutations clustered in two hotspot regions within this protein, encoding residues 641-649 and 1345 to 1365, respectively [4, 17]. The *C. albicans* genome harbors two other *FKS* homologs *FKS2* and *FKS3*, which also play roles in echinocandin resistance. Cells lacking *FKS2* or *FKS3* contain increased amount of glucan and are more resistant to cell wall-damaging agents, suggesting that Fks1 expression is negatively regulated by the other two homologs [18].

The small GTPase Rho1 was first identified as a regulatory subunit of the β-glucan synthase complex in *S. cerevisiae* [19]. In this organism, temperature-sensitive mutants in *ScRHO1* display thermolabile glucan synthase activities, which could be restored by adding purified wild-type ScRho1 protein along with GTP [19]. ScRho1 can be co-purified with the catalytic subunit Fks1, and is co-localized to cell wall remodeling sites, *i.e.,* to the bud tip during growth and to septum during cytokinesis [19]. CaRho1 was later identified to act in the same manner as ScRho1, being the regulatory subunit of glucan synthase [20]. This is also reflected in the sequence similarity between these two species, where CaRho1 shares 82.9% identity to ScRho1 in the amino acid level, and Ca*RHO1* can rescue the glucan synthase activity in a *Scrho1*-deficient mutant.

β (1,6)-glucan is another critical cell wall component and plays an important role in stabilizing fungal cell wall integrity since it acts as linker for the attachment of chitin and mannan proteins to β (1,3)-glucan [8]. It accounts for ~20% of the cell wall weight in yeast form cells and increases to about 27% in hyphae; almost double the amount in *S. cerevisiae* [8]. The biosynthesis of β (1,6)-glucan is not fully understood at the biochemical level, while genetic work reveals that several genes appear to be involved in this process, including the *KRE* family and *BIG1*.

Kre5 and Kre9 are the well-studied members in this family, and they play roles in morphogenesis and fungal virulence. Unlike *ScKRE5*, deletion of both alleles of *CaKRE5* does not affect viability, but the *Cakre5Δ/Δ* mutation does cause aberrant morphology including cell aggregation and enlarged vacuoles. Moreover, the individual null mutants of *kre5Δ/Δ, kre9Δ/Δ*, or *big1Δ/Δ* display a great reduction of β (1,6)-glucan, suggesting they are all required for the polymer's biosynthesis [8, 21, 22]. It is also reported that the remaining β (1,6)-glucan polymer displays characteristic structural features when *KRE5* is disrupted [8]. This suggests that Kre5 might play an indirect role in polysaccharide synthesis.

Chitin

Chitin is an essential polysaccharide in most fungal cell walls [23], and this polymer normally resides at the innermost layer, with a content of less than 10% of cell wall weight [23]. Chitin is a homopolymer of β (1,4)-linked *N*-acetylglucosamine (GlcNAc) [24]. A family of four chitin synthases encoded by *CHS1, CHS2, CHS3* and *CHS8* are responsible for chitin biosynthesis with uridine diphosphate (UDP)-GlcNAc as its substrate (Figure 1. 1). Chs1 is an essential chitin synthase that is required for septa formation, cell wall integrity and virulence [25]. Loss of *CHS1* prevents the daughter cells from separating from the parents, and therefore forms a chain of daughter cells with continuous constrictions but no septa [25]. Chs2 preferentially expresses in hyphal cells, and disruption of *CHS2* decreases chitin by about 40% in hyphal cells compared to the parental strain. The *CHS2* null mutant is also prototrophic and does not exhibit attenuated virulence [26]. Chs3 is not required for growth, germ-tube formation, septation, but is essential for overall chitin synthesis, bud scar formation, virulence and stress responses [27-29]. Chs8 is a non-essential chitin synthase in *C. albicans*, reflected by normal cell growth, morphologies and chitin content in the homozygous knockout mutant [30]. However, individual disruption of *CHS2* and *CHS8* increases the calcofluor white sensitivity and correlates with increased glucan content and decreased mannan content [30]. Two chitin microfibril architectures have been reported: long- and short- chitin fibrils, and they are primarily located at the septa and lateral cell walls,

respectively [31]. Each chitin synthase plays an individual role in synthesizing differing microfibril structures at specific locations in the cell wall [24]. Chitin can also be remodeled through the actions of four chitinases (Cht1-4), which cleave the glycosidic bonds in chitin, therefore the resultant short fibril can be better embedded in the cell wall.

Outer layer

The outer layer of the cell wall consists of highly glycosylated mannoproteins that are modified by *O*-linked and *N*-linked mannosylation and phosphomannosylation [23] (Figure 1. 2). The glycoproteins serve a variety of functions, including adhesion, morphogenesis and biofilm formation. The protein *O*-mannosytransferase (Pmt) family of enzymes catalyze the first step of *O*-mannosylation on secretory proteins at specific serine or threonine residues during transit through the endoplasmic reticulum (ER), and further mannose residues can be added by Mnt1/2 family proteins in the Golgi [32]. Five Pmt isoforms (Pmt1-5) have been identified in *C. albicans*, where Pmt1, Pmt2 and Pmt4 are the major enzymes of *O*-mannosyl transferase, and each of them has different expression profiles to counteract cell wall stress. For instance, when cells are treated with the *N*-glycosylation inhibitor tunicamycin, Pmt1 expression is de-repressed, whereas the other two are inhibited compared to non-stressed conditions, suggesting that *PMT* isoforms play distinct roles in *Candida* cells for better adaptation to cell wall insults [33].

The *N*-glycosylation processes occur as proteins cross through the ER [34]. The *N*-mannan precursor is attached to asparagines at the sequence *N*-X-S/T, where X is any amino acid, and the sugar core is further processed in the ER by glycosidases, resulting in a mature yet shorter *N*-mannan core. As proteins pass through the Golgi, a single mannose is attached to the core by the α (1,6)-mannosyl transferase Och1, and further elaborated with α (1,6)-mannose by what is believed to be two complexes consisting of M-Pol I (Mnn9 and Van1), and then M Pol II (Mnn9, Mnn10, Mnn11, Anp1, and Hoc1), respectively, to form the linear α (1,6)-mannan backbone [35]. The branched chains are built on this backbone

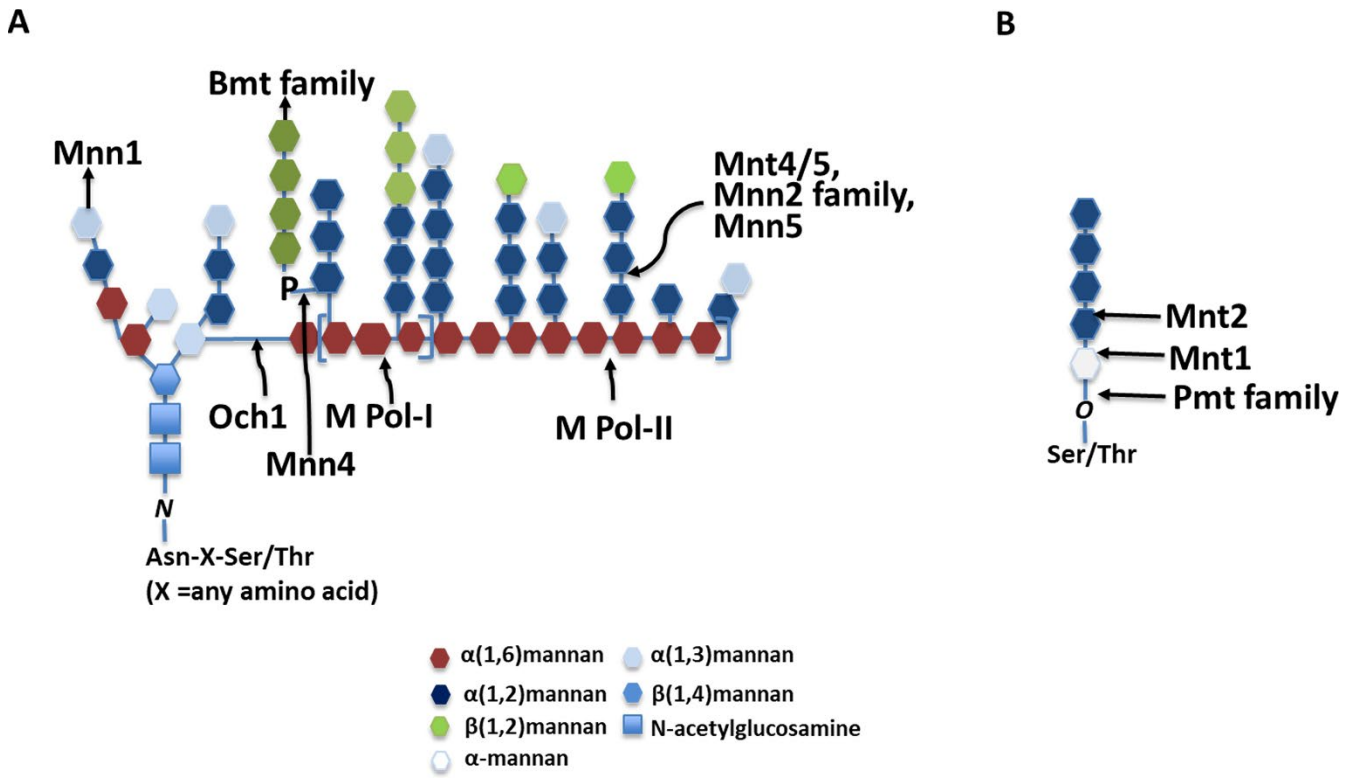


Figure 1. 2. A model of *N*- and *O*-glycosylation of *C. albicans* cell wall proteins.

N-glycosylation initiates at the ER, where the *N*-mannan is attached to asparagines at the sequence *N*-X-S/T. When the protein passes through the Golgi, a single mannose is attached to the core by Och1, and further elaborated with α (1,6)-mannose by M-Pol I and then M Pol II complex to form the linear α (1,6)-mannan backbone. The branched chains consist of α (1,2)- residues that are added by α (1,2)-mannosyl transferases Mnt4 and Mnt5, Mnn2 family, and Mnn5. β (1,2)-mannan and α (1,3)-mannose are found to cap the branched chain by Bmt family and transferase Mnn1, respectively. The Pmt family enzymes catalyze the first step of O-mannosylation at specific serine or threonine residues and mannose residues are further added by Mnt1/2 family proteins in the Golgi. Phosphomannan is attached to the outer chain via a phosphodiester bond through transferase Mnn4 and family members, and capped with β (1,2)-mannan by Bmt family.

and consist of α (1,2)-residues that are added by α (1,2)-mannosyl transferases Mnt4 and Mnt5, Mnn2 family, and Mnn5. In *C. albicans*, β (1,2)-mannan is found to cap the outer chain and is added by β-mannosyl transferases (Bmt1 and Bmt3) and alternatively, α (1,3)-mannose is added by transferases like Mnn1 [35, 36]. Phosphomannan can also be attached to the outer chain via a phosphodiester bond through transferase Mnn4 and family members, and capped with β (1,2)-mannan by Bmt2, Bmt3 and Bmt4 [32].

Most of these proteins are glycosylphosphatidylinositol (GPI) anchored, and GPI-anchored proteins are found ubiquitously across fungi species, and their functions range from enzymatic to antigenic and adhesion [23]. They are linked at the C-terminus through a phosphodiester bond to phosphoethanolamine which is attached to a tri-mannosyl-glucosamine core (Man3-GlcN) that is bound to phosphatidylinostol (PI) in the membrane. Release of the GPI remnant is accomplished by hydrolysis of the mannose-GlcN bond, and this carbohydrate remnant is covalently linked to β (1,6)-glucan in the cell wall [23, 37-39].

Immune-recognition of individual cell wall components

The first step in initiation of an immune response to *Candida* species is the recognition of fungal pathogens through pattern-recognition receptors (PRRs). This process involves the detection of conserved pathogen-associated molecular patterns (PAMPs) by the cognate PRRs, including C-type lectin receptors (CLRs), Toll-like receptors (TLR), and Galectin-3. The distinct structures of various polysaccharides within the *Candida* cell wall represent conserved PAMPs and are further recognized by the corresponding PRRs during the development of anti-fungal immune responses.

β (1,3)-glucan is an important PAMP for detecting fungi

The myeloid-expressed receptor Dectin-1 plays a major role in anti-fungal innate immune responses [40]. It belongs to the C-type signaling lectin family, and specifically recognizes β (1,3)-glucan-containing particles by its single extracellular carbohydrate-recognition-domain (CRD). Upon recognition of β (1,3)-glucan by

CRD, Dectin-1 responds through its tyrosine-based activation motif in the cytoplasmic tail [41, 42], which in turn triggers pro-inflammatory responses including phagocytosis of the fungal pathogen and induces cytokine secretion for fungal clearance [43]. Leukocytes derived from Dectin-1-deficient mice are unable to recognize fungal particles unless opsonized with serum and display impaired immune responses toward fungal pathogens, including reduced recruitment of immune cells and decreased production of pro-inflammatory cytokines. The Dectin-1-deficient mice are therefore susceptible to fungal infection and exhibit significantly enhanced systemic fungal dissemination and reduced survival rates when infected with some strains of *C. albicans* [43]. Leukocytes isolated from wild-type mice are more capable of killing *Candida* cells with exposed β (1,3)-glucan by producing increased levels of pro-inflammatory cytokines compared to that of Dectin-1-deficient mice [44]. In contrast, specific strains of *C. albicans* did not show differences in virulence when infecting Dectin-$1^{-/-}$ mice [45], and there was a correlation between increased chitin levels and decreased dependence on Dectin-1. This suggests that chitin levels can influence the relationship between exposed β (1,3)-glucan and Dectin-1 in the immune response to *C. albicans*. However, even in strains that are more resistant to Dectin-1 in their wild-type state, if they are treated with caspofungin, which exposes β (1,3)-glucan and increases chitin, Dectin-1 is required for clearance by the drug [45]. This suggests that β (1,3)-glucan exposure to Dectin-1 plays an important role in clearance in response to caspofungin.

While Dectin-1 acts as the major receptor for β (1,3)-glucan recognition, several other receptors mediate host immune responses to β (1,3)-glucan-containing particles. TLR2 receptors synergize with Dectin-1 signaling in responses to zymosan, a β (1,3)-glucan preparation from yeast cell wall, by increasing pro-inflammatory cytokines production *in vitro*, in spite of the fact that TLR2 alone does not directly recognize zymosan nor activate immune responses sufficiently when stimulated by zymosan [42]. In neutrophils, phagocytosis of zymosan-containing particles is promoted by Dectin-1 in the absence of serum, but in the presence of serum it is independent [46]. Complement receptor 3 (CR3)

is also a receptor of β (1,3)-glucan on neutrophils. Neutrophils exhibit reduced phagocytosis of zymosan when derived from the patients deficient in CR3, and these patients have higher susceptibility to fungal infections [47].

Fungi can mask β (1,3)-glucan from immune surveillance

Although fungal cells can induce host inflammatory responses by way of β (1,3)-glucan detection, it is not surprising that fungal pathogens have evolved mechanisms for immune avoidance by concealing β (1,3)-glucan in various ways [23]. In *Histoplasma capsulatum*, α-glucan and the glycosyl-hydrolyase Eng1 are important for minimizing β-glucan exposure [48]. Alpha-glucan functions by concealing β-glucan, while Eng1 hydrolyzes β-(1,3)-glycosyl bonds, and therefore removes exposed β-glucans [49]. *Aspergillus fumigatus* Uge3 regulates the biosynthesis of galactosaminogalactan, which masks hyphal β-glucan from immune detection [50]. In *C. albicans*, β (1,3)-glucan is buried underneath the outer layer of glycosylated proteins in a process referred to as masking [23]. During infection, unmasking can occur in response to the host environment (Figure 1. 3). Wheeler *et al.* described a time-course of β (1,3)-glucan exposure during disseminated infection of *C. albicans* in a mouse model [51]. The polymer is masked by the outer layer of glycoprotein at the beginning of infection and becomes exposed after several days [51]. The percentage of cells with β (1,3)-glucan exposed increases from about 20% (early infection, 16 hours post-infection) to 80% (late infection, 7 days post-infection) [51]. The exact mechanism by which unmasking occurs is not fully understood, but it has been observed that when *C. albicans* is exposed to neutrophils *in vitro*, they can damage cell wall and cause β (1,3)-glucan unmasking. Furthermore, caspofungin pretreatment causes significantly higher levels of β (1,3)-glucan exposure in *C. albicans* during systemic infection compared to the non-treated fungal cells. This drug also displays a filament-bias for unmasking [51]. The mechanism by which filaments are more readily unmasked is unknown. However, Lowman *et al.* reported that *C. albicans* hyphal glucan exhibited a novel glucan structure, where hyphal glucan has a unique cyclical structure and an unexpected 2,3 linkage [52]. It has also been

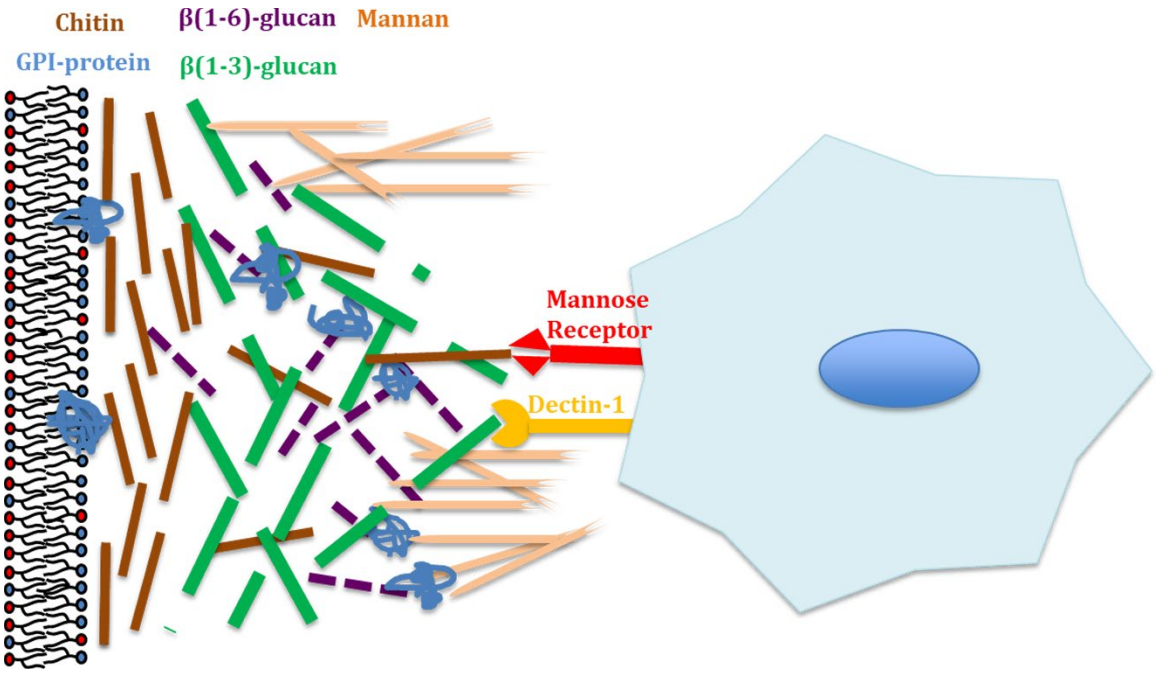


Figure 1. 3. *C. albicans* β (1,3)-glucan is an important PAMP for detecting fungi.
Under certain circumstances, β (1,3)-glucan is exposed at cell surface, and this exposure can be detected by its cognate receptors Dectin-1 on immune cells (macrophages and neutrophils) to stimulate pro-inflammatory immune responses. Chitin is recognized by the Mannose Receptor and further initiates anti-inflammatory responses.

observed that hyphal glucan is more immunogenic than yeast glucan and stimulates higher levels of pro-inflammatory cytokine production from human peripheral blood mononuclear cells (PBMCs) and macrophages by a Dectin-1 dependent mechanism [52]. This finding provides evidence that the glucan architecture can influence how innate immune cells discriminate between the yeast form and hyphal cells of *C. albicans*.

Impacts of β (1,3)-glucan exposure on virulence

β (1,3)-glucan is naturally present in the cell wall of bacteria, fungi and plant, whereas it differs in biomass, structure, solubility and branching structure depending on the source. This polymer can be used as a fiber supplement, cosmetic products or medical uses *etc.* [53]. A large body of work on this polysaccharide is driven by the immunomodulatory effect it has in animals [13, 54]. Particularly in the medical mycology field, the significance of understanding β (1,3)-glucan recognition by Dectin-1 during fungal commensalism and pathogenesis has been appreciated in gastrointestinal (GI) tract colonization model and a systemic infection model [43, 54, 55]. The human GI tract contains a large variety of microbes and the most frequently isolated fungi are *Candida* species. However, the factors impacting fungal cells colonization *in vivo* have not been thoroughly defined yet. Sem *et al.* reports that the cell wall β (1,3)-glucan exposure trait, but not total β (1,3)-glucan level nor other cell wall component levels, critically determines the ability of *Candida* species colonizing the GI tract [55]. Several mutants including the *chs3Δ/Δ* mutant defective in chitin biosynthesis, or the *mnt1Δ/Δmnt2Δ/Δ* double mutant defective in *O*-mannosylation surprisingly colonize in mouse GI tract well, although they individually display cell wall dysfunction. There is no association between in *vivo* fitness and the ability to grow under a variety of stressful environments, which the microbes normally encounter within the host, including organic acid, ox bile, bile salt, low pH, high reactive oxygen species (ROS), high NO. Instead, the fungal cell wall architecture plays an essential role in shaping the *in vivo* fitness, and this trend is also seen in different *Candida* species, including *C. glabrata, C. tropicalis, and C. dubliniensis*. This

highlights the significance of the dynamics of β (1,3)-glucan structure in shaping *Candida* cells as GI commensals or pathogens.

Altogether, considering the crucial immunobiological relevance of β (1,3)-glucan exposure and the mechanisms used by many pathogens to mask it, a new strategy for drug design may be to make fungal pathogens more visible and susceptible to the host immune attack by exposing β (1,3)-glucan. Goals should be set to identify signaling pathways that cause β (1,3)-glucan unmasking as these may serve as targets for novel antifungal drugs to improve adjunctive therapy.

Chitin modulates inflammation based on size and dosage

Chitin plays an important role in modulating pro- and anti-inflammatory immune responses in a concentration- and size-dependent manner. Low concentrations (<10 μg/ml) induce anti-inflammatory cytokine production, including IL-10, while high chitin concentrations (>100 μg/ml) strongly induce TNF-α secretion via Dectin-1 and TLR-2 receptors [56, 57]. Large chitin particles (>70 μm) are immunologically-inert, whilst intermediate sized chitins (40-70 μm) induce pro-inflammatory cytokine production at higher dosages [57]. Small chitin particles (<10 μm) modulate immune responses based on concentration [56, 57]. The majority of chitin particles derived from *C. albicans* are small in size (between 1-10 μm). Fungal chitin particles in this organism are recognized via a mannose receptor located on the membrane of immune cells, and chitin further induces anti-inflammatory responses via intracellular NOD2- and TLR9- signaling [56] (Figure 1. 3). As mentioned above increased chitin levels seems to correlate with decreased Dectin-1 dependence for clearance in mice in *C. albicans* [45]. Conversely, however, increased chitin exposure in response to caspofungin or some mutations, increases Dectin-1 dependent immune responses *in vitro* [45, 51, 58]. Thus, the role of chitin levels in modulating immunity seems to be context dependent and needs further study.

Glycosylated protein recognition

Glycosylation of cell wall proteins is critical in the immune-modulation during fungal infection. *N*-glycan is found to play a dominant role in inducing epithelial immunity *in vitro* [59]. Due to the sophisticated biochemical makeup of mannan layer, several PRRs are involved in the immune recognition. For instance, Mannose receptor primarily detects *N*-mannan and TLR-4 targets *O*-mannan, while Dectin-2 and Galectin-3 participate in α-mannan and β-mannan recognition respectively [59, 60]. However, immune recognition of glycosylated proteins is not relevant for this study, but has been extensively studied/reviewed in [34, 36, 60-62]

β (1,3)-glucan unmasking can be induced by manipulation of cell wall signaling pathways

The process of cell wall biogenesis and remodeling is regulated through complex signaling pathways including several MAP kinase (MAPK) cascades. The MAPK pathways are well-conserved signaling cascades in eukaryotes, which are essential for coping with a wide range of stimuli, including osmotic stress, oxidative stress, cell wall damage, and changes in glycosylation [11, 63-66]. A conserved module of three kinases comprises the MAPK signaling cascade: the MAP kinase kinase kinase (MAPKKK), the MAP kinase kinase (MAPKK) and the MAP kinase (MAPK). After an upstream signal phosphorylates (activates) the MAPKKK, it in turn phosphorylates and activates the MAPKK, followed by MAPK phosphorylation and activation. The activated MAPK further phosphorylates downstream transcription factors to initiate gene expression for better adaption to the environment [66]. Three major cell wall MAPK pathways have been discovered in fungal pathogens, and they each play specific roles in sustaining cell wall architecture.

Central role of Cek1 activity in controlling β (1,3)-glucan masking

Among these MAPK pathways, Ste11-Hst7-Cek1, comprising the Cek1 MAPK cascade, serves pivotal functions in maintaining sophisticated cell wall structure in equilibrium, including masking β (1,3)-glucan from immune detection, thus

sustaining cell wall glycostructures [33, 64, 67] (Figure 1. 4). In this pathway, activated Cek1 phosphorylates downstream transcription factors including Cph1 and Ace2 to initiate compensatory transcriptional activities. The activation of Cph1 further stimulates the upstream Cek1 pathway by a positive feedback mechanism that provides transcription of higher levels of the MAP kinase [68]. Cek1 can be degraded by the proteasome, and is found to be a short-lived protein, as revealed by quick degradation to 25% of the starting levels 30mins after being treated with the protein synthesis inhibitor nourseothricin [69].

Cek1 activity is regulated tightly by multiple stimuli in *C. albicans*. Several conditions induce Cek1 phosphorylation, including disruption of phosphatidylserine synthase Cho1 [70], growth resumption from the overnight culture to fresh media [69], being treated with human salivary fungicidal histatin 5 (Hst5) protein which protects the oral cavity from oropharyngeal candidiasis [71], hyperactivation of upstream GTPases Cdc42 and Rho1 [70], anti-fungal drug caspofungin treatment [65], cleavage of the extracellular domain of signaling mucin Msb2 [72], and activation of filamentous growth [73]. The quorum sensing molecule farnesol, in contrast, is found to inhibit Cek1 phosphorylation, mediated by the transmembrane adaptor protein Sho1 [69]. Besides sustaining cell wall structure, activated Cek1 plays a broad role in establishing fungal infections. Cek1 is involved in the yeast-to-hyphal morphogenesis, a critical step to initiate pathogenicity of *C. albicans*. Loss of *CEK1* impairs morphogenesis on certain agar-invasive hyphal growth media, such as Lee's medium with mannitol as a carbon source instead of glucose and SLAD medium (low nitrogen) [73]. Although 10% serum, a potent inducer for morphogenesis, induces morphologically normal hyphae in a *cek1Δ/Δ* mutant, the growth rate of mycelial colonies of the mutant is affected [73]. Cek1 also controls the virulence in the mouse disseminated infection model and the mouse mastitis model [73, 74]. Furthermore, Cek1 is involved in switching of regular white yeast cells to the mating-competent opaque cells and induction of mating responses in opaque cells through the transcription factor Cph1 [68].

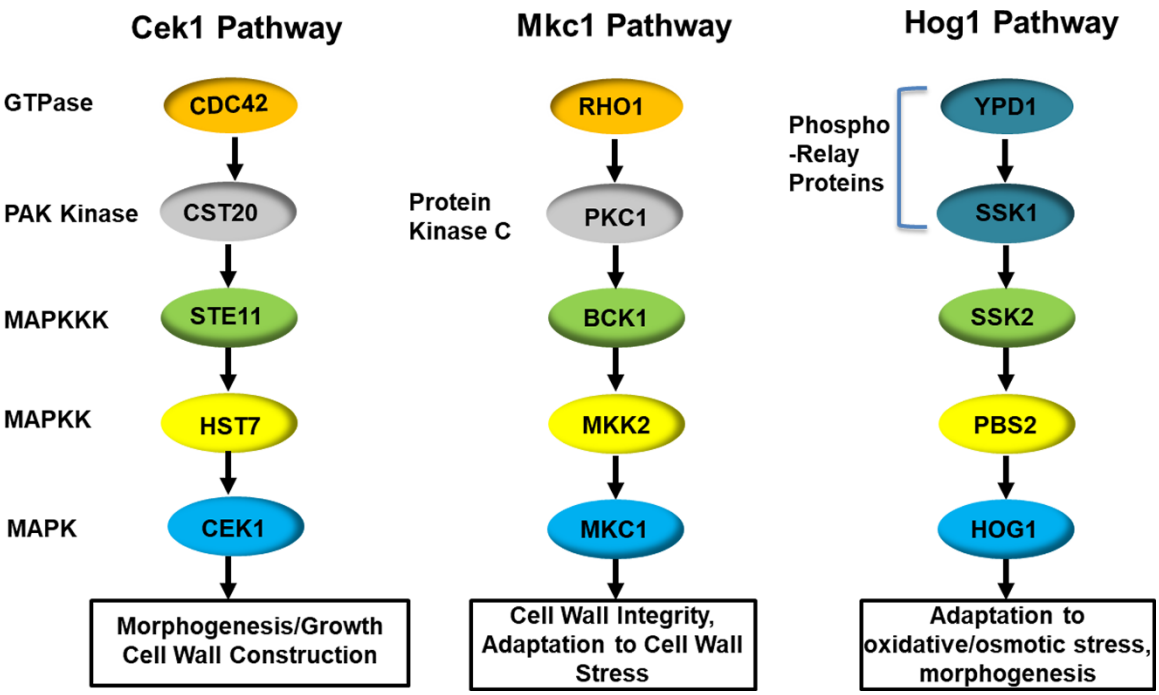


Figure 1. 4. The MAPKs pathways in *C. albicans* that are involved in cell wall construction. The Cek1, Mkc1 and Hog1 MAPK cascades, and their upstream activating proteins are shown. The Cek1 pathway is activated by upstream GTPase Cdc42 and PAK kinase Cst20. The Mkc1 pathway is stimulated by GTPase Rho1 and protein kinase C (Pkc1). Two-component phospho-relay system Ypd1p and Ssk1 regulates the downstream Hog1 pathway activation.

Cek1 disruption causes exposure of cell wall components

The *cek1Δ/Δ* mutant displays unmasking of β (1,3)-glucan and hyper-sensitivity to agents that disturb the cell wall such as Congo red, and the *N*-glycosylation inhibitor tunicamycin [75, 76]. This mutant also exposes α (1,2)- and β (1,2)-mannosides, suggesting the glycostructure is defective in *CEK1* disruption. Exposed β-mannan increases the binding to murine macrophages partially through the recognition receptor galectin-3, although this receptor itself does not account for the mutant's impaired virulence in the mouse systemic infection model [67]. This suggests that pathogen-specific β-mannan is not immunogenic enough to initiate fungal killing. Transcriptome analysis reveals that in the *cek1Δ/Δ* mutant, 27 genes are down-regulated, including genes encoding proteins that respond to drug/chemical stress and cell wall biogenesis. This confirms the significance of the Cek1 pathway in cell wall construction. The transcriptomic profile of the *cek1Δ/Δ* mutant treated with tunicamycin further reveals that a significant number of genes involved in cell wall biogenesis are differentially expressed. The cell wall genes that were specifically defective in this condition include *CHT2*, a chitinase which causes chitin exposure when repressed [77]; *XOG1,* an exo-β (1,3)-glucanase that participates in glucan metabolism [78]; *MSB2,* a cell wall damage sensor protein which induces β (1,3)-glucan exposure when disrupted [72]; and *EFG1,* the key transcription factor for morphogenesis. This differential expression profile might account for the *C. albicans* hypersensitivity to cell wall insults when *CEK1* is disrupted, and also highlights the importance of Cek1 for cell wall stress adaption transcriptionally.

Ace2, another transcription factor downstream of Cek1, has been shown to play an important role in sustaining cell wall structure in *C. albicans*. The *ace2Δ/Δ* mutant displays severe β (1,3)-glucan exposure, and transcripts induced by Ace2 upregulation are thought to be responsible for β (1,3)-glucan masking induced by lactate treatment [67, 79, 80]. This may be related to the role Ace2 plays in controlling cell wall glycostructure [76]. Damage of *N*-glycans is sensed by cell wall proteins Msb2 and Sho1, and results in the activation of Cek1, which in turns activates cell wall repair activities mediated by Ace2 transcriptionally [76]. In

addition, Ace2 controls the expressions of *O*-glycosylation genes in an isoform-specific manner [33]. Under unstressed condition, Ace2 represses Pmt1 expression via transcription factor Zcf21, and de-represses it in response to tunicamycin [76]. It is possible that the cell wall damage induced by *CEK1* deletion is potentially due to loss of Ace2 transcriptional activity.

Cek1 activation promotes exposure of cell wall β (1,3)-glucan

Davis *et al.* reported that the phosphatidylserine (PS) synthase enzyme (Cho1) controls cell wall β (1,3)-glucan exposure [58]. Phospholipids are crucial components of cellular membranes in eukaryotes, and PS is essential for *C. albicans* virulence [6]. A *cho1Δ/Δ* mutant exhibits greater β (1,3)-glucan exposure compared to wild-type [58, 81]. This exposure allows increased recognition by Dectin-1 and elicits a stronger pro-inflammatory response from macrophages [11, 58, 81]. Interestingly, in the *cho1Δ/Δ* mutant, both the Cek1 and Mkc1 MAPKs are constitutively activated [70]. Li *et al.* reported that conditions that induce Cek1, such as incubation with *N*-acetylglucosamine (GlcNAc), cause increased β (1,3)-glucan exposure in *C. albicans* [71]. However, this has the caveat that GlcNAc induces yeast-to-hyphal transition, and filaments have higher level of glucan exposure due to the intrinsic cell wall architecture differences [52]. Chen *et al.* found that hyper-activation of Cek1 via hyperactivation of the upstream Ste11 MAPKKK causes glucan exposure in yeast-form cells in the form of punctate exposed glucan foci along the periphery. This is similar to the phenotype exhibited by the *cho1Δ/Δ* mutant and may be the reason *cho1Δ/Δ* exhibits β (1,3)-glucan exposure. Unmasking from hyperactive Cek1 stimulates an immune response, as up-regulated TNF-α secretion is induced from murine macrophages. The mechanism by which Cek1 is hyperactivated in the *cho1Δ/Δ* mutant is not completely clear but appears to be acting downstream of the small GTPase Cdc42, which is up-regulated in *cho1Δ/Δ* [70]. This indicates a model where PS synthase normally down-regulates Cdc42. This is most likely mediated by synthesis of PS, which has been shown to be an activator of GTPase activating proteins (GAPs) that downregulated Cdc42 in *S. cerevisiae*, such as Rga1 and Rga2 [82]. Thus,

the model is that loss of PS synthesis leads to hyperactivation of Cdc42, which in turn activates Cek1 and causes β (1,3)-glucan exposure. However, the mechanism by which this occurs is not yet known.

Caspofungin, an echinocandin antifungal, causes β (1,3)-glucan exposure even at sublethal concentrations [83], and this correlates with Cek1 activation [65]. This suggests that Cek1 activation might contribute to β (1,3)-glucan exposure, at least partially, after caspofungin treatment. This also indicates that improper activation of Cek1 seems involved in cell wall structure alteration in different situations. The resultant β (1,3)-glucan exposure promoted by Cek1 hyperactivation may be induced by the downstream transcription factor Cph1, an important regulator for *C. albicans* filament development [79], and white-to-opaque switching [68]. Cph1 expression depends on Cek1 and further boosts Cek1 expression in a positive-feedback loop [68]. A Cph1-overexpressing strain under yeast condition induces pseudo-hyphal structure, and several hyphal-specific genes are induced transcriptionally [84]. The overall mechanism by which these proteins might cause unmasking is unknown, but discovery of this could help explain unmasking under cell wall altering conditions.

The Mkc1 pathway does not appear to cause β-glucan unmasking

In *C. albicans*, the Mkc1 MAPK cascade, consisting of Bck1-Mkk2-Mkc1, is activated in response to exogenous cell wall stress, oxidative stimuli, antifungal drugs, or low-temperature shocks [85] (Figure 1. 4). Mkc1 is also important for fungal virulence, as loss of *MKC1* reduces fungal virulence in the mouse systemic infection model [86]. Although Mkc1 is central for cell wall construction in *C. albicans*, this pathway does not appear to be required for β-glucan masking in *C. albicans*, as evidenced by a lack of β-glucan unmasking in *mkc1Δ/Δ* mutants [87]. In addition, while loss of PS synthase in *C. albicans* increases Mkc1 activity, disruption of this MAPK in *cho1Δ/Δ* does not prevent β (1,3)-glucan exposure, but in fact exacerbates the phenotype in the *cho1Δ/Δ mkc1Δ/Δ* double mutant [70]. This suggests that Mkc1 is activated to compensate for cell wall disfunction when PS synthase is deleted, perhaps in response to hyperactivation of Cek1. Finally, a

hyperactive GTP-bound form of Rho1 (*RHO1*^{Q67L}), located upstream of Mkc1 pathway, displays increased β (1,3)-glucan exposure compared to wild-type. Surprisingly it also exhibits increased phosphorylation of Cek1 along with Mkc1 [70]. This further suggests that the increased β-glucan exposure from the hyperactive *RHO1*^{Q67L} mutant may be caused by Cek1 upregulation, instead of Mkc1.

Hog1 activity contributes to neutrophil-driven β (1,3)-glucan exposure

The Hog1 pathway, comprised of Ssk2-Pbs2-Hog1, is well-known for its response to osmotic stress, oxidative stress and morphogenesis [66, 88, 89] (Figure 1. 4). This pathway is also involved in cell wall biosynthesis, as *HOG1* disruption increases resistance to certain cell wall inhibitors such as Congo red (a dye interacting with the fungal cell wall), and Nikkomycin Z (an inhibitor of chitin biosynthesis) [89] and enhances susceptibility to cell wall digestion by β (1,3)-glucanase [88]. Hopke *et al.* discovered that the *C. albicans* Hog1 pathway is necessary for hyphal cells to respond to damage from neutrophils. Hyphae damaged by neutrophils exhibit increased β (1,3)-glucan exposure, but unmasking does not occur in the *hog1Δ/Δ* mutant. [11]. Neutrophil-damage of hyphae results in remodeling during which the outer layer of glycosylated proteins is damaged within seconds upon neutrophils attack, followed by chitin deposition 30 mins post-insult, and β-glucan unmasking takes place even later. *HOG1* disruption greatly impairs the ability of fungal cells to adapt to neutrophil attack [11]. Increased chitin synthesis can rescue fungal cells from cell wall stress, and chitin synthase (Chs) 3 is shown to be essential for most of the localized chitin deposition and glucan unmasking during cell wall remodeling triggered by neutrophils insult. Although Chs2 and Chs8 are also induced, they are not responsible for β-glucan exposure [11]. Of note, the cell wall associated Cek1- and Mkc1- pathways are not required for this response. This demonstrates the specificity of individual MAPK pathways in coping with distinct cell wall stresses.

Caspofungin treatment remodels cell wall architecture

Wheeler *et al.* identified that sub-lethal dosages of caspofungin upset the intricate cell wall structure and causes β (1,3)-glucan exposure without killing the fungi, and this elicits more potent proinflammatory responses including TNF-α secretion from the macrophages through Dectin-1 [83]. A genetic network responsible for masking β (1,3)-glucan was identified in a screen of the *S. cerevisiae* non-essential gene deletion mutant library. Most of the genes within the network are involved in regulating polarized cell wall remodeling, (*i.e.* polarized actin). The *S. cerevisiae* genetic network for concealing β (1,3)-glucan is partially conserved in *C. albicans* [83]. For instance, *CaPHR2*, the sole homolog of *ScGAS1*, a β-glucan transglycosylase, plays a role for concealing glucan in both organisms. In contrast, *ScSLT2*, a homolog of *CaMKC1,* is required for masking in *S. cerevisae*, but does not cause β (1,3)-glucan exposure when deleted in the pathogen.

Badrane *et al.* determined that sublethal concentrations of caspofungin treatment causes fundamental cell wall re-arrangements, but also leads to rapid responses at the plasma membrane in components related to the actin cytoskeleton [90]. This includes redistribution of both phosphatidylinositol-(4,5)-bisphosphate (PI (4,5) P_2), a signaling phospholipid, and septins, which serve as scaffolds for cytokinesis events [90]. PI (4,5) P_2 and septins are re-localized 5 mins after exposure to caspofungin, which represents an early event in response to drug treatment, and further re-direct chitin and cell wall proteins to deposit at the site of co-localization [90]. This response might be important for membrane localized cell wall repair proteins like chitin synthases to fix the damage caused by β (1,3)-glucan biosynthesis inhibition. In fact, chitin production also increases in response to caspofungin treatment [58, 81, 90].

Acidic pH induces cell wall structure remodeling

Sherrington *et al.* revealed that *C. albicans* adaptation to an acidic pH of 2 promotes cell wall remodeling, exposure of both chitin and β (1,3)-glucan, and reduced thickness of the outer mannan layer [77]. The mechanism by which β (1,3)-glucan exposure is induced by acidic pH remains to be fully defined. The

major signaling cascades such as the Mkc1 and Hog1 pathways are not involved in this pH driven glucan unmasking mechanism, suggesting a non-canonical cell wall remodeling pathway is involved. In contrast, Chitinase 2 (Cht2) regulates chitin exposure in a pH-dependent manner by cleaving the glyosidic bonds in the long-chain of chitin and allowing correct incorporation of chitin short fibril into the inner layer for better masking. In alkaline environments, *CHT2* expression is dependent on the transcription factors Bcr1 and Rim101, while in the acidic environment, the Rim101 signaling pathway is inactivated and Cht2 expression is repressed. Decreased Cht2 results in longer chitin fibrils and less efficient remodeling of the inner layer, thus enhancing exposure of chitin at the cell surface.

Physiological-relevant L-lactate enhances β (1,3)-glucan masking

Ballou *et al.* has reported that changes in carbon sources alters cell wall architecture, particularly by using physiologically-relevant levels of L-lactate as a carbon source that stimulates β (1,3)-glucan masking [80]. The G-protein coupled receptor Gpr1, the key lactate binding receptor in *C. albicans*, is found to sense environmental lactate and plays a role in lactate-driven β (1,3)-glucan masking [80]. While Gpr1 is the upstream regulator of cyclic adenosine monophosphate (cAMP) pathway responsible for morphogenesis in this organism, neither this pathway nor other key morphogenetic signaling modules like Efg1 or Cph1, are responsible for the cell wall remodeling in lactate condition [80, 91]. Instead, the transcription factor Crz1, which is important for cell wall integrity, antifungal resistance, and cation homeostasis, is found to mediate masking induced by lactate in a calcineurin-independent manner [80]. This is interesting since in *C. albicans* Crz1 is thought to be regulated by calcineurin when stimulated by exogenous Ca^{2+}, suggesting Gpr1 is another regulator of Crz1 activation that acts independently of the canonical pathway [92]. A significant number of cell wall genes are altered transcriptionally and some of these may facilitate the glucan masking process [80]. Strikingly, major changes in mannan distribution or disruption of several cell wall crosslinking enzymes do not appear to account for lactate-induced β (1,3)-glucan masking. For instance, deletion of *MNN22* (α(1,2)-

mannosyltransferase) or *EXG2* (exo-1,3-beta-glucosidase) does not block masking caused by lactate [80].

Contribution of mannan layer to β (1,3)-glucan (un)cloaking

A number of studies have been conducted to determine if structural alterations in the mannan layer result in β (1,3)-glucan (un)masking under various condition [77, 80]. Thus far, no definitive conclusion has been reached. Mnn4 is the regulator of mannosyl-phosphorylation of *N*-linked mannans to cell wall proteins [32] (Figure 1. 3). It is reasoned that disruption of *MNN4* might reduce the mannan complexity and therefore results in a porous mannan layer, which makes the glucan layer more accessible to the immune detection. However, loss of *MNN4* does not cause higher levels of β (1,3)-glucan exposure compared to the parental strain. Some other characteristics such as cell growth, morphogenesis, and fungal virulence are not affected either [61]. This suggests that loss of phosphomannan is not sufficient to cause glucan exposure. In contrast, the *MNN2* α (1,2)-mannosyltransferase family, responsible for the formation of *N*-glycan outer chains by addition of α (1,2)-mannose to the α (1,6)-backbone (Figure 1. 3), is required for maintaining β (1,3)-glucan cloaking. Loss of this family results in 70% reduction in the relative proportion of mannan in the cell wall, which then unmasks the β (1,3)-glucan, despite no changes in the overall β-glucan levels [34]. Och1, an important α (1,6)-mannosyltransferase that initiates the outer *N*-glycan chain branching (Figure 1. 3), plays a role in masking the underlying β-glucan layer. Loss of *OCH1* results in significantly increased amounts of β-glucan and reduced mannan contents, and also causes β-glucan architectural changes [55, 93]. This further indicates that glycosylation is essential for maintaining *C. albicans* cell wall structure and β (1,3)-glucan masking.

Most of the *N*- and *O*-linked glycosylation are glycosylphosphatidylinositol (GPI) anchored. Due to the requirement of cell wall protein modification by GPI remnants, it provides an important strategy to attack the fungal pathogen. A novel chemical compound named gepinacin is found to specifically inhibit the activity of a critical acyltransferase Gwt1 in yeast, which initiates inositol acylation of GPI

anchor biosynthesis within the ER [94]. Incubation of *C. albicans* wild-type cells with a sublethal dose of gepinacin impairs filamentation, increases β (1,3)-glucan exposure and strain immunogenicity which induces higher levels of TNF-α secretion from murine macrophages [95]. Alternatively, disruption of *CaGPI7*, a protein essential for GPI anchor biosynthesis, blocks the decoration of the second mannose in the glycan with phosphoethanolamine, and subsequently unmasks β (1,3)-glucan [44]. The *gpi7* deletion mutant further promotes the inflammatory response through the recruitment of immune cells in a Dectin1-dependent manner *in vivo* [44].

Summary

My dissertation focuses on the identification of signaling proteins that cause cell wall β (1,3)-glucan exposure in the fungal pathogen *C. albicans*, thus enhancing host immune responses. We previously found disruption of the synthesis of the phospholipid phosphatidylserine in *C. albicans* (*cho1Δ/Δ* mutation), then *cho1Δ/Δ* exhibits significantly increased exposure of β (1,3)-glucan to immune detection compared to wild-type. However, the mechanisms through which *CHO1* disruption results in β (1,3)-glucan exposure are not clear. In Chapter II, I used genetic approaches to evaluate the effect of cell wall associated signaling pathways on β (1,3)-glucan unmasking in *C. albicans*, and found Cek1 MAPK cascade is involved in the modulation of cell wall remodeling when over-activated. In Chapter III, I screened the upstream regulators of Cek1 and characterized the impact of Cek1 activity on fungal virulence. In Chapter IV, I performed RNA-sequencing analysis to assess the whole genome transcription profile when Cek1 is hyper-activated. In Chapter V, I concluded the findings in this dissertation and proposed future directions to continue studying the influence of Cek1 activation on fungal cell wall remodeling.

Reference

1. Bustamante CI. Treatment of Candida infection: a view from the trenches! Curr Opin Infect Dis. 2005;18(6):490-5. PubMed PMID: 16258321.
2. Eggimann P, Garbino J, Pittet D. Management of Candida species infections in critically ill patients. The Lancet Infectious diseases. 2003;3(12):772-85. PubMed PMID: 14652203.
3. Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD. Azole Antifungal Resistance in Candida albicans and Emerging No*N*-albicans Candida Species. Front Microbiol. 2016;7:2173. doi: 10.3389/fmicb.2016.02173. PubMed PMID: 28127295; PubMed Central PMCID: PMCPMC5226953.
4. Perlin DS. Mechanisms of echinocandin antifungal drug resistance. Ann N Y Acad Sci. 2015;1354:1-11. doi: 10.1111/nyas.12831. PubMed PMID: 26190298; PubMed Central PMCID: PMCPMC4626328.
5. Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of Candida albicans by the innate immune system. Nat Rev Microbiol. 2008;6(1):67-78. doi: 10.1038/nrmicro1815. PubMed PMID: 18079743.
6. Chen Y-L, Montedonico AE, Kauffman S, Dunlap JR, Menn F-M, Reynolds TB. Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence inCandida albicans. Molecular microbiology. 2010;75(5):1112-32. doi: 10.1111/j.1365-2958.2009.07018.x.
7. Norice CT, Smith FJ, Jr., Solis N, Filler SG, Mitchell AP. Requirement for Candida albicans Sun41 in biofilm formation and virulence. Eukaryotic cell. 2007;6(11):2046-55. doi: 10.1128/EC.00314-07. PubMed PMID: 17873081; PubMed Central PMCID: PMCPMC2168420.
8. Herrero AB, Magnelli P, Mansour MK, Levitz SM, Bussey H, Abeijon C. KRE5 gene null mutant strains of Candida albicans are avirulent and have altered cell wall composition and hypha formation properties. Eukaryotic cell. 2004;3(6):1423-32. doi: 10.1128/EC.3.6.1423-1432.2004. PubMed PMID: 15590817; PubMed Central PMCID: PMCPMC539026.
9. Ghannoum MA, Spellberg B, Saporito-Irwin SM, Fonzi WA. Reduced virulence of Candida albicans PHR1 mutants. Infection and immunity. 1995;63(11):4528-30. PubMed PMID: 7591097; PubMed Central PMCID: PMCPMC173646.
10. Mora-Montes HM, Netea MG, Ferwerda G, Lenardon MD, Brown GD, Mistry AR, et al. Recognition and blocking of innate immunity cells by Candida albicans chitin. Infection and immunity. 2011;79(5):1961-70. doi: 10.1128/IAI.01282-10. PubMed PMID: 21357722; PubMed Central PMCID: PMC3088140.
11. Hopke A, Nicke N, Hidu EE, Degani G, Popolo L, Wheeler RT. Neutrophil Attack Triggers Extracellular Trap-Dependent Candida Cell Wall Remodeling and Altered Immune Recognition. PLoS pathogens. 2016;12(5):e1005644. doi: 10.1371/journal.ppat.1005644. PubMed PMID: 27223610; PubMed Central PMCID: PMC4880299.
12. Netea MG, Joosten LAB, van der Meer JWM, Kullberg BJ, van de Veerdonk FL. Immune defence against Candida fungal infections. Nature Reviews Immunology. 2015;15(10):630-42. doi: 10.1038/nri3897. PubMed PMID: WOS:000361912600009.

13. Gow NA, Netea MG, Munro CA, Ferwerda G, Bates S, Mora-Montes HM, et al. Immune recognition of Candida albicans beta-glucan by Dectin-1. The Journal of infectious diseases. 2007;196(10):1565-71. doi: 10.1086/523110. PubMed PMID: 18008237; PubMed Central PMCID: PMCPMC2655640.
14. Levin DE. Regulation of cell wall biogenesis in Saccharomyces cerevisiae: the cell wall integrity signaling pathway. Genetics. 2011;189(4):1145-75. doi: 10.1534/genetics.111.128264. PubMed PMID: 22174182; PubMed Central PMCID: PMC3241422.
15. Orlean PA. (1,3)-beta-D-Glucan synthase from budding and filamentous cultures of the dimorphic fungus Candida albicans. Eur J Biochem. 1982;127(2):397-403. PubMed PMID: 6216107.
16. Mio T, Adachi-Shimizu M, Tachibana Y, Tabuchi H, Inoue SB, Yabe T, et al. Cloning of the Candida albicans homolog of Saccharomyces cerevisiae GSC1/FKS1 and its involvement in beta-1,3-glucan synthesis. Journal of bacteriology. 1997;179(13):4096-105. PubMed PMID: 9209021; PubMed Central PMCID: PMC179227.
17. Douglas CM, D'Ippolito JA, Shei GJ, Meinz M, Onishi J, Marrinan JA, et al. Identification of the FKS1 gene of Candida albicans as the essential target of 1,3-beta-D-glucan synthase inhibitors. Antimicrobial agents and chemotherapy. 1997;41(11):2471-9. PubMed PMID: 9371352; PubMed Central PMCID: PMC164147.
18. Suwunnakorn S, Wakabayashi H, Kordalewska M, Perlin DS, Rustchenko E. FKS2 and FKS3 Genes of Opportunistic Human Pathogen Candida albicans Influence Echinocandin Susceptibility. Antimicrobial agents and chemotherapy. 2018;62(4). doi: 10.1128/AAC.02299-17. PubMed PMID: 29358288; PubMed Central PMCID: PMCPMC5913916.
19. Qadota H, Python CP, Inoue SB, Arisawa M, Anraku Y, Zheng Y, et al. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. Science. 1996;272(5259):279-81. PubMed PMID: 8602515.
20. Kondoh O, Tachibana Y, Ohya Y, Arisawa M, Watanabe T. Cloning of the RHO1 gene from Candida albicans and its regulation of beta-1,3-glucan synthesis. Journal of bacteriology. 1997;179(24):7734-41. PubMed PMID: 9401032; PubMed Central PMCID: PMC179736.
21. Lussier M, Sdicu AM, Shahinian S, Bussey H. The Candida albicans KRE9 gene is required for cell wall beta-1, 6-glucan synthesis and is essential for growth on glucose. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(17):9825-30. doi: 10.1073/pnas.95.17.9825. PubMed PMID: 9707560; PubMed Central PMCID: PMCPMC21421.
22. Umeyama T, Kaneko A, Watanabe H, Hirai A, Uehara Y, Niimi M, et al. Deletion of the CaBIG1 gene reduces beta-1,6-glucan synthesis, filamentation, adhesion, and virulence in Candida albicans. Infection and immunity. 2006;74(4):2373-81. doi: 10.1128/IAI.74.4.2373-2381.2006. PubMed PMID: 16552067; PubMed Central PMCID: PMCPMC1418944.
23. Gow NAR, Latge JP, Munro CA. The Fungal Cell Wall: Structure, Biosynthesis, and Function. Microbiology spectrum. 2017;5(3). doi: 10.1128/microbiolspec.FUNK-0035-2016. PubMed PMID: 28513415.

24. Lenardon MD, Whitton RK, Munro CA, Marshall D, Gow NA. Individual chitin synthase enzymes synthesize microfibrils of differing structure at specific locations in the Candida albicans cell wall. Molecular microbiology. 2007;66(5):1164-73. doi: 10.1111/j.1365-2958.2007.05990.x. PubMed PMID: 17971081; PubMed Central PMCID: PMCPMC2780561.
25. Munro CA, Winter K, Buchan A, Henry K, Becker JM, Brown AJP, et al. Chs1 of Candida albicans is an essential chitin synthase required for synthesis of the septum and for cell integrity. Molecular microbiology. 2001;39(5):1414-26. doi: DOI 10.1046/j.1365-2958.2001.02347.x. PubMed PMID: WOS:000167644400027.
26. Gow NA, Robbins PW, Lester JW, Brown AJ, Fonzi WA, Chapman T, et al. A hyphal-specific chitin synthase gene (CHS2) is not essential for growth, dimorphism, or virulence of Candida albicans. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(13):6216-20. PubMed PMID: 8016141; PubMed Central PMCID: PMCPMC44169.
27. Bulawa CE, Miller DW, Henry LK, Becker JM. Attenuated virulence of chiti*N*-deficient mutants of Candida albicans. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(23):10570-4. PubMed PMID: 7479842; PubMed Central PMCID: PMCPMC40653.
28. Mio T, Yabe T, Sudoh M, Satoh Y, Nakajima T, Arisawa M, et al. Role of three chitin synthase genes in the growth of Candida albicans. Journal of bacteriology. 1996;178(8):2416-9. doi: 10.1128/jb.178.8.2416-2419.1996. PubMed PMID: 8636047; PubMed Central PMCID: PMCPMC177954.
29. Walker LA, Munro CA, de Bruijn I, Lenardon MD, McKinnon A, Gow NA. Stimulation of chitin synthesis rescues Candida albicans from echinocandins. PLoS pathogens. 2008;4(4):e1000040. doi: 10.1371/journal.ppat.1000040. PubMed PMID: 18389063; PubMed Central PMCID: PMC2271054.
30. Munro CA, Whitton RK, Hughes HB, Rella M, Selvaggini S, Gow NA. CHS8-a fourth chitin synthase gene of Candida albicans contributes to in vitro chitin synthase activity, but is dispensable for growth. Fungal genetics and biology : FG & B. 2003;40(2):146-58. PubMed PMID: 14516767.
31. Gow NAR, Gooday GW, Newsam RJ, Gull K. Ultrastructure of the Septum in Candida-Albicans. Curr Microbiol. 1980;4(6):357-9. doi: Doi 10.1007/Bf02605377. PubMed PMID: WOS:A1980LB58000008.
32. Murciano C, Moyes DL, Runglall M, Islam A, Mille C, Fradin C, et al. Candida albicans cell wall glycosylation may be indirectly required for activation of epithelial cell proinflammatory responses. Infection and immunity. 2011;79(12):4902-11. doi: 10.1128/IAI.05591-11. PubMed PMID: 21930756; PubMed Central PMCID: PMCPMC3232641.
33. van Wijlick L, Swidergall M, Brandt P, Ernst JF. Candida albicans responds to glycostructure damage by Ace2-mediated feedback regulation of Cek1 signaling. Molecular microbiology. 2016;102(5):827-49. doi: 10.1111/mmi.13494. PubMed PMID: 27589033.
34. Hall RA, Bates S, Lenardon MD, Maccallum DM, Wagener J, Lowman DW, et al. The Mnn2 mannosyltransferase family modulates mannoprotein fibril length, immune recognition and virulence of Candida albicans. PLoS pathogens.

2013;9(4):e1003276. doi: 10.1371/journal.ppat.1003276. PubMed PMID: 23633946; PubMed Central PMCID: PMCPMC3636026.
35. Martinez-Duncker I, Diaz-Jimenez DF, Mora-Montes HM. Comparative Analysis of Protein Glycosylation Pathways in Humans and the Fungal Pathogen Candida albicans. Int J Microbiol. 2014;2014:267497. doi: 10.1155/2014/267497. PubMed PMID: 25104959; PubMed Central PMCID: PMCPMC4106090.
36. Fabre E, Hurtaux T, Fradin C. Mannosylation of fungal glycoconjugates in the Golgi apparatus. Current opinion in microbiology. 2014;20:103-10. doi: 10.1016/j.mib.2014.05.008. PubMed PMID: 24934559.
37. Richard ML, Plaine A. Comprehensive analysis of glycosylphosphatidylinositol-anchored proteins in Candida albicans. Eukaryotic cell. 2007;6(2):119-33. doi: 10.1128/EC.00297-06. PubMed PMID: 17189485; PubMed Central PMCID: PMCPMC1797948.
38. Chaffin WL. Candida albicans cell wall proteins. Microbiology and molecular biology reviews : MMBR. 2008;72(3):495-544. doi: 10.1128/MMBR.00032-07. PubMed PMID: 18772287; PubMed Central PMCID: PMCPMC2546859.
39. Orlean P. Architecture and biosynthesis of the Saccharomyces cerevisiae cell wall. Genetics. 2012;192(3):775-818. doi: 10.1534/genetics.112.144485. PubMed PMID: 23135325; PubMed Central PMCID: PMCPMC3522159.
40. <1-s2.0-016756999290111J-main.pdf>.
41. Ariizumi K, Shen GL, Shikano S, Xu S, Ritter R, 3rd, Kumamoto T, et al. Identification of a novel, dendritic cell-associated molecule, Dectin-1, by subtractive cDNA cloning. The Journal of biological chemistry. 2000;275(26):20157-67. doi: 10.1074/jbc.M909512199. PubMed PMID: 10779524.
42. Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative induction of inflammatory responses by Dectin-1 and Toll-like receptor 2. J Exp Med. 2003;197(9):1107-17. doi: 10.1084/jem.20021787. PubMed PMID: 12719479; PubMed Central PMCID: PMCPMC2193968.
43. Gantner BN, Simmons RM, Underhill DM. Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. Embo Journal. 2005;24(6):1277-86. doi: 10.1038/sj.emboj.7600594. PubMed PMID: WOS:000228327000018.
44. Shen H, Chen SM, Liu W, Zhu F, He LJ, Zhang JD, et al. Abolishing Cell Wall Glycosylphosphatidylinositol-Anchored Proteins in Candida albicans Enhances Recognition by Host Dectin-1. Infection and immunity. 2015;83(7):2694-704. doi: 10.1128/IAI.00097-15. PubMed PMID: 25895969; PubMed Central PMCID: PMC4468527.
45. Marakalala MJ, Vautier S, Potrykus J, Walker LA, Shepardson KM, Hopke A, et al. Differential adaptation of Candida albicans in vivo modulates immune recognition by Dectin-1. PLoS pathogens. 2013;9(4):e1003315. doi: 10.1371/journal.ppat.1003315. PubMed PMID: 23637604; PubMed Central PMCID: PMC3630191.
46. Kennedy AD, Willment JA, Dorward DW, Williams DL, Brown GD, DeLeo FR. Dectin-1 promotes fungicidal activity of human neutrophils. Eur J Immunol. 2007;37(2):467-78. doi: 10.1002/eji.200636653. PubMed PMID: 17230442.

47. van Bruggen R, Drewniak A, Jansen M, van Houdt M, Roos D, Chapel H, et al. Complement receptor 3, not Dectin-1, is the major receptor on human neutrophils for beta-glucan-bearing particles. Mol Immunol. 2009;47(2-3):575-81. doi: 10.1016/j.molimm.2009.09.018. PubMed PMID: 19811837.
48. Rappleye CA, Eissenberg LG, Goldman WE. Histoplasma capsulatum alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(4):1366-70. doi: 10.1073/pnas.0609848104. PubMed PMID: 17227865; PubMed Central PMCID: PMCPMC1783108.
49. Garfoot AL, Shen Q, Wuthrich M, Klein BS, Rappleye CA. The Eng1 beta-Glucanase Enhances Histoplasma Virulence by Reducing beta-Glucan Exposure. MBio. 2016;7(2):e01388-15. doi: 10.1128/mBio.01388-15. PubMed PMID: 27094334; PubMed Central PMCID: PMCPMC4850272.
50. Gravelat FN, Beauvais A, Liu H, Lee MJ, Snarr BD, Chen D, et al. Aspergillus galactosaminogalactan mediates adherence to host constituents and conceals hyphal beta-glucan from the immune system. PLoS pathogens. 2013;9(8):e1003575. doi: 10.1371/journal.ppat.1003575. PubMed PMID: 23990787; PubMed Central PMCID: PMCPMC3749958.
51. Wheeler RT, Kombe D, Agarwala SD, Fink GR. Dynamic, morphotype-specific Candida albicans beta-glucan exposure during infection and drug treatment. PLoS pathogens. 2008;4(12):e1000227. doi: 10.1371/journal.ppat.1000227. PubMed PMID: 19057660; PubMed Central PMCID: PMC2587227.
52. Lowman DW, Greene RR, Bearden DW, Kruppa MD, Pottier M, Monteiro MA, et al. Novel structural features in Candida albicans hyphal glucan provide a basis for differential innate immune recognition of hyphae versus yeast. The Journal of biological chemistry. 2014;289(6):3432-43. doi: 10.1074/jbc.M113.529131. PubMed PMID: 24344127; PubMed Central PMCID: PMCPMC3916545.
53. Majtan J, Jesenak M. beta-Glucans: Multi-Functional Modulator of Wound Healing. Molecules. 2018;23(4). doi: 10.3390/molecules23040806. PubMed PMID: 29614757; PubMed Central PMCID: PMCPMC6017669.
54. Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, et al. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol. 2007;8(1):31-8. doi: DOI 10.1038/ni1408. PubMed PMID: WOS:000242934400011.
55. Sem X, Le GT, Tan AS, Tso G, Yurieva M, Liao WW, et al. beta-glucan Exposure on the Fungal Cell Wall Tightly Correlates with Competitive Fitness of Candida Species in the Mouse Gastrointestinal Tract. Front Cell Infect Microbiol. 2016;6:186. doi: 10.3389/fcimb.2016.00186. PubMed PMID: 28066722; PubMed Central PMCID: PMCPMC5177745.
56. Wagener J, Malireddi RK, Lenardon MD, Koberle M, Vautier S, MacCallum DM, et al. Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. PLoS pathogens. 2014;10(4):e1004050. doi: 10.1371/journal.ppat.1004050. PubMed PMID: 24722226; PubMed Central PMCID: PMC3983064.

57. Da Silva CA, Chalouni C, Williams A, Hartl D, Lee CG, Elias JA. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. J Immunol. 2009;182(6):3573-82. doi: 10.4049/jimmunol.0802113. PubMed PMID: 19265136.
58. Davis SE, Hopke A, Minkin SC, Jr., Montedonico AE, Wheeler RT, Reynolds TB. Masking of beta(1-3)-glucan in the cell wall of Candida albicans from detection by innate immune cells depends on phosphatidylserine. Infection and immunity. 2014. doi: 10.1128/IAI.01612-14. PubMed PMID: 25114110.
59. Wagener J, Weindl G, de Groot PW, de Boer AD, Kaesler S, Thavaraj S, et al. Glycosylation of Candida albicans cell wall proteins is critical for induction of innate immune responses and apoptosis of epithelial cells. PloS one. 2012;7(11):e50518. doi: 10.1371/journal.pone.0050518. PubMed PMID: 23226301; PubMed Central PMCID: PMCPMC3511564.
60. Netea MG, Gow NA, Munro CA, Bates S, Collins C, Ferwerda G, et al. Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J Clin Invest. 2006;116(6):1642-50. doi: 10.1172/JCI27114. PubMed PMID: 16710478; PubMed Central PMCID: PMCPMC1462942.
61. Hobson RP, Munro CA, Bates S, MacCallum DM, Cutler JE, Heinsbroek SE, et al. Loss of cell wall mannosylphosphate in Candida albicans does not influence macrophage recognition. The Journal of biological chemistry. 2004;279(38):39628-35. doi: 10.1074/jbc.M405003200. PubMed PMID: 15271989.
62. Hall RA, Gow NA. Mannosylation in Candida albicans: role in cell wall function and immune recognition. Molecular microbiology. 2013;90(6):1147-61. doi: 10.1111/mmi.12426. PubMed PMID: 24125554; PubMed Central PMCID: PMCPMC4112839.
63. Cullen PJ, Edgerton M. Unmasking fungal pathogens by studying MAPK-dependent cell wall regulation in Candida albicans. Virulence. 2016;7(5):502-5. doi: 10.1080/21505594.2016.1177695. PubMed PMID: 27088569; PubMed Central PMCID: PMC5026791.
64. Galan-Diez M, Arana DM, Serrano-Gomez D, Kremer L, Casasnovas JM, Ortega M, et al. Candida albicans beta-glucan exposure is controlled by the fungal CEK1-mediated mitogen-activated protein kinase pathway that modulates immune responses triggered through Dectin-1. Infection and immunity. 2010;78(4):1426-36. doi: 10.1128/IAI.00989-09. PubMed PMID: 20100861; PubMed Central PMCID: PMC2849429.
65. Roman E, Cottier F, Ernst JF, Pla J. Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in Candida albicans. Eukaryotic cell. 2009;8(8):1235-49. doi: 10.1128/EC.00081-09. PubMed PMID: 19542310; PubMed Central PMCID: PMC2725568.
66. Monge RA, Roman E, Nombela C, Pla J. The MAP kinase signal transduction network in Candida albicans. Microbiology. 2006;152(Pt 4):905-12. doi: 10.1099/mic.0.28616-0. PubMed PMID: 16549655.
67. Roman E, Correia I, Salazin A, Fradin C, Jouault T, Poulain D, et al. The Cek1-mediated MAP kinase pathway regulates exposure of-1,2 and-1,2-mannosides in the cell wall of Candida albicans modulating immune recognition.

Virulence. 2016;7(5):558-77. doi: 10.1080/21505594.2016.1163458. PubMed PMID: WOS:000380007500011.
68. Ramirez-Zavala B, Weyler M, Gildor T, Schmauch C, Kornitzer D, Arkowitz R, et al. Activation of the Cph1-dependent MAP kinase signaling pathway induces white-opaque switching in Candida albicans. PLoS pathogens. 2013;9(10):e1003696. doi: 10.1371/journal.ppat.1003696. PubMed PMID: 24130492; PubMed Central PMCID: PMC3795047.
69. Roman E, Alonso-Monge R, Gong Q, Li D, Calderone R, Pla J. The Cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen Candida albicans. FEMS yeast research. 2009;9(6):942-55. doi: 10.1111/j.1567-1364.2009.00545.x. PubMed PMID: 19656200.
70. Chen T, Jackson JW, Tams RN, Davis SE, Sparer TE, Reynolds TB. Exposure of Candida albicans beta (1,3)-glucan is promoted by activation of the Cek1 pathway. PLoS genetics. 2019;15(1):e1007892. doi: 10.1371/journal.pgen.1007892. PubMed PMID: 30703081.
71. Li R, Puri S, Tati S, Cullen PJ, Edgerton M. Candida albicans Cek1 mitogen-activated protein kinase signaling enhances fungicidal activity of salivary histatin 5. Antimicrobial agents and chemotherapy. 2015;59(6):3460-8. doi: 10.1128/AAC.00214-15. PubMed PMID: 25824232; PubMed Central PMCID: PMC4432120.
72. Puri S, Kumar R, Chadha S, Tati S, Conti HR, Hube B, et al. Secreted aspartic protease cleavage of Candida albicans Msb2 activates Cek1 MAPK signaling affecting biofilm formation and oropharyngeal candidiasis. PloS one. 2012;7(11):e46020. doi: 10.1371/journal.pone.0046020. PubMed PMID: 23139737; PubMed Central PMCID: PMC3491010.
73. Csank C, Schroppel K, Leberer E, Harcus D, Mohamed O, Meloche S, et al. Roles of the Candida albicans mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infection and immunity. 1998;66(6):2713-21. PubMed PMID: 9596738; PubMed Central PMCID: PMCPMC108260.
74. Guhad FA, Jensen HE, Aalbaek B, Csank C, Mohamed O, Harcus D, et al. Mitogen-activated protein kinase-defective Candida albicans is avirulent in a novel model of localized murine candidiasis. FEMS microbiology letters. 1998;166(1):135-9. PubMed PMID: 9741091.
75. Galan-Diez M, Arana DM, Serrano-Gomez D, Kremer L, Casasnovas JM, Ortega M, et al. Candida albicans beta-Glucan Exposure Is Controlled by the Fungal CEK1-Mediated Mitogen-Activated Protein Kinase Pathway That Modulates Immune Responses Triggered through Dectin-1. Infection and immunity. 2010;78(4):1426-36. doi: 10.1128/iai.00989-09.
76. Cantero PD, Ernst JF. Damage to the glycoshield activates PMT-directed O-mannosylation via the Msb2-Cek1 pathway in Candida albicans. Molecular microbiology. 2011;80(3):715-25. doi: 10.1111/j.1365-2958.2011.07604.x. PubMed PMID: 21375589.
77. Sherrington SL, Sorsby E, Mahtey N, Kumwenda P, Lenardon MD, Brown I, et al. Adaptation of Candida albicans to environmental pH induces cell wall remodelling and enhances innate immune recognition. PLoS pathogens.

2017;13(5):e1006403. doi: 10.1371/journal.ppat.1006403. PubMed PMID: 28542528; PubMed Central PMCID: PMC5456412.
78. Tsai PW, Yang CY, Chang HT, Lan CY. Characterizing the role of cell-wall beta-1,3-exoglucanase Xog1p in Candida albicans adhesion by the human antimicrobial peptide LL-37. PloS one. 2011;6(6):e21394. doi: 10.1371/journal.pone.0021394. PubMed PMID: 21713010; PubMed Central PMCID: PMCPMC3119699.
79. Liu H, Kohler J, Fink GR. Suppression of hyphal formation in Candida albicans by mutation of a STE12 homolog. Science. 1994;266(5191):1723-6. PubMed PMID: 7992058.
80. Ballou ER, Avelar GM, Childers DS, Mackie J, Bain JM, Wagener J, et al. Lactate signalling regulates fungal beta-glucan masking and immune evasion. Nature microbiology. 2016;2:16238. doi: 10.1038/nmicrobiol.2016.238. PubMed PMID: 27941860.
81. Hasim S, Allison DP, Retterer ST, Hopke A, Wheeler RT, Doktycz MJ, et al. beta-(1,3)-Glucan Unmasking in Some Candida albicans Mutants Correlates with Increases in Cell Wall Surface Roughness and Decreases in Cell Wall Elasticity. Infection and immunity. 2017;85(1). doi: 10.1128/IAI.00601-16. PubMed PMID: 27849179; PubMed Central PMCID: PMC5203643.
82. Saito K, Fujimura-Kamada K, Hanamatsu H, Kato U, Umeda M, Kozminski KG, et al. Transbilayer phospholipid flipping regulates Cdc42p signaling during polarized cell growth via Rga GTPase-activating proteins. Developmental cell. 2007;13(5):743-51. doi: 10.1016/j.devcel.2007.09.014. PubMed PMID: 17981141.
83. Wheeler RT, Fink GR. A Drug-Sensitive Genetic Network Masks Fungi from the Immune System. PLoS pathogens. 2006;2(4):e35. doi: 10.1371/journal.ppat.0020035.
84. Huang H, Harcus D, Whiteway M. Transcript profiling of a MAP kinase pathway in C. albicans. Microbiol Res. 2008;163(4):380-93. doi: 10.1016/j.micres.2008.03.001. PubMed PMID: 18467082.
85. Kamada Y, Qadota H, Python CP, Anraku Y, Ohya Y, Levin DE. Activation of yeast protein kinase C by Rho1 GTPase. The Journal of biological chemistry. 1996;271(16):9193-6. PubMed PMID: 8621575.
86. Diez-Orejas R, Molero G, Navarro-Garcia F, Pla J, Nombela C, Sanchez-Perez M. Reduced virulence of Candida albicans MKC1 mutants: a role for mitogen-activated protein kinase in pathogenesis. Infection and immunity. 1997;65(2):833-7. PubMed PMID: 9009353; PubMed Central PMCID: PMCPMC176136.
87. Roman E, Alonso-Monge R, Miranda A, Pla J. The Mkk2 MAPKK Regulates Cell Wall Biogenesis in Cooperation with the Cek1-Pathway in Candida albicans. PloS one. 2015;10(7):e0133476. doi: 10.1371/journal.pone.0133476. PubMed PMID: 26197240; PubMed Central PMCID: PMC4509911.
88. Alonso-Monge R, Real E, Wojda I, Bebelman JP, Mager WH, Siderius M. Hyperosmotic stress response and regulation of cell wall integrity in Saccharomyces cerevisiae share common functional aspects. Molecular microbiology. 2001;41(3):717-30. PubMed PMID: 11532139.

89. Alonso-Monge R, Navarro-Garcia F, Molero G, Diez-Orejas R, Gustin M, Pla J, et al. Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of Candida albicans. Journal of bacteriology. 1999;181(10):3058-68. PubMed PMID: 10322006; PubMed Central PMCID: PMCPMC93760.
90. Badrane H, Nguyen MH, Blankenship JR, Cheng S, Hao B, Mitchell AP, et al. Rapid redistribution of phosphatidylinositol-(4,5)-bisphosphate and septins during the Candida albicans response to caspofungin. Antimicrobial agents and chemotherapy. 2012;56(9):4614-24. doi: 10.1128/AAC.00112-12. PubMed PMID: 22687514; PubMed Central PMCID: PMCPMC3421880.
91. Miwa T, Takagi Y, Shinozaki M, Yun CW, Schell WA, Perfect JR, et al. Gpr1, a putative G-protein-coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus Candida albicans. Eukaryotic cell. 2004;3(4):919-31. doi: 10.1128/EC.3.4.919-931.2004. PubMed PMID: 15302825; PubMed Central PMCID: PMCPMC500877.
92. Karababa M, Valentino E, Pardini G, Coste AT, Bille J, Sanglard D. CRZ1, a target of the calcineurin pathway in Candida albicans. Molecular microbiology. 2006;59(5):1429-51. doi: 10.1111/j.1365-2958.2005.05037.x. PubMed PMID: 16468987.
93. Bates S, Hughes HB, Munro CA, Thomas WP, MacCallum DM, Bertram G, et al. Outer chain *N*-glycans are required for cell wall integrity and virulence of Candida albicans. The Journal of biological chemistry. 2006;281(1):90-8. doi: 10.1074/jbc.M510360200. PubMed PMID: 16263704.
94. Umemura M, Okamoto M, Nakayama K, Sagane K, Tsukahara K, Hata K, et al. GWT1 gene is required for inositol acylation of glycosylphosphatidylinositol anchors in yeast. The Journal of biological chemistry. 2003;278(26):23639-47. doi: 10.1074/jbc.M301044200. PubMed PMID: 12714589.
95. McLellan CA, Whitesell L, King OD, Lancaster AK, Mazitschek R, Lindquist S. Inhibiting GPI anchor biosynthesis in fungi stresses the endoplasmic reticulum and enhances immunogenicity. ACS Chem Biol. 2012;7(9):1520-8. doi: 10.1021/cb300235m. PubMed PMID: 22724584.

CHAPTER II: Unmasking of *Candida albicans* β (1,3)-glucan Is Promoted by Activation of the Cek1 Pathway

A version of this chapter was originally published by Tian Chen, Joseph W. Jackson, Robert N. Tams, Sarah E. Davis, Timothy E. Sparer, Todd B. Reynolds

Tian Chen, Joseph W. Jackson, Robert N. Tams, Sarah E. Davis, Timothy E. Sparer, Todd B. Reynolds*. Unmasking of *Candida albicans* β (1,3)-glucan is promoted by activation of the Cek1 pathway. *PLoS Genetics* 2019,15(1): e1007892.

This article was not revised for inclusion in the present dissertation. The author contributions are as follows: Conceptualization: TC, TBR. Formal analysis: TC, TBR. Funding acquisition: TBR. Investigation: TC, SED. Methodology: TC, JWJ, SED, TES. Project administration: TBR. Resources: RNT, TES, TBR. Supervision: TBR. Validation: TC. Visualization: TC. Writing – original draft: TC. Writing – review & editing: TC, TBR. In particular, flow cytometry in Figure 2. 3B and C were performed by JWJ, ELISA in Figure 2. 3D was performed by RNT. All the other experiments were performed by TC.

Abstract

Candida albicans is among the most common causes of human fungal infections and is an important source of mortality. *C. albicans* is able to diminish its detection by innate immune cells through masking of β (1,3)-glucan in the inner cell wall with an outer layer of heavily glycosylated mannoproteins (mannan). However, mutations or drugs that disrupt the cell wall can lead to exposure of β (1,3)-glucan (unmasking) and enhanced detection by innate immune cells through receptors like Dectin-1, the C-type signaling lectin. Previously, our lab showed that the pathway for synthesizing the phospholipid phosphatidylserine (PS) plays a role in β (1,3)-glucan masking. The homozygous PS synthase knockout mutant, *cho1Δ/Δ*, exhibits increased exposure of β (1,3)-glucan. Several Mitogen Activated Protein Kinase (MAPK) pathways and their upstream Rho-type small GTPases are important for regulating cell wall biogenesis and remodeling. In the *cho1Δ/Δ* mutant, both the Cek1 and Mkc1 MAPKs are constitutively activated, and they act downstream of the small GTPases Cdc42 and Rho1, respectively. In addition, Cdc42 activity is up-regulated in *cho1Δ/Δ*. Thus, it was hypothesized that activation of Cdc42 or Rho1 and their downstream kinases cause unmasking. Disruption of *MKC1* does not decrease unmasking in *cho1 Δ/Δ*, and hyperactivation of Rho1 in wild-type cells increases unmasking and activation of both Cek1 and Mkc1. Moreover, independent hyperactivation of the MAP kinase kinase kinase Ste11 in wild-type cells leads to Cek1 activation and increased β (1,3)-glucan unmasking. Thus, upregulation of the Cek1 MAPK pathway causes unmasking, and may be responsible for unmasking in *cho1 Δ/Δ*.

Introduction

Candida albicans is a human commensal that is part of the natural flora of the oral, genital and gastrointestinal tracts. *Candida* species are also the most common fungal pathogens of humans and cause diseases ranging from superficial infections of mucosal surfaces to severe systemic bloodstream infections in immune-compromised patients [1-4], with a mortality rate of approximately 30% [2]. Three major classes of antifungals are used to treat systemic infections including azoles, echinocandins, and polyenes [5-7]. However, drug resistance or toxicity has put limits on these agents.

The *C. albicans* cell wall is considered a good therapeutic drug target due to its role in fungal pathogenicity as it presents important virulence factors, antigenic cell wall proteins and polysaccharides, and serves as the intermediate for fungal-host interactions [3, 8, 9]. One potential method for improving anti-fungal strategies could be to enhance the detection of fungal cell wall antigens by host immune cells. A major innate immune receptor for fungi like *C. albicans* is Dectin-1, a C-type signaling lectin that can recognize β (1,3)-glucan, which is an important component of fungal cell walls [8, 10, 11]. This recognition can initiate protective antifungal immune responses in innate immune cells like macrophages, dendritic cells and neutrophils. The fungal cell wall consists of an inner layer that is enriched in β (1,3)-glucan and underlying chitin, and an outer layer of mannosylated proteins [8]. Under normal conditions, *C. albicans* masks β (1,3)-glucan from Dectin-1 detection via the outer layer of mannosylated proteins [12, 13]. However, unmasking of β (1,3)-glucan can be induced through treatments with drugs such as echinocandins [12] or by certain genetic mutations that disrupt cell wall integrity [12-15].

It has been previously reported that the phosphatidylserine (PS) synthase enzyme (Cho1) controls cell wall β (1,3)-glucan exposure [13]. Phospholipids are crucial components of cellular membranes in eukaryotes. Cho1 synthesizes PS that can act as an end product, but also can be further decarboxylated to form phosphatidylethanolamine (PE). PS and PE are both essential for *C. albicans* virulence [16]. We found that the homozygous *CHO1* mutant, *cho1Δ/Δ,* exhibits

greater β (1,3)-glucan exposure compared to wild-type [13, 14]. This exposure allows increased recognition by Dectin-1 and elicits a stronger pro-inflammatory response [13, 14, 17]. However, the detailed mechanism by which β (1,3)-glucan exposure is caused by *CHO1* disruption remains unknown.

The process of cell wall biogenesis and remodeling is governed through complex signaling pathways, including several mitogen-activated protein kinase (MAPK) cascades and their upstream Rho-type GTPases (Figure 2. 1). MAPK pathways are conserved signaling cascades in eukaryotes that are important for dealing with a wide range of stimuli, including osmotic stress, oxidative stress, cell wall damage, and changes in glycosylation [9, 15, 17-19]. This signaling cascade is composed of a conserved module of three kinases: the MAP kinase kinase kinase (MAPKKK), the MAP kinase kinase (MAPKK) and the MAP kinase (MAPK). The MAPK activates downstream transcription factors and effectors to initiate gene expression for better adaptation to the environment [19]. Among these MAPK pathways, Ste11-Hst7-Cek1 composes the Cek1 MAPK cascade, and is reported to control β (1,3)-glucan masking in *C. albicans* [15, 20, 21]. *CEK1* null mutants display unmasking of β (1,3)-glucan and hyper-sensitivity to agents that disturb the cell wall such as Congo red [15]. The Mkc1 MAPK route, consisting of Bck1-Mkk2-Mkc1, is primarily involved in cell wall construction, as well as responding to exogenous cell wall stress, oxidative stimuli, antifungal drugs, and low-temperature shocks [22, 23]. Yet, this pathway does not appear to be required for masking in *C. albicans* [24], although it is hypersensitive to specific cell wall insults such as echinocandins or calcofluor white.

The upstream small GTPases Cdc42 and Rho1 transmit the signal toward Cek1- and Mkc1- associated MAPK cascades, respectively (Figure 2. 1) [18, 23]. They are also important in remodeling the rigid structure of the cell wall during vegetative growth and during pheromone-induced morphogenesis [25]. Rho1 is a well-known major regulator of the cell wall integrity signaling cascade through several downstream effectors [23, 25-30]. Rho1 is also the regulatory subunit of β (1,3)-glucan synthase, and therefore directly controls cell wall biosynthesis via the binding and activation of its catalytic subunits, such as Fks1 [26, 31]. Cdc42 is

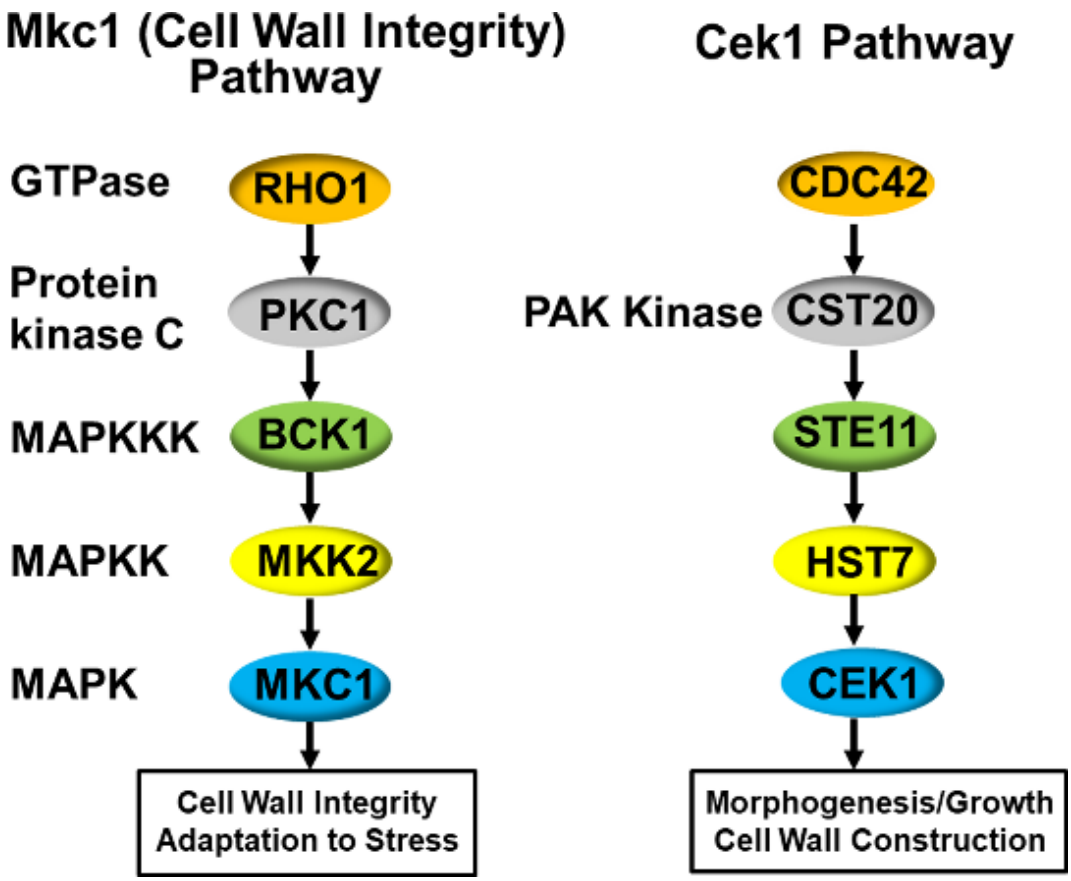


Figure 2. 1. The Cek1 and Mkc1 MAP kinase signaling cascades in *C. albicans* are involved in cell wall biogenesis.

The Cek1 and Mkc1 MAP kinase cascades, and their respective upstream activator signaling proteins are shown. Rho1 activates protein kinase C (Pkc1), which activates the Mkc1 MAP kinase cascade. Cdc42 activates the PAK kinase Cst20 which activates the Cek1 MAP kinase cascade.

essential for cellular polarized growth through a variety of downstream effector proteins in *C. albicans*, including the actin cytoskeleton regulator Bni1, and kinases such as PAK kinase family members Cst20/Cla4 [32-37].

Given the role of the GTPase-associated signaling pathways in cell wall remodeling and regulation, we studied the impact of these signaling routes in affecting β (1,3)-glucan masking in the *C. albicans cho1Δ/Δ* PS synthase mutant. We found that in the *cho1Δ/Δ* mutant there is upregulation of the activity of both Cek1 and Mkc1 MAPKs. Furthermore, we present data indicating that activation of the Cek1 pathway, in particular, is sufficient to cause β (1,3)-glucan exposure in the *cho1 Δ/Δ* mutant.

Methods

Strains and growth media

All of the strains and plasmids used for these experiments are described in Table S.2. 1 and Table S.2. 2. All the primers used in this study were described in Table S.2. 3. The medium used to culture strains was yeast extract-peptone-dextrose (YEPD) medium (1% yeast extract, 2% peptone, and 2% dextrose (Thermo Fisher Scientific) (unless otherwise stated) [38]. To express the gene from the promoter of the gene for ATP sulfurylase (*MET3*), SD minimal medium (2% dextrose, 0.67% Yeast nitrogen base without amino acids) with 1mM ethanolamine (to support *cho1Δ/Δ*) was used [39]. For the induction of genes under the control of the *MAL2* maltase promoter, YPM (1% yeast extract, 2% peptone, and 2% maltose (Thermo Fisher Scientific)) [40] was used. To induce hyphal formation, cells were sub-cultured in Gibco RPMI 1640 medium (Thermo Fisher Scientific).

Strain construction

For the generation of strains that conditionally express green fluorescent protein (GFP) tagged Cdc42 under the regulation of P_{MET3}, we used the plasmid pTC1 composed of P_{MET3}-*yEGFP-CDC42*, which was constructed as follows. *yEGFP* was amplified with primers TRO988+TRO989 with *BamHI* flanking at both ends, and *CDC42* was amplified from SC5314 genomic DNA with primers

TRO995+TRO996 with *SacI* flanking both ends. Both fragments were cloned into pYLC314 [41] 3-end to the *MET3* promoter, where *yEGFP* is in frame and 5-end to *CDC42* and connected by a sequence that encodes an 8-alanine linker. $CDC42^{K183\text{-}187Q}$ was chemically synthesized by Genescript Inc. with SacI sites. It was used to replace wild-type *CDC42* in pTC1 to create pTC14. The pYLC314 plasmid was made by amplifying a $SAT1\text{-}P_{MET3}$ fragment from pYLC229 [41] with primers JCO165 + JCO166 that introduced *EcoRI* sites and cloning it into the *EcoRI* site in pBluescript II SK+. Both pTC1 and pTC14 were linearized within P_{MET3} using *AflII*, and then transformed into the strains of interests by electroporation. The transformation methods have been previously described [42]. TCO1+TCO2 were used to validate if the fragment was integrated into P_{MET3} locus in the chromosome.

To generate the strain CaTC11 that conditionally expresses $STE11^{\Delta N467}$ under the regulation of *MAL2* promoter, we constructed the plasmid pTC20 that consists of P_{MAL2}- $STE11^{\Delta N467}$-T_{TEF3}. First, pTC19 containing the P_{MAL2} promoter was generated from pYLC314 [41]. The P_{MET3} was removed from pYLC314 by restriction with *PstI*. The regulatable promoter P_{MAL2} was amplified from wild-type *C. albicans* genomic DNA using the primers TCO36+TCO37. The promoter was then cloned into the *PstI* site of the linearized pYLC314 to create pTC19. Then, the hyperactive domain of $STE11^{\Delta N467}$ with a *TEF3* terminator was amplified from the $P_{TET\text{-}ON}$ - $STE11^{\Delta N467}$ plasmid (generously provided by Dr. Joachim Morschhausser) [43] with primers TCO42+TCO43, with BamHI at both ends and it was cloned into pTC19. The resultant pTC20 was then linearized with *XcmI* and transformed into wild-type SC5314.

To generate the heterozygous deletion of *PKC1*, ~500 bp 5- and 3-untranslated regions (UTRs) flanking *PKC1* were amplified as *KpnI-Xho*I and *NotI-SacI* fragments, by using the primers TCO59+TCO60 and TCO62+TCO110, respectively. The 5- and 3- UTR fragments were cloned into the corresponding sites of the SAT1 flipper in the plasmid pSFS2A [44], respectively. The resulting plasmid pTC041 was digested with *KpnI* and *SacI*, and the larger fragment was gel purified (Qiagen Inc.) and transformed into the strains of interest. The

nourseothricin-resistant transformants were further cultured in medium containing maltose as a carbon source to induce *caFLP* expression, which is under *MAL2* promoter regulation. The confirmation of a *PKC1* allele being deleted was verified with TCO63+JCO95.

To introduce the Q67L mutation into *RHO1,* first the *RHO1* gene was PCR amplified with primers TCO8+TCO72 from SC5314 genomic DNA as a *SacI-NotI* fragment and cloned into vector pBT1 to generate pTC35. To generate the pBT1 plasmid, the *MET3* promoter was removed from pYLC314 by cutting with PstI. The *ENO1* promoter was amplified with primers BTO30+BTO31 as a *BamHI-NotI* fragment and cloned into pYLC314 to generate pBT1. Then primers TCO74+TCO75 were used to create pTC38 from pTC35 by point mutating *RHO1* using site-directed mutagenesis. A similar site directed mutagenesis protocol was used to introduce the G12V mutation into *CDC42.* TRO996+TCO101 were used for *CDC42* PCR amplification and it was cloned into pBT1 to create pTC34. Then TCO76+TCO77 were used to introduce the G12V mutation to *CDC42* to result in pTC37 which expresses P_{ENO1}-$CDC42^{G12V}$. pTC37 and pTC38 were linearized with *MscI* and transformed into SC5314.

To create the strain that has GFPCRIB (Cdc42-Rac1 interactive binding motif) to probe the localization of GTP-Cdc42, *RAC1* was disrupted in SC5314 using *C. albicans* CRISPR-Cas9. This was to exclude GFPCRIB binding with Rac1. Primers TCO38+TCO39 were annealed to generate a *RAC1* sgRNA flanked with BsmbI, which were then ligated with the deletion construct pV1393 [45] to create the plasmid pTC18. TCO40+TCO41 were used to generate the repair template that spanned the Cas9 cut site in order to introduce 3 stop codons to prevent *RAC1* expression and a new *HindIII* cut site to allow quick genotyping of the transformants. pTC18 was digested with *KpnI* and *SacI*, and the larger fragment was transformed into both SC5314 and *cho1Δ/Δ* together with repair template. TCO51+TCO52 were used for colony PCR to test if the repair template had integrated into the Cas9 cut site. The positive *rac1Δ/Δ* transformant was then transformed with linearized pTC029. The pTC029 plasmid was created from pExpArg-pADH1CRIBGFP [46] by cutting it with NotI and ligating it with a *Candida-*

adapted hygromycin B resistance marker amplified with primers TCO92/TCO93 from pRB436 (a gift from Dr. Richard Bennett). CRISPR/Cas9 was also utilized to knock out *MKC1* and *PKC1* in the strains of interests. TCO32+TCO33 and TCO44+TCO45 were used to generate the sgRNA for *MKC1* and *PKC1*, respectively. TCO34+TCO35, TCO46+TCO47 were used to create the repair templates for *MKC1* and *PKC1* with *EcoRI* and *BglII* introduced, respectively. TCO28+TCO29 and TCO49+TCO50 were used for colony PCR on transformants respectively to check if the repair template integrated into the Cas9-cut region.

To generate strains that have GFPRID (Rho1 Interactive Domain) to probe the localization of GTP-Rho1, each strain was transformed with pTC033. The pTC033 plasmid consists of pExpArg-pACT1GFPRID [46] with a SAT1 marker cloned into the Not I restriction site following amplification with TCO15+TCO16. The pTC033 plasmid was linearized with StuI and transformed into the strains of interest.

Western blotting

Cells were grown overnight in liquid YPD at 30°C, diluted to an OD_{600} of 0.2 in fresh YPD medium and allowed to grow for 3 hours. For the *STE11*$^{\Delta N467}$ strain under the *MAL2* promoter regulation, cells were grown overnight in liquid YPM at 30°C and diluted back to OD_{600} of 0.1 into fresh YPM medium and grown to log phase. Cells were pelleted by centrifugation and resuspended in 250µl phosphate buffered saline (PBS) supplemented with protease inhibitor cocktail (PMSF, leupeptin, and pepstatin (RPI, Corp., Mount Prospect), complete Protease Inhibitor tablet and PhosStop Phosphatase Inhibitor tablet (Roche Diagnostics GmbH, Mannheim, Germany). An equal volume of 150-212µm acid-washed beads (Sigma Aldrich, MO, USA) was added to each tube. Cells were mechanically disrupted in a Biospec Mini-BeadBeater (Bio Spec Product Inc., USA) with 6 rounds of 1min homogenization at 4°C and 1min intervals for each cycle. Samples were centrifuged at 5,000×rpm for 10 min at 4°C, the supernatant was collected, and the protein concentration was quantified using the Bradford protein assay (Bio-Rad Laboratories Inc., USA). Extracts were heated for 3 min at 95°C, and equal

amounts of protein from each sample were separated on an SDS-12% polyacrylamide gel. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane with a Hoefer MiniVE vertical electrophoresis unit (Amersham Biosciences Inc., USA). Membranes were blocked in blocking buffer (LI-COR biosciences Inc., USA) at room temperature for 1hour and subsequently incubated overnight at 4°C with Anti-phospho-p44/p42 MAPK (Thr202/Tyr204) antibody at a 1:2000 dilution (Cell Signaling Technology, Inc., USA) to detect phosphorylated Mkc1 and Cek1 MAPKs. The expression of total Mkc1 was detected with the primary antibody against total Mkc1. The expression of total Cek1 was measured with an antibody to total Cek1. The secondary antibody against Phospho-p44/42 Ab, Mkc1 Ab and Cek1 Ab was IRye800CW goat anti–rabbit IgG (H+L) conjugate (green, 1:10,000 dilution; LI-COR Biosciences) incubated in the dark followed by extensive washing and quantitation using an Odyssey IR imaging system (LI-COR Biosciences). Phosphorylated and total proteins levels were quantitated using ImageJ (National Institutes of Health, Bethesda, MD). As a control protein, tubulin was detected with rat anti-tubulin primary antibody (Bio-Rad Laboratories Inc., USA) at a 1:1000 dilution and IRDye 680RD Goat-anti-Rat IgG (H+L) (red, 1:10,000 dilution; LI-COR Biosciences).

Pull-down assay for active Cdc42

Cells were grown in YPD to log phase, and pelleted by centrifugation, and re-suspended in Lysis/Binding/Wash buffer, provided by Active Cdc42 Pull-Down and Detection Kit (Thermo Fisher Scientific) with protease inhibitors cocktail (PMSF, leupeptin, and pepstatin) (RPI, Corp., Mount Prospect) and complete phosphatase inhibitor tablet (Roche Diagnostics GmbH, Mannheim, Germany), and cells were disrupted with acid-washed glass beads (Sigma-Aldrich Co. LLC., USA) in a Biospec Mini-Bead Beater with 6 rounds of 1min homogenization at 4°C and 1min interval for each cycle. The protein concentration was quantified using the Bradford protein assay (Bio-Rad Laboratories Inc., USA).

1,500 µg of total protein were used for the pull-down procedure following the instruction from Active Cdc42 Pull-Down and Detection Kit (Thermo Fisher

Scientific). 50ul of the pull-down samples containing active Cdc42 were separated by SDS-PAGE, transferred to PVDF with the Hoefer MiniVE vertical electrophoresis unit (Amersham Biosciences Inc., USA), and detected with mouse monoclonal anti-Cdc42 antibody at a 1:250 dilution (Cytoskeleton Inc., USA), followed by secondary detection with IRye800CW goat anti–mouse IgG (H+L) conjugate (1:10,000; LI-COR biosciences). As a control protein, tubulin was detected with rat anti-tubulin primary antibody (Bio-Rad Laboratories Inc., USA) and IRDye 680RD Goat-anti-Rat IgG (H+L) (LI-COR biosciences). Densitometry quantification of Cdc42 bands was performed with ImageJ (National Institutes of Health, Bethesda, MD).

Immunofluorescent imaging of β (1,3)-glucan exposure

This procedure was done as described in [13] with minor modification. *C. albicans* cells were grown overnight in YPD or YPM medium at 30°C. Mouse 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 µL of PBS and visualized with LEICA DM5500B epi-fluorescent microscope with Hamamatsu Orca-ER CCD digital camera (Model#C4742-80-12AG). The pictures were taken through Leica Application Suite AF (Advanced Fluorescence) software.

Fluorescence imaging

For imaging GFP-Cdc42 expressed under the *MET3* promoter, *Candida* cells were cultured overnight in SD minimal medium plus 1mM ethanolamine at 30°C, diluted to an OD_{600} of 0.2 in the fresh SD medium and allowed to grow for about 4-5 hours to reach the OD_{600} of 0.6-0.8. Cells carrying the CRIB-GFP or GFP-RID constructs (the expression of each is under the constitutive *ADH1* and *ACT1* promoters, respectively), were cultured in YPD medium. The overnight culture at 30°C was diluted back to an OD_{600} of 0.2 in fresh YPD medium and grown for 3 hours to reach log phase. 1mL of cells was collected and re-suspended in 100µl of PBS.

3µl of samples were mounted on the slide and observed under Leica DM RXA epi-fluorescent microscope with Leica DFC365FX CCD camera (Vashaw Scientific, Inc.). The pictures were taken through Leica Application Suite (LAS) V4.4 software.

Flow cytometry

To stain the *STE11*$^{\Delta N467}$ strain (P_{MAL} promoter) and its controls, overnight cultures in YPM or YPD were collected and blocked in PBS plus 3% bovine serum albumin (BSA, Thermo Fisher Scientific, USA) for 30mins. Primary and secondary antibody incubations occurred on ice in PBS plus 3% BSA for 1.5 h and 20mins, respectively. Soluble human Dectin-1–Fc (sDectin-1-Fc) [8] at 16.5 µg/ml was used to detect exposed β (1,3) glucan and mouse anti-Als3 antibody with 1:800 dilution was used for staining Als3 on hyphal cells. The Donkey anti-human IgG (H+L) Alexa Fluor 488 (Jackson Immuno Research) and goat anti-mouse antibody conjugated to R-Phycoerythrin (R-PE) were used as secondary antibodies, respectively.

To stain exposed β (1,3)-glucan on *CDC42*^{G12V} cells, overnight cultures were collected, and mouse anti-β (1,3)-glucan antibody at a 1:800 dilution and rabbit anti-mouse IgG (H+L) Alexa Fluor 488 (Jackson Immuno Research) were utilized as primary and secondary antibodies, respectively. 5ul of eBioscience™ propidium iodide dye (Thermos fisher) was then added to the solution for the live/dead staining and incubated for 5min at room temperature.

To stain β (1,3)-glucan in *Candida* cells with *MKC1* deleted, the overnight culture was incubated with mouse anti-β (1,3)-glucan antibody at a 1:800 dilution as primary antibody and followed by goat anti-mouse antibody conjugated to R-Phycoerythrin (R-PE) at 1:300 dilution (Jackson ImmunoResearch) as a secondary antibody. The staining process for *RHO1*^{Q67L} strains was the same except that the overnight cultures were diluted back to OD_{600} at 0.1 and the log phage cells were collected after 3hrs growth for staining.

For all of the above conditions, after staining, cells were processed by washing five times with PBS, and samples were resuspended in 500µl of FACS buffer (PBS, 1% serum, 0.1% sodium azide) for flowcytometry in a FACSCalibur

LSR II flow cytometer (Becton Dickinson). Singlets were gated by using a forward scatter area (FSC-A) versus side scatter area (SSA) plot, followed by forward scatter width (FSC-W) versus forward scatter area (FSC-A) density plot, as well as a side scatter width (SSC-W) versus side scatter area (SSC-A) plot to exclude clumping cells. We further compare the PE fluorescence intensity from the P3 singlets population in different *Candida* cells. Flow cytometry data were obtained for 100,000 gated events per strain and experiments were performed in triplicate and analyzed using FlowJo software package with version 10.11 (FlowJo LLC, OR, USA).

Enzyme-linked immunosorbent assay (ELISA) of TNF-α

RAW264.7 macrophages were plated the day prior at 5×10^5/well in a 24-well plate. To activate $STE11^{\Delta N467}$ expression under P_{MAL} regulation, $STE11^{\Delta N467}$ mutant cells were grown at YPM. Overnight cultures were washed and diluted to an OD_{600} of 1.25 in 5ml PBS/well in a 6-well plate for UV-kill. To do this, the 6-well plate was placed in the Spectrolinker XL-1000 UV Crosslinker (Spectroline Inc., USA) and the ENERGY mode was set to 100,000 μJ/cm^2. The UV-killing process was repeated 5 times. UV-killed *Candida* cells were then added to the RAW264.7 macrophages and coincubated at a 1:10 ratio for 4 h at 37°C and 5% CO_2. The supernatant of each well was collected and filtered through a syringe filter with 0.2μm pore size (Millipore Sigma, US) to exclude the macrophage debris. The ELISA kit instructions from the manufacturer (R&D Systems) were followed. Each sample has three individual wells, and the statistical analysis was performed by using Two-way analysis of variance ANOVA (GraphPad Prism, v7.04 software).

Results

C. albicans cho1Δ/Δ exhibits activated MAPKs

Given the strong cell wall phenotypes seen in *cho1Δ/Δ*, we hypothesized that this mutant might exhibit increased activation of cell wall signaling pathways such as Cek1 and Mkc1 MAP kinase cascades. As shown in Figure 2. 2A, Western blots with the Phospho-p42/44 antibody, that labels the phosphorylated (activated)

forms of both Cek1 and Mkc1, revealed that these kinases were constitutively phosphorylated in *cho1Δ/Δ* compared to wild-type and other test strains. No significant difference was found between the *psd1Δ/Δpsd2Δ/Δ* mutant (synthesizes PE from PS) and wild-type (Figure 2. 2A). This indicates that disruption of the PS synthase specifically up-regulates the activity of both cell wall MAPK cascades. Similar trends were also seen under hyphal induction conditions. When cells were sub-cultured in RPMI 1640 medium (induces filamentation [47]), *cho1Δ/Δ* exhibited greater phosphorylation of Cek1 and Mkc1 than wild-type and other test strains (Figure 2. 2B). Collectively, these results indicate that loss of Cho1 activates the Cek1 and Mkc1 MAPK pathways.

Activation of the Cek1 pathway causes β (1,3)-glucan masking

Galán-Díez *et al.* observed that a *cek1Δ/Δ* homozygous deletion mutant exhibits β (1,3)-glucan exposure in *C. albicans* [15]. In contrast, Li *et al.* reported that Cek1-inducing conditions, such as incubation with *N*-acetylglucosamine (GlcNAc) in the media, causes increased β (1,3)-glucan exposure in *C. albicans* [48]. To further investigate if activation of the Cek1 pathway increases exposure of β (1,3)-glucan in *C. albicans* yeast-form cells, we constructed a strain that expresses a hyper-active allele of *STE11* (*STE11*$^{\Delta 467}$) under the regulation of the maltose promoter (P_{MAL2}). Deletion of 467 N-terminal amino acids, including the inhibitory domain of Ste11, hyper-activates this kinase [43]. Ste11 is upstream of Cek1 and activates it via sequential phosphorylation through Hst7 (Figure 2. 1). Expression of the *STE11*$^{\Delta N467}$ allele in YP maltose (YPM) media results in greater phosphorylation of Cek1 compared to growth of this strain in YPD (represses *STE11*$^{\Delta N467}$ expression) (Figure 2. 3A). The *STE11*$^{\Delta N467}$ expressing strain exhibited greater β (1,3)-glucan exposure in YPM than YPD when stained with anti-β (1,3)-glucan antibody (Figure S.2. 1).

The Cek1 pathway is involved in inducing the yeast-to-hyphae transition. A small subset of cells forms filaments in the hyper-activated *STE11*$^{\Delta N467}$ strain in YPM, and hyphae exhibit β (1,3)-glucan unmasking more readily than yeast-form cells [12, 13]. To determine if the yeast-form cells themselves exhibited greater

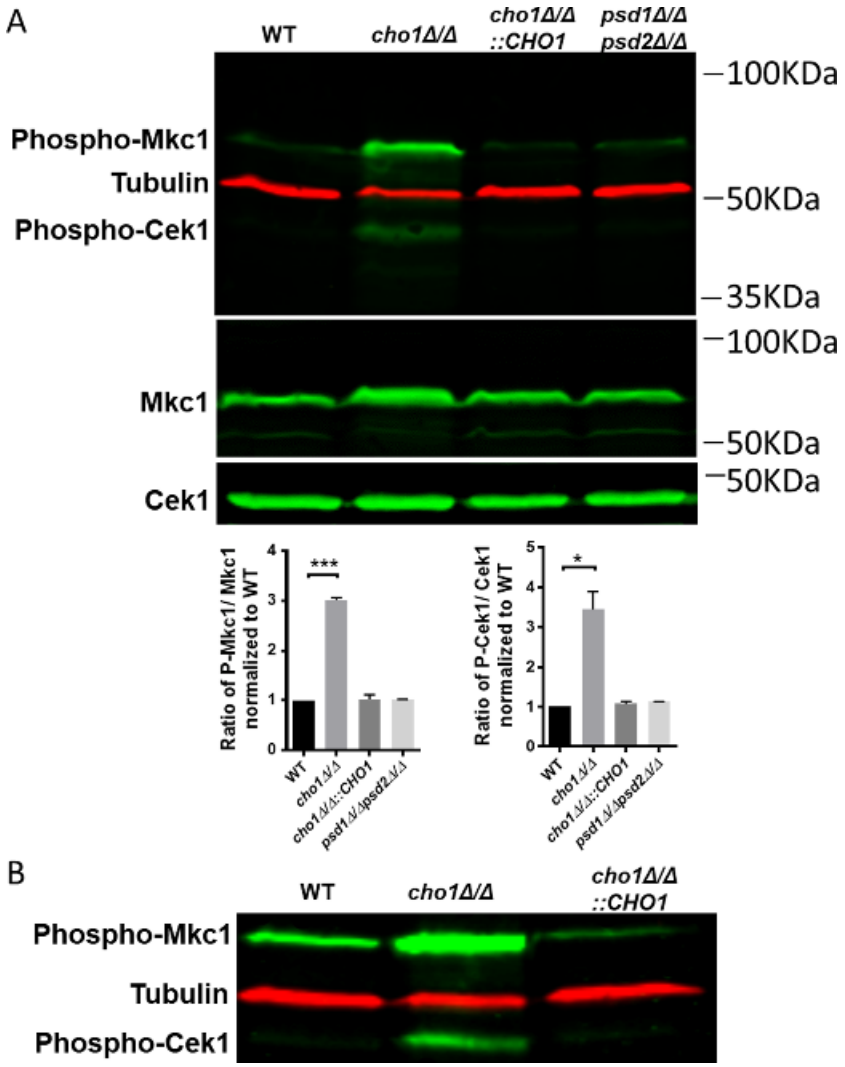


Figure 2. 2. Cek1 and Mkc1 MAPKs exhibit increased activation in *cho1Δ/Δ* yeast cells compared to wild-type.

(A) Proteins from yeast-form cells growing in log phase in YPD media were extracted and Western blotting was performed with anti-phospho-p44/42 antibody to detect Phospho-Mkc1 and Phospho-Cek1. Anti-Mkc1 was used for total Mkc1, anti-Cek1 for total Cek1, and anti-tubulin as a loading control. Graphs of quantification by Image J of the phosphorylated forms of each kinase are expressed as a percent of the wild-type control after being normalized to the total kinase blot for each respective MAP kinase and the tubulin loading controls for each gel. Quantification is based on three biological replicates. The statistical analysis was performed by using One-way ANOVA *, p=0.0308. ***, p=0.0005. (B) Western blotting was performed on extracts from cells grown as hyphae in RPMI media and probed with antibodies detecting Phospho-Cek1 and Phospho-Mkc1 as well as the tubulin loading control.

unmasking, we used flow cytometry with a second hyphal-specific probe to gate out hyphal cells while measuring β (1,3)-glucan exposure. In particular, we stained strains with soluble Dectin-1 (sDectin-1) protein which binds with exposed β (1,3)-glucan and anti-Als3 antibody, which stains the hyphal-specific protein Als3. Thus, Als3 staining was used as a marker to gate out hyphae by flow cytometry allowing us to focus on yeast-form cells. This double staining revealed that wild-type yeast-form cells expressing hyper-activated Ste11 (*STE11$^{\Delta N467}$*) in YPM have significantly increased unmasking compared to yeast cells in YPD (Figure 2. 3B); compare the 1st quadrants (Q1) of the plots of *STE11$^{\Delta 467}$* grown in both YPM and YPD (bottom two plots).

β-1,3-glucan exposure is more intense at bud scars, which presented the possibility that the higher glucan exposure in *STE11$^{\Delta N467}$*-YPM is associated with more bud scars provided that maltose increases growth rate. In fact, a growth curve demonstrated that both wild-type and *STE11$^{\Delta N467}$* cells cultured in YPM grew slightly better compared to corresponding strains in YPD culture (Figure S.2. 2). However, when we co-stained cells with β (1,3)-glucan antibody and calcofluor white, a dye that stains the chitin that is normally concentrated at the bud scar [49], the exposed β (1,3)-glucan in *STE11$^{\Delta N467}$* is scattered along the cell periphery, whereas the calcofluor white staining is constricted to the division sites (*e. g.* bud scars), revealing little overlap (Figure S.2. 3). Furthermore, *STE11$^{\Delta N467}$* and wild-type have similar growth rates in YPM (Figure S.2. 2), but *STE11$^{\Delta 467}$* has significantly elevated β (1,3)-glucan unmasking in this medium (Figure 2. 3B and C). Conversely, the strains have similar rates of growth and β (1,3)-glucan exposure in YPD (Figure 2. 3B and C), a condition where Cek1 is not hyperactived (Figure 2. 3A). Moreover, wild-type replicates more rapidly in YPM than YPD, but β (1,3)-glucan exposure is comparable for wild-type in both media (Figure 2. 3B). Altogether, these data indicate that hyperactivation of the Cek1 pathway leads to increased β (1,3)-glucan exposure that is not based on increased numbers of bud scars.

The correlation between increased β (1,3)-glucan exposure and enhanced immune responses such as upregulated tumor necrosis factor alpha (TNF-α)

secretion has been studied intensively [12-14, 17, 50, 51]. Exposed β (1,3)-glucan is recognized by the receptor Dectin-1 on the surface of immune cells including macrophages and neutrophils, and this recognition activates the host immune response for fungal clearance including the secretion of TNF-α [12]. To determine if the increased β (1,3)-glucan exposure in the *STE11*$^{\Delta N467}$ strain is immunologically relevant, we performed an enzyme-linked immunosorbent assay (ELISA) to quantify TNF-α secretion from RAW264.7 macrophages exposed to this strain. As seen in Figure 2. 3D, TNF-α secretion was significantly upregulated when the Cek1 MAPK pathway was hyper-activated (*STE11*$^{\Delta N467}$ in YPM). It should be considered when examining the data in Figure 2. 3D that production of TNF-α is reduced in all strains that were grown in YPM, including wild-type and *cho1ΔΔ*. Thus, while the increase in TNF-α of cultures of *STE11*$^{\Delta N467}$ grown in YPM is ~35% greater than that in YPD, the increase of *STE11*$^{\Delta N467}$ over wild-type, both grown in YPM, is 2-fold. Thus, increased β (1,3)-glucan exposure in the *STE11*$^{\Delta N467}$ strain increases proinflammatory responses from macrophages.

CHO1 disruption upregulates Cdc42 activity in *C. albicans*

The above results indicate that hyper-activation of Ste11 can cause unmasking, and since the Cek1 MAPK pathway, which acts downstream of Cdc42 [18], is constitutively activated in *cho1Δ/Δ*, this suggests that Cdc42 activity might be upregulated in *cho1Δ/Δ* (Figure 2. 1). To test this possibility, Cdc42 activity was measured by monitoring the amount of active Cdc42 (GTP-bound) in cells. GTP-bound Cdc42 was isolated using agarose beads coated with Cdc42/Rac1 interactive binding (CRIB) domain [46]. As seen in Figure 2. 4A, the concentration of GTP-bound Cdc42 in *cho1Δ/Δ* is higher than that in the other two strains. This confirms our hypothesis that *cho1Δ/Δ* has a higher concentration of active Cdc42 than wild-type. Thus, Cho1 or its biochemical product PS may impact Cdc42 activity negatively in wild-type cells, although the regulation may be indirect.

We then compared the localization of active Cdc42 in *cho1Δ/Δ* and wild-type by using a CaCRIB-GFP probe [46]. This motif binds with both Cdc42 and Rac1 GTPases. As seen in Figure 2. 4B, both wild-type and *cho1Δ/Δ* cells have

Figure 2. 3. Hyperactive Ste11 (*STE11$^{\Delta N467}$*) causes significant increases in β (1,3)-glucan exposure and TNF-α secretion.

(A) The Cek1 MAPK is hyper-activated by transforming wild-type cells with a hyperactive allele of *STE11* (*P_{MAL}-STE11$^{\Delta N467}$*), which is induced by adding maltose as a carbon source. (B) Both wild-type and *STE11$^{\Delta N467}$* expressing cells were cultured overnight (16hrs) in YPD or YPM individually and then were doubly stained. The Y-axis represents staining with soluble Dectin-1-Fc (sDectin-1-Fc) that binds to exposed β (1,3)-glucan, and the X-axis represents anti-Als3 antibody, which binds to the hyphal-specific protein Als3. Flow cytometry was performed to quantify β (1,3)-glucan exposure from the yeast-form population (Q1: sDectin-1 single positive staining; Q2: sDectin-1 & Als3 double positive staining; Q3: Als3 single positive staining; Q4: double negative staining). Gates were established with an unstained control where 97% of unstained cells gated within Q4. Gating strategies are further described in the Methods. (C) Left graph: Comparison of β (1,3)-glucan exposure from the yeast-form population of *STE11$^{\Delta N467}$* versus that of wild-type, both of which are cultured in YPM. *, p=0.0289. Right graph: β (1,3)-glucan exposure was compared between these two strains when grown in YPD medium. (D) Expression of *STE11$^{\Delta N467}$* significantly induces TNF-α secretion after growing in YPM overnight. RAW264.7 macrophages were challenged with various C. albicans stains. C. albicans strains were grown in YPD or YPM, washed, UV-inactivated, and then add to the macrophages for 4hrs. The macrophage supernatant was collected and assayed by ELISA to quantify TNF-α production. **, P=0.0030; ***, P=0.0002; ****, p<0.0001.

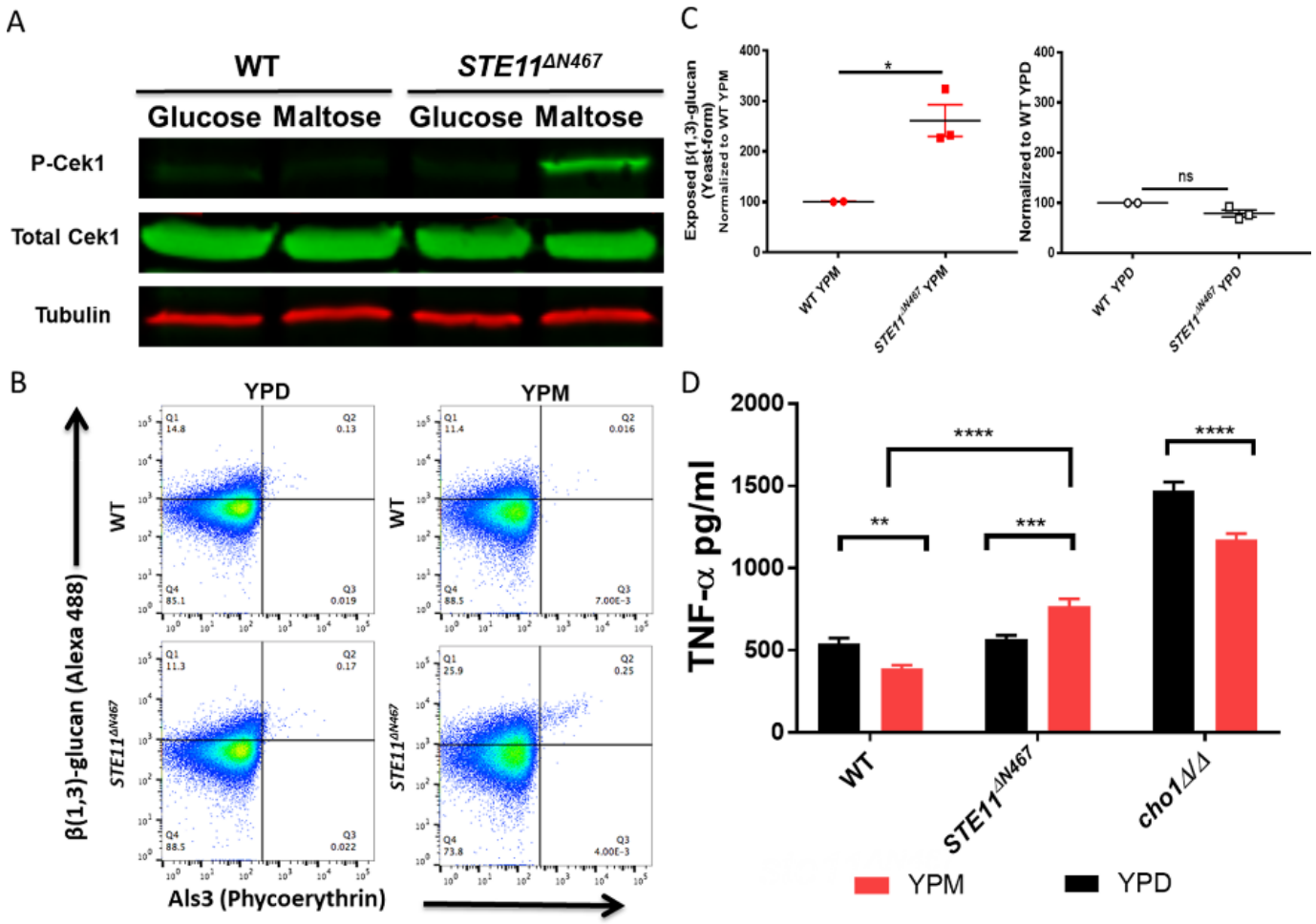


Figure 2. 3 Continued

similarly localized active Cdc42 with the CRIB-GFP probe concentrated at the growth sites (buds). CRIB-GFP can bind both Rac1 and Cdc42, so to measure Cdc42 localization alone, we disrupted *RAC1* in both wild-type and *cho1Δ/Δ* by using a *C. albicans* CRISPR-Cas9 system [45]. Both *rac1Δ/Δ* and *cho1Δ/Δ rac1Δ/Δ* mutant cells have a similar pattern of CRIB-GFP localization during budding growth compared to wild-type and *cho1Δ/Δ* (Figure 2. 4B and C). This suggests that active Cdc42 is found in its normal localization in *cho1Δ/Δ* cells.

These results were in potential contrast to those for Cdc42 in a *S. cerevisiae cho1Δ* mutant, where PS disruption causes impaired Cdc42 polarization [52]. However, in this study, Fairn *et al*. used a GFP-Cdc42 construct to examine localization, which visualizes total Cdc42 rather than just active Cdc42. Therefore, we examined localization of total GFP-Cdc42 in *C. albicans cho1Δ/Δ* to determine how total Cdc42 responds to PS deficiency (Figure 2. 5A). In the wild-type and reintegrated strains (*cho1Δ/Δ::CHO1*), Cdc42 is localized to the plasma membrane and internal membranes, and accumulates in bud necks and bud tips. The *cho1Δ/Δ* mutant has impaired polarization of GFP-Cdc42 to bud necks and tips. There is an overall decrease in plasma membrane binding of GFP-Cdc42, and instead GFP-Cdc42 accumulates in the cytoplasm. Approximately 80% of wild-type yeast cells have polarized Cdc42 localization, while only 20% of *cho1Δ/Δ* cells show polarized localization (Figure 2. 5B). This result indicates that *CHO1* is necessary for the proper localization of total GFP-Cdc42 in *C. albicans.*

We next examined the mechanism by which PS may impact GFP-Cdc42 localization to buds and bud necks. PS is the most abundant anionic phospholipid of the plasma membrane, and it is largely restricted to the inner leaflet [52, 53]. A C-terminal polybasic region in some Rho-family small GTPases is a crucial domain for lipid interaction, where several positively charged amino acid residues promote plasma membrane localization and have been suggested to do so via electrostatic interactions with negatively charged phospholipids including PS [54, 55]. To elucidate if this domain is crucial for localization of Cdc42 in *C. albicans*, we constructed a GFP-Cdc42 mutant where the four C-terminal lysines were mutated to glutamines (GFP-Cdc42$^{K183\text{-}187Q}$) and observed its localization in *C. albicans*

Figure 2. 4. Cdc42 activity is upregulated in *cho1Δ/Δ* compared to wild-type.

(A) GTP-Cdc42 was pulled-down with beads conjugated with GST-CRIB, which specifically binds with active GTP-Cdc42/Rac1. Cdc42 that was pulled down was then detected via Western blotting with anti-Cdc42 antibody. The amount of total Cdc42 in the extract was also probed as a control. The GTP-Cdc42/Total-Cdc42 ratio is expressed as a percentage of wild-type. (B) CaCRIB-GFP localization is not altered upon *CHO1* deletion. The CaCRIB-GFP probe was transformed into Candida strains to investigate the active GTP-Cdc42 localization. The scale bar represents 10μm. (C) Cells from Figure 2. 4B were analyzed by microscopy for the number that exhibited CaCRIB-GFP localization to buds. Quantification is of three biological replicates, and each replicate has at least 50 cells.

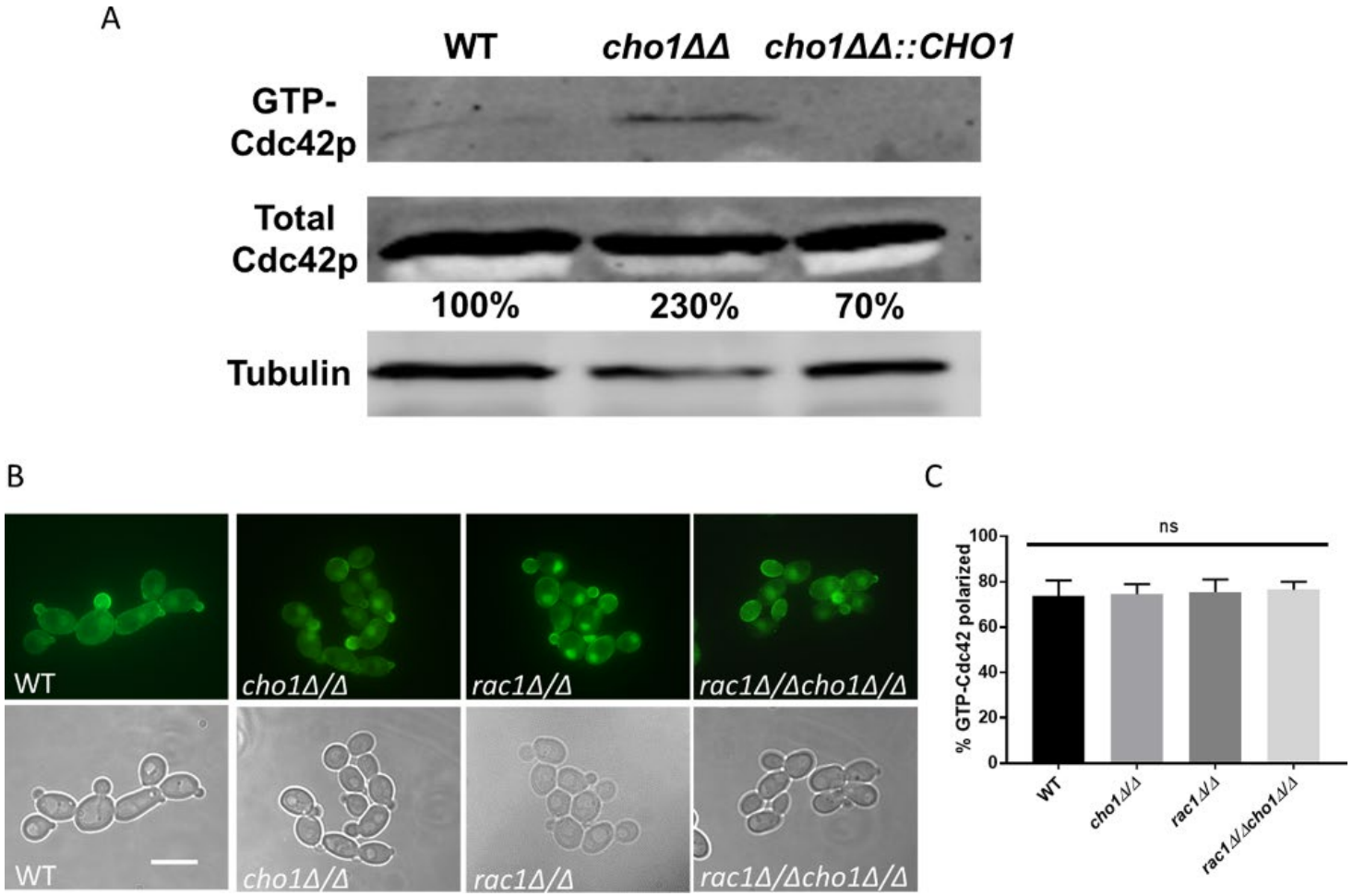


Figure 2. 4 Continued

wild-type cells. As shown in Figure 2. 5A, most of the GFP-Cdc42$^{K183\text{-}187Q}$ was associated with endomembrane structures instead of the plasma membrane. Of note, the preferential accumulation of GFP-Cdc42 seen in the buds of normal wild-type yeast was absent in the mutated Cdc42$^{K183\text{-}187Q}$ protein. This indicates that the C-terminal polybasic region of Cdc42p is important for association of total GFP-Cdc42 with plasma membrane. However, this does not show if the C-terminal domain is regulating localization by directly interacting with PS, although that is one possibility.

In contrast, as observed in Figure 2. 4, GTP-bound unmodified Cdc42 is still able to associate with the bud necks and tips in the absence of *CHO1*, indicating that active Cdc42 can still localize to the appropriate places in the cell. The discrepancy we see between total GFP-Cdc42 localization and localization of GTP-bound native Cdc42 could be caused by the GFP or reflect differences between total versus active Cdc42 populations.

Activation of Cdc42 activity contributes to cell wall unmasking

The above results indicate that the GTPase Cdc42 has increased activation in *cho1Δ/Δ* (Figure 2. 4). To further investigate if this up-regulated Cdc42 activity contributes to β (1,3)-glucan exposure, we constructed a mutant strain that ectopically expresses a *CDC42* hyperactive allele (*CDC42^{G12V}*) in wild-type. Introduction of *CDC42^{G12V}* decreases the intrinsic GTPase activity, therefore increasing the proportion of Cdc42 in an active GTP-bound state [18]. Cells overexpressing *CDC42^{G12V}* exhibited decreased proliferation in YPD liquid and poor growth on YPD agar plates [32]. Similarly, a hyper-activated *CDC42^{G12V}* mutant was dominant lethal in *S. cerevisiae* [56]. Our strain is viable, but does exhibit growth defects, so we measured β (1,3)-glucan exposure in the *CDC42^{G12V}* mutant by staining with anti-β (1,3)-glucan antibody, but also co-stained cells with propidium iodide to control for cell-viability. Propidium iodide staining revealed that the overnight *CDC42^{G12V}* culture contained fewer live cells compared to wild-type (Figure S.2. 4A). However, within the live cell populations for both strains, there was a much greater level of β (1,3)-glucan exposure in the *CDC42^{G12V}* cells

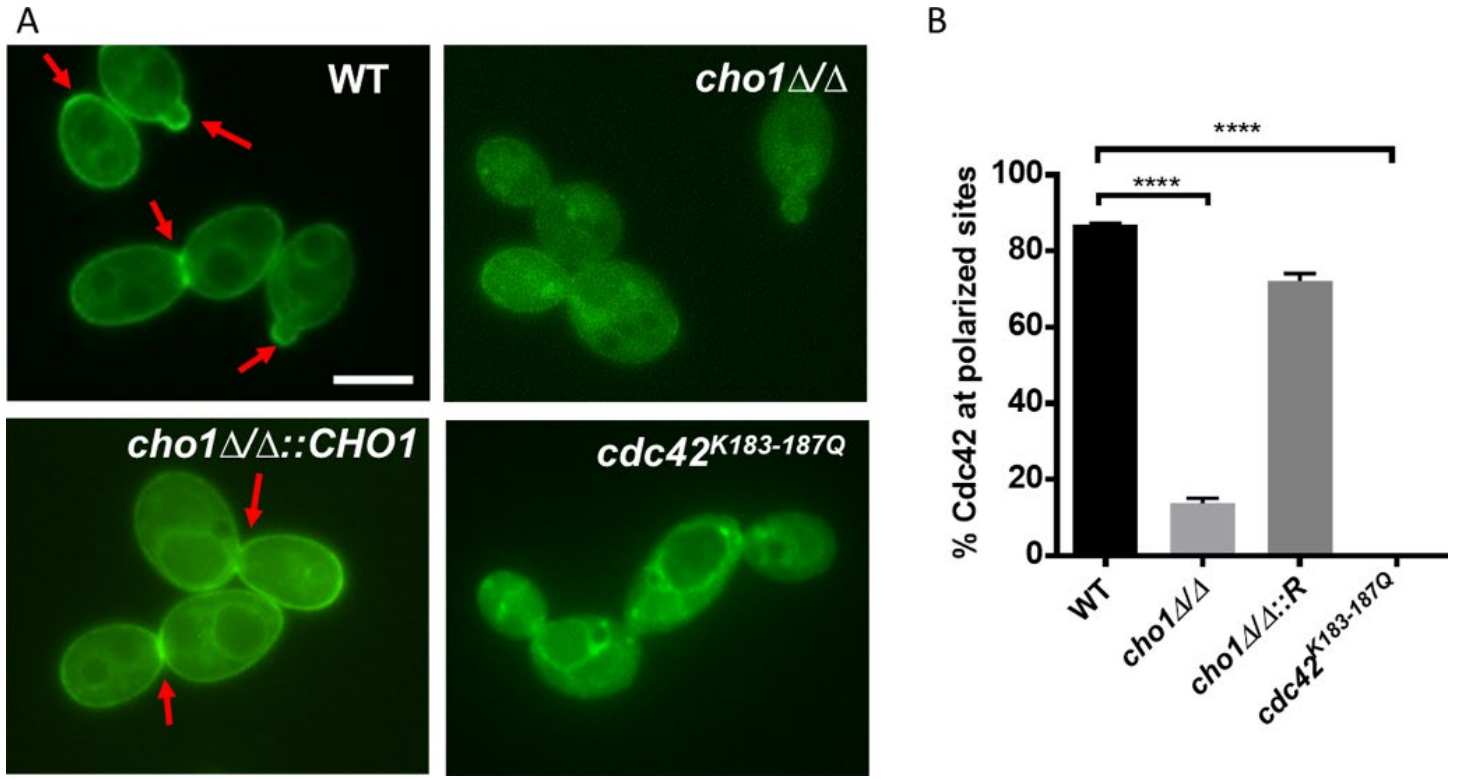


Figure 2. 5. Cho1 is essential for GFP-Cdc42 polarization at the plasma membrane.

(A) GFP-Cdc42 localization is examined for each strain by microscopy. The red arrows indicate the fluorescence concentrated at the bud tips or bud necks. The scale bar represents 5μm. (B) Quantification of the degree of polarization of GFP-Cdc42 in Candida cells. A minimum of 50 cells was counted for each strain and the imaging experiment was repeated three times. The statistical analysis was carried out by One-way ANOVA. ****, $p<0.0001$.

compared to wild-type (Figure S.2. 4B). This suggests that increased Cdc42 activity causes β (1,3)-glucan masking, with the caveat that *CDC42*^{G12V} is clearly having pleiotropic effects.

The Rho1-associated signaling pathway does not have a clear role in causing β (1,3)-glucan exposure

Our data indicate that the Cek1 pathway can cause β (1,3)-glucan exposure when hyper-activated, and this may help explain the increased β (1,3)-glucan exposure seen in the *cho1Δ/Δ* mutant. However, the Mkc1 pathway is also upregulated in *cho1Δ/Δ* (Figure 2. 2), and we wanted to determine if activation of this pathway plays a role in β (1,3)-glucan exposure as well. First, both *MKC1* alleles were disrupted via the *C. albicans* CRISPR-cas9 system [45] in wild-type and *cho1Δ/Δ*. Western blotting was performed to confirm that Mkc1 was not expressed in the mutants with both *MKC1* alleles disrupted (Figure S.2. 5). Immunostaining with anti-β (1,3)-glucan antibody on wild-type, *cho1Δ/Δ, mkc1Δ/Δ* and *cho1Δ/Δmkc1Δ/Δ* strains showed that deletion of *MKC1* did not rescue the β (1,3)-glucan exposure phenotype in the *cho1Δ/Δ* mutant (Figure 2. 6). In fact, flow cytometry demonstrated that the *mkc1Δ/Δcho1Δ/Δ* double mutant cells exhibited increased levels β (1,3)-glucan exposure compared to *cho1Δ/Δ* (Figure 2. 6B). This suggests that Mkc1 MAPK probably plays a role in sustaining cell wall organization when *CHO1* is disrupted.

Pkc1 acts as a signaling module to connect Rho1 to the Mkc1 MAPK cascade [25-27]. We deleted one *PKC1* allele in *cho1Δ/Δ*, and this did not suppress the β (1,3)-glucan exposure phenotype (Figure S.2. 6). Attempts to make a complete *cho1Δ/Δ pkc1Δ/Δ* double mutant failed. This does not completely test for a role for Pkc1 in unmasking but is consistent with those above indicating that increased activation of the Mkc1 pathway does not cause β (1,3)-glucan exposure.

We then examined if Rho1 might play a role in increased β (1,3)-glucan exposure in *cho1Δ/Δ*. Total, but not active, Cdc42 is mis-localized in the *cho1Δ/Δ* (Figure 2. 4 and Figure 2. 5), therefore, we tested the distribution of active Rho1.

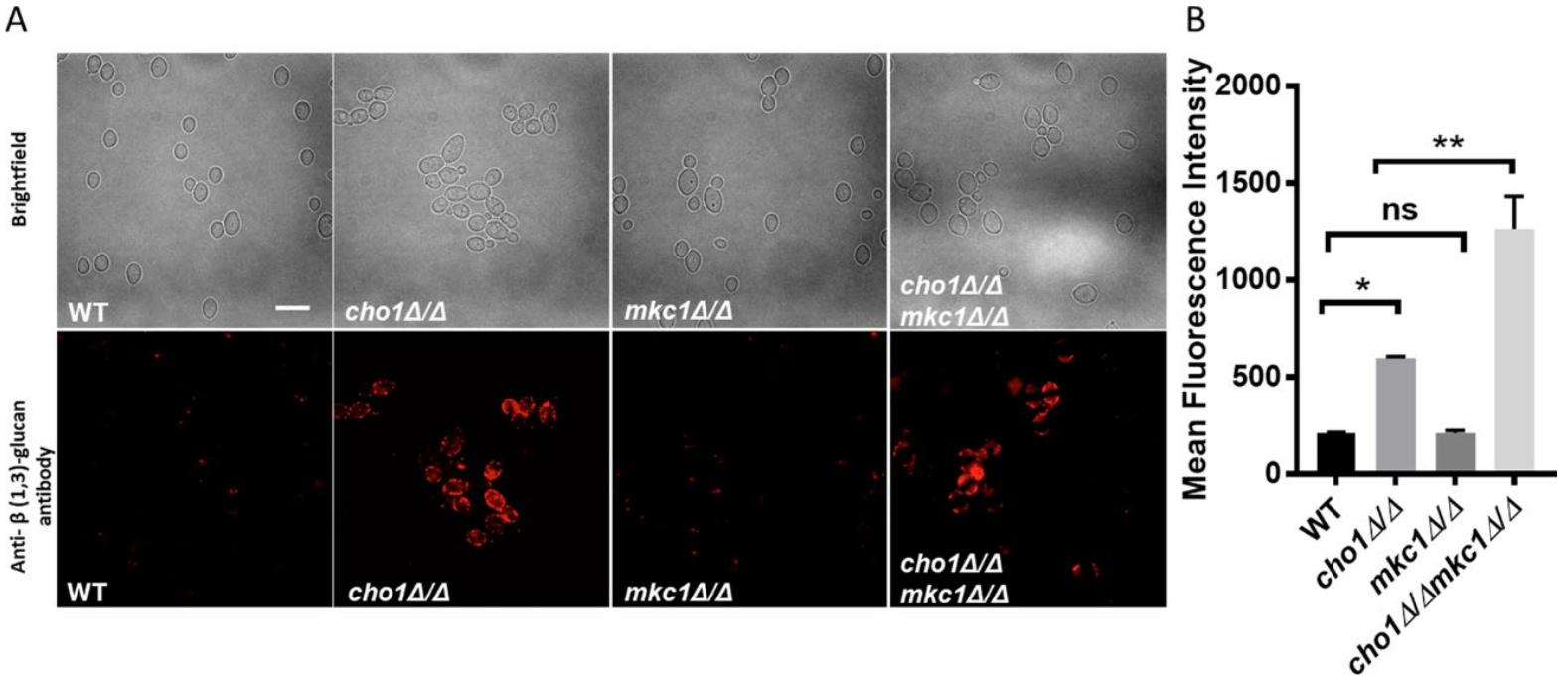


Figure 2. 6 Deletion of *MKC1* in *cho1Δ/Δ* does not diminish β (1,3)-glucan exposure.

(A) Cells were stained with primary anti-β (1,3)-glucan antibody and Cy3-conjugated secondary antibody and imaged by epi-fluorescent microscopy. The scale bar indicates 10μm. (B) Flow cytometry was carried out to quantify β (1,3)-glucan exposure. Cells were incubated with primary anti-β (1,3)-glucan antibody and PE-conjugated secondary antibody. The statistical analysis was carried out by doing One-way ANOVA analysis. *, P=0.0485; **, P=0.0024.

This was achieved using a probe for active Rho1, that consists of a GFP tagged *C. albicans* Pkc1 Rho Interactive Domain (GFP-RID) [46]. In wild-type and *cho1Δ/Δ::CHO1,* GFP-RID is localized to the growth sites (i.e. buds and sites of cell division) (Figure 2. 7), however the signal in *cho1Δ/Δ* is delocalized. This suggests that the Rho1 cell wall remodeling system might be re-localized when Cho1 is disrupted. Rho1 also has multiple lysines on its extreme C-terminus, similar to Cdc42, thus its mislocalization in *cho1Δ/Δ* could be affected for similar reasons as observed for total GFP-Cdc42 (Figure 2. 5). Due to the lack of GFP-Rho1, we have not examined GFP-Rho1 to find the exact localization of total Rho1 in *cho1Δ/Δ*. The increased activation of Mkc1 in *cho1Δ/Δ* suggests that its upstream regulator, Rho1, might exhibit a similar increase in activation. To test if up-regulated Rho1 can cause β (1,3)-glucan exposure, we constructed a strain that ectopically expresses a hyperactive allele of $RHO1^{Q67L}$ in wild-type. Introduction of the $RHO1^{Q67L}$ allele decreases the ability of Rho1 to cleave GTP to GDP, therefore increasing the level of GTP-Rho1 [25]. As shown in Figure 2. 8A and B, hyper-activated $RHO1^{Q67L}$ did cause a significant increase in cell wall unmasking compared to wild-type, but not as great as that seen with $STE11^{\Delta N467}$. However, examination of MAPK phosphorylation revealed that active Cek1 was unexpectedly upregulated along with active Mkc1 (Figure 2. 8C). Thus, the β (1,3)-glucan exposure in the $RHO1^{Q67L}$ strain could be due at least in part to Cek1 activation rather than Mkc1 (Figure 2. 8C).

Discussion

Previously, our lab showed that the enzyme for synthesizing PS, Cho1, plays a role in controlling β (1,3)-glucan exposure [13]. The homozygous PS synthase knockout mutant, *cho1Δ/Δ*, exhibits increased β (1,3)-glucan exposure compared to wild-type [13]. However, the mechanism by which *cho1Δ/Δ* displays the β (1,3)-glucan exposure phenotype was unclear.

In this report, we identify two MAPK signaling pathways (Cek1 and Mkc1) that are activated in the *cho1Δ/Δ* mutant (Figure 2. 2), and we hypothesized that one or both may contribute to increased β (1,3)-glucan exposure in *cho1Δ/Δ*.

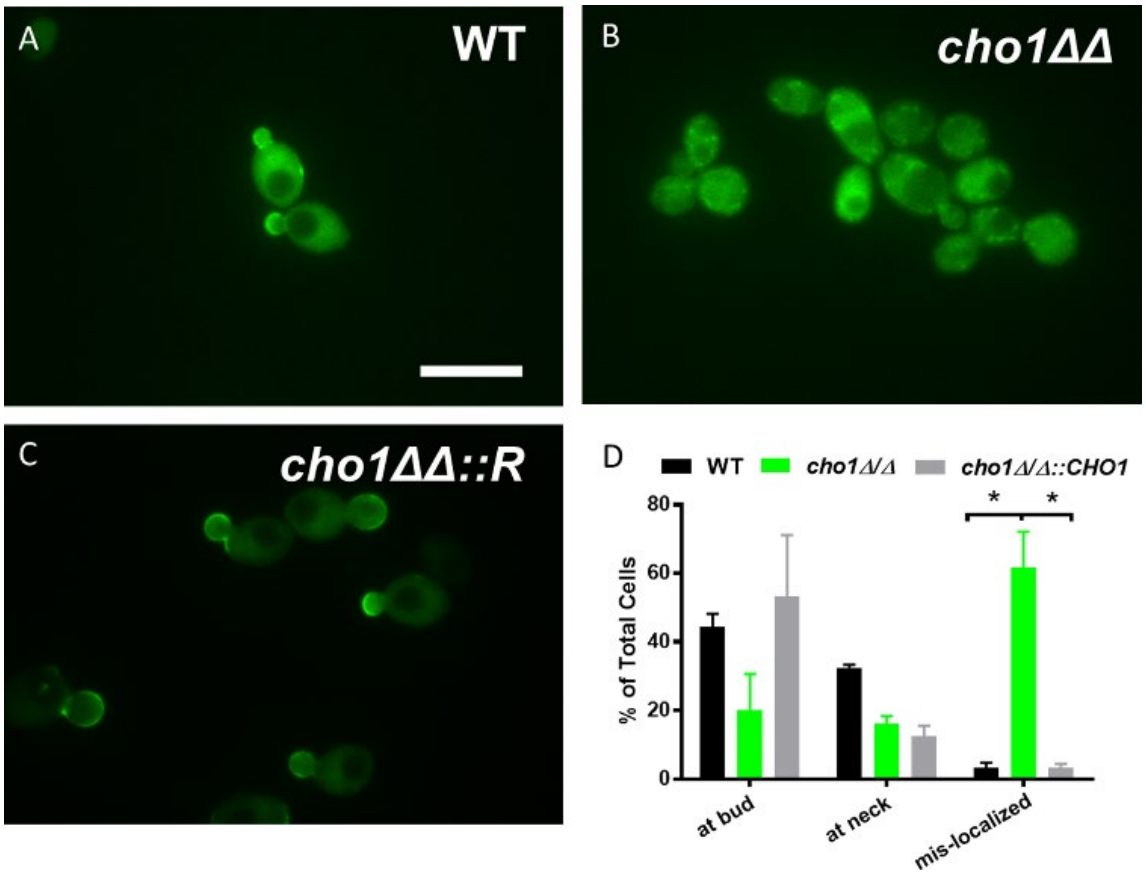


Figure 2. 7. Active GTP-Rho1 is de-localized in *cho1Δ/Δ*.

(A-C) GFP-RID localization was used as proxy for active (GTP-bound) Rho1 in cells and was analyzed by epifluorescent microscopy. (D) Quantification of the degree of polarization of GTP-Rho1 in Candida cells. A minimum of 50 cells were counted for each strain and this repeated three times. The statistical analysis was done by One-way ANOVA. *, $p<0.019$.

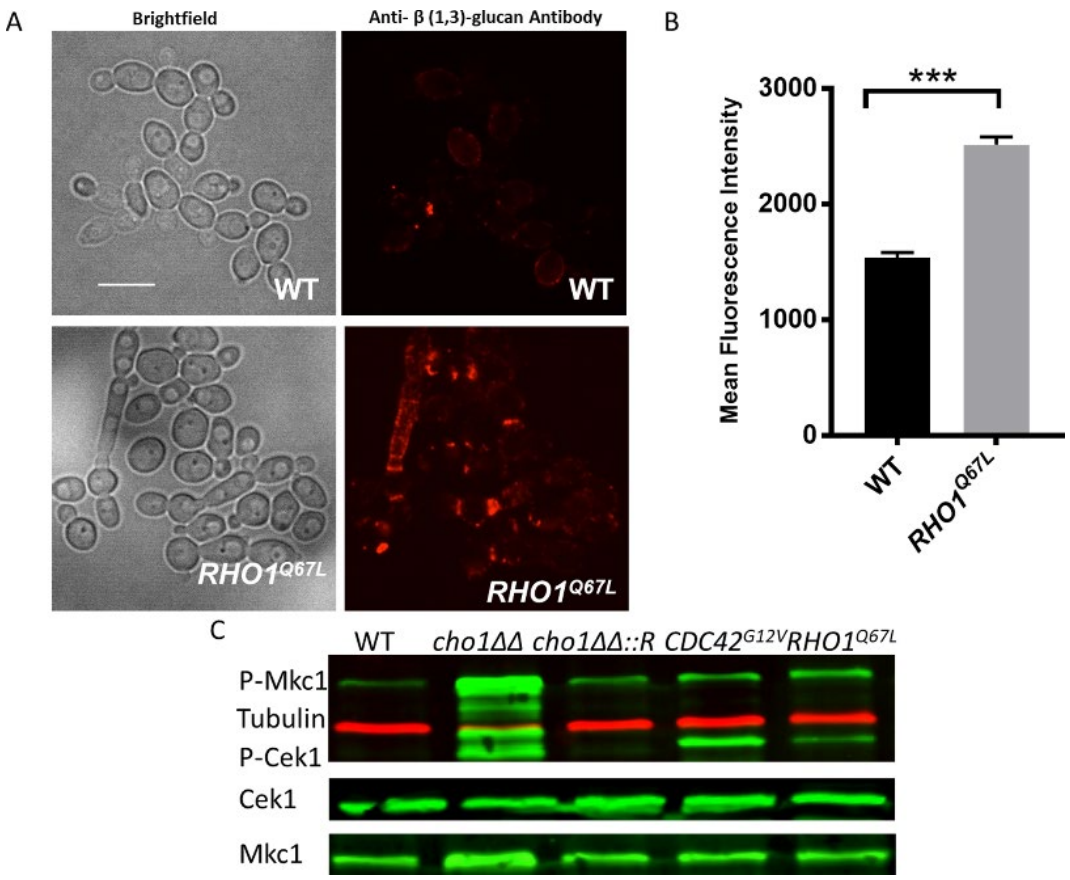


Figure 2. 8. Hyper-activated Rho1 causes β (1,3)-glucan exposure.

(A-B) Candida cells were stained for β (1,3)-glucan exposure as described in Figure 2. 6. The scale bar represents 10μm. ***, p=0.0003. (C) Western blotting was performed to examine the effect of expressing hyperactive $RHO1^{Q67L}$ on the regulation of downstream MAPKs activities. Phospho-p44/42 antibody was used to detect Phospho-Cek1 and Phospho-Mkc1 and anti-tubulin, anti-Mkc1, and anti-Cek1 antibodies were used as controls.

MAPK signal transduction cascades are essential pathways for *C. albicans* adaptation to the host environment [35, 57]. Cek1 and Mkc1 are major MAPK pathways in this organism that play roles in cell wall regulation. The Mkc1-associated pathway is primarily responsible for cell wall integrity, while the Cek1-mediated signaling cascade is important for cell wall construction and hyphal formation [19, 58-60].

Cdc42-Cek1 MAPK pathway activation can increase β (1,3)-glucan exposure

We tested the hypothesis that one or both of these pathways can cause β (1,3)-glucan exposure in *cho1Δ/Δ* by determining if they could contribute to this phenotype independently of loss of PS. We found confirming evidence for the Cek1 pathway. In particular, a hyperactive form of Ste11 (*STE11$^{\Delta N467}$*), the MAPKKK that activates Cek1 (Figure 2. 1), stimulates significant β (1,3)-glucan exposure in yeast-form cells compared to wild-type cells (Figure 2. 3). This confirms an assertion that β (1,3)-glucan can be unmasked in Cek1 inducing conditions [48]. The cells with *STE11$^{\Delta N467}$*-induced unmasking also exhibit more TNF-α secretion from the macrophages (Figure 2. 3D).

Ste11 is downstream of the small GTPase Cdc42 (Figure 2. 1), which has been well-studied in *C. albicans* [18, 32, 46, 61]. Cdc42 is involved in cellular proliferation and bud emergence and activates the downstream protein kinase Cst20 [32], which also controls the activation of the Cek1 MAPK cascade including Ste11 [15, 18, 58]. To control accurate cellular function, Cdc42 cycles between an active GTP-bound and inactive GDP-bound state [61]. By performing pull-downs of GTP-Cdc42 with CaCRIB-GST and Western blotting, we have evidence that the level of active GTP-Cdc42 is higher in *cho1Δ/Δ* compared to wild-type (Figure 2. 4A). This might be responsible for the increased activation of the downstream Ste11-associated cascade. We did not test to see if disruption of *CEK1* in the *cho1Δ/Δ* strain would decrease β (1,3)-glucan exposure because *cek1Δ/Δ* also exhibits more exposed β (1,3)-glucan than wild-type [15], and this would be uninterpretable.

Possible roles of PS in regulating Cdc42 activity and localization

The impact of PS on β (1,3)-glucan exposure is likely indirect, but may be occurring through its role in regulating Cdc42. The loss of PS correlates with increased Cdc42 activity, which in turn can lead to activation of the Cek1 pathway, which does cause β (1,3)-glucan exposure when activated (Figure 2. 3). However, the mechanism by which loss of PS causes Cdc42 activation is currently unclear, but possibilities are discussed below.

PS may impact Cdc42 activity indirectly by regulating the GTPase activating proteins (GAPs) for Cdc42. These GAPs act as repressors of Cdc42 activity. Previous investigations identified that PS stimulates the GAP activity of Rga1 and Rga2 toward Cdc42 in *S. cerevisiae* [62]. Given that *C. albicans cho1Δ/Δ* lacks PS [16], this may result in less inhibition of the GAP activity, and in turn results in less inhibition of Cdc42 activity.

Alternatively, there are data indicating that PS can control the localization of a subpopulation of Cdc42. For example, we found that GFP-tagged Cdc42 is mislocalized in *C. albicans cho1Δ/Δ*. Moreover, mutating the C-terminal lysines to glutamine in GFP-Cdc42 led to mislocalization of GFP-Cdc42$^{K183\text{-}187Q}$ in wild-type cells (Figure 2. 5). This is similar to what has been observed in *S. cerevisiae*, where Cdc42 localization is affected by both PS and the basic lysine residues at the C-terminal domain of Cdc42 [52, 55]. However, in contrast to this, the localization of active GTP-Cdc42 in *C. albicans*, as measured by CaCRIB-GFP (binds to GTP-Cdc42/Rac1) appears to be focused in the bud necks and tips like wild-type (Figure 2. 4B). Therefore, PS might control only a subpopulation of Cdc42 localization. It is also possible that GFP-Cdc42 does not fully represent endogenous Cdc42 in its activated state.

The mechanism by which PS controls Cdc42 localization in *C. albicans* remains to be fully elucidated. One model suggests that Cdc42 localization is controlled in part through the interaction between the negatively charged PS head group and the lysines at the C-terminus of Cdc42 (Figure 2. 5). However, this is only a model at this point and remains to be tested, as the impact of PS on GFP-Cdc42 may be indirect. These lysines may interact with another protein that is

required to localize Cdc42 that itself is impacted by PS. In addition, the correct localization of active Cdc42 in *cho1Δ/Δ* indicates that other factors, perhaps GEFs or GAPs, play an important role in Cdc42 localization, independently of PS (Figure 2. 4).

Activation of the Mkc1 MAPK pathway does not appear to be sufficient to cause unmasking

The other MAPK pathway upregulated in *cho1Δ/Δ* is the Mkc1 pathway (Figure 2. 1 and Figure 2. 2). We tested for its role in *cho1Δ/Δ*-dependent β (1,3)-glucan exposure by generating a *cho1Δ/Δmkc1Δ/Δ* double mutant, and this did not diminish β (1,3)-glucan exposure (Figure 2. 6). Moreover, we disrupted one allele of the upstream kinase Pkc1, and this also did not diminish β (1,3)-glucan exposure (Figure S.2. 6). Finally, a hyperactive GTP-bound form of Rho1 (*RHO1*^{Q67L}) was generated, and it did lead to modest β (1,3)-glucan exposure compared to wild-type, however surprisingly it also led to increased phosphorylation of Cek1 as well as Mkc1 (Figure 2. 8), thus the increase may be caused by Cek1 upregulation.

An alternative role for Mkc1 may be to diminish β (1,3)-glucan exposure in stress conditions. For example, the *mkc1Δ/Δ* mutant did not exhibit enhanced β (1,3)-glucan exposure compared to wild-type, but the *cho1Δ/Δ mkc1Δ/Δ* double mutant exhibited greater β (1,3)-glucan exposure than *cho1Δ/Δ* alone. This coupled with the mislocalization of active Rho1 in *cho1Δ/Δ* (Figure 2. 7), may indicate that Mkc1 is activated to compensate for cell wall disfunction that is caused by the *cho1Δ/Δ* mutation, perhaps even due to upregulated Cek1.

Surprisingly, our results with the Mkc1 pathways relationship to PS contrast with what is observed for the orthologous pathway in *S. cerevisiae*. In baker yeast, PS has been shown to be necessary for the activation of the *C. albicans* Mkc1 homolog Slt2 in *S. cerevisiae* [22, 63]. However, we observed that loss of PS synthase in *C. albicans* causes increased Mkc1 activity (Figure 2. 2), suggesting that there are fundamental differences in the manner through which the Mkc1-associated cascade is regulated in pathogenic versus non-pathogenic yeasts. This

report also sets the stage for better understanding how the phospholipid PS synthase influences GTPase activity and localization in this pathogenic organism.

Conclusions

Candida albicans is able to diminish its detection by innate immune cells through masking of β (1,3)-glucan in the inner cell wall with an outer layer of heavily glycosylated mannoproteins (mannan) [12, 64, 65]. Once exposed, this glucose polymer antigen can be detected by Dectin-1, a C-type signaling lectin found on host immune cells [10, 11]. However, it usually takes several days after infection before β (1,3)-glucan is exposed to the immune system [64]. Therefore, if the β (1,3)-glucan exposure process could be induced more rapidly, the immune responses would be expected to improve and clear fungal pathogens more effectively [12, 64, 66-68].

Identification of specific pathways that contribute to β (1,3)-glucan exposure when activated could help elucidate future drug targets that can induce β (1,3)-glucan exposure to improve immune response. Thus, compounds that specifically activate Cek1 may be useful in this regard. If such compounds were combined with the current azole class of antifungals, which act statically, and immune detection were simultaneously enhanced, this could potentially enhance the clearance of fungi.

References

1. Naglik JR, Moyes D. Epithelial cell innate response to Candida albicans. Advances in dental research. 2011;23(1):50-5. doi: 10.1177/0022034511399285. PubMed PMID: 21441481; PubMed Central PMCID: PMC3144045.
2. Morrell M, Fraser VJ, Kollef MH. Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. Antimicrobial agents and chemotherapy. 2005;49(9):3640-5. doi: 10.1128/AAC.49.9.3640-3645.2005. PubMed PMID: 16127033; PubMed Central PMCID: PMC1195428.
3. Eggimann P, Garbino J, Pittet D. Management of Candida species infections in critically ill patients. The Lancet Infectious diseases. 2003;3(12):772-85. PubMed PMID: 14652203.
4. Bustamante CI. Treatment of Candida infection: a view from the trenches! Curr Opin Infect Dis. 2005;18(6):490-5. PubMed PMID: 16258321.
5. Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD. Azole Antifungal Resistance in Candida albicans and Emerging Non-albicans Candida Species. Front Microbiol. 2016;7:2173. doi: 10.3389/fmicb.2016.02173. PubMed PMID: 28127295; PubMed Central PMCID: PMCPMC5226953.
6. Perlin DS. Mechanisms of echinocandin antifungal drug resistance. Ann N Y Acad Sci. 2015;1354:1-11. doi: 10.1111/nyas.12831. PubMed PMID: 26190298; PubMed Central PMCID: PMCPMC4626328.
7. Barker KS, Rogers PD. Recent insights into the mechanisms of antifungal resistance. Curr Infect Dis Rep. 2006;8(6):449-56. PubMed PMID: 17064638.
8. Ruiz-Herrera J, Elorza MV, Valentin E, Sentandreu R. Molecular organization of the cell wall of Candida albicans and its relation to pathogenicity. FEMS yeast research. 2006;6(1):14-29. doi: 10.1111/j.1567-1364.2005.00017.x. PubMed PMID: 16423067.
9. Cullen PJ, Edgerton M. Unmasking fungal pathogens by studying MAPK-dependent cell wall regulation in Candida albicans. Virulence. 2016;7(5):502-5. doi: 10.1080/21505594.2016.1177695. PubMed PMID: 27088569; PubMed Central PMCID: PMC5026791.
10. Brown GD, Gordon S. Immune recognition. A new receptor for beta-glucans. Nature. 2001;413(6851):36-7. doi: 10.1038/35092620. PubMed PMID: 11544516.
11. Dennehy KM, Brown GD. The role of the beta-glucan receptor Dectin-1 in control of fungal infection. J Leukoc Biol. 2007;82(2):253-8. doi: 10.1189/jlb.1206753. PubMed PMID: 17475782.
12. Wheeler RT, Kombe D, Agarwala SD, Fink GR. Dynamic, morphotype-specific Candida albicans beta-glucan exposure during infection and drug treatment. PLoS pathogens. 2008;4(12):e1000227. doi: 10.1371/journal.ppat.1000227. PubMed PMID: 19057660; PubMed Central PMCID: PMC2587227.
13. Davis SE, Hopke A, Minkin SC, Jr., Montedonico AE, Wheeler RT, Reynolds TB. Masking of beta(1-3)-glucan in the cell wall of Candida albicans from detection by innate immune cells depends on phosphatidylserine. Infection and immunity. 2014. doi: 10.1128/IAI.01612-14. PubMed PMID: 25114110.

14. Hasim S, Allison DP, Retterer ST, Hopke A, Wheeler RT, Doktycz MJ, et al. beta-(1,3)-Glucan Unmasking in Some Candida albicans Mutants Correlates with Increases in Cell Wall Surface Roughness and Decreases in Cell Wall Elasticity. Infection and immunity. 2017;85(1). doi: 10.1128/IAI.00601-16. PubMed PMID: 27849179; PubMed Central PMCID: PMC5203643.
15. Galan-Diez M, Arana DM, Serrano-Gomez D, Kremer L, Casasnovas JM, Ortega M, et al. Candida albicans beta-glucan exposure is controlled by the fungal CEK1-mediated mitogen-activated protein kinase pathway that modulates immune responses triggered through dectin-1. Infection and immunity. 2010;78(4):1426-36. doi: 10.1128/IAI.00989-09. PubMed PMID: 20100861; PubMed Central PMCID: PMC2849429.
16. Chen Y-L, Montedonico AE, Kauffman S, Dunlap JR, Menn F-M, Reynolds TB. Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence inCandida albicans. Molecular microbiology. 2010;75(5):1112-32. doi: 10.1111/j.1365-2958.2009.07018.x.
17. Hopke A, Nicke N, Hidu EE, Degani G, Popolo L, Wheeler RT. Neutrophil Attack Triggers Extracellular Trap-Dependent Candida Cell Wall Remodeling and Altered Immune Recognition. PLoS pathogens. 2016;12(5):e1005644. doi: 10.1371/journal.ppat.1005644. PubMed PMID: 27223610; PubMed Central PMCID: PMC4880299.
18. Roman E, Cottier F, Ernst JF, Pla J. Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in Candida albicans. Eukaryotic cell. 2009;8(8):1235-49. doi: 10.1128/EC.00081-09. PubMed PMID: 19542310; PubMed Central PMCID: PMC2725568.
19. Monge RA, Roman E, Nombela C, Pla J. The MAP kinase signal transduction network in Candida albicans. Microbiology. 2006;152(Pt 4):905-12. doi: 10.1099/mic.0.28616-0. PubMed PMID: 16549655.
20. Kohler JR, Fink GR. Candida albicans strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(23):13223-8. PubMed PMID: 8917572; PubMed Central PMCID: PMCPMC24074.
21. Liu H, Kohler J, Fink GR. Suppression of hyphal formation in Candida albicans by mutation of a STE12 homolog. Science. 1994;266(5191):1723-6. PubMed PMID: 7992058.
22. Kamada Y, Qadota H, Python CP, Anraku Y, Ohya Y, Levin DE. Activation of yeast protein kinase C by Rho1 GTPase. The Journal of biological chemistry. 1996;271(16):9193-6. PubMed PMID: 8621575.
23. Levin DE. Regulation of cell wall biogenesis in Saccharomyces cerevisiae: the cell wall integrity signaling pathway. Genetics. 2011;189(4):1145-75. doi: 10.1534/genetics.111.128264. PubMed PMID: 22174182; PubMed Central PMCID: PMC3241422.
24. Roman E, Alonso-Monge R, Miranda A, Pla J. The Mkk2 MAPKK Regulates Cell Wall Biogenesis in Cooperation with the Cek1-Pathway in Candida albicans. PloS one. 2015;10(7):e0133476. doi: 10.1371/journal.pone.0133476. PubMed PMID: 26197240; PubMed Central PMCID: PMC4509911.

25. Xie JL, Grahl N, Sless T, Leach MD, Kim SH, Hogan DA, et al. Signaling through Lrg1, Rho1 and Pkc1 Governs Candida albicans Morphogenesis in Response to Diverse Cues. PLoS genetics. 2016;12(10):e1006405. doi: 10.1371/journal.pgen.1006405. PubMed PMID: 27788136; PubMed Central PMCID: PMC5082861.
26. Delley PA, Hall MN. Cell wall stress depolarizes cell growth via hyperactivation of RHO1. The Journal of cell biology. 1999;147(1):163-74. PubMed PMID: 10508863; PubMed Central PMCID: PMC2164985.
27. Valdivia RH, Schekman R. The yeasts Rho1p and Pkc1p regulate the transport of chitin synthase III (Chs3p) from internal stores to the plasma membrane. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(18):10287-92. doi: 10.1073/pnas.1834246100. PubMed PMID: 12928491; PubMed Central PMCID: PMC193553.
28. Lenardon MD, Milne SA, Mora-Montes HM, Kaffarnik FA, Peck SC, Brown AJ, et al. Phosphorylation regulates polarisation of chitin synthesis in Candida albicans. Journal of cell science. 2010;123(Pt 13):2199-206. doi: 10.1242/jcs.060210. PubMed PMID: 20530569; PubMed Central PMCID: PMC2886742.
29. Lenardon MD, Whitton RK, Munro CA, Marshall D, Gow NA. Individual chitin synthase enzymes synthesize microfibrils of differing structure at specific locations in the Candida albicans cell wall. Molecular microbiology. 2007;66(5):1164-73. doi: 10.1111/j.1365-2958.2007.05990.x. PubMed PMID: 17971081; PubMed Central PMCID: PMCPMC2780561.
30. Munro CA, Selvaggini S, de Bruijn I, Walker L, Lenardon MD, Gerssen B, et al. The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in Candida albicans. Molecular microbiology. 2007;63(5):1399-413. doi: 10.1111/j.1365-2958.2007.05588.x. PubMed PMID: 17302816; PubMed Central PMCID: PMCPMC2649417.
31. Qadota H, Python CP, Inoue SB, Arisawa M, Anraku Y, Zheng Y, et al. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. Science. 1996;272(5259):279-81. PubMed PMID: 8602515.
32. Ushinsky SC, Harcus D, Ash J, Dignard D, Marcil A, Morchhauser J, et al. CDC42 is required for polarized growth in human pathogen Candida albicans. Eukaryotic cell. 2002;1(1):95-104. PubMed PMID: 12455975; PubMed Central PMCID: PMC118047.
33. Brand AC, Morrison E, Milne S, Gonia S, Gale CA, Gow NA. Cdc42 GTPase dynamics control directional growth responses. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(2):811-6. doi: 10.1073/pnas.1307264111. PubMed PMID: 24385582; PubMed Central PMCID: PMCPMC3896204.
34. Bassilana M, Hopkins J, Arkowitz RA. Regulation of the Cdc42/Cdc24 GTPase module during Candida albicans hyphal growth. Eukaryotic cell. 2005;4(3):588-603. doi: 10.1128/EC.4.3.588-603.2005. PubMed PMID: 15755921; PubMed Central PMCID: PMCPMC1087799.
35. Csank C, Schroppel K, Leberer E, Harcus D, Mohamed O, Meloche S, et al. Roles of the Candida albicans mitogen-activated protein kinase homolog,

Cek1p, in hyphal development and systemic candidiasis. Infection and immunity. 1998;66(6):2713-21. PubMed PMID: 9596738; PubMed Central PMCID: PMCPMC108260.
36. Leberer E, Ziegelbauer K, Schmidt A, Harcus D, Dignard D, Ash J, et al. Virulence and hyphal formation of Candida albicans require the Ste20p-like protein kinase CaCla4p. Current biology : CB. 1997;7(8):539-46. PubMed PMID: 9259554.
37. Gulli MP, Jaquenoud M, Shimada Y, Niederhauser G, Wiget P, Peter M. Phosphorylation of the Cdc42 exchange factor Cdc24 by the PAK-like kinase Cla4 may regulate polarized growth in yeast. Mol Cell. 2000;6(5):1155-67. PubMed PMID: 11106754.
38. Styles C. How to set up a yeast laboratory. Methods in enzymology. 2002;350:42-71. PubMed PMID: 12073328.
39. Care RS, Trevethick J, Binley KM, Sudbery PE. The MET3 promoter: a new tool for Candida albicans molecular genetics. Molecular microbiology. 1999;34(4):792-8. PubMed PMID: 10564518.
40. Backen AC, Broadbent ID, Fetherston RW, Rosamond JD, Schnell NF, Stark MJ. Evaluation of the CaMAL2 promoter for regulated expression of genes in Candida albicans. Yeast. 2000;16(12):1121-9. doi: 10.1002/1097-0061(20000915)16:12<1121::AID-YEA614>3.0.CO;2-U. PubMed PMID: 10953084.
41. Chen YL, Kauffman S, Reynolds TB. Candida albicans uses multiple mechanisms to acquire the essential metabolite inositol during infection. Infection and immunity. 2008;76(6):2793-801. doi: 10.1128/IAI.01514-07. PubMed PMID: 18268031; PubMed Central PMCID: PMC2423082.
42. De Backer MD, Maes D, Vandoninck S, Logghe M, Contreras R, Luyten WH. Transformation of Candida albicans by electroporation. Yeast. 1999;15(15):1609-18. doi: 10.1002/(SICI)1097-0061(199911)15:15<1609::AID-YEA485>3.0.CO;2-Y. PubMed PMID: 10572258.
43. Ramirez-Zavala B, Weyler M, Gildor T, Schmauch C, Kornitzer D, Arkowitz R, et al. Activation of the Cph1-dependent MAP kinase signaling pathway induces white-opaque switching in Candida albicans. PLoS pathogens. 2013;9(10):e1003696. doi: 10.1371/journal.ppat.1003696. PubMed PMID: 24130492; PubMed Central PMCID: PMC3795047.
44. Reuss O, Vik A, Kolter R, Morschhauser J. The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene. 2004;341:119-27. doi: 10.1016/j.gene.2004.06.021. PubMed PMID: 15474295.
45. Vyas VK, Barrasa MI, Fink GR. A Candida albicans CRISPR system permits genetic engineering of essential genes and gene families. Science advances. 2015;1(3):e1500248. doi: 10.1126/sciadv.1500248. PubMed PMID: 25977940; PubMed Central PMCID: PMCPMC4428347.
46. Corvest V, Bogliolo S, Follette P, Arkowitz RA, Bassilana M. Spatiotemporal regulation of Rho1 and Cdc42 activity duringCandida albicansfilamentous growth. Molecular microbiology. 2013;89(4):626-48. doi: 10.1111/mmi.12302.
47. Phan QT, Belanger PH, Filler SG. Role of hyphal formation in interactions of Candida albicans with endothelial cells. Infection and immunity.

2000;68(6):3485-90. PubMed PMID: 10816502; PubMed Central PMCID: PMCPMC97632.
48. Li R, Puri S, Tati S, Cullen PJ, Edgerton M. Candida albicans Cek1 mitogen-activated protein kinase signaling enhances fungicidal activity of salivary histatin 5. Antimicrobial agents and chemotherapy. 2015;59(6):3460-8. doi: 10.1128/AAC.00214-15. PubMed PMID: 25824232; PubMed Central PMCID: PMC4432120.
49. Baggett JJ, Shaw JD, Sciambi CJ, Watson HA, Wendland B. Fluorescent labeling of yeast. Curr Protoc Cell Biol. 2003;Chapter 4:Unit 4 13. doi: 10.1002/0471143030.cb0413s20. PubMed PMID: 18228435.
50. Hopke A, Brown AJP, Hall RA, Wheeler RT. Dynamic Fungal Cell Wall Architecture in Stress Adaptation and Immune Evasion. Trends in microbiology. 2018;26(4):284-95. doi: 10.1016/j.tim.2018.01.007. PubMed PMID: 29452950; PubMed Central PMCID: PMC5869159.
51. Sherrington SL, Sorsby E, Mahtey N, Kumwenda P, Lenardon MD, Brown I, et al. Adaptation of Candida albicans to environmental pH induces cell wall remodelling and enhances innate immune recognition. PLoS pathogens. 2017;13(5):e1006403. doi: 10.1371/journal.ppat.1006403. PubMed PMID: 28542528; PubMed Central PMCID: PMC5456412.
52. Fairn GD, Hermansson M, Somerharju P, Grinstein S. Phosphatidylserine is polarized and required for proper Cdc42 localization and for development of cell polarity. Nature cell biology. 2011;13(12):1424-30. doi: 10.1038/ncb2351.
53. Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A, Grinstein S. Membrane phosphatidylserine regulates surface charge and protein localization. Science. 2008;319(5860):210-3. doi: 10.1126/science.1152066. PubMed PMID: 18187657.
54. Finkielstein CV, Overduin M, Capelluto DG. Cell migration and signaling specificity is determined by the phosphatidylserine recognition motif of Rac1. The Journal of biological chemistry. 2006;281(37):27317-26. doi: 10.1074/jbc.M605560200. PubMed PMID: 16861229.
55. Das A, Slaughter BD, Unruh JR, Bradford WD, Alexander R, Rubinstein B, et al. Flippase-mediated phospholipid asymmetry promotes fast Cdc42 recycling in dynamic maintenance of cell polarity. Nature cell biology. 2012;14(3):304-10. doi: 10.1038/ncb2444. PubMed PMID: 22344035; PubMed Central PMCID: PMC3534761.
56. Davis CR, Richman TJ, Deliduka SB, Blaisdell JO, Collins CC, Johnson DI. Analysis of the mechanisms of action of the Saccharomyces cerevisiae dominant lethal cdc42G12V and dominant negative cdc42D118A mutations. The Journal of biological chemistry. 1998;273(2):849-58. PubMed PMID: 9422741.
57. Diez-Orejas R, Molero G, Navarro-Garcia F, Pla J, Nombela C, Sanchez-Perez M. Reduced virulence of Candida albicans MKC1 mutants: a role for mitogen-activated protein kinase in pathogenesis. Infection and immunity. 1997;65(2):833-7. PubMed PMID: 9009353; PubMed Central PMCID: PMCPMC176136.
58. Roman E, Nombela C, Pla J. The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen Candida albicans. Molecular and cellular biology. 2005;25(23):10611-27. doi:

10.1128/MCB.25.23.10611-10627.2005. PubMed PMID: 16287872; PubMed Central PMCID: PMC1291223.
59. Guhad FA, Jensen HE, Aalbaek B, Csank C, Mohamed O, Harcus D, et al. Mitogen-activated protein kinase-defective Candida albicans is avirulent in a novel model of localized murine candidiasis. FEMS microbiology letters. 1998;166(1):135-9. PubMed PMID: 9741091.
60. Roman E, Alonso-Monge R, Gong Q, Li D, Calderone R, Pla J. The Cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen Candida albicans. FEMS yeast research. 2009;9(6):942-55. doi: 10.1111/j.1567-1364.2009.00545.x. PubMed PMID: 19656200.
61. Bassilana M, Arkowitz RA. Rac1 and Cdc42 have different roles in Candida albicans development. Eukaryotic cell. 2006;5(2):321-9. doi: 10.1128/EC.5.2.321-329.2006. PubMed PMID: 16467473; PubMed Central PMCID: PMC1405900.
62. Saito K, Fujimura-Kamada K, Hanamatsu H, Kato U, Umeda M, Kozminski KG, et al. Transbilayer phospholipid flipping regulates Cdc42p signaling during polarized cell growth via Rga GTPase-activating proteins. Developmental cell. 2007;13(5):743-51. doi: 10.1016/j.devcel.2007.09.014. PubMed PMID: 17981141.
63. Nomura W, Ito Y, Inoue Y. Role of phosphatidylserine in the activation of Rho1-related Pkc1 signaling in Saccharomyces cerevisiae. Cellular signalling. 2017;31:146-53. doi: 10.1016/j.cellsig.2017.01.002. PubMed PMID: 28065784.
64. Wheeler RT, Fink GR. A Drug-Sensitive Genetic Network Masks Fungi from the Immune System. PLoS pathogens. 2006;2(4):e35. doi: 10.1371/journal.ppat.0020035.
65. Shen H, Chen SM, Liu W, Zhu F, He LJ, Zhang JD, et al. Abolishing Cell Wall Glycosylphosphatidylinositol-Anchored Proteins in Candida albicans Enhances Recognition by Host Dectin-1. Infection and immunity. 2015;83(7):2694-704. doi: 10.1128/IAI.00097-15. PubMed PMID: 25895969; PubMed Central PMCID: PMC4468527.
66. Mitchell AP, Wheeler RT, Kombe D, Agarwala SD, Fink GR. Dynamic, Morphotype-Specific Candida albicans β-Glucan Exposure during Infection and Drug Treatment. PLoS pathogens. 2008;4(12):e1000227. doi: 10.1371/journal.ppat.1000227.
67. Khan AA, Wheeler JM, Cunningham C, George B, Kettlewell M, Mortensen NJ. The management and outcome of anastomotic leaks in colorectal surgery. Colorectal Dis. 2008;10(6):587-92. Epub 2007/12/12. doi: CDI1417 [pii]
10.1111/j.1463-1318.2007.01417.x. PubMed PMID: 18070185.
68. Marakalala MJ, Vautier S, Potrykus J, Walker LA, Shepardson KM, Hopke A, et al. Differential adaptation of Candida albicans in vivo modulates immune recognition by dectin-1. PLoS pathogens. 2013;9(4):e1003315. doi: 10.1371/journal.ppat.1003315. PubMed PMID: 23637604; PubMed Central PMCID: PMC3630191.
69. Gillum AM, Tsay EY, Kirsch DR. 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. 1984;198(2):179-82. PubMed PMID: 6394964.

Appendix

Table S.2. 1. *C. albicans* strains used in this study

Strain	Parent	Genotype	Source or reference
SC5314	Clinical isolate	Prototrophic wild type	[69]
YLC337	SC5314	*cho1Δ/Δ*	[16]
SED022	YLC337	*cho1Δ/Δ::CHO1*	[13]
YLC375	SC5314	*psd1Δ/Δ psd2Δ/Δ*	[16]
TC001	SC5314	Wild-type P_{MET3}-*GFP-CDC42*	This study
TC002	YLC337	*cho1Δ/Δ* P_{MET3}-*GFP-CDC42*	This study
TC003	SED022	*cho1Δ/Δ::CHO1* P_{MET3}-*GFP-CDC42*	This study
TC004	YLC375	*psd1Δ/Δpsd2Δ/Δ* P_{MET3}-*GFP-CDC42*	This study
TC005	SC5314	*mkc1Δ/Δ*	This study
TC006	SC5314	Wild-type P_{MET3}-*GFP-CDC42*$^{K183\text{-}187Q}$	This study
TC007	SC5314	*rac1Δ/Δ*	This study
TC011	SC5314	Wild-type P_{MAL}-*STE11*$^{\Delta N467}$	This study
TC033	YLC337	*cho1Δ/Δrac1Δ/Δ*	This study
TC034	YLC337	*cho1Δ/Δmkc1Δ/Δ*	This study
TC046	SC5314	Wild-type *GFP-RID*	This study
TC064	TC033	*cho1Δ/Δrac1Δ/Δ* CRIBGFP	This study
TC071	YLC337	*cho1Δ/Δ GFPRID*	This study
TC072	SED022	*cho1Δ/Δ::CHO1* GFPRID	This study
TC073	TC007	*rac1Δ/Δ* CRIBGFP	This study
TC078	SC5314	Wild-type P_{ENO1}-*CDC42*^{G12V}	This study
TC083	SC5314	Wild-type P_{ENO1}-*RHO1*^{Q67L}	This study
TC107	SC5314	Wild-type *PKC1/pkc1Δ*	This study
TC110	YLC337	*cho1Δ/Δ PKC1/pkc1Δ*	This study

Table S.2. 2. Plasmids used in this study

Strain Name	Description	Source
pYLC314	*CaNAT-$P_{MET3,}$ ampR*	[41]
pTC001	*CaNAT-P_{MET3}-GFP-CDC42, ampR,*	This study
pYLC146	FLP-CaNAT, *chloraR*	This study
pBT1	*CaNAT-P_{ENO1}*, *ampR*	This study
pExpArg-pADH1CRIBGFP	pExpArg-pADH1CRIBGFP	[46]
pTC009	pExpArg-pADH1CRIBGFP+HYG^R	This study
pTC014	*CaNAT-P_{MET3}-GFP-$CDC42^{K183\text{-}187Q}$, ampR*	This study
pV1393	*FLP-CAS9-CaNAT-SNR52p-sgRNA*, *ampR*	[45]
pTC015	pV1393 *CaMKC1-sgRNA, ampR*	This study
pTC018	pV1393 *CaRAC1-sgRNA*, *ampR*	This study
pTC019	CaNAT-P_{MA}L, *ampR,*	This study
pTC020	pTC019 *$CaSTE11^{\Delta N467}$,* ampR	This study
pTC029	pExpArg-pADH1CRIBGFP+Hyg^R	This study
pExpArg-pACT1GFPRID	pExpArg-pACT1GFPRID, *ampR*	[46]
pTC033	pExpArg-pACT1GFPRID+NAT^R	This study
pTC034	pBT1 *CaCDC42*, *ampR*	This study
pTC035	pBT1 *CaRHO1*, *ampR*	This study
pTC037	pBT1 *$CaCDC42^{G12V}$*, *ampR*	This study
pTC038	pBT1 *$CaRHO1^{Q67L}$*, *ampR*	This study
pTC041	pYLC146 CaPKC1-KO, *chloraR*	This study

Table S.2. 3. Primers used in this study

Name	Sequence	Description
TCO1	GATTGTGGCTCATAGTAAC	Primer-F to check if pTC1/pTC14 integrated into pMET3 locus
TCO2	AGTAGCATCACCTTCACCT	Primer-R to check if pTC1/pTC14 integrated into pMET3 locus
TCO8	AAAAGAGCTCAAAAGTATAGGATAAAACATAAT	Primer-R for CaRHO1 amplification with SacI flanking
TCO15	AAAGCGGCCGCCGTCAAAACTAGAGAATAATAAG	Primer-F to amplify SAT1 resistance gene with NotI flanking
TCO16	AAAGCGGCCGCGACCACCTTTGATTGTAAA	Primer-R to amplify SAT1 resistance gene with NotI flanking
TCO28	TATATTATGGCAGATCCGTAA	Primer-R at CaMKC1+23bp for colony PCR to detect if the MKC1 repair template integrated into the chromosome
TCO29	AACCCATCTACAGTCTCAAA	Primer-R at CaMKC1+1097 for colony PCR to detect if the MKC1 repair template integrated into the chromosome
TCO32	ATTTGAGAACCCCGACGAAAATGCTG	Primer-F at CaMKC1+602 to amplify MKC1 SgRNA
TCO33	AAAACAGCATTTTCGTCGGGGTTCTC	Primer-R at CaMKC+621 to amplify MKC1 SgRNA
TCO36	AAAACTGCAGTTGATATTTTTGTCTAGTACC	Primer-F at CaMAL2-543 to amplify MAL2 promoter
TCO37	AAAACTGCAGTGTAGTTGATTATTAGTTAAAC	Primer-R at CaMAL2-1 to amplify MAL2 promoter
TCO38	ATTTGAACCAATCAAATTAGGATTAG	Primer-F at CaRAC1 to amplify RAC1 SgRNA
TCO39	AAAACTAATCCTAATTTGATTGGTTC	Primer-R at CaRAC1 to amplify RAC1 SgRNA
TCO42	AAAAGGATCCATGGATTCGAGATCCATAAT	Primer-F to amplify *CaSTE11*$^{\Delta N467}$ with BamHI flanking
TCO43	AAAAGGATTCCTCGAGGGAAATAACTACTA	Primer-R to amplify *CaSTE11*$^{\Delta N467}$ with BamHI flanking
TCO44	ATTTGAGTCGAAAATAAGTATTCCGG	Primer-F at CaPKC1 to amplify PKC1 SgRNA
TCO45	AAAACCGGAATACTTATTTTCGACTC	Primer-R at CaPKC1 to amplify PKC1 SgRNA
TCO48	AAGACACGGTATAAAAATGG	Primer-F at pMAL2 region of pTC19 to check if pTC19 reintegrated into the chromosome
TCO49	ACAAGTTGTCAATGATATTAG	Primer-F for colony PCR to detect if the PKC1 repair template integrated into the chromosome

Table S.2. 3. Continued

Name	Sequence	Description
TCO50	ATCACTTGAAGGAGATACTG	Primer-R for colony PCR to detect if the PKC1 repair template integrated into the chromosome
TCO51	AGCATTAAATCAGTCGTAGT	Primer-F at CaRAC1+7bp for colony PCR to detect if the RAC1 repair template integrated into the chromosome
TCO52	TCTTTTCTTACCAGTACCAG	Primer-R at CaRAC1+675bp for colony PCR to detect if the RAC1 repair template integrated into the chromosome
TCO57	TTTCCACAATCAAACATCCAA	Primer-R at *CaSTE11*$^{\Delta N467}$ region of pTC19 to check if pTC19 reintegrated into the chromosome
TCO59	AAAAGGTACCTACAAGAAAGTAAAGCCAAAG	Primer-F at CaPKC1-501bp to amplify CaPKC1 promoter with KpnI flanking
TCO60	AAAACTCGAGATTGGAATTACACTGATCTC	Reverse primer at CaPKC1-1bp to amplify CaPKC1 promoter with XhoI flanking
TCO62	AAAAGAGCTCGATACAAATAGAGAAGTTTCC	Primer-R at +700bp of CaPKC1 3'NTR to amplify CaPKC1 terminator with SacI flanking
TCO63	GCAATAGTAAGTAGCGAATAA	Primer-F at CaPKC1-688 to check if the linearized pTC41 integrated into chromosome
TCO72	AAAAGCGGCCGCATGGTTAACGGTCCAGCTGA	Primer-F for CaRHO1 amplification with NotI flanking
TCO74	ATTATGGGATACTGCTGGTCTAGAAGATTATGATAGATTAA	Primer-F for point mutation of *CaRHO1*^{R67L}
TCO75	TTAATCTATCATAATCTTCTAGACCAGCAGTATCCCATAAT	Primer-R for point mutation of *CaRHO1*^{R67L}
TCO76	TGTGTTGTTGTCGGTGATGTTGCCGTTGGTAAAACTTGCT	Primer-F for point mutation of *CaCDC42*^{G12V}
TCO77	AGCAAGTTTTACCAACGGCAACATCACCGACAACAACACA	Primer-R for point mutation of *CaCDC42*^{G12V}
TCO92	AAAGCGGCCGCTCGATATTGCTAGAGGCAAA	Primer-F to amplify Hygromycin resistant gene
TCO93	AAAGCGGCCGCATTTTATGATGGAATGAATGG	Primer-R to amplify Hygromycin resistant gene
TCO101	AAAAGCGGCCGCATGCAAACTATAAAATGTGTTG	Primer-F to amplify *CaCDC42* with NotI flanking
TCO110	AAAAGCGGCCGCTGATTAAAGTAGATATATAATAATA	Primer-F at +1bp of CaPKC1 3'NTR to amplify CaPKC1 terminator with NotI flanking
TCO988	AAAAGGATCCATGTCTAAAGGTGAAGAATTA	Primer-F to amplify EGFP with BamHI flanking

Table S.2. 3. Continued

Name	Sequence	Description
TRO989	AAAAGGATTCTTTGTACAATTCATCCATAC	Primer-R to amplify EGFP with BamHI flanking
TRO993	TTGGTGATGGTCCAGTCTTGT	Primer-F at the EGFP region of pTC1 to check if EGFP and CDC42 ligated in a correct orientation
TRO994	CAGAACACTCAACATACTTGACAGC	Primer-R at CaCDC42+463bp to check if EGFP and CDC42 ligated in a correct orientation
TRO995	AAAAGAGCTCGCAGCTGCAGCTGCAGCTGCAGCTCAAACTA TAAAATGTGTTGT	Primer-F to amplify Cdc42 with SacI flanking
TRO996	AAAAGAGCTCTGATTTATATTATGTCTAGAGT	Primer-R to amplify CaCDC42 with SacI flanking
JCO95	GATTATTAGTTAAACCACTGC	Primer-R at pMAL2 of pTC41 to check if the linearized pTC41 integrated into chromosome
BTO30	AAAAGGATTCAGGTCTGTCATATTTCTATC	Primer-F to amplify ENO1 promoter
BTO31	AAAAGCGGCCGCGTTGTAATATTCCTGAATTATC	Primer-R to amplify ENO1 promoter
JCO165	AAAGAATTCCGTCAAAACTAGAGAATAATAAAG	Primer-F to amplify pMET3-SAT1
JCO166	AAAGAATTCGTTTTCTGGGGAGGGTATTT	Primer-R to amplify pMET3-SAT1
TCO34	CTTAAAATTTGTGACTTTGGTTTAGCAAGAGGGTTTTCTGAG AACCCCTAAGAATAAGCTT	CaMKC1-F for crispr-cas9 repair template
TCO35	CCCTGTACCATCTGGTGGCAACATACTCTGTCATAAACTATT AAGCTTATTCTTAGGGGTTCT	CaMKC1-R for crispr-cas9 repair template
TCO40	GTTTTTGATAATTATTCAGCCTCAGTTATGATTGATGGCGAAC CAATCTAATAAGGATAAGCTT	CaRAC1-F for crispr-cas9 repair template
TCO41	GTCTTAATCTATCATATTCTGATTGACCAGCGGTATAAGCTTA TCCTTATTAGATTGGTT	CaRAC1-R for crispr-cas9 repair template
TCO46	AGATTCAATTTATGTTGCAATATTTGGAATTCAAACTCCAGAT CTAAAATAAGTAATCCTAA	CaPKC1-F for crispr-cas9 repair template
TCO47	CCATCCATAAGATAAAGATGAGACAATTTTTTATTGGCTTAG GATTACTTATTTTAGATCT	CaPKC1-R for crispr-cas9 repair template

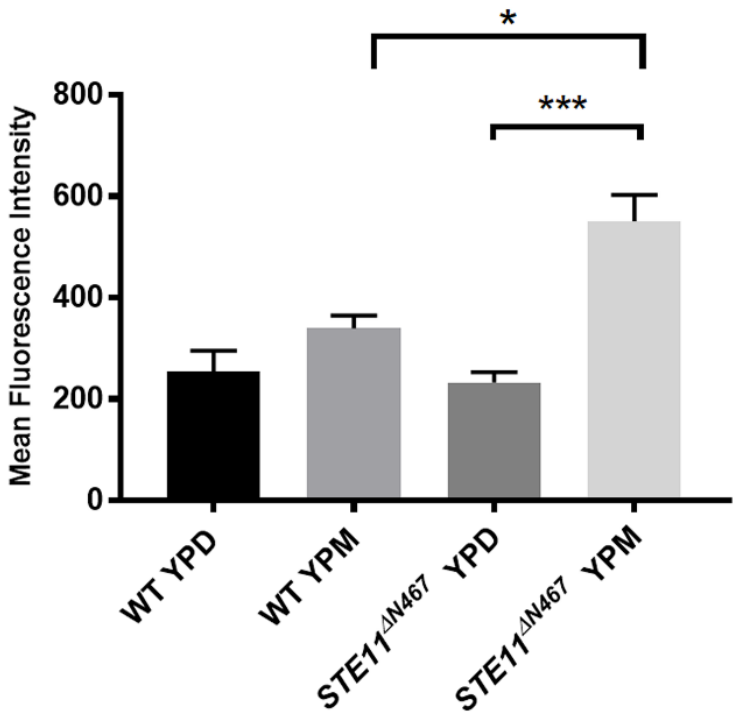


Figure S.2. 1. *STE11*$^{\Delta N467}$ exhibited significantly increased β (1,3)-glucan exposure compared to wild-type cells.

Overnight cultures of Candida cells were incubated with anti-β (1,3)-glucan primary antibody and PE-conjugated secondary antibody, followed by flow cytometry to quantify the fluorescence intensity. Data represent three biological replicates. The statistical analysis was done by One-way ANOVA. ***, P=0.0004; *, p=0.0137.

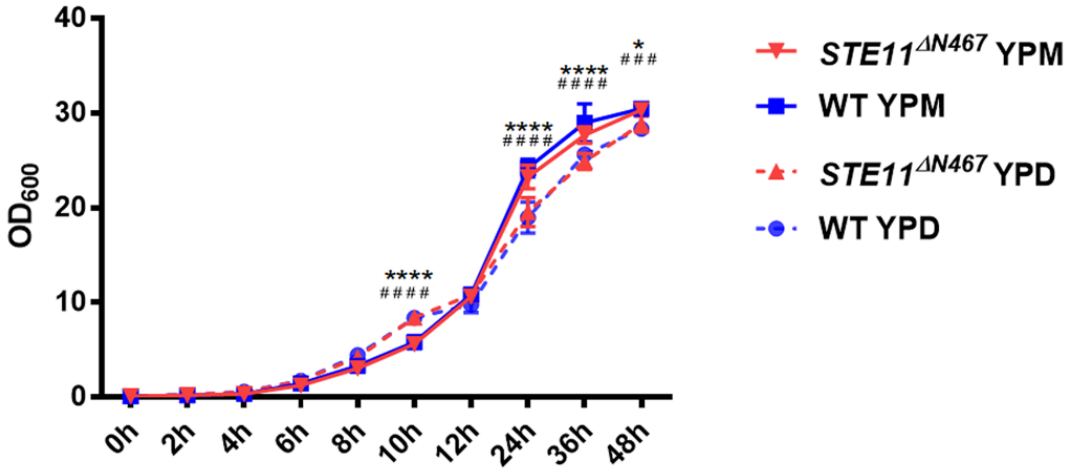


Figure S.2. 2. Growth curves were measured to determine the growth rate of strains in YPD vs YPM.

Cells were grown overnight in YPD, diluted back to 0.1 OD600 and transferred to fresh YPD or YPM. A growth curve was performed with three replicates per condition, and plotted based on the growth rate of different strains measured in 48 hrs. The growth at each time-point between YPD and YPM cultures of *STE11*$^{\Delta N467}$ were compared by Two-way ANOVA (****, p<0.0001; *, p=0.0286). The same comparison was made between wild-type YPD and YPM culture (####, p<0.0001; ###, p=0.0007).

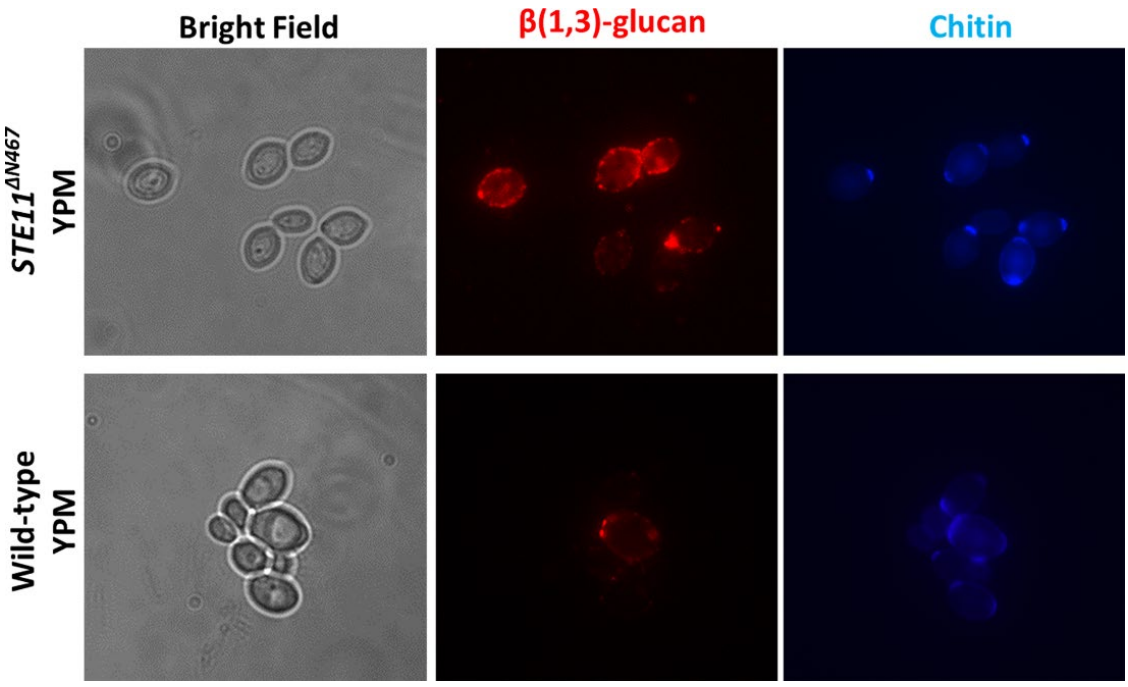


Figure S.2. 3. The exposed β (1,3)-glucan in *STE11*$^{\Delta N467}$ YPM cells was not restricted to bud scars.

Overnight cultures of wild-type and *STE11*$^{\Delta N467}$ grown in YPM were co-stained with anti-β (1,3)-glucan antibody and Cy3 secondary to visualize exposed β (1,3)-glucan and calcofluor white to visualize chitin.

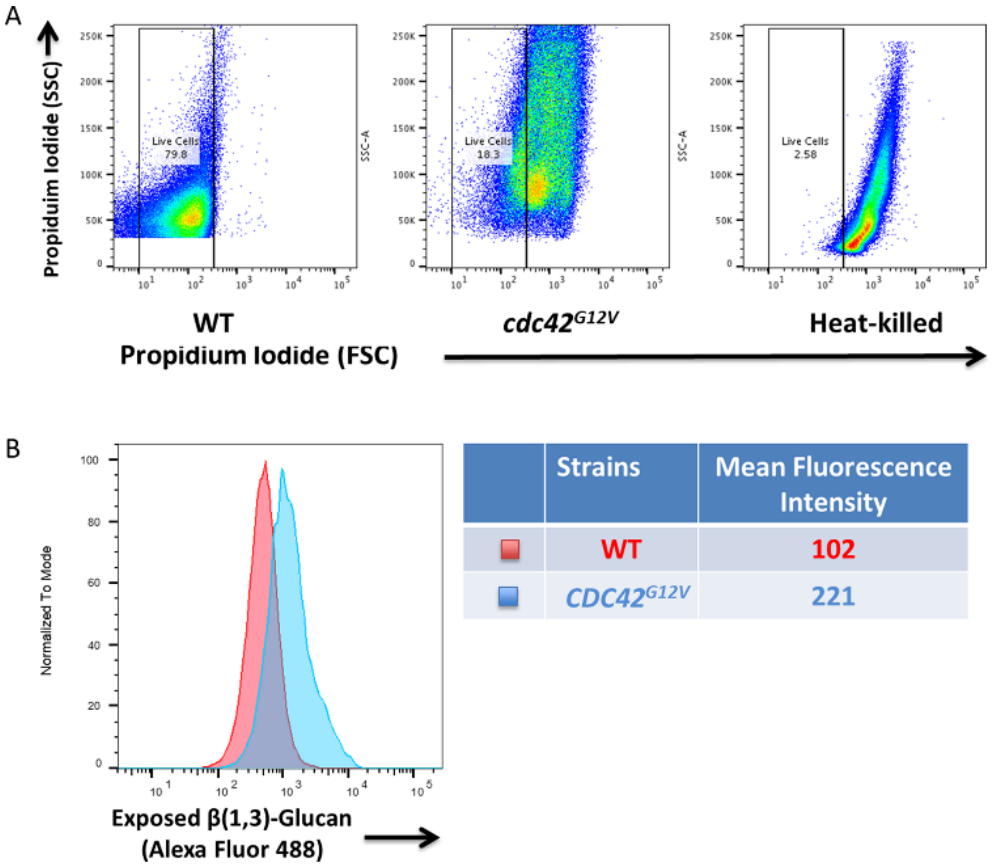


Figure S.2. 4. *CDC42*^{G12V} increases β (1,3)-glucan exposure, but also reduces the viable cell population.

(A) Propidium iodide staining was performed to quantify the live cells in *Candida* strains. (B) β (1,3)-glucan exposure in live (gated for propidium iodide negative cells) wild-type and *CDC42*^{G12V} populations was measured by flow cytometry.

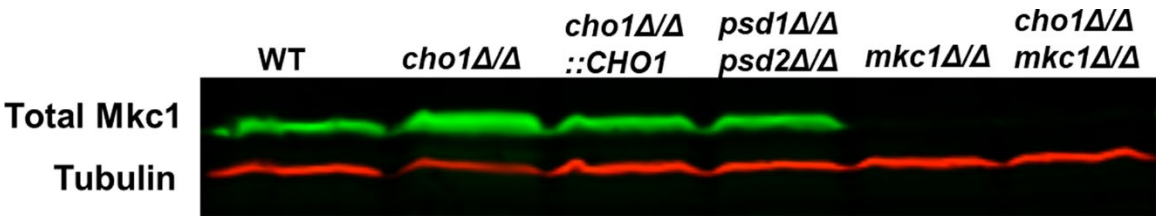


Figure S.2. 5. *MKC1* was knocked out in *C. albicans* via CRISPR-Cas9.

Western blotting was performed using anti-Mkc1 antibody to confirm the absence of *MKC1* in the *MKC1* knockout mutants compared to wild-type (WT) and other strains. Tubulin was probed with anti-tubulin antibody as a loading control.

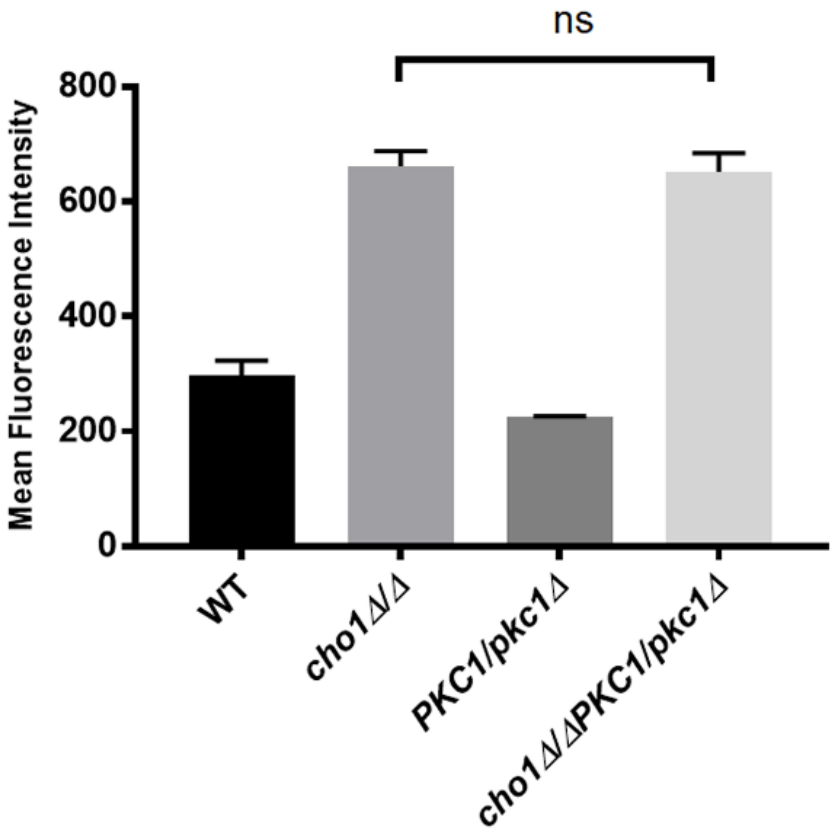


Figure S.2. 6. Deleting one *PKC1* allele in *cho1Δ/Δ* did not rescue β (1,3)-glucan exposure. One *PKC1* allele was deleted through SAT1-flipper method. Cells were then stained with anti-β (1,3)-glucan primary antibody and phycoerythrin (PE)-conjugated secondary antibody. The statistical analysis was carried out by doing One-way ANOVA.

CHAPTER III: Hyperactivation of Cek1 Attenuates Virulence in *Candida albicans*

This chapter will eventually be submitted for publication.

The tentative title is: Hyperactivation of Cek1 Attenuates Virulence in *Candida albicans.* Contributing authors are: Tian Chen[1], Andrew S. Wagner[1], Robert N. Tams[1], James E. Eyer[2], Sarah J. Kauffman[1], Eric R. Gann[1], Elias Fernandez[2], Todd B. Reynolds[1] *

[1]Department of Microbiology, University of Tennessee, Knoxville, TN 37996, USA
[2]Department of Biochemistry & Cellular and Molecular Biology, University of Tennessee, Knoxville, TN, 37996, USA

Author contributions are as follows: Conceptualization: TC, TBR. Formal analysis: TC, TBR. Funding acquisition: TBR. Investigation: TC. Methodology: TC, ASW, RNT, JEE, SJK, ERG. Project administration: TBR. Resources: EF, TBR. Supervision: TBR. Validation: TC. Visualization: TC. Writing – original draft: TC. Writing – review & editing: TC, TBR. In particular, ELISA in Figure 3. 5 was performed by ASW. Animal experiment was done by ASW, RNT, SJK, ERG. JEE helped with protein purification. All the other experiments were done by TC.

Abstract

Candida albicans is among the most prevalent opportunistic human fungal pathogens and is an important source of mortality, particularly in immune-compromised patients. The ability to mask the immunogenic polysaccharide β (1,3)-glucan from immune detection via an outer layer of heavily mannosylated proteins is a key virulence factor of *C. albicans*. We previously reported that hyperactivation of the Cek1 Mitogen Activated Protein Kinase (MAPK) pathway promotes β (1,3)-glucan exposure and enhances detection of cells by macrophages. In this communication, we report a novel upstream regulator of Cek1 activation and characterize the impact of Cek1 activity on fungal virulence. Lrg1 encodes a GTPase activating protein (GAP) homolog that has been reported to inhibit the GTPase Rho1. We find that disruption of *LRG1* causes Cek1 hyperactivation and β (1,3)-glucan unmasking, but when GTPase activation is measured for a panel of cellular small GTPases, the *lrg1Δ/Δ* mutant exhibits increased activation of the GTPases Cdc42 and Ras1, but not Rho1 or Rac1. Unmasking and Cek1 activation in *lrg1Δ/Δ* can be blocked by inhibition of the Ste11 MAPKKK, indicating that Lrg1 acts through the canonical Cek1 MAPK cascade. In order to determine how Cek1 hyper-activation specifically impacts virulence, a doxycycline repressible hyperactive Ste11$^{\Delta N467}$ was generated in *C. albicans* and was found to induce production of the pro-inflammatory cytokine TNF-α from murine macrophages. This *in vitro* phenotype correlates with decreased colonization and virulence in a mouse model of systemic infection.

Introduction

Candida spp. species are the fourth most common cause of bloodstream infections, and the leading causes of invasive fungal infections in hospitalized patients in the United States [1]. Despite the presence of several effective antifungals, the mortality rate of these infections still exceeds 40% [1]. Current anti-fungal drugs for *Candida* include polyenes, azoles, and echinocandins, however mortality rates are unacceptably high even after accounting for limitations like drug resistance or toxicity. This suggests that other therapeutic approaches need to be used in conjunction with anti-infectives. One approach is to improve the residual immune response of patients to these pathogens [2-4]. This could be improved by making fungal pathogens more visible to the immune system's sensory cells. The fungal cell wall is a major focus for this as it is the main interface between the immune system and the fungus [5].

The *C. albicans* cell wall is composed of three major layers, where the outer layer is enriched for glycosylated proteins (mannan), and the inner layer consists of chitin, β (1,6)-glucan and the highly immunogenic β (1,3)-glucan. Fungal pathogens have a diversity of mechanisms to manipulate cell wall architecture to mask pro-inflammatory β (1,3)-glucan epitopes from host recognition. In *Histoplasma capsulatum*, α-glucan serves a masking function by concealing the β-glucan, while the Eng1 exoglucanase hydrolyzes β-(1,3)-glycosyl bonds and removes exposed β-glucans not covered by α-glucan [6, 7]. *Aspergillus fumigatus* Uge3 regulates the biosynthesis of galactosaminogalactan, a polymer that covers hyphal β-glucan from immune detection [8]. In *C. albicans*, β (1,3)-glucan is masked by the outer layer of mannan. In this organism, certain genetic mutations, treatment with the cell wall inhibitor caspofungin, or damage by neutrophils can expose *C. albicans* β (1,3)-glucan [9-11]. This exposure facilitates recognition by host immune cells through receptors like Dectin-1, and therefore launches immune responses more efficiently and rapidly, including induction of pro-inflammatory cytokines like tumor necrosis factor (TNF)-α for anti-fungal clearance [12]. The importance of β (1,3)-glucan exposure and resultant enhancement of immune

detection is becoming better appreciated in medical mycology and may lead to discoveries that will improve adjunctive therapy approaches [9, 11, 13-16].

We previously identified that the activated GTPase Rho1 (*RHO1*^{Q67L}) exhibited β (1,3)-glucan unmasking in *C. albicans* [17]. The most well-known contribution of Rho1 in this organism is to maintain sophisticated cell wall architecture through regulating several downstream effectors including the β (1,3)-glucan synthase Fks1, protein kinase C (Pkc1), and the cell wall integrity MAPK cascade containing Mkc1 [18, 19]. To function accurately, Rho1 serves as a molecular switch by cycling between active GTP-bound and inactive GDP-bound state [20]. The regulatory proteins of GTPases generically include guanosine exchange factors (GEFs), which stimulate GTP to be loaded onto the enzyme and keep it in the active GTP-bound state, and GTPase-activating proteins (GAPs), which promote the hydrolysis of GTP and convert the enzyme into the inactive GDP-bound state [21-24]. In *C. albicans*, Lrg1 is proposed to act as the Rho1 GAP based on the evidence that *lrg1Δ/Δ* mutant and hyper-activated *RHO1*^{Q67L} mutants both induce hyphal formation individually and ScLrg1 is a GAP for ScRho1 in *Saccharomyces cerevisiae*, suggesting that Lrg1 negatively regulates Rho1 activity [25, 26]. However, GAP activity towards Rho1 *in vitro* has not been evaluated in *C. albicans* [25].

Cdc42 is also an essential GTPase in *C. albicans*, and it is required for cell viability, polarized growth, yeast-to-hyphal morphogenesis *etc.* [27, 28]. Like other GTPases, CaCdc42 activity is regulated by GAPs negatively and the GEFs positively [23]. The activated Cdc42 turns on downstream effectors including PAK kinase Cst20, which further initiates hyphal formation by mediating Cek1 MAPK cascade signaling [17, 27]. The Cek1 MAPK cascade, comprised of Ste11-Hst7-Cek1, is primarily responsible for gene transcription involved in morphogenesis, cell wall stress adaption, and cell growth [17, 29-31]. In *C. albicans*, Cek1 has been reported to respond to several GTPases including Cdc42, Rho1, Ras1, and Rac1, although most of this data is genetic in nature [17, 32, 33]. Here, we demonstrate for the first time that in *C. albicans*, Lrg1 negatively controls Cdc42 and Ras1 activity *in vivo*, but not Rho1 or Rac1. Moreover, activation of Cek1 downstream of

lrg1Δ/Δ occurs through the canonical Ste11 MAPKKK. Furthermore, we reveal that hyperactivation of Cek1 by a Ste11$^{\Delta N467}$ allele compromises fungal virulence in the mouse model of systemic infection.

Methods

Strains and growth media

All the strains and plasmids in this study are described in Table S.3. 1 and Table S.3. 2, respectively. Primers used in this study were described in Table S.3. 3. The media used to culture strains was yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose (Thermo Fisher Scientific)) [34]. YPM medium (1% yeast extract, 2% peptone, and 2% maltose (Thermo Fisher Scientific)) was used to flip out SAT1-flipper which is under the control of maltose (*MAL2*) promoter. Doxycycline (Sigma-Aldrich, USA) was added with the working concentration of 0.5 µg/mL as the repressor for the tetracycline-repressing promoter.

Strain construction

To generate the *LRG1* reintegrated strain, primers TCO68 and TCO69 were used to amplify the *LRG1* open reading frame (ORF) and 200bp of terminator as a SacI-NotI fragment, which was cloned into the pBT1 vector [35] between the constitutive *ENO1* promoter and the SAT1 selective marker. The resultant pTC7 was cut with MscI and the linearized fragment was transformed to the *lrg1Δ/Δ* competent cells by electroporation to create the TC14 mutant. The transformation method has been described in [36].

To generate the homozygous deletion of *STE11*, the same protocol was utilized as described in [37] with minor modification. ~500 bp of 5' promoter and 3' terminator flanking the *STE11* ORF were amplified as KpnI-XhoI and NotI-SacI fragments, by using the primers TCO155+TCO156 and TCO157+TCO158, respectively. These two fragments were cloned to the corresponding ends of the SAT1-flipper plasmid pSFS2A, respectively [38]. The resulting plasmid pTC71 was digested with KpnI and SacI, and the larger fragment was transformed into *lrg1Δ/Δ*.

The nourseothricin-resistant transformants were further cultured in YPM medium containing maltose as a carbon source to induce *caFLP* expression, which is under *MAL2* promoter regulation. Successful deletion of one *STE11* allele was confirmed by PCR with the primers TCO159+JCO95. To delete the second *STE11* allele in the resultant *Candida* strain TC41, primers TCO162+TCO163 were used to amplify the tetracycline-repressing promoter P_{tetOFF} with the template of plasmid pWTF1 [39]. The P_{tetOFF} N-terminal was flanked by 60bp of homology to the *STE11* promoter and the C-terminal was flanked by 60 bp of homology to the *STE11* open reading frame (ORF) located at 1402 -1461 bp. The PCR product was gel-purified (Qiagen Inc., Germany), and transformed into the competent *Candida* TC41 cells, and plated on YPD plates with 2mg/ml hygromycin B. The primers TCO181+TCO57 were utilized for colony PCR to confirm if the pWTF1 fragment replaced the N-terminal 1401bp of *STE11* ORF on the chromosome.

To generate the *Candida* strain which expresses the doxycycline regulated P_{tetOFF}–$STE11^{\Delta N467}$, primers TCO162+TCO163 were utilized as mentioned above to amplify the tetracycline-repressing promoter from the template of plasmid pWTF1 [39]. The PCR product was transformed to the wild-type DAY286 competent cells, and then plated on the YPD plates with 2mg/ml hygromycin B. TCO168+TCO169 were used to check if pWTF1 was integrated into the chromosome.

To generate the GST-RID-6×His construct, primers TCO139+TCO140 were used to amplify the RID domain located at 1111-1908bp of the *CaPKC1* ORF as a *BamHI-SalI* fragment by using *C. albicans* genomics DNA as template, which was then cloned into the expression vector pGEX to create pTC55. The resultant plasmid was transformed into competent *E. coli* BL21 cells for high protein expression. Primers TCO165+TCO92 were used to amplify a *Candida*-adapted hygromycin B resistance marker from pRB436 as a NotI-XbaI fragment. The PCR product was ligated to the corresponding sites in pYGS1244 [24], in which Rho1 was tagged with 6×*myc* at the N-terminus. The resultant pTC73 was transformed into the *Candida* strains of interest.

To tag the N-terminus of Rac1 with GFP, TCO170+TCO175 were used to amplify the *RAC1* ORF flanked by RsrII and Mlu, from genomic DNA extracted from the *C. albicans* DAY286 strain. The PCR product was digested and inserted into the corresponding locus of the plasmid pTC33 to replace the RID domain. TRO993 was used for sequencing to check if the *RAC1* was ligated into the plasmid in the correct orientation. The resultant plasmid pTC76 was linearized by StuI restriction enzyme, followed by electroporation into *Candida* competent cells. The positive transformants selected on YPD with nourseothricin were further screened for GFP fluorescence by performing microscopy with a LEICA DM5500B epi-fluorescent microscope with a Hamamatsu Orca-ER CCD digital camera (Model#: C4742-80-12AG).

To generate the hyperactive $RAS1^{G13V}$ allele, primers TCO185+TCO186 were used to PCR amplify the $RAS1^{G13V}$ allele from the genome of the Ca79 strain [40].and introduce NotI and SacI restriction sites. The PCR product was digested and ligated into the plasmid pBT1. TCO185 was used for sequencing to check if the $RAS1^{G13V}$ allele was inserted with the correct orientation. The resultant plasmid pTC78 was linearized with BglII and transformed to the *C. albicans* competent cells of interest. The positive colonies were selected on YPD plates with nourseothricin and TCO185 and TCO186 were used to check if the fragment was located on the chromosome.

Protein purification

To measure Rho1 activity, we first generated a GST-RID-6×His (Rho1 Interactive Domain) construct that binds with GTP-Rho1 and expressed it in *E. coli* BL21 strain. The overnight culture was diluted to 1:100 ratio in fresh LB medium and grown to OD_{600} 0.6-0.9. IPTG (Sigma-Aldrich) was added to the final concentration of 5mM to induce GST-RID expression at 20°C for 20hrs. The culture was pelleted by centrifugation and resuspended into cell lysis buffer (50mM Tris-HCl, 500mM NaCl, 30mM Imidazole, 400ul of 0.25mM PMSF (phenylmethylsulfonyl fluoride), 100ul of BME, 10mM MgCl2, and 1 protease inhibitor tablet (Roche Diagnostics GmbH, Mannheim, Germany)) was agitated by sonication (Sonic Dismembrator

F550 Ultrasonic Homogenizer, Fisher Scientific). The liquid was centrifuged for 1hr at 17,000rpm at 4°C. The protein is soluble and was thus located in the supernatant. The solution was then slowly run through a Ni-NTA column (QIAGEN Inc., Germany) for binding, and the beads were then gently washed with wash buffer (50mM Tris-HCl, PH8.0, 500mM NaCl, 30mM imidazole) to remove the nonspecific binding protein. To elute out the His-tagged protein, 6 rounds of 250ul of elution buffer (the same as wash buffer except with 300mM imidazole) was added. The eluted fractions were then run through a PD-10 column (GE Healthcare) to remove the imidazole. The product was applied to an Amicon ultra 0.5ml-centrifugal filter unit (Merck KGaA, Darmstadt, Germany) to concentrate the protein.

Western Blotting

Western Blotting was performed as previously described [17]. To detect the phosphorylation of Cek1 and Mkc1 MAPKs, rabbit anti-Phospho-p44/42 antibody (Cell Signaling Technology, Inc., USA) at 1:2,000 dilution was utilized. The expression of total Mkc1 was detected with the primary rabbit-anti-total Mkc1 Ab at a 1:1000 dilution. The expression of total Cek1 was measured with a rabbit-anti-total Cek1 Ab at a 1:1000 dilution. The secondary antibody against Phospho-p44/42 Ab, Mkc1 Ab and Cek1 Ab was IRye800CW goat anti–rabbit IgG (H+L) conjugate (green, 1:10,000 dilution; LI-COR Biosciences). Tubulin was detected as a control with rat anti-tubulin primary antibody (Bio-Rad Laboratories Inc., USA) at a 1:1000 dilution and IRDye 680RD Goat-anti-Rat IgG (H+L) (red, 1:10,000 dilution; LI-COR Biosciences).

Pull-down assay for active GTPases

GTPase activity assay was performed as previously described [17]. The procedure of cell wall disruption and protein concentration determination followed the above-mentioned Western Blotting protocol. To detect Cdc42 activity, 1,500 μg of total protein was used for the pull-down procedure following the instruction from the Active Cdc42 Pull-Down and Detection Kit (Thermo Fisher Scientific, USA). The

same kit was used to pull down the active GTP-bound GFP-tagged-Rac1 (See Strain Construction), since the GST-Pak1 provided in the kit can also bind with active Rac1. GTPase Ras1 activity was evaluated following the protocol from the Active Ras1 Pull-Down and Detection Kit (Thermo Fisher Scientific, USA). To pull-down the active GTP-bound *c-myc*-tagged Rho1 (See Strain Construction), the Active Rho Pull-Down and Detection Kit (Thermo Fisher Scientific, USA) was purchased and the instruction was followed, except that the purified GST-Rhotekin-RBD peptides provided in the kit was replaced with the purified GST-RID-6×His that we generated (See Protein Purification above) for optimized binding capacity between GTP-Rho1 and the RID domain.

The antibody used to detect Cdc42 was rabbit polyclonal anti-*S. cerevisiae* Cdc42 (kindly sent by Dr. Doug Kellogg at University of California, Santa Cruz). The antibody to detect Ras1 was mouse monoclonal anti-Ras1 antibody (Anti-Ras, clone RAS10, Millipore Sigma, USA) with the working concentration at 1.5μg/ml. The antibody for c-*myc*-Rho1 detection was mouse anti-c-Myc Monoclonal Antibody (9E10) (Thermo Fisher Scientific, USA) at 1:1000 dilution. The rabbit anti-GFP antibody was used for the GFP tagged-Rac1 detection (Sigma Inc., USA). The antibody to recognize the loading control tubulin was rat anti-tubulin (Bio-Rad Inc., USA). The secondary antibody included IRye800CW goat anti–mouse IgG (H+L) conjugate (1:10,000; LI-COR biosciences), IRye800CW goat anti-rabbit IgG(H+L) conjugate (1:10,000; LI-COR biosciences), and IRye600RD goat anti-rat IgG(H+L) conjugate (1:10,000; LI-COR biosciences). Densitometry quantification of active GTP-bound GTPases versus the total GTPases input bands was performed with ImageJ (National Institutes of Health, Bethesda, MD).

Immunofluorescent imaging of β (1,3)-glucan exposure

To stain the *lrg1Δ/Δ* mutant, *Candida* cells were grown overnight in YPD at 30 °C. The culture was collected and processed to immunostaining. The staining and imaging protocol were described as [17]

Flow cytometry

To stain the *STE11*$^{\Delta N467}$ strain under the regulation of P_{tetOFF} promoter over-time, overnight cultures in YPD with doxycycline were diluted back to 0.2, and cells were collected after 1h, 2h, 4h and 6h grown in the fresh YPD medium without doxycycline. To stain the *lrg1Δ/Δ* mutant, overnight culture in YPD was collected for immunostaining. The staining protocol and gating strategy was followed as described in [17]. Flow cytometry data were obtained for 100,000 gated events per strain and experiments were performed in triplicate, and the data were analyzed using FlowJo software package with version 10.11 (FlowJo LLC, OR, USA).

Enzyme-linked immunosorbent assay (ELISA) of TNF-α

To activate Ste11$^{\Delta N467}$ expression under the P_{tetOFF} regulation, the P_{tetOFF}-*STE11*$^{\Delta N467}$ mutant was grown in YPD without doxycycline overnight. RAW264.7 murine macrophages were in used in this assay. ELISA kit instructions (R&D Systems, USA) were followed. Each sample has three individual wells, and the statistical analysis was performed by using Two-way analysis of variance ANOVA (GraphPad Prism, v7.04 software).

Mouse model

Outbred male ICR mice were used in this study. *C. albicans* wild-type DAY286 and P_{tetOFF}–*STE11*$^{\Delta N467}$ strains were cultured overnight at 30°C in 50ml YPD with 0.5 µg/ml of doxycycline to repress Ste11$^{\Delta N467}$ expression. The overnight culture was counted via hemocytometer and diluted to 10^7 cells/ml. The diluted fungal cells were plated on YPD plate to test the viability. Mice were injected via the lateral tail vein with 0.1 ml of the fungal cells. Mice were given drinking water either co-supplemented with 2 mg/ml doxycycline plus 5% sucrose to cover the bitter taste of the antibiotics [41] or given 5% sucrose water alone. Mice were monitored closely for 21 days for signs of illness. For the fungal burden counting experiment, mice were sacrificed 4 days post infection. Kidneys were harvested, homogenized and serial diluted in water, and plated on YPD plate. The plates were incubated at 30°C for 2 days to determine cfu per gram of kidney.

Ethics statement

All mouse model experiments in this study were performed under an animal protocol (1083) 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.

Results

The *lrg1Δ/Δ* mutation causes β (1,3)-glucan exposure in *C. albicans*

Lrg1 homologs impact several cell wall-related functions in fungi (*e.g.* cell wall integrity, cell fusion, and morphogenesis) [25, 26, 42, 43]. In *S. cerevisiae*, Lrg1 stimulates the intrinsic GTPase activity of Rho1, and therefore converts Rho1 to its inactive, GDP-bound state [26, 42, 43]. We previously showed that hyperactive Rho1 exposes β (1,3)-glucan in the cell wall, but appeared to act through the Cek1 MAPK rather than Mkc1 [17]. Since Lrg1 has been described as the Rho1 GAP in *C. albicans*, we hypothesized that disruption of *LRG1* would cause β (1,3)-glucan exposure. To this end, we performed immunofluorescence staining with anti-β (1,3)-glucan antibody to examine if the *lrg1Δ/Δ* mutant exhibits increased β (1,3)-glucan exposure. As shown in Figure 3. 1A, β (1-3)-glucan is noticeably more exposed in *lrg1Δ/Δ* cells than in wild-type or the complemented strain, where Lrg1 expression is under the regulation of constitutive promoter P_{ENO1}. Flow cytometry confirms that *lrg1Δ/Δ* has significantly increased levels of β (1,3)-glucan unmasking compared to control strains (Figure 3. 1B). This reveals that Lrg1 acts as a repressor of cell wall β (1,3)-glucan unmasking in *C*. *albicans*.

LRG1 disruption up-regulates the Cek1 MAPK activity

Several MAPKs pathways are involved in the regulation of cell wall architecture in *C. albicans* [29, 44], and improper activation of the Cek1 MAPK by upstream GTPases such as Cdc42 or Rho1 causes β (1,3)-glucan exposure [17]. Lrg1 is a proposed Rho1 GAP in *C. albicans* [25], which should upregulate Mkc1 and possibly Cek1. To this purpose, we performed Western blotting to detect the activated (phosphorylated) forms of Cek1 and Mkc1 in *C. albicans.* As seen in

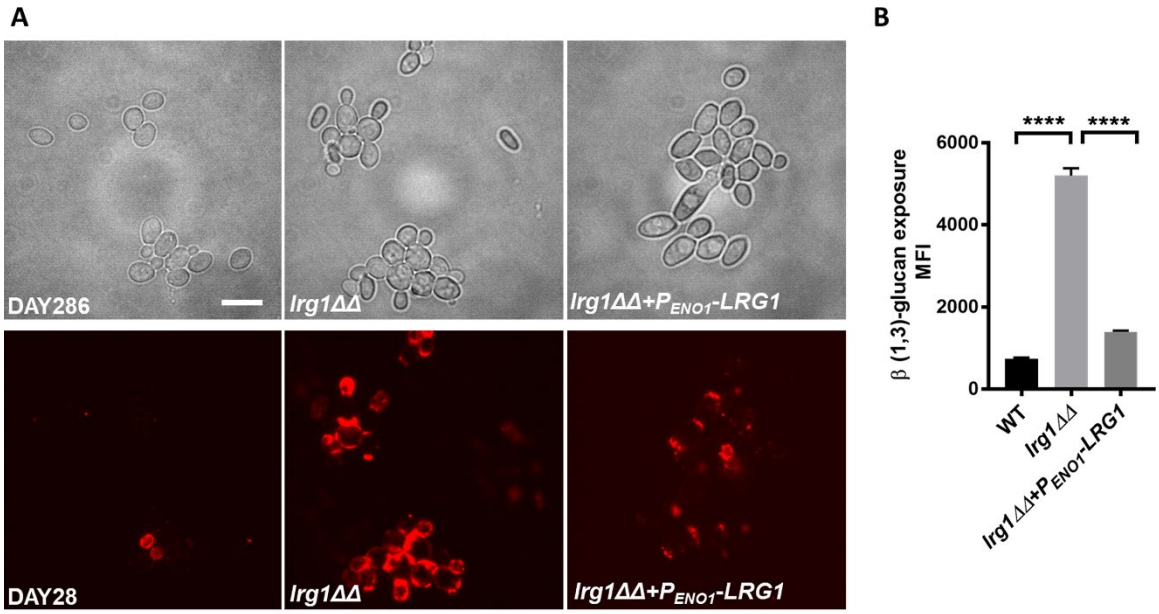


Figure 3. 1. Lrg1 represses β (1,3)-glucan unmasking in *C. albicans*.

(A) Cells cultured overnight in YPD medium were stained with anti-β (1,3)-glucan antibody and Cy3-conjugated secondary antibody. Cells were imaged by epi-fluorescent microscopy. (B) Cells were stained with anti-β (1,3)-glucan antibody and Phycoerythrin (PE) –conjugated secondary antibody. Flow cytometry was performed to quantify β (1,3)-glucan exposure in different *Candida* cells. The statistical analysis was performed by doing One-way ANOVA. ****, $p<0.0001$

Figure 3. 2, *lrg1Δ/Δ* does not visibly increase the intensity of phosphorylated Mkc1, but instead Cek1 was hyper-phosphorylated up to 15-fold compared to wild-type. This indicates that Lrg1 represses the activity of Cek1 instead of Mkc1 in *C. albicans*, and this might be responsible for the cell wall exposure in the *lrg1Δ/Δ* mutant.

LRG1 disruption hyper-activates the small GTPases Cdc42 and Ras1

The small GTPase Cdc42 sits upstream of Cek1, and is essential for many cellular functions, including cellular polarized growth and bud emergence in *C. albicans* [27, 45]. Regulation of the GTP/GDP-binding state controls the Cdc42 activation state [27]. Given that Cdc42 is known to control Cek1 MAPK activity in *C. albicans*, we hypothesized that the *lrg1Δ/Δ* disruption activates Cek1 through Cdc42. To test this, we measured Cdc42 activity by quantifying the amount of GTP-bound active Cdc42 *in vivo*. As seen in Figure 3. 3A and B, the concentration of GTP-bound Cdc42 was clearly up-regulated compared to that of wild-type DAY286 and the reintegrated strain. Thus, this suggests that Lrg1 controls Cdc42 activity negatively in *C. albicans*. Cdc42 has been shown to control Cek1 phosphorylation in this organism [17], so *LRG1* disruption-dependent Cek1 hyper-activation may be mediated by Cdc42.

There is a possibility that Lrg1 impacts other Cek1-associated GTPases. To address this, we measured the activity of the GTPase Rac1, which has been implied to function upstream of Cek1 [33]. Rac1 activity was assessed by pulling down the activated GTP-bound GFP-tagged Rac1. The Cdc42/Rac1 interactive binding (CRIB) protein used in the Cdc42 pull-down assay were used as the probe to isolate GTP-bound Rac1, while GFP antibody was utilized to detect the amount of GFP-Rac1 specifically. As shown in Figure S.3. 1, the level of GTP-bound GFP-Rac1 in the *lrg1Δ/Δ* mutant is reduced compared to that of wild-type. This suggests that Rac1 and Cdc42 might work antagonistically when Lrg1 is disrupted in *C. albicans*.

Ras1 is an important GTPase for filamentation and controls the cyclic AMP signaling cascade and protein kinase A. It has been suggested to act upstream of

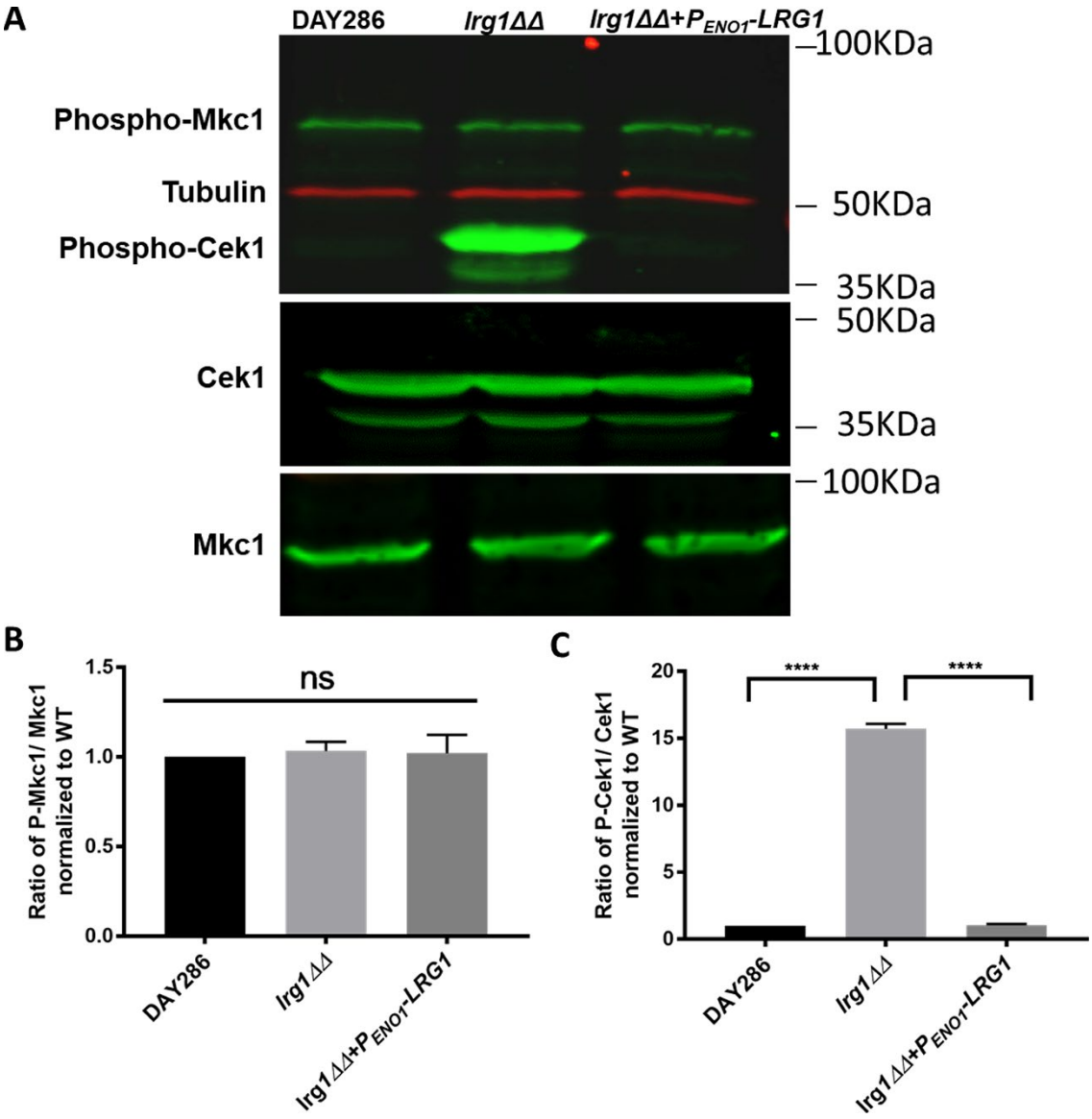


Figure 3. 2 .The Cek1p MAPK is hyper-phosphorylated in *lrg1Δ/Δ* cells compared to wild-type

(A). Proteins were isolated from *Candida* cells sub-cultured 3 hrs into log phases after grown overnight in YPD at 30°C. Western blotting was performed with anti-phospho-p44/42 antibody, which stains both phosphorylated Mkc1 and Cek1, as well as anti-Mkc1 and -Cek1 antibodies, which stain total Mkc1 and Cek1 proteins, respectively. Phospho-MAPKs signal was expressed as a percent of wild-type (WT) control after being normalized to the tubulin loading control. (B). The P-Mkc1 bands were quantified and normalized based on the total Mkc1 bands and tubulin. (C). The P-Cek1 bands were quantified and normalized based on the total Cek1 bands and tubulin. The graphs in both cases were based on quantification of 3 blots, and statistical analysis was performed by doing one-way ANOVA. ****, $p<0.0001$

Cek1 in *C. albicans* [32]. Since Cek1 is hyper-active in the *lrg1Δ/Δ* mutant (Figure 3. 2), it is reasonable to evaluate if Ras1 activity is upregulated when *LRG1* is disrupted. We performed pull-down assay to isolate active GTP-Ras1 by using its downstream effector Raf1 as a probe. The ratio of GTP-Ras1 to total Ras1 is significantly higher in *lrg1Δ/Δ* than that in other strains, indicating that *LRG1* disruption induces Ras1 activity (Figure 3. 3C and D). To biochemically test if Ras1 can cause Cek1 hyperactivation, we performed Western blotting on the P_{MAL}-$RAS1^{G13V}$ mutant (hyperactive allele) with Phospho-p44/42 antibody recognizing the phosphorylated Cek1 and Mkc1. Unexpectedly, as seen in Figure 3. 3E, hyperactivated $Ras1^{G13V}$ does not induce Cek1 activation under the inducing conditions of using maltose as the carbon source. Similar results were seen in the $RAS1^{G13V}$ mutant under the constitutive promoter P_{ENO1} (Figure S.3. 2). These results indicate that in *C. albicans* Ras1 is not activating Cek1 downstream of the *lrg1Δ/Δ* mutation.

Lrg1 does not act as the Rho1 GAP in *C. albicans*

In *S. cerevisiae*, Lrg1 represses Rho1, and this has been implied by genetic data in *C. albicans*, therefore, we measured Rho1 activity in the *lrg1Δ/Δ* mutant. Due to lack of a commercial reagent to pull down active GTP-Rho1, we expressed and purified a GST-tagged Rho1 interactive domain (RID) protein specific for *C. albicans* GTP-Rho1 [46]. Due to lack of available commercial CaRho1 antibody, CaRho1 was tagged with the *c-myc* epitope tag for the Western blot detection [24]. However, as shown in Figure 3. 3F and G, the *lrg1Δ/Δ* mutant does not contain a higher concentration of GTP-Rho1 compared to wild-type. GTPγS is a non-hydrolysable substrate for these GTPases and therefore acts as positive control to evaluate if our reagents work. The lack of an induction of Rho1 activity in the *lrg1Δ/Δ* mutant suggests that Lrg1 does not act as the Rho1 GAP in *C. albicans*.

Figure 3. 3. Lrg1 inhibits the activity of GTPases Cdc42 and Ras1, but not Rho1.

(A). GTP-Cdc42 was pulled-down by glutathione beads conjugated with glutathione *S*-transferase (GST)-PAK1, which specifically binds active GTP-Cdc42/Rac1. Western blotting was performed on the isolated GTP-Cdc42 portion with anti-Cdc42 antibody and tubulin was used as loading control. (B). The GTP-bound Cdc42/Total-Cdc42 ratio compared to WT was calculated. The densitometry of active GTP-Cdc42 band and total Cdc42 band was quantified by Image J based on 2 blots. *, p=0.0261. (C). The active GTP-Ras1 was pulled down by using the Ras1 binding domain (RBD) within Raf1 as a probe, followed by Western blotting using anti-Ras1 antibody to evaluate the amount of active Ras1. (D). The ratio of GTP-bound Ras1/Total-Ras1 compared to WT was calculated as shown in B. ***, P=0.0006. (E). Western blotting was performed with anti-P-p44/42 antibody to assess the phosphorylation of Cek1 in the hyperactive *RAS1*^{G13V} mutant under the regulation of maltose promoter. (F). The active *c*-myc-tagged Rho1 was isolated by incubating with the purified GST-RID protein. The mixture was incubated with glutathione beads. Western blotting was performed with anti-*c*-myc antibody to demonstrate the amount of GTP-bound Rho1 in the pulled-down portion. Total Rho1 was determined by performing Western blotting on the total protein extract. (G). The ratio of GTP-bound Rho1/Total-Rho1 compared to WT was calculated as shown in B. NS, no significance.

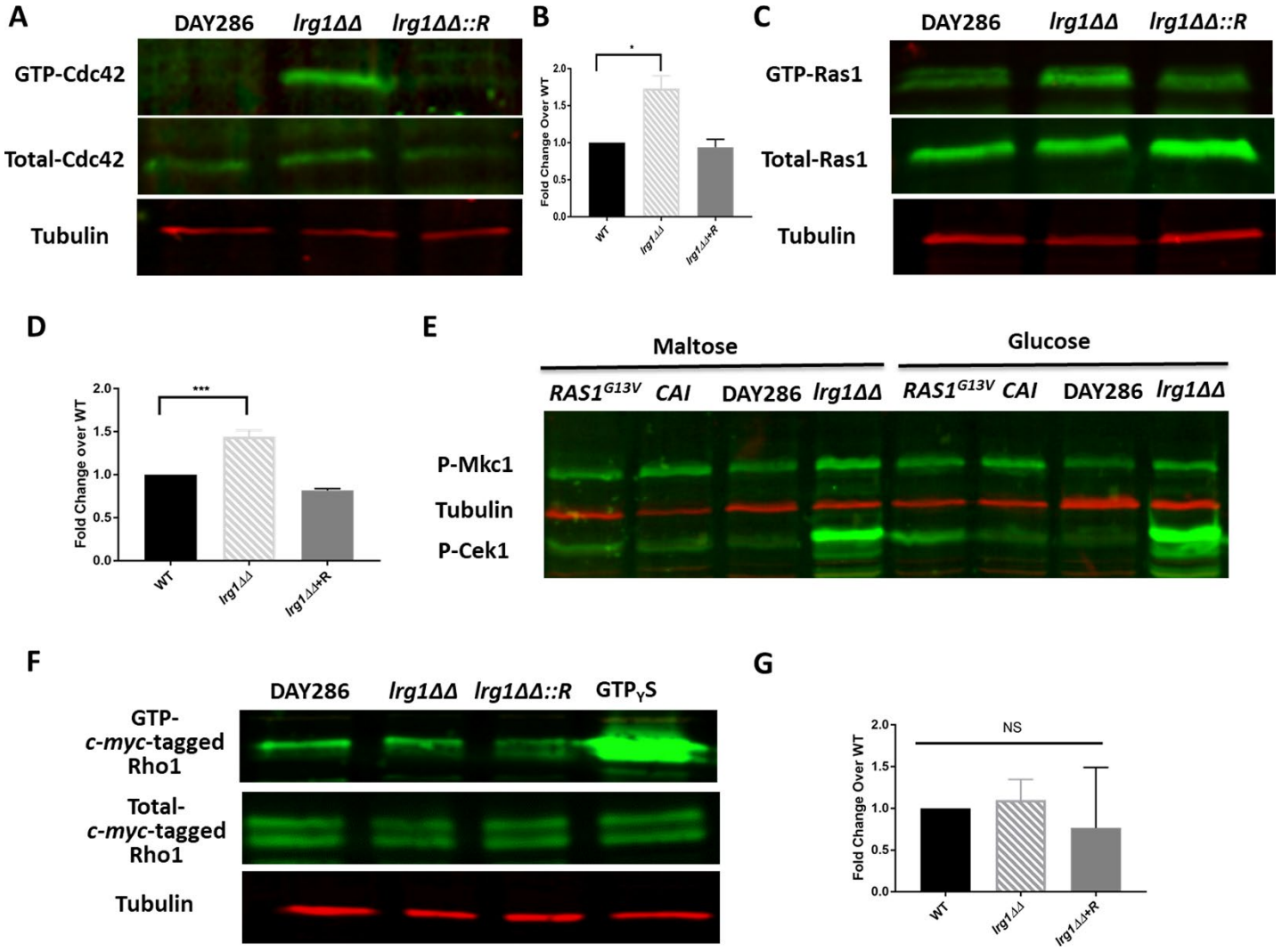


Figure 3. 3 Continued

Ste11 disruption blocks Cek1 hyper-phosphorylation and β (1,3)-glucan exposure in *lrg1Δ/Δ*

Our results indicate that Lrg1 negatively controls Cek1 phosphorylation through Cdc42 (Figure 3. 3), but it is was not clear if the pathway acts through the canonical Cek1 MAPK cascade. Therefore, we next elucidated which upstream kinase participates in this signal transduction pathway. The Ste11 MAPKKK sits at the top of the Cek1 MAPK module, therefore we disrupted *STE11* in *lrg1Δ/Δ* to determine if this would prevent Cek1 activation. One *STE11* allele was deleted in *lrg1Δ/Δ* using SAT1-flipper [37]. However, we could not disrupt the second *STE11* allele in *lrg1Δ/Δ*. Thus, we replaced the second allele with the hyperactive $STE11^{\Delta N467}$ allele under the regulation of tetracycline-repressible promoter (P_{tetOFF}-$STE11^{\Delta N467}$). This resulted in an *lrg1Δ/Δste11Δ/Δ::*P_{tetOFF}-$STE11^{\Delta N467}$ strain, indicating that *STE11* is not essential in the *lrg1Δ/Δ* background, but may be difficult to recover from transformation conditions. The *lrg1Δ/Δste11Δ/Δ::*$P_{tet\text{-}OFF}$-$STE11^{\Delta N467}$ mutant was treated with 0.5μg/ml of doxycycline overnight to repress *STE11* expression, followed by sub-culture in fresh YPD medium ±doxycycline for 3 hours. Western blotting was performed to measure Cek1 and Mkc1 activation.

As seen in Figure 3. 4A, failure to express Ste11 in *lrg1Δ/Δ* (*lrg1Δ/Δste11Δ/Δ::*P_{tetOFF}-$STE11^{\Delta N467}$ +doxycycline) blocks Cek1 phosphorylation. This result indicates that Lrg1 negatively controls Cek1 activity through Ste11. We next investigated if β (1,3)-glucan exposure can be suppressed in *lrg1Δ/Δ* when Ste11 expression is repressed. The overnight cultured *Candida* cells (±doxycycline) were stained with anti-β (1,3)-glucan antibody, and then measured by flow cytometry to quantify the unmasking. As seen in Figure 3. 4B, inhibition of Ste11 expression by adding doxycycline (*lrg1Δ/Δste11Δ/Δ::*P_{tetOFF}-$STE11^{\Delta N467}$ +doxycycline) completely blocked β (1,3)-glucan unmasking. This indicates that hyper-activation of the Cek1 MAPK pathway is responsible for exposing β (1,3)-glucan in the *lrg1Δ/Δ* mutant.

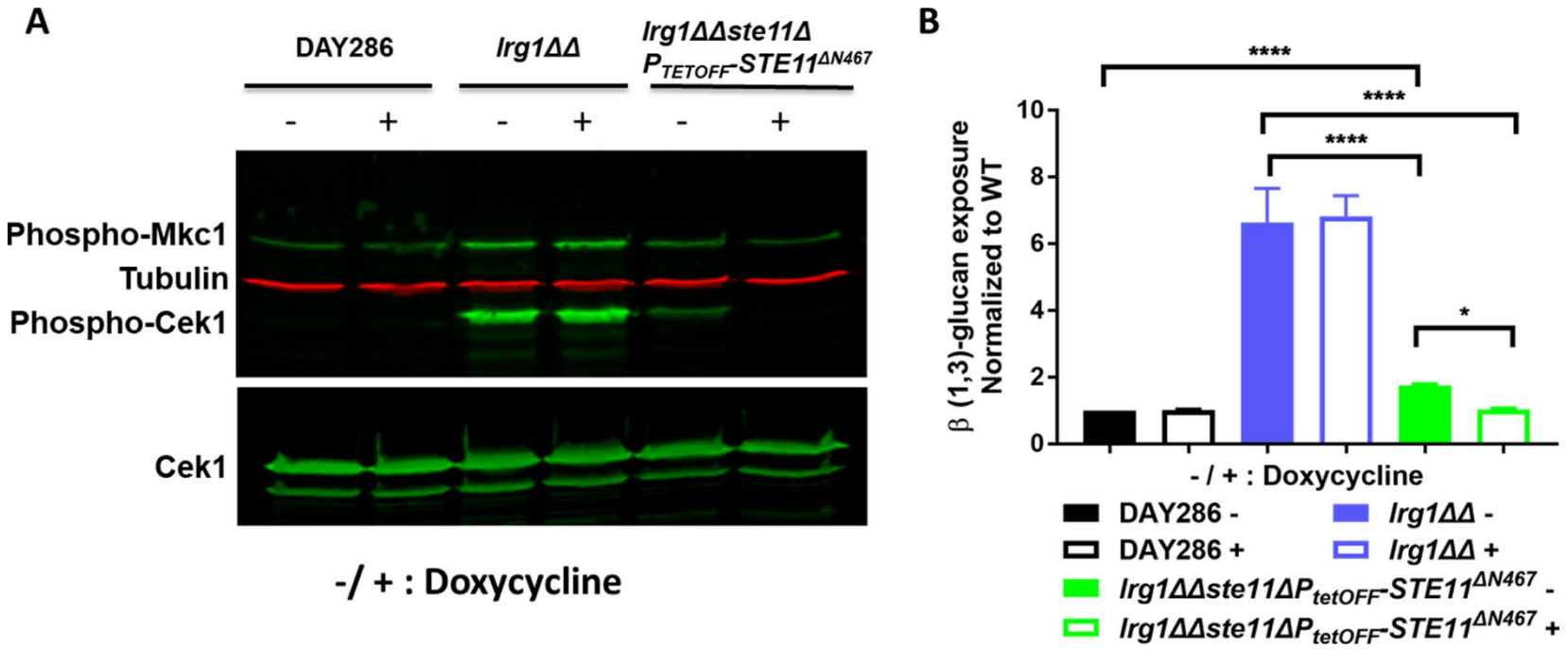


Figure 3. 4. Disruption of *LRG1* causes Cek1 over-activation through Ste11 MAPKKK

A. One *STE11* allele was deleted in *lrg1Δ/Δ* using the well-established SAT1-flipper method. The second *STE11* allele was replaced by the P_{tetOFF}-$STE11^{\Delta N467}$ allele. The *Candida* cells were treated with 0.5μg/ml of doxycycline overnight at 30°C, followed by sub-culture in fresh YPD medium for 3 hours at 30°C into log phase with or without doxycycline. Western blotting was performed with the anti-P-p44/42 antibody. B. The *Candida* cells were cultured overnight with or without doxycycline, followed by immunofluorescent staining with anti-β (1,3)-glucan antibody and PE-conjugated secondary antibody. The stained cells were measured by flow cytometry for the quantification of β (1,3)-glucan exposure. Strains were tested three times with 2 technical replicates each time, and statistical analysis was performed by doing one-way ANOVA. ****, $p<0.0001$. *, $p=0.0142$.

Cells with a *lrg1Δ/Δ* mutation or expressing the hyperactive *STE11$^{\Delta N467}$* allele induce TNF-α secretion from RAW264.7 macrophages

The relationship between the increased β (1,3)-glucan exposure and more TNF-α secretion has been studied intensively [9, 11, 13, 15, 17, 30]. Due to the strong β (1,3)-glucan exposure exhibited by *lrg1Δ/Δ* (Figure 3. 1), we performed enzyme-linked immunosorbent assays (ELISAs) to study if unmasking in *lrg1Δ/Δ* correlates with increased TNF-α production. As seen in Figure 3. 5A, loss of *LRG1* significantly up-regulates TNF-α secretion released from RAW264.7 murine macrophages. The *cho1Δ/Δ* mutant in Figure 3. 5A served as a positive control, which has shown to cause upregulation of TNF-α production compared to the wild-type SC5314 strain [11, 15, 17]. Cek1 is hyperactivated in multiple conditions associated with β (1,3)-glucan exposure including caspofungin treatment [47], *cho1Δ/Δ* [11], hyperactivation of Cdc42, [17], and *lrg1Δ/Δ* (Figure 3. 1 and Figure 3. 2). Furthermore, hyperactivation of Cek1 via an activated allele of *STE11* (*STE11$^{\Delta N467}$*) causes unmasking [17]. We suspect that this hyperactivation of Cek1 will lead to decreased virulence. Many of the mutants we have described such as hyperactive Cdc42 or Rho1, *lrg1Δ/Δ*, or *cho1Δ/Δ* have pleotropic effects, which makes it challenging to assess specific roles in virulence. However, the *STE11$^{\Delta N467}$* mutation causes a more specific upregulation of Cek1 activation [17].

Thus, to test this *in vivo,* we overexpressed *STE11$^{\Delta N467}$* from the doxycycline repressible promoter (*P$_{tetOFF}$*) [39], so we could then use this in the mouse model of infection and turn the gene on or off in the host after infection. We transformed the *P$_{tetOFF}$-STE11$^{\Delta N467}$* allele into the wild-type *Candida* strain DAY286 cells and confirmed that in the absence of doxycycline Cek1 is hyperactivated (Figure S.3. 3), but in the presence of the drug the strain behaved like wild-type. Previous overexpression experiments with *STE11$^{\Delta N467}$* using the *P$_{MAL2}$-STE11$^{\Delta N467}$* construct showed hyperactivation of Cek1, but not Mkc1, however when we upregulated *STE11$^{\Delta N467}$* with the *P$_{tetOFF}$-STE11$^{\Delta N467}$* construct, we observed stronger hyperactivation of Cek1 and Mkc1. This may be because a strong enough activation of Cek1 causes a compensatory activation of Mkc1 [17], although this is not certain.

Immunofluorescent staining of the P_{tetOFF}-$STE11^{\Delta N467}$ strain for β (1,3)-glucan exposure indicates that when cultured in YPD medium overnight without doxycycline, the mutant exhibits significantly increased β (1,3)-glucan exposure (Figure S.3. 4). To evaluate the correlation between β (1,3)-glucan exposure exhibited by $STE11^{\Delta N467}$ expression from the P_{tetOFF} promoter and TNF-α secretion, we performed ELISAs on overnight cultures of wild-type DAY286 and the P_{tetOFF}-$STE11^{\Delta N467}$ strain in the presence or absence of doxycycline. As seen in Figure 3. 5B, when $Ste11^{\Delta N467}$ is induced in the P_{tetOFF}-$STE11^{\Delta N467}$ strain (−doxycycline), TNF-α production is significantly up-regulated from RAW246.7 macrophages.

Hyperactivation of Cek1 results in decreased fungal virulence *in vivo*

We predict that due to increased unmasking, activation of P_{tetOFF}-$STE11^{\Delta N467}$ in mice will lead to a loss of virulence. To test this, wild-type and P_{tetOFF}-$STE11^{\Delta N467}$ strains were cultured in YPD overnight with doxycycline to repress $Ste11^{\Delta N467}$ expression, and then injected into the tail vein of outbred ICR mice. The mice injected with the P_{tetOFF}-$STE11^{\Delta N467}$ strain and provided with doxycycline succumbed to fungal infection in ~10 days. This is similar to mice injected with wild-type ±doxycycline. However, mice infected with P_{tetOFF}-$STE11^{\Delta N467}$ without doxycycline (where $STE11^{\Delta N467}$ is overexpressed), survived significantly longer than mice in the other groups (Figure 3. 6).

Furthermore, when mice were sacrificed at day 4 post-infection, mice infected with P_{tetOFF}-$STE11^{\Delta N467}$ minus doxycycline exhibited (2.43±1.07) $\times 10^2$ cfu g^{-1} kidney, while the mice infected with the same strain with doxycycline exhibited (2.51±0.65) $\times 10^4$ cfu g^{-1} of kidney (Figure 3. 7). Thus, there was a decrease of ~ 2 logs (P=0.0006) in kidney fungal burden when the gene was expressed. A growth curve *in vitro* demonstrated that wild-type and $STE11^{\Delta N467}$ had a similar growth pattern without doxycycline, and addition of doxycycline did increase the cell division rate modestly from 90 minutes to ~103 minutes in early log phase, however, the strain rapidly catches up to wild-type, suggesting only a temporary decrease in growth as the $STE11^{\Delta N467}$ is turned on (Figure S.3. 5). This suggests

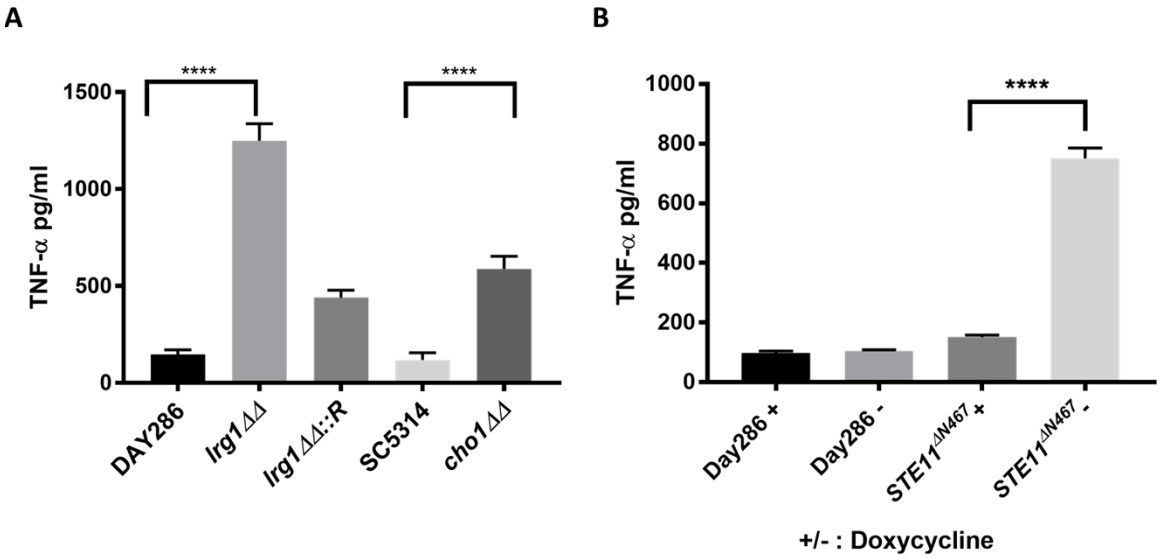


Figure 3. 5. *LRG1* disruption significantly induces TNF-α secretion.

(A). *C. albicans* cells were grown in YPD overnight at 30°C, UV-inactivated, and then co-cultured with RAW264.7 macrophages for 4hrs. The RAW264.7 macrophage supernatant was collected and filtered through a 0.22μm syringe filter to remove cell debris. The filtrates were assayed by ELISA to quantify the TNF-α production. Samples were tested in triplicate three times for an n of 9. The statistical analysis was done by one-way ANOVA. ****, P<0.0001. (B). *C. albicans* wild-type and P_{tetOFF}-$STE11^{\Delta N467}$ strains were cultured overnight in YPD at 30°C, plus or minus doxycycline. The UV-kill, incubation with macrophages RAW264.7, filtration and ELISA procedures were the same as described in A. ****, P<0.0001.

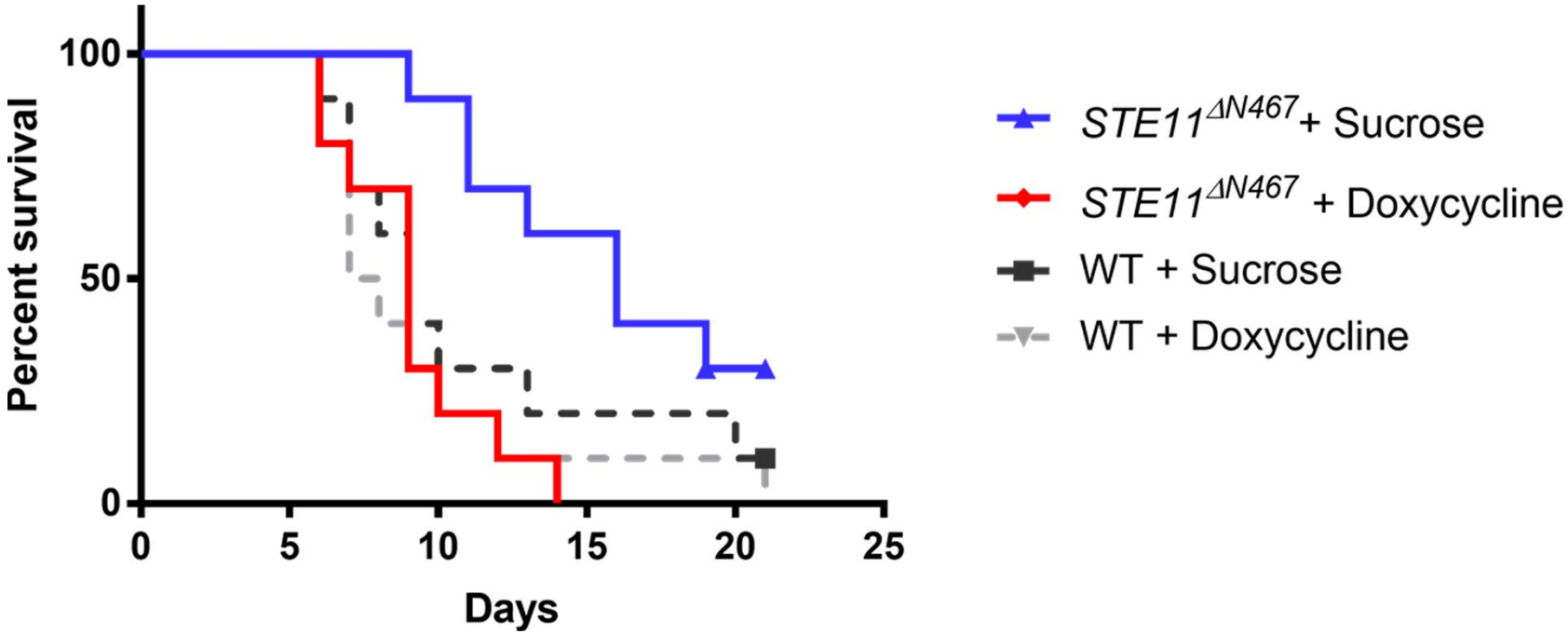


Figure 3. 6. Activated Cek1 leads to attenuated fungal virulence in the mouse model of systemic infection.

C. albicans strains wild-type (DAY286) and P_{tetOFF}-*STE11*$^{\Delta N467}$ were cultured overnight at 30°C with doxycycline to repress Ste11$^{\Delta N467}$ expression. The overnight culture was diluted to 10^6 cells/ml, and 100 µl of the suspension were injected into the lateral tail vein of outbred ICR mice. Mice were given 2 mg/ml of sucrose in their drinking water either without doxycycline or with doxycycline to repress Ste11$^{\Delta N467}$ expression *in vivo*. The symptoms of illness were monitored over 21 days. Each group has 10 ICR mice. *, p=0.016

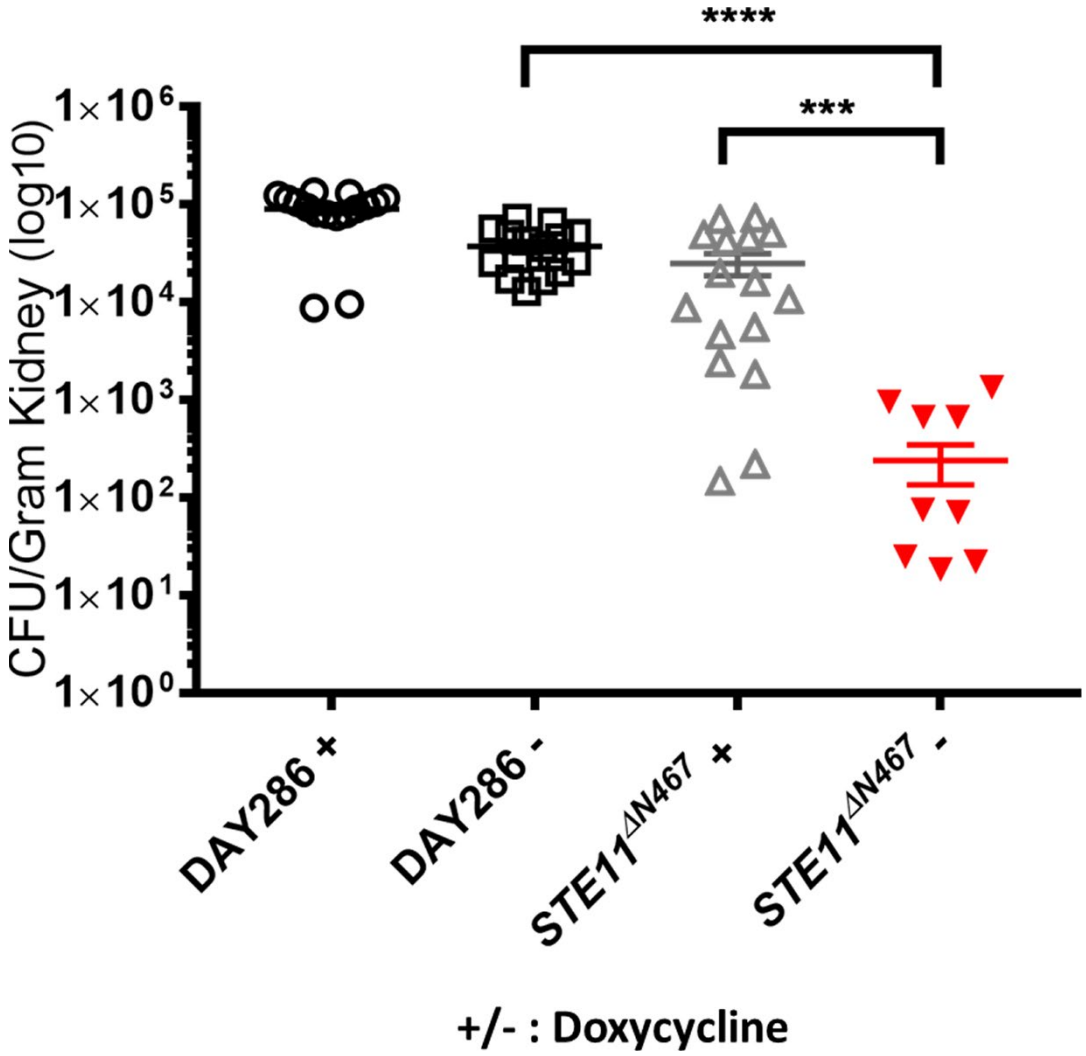


Figure 3. 7. Ste11 hyper-activation causes decreased kidney fungal burden in the mouse model of systemic infection.

C. albicans strains wild-type DAY286 and P_{tetOFF}-*STE11*$^{\Delta N467}$ were cultured overnight at 30°C with doxycycline to repress Ste11$^{\Delta N467}$ expression. The overnight culture was diluted to 10^7 cells/ml, and 100 µl of the suspension was injected to the lateral tail vein of outbred ICR mice. Mice were given sucrose drinking water either with doxycycline to repress Ste11$^{\Delta N467}$ expression *in vivo* or given sucrose water alone. The mice were sacrificed 4 days post-infection and the kidneys from each mouse were taken and homogenized. The homogenates were diluted to 10^{-3} by performing serial dilution. 1 ml of the diluted homogenates from each dilution were plated on the YPD plates and cultured in 30°C for two days. The colony forming unit was counted on each plate. Eight mice were tested for each strain. ***, p=0.0006; ****, p<0.0001.

that β (1,3)-glucan exposure driven by P_{tetOFF}-*STE11*$^{\Delta N467}$ activates the host immunity more efficiently, which further clears the fungal pathogen more rapidly.

Discussion

Previously our lab determined that hyper-activation of Cek1 promotes β (1,3)-glucan exposure in response to loss of phosphatidylserine synthase [17]. This process involves the activation of the GTPase Cdc42 which positively regulates Cek1 MAPK activity [17]. In this communication, we characterized a novel upstream regulator of Cek1 by identifying that the Lrg1 GAP negatively regulates Cek1 activity. Lrg1 acts through the GTPase Cdc42, but not the GTPase Rho1, which has been reported as a target of Lrg1 in the literature. Lrg1 disruption stimulates significantly increased β (1,3)-glucan unmasking, which further induces a higher level of TNF-α secretion from murine macrophages**.**

The Lrg1-Cdc42 cascade modulates Cek1 MAPK activity via Ste11 in *C. albicans*

In *S. cerevisiae*, Lrg1 has been shown to interact with the activated form of Rho1 and act as GAP [26, 48]. However, there are discrepancies concerning the negative regulation of other GTPases by Lrg1 in different studies. Roumanie *et. al*. found that Lrg1 acts as GAP for two other GTPases, Rho2 and Cdc42 by performing spectroscopic measurement *in vitro* based on 7-methyl-6-thioguanosine (MESG)/phosphorylase system [49]. In contrast, Fitch *et al.* found Lrg1 is a Rho1-specific GAP *in vitro* by measuring the amount of Rho1 bound with [α-^{32}P]-labeled GDP or GTP, after stimulation with purified Lrg1 [26]. Regarding the effect of Lrg1 on Rho1-mediated cell wall processes, Lrg1 is reported to negatively regulate glucan synthase activity, whilst controversy appears on its role in activation of the cell wall integrity Mkc1 pathway [42, 48]. These opposing results could be due to the different backgrounds of *S. cerevisiae* strains used. In *Neurospora crassa*, Lrg1 was shown to serve as a Rho1-specific GAP and impact several downstream pathways of Rho1 [43]. In *C. albicans*, the *lrg1Δ/Δ* mutant increases hyphal formation and biofilm development, which are phenotypes

shared with a *RHO1*^{Q67L} gain of function mutant. However, in this study there was no biochemical evidence to demonstrate that Lrg1 acts as Rho1 GAP [25, 50]. In this communication, we provided evidence that in *C. albicans* loss of Lrg1 increases β (1,3)-glucan masking (Figure 3. 1), and this is likely mediated by hyper-activated Cek1 (Figure 3. 2). Our results also suggest that Lrg1 does not act as a Rho1 GAP in *C. albicans*. Lrg1 does not show Rho1 inhibitory activity *in vivo*, nor does it inhibit Rac1 activity (Figure 3. 3 and Figure S.3. 1). Instead, Lrg1 exhibits repression of the activities of both Cdc42 and Ras1, as indicated by significant induction of both GTPase activities *in vivo* in the *lrg1Δ/Δ* mutant (Figure 3. 3). The unexpected results imply signal rewiring occurs in the pathogenic *C. albicans* versus the non-pathogenic *S. cerevisiae*. Ras1 plays pivotal roles in fungal morphogenesis, and as a result contributes to virulence [32]. Loss of *RAS1* causes defects in yeast-to-hyphal transition, which nonetheless can be restored by overexpressing signaling component in the Cek1 pathway in the *ras1Δ/Δ* mutant [32]. Although this suggests that Ras1 might be upstream of Cek1, we cannot rule out the possibility that Cek1 and Ras1 act in a parallel manner. Our western blot result shows that hyperactive *RAS1*^{G13V} mutant does not display Cek1 overactivation (Figure 3. 3E and Figure S.3. 2), suggesting that hyperactivation of Ras1 is not sufficient to induce Cek1 phosphorylation, and that these two proteins are not in a linear signaling cascade.

The Ste11 MAPKKK is a well-known upstream regulator of Cek1 activity and the hyperactivated *STE11*$^{\Delta N467}$ mutant causes β (1,3)-glucan exposure in *C. albicans* [17]. Here we show that Ste11 is also involved in the Lrg1-dependent Cek1 hyperactivation, given that disruption of *STE11* rescues both β (1,3)-glucan exposure and Cek1 hyper-phosphorylation in the *lrg1Δ/Δ* mutant (Figure 3. 4). Given the established effect of Cdc42 on Cek1 activation [17], our results indicate that Lrg1 negatively modulates Cek1 activity via the GTPase Cdc42 (Figure 3. 8).

β (1,3)-glucan exposure compromises fungal virulence

Several cell wall defective mutants have exhibited attenuated fungal virulence in the mouse model of systemic infection, including the phosphatidylserine synthase

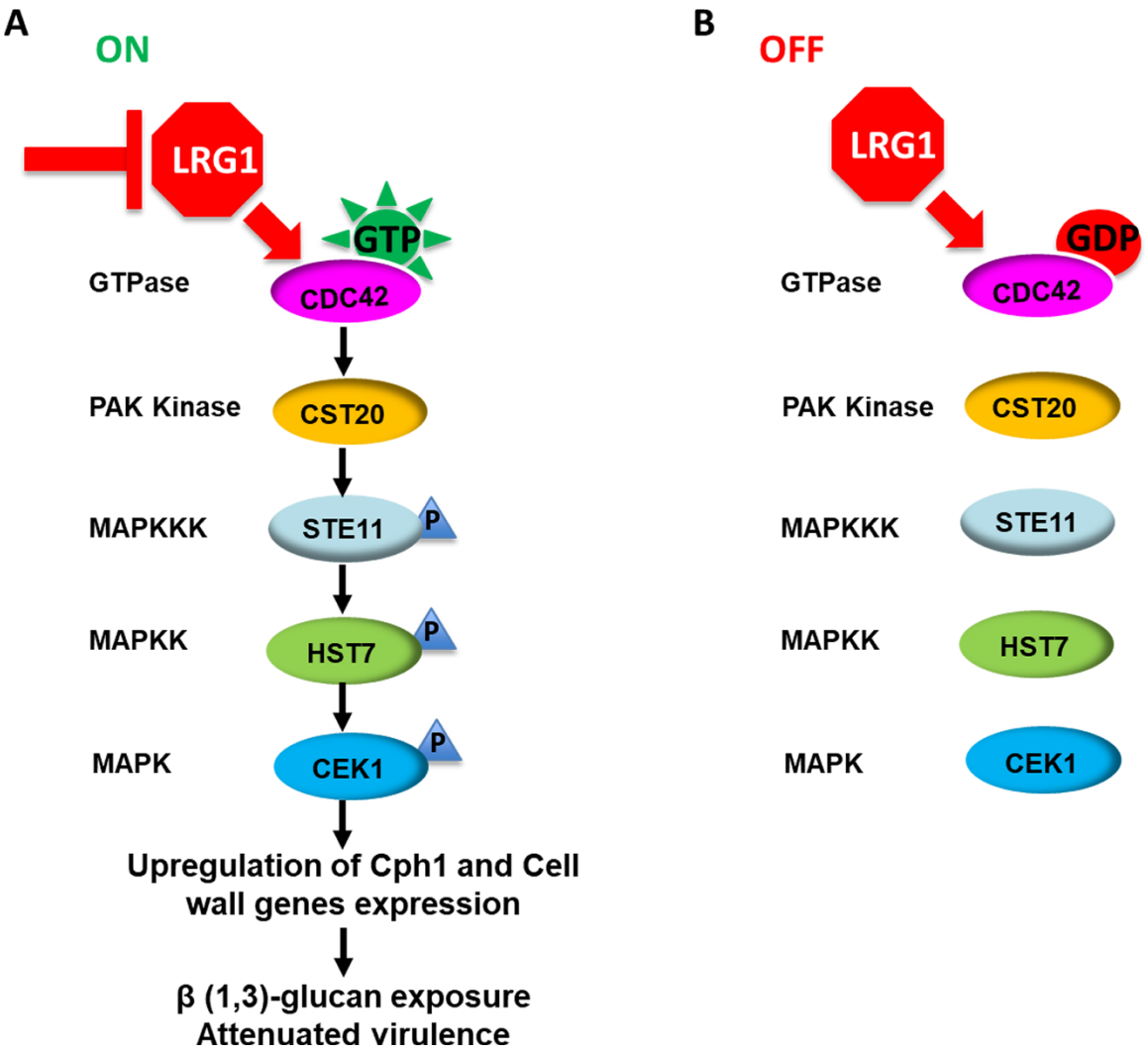


Figure 3. 8. A model shows that Lrg1 acts as a molecular switch regulating Cek1 activity negatively via the GTPase Cdc42.

(A). Lrg1 disruption turns on GTPase Cdc42 activity *in vivo*, which is represented by higher level of GTP-bound Cdc42. This further phosphorylates/activates the canonical downstream Ste11-Hst7-Cek1 cascade. Hyperactivated Cek1 is shown in this study to stimulate β (1,3)-glucan exposure and compromise fungal virulence. (B). Lrg1 is speculated to act as GTPase Cdc42 GAP, which stimulates its intrinsic GTPase activity. Cdc42 is therefore favorably disposed to GDP-bound state to suppress Cek1 phosphorylation.

mutant (*cho1Δ/Δ*), *ace2Δ/Δ, cek1Δ/Δ*, *kre5 Δ/Δ, phr1 Δ/Δ* [11, 15, 30, 51, 52]. However, all these mutants display pleotropic phenotypes. Therefore, it is hard to differentiate the effect of β (1,3)-glucan exposure versus the role that other types of defects play on fungal virulence. Also, β (1,3)-glucan of the mutants is exposed even before the fungal cells are injected into the mouse tail. This raises a problem that the mutants with β (1,3)-glucan exposed might be recognized and cleared by the immune systems more rapidly, even before the systemic infection is established.

In this communication, we made the P_{tetOFF}-$STE11^{\Delta N467}$ mutant that addressed the above-mentioned disadvantages. Ste11 sits upstream and specifically regulates Cek1 activity in *C. albicans* [53]. The truncated $STE11^{\Delta N467}$ mutant without the N-terminal inhibitory domain displays great induction of Cek1 activity and higher level of β (1,3)-glucan exposure [17]. The P_{tetOFF} promoter can be further used to control $Ste11^{\Delta N467}$ expression, by adding/removing the inhibitor doxycycline as needed. The $STE11^{\Delta N467}$ mutant when induced to hyper-activate Cek1 has decreased virulence *in vivo*, reflected by significantly greater mouse survival over 21 days compared to other control groups, and an ~100 fold decreased in fungal burdens in mouse kidneys (Figure 3. 6 and Figure 3. 7). This suggests that the exposed β (1,3)-glucan might contribute to the attenuated fungal virulence, due to its higher visibility to immune receptors via Dectin-1 potentially. Regardless of the mechanism, virulence is compromised, thus hyperactivation of Cek1 via a variety of different pathways may be useful for improving immune recognition of *C. albicans* and adjunctive therapy.

Reference

1. Zaoutis TE, Argon J, Chu J, Berlin JA, Walsh TJ, Feudtner C. The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2005;41(9):1232-9. doi: 10.1086/496922. PubMed PMID: 16206095.
2. Netea MG, Joosten LA, van der Meer JW, Kullberg BJ, van de Veerdonk FL. Immune defence against Candida fungal infections. Nat Rev Immunol. 2015;15(10):630-42. doi: 10.1038/nri3897. PubMed PMID: 26388329.
3. Cheng SC, Joosten LA, Kullberg BJ, Netea MG. Interplay between Candida albicans and the mammalian innate host defense. Infection and immunity. 2012;80(4):1304-13. doi: 10.1128/IAI.06146-11. PubMed PMID: 22252867; PubMed Central PMCID: PMCPMC3318407.
4. Casadevall A, Pirofski LA. Adjunctive immune therapy for fungal infections. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2001;33(7):1048-56. doi: 10.1086/322710. PubMed PMID: 11528579.
5. Gow NAR, Latge JP, Munro CA. The Fungal Cell Wall: Structure, Biosynthesis, and Function. Microbiology spectrum. 2017;5(3). doi: 10.1128/microbiolspec.FUNK-0035-2016. PubMed PMID: 28513415.
6. Rappleye CA, Eissenberg LG, Goldman WE. Histoplasma capsulatum alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(4):1366-70. doi: 10.1073/pnas.0609848104. PubMed PMID: 17227865; PubMed Central PMCID: PMCPMC1783108.
7. Garfoot AL, Shen Q, Wuthrich M, Klein BS, Rappleye CA. The Eng1 beta-Glucanase Enhances Histoplasma Virulence by Reducing beta-Glucan Exposure. MBio. 2016;7(2):e01388-15. doi: 10.1128/mBio.01388-15. PubMed PMID: 27094334; PubMed Central PMCID: PMCPMC4850272.
8. Gravelat FN, Beauvais A, Liu H, Lee MJ, Snarr BD, Chen D, et al. Aspergillus galactosaminogalactan mediates adherence to host constituents and conceals hyphal beta-glucan from the immune system. PLoS pathogens. 2013;9(8):e1003575. doi: 10.1371/journal.ppat.1003575. PubMed PMID: 23990787; PubMed Central PMCID: PMCPMC3749958.
9. Wheeler RT, Kombe D, Agarwala SD, Fink GR. Dynamic, morphotype-specific Candida albicans beta-glucan exposure during infection and drug treatment. PLoS pathogens. 2008;4(12):e1000227. doi: 10.1371/journal.ppat.1000227. PubMed PMID: 19057660; PubMed Central PMCID: PMC2587227.
10. Hopke A, Nicke N, Hidu EE, Degani G, Popolo L, Wheeler RT. Neutrophil Attack Triggers Extracellular Trap-Dependent Candida Cell Wall Remodeling and Altered Immune Recognition. PLoS pathogens. 2016;12(5):e1005644. doi: 10.1371/journal.ppat.1005644. PubMed PMID: 27223610; PubMed Central PMCID: PMC4880299.
11. Davis SE, Hopke A, Minkin SC, Jr., Montedonico AE, Wheeler RT, Reynolds TB. Masking of beta(1-3)-glucan in the cell wall of Candida albicans from

detection by innate immune cells depends on phosphatidylserine. Infection and immunity. 2014. doi: 10.1128/IAI.01612-14. PubMed PMID: 25114110.
12. Gantner BN, Simmons RM, Underhill DM. Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. Embo Journal. 2005;24(6):1277-86. doi: 10.1038/sj.emboj.7600594. PubMed PMID: WOS:000228327000018.
13. Hopke A, Brown AJP, Hall RA, Wheeler RT. Dynamic Fungal Cell Wall Architecture in Stress Adaptation and Immune Evasion. Trends in microbiology. 2018;26(4):284-95. doi: 10.1016/j.tim.2018.01.007. PubMed PMID: 29452950; PubMed Central PMCID: PMC5869159.
14. Wester MJ, Lin J, Neumann AK. A computational model for regulation of nanoscale glucan exposure in Candida albicans. PloS one. 2017;12(12):e0188599. doi: 10.1371/journal.pone.0188599. PubMed PMID: 29232689; PubMed Central PMCID: PMC5726713.
15. Hasim S, Allison DP, Retterer ST, Hopke A, Wheeler RT, Doktycz MJ, et al. beta-(1,3)-Glucan Unmasking in Some Candida albicans Mutants Correlates with Increases in Cell Wall Surface Roughness and Decreases in Cell Wall Elasticity. Infection and immunity. 2017;85(1). doi: 10.1128/IAI.00601-16. PubMed PMID: 27849179; PubMed Central PMCID: PMC5203643.
16. Sherrington SL, Sorsby E, Mahtey N, Kumwenda P, Lenardon MD, Brown I, et al. Adaptation of Candida albicans to environmental pH induces cell wall remodelling and enhances innate immune recognition. PLoS pathogens. 2017;13(5):e1006403. doi: 10.1371/journal.ppat.1006403. PubMed PMID: 28542528; PubMed Central PMCID: PMC5456412.
17. Chen T, Jackson JW, Tams RN, Davis SE, Sparer TE, Reynolds TB. Exposure of Candida albicans beta (1,3)-glucan is promoted by activation of the Cek1 pathway. PLoS genetics. 2019;15(1):e1007892. doi: 10.1371/journal.pgen.1007892. PubMed PMID: 30703081.
18. Qadota H, Python CP, Inoue SB, Arisawa M, Anraku Y, Zheng Y, et al. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. Science. 1996;272(5259):279-81. PubMed PMID: 8602515.
19. Delley PA, Hall MN. Cell wall stress depolarizes cell growth via hyperactivation of RHO1. The Journal of cell biology. 1999;147(1):163-74. PubMed PMID: 10508863; PubMed Central PMCID: PMC2164985.
20. Kondoh O, Tachibana Y, Ohya Y, Arisawa M, Watanabe T. Cloning of the RHO1 gene from Candida albicans and its regulation of beta-1,3-glucan synthesis. Journal of bacteriology. 1997;179(24):7734-41. PubMed PMID: 9401032; PubMed Central PMCID: PMC179736.
21. Gulli MP, Jaquenoud M, Shimada Y, Niederhauser G, Wiget P, Peter M. Phosphorylation of the Cdc42 exchange factor Cdc24 by the PAK-like kinase Cla4 may regulate polarized growth in yeast. Mol Cell. 2000;6(5):1155-67. PubMed PMID: 11106754.
22. Hope H, Bogliolo S, Arkowitz RA, Bassilana M. Activation of Rac1 by the guanine nucleotide exchange factor Dck1 is required for invasive filamentous growth in the pathogen Candida albicans. Molecular biology of the cell.

2008;19(9):3638-51. doi: 10.1091/mbc.E07-12-1272. PubMed PMID: 18579689; PubMed Central PMCID: PMCPMC2526690.
23. Grahl N, Demers EG, Lindsay AK, Harty CE, Willger SD, Piispanen AE, et al. Mitochondrial Activity and Cyr1 Are Key Regulators of Ras1 Activation of C. albicans Virulence Pathways. PLoS pathogens. 2015;11(8):e1005133. doi: 10.1371/journal.ppat.1005133. PubMed PMID: 26317337; PubMed Central PMCID: PMC4552728.
24. Yang SL, Zeng G, Chan FY, Wang YM, Yang D, Wang Y. Sac7 and Rho1 regulate the white-to-opaque switching in Candida albicans. Scientific reports. 2018;8(1):875. doi: 10.1038/s41598-018-19246-9. PubMed PMID: 29343748; PubMed Central PMCID: PMCPMC5772354.
25. Xie JL, Grahl N, Sless T, Leach MD, Kim SH, Hogan DA, et al. Signaling through Lrg1, Rho1 and Pkc1 Governs Candida albicans Morphogenesis in Response to Diverse Cues. PLoS genetics. 2016;12(10):e1006405. doi: 10.1371/journal.pgen.1006405. PubMed PMID: 27788136; PubMed Central PMCID: PMC5082861.
26. Fitch PG, Gammie AE, Lee DJ, de Candal VB, Rose MD. Lrg1p Is a Rho1 GTPase-activating protein required for efficient cell fusion in yeast. Genetics. 2004;168(2):733-46. doi: 10.1534/genetics.104.028027. PubMed PMID: 15514049; PubMed Central PMCID: PMC1448843.
27. Ushinsky SC, Harcus D, Ash J, Dignard D, Marcil A, Morchhauser J, et al. CDC42 is required for polarized growth in human pathogen Candida albicans. Eukaryotic cell. 2002;1(1):95-104. PubMed PMID: 12455975; PubMed Central PMCID: PMC118047.
28. Bassilana M, Hopkins J, Arkowitz RA. Regulation of the Cdc42/Cdc24 GTPase module during Candida albicans hyphal growth. Eukaryotic cell. 2005;4(3):588-603. doi: 10.1128/EC.4.3.588-603.2005. PubMed PMID: 15755921; PubMed Central PMCID: PMCPMC1087799.
29. Roman E, Correia I, Salazin A, Fradin C, Jouault T, Poulain D, et al. The Cek1-mediated MAP kinase pathway regulates exposure of-1,2 and-1,2-mannosides in the cell wall of Candida albicans modulating immune recognition. Virulence. 2016;7(5):558-77. doi: 10.1080/21505594.2016.1163458. PubMed PMID: WOS:000380007500011.
30. Galan-Diez M, Arana DM, Serrano-Gomez D, Kremer L, Casasnovas JM, Ortega M, et al. Candida albicans beta-glucan exposure is controlled by the fungal CEK1-mediated mitogen-activated protein kinase pathway that modulates immune responses triggered through dectin-1. Infection and immunity. 2010;78(4):1426-36. doi: 10.1128/IAI.00989-09. PubMed PMID: 20100861; PubMed Central PMCID: PMCPMC2849429.
31. Roman E, Alonso-Monge R, Gong Q, Li D, Calderone R, Pla J. The Cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen Candida albicans. FEMS yeast research. 2009;9(6):942-55. doi: 10.1111/j.1567-1364.2009.00545.x. PubMed PMID: 19656200.
32. Leberer E, Harcus D, Dignard D, Johnson L, Ushinsky S, Thomas DY, et al. Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and

cAMP signalling pathways in the pathogenic fungus Candida albicans. Molecular microbiology. 2001;42(3):673-87. PubMed PMID: 11722734.
33. Hope H, Schmauch C, Arkowitz RA, Bassilana M. The Candida albicans ELMO homologue functions together with Rac1 and Dck1, upstream of the MAP Kinase Cek1, in invasive filamentous growth. Molecular microbiology. 2010;76(6):1572-90. doi: 10.1111/j.1365-2958.2010.07186.x. PubMed PMID: 20444104.
34. Cassilly CD, Maddox MM, Cherian PT, Bowling JJ, Hamann MT, Lee RE, et al. SB-224289 Antagonizes the Antifungal Mechanism of the Marine Depsipeptide Papuamide A. PloS one. 2016;11(5):e0154932. doi: 10.1371/journal.pone.0154932. PubMed PMID: 27183222; PubMed Central PMCID: PMC4868317.
35. Tams RN, Cassilly CD, Anaokar S, Brewer WT, Dinsmore JT, Chen YL, et al. Overproduction of Phospholipids by the Kennedy Pathway Leads to Hypervirulence in Candida albicans. Front Microbiol. 2019;10:86. doi: 10.3389/fmicb.2019.00086. PubMed PMID: 30792701; PubMed Central PMCID: PMCPMC6374345.
36. De Backer MD, Maes D, Vandoninck S, Logghe M, Contreras R, Luyten WH. Transformation of Candida albicans by electroporation. Yeast. 1999;15(15):1609-18. doi: 10.1002/(SICI)1097-0061(199911)15:15<1609::AID-YEA485>3.0.CO;2-Y. PubMed PMID: 10572258.
37. Reuss O, Vik A, Kolter R, Morschhauser J. The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene. 2004;341:119-27. doi: 10.1016/j.gene.2004.06.021. PubMed PMID: 15474295.
38. Chen Y-L, Montedonico AE, Kauffman S, Dunlap JR, Menn F-M, Reynolds TB. Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence inCandida albicans. Molecular microbiology. 2010;75(5):1112-32. doi: 10.1111/j.1365-2958.2009.07018.x.
39. Lai WC, Sun HF, Lin PH, Ho Lin HL, Shieh JC. A new rapid and efficient system with dominant selection developed to inactivate and conditionally express genes in Candida albicans. Current genetics. 2016;62(1):213-35. doi: 10.1007/s00294-015-0526-6. PubMed PMID: 26497136.
40. Feng QH, Summers E, Guo B, Fink G. Ras signaling is required for serum-induced hyphal differentiation in Candida albicans. Journal of bacteriology. 1999;181(20):6339-46. PubMed PMID: WOS:000083006500016.
41. Tremblay P, Meiner Z, Galou M, Heinrich C, Petromilli C, Lisse T, et al. Doxycycline control of prion protein transgene expression modulates prion disease in mice. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(21):12580-5. PubMed PMID: 9770528; PubMed Central PMCID: PMCPMC22873.
42. Lorberg A, Schmitz HP, Jacoby JJ, Heinisch JJ. Lrg1p functions as a putative GTPase-activating protein in the Pkc1p-mediated cell integrity pathway in Saccharomyces cerevisiae. Molecular genetics and genomics : MGG. 2001;266(3):514-26. doi: 10.1007/s004380100580. PubMed PMID: 11713681.
43. Vogt N, Seiler S. The RHO1-specific GTPase-activating protein LRG1 regulates polar tip growth in parallel to Ndr kinase signaling in Neurospora.

Molecular biology of the cell. 2008;19(11):4554-69. doi: 10.1091/mbc.E07-12-1266. PubMed PMID: 18716060; PubMed Central PMCID: PMC2575149.
44. Roman E, Alonso-Monge R, Miranda A, Pla J. The Mkk2 MAPKK Regulates Cell Wall Biogenesis in Cooperation with the Cek1-Pathway in Candida albicans. PloS one. 2015;10(7):e0133476. doi: 10.1371/journal.pone.0133476. PubMed PMID: 26197240; PubMed Central PMCID: PMC4509911.
45. Bassilana M, Arkowitz RA. Rac1 and Cdc42 have different roles in Candida albicans development. Eukaryotic cell. 2006;5(2):321-9. doi: 10.1128/EC.5.2.321-329.2006. PubMed PMID: 16467473; PubMed Central PMCID: PMC1405900.
46. Corvest V, Bogliolo S, Follette P, Arkowitz RA, Bassilana M. Spatiotemporal regulation of Rho1 and Cdc42 activity duringCandida albicansfilamentous growth. Molecular microbiology. 2013;89(4):626-48. doi: 10.1111/mmi.12302.
47. Wheeler RT, Fink GR. A Drug-Sensitive Genetic Network Masks Fungi from the Immune System. PLoS pathogens. 2006;2(4):e35. doi: 10.1371/journal.ppat.0020035.
48. Watanabe D, Abe M, Ohya Y. Yeast Lrg1p acts as a specialized RhoGAP regulating 1,3-beta-glucan synthesis. Yeast. 2001;18(10):943-51. doi: Doi 10.1002/Yea.742. PubMed PMID: WOS:000169965500007.
49. Roumanie O, Weinachter C, Larrieu I, Crouzet M, Doignon F. Functional characterization of the Bag7, Lrg1 and Rgd2 RhoGAP proteins from Saccharomyces cerevisiae. FEBS letters. 2001;506(2):149-56. PubMed PMID: 11591390.
50. Seneviratne CJ, Zeng G, Truong T, Sze S, Wong W, Samaranayake L, et al. New "haploid biofilm model" unravels IRA2 as a novel regulator of Candida albicans biofilm formation. Scientific reports. 2015;5:12433. doi: 10.1038/srep12433. PubMed PMID: 26202015; PubMed Central PMCID: PMCPMC5378891.
51. Ballou ER, Avelar GM, Childers DS, Mackie J, Bain JM, Wagener J, et al. Lactate signalling regulates fungal beta-glucan masking and immune evasion. Nature microbiology. 2016;2:16238. doi: 10.1038/nmicrobiol.2016.238. PubMed PMID: 27941860.
52. Ghannoum MA, Spellberg B, Saporito-Irwin SM, Fonzi WA. Reduced virulence of Candida albicans PHR1 mutants. Infection and immunity. 1995;63(11):4528-30. PubMed PMID: 7591097; PubMed Central PMCID: PMCPMC173646.
53. Cheetham J, Smith DA, da Silva Dantas A, Doris KS, Patterson MJ, Bruce CR, et al. A single MAPKKK regulates the Hog1 MAPK pathway in the pathogenic fungus Candida albicans. Molecular biology of the cell. 2007;18(11):4603-14. doi: 10.1091/mbc.E07-06-0581. PubMed PMID: 17804815; PubMed Central PMCID: PMC2043575.
54. Gillum AM, Tsay EY, Kirsch DR. 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. 1984;198(2):179-82. PubMed PMID: 6394964.

55. Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in Candida albicans. Genetics. 1993;134(3):717-28. PubMed PMID: 8349105; PubMed Central PMCID: PMCPMC1205510.

Appendix

Table S.3. 1. *C. albicans* strains used in this study

Strain	Parent	Genotype	Source or reference
SC5314	Clinical isolate	Prototrophic wild type	[54]
YLC337	SC5314	*cho1Δ/Δ*	[38]
DAY286	SC5314	*ura3::imm434/ura3::imm434 iro1/iro1::imm434 his1::hisG/his1::hisG arg4/arg4*	[55]
lrg1Δ/Δ	DAY286	*ura3::imm434/ura3::imm434 iro1/iro1::imm434 his1::hisG/his1::hisG arg4/arg4 lrg1::Tn7-UAU1/lrg1::Tn7-URA3*	[25]
TC14	*lrg1Δ/Δ*	*lrg1Δ/Δ*:: P_{ENO1}-*LRG1*	This study
TC41	*lrg1Δ/Δ*	*lrg1Δ/Δste11Δ/STE11*	This study
TC48	TC41	*lrg1Δ/Δste11Δ/Δ::*P_{tetOFF}-$STE11^{\Delta N467}$	This study
TC128	DAY286	DAY286 with 6×myc-Rho1	This study
TC131	*lrg1Δ/Δ*	*lrg1Δ/Δ* with 6×myc-Rho1	This study
TC132	*lrg1Δ/Δ::R*	*lrg1Δ/Δ:: R* with 6×myc-Rho1	This study
TC147	DAY286	DAY286+P_{tetOFF}-$STE11^{\Delta N467}$	This study
TC44	DAY286	DAY286 with GFP-Rac1	This study
TC158	*lrg1Δ/Δ*	*lrg1Δ/Δ* with GFP-Rac1	This study
CAF2-1	SC5314	*URA3/ura3*::imm434 *IRO1/iro1*::imm434	[55]
Ca79	*CAF2-1*	*RAS1/RAS1/*P_{MAL2}-$RAS1^{G13V}$	[40]
TC159	DAY286	DAY286+P_{ENO1}-$RAS1^{G13V}$-*1*	This study
TC160	DAY286	DAY286+ P_{ENO1}-$RAS1^{G13V}$-*2*	This study

Table S.3. 2. Plasmids used in this study

Strain Name	Description	Source
pYLC146	FLP-CaNAT, *chloraR*	This study
pBT1	*CaNAT-*P_{ENO1}, *ampR*	[35]
pTC7	pENO1+*CaLRG1*, *ampR*	This study
pExpArg-pACT1GFPRID	pExpArg-pACT1GFPRID	[46]
pTC33	pExpArg-pACT1GFPRID+NAT^R	[17]
pTC55	pGEX+RIDHIS in *DH5α*, *ampR*	This study
pTC57	pGEX+RIDHIS in *BL21*, *ampR*	This study
pTC71	*pYLC146+STE11-KO, chloraR*	This study
pYGS1244	TetO-Myc-RHO1-UTR*-TetR/CIP10U	[24]
pTC73	pYGS1244+$HYGRO^R$	This study
pTC76	pExpArg-pACT1GFPRAC1+NAT^R	This study
pTC78	pENO1+$CaRAS1^{G13V}$, *ampR*	This study
pWTF1	P_{tetOFF}+$HYGRO^R$	[39]

Table S.3. 3. Primers used in this study

Name	Sequence	Description
TCO57	TTTCCACAATCAAACATCCAA	Primer-R sits at the C-terminus of *STE11* to check if P_{TETOFF}- $STE11^{\Delta N467}$ is replaced the 2nd allele of *STE11* in TC41
TCO69	AAAAGCGGCCGCATGAAGCATTCGTTTGATAC	Primer-F to amplify *CaLRG* ORF and Terminator
TCO68	AAAAGAGCTCGGTTTAGATTAAGCAGAGTT	Primer-R to amplify *CaLRG* ORF and Terminator
TCO92	AAAGCGGCCGCTCGATATTGCTAGAGGCAAA	Primer-F to amplify Hygromycin resistance B gene with NotI flanking
TCO93	AAAGCGGCCGCATTTTATGATGGAATGAATGG	Primer-R to amplify Hygromycin resistance B gene with NotI flanking
TCO139	AAAAGGATCCTTAGAACCAGGTGGTCAAAT	Primer-F to amplify RID domain located at 1111-1908bp of *CaPKC1* ORF flanking with BamHI
TCO140	AAAAGTCGACCCGACGTCTAGTAGAAGGA	Primer-R to amplify RID domain located at 1111-1908bp of CaPKC1 ORF flanking with SalI
TCO155	AAAAGGTACCGATGAACAGGTATTAGTATCA	Primer-F to amplify 500bp of CaSTE11 promoter with KpnI flanking
TCO156	AAAACTCGAGGACTGTTTGTTAGATTTGAGT	Primer-R to amplify 500bp of CaSTE11 promoter with XhoI flanking
TCO157	AAAAGCGGCCGCCCATTAATTATGTCGAAACAA	Primer-F to amplify 500bp of CaSTE11 terminator with NotI flanking
TCO158	AAAAGAGCTCCAAGTAGGTTCACTGTAGAT	Primer-R to amplify 500bp of CaSTE11 terminator with SacI flanking
TCO159	AGGAGAGATTAAAACTTGGA	Primer-F to check if the pYLC146+STE11 KO construct was integrated into chromosome
TCO162	ATATAGAAAACCTAATACATTAGTTCGTGTGTAT ATGGTACTCAAATCTAACAAACAGTCCTTGGACT CTTGAATCCGCGG	Primer -F to amplify tetracycline-repressing promoter flanking by 60-mer homology of *STE11* promoter
TCO163	AATCCTATGCAATGCAGTTCCCCCACTTAATGAT TTTGACGATATTATGGATCTCGAATCCATGACTG CACCAGCTCCGGTACCACT	Primer -R to amplify tetracycline-repressing promoter flanking by 60-mer homology of STE11 ORF located at 1402-1461bp
TCO181	CTACTCGATTGAGTAATAGA	Primer-F sits at $P_{miniOP4}$ of pWTF1 to check if pWTF1 replaced the *STE11* promoter and the N-terminal 467 aa.

Table S.3. 3. Continued

Name	Sequence	Description
TCO57	TTTCCACAATCAAACATCCAA	Primer-R sits at the C-terminal of *STE11* to check if pWTF1 replaced the *STE11* promoter and the N-terminal 467 aa.
TCO165	AAATCTAGAATTTTATGATGGAATGAATGG	Primer-R to amplify Hygronyocin B resistance gene with XbaI flanking
TCO168	CTGTTCTTTCATGGTATTCT	Primer-F sits at -117bp of the *STE11* promoter to check if P_{TETOFF}-STE11ΔN467 was integrated into the chromosome
TCO169	ACTGATTTATTCAATTGATGG	Primer-R sits within the pTDH3 of pWTF, and is to check if P_{TETOFF}-STE11ΔN467 was integrated into the chromosome
TCO170	AAAACGGACCGTGTACCCATACGATGTCCCAGACTACGCAATGAGAAGCATTAAATCAGTCGTAG	Primer-F to amplify *RAC1* ORF with RsrII flanking
TCO175	AAAAACGCGTCCCATGATTATAATATAGTACATTTTTTAGCTCTC	Primer-R to amplify *RAC1* ORF with MluI flanking
TRO993	TTGGTGATGGTCCAGTCTTGT	Primer-F at the EGFP region of pTC76 to check if EGFP and RAC1 ligated in a correct orientation

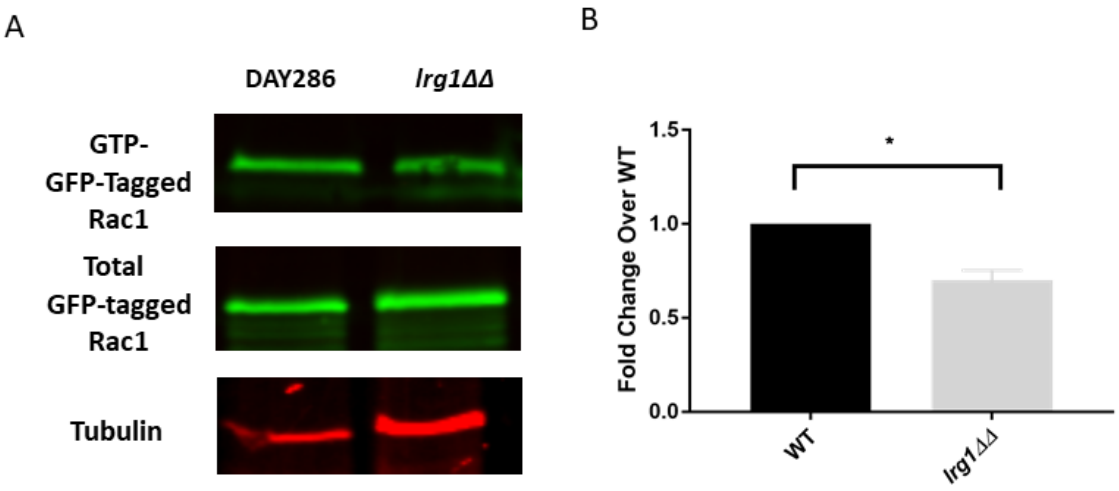


Figure S.3. 1. Loss of Lrg1 decreases GTPase Rac1 activity.

(A) Active GTP-bound GFP-tagged Rac1 was pulled-down using GST-CRIB as a probe, followed by Western blotting with anti-GFP antibody. The amount of total Rac1 was also detected from whole cell lysates by using anti-GFP antibody. (B). The GFP-Rac1 band in the pull-down lanes was quantified by Image J and normalized to the total GFP-Rac1. This data represents 2 blots. *, P=0.0154.

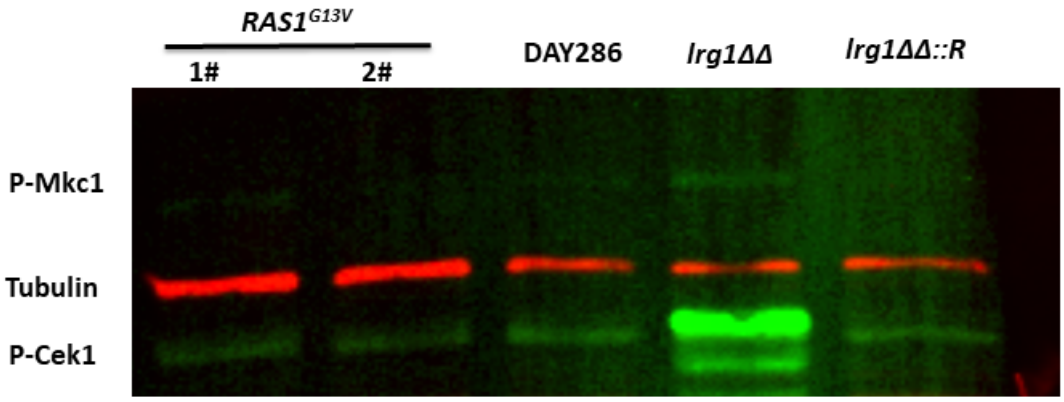


Figure S.3. 2. The hyperactive *Ras1^{G13V}* does not display Cek1 phosphorylation.

The *RAS1^{G13V}* mutant under regulation of constitutively expressing enolase promoter was cultured overnight in YPD at 30 °C and diluted back to OD_{600} at 0.2 for 3 hours growth. Cells were collected, and Western blotting was performed with anti-P-p44/42 antibody to assess Cek1 phosphorylation.

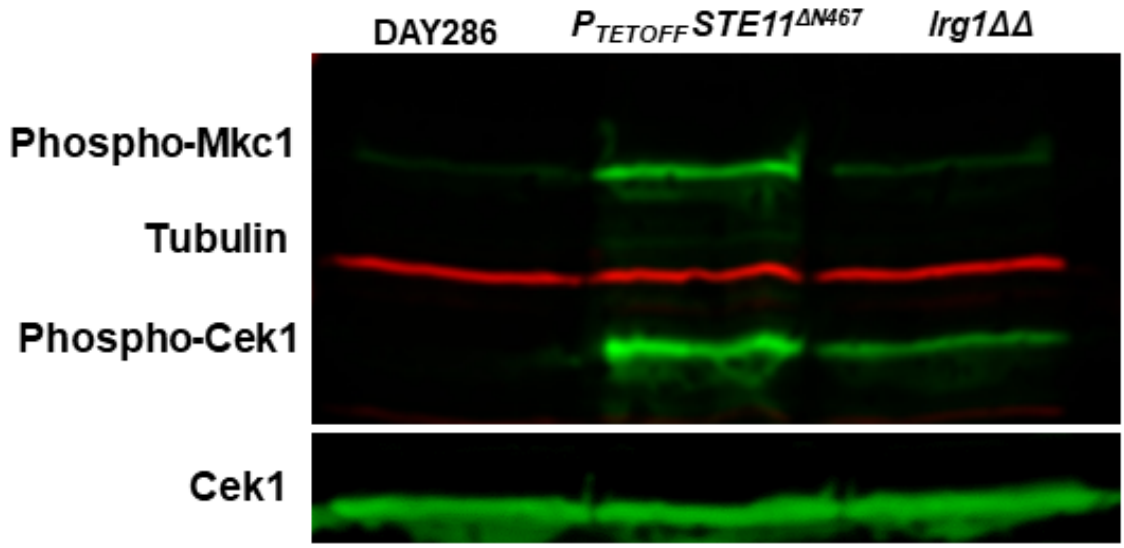


Figure S.3. 3. Cek1 MAPK is hyper-phosphorylated when the P_{tetOFF}-$STE11^{\Delta N467}$ construct is induced.

The P_{tetOFF}-$STE11^{\Delta N467}$ strain was cultured overnight in YPD medium at 30°C in the presence of doxycycline, washed with PBS, diluted back to 0.2 and grown for 3 hours into log phase. Western blotting was performed on the *Candida* cell extracts to evaluate the activation state of MAPKs. Phospho-p44/42 antibody was used as the primary antibody to detect the level of phosphorylated Cek1 and Mkc1 MAPKs. The *lrg1Δ/Δ* mutant was used as a control. The overnight *lrg1Δ/Δ* culture without doxycycline was diluted back to OD_{600} 0.2 and grown for 3 hours.

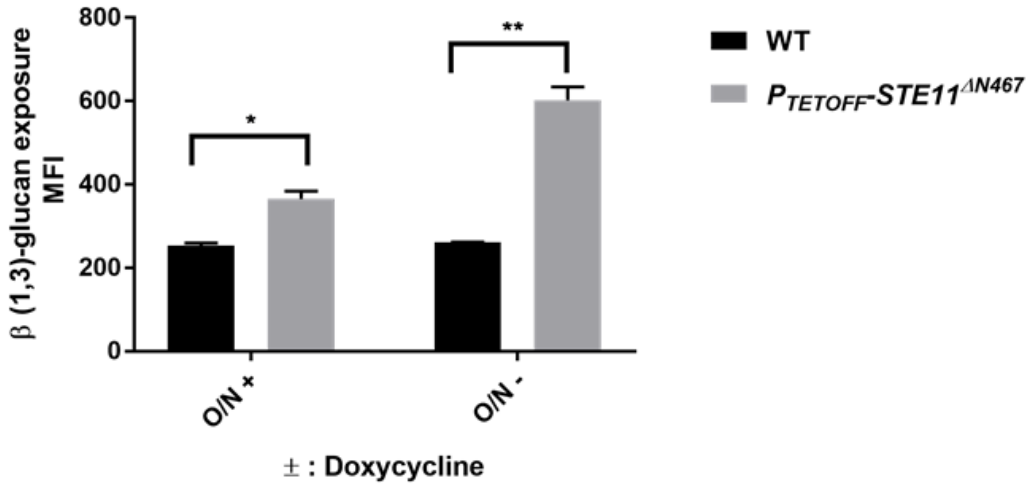


Figure S.3. 4. An overnight culture of P_{tetOFF}-STE11$^{\Delta N467}$ exhibits increased β (1,3)-glucan exposure when induced.

The P_{tetOFF}-STE11$^{\Delta N467}$ strain was cultured overnight in YPD medium at 30°C in the presence or absence of doxycycline. The overnight culture was stained with anti-β (1,3)-glucan antibody and PE-conjugated secondary antibody. Flow cytometry was then performed to quantify the level of β (1,3)-glucan exposure shown in the mutants. **, P=0.0043, *, P=0.0158.

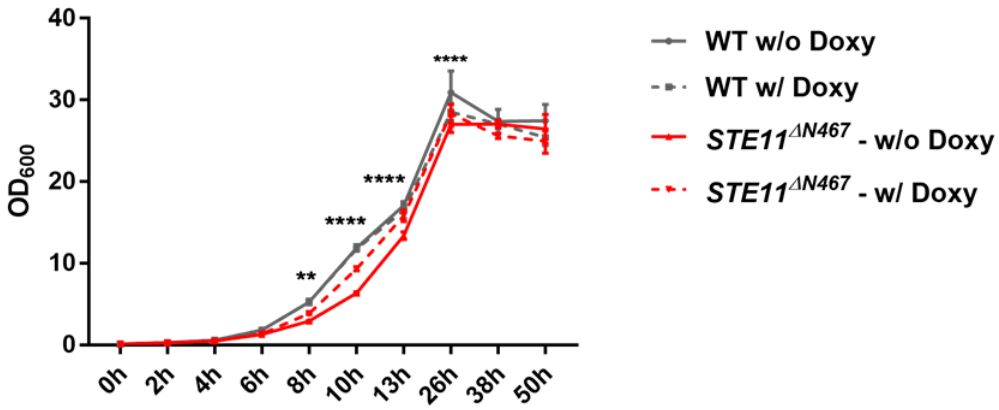


Figure S.3. 5. The growth rate of different *Candida* strains was measured to determine if hyperactivation of Cek1 MAPK affects cellular growth.

Cells were grown overnight in YPD at 30°C plus doxycycline, diluted back to OD_{600} of 0.1 and transferred to fresh YPD with or without doxycycline. A growth curve was generated with three replicates for each condition. The growth rate of *STE11*$^{\Delta N467}$ at each timepoint without (w/o) doxycycline was compared with that of wild-type under the same condition by using Two-way ANOVA (**, p=0.0024; ****, p<0.0001).

CHAPTER IV: Genetic Analysis of Genes Expressed during β (1,3)-glucan Exposure Induced by Cek1 Hyperactivation

This chapter is part of a manuscript that includes Chapter III that we are preparing to submit.

Contributing authors to this study include: Tian Chen, Todd B. Reynolds

Author contributions are as follows: Conceptualization: TC, TBR. Formal analysis: TC, TBR. Funding acquisition: TBR. Investigation: TC. Methodology: TC. Project administration: TBR. Resources: TBR. Supervision: TBR. Validation: TC. Visualization: TC. Writing – original draft: TC. Writing – review & editing: TC, TBR. TC performed all the experiments in this chapter.

Abstract

A full molecular model to explain β (1,3)-glucan exposure has not yet been developed, although a growing body of data indicates it correlates with cell wall repair processes. Activation of Cek1 is consistent with this. To investigate how Cek1 activation could contribute to this, the gene expression profile controlled by hyperactive Cek1 needs to be determined. To achieve this goal, we utilized the Cek1-hyperactivating mutant (P_{tetOFF}-$STE11^{\Delta N467}$), where the N-terminus inhibitory domain of Ste11 is deleted, and the C-terminus is under the regulation of the tetracycline repressible promoter. We found that the $STE11^{\Delta N467}$ mutant displays a statistically significant increasing β (1,3)-glucan unmasking 4 hours after sub-culturing into conditions without doxycycline. Total RNA was thus extracted from wild-type and P_{tetOFF}-$STE11^{\Delta N467}$ strains based on timing parameter. The RNA samples were sequenced via the Illumina MiSeq platform, and 109 genes were identified to be differentially expressed more than 2-fold in $STE11^{\Delta N467}$ inducing condition compared to other control conditions. These genes are enriched for cell wall construction/repair, including chitin production, and the β-glucan biosynthesis process. Several signal transduction genes were also found to be expressed differentially. Of note, the Cek1-regulated transcription factor Cph1, required for hyphal development, is induced ~9-folds under Cek1-inducing condition. This suggests that Cph1 might be the downstream effector mediating cell wall unmasking induced by Cek1 hyperactivation. Moreover, Cek1 is induced to be hyper-phosphorylated when treated with the anti-fungal caspofungin, and disruption of *CPH1* decreases the level of β (1,3)-glucan exposure caused by the drug. This highlights the significance of Cek1-Cph1 in cell wall remodeling for therapeutic applications. Our data also provides groundwork to further assess potential cell wall proteins causing β (1,3)-glucan exposure when overexpressed/repressed, which can then be considered as future drug targets.

Introduction

Cek1 is a well-known MAP kinase involved in cell wall biogenesis, morphological alterations, and fungal virulence [1-5]. Disruption of *CEK1* attenuates hyphal development on certain agar media when mannitol is used as the carbon source, or on glucose media while the nitrogen source is limited. The *cek1Δ/Δ* mutation also adversely affects the serum-induced mycelial colony diameters and growth rate [1] and compromises fungal virulence in the mouse mastitis and systemic infection models [1, 2].

Recently, Cek1 activity was identified to be key to controlling β (1,3)-glucan exposure. Loss of Cek1 causes exposure of certain cell wall components, including β (1,3)-glucan, α (1,2)- and β (1,2)-mannosides, via transcriptionally regulating a significant number of cell wall biogenesis genes (*PGA13*, *IHD1*, *etc.*) and stress response genes (*HSP21*, *DDR48, etc.*) [6, 7]. The *cek1Δ/Δ* mutant stimulates the host immune response more effectively compared to the wild-type, via a dectin-1 receptor-dependent fungal recognition mechanism [7]. This mutant is also better recognized by the galectin-3 receptor, a member of β-galactoside-binding protein family [8]. On the other hand, we previously identified that hyper-activated Cek1 promotes β (1,3)-glucan exposure [9]. Like cek1*Δ/Δ*, the mutant containing Cek1 hyperactivation is recognized more effectively by murine macrophages than wild-type, and fungal virulence is attenuated in the mouse model of systemic infection (Figure 3. 5, Figure 3. 6, and Figure 3. 7). However, the genetic mechanisms causing β (1,3)-glucan exposure in this mutant are unknown.

Cek1 has two downstream transcription factors Cph1 and Ace2, where Cph1 impacts hyphal growth and white-opaque switching and Ace2 affects cell wall glycosylation [1, 8]. Disruption of *ACE2* was reported to cause severe β (1,3)-glucan exposure [10]. Conversely, transcriptional upregulation of *ACE2* is thought to be responsible for increased β (1,3)-glucan masking driven by lactate treatment [10]. This is possibly due to the regulation of Ace2 on homeostasis of cell wall glycostructure in order to cover the glucan layer underneath [8]. Cph1 is also involved in *C. albicans* cell wall construction [11-13]. The *CPH1* over-expressing mutant exhibit pseudo-filament formation under yeast growth condition, and

several transcriptionally upregulated cell wall genes driven by Cph1 hyperexpression under this condition were also induced in a wild-type strain grown under hyphal-culturing condition [13]. Given that *C. albicans* hyphae intrinsically display a higher level of β (1,3)-glucan exposure, it is possible that Cph1 overexpression under yeast condition might drive cell wall to transition to a hyphal-like structure. Altogether, this indicates that the cell wall dysfunction resulted from Cek1 activity imbalance might be mediated through these two downstream effectors transcriptionally.

To determine the genetic profile induced by Cek1 hyperactivation which might result in β (1,3)-glucan exposure, we performed RNA-seq on the P_{tetOFF}-$STE11^{\Delta N467}$ mutant which has been confirmed to hyper-activate Cek1 when induced (Figure S.3. 3). Our transcriptome analysis identified a group of genes that are enriched for cell wall synthesis and responses to chemicals/stress and are increased in expression by more than 2-fold in the hyperactive Cek1 mutant compared to control groups. The transcription factor Cph1 is among them and is up-regulated ~9-fold, which might impact cell wall architecture.

Methods

Strains and plasmids

All the strains in this chapter were described in Table S.4. 1.

Growth conditions

The media used to culture *Candida* cells was yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose (Thermo Fisher Scientific)) [14]. Doxycycline (Sigma-Aldrich, USA) was added at the working concentration of 0.5 μg/mL as the repressor for the tetracycline-repressing promoter.

Western blotting

To extract proteins from P_{tetOFF}-$STE11^{\Delta N467}$ mutant, the strain was cultured overnight in the presence of doxycycline, and sub-cultured in fresh YPD

±doxycycline and grown for 4 hours to mid-log phase. To extract proteins from *Candida* cells being treated with caspofungin, the wild-type DAY286 cells were cultured without caspofungin overnight, and then transferred to in the fresh YPD medium without the drug for 3 hours into log phase. Then, a ½ MIC of caspofungin (63ng/ml) was added to the culture for further incubation for 30min, 1 hr and 2 hrs. Western Blotting was performed as previously described [9].

To detect the phosphorylation of Cek1 and Mkc1 MAPKs, rabbit anti-Phospho-p44/42 antibody (Cell Signaling Technology, Inc., USA) at 1:2000 dilution was utilized. The expression of total Mkc1 was detected with the primary rabbit-anti- Mkc1 Ab at a 1:1000 dilution (provided by Dr. Jesus Pla at Universidad Complutense de Madrid, Spain). The expression of total Cek1 was measured with a rabbit-anti- Cek1 Ab at a 1:1000 dilution (a gift from Dr. Mira Edgerton at The State University of New York). The secondary antibody for Phospho-p44/42 Ab, Mkc1 Ab and Cek1 Ab was IRye800CW goat anti–rabbit IgG (H+L) conjugate (green, 1:10,000 dilution; LI-COR Biosciences). Tubulin was probed as a control with rat anti-tubulin primary antibody (Bio-Rad Laboratories Inc., USA) at a 1:1000 dilution and IRDye 680RD Goat-anti-Rat IgG (H+L) (red, 1:10,000 dilution; LI-COR Biosciences) as a secondary antibody.

Flow cytometry

To stain the *STE11*$^{\Delta N467}$ strain under the regulation of P_{tetOFF} promoter over-time, overnight cultures in YPD with doxycycline were diluted back to 0.2, and cells were collected after 1hr, 2hrs, 4hrs and 6hrs grown in the fresh YPD medium without doxycycline. To stain *Candida* cells treated with caspofungin, the overnight culture without the drug was sub-cultured for 3 hours. The staining protocol and gating strategy was followed as described in [9]. Flow cytometry data were obtained for 100,000 gated events per strain and experiments were performed in triplicate, and the data were analyzed using the FlowJo software package with version 10.11 (FlowJo LLC, OR, USA).

RNA extraction

The RNA extraction protocol was modified from [15]. 15 ml of yeast overnight culture were collected and washed 3 times with PBS. 750 µl of TES buffer (Tris-Cl (10 mM, pH 7.6), EDTA (10 mM), Sodium dodecyl sulfate (SDS) (0.5%, w/v)), which was prepared with RNAse-free water (RPI, Corp. USA) and 750 µl of acid phenol at pH 4.5 (Thermo Fisher, USA) were added to the pellet and an equal volume of 150-212µm acid-washed glass beads (Millipore-Sigma, USA) were added to each tube. Cells were mechanically disrupted in a Biospec Mini-BeadBeater (Bio Spec Product Inc., USA) with 4 rounds of 1 min homogenization at 4°C and 2 min intervals for each cycle on ice. Samples were placed in a 65°C heat block for 30min and vortexed thoroughly every 10mins. The mixture was centrifuged for 5 min at 13,000 ×rpm at room temperature, and the aqueous layer was transferred to a fresh tube containing 700 µl of acid phenol at pH 4.5 and spun for 5 mins. The aqueous layer was transferred and washed twice with 600 µl of neutral pH phenol (Thermo Fisher, USA), followed by washing with 600 µl of chloroform until the interface was clean. The supernatant was further transferred to the tube containing 150 µl of 3M sodium acetate, followed by adding 1 ml 100% ethanol, and the mixture was placed in -80°C freezer overnight to precipitate the nucleic acid out of solution. The pellet was collected by centrifugation at 13,000×rpm at 4°C for 10mins, and further washed with 500 µl of 70% ice-cold ethanol. The pellet was dried at room temperature and resuspended in 100 µl of RNase-free water. DNA was removed by using Turbo DNA-free kit (Thermo Fisher, USA) following manufacturer's instructions.

RNA Samples were analyzed by the 2100 series bioanalyzer (Agilent Technologies, USA) for quality at the University of Tennessee Genomics Core, followed by cDNA library preparation and sequencing. Barcoded cDNA libraries were prepared with a TruSeq Stranded mRNA Sample Preparation Illumina kit according to manufacturer's specifications (Illumina, Inc., USA). The libraries' qualities were validated by bioanalyzer (Agilent Technologies, USA), and then arranged on a flow cell from the MiSeq reagent kit v3 (150×cycle) (Illumina, Inc., USA), and sequenced on the Illumina MiSeq M04398 machine.

Bioinformatic Analysis

All the operations were performed on CLC Genomics Workbench v.12.0 software (Qiagen, Germany). Trimming was performed with a quality score limit set as 0.01 and ambiguity set as 0. The read length to be discarded is set to below 50. The hits were mapped to the assembly 22 of the *C. albicans* reference strain SC5314 [16]. Genome annotations were performed using the corresponding GFF file and annotated with the GFF file tool which is available on CLC Genomics Workbench v.12.0 software. The mapping parameters for mismatch cost, insertion cost, deletion cost, length fraction, and similarity fraction used at default values, which were 2, 3, 3, 0.8, 0,8, respectively. Expression values for each gene were calculated from unique gene reads (maximum number of hits for a read is set to 1) and normalized by gene length and sequencing depth yielding the expression value of transcripts per million (TPM). To determine if gene expression values are correct, the TPM reads of five housekeeping genes (*TEF1*, *ACT1*, *TUB1*, *ENO1*, and *PMA1*) were evaluated to calculate the average for each condition, which was further plotted as shown in [17]. To identify differentially expressed genes between treatment and control, P-values of individual genes were adjusted for false discovery rate (FDR), and genes with an FDR adjusted p-value < 0.05 in the treatment group were considered differentially expressed. Expression values exceeding a factor of 2 (fold change), either higher or lower than that in control were considered of significantly different expression.

Results

The P_{tetOFF}-$STE11^{\Delta N467}$ mutant displays increased β (1,3)-glucan over-time

To investigate how Cek1 activation could contribute to β (1,3)-glucan exposure, the gene expression profile resulting from hyper-activated Cek1 was measured. First, we determined the parameters for this experiment. The P_{tetOFF}-$STE11^{\Delta N467}$ strain ±doxycycline was compared to the isogenic wild-type in identical conditions. To determine the time-point when the $STE11^{\Delta N467}$ mutant starts to display β (1,3)-glucan exposure, we cultured *Candida* cells overnight with doxycycline to repress $Ste11^{\Delta N467}$ expression, followed by dilution into fresh YPD medium without

doxycycline to switch on *STE11$^{\Delta N467}$* expression up to 6 hours. The *Candida* cells were then subjected to immunofluorescent staining by using anti-β (1,3)-glucan antibody, and subsequent flow cytometry to quantify the exposure level over time. As seen in Figure 4. 1A, the *STE11$^{\Delta N467}$* mutant began to display statistically significant increased unmasking 4 hours sub-cultured in the medium without doxycycline. Interestingly, the time-point when unmasking became significant correlates well with the Western blotting results where Cek1 began to display increased phosphorylation after doxycycline was removed 4 hours (Figure 4. 1B). on this, we extracted total RNA from the wild-type and *P$_{tetOFF}$-STE11$^{\Delta N467}$* strains 4 hours after sub-culturing into ±doxycycline conditions.

β (1,3)-glucan exposure induced by hyper-activation of the Cek1 cascade correlates with upregulation of cell wall synthesis genes

Based on these timing parameters, we extracted total RNA from the wild-type and *P$_{tetOFF}$-STE11$^{\Delta N467}$* strains 4 hours after sub-culturing into ±doxycycline conditions, with each condition represented by three biological replicates (12 total samples). RNA sequencing was performed using the Illumina MiSeq platform, which generated a total of 59 million 75bp paired-end reads from 12 libraries. A principal component plot (PCA) was created by using the RNA Analysis Package in CLC Genomics Workbench software to determine the variabilities between replicates Based and different treatment samples. As seen in Figure 4. 2, the two wild-type groups (±doxycycline) clustered tightly, suggesting that doxycycline does not cause undesired effect on wild-type. The *P$_{tetOFF}$-STE11$^{\Delta N467}$* strain was found in two distinct clusters based on the presence and absence of doxycycline, which both separated from wild-type. The *STE11$^{\Delta N467}$* strain +doxycycline grouped away from wild-type and this may be because of basal expression of *STE11$^{\Delta N467}$*, which would explain the modest increase in TNF-α elicited by macrophages by these cells compared to wild-type, even in the presence of doxycycline (Figure 3. 5B). To be certain that the expression levels of genes overall were consistent, we examined 5 housekeeping genes that are known to have differential expression levels. We found that those housekeeping genes were highly consistent between

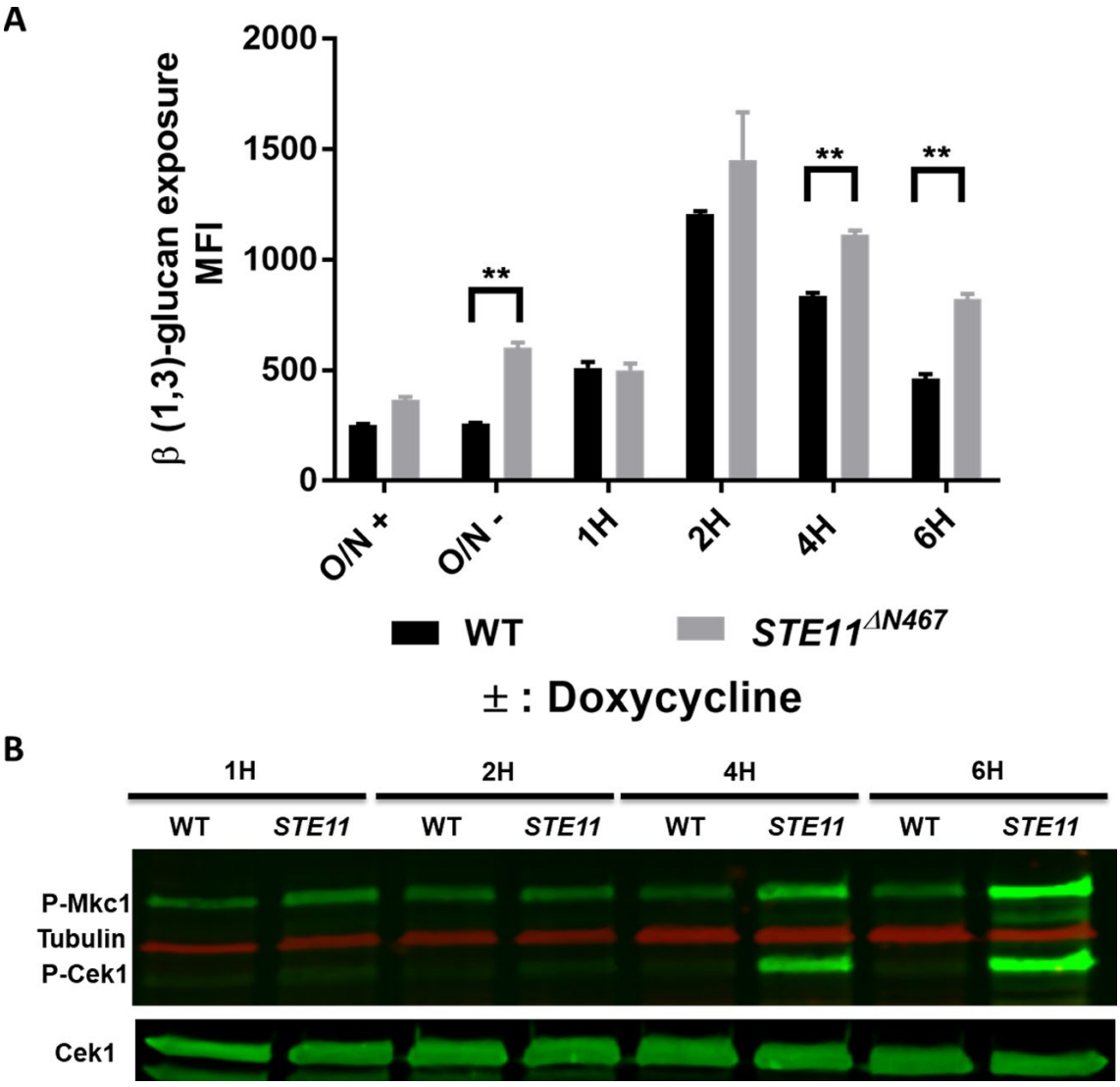


Figure 4. 1. The P_{tetOFF} -$STE11^{\Delta N467}$ strain exhibits β (1,3)-glucan exposure in a time-dependent manner in the absence of doxycycline.

A. The $STE11^{\Delta N467}$ mutant was cultured overnight in YPD at 30°C in the presence or absence of doxycycline. Aliquots of the overnight culture (O/N+ and O/N-) were taken as 0 timepoint controls and stained with mouse anti-β (1,3)-glucan antibody and goat-anti-mouse PE-conjugated secondary antibody. An aliquot of the overnight P_{tetOFF}-$STE11^{\Delta N467}$ culture treated with doxycycline was washed three times with PBS and diluted back to 0.2 in fresh YPD without doxycycline to switch on $Ste11^{\Delta N467}$ expression and was grown for 1hr, 2hrs, 4hrs, and 6hrs. Cells were collected at the indicated time points and stained as described above. All the stained cells were subject to the flow cytometer to quantify the β (1,3)-glucan exposure level. Samples were tested three times with 2 replicates each time. **, P<0.008. B. Cells were cultured as described in A, and then total protein was isolated, and Western blotting was performed to evaluate the MAPKs phosphorylation by using phospho-p44/42 antibody and total Cek1 and tubulin as controls.

Figure 4. 2. PCA plot displays the clustering of samples within each group.

PCA plot was created by using RNA Analysis Package in CLC Genomics Workbench software (V12.0) to determine the variabilities between replicates and different treatment samples.

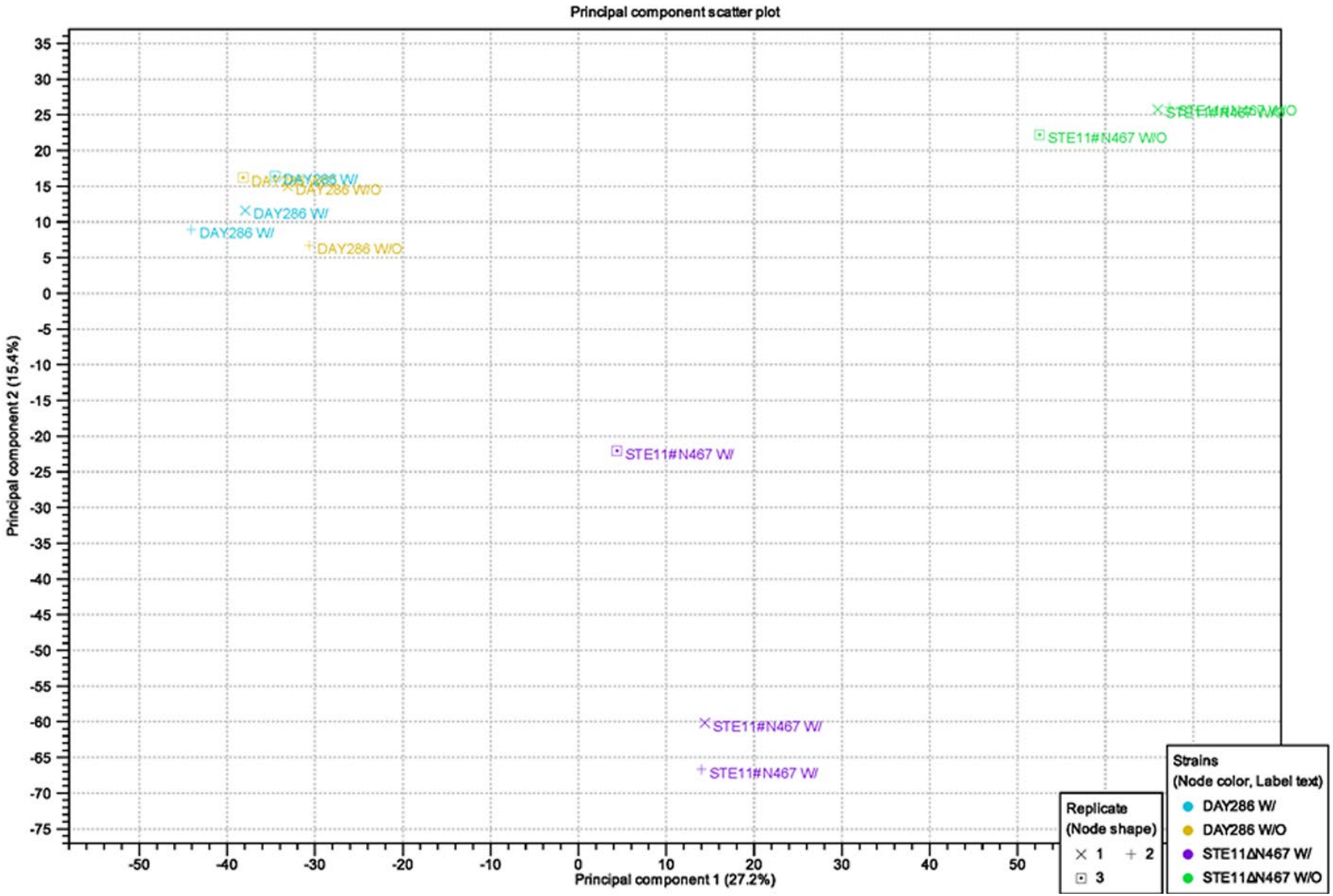


Figure 4. 2. Continued

the samples (Figure 4. 3). In *STE11$^{\Delta N467}$* inducing-condition (-doxycycline), 109 genes are commonly regulated by a > 2-fold change in expression value, compared to the other three conditions (wild-type±doxycycline and *STE11$^{\Delta N467}$* +doxycycline) (Figure 4. 4A). Among the commonly regulated 109 genes, 16 genes are down-regulated, and 93 genes are up-regulated (Figure 4. 4B). The commonly regulated genes were listed in Table S.4. 2. Although Cek1 activation is associated with hyphal formation, there are only a few genes that are conventional hyphal-specific proteins. A number of genes commonly associated with hyphal formation like members of the Als family and Ece1 are not upregulated. However, 13 genes that are involved in cell wall construction are upregulated (Figure 4. 4B and Table 4. 1). These genes are enriched for cell wall or extracellular genes (14% of upregulated genes were secreted genes compared to 5% secreted genes in the total *Candida* genome, indicating that there was 2× fold enrichment in cell wall genes in our data set). 26 genes are responsive to chemicals and stress, including *OPY2* and *RBT4* and 10 genes encode signal transduction proteins (*CPP1*, *BUD5*, *RGA2*, *HAC1*, *WSC2*, *CPH1 etc.*). In the category of down-regulated genes, it is worth noting that Eng1 expression was decreased ~2 fold in the *STE11$^{\Delta N467}$*-inducing condition. Eng1 is an endo-β (1,3)-glucanase, which has been reported to keep glucan masked by removing the exposed β (1,3)-glucan in the fungal pathogen *Histoplasma capsulatum*. Altogether, these data suggest that the β (1,3)-glucan unmasking could be caused by Cek1 hyper-activation through inappropriate expression of cell wall repair machinery.

In addition, our data suggest that Cph1 is likely responsible for these effects. Cek1 has two known downstream transcription factors, Cph1 and Ace2, but they display distinct profiles in our data. Cph1 is up-regulated 8.4-fold in *STE11$^{\Delta N467}$* yeast condition compared to that of wild-type. Cph1 is important for *Candida albicans* filament development on certain solid media [18] and is involved in the switching from white cells to the mating-competent opaque cells [19]. In contrast, Ace2, involved in cell wall glycosylation, morphogenesis and virulence [8, 20] does not display altered transcripts when Cek1 is hyperactivated. This indicates Cph1 is likely the transcription factor responsive to Cek1-inducing condition.

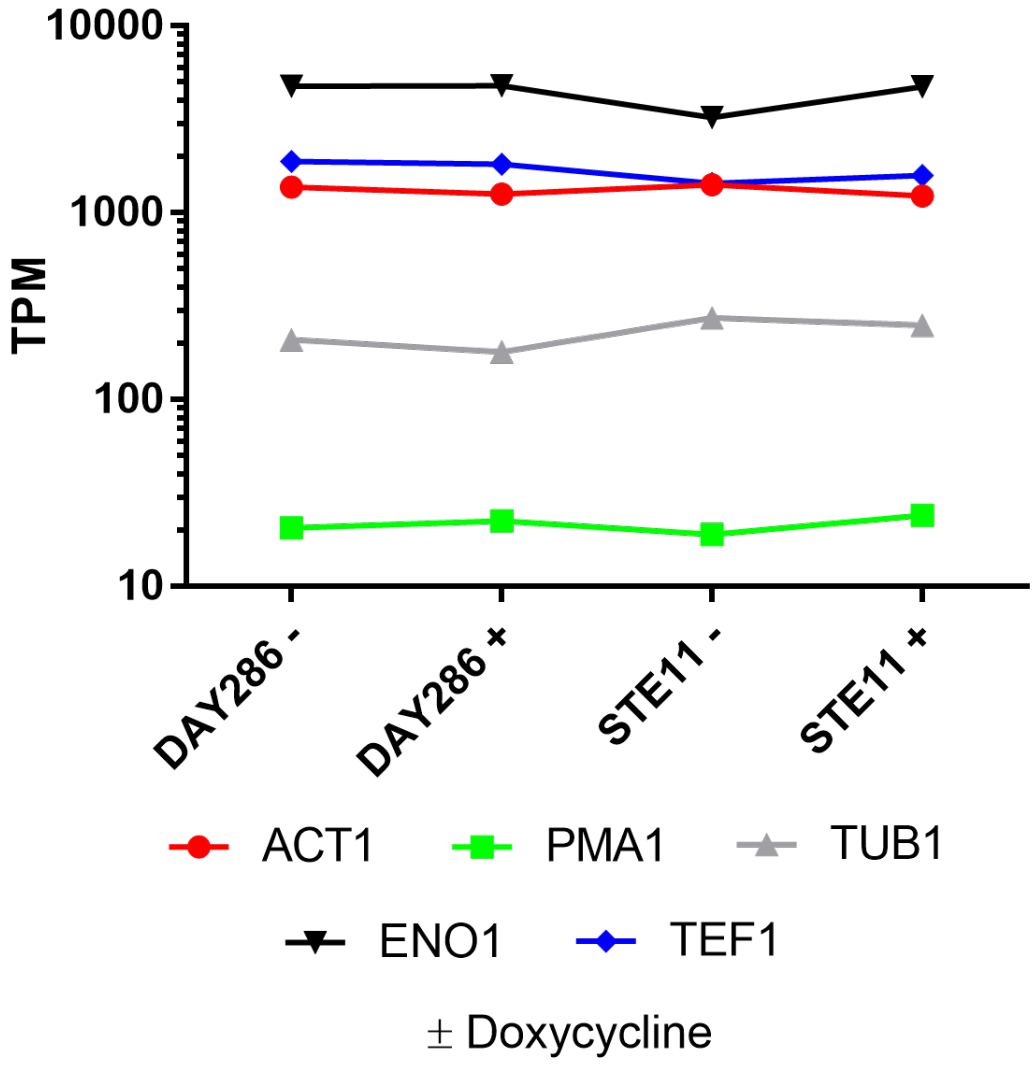


Figure 4. 3. Housekeeping genes expression was similar among different *Candida* strains. The TPM reads of five housekeeping genes (*TEF1, ACT1, TUB1, ENO1*, and *PMA1*) were used to calculate the average for each condition to determine if gene expression values are correct.

Figure 4. 4. Differentially expressed genes in *STE11$^{\Delta N467}$*-inducing condition are enriched for cell wall repair genes.

(A). Venn diagram shows that 109 genes are differentially expressed in the *P$_{tetOFF}$-STE11$^{\Delta N467}$* mutant when Cek1 is induced (*STE11* –doxycycline) compared to the other three control groups (wild-type± and STE11+doxycycline). Genes with a false discovery rate (FDR) adjusted p-value < 0.05 were considered differentially expressed. (B). Heat-maps were generated for genes with expression values exceeding a factor of 2 (fold change) in *STE11$^{\Delta N467}$* inducing condition (STE11 –doxycycline) compared to the other three controls

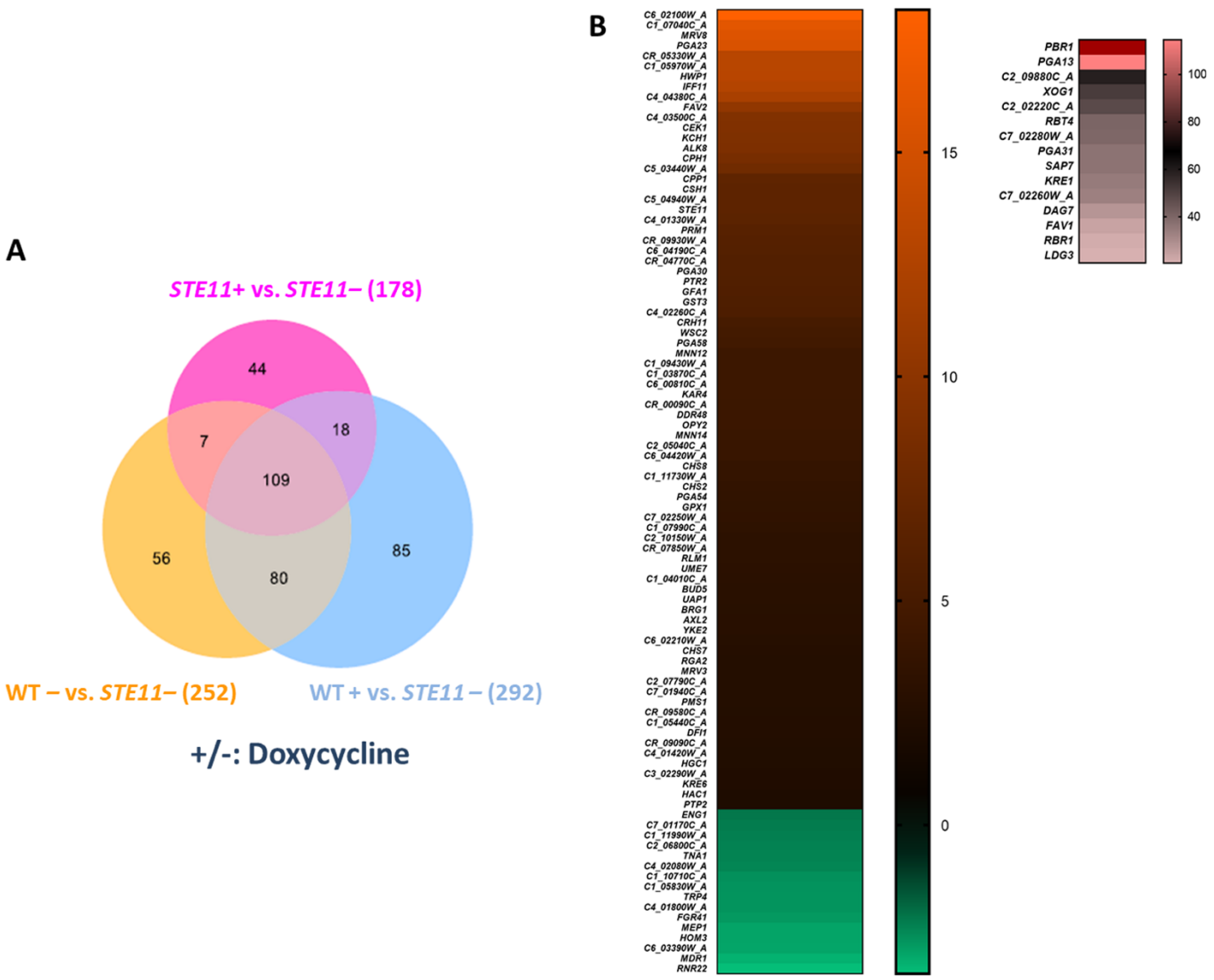


Figure 4. 4. Continued

Table 4. 1: Commonly regulated genes induced by *STE11$^{\Delta N467}$* –doxycycline vs. Wild-type± and *STE11$^{\Delta N467}$* +doxycycline

Gene Ontology terms	Names
• **Function**	
Cell Wall Organization	CEK1, CHS2, CPH1, CRH11, ENG1*, HAC1, HWP1, KRE6, PGA13, PGA31, RLM1, WSC2, XOG1
Stress/Chemical Response	BRG1, C1_03870C_A, C1_10710C_A*, C3_02290W_A, CEK1, CPH1, CPP1, DDR48, FGR41*, GPX1, HAC1, MDR1*, MEP1, OPY2, PMS1, RLM1, STE11, WSC2, C7_01170C_A*, DAG7, GFA1, PGA23, PGA31, RBT4, RLM1, TNA1*
• **Component**	
Cell Wall and Extracellular Regions	CRH11, CSH1, DDR48, ENG1*, HWP1, IFF11, KRE1, PGA13, PGA30, PGA31, XOG1, C4_01800W_A*, DAG7, FGR41*, RBT4,SAP7

*: transcriptionally down-regulated genes

Cek1 activity is induced by caspofungin treatment

β (1,3)-glucan exposure has been reported to correlate with activation of Cek1 pathway under various conditions, such as phosphatidylserine synthase (Cho1) disruption and hyperactivation of the GTPases Cdc42 and Rho1 [9]. Caspofungin, an antifungal drug inhibiting β (1,3)-glucan biosynthesis, causes β (1,3)-glucan exposure even at sublethal condition [21]. However, the mechanism regarding β (1,3)-glucan exposure induced by caspofungin insult has not yet been discovered. Here we evaluated if the correlation between β (1,3)-glucan unmasking and Cek1 phosphorylation holds true under caspofungin treatment. As shown in Figure 4. 5, the Cek1 MAPK is induced to be activated 30 mins after incubation with ½ MIC of caspofungin, and the induction increases over-time. This suggests that Cek1 activation might contribute to β (1,3)-glucan exposure, at least partially, under the caspofungin insult. This further indicates that improper activation of Cek1 seems involved in cell wall remodeling in different situations.

Discussion

In this chapter, we provided the transcriptome analysis to address the genetic mechanism regarding β (1,3)-glucan exposure induced by Cek1 hyperactivation. Our RNA-Seq results reveal that several categories of genes are expressed differentially when Ste11-Cek1 are hyperactivated, and they include cell walls genes, signal transduction genes, and stress genes (Table S.4. 2). Cph1, one of two well-known downstream transcription factors that is phosphorylated/activated by Cek1, is significantly up-regulated in *STE11*$^{\Delta N467}$ inducing conditions. Cph1 is important for *C. albicans* filament development [18], and white-to-opaque switching [19]. The Cph1-overexpressing mutant under yeast conditions induces pseudo-hyphal structure, and several hyphal-specific genes are also induced transcriptionally [13]. Lowman *et al.* reported that *C. albicans* hyphal glucan exhibited a novel glucan structure, where hyphal glucan has a unique cyclical structure which intrinsically display higher levels of β (1,3)-glucan exposure and immunogenicity [11]. Our data shows that *CPH1* is induced ~9 fold, and it is possible that the β (1,3)-glucan structure might transition to a more hyphal glucan

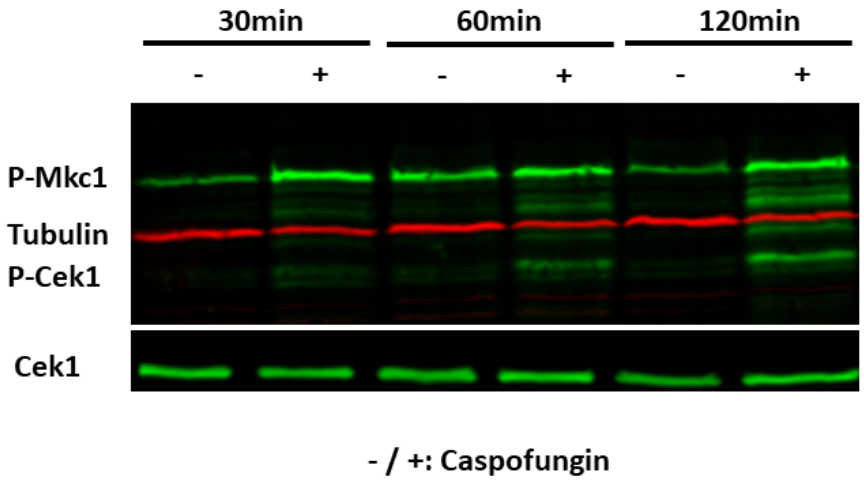


Figure 4. 5. Caspofungin treatment over-activates the Cek1 MAPK.

C. albicans wild-type DAY286 strain was cultured at 30°C in YPD medium overnight. The next day the overnight culture was diluted back to OD_{600} of 0.2 in fresh YPD medium for 3 hours growth into the log phase, followed by ½ MIC of caspofungin treatment (+) for 30 min, 1 hr and 2hrs, respectively. The culture was collected and proceeded to the Western blotting procedure. The antibody to detect phosphorylated Cek1 was phospho-p44/42, which can also detect the phosphorylated Mkc1. Tubulin was detected as the loading control.

structure even under yeast-conditions, and therefore display more glucan exposure. Alternatively, Cek1 is upregulated in response to caspofungin treatment and the changes in exposure may be related to aberrantly induced cell wall repair.

The *cek1ΔΔ* mutant displays β (1,3)-glucan exposure, hyper-sensitivity to agents that disturb the cell wall such as Congo red, and the *N*-glycosylation inhibitor tunicamycin [7, 22, 23]. This mutant also exhibits α (1,2)- and β (1,2)-mannosides exposure, suggesting the glycostructure is defective upon *CEK1* disruption. This characteristic facilitates the immune recognition by the receptor galectin-3. Ace2, a second downstream transcription factor of Cek1, is required for cell wall glycosylation, and causes β (1,3)-glucan exposure when disrupted [8, 10]. Interestingly, Ace2 is not induced transcriptionally when Cek1 is hyperactivated. Thus, Cek1 may be able to deploy specific transcription factors for unique adaptation responses based on the nature of the insult.

Cek1 activity is regulated tightly by multiple stimuli in *C. albicans*. Several conditions induce Cek1 phosphorylation, including disruption of the phosphatidylserine synthase Cho1[9], growth resumption from the overnight culture to fresh media [24], being treated with fungicidal salivary histatin 5 (Hst5) protein [25]. We found that *Candida* cells treated with caspofungin also exhibit Cek1 hyperphosphorylation in a time-dependent manner (Figure 4. 5). Given the correlation of Cek1 activation and resultant cell wall remodeling [9], this might explain, at least partially, the mechanism through which β (1,3)-glucan is unmasked stimulated by caspofungin. This indicates the significance of Cek1 activity in regulating cell wall structure.

Overall, our RNA-seq profile on the Cek1-overactivating mutant provides groundwork for further understanding the genetic mechanisms of β (1,3)-glucan exposure caused by this mutation. We hypothesize that differential expression of some of those genes cause β (1,3)-glucan exposure, including Cph1. Genetic work can be done by Cph1 overexpression to evaluate if the mutation leads to cell wall remodeling and increases immunogenicity. Several cell wall associated proteins are also of interest, including chitin synthases (Chs2, 3 and 8), given the correlation between increased chitin levels and β (1,3)-glucan exposure [26, 27]. The same

genetic strategy can be performed on these target genes by overexpression to assess their involvement in causing cell wall unmasking.

Reference

1. Csank C, Schroppel K, Leberer E, Harcus D, Mohamed O, Meloche S, et al. Roles of the Candida albicans mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infection and immunity. 1998;66(6):2713-21. PubMed PMID: 9596738; PubMed Central PMCID: PMCPMC108260.
2. Guhad FA, Jensen HE, Aalbaek B, Csank C, Mohamed O, Harcus D, et al. Mitogen-activated protein kinase-defective Candida albicans is avirulent in a novel model of localized murine candidiasis. FEMS microbiology letters. 1998;166(1):135-9. PubMed PMID: 9741091.
3. Monge RA, Roman E, Nombela C, Pla J. The MAP kinase signal transduction network in Candida albicans. Microbiology. 2006;152(Pt 4):905-12. doi: 10.1099/mic.0.28616-0. PubMed PMID: 16549655.
4. Roman E, Nombela C, Pla J. The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen Candida albicans. Molecular and cellular biology. 2005;25(23):10611-27. doi: 10.1128/MCB.25.23.10611-10627.2005. PubMed PMID: 16287872; PubMed Central PMCID: PMC1291223.
5. Roman E, Arana DM, Nombela C, Alonso-Monge R, Pla J. MAP kinase pathways as regulators of fungal virulence. Trends in microbiology. 2007;15(4):181-90. doi: 10.1016/j.tim.2007.02.001. PubMed PMID: 17321137.
6. Roman E, Correia I, Salazin A, Fradin C, Jouault T, Poulain D, et al. The Cek1mediated MAP kinase pathway regulates exposure of alpha1,2 and beta1,2mannosides in the cell wall of Candida albicans modulating immune recognition. Virulence. 2016;7(5):558-77. doi: 10.1080/21505594.2016.1163458. PubMed PMID: 27191378; PubMed Central PMCID: PMCPMC5038168.
7. Galan-Diez M, Arana DM, Serrano-Gomez D, Kremer L, Casasnovas JM, Ortega M, et al. Candida albicans beta-glucan exposure is controlled by the fungal CEK1-mediated mitogen-activated protein kinase pathway that modulates immune responses triggered through dectin-1. Infection and immunity. 2010;78(4):1426-36. doi: 10.1128/IAI.00989-09. PubMed PMID: 20100861; PubMed Central PMCID: PMC2849429.
8. van Wijlick L, Swidergall M, Brandt P, Ernst JF. Candida albicans responds to glycostructure damage by Ace2-mediated feedback regulation of Cek1 signaling. Molecular microbiology. 2016;102(5):827-49. doi: 10.1111/mmi.13494. PubMed PMID: 27589033.
9. Chen T, Jackson JW, Tams RN, Davis SE, Sparer TE, Reynolds TB. Exposure of Candida albicans beta (1,3)-glucan is promoted by activation of the Cek1 pathway. PLoS genetics. 2019;15(1):e1007892. doi: 10.1371/journal.pgen.1007892. PubMed PMID: 30703081.
10. Ballou ER, Avelar GM, Childers DS, Mackie J, Bain JM, Wagener J, et al. Lactate signalling regulates fungal beta-glucan masking and immune evasion. Nature microbiology. 2016;2:16238. doi: 10.1038/nmicrobiol.2016.238. PubMed PMID: 27941860.
11. Lowman DW, Greene RR, Bearden DW, Kruppa MD, Pottier M, Monteiro MA, et al. Novel structural features in Candida albicans hyphal glucan provide a

basis for differential innate immune recognition of hyphae versus yeast. The Journal of biological chemistry. 2014;289(6):3432-43. doi: 10.1074/jbc.M113.529131. PubMed PMID: 24344127; PubMed Central PMCID: PMCPMC3916545.
12. Lane S, Birse C, Zhou S, Matson R, Liu H. DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in Candida albicans. The Journal of biological chemistry. 2001;276(52):48988-96. doi: 10.1074/jbc.M104484200. PubMed PMID: 11595734.
13. Huang H, Harcus D, Whiteway M. Transcript profiling of a MAP kinase pathway in C. albicans. Microbiol Res. 2008;163(4):380-93. doi: 10.1016/j.micres.2008.03.001. PubMed PMID: 18467082.
14. Cassilly CD, Maddox MM, Cherian PT, Bowling JJ, Hamann MT, Lee RE, et al. SB-224289 Antagonizes the Antifungal Mechanism of the Marine Depsipeptide Papuamide A. PloS one. 2016;11(5):e0154932. doi: 10.1371/journal.pone.0154932. PubMed PMID: 27183222; PubMed Central PMCID: PMC4868317.
15. Wen J, Won D, Fozo EM. The ZorO-OrzO type I toxin-antitoxin locus: repression by the OrzO antitoxin. Nucleic Acids Res. 2014;42(3):1930-46. doi: 10.1093/nar/gkt1018. PubMed PMID: 24203704; PubMed Central PMCID: PMCPMC3919570.
16. Skrzypek MS, Binkley J, Binkley G, Miyasato SR, Simison M, Sherlock G. The Candida Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and visualization of high throughput sequencing data. Nucleic Acids Res. 2017;45(D1):D592-D6. doi: 10.1093/nar/gkw924. PubMed PMID: 27738138; PubMed Central PMCID: PMCPMC5210628.
17. Wilhelm SW, Bird JT, Bonifer KS, Calfee BC, Chen T, Coy SR, et al. A Student's Guide to Giant Viruses Infecting Small Eukaryotes: From Acanthamoeba to Zooxanthellae. Viruses. 2017;9(3). doi: 10.3390/v9030046. PubMed PMID: 28304329.
18. Liu H, Kohler J, Fink GR. Suppression of hyphal formation in Candida albicans by mutation of a STE12 homolog. Science. 1994;266(5191):1723-6. PubMed PMID: 7992058.
19. Ramirez-Zavala B, Weyler M, Gildor T, Schmauch C, Kornitzer D, Arkowitz R, et al. Activation of the Cph1-dependent MAP kinase signaling pathway induces white-opaque switching in Candida albicans. PLoS pathogens. 2013;9(10):e1003696. doi: 10.1371/journal.ppat.1003696. PubMed PMID: 24130492; PubMed Central PMCID: PMC3795047.
20. Kelly MT, MacCallum DM, Clancy SD, Odds FC, Brown AJ, Butler G. The Candida albicans CaACE2 gene affects morphogenesis, adherence and virulence. Molecular microbiology. 2004;53(3):969-83. doi: 10.1111/j.1365-2958.2004.04185. x. PubMed PMID: 15255906.
21. Wheeler RT, Fink GR. A Drug-Sensitive Genetic Network Masks Fungi from the Immune System. PLoS pathogens. 2006;2(4): e35. doi: 10.1371/journal.ppat.0020035.
22. Galan-Diez M, Arana DM, Serrano-Gomez D, Kremer L, Casasnovas JM, Ortega M, et al. Candida albicans -Glucan Exposure Is Controlled by the Fungal

CEK1-Mediated Mitogen-Activated Protein Kinase Pathway That Modulates Immune Responses Triggered through Dectin-1. Infection and immunity. 2010;78(4):1426-36. doi: 10.1128/iai.00989-09.
23. Cantero PD, Ernst JF. Damage to the glycoshield activates PMT-directed O-mannosylation via the Msb2-Cek1 pathway in Candida albicans. Molecular microbiology. 2011;80(3):715-25. doi: 10.1111/j.1365-2958.2011.07604.x. PubMed PMID: 21375589.
24. Roman E, Alonso-Monge R, Gong Q, Li D, Calderone R, Pla J. The Cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen Candida albicans. FEMS yeast research. 2009;9(6):942-55. doi: 10.1111/j.1567-1364.2009.00545.x. PubMed PMID: 19656200.
25. Li R, Puri S, Tati S, Cullen PJ, Edgerton M. Candida albicans Cek1 mitogen-activated protein kinase signaling enhances fungicidal activity of salivary histatin 5. Antimicrobial agents and chemotherapy. 2015;59(6):3460-8. doi: 10.1128/AAC.00214-15. PubMed PMID: 25824232; PubMed Central PMCID: PMC4432120.
26. Davis SE, Hopke A, Minkin SC, Jr., Montedonico AE, Wheeler RT, Reynolds TB. Masking of beta (1-3)-glucan in the cell wall of Candida albicans from detection by innate immune cells depends on phosphatidylserine. Infection and immunity. 2014. doi: 10.1128/IAI.01612-14. PubMed PMID: 25114110.
27. Hasim S, Allison DP, Retterer ST, Hopke A, Wheeler RT, Doktycz MJ, et al. beta-(1,3)-Glucan Unmasking in Some Candida albicans Mutants Correlates with Increases in Cell Wall Surface Roughness and Decreases in Cell Wall Elasticity. Infection and immunity. 2017;85(1). doi: 10.1128/IAI.00601-16. PubMed PMID: 27849179; PubMed Central PMCID: PMC5203643.
28. Wilson RB, Davis D, Mitchell AP. Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions. Journal of bacteriology. 1999;181(6):1868-74. PubMed PMID: 10074081; PubMed Central PMCID: PMCPMC93587.
29. Noble SM, French S, Kohn LA, Chen V, Johnson AD. Systematic screens of a Candida albicans homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat Genet. 2010;42(7):590-8. doi: 10.1038/ng.605. PubMed PMID: 20543849; PubMed Central PMCID: PMCPMC2893244.

Appendix

Table S.4. 1. *C. albicans* strains used in this study

Strains	Parent	Genotype	Source or reference
DAY286	Wild-type	*ura3::imm434/ura3::imm434 iro1/iro1::imm434 his1::hisG/his1::hisG arg4/arg4*	[28]
TC147	DAY286	DAY286+P_{tetOFF}-*STE11*$^{\Delta N467}$	Table S.3. 1
SN50	Wild-type	*ura3::imm434/ura3::imm434 iro1/iro1::imm434 his1::hisG/his1::hisG arg4/arg4*	[29]
cph1ΔΔ	SN50	SN50-*cph1ΔΔ*	[29]

Table S.4. 2. Genes that were differentially regulated in *STE11$^{\Delta N467}$* –doxycycline

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11$^{\Delta N467}$*+doxy
PBR1	Protein of unknown function; required for cohesion, adhesion, and RPMI biofilm formation; induced by alpha pheromone in white cells; fluconazole-induced; Spider biofilm induced	1,269.77	1,276.88	333.46
PGA13	GPI-anchored cell wall protein involved in cell wall synthesis; required for normal cell surface properties; induced in oralpharyngeal candidasis; Spider biofilm induced; Bcr1-repressed in RPMI a/a biofilms	114.57	93.25	22.48
C2_09880C_A	Putative protein of unknown function; Plc1-regulated; transcript induced by Mnl1 under weak acid stress; flow model, rat catheter, Spider biofilm induced	59.07	32.66	14.68
XOG1	Exo-1,3-beta-glucanase; 5 glycosyl hydrolase family member; affects sensitivity to chitin and glucan synthesis inhibitors; not required for yeast-to-hypha transition or for virulence in mice; Hap43-induced; Spider biofilm induced	51.34	55.89	37.56
C2_02220C_A	Protein of unknown function; F-12/CO2 early biofilm induced	47.72	46.24	18.21
RBT4	Pry family protein; required for virulence in mouse systemic/rabbit corneal infections; not filamentation; mRNA binds She3, is localized to hyphal tips; Hap43-induced; in both yeast and hyphal culture supernatants; Spider biofilm induced	40.37	31.85	43.55
C7_02280W_A	Ortholog of C. parapsilosis CDC317: CPAR2_808370, C. dubliniensis CD36: Cd36_72070, Candida orthopsilosis Co 90-125: CORT_0C00800 and Candida albicans WO-1 : CAWG_05577	39.92	43.07	19.01
PGA31	Cell wall protein; putative GPI anchor; expression regulated upon white-opaque switch; induced by Congo Red and cell wall regeneration; Bcr1-repressed in RPMI a/a biofilms	36.75	44.22	17.82
SAP7	Pepstatin A-insensitive secreted aspartyl protease; self-processing; expressed in human oral infection; Ssn6p-regulated; role in murine intravenous infection; induced during, but not required for, murine vaginal infection; N-glycosylated	36.55	16.76	14.56
KRE1	Cell wall glycoprotein; beta glucan synthesis; increases glucan content in S. cerevisiae kre1, complements killer toxin sensitivity; caspofungin induced; Spider/rat catheter/flow model biofilm induced; Bcr1-repressed in RPMI a/a biofilms	34.49	25.71	18.03

Table S.4. 2. Continued

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11*$^{\Delta N467}$+doxy
C7_02260W_A	Ortholog of C. parapsilosis CDC317: CPAR2_808350, C. dubliniensis CD36: Cd36_72060, Candida orthopsilosis Co 90-125: CORT_0C00820 and Candida albicans WO-1 : CAWG_05575	33.04	31.96	17.09
DAG7	Secretory protein; a-specific, alpha-factor induced; mutation confers hypersensitivity to toxic ergosterol analog; fluconazole-induced; induced during chlamydospore formation in C. albicans and C. dubliniensis	27.47	28.28	24.06
FAV1	Protein with weak similarity to S. cerevisiae Fus2p; induced by alpha pheromone mating factor in MTLa/MTLa opaque cells	23.68	36.82	8.76
RBR1	Glycosylphosphatidylinositol (GPI)-anchored cell wall protein; required for filamentous growth at acidic pH; expression repressed by Rim101 and activated by Nrg1; Hap43-induced	21.41	28.04	5.53
LDG3	Putative LDG family protein; F-12/CO2 early biofilm induced	20.45	23.8	11.02
C6_02100W_A	Secreted potein; Hap43-repressed; fluconazole-induced; regulated by Tsa1, Tsa1B under H2O2 stress conditions; induced by Mnl1p under weak acid stress; Spider biofilm induced	18.17	20.69	8.74
C1_07040C_A	Pry family pathogenesis-related protein; oral infection upregulated gene; mutant has reduced capacity to damage oral epithelial cells	16.15	13.23	13.55
MRV8	Protein of unknown function; Spider biofilm induced	15.68	15.16	11.93
PGA23	Putative GPI-anchored protein of unknown function; Rim101-repressed; Cyr1-regulated; colony morphology-related gene regulation by Ssn6	15.46	17.02	12.49
CR_05330W_A	Protein of unknown function; Spider biofilm repressed	13.1	13	7.97
C1_05970W_A	Putative adhesin-like protein; macrophage-induced gene	13.06	14.56	14.35
HWP1	Hyphal cell wall protein; host transglutaminase substrate; opaque-, a-specific, alpha-factor induced; at MTLa side of conjugation tube; virulence complicated by URA3 effects; Bcr1-repressed in RPMI a/a biofilms; Spider biofilm induced	13.05	6.29	4.22
IFF11	Secreted protein required for normal cell wall structure and for virulence; member of the IFF family; Hap43p-repressed gene	12.7	17.03	9.93
C4_04380C_A	Protein of unknown function; Spider biofilm induced	12.04	12.56	11.25

Table S.4. 2. Continued

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11*$^{\Delta N467}$+doxy
FAV2	Adhesin-like protein; induced by mating factor in MTLa/a opaque cells, in cyr1 null, in filaments; regulated by Nrg1, Rfg1, Tup1, Tec1, Efg1, Ntd80, Rob1, Brg1; induced in oralpharyngeal candidasis; Spider biofilm induced	10.33	7.52	10.97
C4_03500C_A	Protein of unknown function; regulated by Tsa1, Tsa1B in minimal media at 37 degrees C	9.13	8.58	36.84
KCH1	Ortholog of Kch1 a potassium transporter; mediates K+ influx and activates high-affinity Ca2+ influx system during mating pheromone response in S. cerevisiae; induced by alpha pheromone in SpiderM medium	8.95	12.59	9.23
ALK8	Alkane-inducible cytochrome P450; catalyzes hydroxylation of lauric acid to hydroxylauric acid; overproduction causes fluconazole resistance in WT and causes multidrug resistance in a cdr1 cdr2 double mutant; rat catheter biofilm repressed	8.84	7.53	4.37
CPH1	Transcription factor; for mating, filamentation on solid media, pheromone-stimulated biofilms; in pathway with Ess1, Czf1; required with Efg1 for host cytokine response; regulates galactose metabolism genes; rat cathether biofilm repressed	8.46	9.24	7.52
C5_03440W_A	Protein of unknown function	7.98	8.3	6.92
CPP1	VH1 family MAPK phosphatase; regulates Cst20-Hst7-Cek1-Cph1 filamentation pathway; negatively regulates mating, represses yeast-hyphal switch; required for virulence in mice; yeast-enriched; induced by alpha pheromone in SpiderM medium;	6.7	8.03	6.9
CSH1	Aldo-keto reductase; role in fibronectin adhesion, cell surface hydrophobicity; regulated by temperature, growth phase, benomyl, macrophage interaction; azole resistance associated; Spider biofilm induced; rat catheter biofilm repressed	6.61	8.4	5.01
C5_04940W_A	Maltase; induced during growth on sucrose; induced by alpha pheromone in SpiderM medium; early-stage flow model biofilm indced	6.57	6.29	5.56
STE11	Protein similar to S. cerevisiae Ste11p; mutants are sensitive to growth on H2O2 medium	6.56	6.02	8.53
C4_01330W_A	Protein of unknown function; Spider biofilm induced	6.45	4.56	2.52

Table S.4. 2. Continued

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11*$^{\Delta N467}$+doxy
PRM1	Putative membrane protein with a predicted role in membrane fusion during mating; Hap43p-repressed gene; protein induced during the mating process	6.16	5.83	7.85
CR_09930W_A	Protein of unknown function; induced by alpha pheromone in SpiderM medium	5.99	5.11	4.88
C6_04190C_A	Protein of unknown function; Spider biofilm induced	5.92	6.05	3.84
CR_04770C_A	Ortholog of C. dubliniensis CD36 : Cd36_30140, C. parapsilosis CDC317 : CPAR2_204040, Candida tenuis NRRL Y-1498 : CANTEDRAFT_114703 and Debaryomyces hansenii CBS767 : DEHA2G22880g	5.83	6.28	2.89
PTR2	Oligopeptide transporter involved in uptake of di-/tripeptides; highly induced during chlamydospore formation in both C. albicans and C. dubliniensis	5.73	5.02	2.27
GFA1	Glucosamine-6-phosphate synthase, homotetrameric enzyme of chitin/hexosamine biosynthesis; inhibited by UDP-GlcNAc, FMDP, N-acyl peptide, kanosamine-6-P; functional homolog of S. cerevisiae Gfa1p; Cagrowth-phase regulated; catalytic Cys	5.65	7.04	4.98
GST3	Glutathione S-transferase; expression regulated upon white-opaque switch; induced by human neutrophils; peroxide-induced; induced by alpha pheromone in SpiderM medium; Spider biofilm induced	5.52	5.73	2.45
C4_02260C_A	Protein of unknown function; repressed by prostaglandins; Hap43-induced, Spider biofilm induced	5.48	17.53	6.59
CRH11	GPI-anchored cell wall transglycosylase, putative ortholog of S. cerevisiae Crh1p; predicted glycosyl hydrolase domain; similar to Csf4p and to antigenic A. fumigatus Aspf9; predicted Kex2p substrate; caspofungin-induced	4.95	4.45	3.31
WSC2	Putative cell wall integrity and stress response protein; mRNA binds She3; Spider biofilm induced	4.74	5.31	2.99
PGA58	Putative GPI-anchored protein; transcription is positively regulated by Tbf1p	4.59	4.42	2.97
MNN12	Predicted alpha-1,3-mannosyltransferase activity with a role in protein glycosylation	4.25	4.88	3.61

Table S.4. 2. Continued

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11*$^{\Delta N467}$+doxy
C1_09430W_A	Putative membrane protein; induced by alpha pheromone in SpiderM medium; Hap4-induced gene; Spider biofilm induced	4.24	5.89	4.82
C1_03870C_A	Predicted heme-binding stress-related protein; Tn mutation affects filamentous growth; induced during chlamydospore formation in C. albicans and C. dubliniensis; Spider biofilm induced	4.23	4.46	4.99
C6_00810C_A	Protein of unknown function; Hap43-repressed gene	4.21	2.98	3.56
KAR4	Transcription factor; required for gene regulation in response to pheromones; ortholog of S. cerevisiae Kar4; role in karyogamy; opaque-specific, a-specific; induced by alpha factor	4.21	3.62	2.99
CR_00090C_A	Protein of unknown function; stationary phase enriched protein; induced upon yeast-hypha transition; benomyl or caspofungin induced; Hap43-repressed; Spider biofilm induced	4.17	3.76	2.01
OPY2	Predicted transmembrane protein; role in cell wall biogenesis; required for Cek1 phosphorylation; Spider biofilm induced	4.14	4.11	2.81
MNN14	Predicted alpha-1,3-mannosyltransferase activity with a role in protein glycosylation; Hap43-repressed; Spider biofilm induced	4	3.91	2.57
C2_05040C_A	Ortholog(s) have cellular bud neck, fungal-type vacuole localization	3.94	3.83	3.47
C6_04420W_A	Protein of unknown function; GlcNAc-induced protein; Spider biofilm induced; rat catheter biofilm repressed	3.94	2.64	2.84
CHS8	Chitin synthase required for synthesis of long-chitin fibrils; nonessential; 8 or 9 membrane spanning regions; mRNA present in yeast and hyphae; induced during cell wall regeneration; flow model biofilm repressed	3.65	3.74	2.66
C1_11730W_A	Protein with SEL-1 like protein domain; early-stage flow model biofilm induced	3.64	3.63	2.81
CHS2	Chitin synthase; nonessential; required for wild-type chitin deposition in hyphae; transcript regulated during dimorphic transition; Chs1 and Chs2, but not Chs3, are inhibited by the protoberberine HWY-289; flow model biofilm repressed	3.56	4.08	3.26
PGA54	GPI-anchored protein; Hog1-repressed; induced in cyr1 or efg1 mutant or in hyphae; colony morphology-related gene regulation by Ssn6; induced in RHE model; mRNA binds She3; regulated in Spider biofilms by Tec1, Egf1, Ntd80, Rob1, Brg1	3.47	3.69	3.42

Table S.4. 2. Continued

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11*$^{\Delta N467}$+doxy
GPX1	Putative thiol peroxidase; rat catheter and Spider biofilm induced	3.42	3.75	2.03
C7_02250W_A	Ortholog of C. dubliniensis CD36 : Cd36_72050, C. parapsilosis CDC317 : CPAR2_301140, Candida tenuis NRRL Y-1498 : CANTEDRAFT_135055 and Debaryomyces hansenii CBS767 : DEHA2E07678g	3.35	4.17	2.87
C1_07990C_A	Similar to cell-wall mannoproteins; induced in low iron; induced in cyr1 homozygous null; regulated by osmotic and oxidative stress via Hog1; Spider biofilm induced	3.25	3.7	3.17
C2_10150W_A	Secreted protein; fluconazole-induced	3.1	2.84	2.92
CR_07850W_A	Ortholog of C. dubliniensis CD36 : Cd36_33530, C. parapsilosis CDC317 : CPAR2_201980, Candida tenuis NRRL Y-1498 : CANTEDRAFT_134293 and Debaryomyces hansenii CBS767 : DEHA2A10164g	3.1	2.65	2.03
RLM1	Putative transcription factor; required for wild-type resistance to cell wall perturbation, caspofungin treatment; regulates caspofungin induction of PGA13	3.07	3.23	2.31
C1_04010C_A	Protein with a NADP-dependent oxidoreductase domain; transcript induced by ketoconazole; rat catheter and Spider biofilm induced	2.99	3.89	2.27
BUD5	Predicted GTP/GDP exchange factor for Rsr1; rat catheter biofilm induced	2.91	3.18	2.1
UAP1	UDP-N-acetylglucosamine pyrophosphorylase, catalyzes biosynthesis of UDP-N-acetylglucosamine from UTP and N-acetylglucosamine 1-phosphate; functional homolog of S. cerevisiae Qri1p; alkaline upregulated	2.9	4.31	3.82
BRG1	Transcription factor; recruits Hda1 to hypha-specific promoters; Tn mutation affects filamentation; Hap43-repressed; Spider and flow model biofilm induced; required for Spider biofilm formation; Bcr1-repressed in RPMI a/a biofilms	2.85	4.05	2.76
AXL2	Ortholog of S. cerevisiae Axl2; a plasma membrane protein involved in determination of budding pattern; O-glycosylated by Pmt4; mutant is viable	2.8	4.24	3.54

Table S.4. 2. Continued

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11*$^{\Delta N467}$+doxy
YKE2	Possible heterohexameric Gim/prefoldin protein complex subunit; role in folding alpha-tubulin, beta-tubulin, and actin; transcript induced by yeast-to-hypha switch; regulated by Nrg1, Tup1; Spider and flow model biofilm induced	2.78	3.28	2.18
C6_02210W_A	Protein of unknown function; oxidative stress-induced via Cap1; induced by alpha pheromone in SpiderM medium	2.67	2.4	2.21
CHS7	Protein required for wild-type chitin synthase III activity; similar to (but not functional homolog of) S. cerevisiae Chs7p, which effects ER export of Chs3p; induced cyr1 mutant hyphae and ras1 yeast-form cells; Spider biofilm induced	2.58	2.6	2.68
RGA2	Putative GTPase-activating protein (GAP) for Rho-type GTPase Cdc42; involved in cell signaling pathways controlling cell polarity; induced by low-level peroxide stress; flow model biofilm induced	2.54	2.55	2.4
MRV3	Ortholog of Candida albicans WO-1 : CAWG_04793	2.54	2.42	2.08
C2_07790C_A	Protein of unknown function; induced by alpha pheromone in SpiderM medium	2.53	3.05	2.97
C7_01940C_A	Pheromone-regulated protein (Prm10) of S. cerevisiae; colony morphology-related gene regulation by Ssn6; induced by Mnl1 under weak acid stress; possibly essential gene, disruptants not obtained by UAU1 method; Spider biofilm induced	2.53	2.37	2.26
PMS1	Putative DNA mismatch repair factor; ortholog of S. cerevisiae PMS1 which is an ATP-binding protein involved in DNA mismatch repair	2.5	3.33	2.94
C1_05440C_A	Protein of unknown function; flow model biofilm induced; Spider biofilm induced	2.45	2.14	2.01
DFI1	Cell-surface associated glycoprotein; promotes activation of Cek1 in a matrix-dependent manner; N-glycosylated; Spider biofilm induced	2.43	2.63	2.28
CR_09090C_A	Ortholog of C. dubliniensis CD36 : Cd36_34510, Candida tropicalis MYA-3404 : CTRG_05938 and Candida albicans WO-1 : CAWG_02183	2.36	2.92	2.01
C4_01420W_A	Ortholog of C. dubliniensis CD36 : Cd36_41430, Candida tropicalis MYA-3404 : CTRG_00187 and Candida albicans WO-1 : CAWG_03642	2.3	2.59	2.15

Table S.4. 2. Continued

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11*$^{\Delta N467}$+doxy
HGC1	Hypha-specific G1 cyclin-related protein involved in regulation of morphogenesis, biofilm formation; Cdc28-Hgc1 maintains Cdc11 S394 phosphorylation during hyphal growth; required for virulence in mice; regulated by Nrg1, Tup1, farnesol	2.29	3.29	2.56
C3_02290W_A	Protein similar to S. cerevisiae Ydr282cp; transposon mutation affects filamentous growth; Hap43p-repressed gene	2.25	2.18	2.61
KRE6	Essential beta-1,6-glucan synthase subunit; change in mRNA length, not abundance, at yeast-hypha transition; alkaline induced by Rim101, on cell wall regeneration; Spider biofilm induced; Bcr1-repressed in RPMI a/a biofilms	2.24	2.7	2.34
HAC1	bZIP transcription factor; role in unfolded protein response and control of morphology; transcript undergoes atypical splicing at C terminus under ER stress; induced during mating and by caspofungin; mRNA binds She3; Spider biofilm induced	2.12	2.32	2.59
PTP2	Predicted protein tyrosine phosphatase; involved in regulation of MAP kinase Hog1 activity; induced by Mnl1 under weak acid stress; rat catheter and Spider biofilm induced	2.11	2.22	2.45
ENG1	Endo-1,3-beta-glucanase; ortholog of S. cerevisiae Dse4 needed for cell separation; caspofungin, fluconazole repressed; repressed by alpha pheromone in SpiderM medium; flow model biofilm induced; rat catheter biofilm repressed	-2.09	-2.14	-2.79
C7_01170C_A	Putative oxidoreductase; mutation confers hypersensitivity to toxic ergosterol analog; rat catheter and Spider biofilm induced	-2.17	-4.89	-4.56
C1_11990W_A	Putative cell wall adhesin-like protein; repressed in core caspofungin response and by alpha pheromone in SpiderM medium; transcript reduced in ace2 mutant; flow model, rat catheter and Spider biofilm repressed	-2.22	-2.06	-3.17
C2_06800C_A	Protein of unknown function; Spider biofilm induced	-2.28	-2.94	-2.31
C4_02080W_A	Protein with a mitochondrial carrier protein domain; possibly an essential gene, disruptants not obtained by UAU1 method; Spider biofilm repressed	-2.35	-3.3	-3.21
C1_10710C_A	Protein similar to S. cerevisiae Yor378w; MFS family transporter; transposon mutation affects filamentous growth; null mutants are viable; fungal-specific (no human or murine homolog)	-2.52	-2.4	-2.35

Table S.4. 2. Continued

Gene Name	Function	Fold Change		
		vs. WT. –doxy	vs. WT +doxy	vs. *STE11*$^{\Delta N467}$+doxy
C1_05830W_A	Ortholog(s) have trans-aconitate 3-methyltransferase activity and cytosol localization	-2.58	-4.53	-3.84
TRP4	Predicted enzyme of amino acid biosynthesis; upregulated in biofilm; regulated by Gcn2p and Gcn4p; S. cerevisiae ortholog is Gcn4p regulated	-2.59	-2.91	-2.15
C4_01800W_A	Protein with a dienelactone hydrolase domain; Hap43-repressed gene	-2.59	-2.9	-3.18
FGR41	Putative GPI-anchored adhesin-like protein; transposon mutation affects filamentous growth; Spider biofilm repressed	-2.68	-2.85	-2.71
MEP1	Ammonium permease; Mep1 more efficient permease than Mep2, Mep2 has additional regulatory role; 11 predicted transmembrane regions; low mRNA abundance; hyphal downregulated; flow model biofilm induced	-2.87	-3.76	-2.46
HOM3	Putative L-aspartate 4-P-transferase; fungal-specific (no human or murine homolog); regulated by Gcn2 and Gcn4; early-stage flow model biofilm induced	-2.87	-3.4	-3.53
C6_03390W_A	Mitochondrial dicarboxylate transporter; possibly an essential gene, disruptants not obtained by UAU1 method	-2.89	-2.5	-2.78
MDR1	Plasma membrane MDR/MFS multidrug efflux pump; methotrexate is preferred substrate; overexpression in drug-resistant clinical isolates confers fluconazole resistance; repressed in young biofilms; rat catheter biofilm induced	-3.12	-3.09	-2.47
RNR22	Putative ribonucleoside diphosphate reductase;colony morphology-related gene regulation by Ssn6; transcript regulated by tyrosol and cell density; Hap43-repressed; Spider biofilm induced	-3.32	-2.98	-3.71

CHAPTER V: Conclusion and Future Direction

Conclusion

Altogether, the findings in this dissertation demonstrate the central role of the Cek1 MAPK in regulating cell wall β (1,3)-glucan masking in *C. albicans*. Davis *et al.* identified that loss of the phosphatidylserine (PS) synthase enzyme (Cho1) results in β (1,3)-glucan exposure in this organism. However, the mechanism by which loss of PS synthase leads to cell wall unmasking remained to be defined. To address this, in Chapter II I began using biochemical methods to identify the potential protein(s) causing this phenotype. Several proteins comprising molecular signaling pathways, which regulate fungal cell wall synthesis, are hyper-activated when the PS synthesis enzyme is disrupted. We hypothesized that one or a few of these signaling proteins may contribute to increased β (1,3)-glucan exposure in the *cho1Δ/Δ* mutant. I further discovered that when the protein kinase Cek1 is activated by itself, β (1,3)-glucan is exposed. The increased β (1,3)-glucan exposure exhibited from the strain containing hyper-activated Cek1 is shown to be immunologically relevant, reflected by stronger pro-inflammatory anti-fungal immune responses induced from murine macrophages. The GTPase Cdc42 is a central regulator of cell growth and morphological development. Cdc42 activity is found to be induced in the *cho1Δ/Δ* mutant, and genetic work demonstrated that hyperactivated Cdc42 results in the up-regulation of Cek1 activity. Based on these observations, we propose a model that Cho1 or its biochemical product PS impacts GTPase Cdc42 activity, which controls downstream Cek1 phosphorylation and thus induces β (1,3)-glucan exposure.

Chapter III demonstrated a novel signaling cascade controls Cek1 activity, which is composed of a protein called Lrg1, which represses β (1,3)-glucan exposure and Cek1 activity, and its cognate small GTPase is Cdc42. The discovery that Lrg1 inhibits Cdc42 activity in *C. albicans* is novel and unexpected because Lrg1 was thought to act on another GTPase Rho1, a major regulator for cell wall biosynthesis. Activated Cdc42 further relays the activation signal to the downstream Cek1 pathway. This work uncovers a new aspect of basic biology of signaling transduction leading to Cek1 activation and β (1,3)-glucan exposure in *C. albicans*. The mouse model of systemic infection, which mimics the human

disseminated fungal disease, revealed that the hyper-activation of Cek1 attenuates fungal virulence within the host and leads to better fungal clearance from the mouse kidney, which is the primary organ that *C. albicans* infects. Thus, we propose a model where loss of *LRG1* results in Cek1 over-activation, which further leads to more β (1,3)-glucan exposure, and the cell wall remodeling increases immune detection within the host and results in more rapid immune clearance.

Chapter IV described the genetic mechanisms responsible for the modulation of Cek1 activation on β (1,3)-glucan exposure in *C. albicans*. RNA-seq analysis was used to elucidate the whole genome transcriptional profiles regulated by activated Cek1 during β (1,3)-glucan unmasking. This strategy demonstrates that several cell wall associated genes are differentially manipulated transcriptionally when Cek1 is activated. Of interest, chitin production is significantly induced at the genetic level, *e.g. CHS2*, *CHS3*, *CHS8* and *PGA31* are upregulated. *PGA31* is a chitin production-relevant gene [1]. Given the relationship of chitin hyper-production and (1,3)-glucan exposure [2], this implies that chitin levels might impact cell wall unmasking in the Cek1-overactivating mutant. Cek1 downstream transcription factor Cph1 is also suggested to mediate cell wall dysfunction transcriptionally, as its transcript is induced significantly in the *CEK1* hyper-activating mutant. However, this is speculation and further research will be conducted to assess these hypotheses.

Future direction

To determine genetic target(s) downstream Cek1 causing β (1,3)-glucan exposure

The work in this dissertation has described an entirely new physiological role of Cek1 activation in regulating cell wall architecture and the resultant immunomodulation. Cek1 disruption has already been proved to impact *C. albicans* cell wall structure by exposing polysaccharides including β (1,3)-glucan, β (1,2)-mannosides to their cognate immune receptors, and thus boost immune responses that aid in clearance of the pathogen more rapidly [3, 4]. In this

dissertation, we provide evidence that hyper-activated Cek1 is also able to cause cell wall structure problems, as evidenced by massive β (1,3)-glucan exposure, which causes induction of immune detection, as confirmed by *in vitro* cytokine secretion (Figure 2. 3 and Figure 3. 5), and efficient pathogen clearance as suggested by the *in vivo* mouse model (Figure 3. 6 and Figure 3. 7). These observations are quite interesting, but also raise some important questions, *e.g.* what major regulating factors are involved in mediating cell wall architecture defects driven by unbalanced Cek1 activity? Research is still underway to pinpoint the gene(s) causing β (1,3)-glucan exposure induced by Cek1 activation, but our transcriptome analysis does reveal a few potential targets (Figure 4. 4). Of particular interests, Cph1, a Cek1 downstream transcription factor, is induced ~9 fold in the Cek1 activating mutant. Given the fact that the Cph1-overexpressing mutant exhibits pseudohyphal structure under yeast-condition (cultured in YPD liquid at 30°C), and filaments have more β (1,3)-glucan exposure naturally [5, 6], it seems likely that the increased cell wall unmasking induced by Cek1 overactivation under yeast condition results from the transcriptional regulation mediated by Cph1. To test this, a Cph1-overexpressing mutant would need to be created to evaluate its impact on β (1,3)-glucan exposure. Second, the hyphal glucan of *C. albicans* was reported to exhibit a unique biochemical structure, which has “cyclical chain” architecture and an unexpected 2,3-linkage [6]. While the structural significance of the cyclical chain is not fully understood, it was speculated that this might be partially responsible for higher levels of cell wall unmasking. We hypothesize that the β (1,3)-glucan structure under the yeast condition when Cek1 is hyperactivated is transitioned to the hyphal β (1,3)-glucan structure. This can be determined by using nuclear magnetic resonance (NMR) and gas chromatography–mass spectrometry (GC-MS), and this work can be achieved potentially by collaboration with Dr. Michael Kruppa at East Tennessee State University.

It was reported that *C. albicans* hyphae damaged by neutrophils exhibit increased β (1,3)-glucan, and this is mediated through delocalized *CHS3* [2]. There is also a correlation between chitin overproduction and β (1,3)-glucan unmasking [7, 8]. Based on this, it seems likely that several transcriptionally up-regulated chitin

synthases might impact cell wall structure when Cek1 is hyperactivated. To address this speculation, it is necessary to perform genetic analysis by overexpression of those targets individually independent of Cek1. The level of β (1,3)-glucan exposure would be assessed by performing immunofluorescent staining with anti- β (1,3)-glucan antibody. If individual mutation results in mild but not significant increased unmasking, it is possibly that these proteins need to be together to have a synergistic effect. To test this possibility, we can try to overexpress those targets together (Chs2, Chs3 and Chs8) in wild-type, and the immunofluorescent assay would be performed to evaluate the impact on cell wall exposure. Second, the endo-β (1,3)-glucanase (Eng1) was determined to enhance the virulence of fungal pathogen *H. capsulatum* by removing surface-exposed β (1,3)-glucan from immune detection [9]. The transcript of *C. albicans ENG1* homolog is down-regulated by 2-fold when Cek1 is hyperactivated (Figure 4. 4 and Table 4. 1). Although CaEng1 is primarily responsible for cell separation during budding [10], we cannot rule out the possibility that it might also impact β (1,3)-glucan structure as HcEng1 does. To assess this, it is necessary to quantify the level of β (1,3)-glucan exposure between the *ENG1* disrupted mutant vs. wildtype.

To identify signals involved in caspofungin-driven β (1,3)-glucan exposure

Caspofungin is a well-known fungicidal drug with the mechanism of inhibiting β (1,3)-glucan biosynthesis. It also exposes this polysaccharide to the surface and therefore facilitates immune detection even at a sub-lethal concentration. However, the direct mechanisms by which cell wall structure is disordered by caspofungin treatment are unclear. Our results imply that the Cek1 signaling cascade is one signaling pathway responding to casponfugin. This was suggested by hyperactivation of Cek1 when incubated with caspofungin at ½ MIC, and the induction gets more pronounced over time (Figure 4.5). This is promising as it reveals one potential target leading to β (1,3)-glucan exposure induced by caspofungin. Future work can be done to evaluate if two downstream transcription factors (Ace2 and Cph1) are involved in cell wall unmasking responsive to caspofungin.

Of interest, a genome-wide expression profile based on a microarray assay reveals that after *Candida* cells are exposed to ½ MIC of caspofugin for 3 hours, 480 gene were found to be regulated in response to the drug, and 3% of the genes are involved in cell wall maintenance [11]. Several targets overlap with our RNA-Seq data when Cek1 is hyperactivated, *e.g. CRH11, PHR1,* and *KRE1* whose transcripts are upregulated, while *SCW11* and *ENG1* are down-regulated in both studies. Moreover, Xu *et al.* studied the *in vivo* response to caspofungin, where the fungal gene expression profile was measured 2hr after drug administration in mice that had already been infected by *C. albicans* for 24hr. 12 genes were found to be induced that are enriched for cell wall or secreted proteins, particularly of *GCA2, PGA13, PGA31, PIR1* and *RBR2* were also found in our RNA-Seq data when Cek1 is hyperactivated. This highlights the significance of the Cek1 hyperactivation in restructuring the cell wall architecture in response to caspofungin. The abovementioned targets would need to be manipulated genetically in a wild-type background based on their expression profile. For instance, the individual deletion mutant would be made for those displaying upregulated transcripts in both caspofungin treatment and Cek1 overactivation situations. Further research would assess if the mutant rescues cell wall β (1,3)-glucan exposure after being exposed to caspofungin.

Several other signaling proteins are known to be responsive to caspofungin treatment, *e.g.* Mkc1, and Hog1 MAPKs. Therefore, it would be necessary to assay the rescue effect of the *HOG1* or *MKC1* disrupted mutant on β (1,3)-glucan exposure when induced by caspofungin. However, if none of those targets are involved in the caspofungin-driven β (1,3)-exposure, we would set off an extensive screen on the deletion mutant library on hand with ~700 nonessential gene mutants. The goal is to find the genetic network that is responsible for cell wall re-structure stimulated by caspofungin.

To disentangle signaling rewiring that involves GTPases and associated molecules

The findings of this dissertation have provided a greater general understanding of signaling transduction/rewiring in pathogenic *C. albicans.* There are several unanticipated findings in Chapters II and III. For instance, Cdc42 activity was shown to be stimulated when PS synthase enzyme Cho1 was deleted (Figure 2. 4). We originally hypothesized that Cdc42 activity might be down-regulated due to the delocalization of GFP-Cdc42 in the *cho1Δ/Δ* mutant (Figure 2. 5). This phenotype was also seen in the model yeast *S. cerevisiae* where loss of Cho1 delocalizes Cdc42 from polarized growth site to an internal membrane [12]. However, an *in vitro* study in *S. cerevisiae* showed that PS stimulates the GAP activity for ScCdc42, suggesting that loss of PS has less stimulation on Cdc42 GAP activity and thus results in the increased Cdc42 activity [13]. Likewise, the mechanisms through which Cdc42 activity is stimulated by *CHO1* deletion in *C. albicans* could be investigated in a similar way *in vitro*. To do this, radio-labeled [γ-^{32}P] GTP-bound CaCdc42 needs to be incubated with PS, along with its GAPs, *e.g.* Rga1, or Rga2. The GTP hydrolysis would then be assayed by measuring the radioactivity of [γ-^{32}P] GTP-bound Cdc42 after incubation. Some other phospholipids such as phosphatidylethanolamine and phosphatidylinositol can be used as controls to assess if the stimulation effect on Cdc42 GAPs activity is PS-dependent. Moreover, other GTPase GAPs, *e.g.* Sac7 as GAP for Rho1, can also be assayed to evaluate if PS is a universal inhibitor on GAPs.

In Chapter III, Lrg1 inhibits GTPases Cdc42 and Ras1 activity *in vivo* (Figure 3. 3), even though Lrg1 was originally suggested to act as a Rho1 activity repressor in this organism [14]. Research would demonstrate if Lrg1 functions as GAP for Cdc42, Ras1 and/or Rho1 by performing *in vitro* GTPases assay. In this experiment, Lrg1 protein will be incubated with [γ-^{32}P] GTP-bound GTPases, and the radioactivity of [γ-^{32}P] GTP-bound GTPases after incubation will be measured. To analyze if the CaLrg1 GAP homolog domain (representing amino acids 1183-1375) exhibits true GAP activity, genetic manipulation will delete the rhoGAP domain and the inhibition on GTPase activity of Cdc42 or Ras1 by the

lrg1ΔrhoGAP mutant will be analyzed via biochemical assay *in vitro* as abovementioned. Furthermore, we found that hyper-activated Ras1 does not induce Cek1 phosphorylation (Figure 3. 3), although the epistasis evidence suggests Ras1 acts upstream of Cek1 [15].

To explore the roles of Lrg1 in regulating *C. albicans* cell wall architecture

Besides acting as a rho-GAP, Lrg1 has been shown to impact cell polarity, cell fusion, Mkc1 MAPK activity and β (1,3)-glucan biosynthesis *etc.* in *S. cerevisiae* [16, 17]. In *N. crassa, LRG1* have also been studied and suggested to regulate polar tip growth [18]. In contrast, little is known about the function of Lrg1 in the pathogenic *C. albicans*. It was described that Lrg1 negatively controls filamentation in this organism [14]. In this dissertation, we further elaborated that CaLrg1 controls β (1,3)-glucan exposure by negatively regulating Cek1 MAPK cascade, and the *lrg1Δ/Δ* mutant displays higher immunogenicity suggested by stronger pro-inflammatory cytokines production from murine macrophages (Figure 3. 1, Figure 3. 2, and Figure 3. 5). It seems likely that Lrg1 influences cell wall structure; however, questions remain regarding the mechanisms by which Lrg1 does so at the biochemical level. For instance, while loss of *LRG1* induces β (1,3)-glucan exposure, we are not clear if it is due to a greater level β (1,3)-glucan being produced. Also, it is not clear if loss of *LRG1* impacts chitin synthesis or localization in *C. albicans*, although it does so in *N. crassa.* Research can be done by staining *Candida* cells with aniline blue for β (1,3)-glucan and calcofluor white for chitin, respectively, to analyze their contents and localization. Moreover, it would be interesting to analyze the localization of Lrg1 *in vivo* by tagging it with GFP, and probe the deliver machinery of cell growth components, *e.g.* actin cytoskeleton simultaneously, which would help understand the correlation between Lrg1 localization and new cell wall component deposition. Last, it was predicted by SMART.EMBL.DE that *Ca*Lrg1 contains two Zinc-binding LIM domains which generically help bind protein partners in eukaryotes; however, it not known how these two domains act specifically in *C. albicans*. It was suggested that *Nc*Lrg1 contains three LIM domains which are required for localizing *LRG1* to the growth

sites. It is possibly that these two domains act similarly in *C. albicans*, but research would need to be conducted by deleting these two LIM domains in *CaLRG1*, and localization of the mutated *LRG1* can be assessed by tagging with GFP.

To elaborate the mechanisms by which β (1,3)-glucan exposure impacts fungal virulence *in vivo*

As described in Figure 3. 6 we determined that increased β (1,3)-glucan exposure seems to compromise fungal virulence, as evidenced by significantly longer mouse survival rates and faster pathogen clearance in mouse kidneys. This suggests that the phenotype of β (1,3)-glucan exposure is immunologically relevant. To demonstrate if the enhanced killing ability *in vivo* is due to higher levels of unmasked β (1,3)-glucan exhibited from the Cek1-hyperactivating mutant, *ex vivo* staining by using anti-β (1,3)-glucan antibody on homogenized tissues, *e.g.* kidney, liver would need to be done. Moreover, immune cell recruitment and cytokine profiles by flow cytometry are also necessary to demonstrate if immune responses are boosted in response to the exposed β (1,3)-glucan. This work can be achieved potentially through collaboration with Dr. Sparer, whose lab has established the techniques at the Microbiology Department at UT. Finally, it is valuable to evaluate if the enhanced immune responses are dependent on Dectin-1 and this can be studied by using dectin-1 deficient mice.

Reference

1. Plaine A, Walker L, Da Costa G, Mora-Montes HM, McKinnon A, Gow NA, et al. Functional analysis of Candida albicans GPI-anchored proteins: roles in cell wall integrity and caspofungin sensitivity. Fungal genetics and biology : FG & B. 2008;45(10):1404-14. doi: 10.1016/j.fgb.2008.08.003. PubMed PMID: 18765290; PubMed Central PMCID: PMCPMC2649418.
2. Hopke A, Nicke N, Hidu EE, Degani G, Popolo L, Wheeler RT. Neutrophil Attack Triggers Extracellular Trap-Dependent Candida Cell Wall Remodeling and Altered Immune Recognition. PLoS pathogens. 2016;12(5):e1005644. doi: 10.1371/journal.ppat.1005644. PubMed PMID: 27223610; PubMed Central PMCID: PMC4880299.
3. Galan-Diez M, Arana DM, Serrano-Gomez D, Kremer L, Casasnovas JM, Ortega M, et al. Candida albicans beta-glucan exposure is controlled by the fungal CEK1-mediated mitogen-activated protein kinase pathway that modulates immune responses triggered through dectin-1. Infection and immunity. 2010;78(4):1426-36. doi: 10.1128/IAI.00989-09. PubMed PMID: 20100861; PubMed Central PMCID: PMCPMC2849429.
4. Roman E, Correia I, Salazin A, Fradin C, Jouault T, Poulain D, et al. The Cek1mediated MAP kinase pathway regulates exposure of alpha1,2 and beta1,2mannosides in the cell wall of Candida albicans modulating immune recognition. Virulence. 2016;7(5):558-77. doi: 10.1080/21505594.2016.1163458. PubMed PMID: 27191378; PubMed Central PMCID: PMCPMC5038168.
5. Huang H, Harcus D, Whiteway M. Transcript profiling of a MAP kinase pathway in C. albicans. Microbiol Res. 2008;163(4):380-93. doi: 10.1016/j.micres.2008.03.001. PubMed PMID: 18467082.
6. Lowman DW, Greene RR, Bearden DW, Kruppa MD, Pottier M, Monteiro MA, et al. Novel structural features in Candida albicans hyphal glucan provide a basis for differential innate immune recognition of hyphae versus yeast. The Journal of biological chemistry. 2014;289(6):3432-43. doi: 10.1074/jbc.M113.529131. PubMed PMID: 24344127; PubMed Central PMCID: PMCPMC3916545.
7. Hasim S, Allison DP, Retterer ST, Hopke A, Wheeler RT, Doktycz MJ, et al. beta-(1,3)-Glucan Unmasking in Some Candida albicans Mutants Correlates with Increases in Cell Wall Surface Roughness and Decreases in Cell Wall Elasticity. Infection and immunity. 2017;85(1). doi: 10.1128/IAI.00601-16. PubMed PMID: 27849179; PubMed Central PMCID: PMC5203643.
8. Davis SE, Hopke A, Minkin SC, Jr., Montedonico AE, Wheeler RT, Reynolds TB. Masking of beta (1-3)-glucan in the cell wall of Candida albicans from detection by innate immune cells depends on phosphatidylserine. Infection and immunity. 2014. doi: 10.1128/IAI.01612-14. PubMed PMID: 25114110.
9. Garfoot AL, Shen Q, Wuthrich M, Klein BS, Rappleye CA. The Eng1 beta-Glucanase Enhances Histoplasma Virulence by Reducing beta -Glucan Exposure. MBio. 2016;7(2): e01388-15. doi: 10.1128/mBio.01388-15. PubMed PMID: 27094334; PubMed Central PMCID: PMCPMC4850272.
10. Esteban PF, Rios I, Garcia R, Duenas E, Pla J, Sanchez M, et al. Characterization of the CaENG1 gene encoding an endo-1,3-beta-glucanase

involved in cell separation in Candida albicans. Curr Microbiol. 2005;51(6):385-92. doi: 10.1007/s00284-005-0066-2. PubMed PMID: 16328626.
11. Liu TT, Lee RE, Barker KS, Lee RE, Wei L, Homayouni R, et al. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in Candida albicans. Antimicrobial agents and chemotherapy. 2005;49(6):2226-36. doi: 10.1128/AAC.49.6.2226-2236.2005. PubMed PMID: 15917516; PubMed Central PMCID: PMCPMC1140538.
12. Fairn GD, Hermansson M, Somerharju P, Grinstein S. Phosphatidylserine is polarized and required for proper Cdc42 localization and for development of cell polarity. Nature cell biology. 2011;13(12):1424-30. doi: 10.1038/ncb2351.
13. Saito K, Fujimura-Kamada K, Hanamatsu H, Kato U, Umeda M, Kozminski KG, et al. Transbilayer phospholipid flipping regulates Cdc42p signaling during polarized cell growth via Rga GTPase-activating proteins. Developmental cell. 2007;13(5):743-51. doi: 10.1016/j.devcel.2007.09.014. PubMed PMID: 17981141.
14. Xie JL, Grahl N, Sless T, Leach MD, Kim SH, Hogan DA, et al. Signaling through Lrg1, Rho1 and Pkc1 Governs Candida albicans Morphogenesis in Response to Diverse Cues. PLoS genetics. 2016;12(10):e1006405. doi: 10.1371/journal.pgen.1006405. PubMed PMID: 27788136; PubMed Central PMCID: PMC5082861.
15. Leberer E, Harcus D, Dignard D, Johnson L, Ushinsky S, Thomas DY, et al. Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus Candida albicans. Molecular microbiology. 2001;42(3):673-87. PubMed PMID: 11722734.
16. Fitch PG, Gammie AE, Lee DJ, de Candal VB, Rose MD. Lrg1p Is a Rho1 GTPase-activating protein required for efficient cell fusion in yeast. Genetics. 2004;168(2):733-46. doi: 10.1534/genetics.104.028027. PubMed PMID: 15514049; PubMed Central PMCID: PMC1448843.
17. Watanabe D, Abe M, Ohya Y. Yeast Lrg1p acts as a specialized RhoGAP regulating 1,3-beta-glucan synthesis. Yeast. 2001;18(10):943-51. doi: Doi 10.1002/Yea.742. PubMed PMID: WOS:000169965500007.
18. Vogt N, Seiler S. The RHO1-specific GTPase-activating protein LRG1 regulates polar tip growth in parallel to Ndr kinase signaling in Neurospora. Molecular biology of the cell. 2008;19(11):4554-69. doi: 10.1091/mbc. E07-12-1266. PubMed PMID: 18716060; PubMed Central PMCID: PMC2575149.

VITA

Tian Chen was born and raised at Suining City, Sichuan Province in China by her parents Xuefeng Chen and Leying Tang. She earned her B.S. and M.S. in a nearby city called Chongqing, where her grandparents, aunt, and uncles live. She thought she would settle down there to be close to her family. But apparently, she was wrong. She decided to pursue her Ph.D. in the United States after being motivated from a collaborative summer research program in Minnesota. She was thrilled to join the Department of Microbiology at the University of Tennessee in August of 2013, where she studied under the supervision of Dr. Todd Reynolds until she graduated in 2019. She is content that she will be working at Shandong University in China as a microbiologist with her Ph.D. degree, a place where her husband Xu works as a chemist. Her little boy Ryan also attends the university-affiliated daycare there. Tian is excited that her lifelong dream is about to come true: having a Ph.D. degree and being together with her family! Woohoo!