Stress Response Pathways Regulate Drug Resistance and Morphogenesis in the Human Fungal Pathogen *Candida albicans*

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Molecular Genetics
University of Toronto

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Department of Molecular Genetics

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Abstract

The dimorphic yeast *Candida albicans* is a leading causative agent of fungal infections in humans. Critical to *C. albicans* pathogenesis is its ability to transition between yeast and filamentous forms. Currently, a major clinical problem is the frequent emergence of resistance to existing antifungal drugs. Development of new antifungal agents remains a difficult process, partly due to the conservation of many potential therapeutic targets between *C. albicans* and humans. Moreover, stress responses in *C. albicans* enhance antifungal tolerance and enable drug resistance. Therefore, tactical targeting of specific stress response pathways that regulate drug resistance and morphogenesis in combination with known antifungal agents may provide a viable strategy to enhance the efficacy of antifungals and suppress the emergence of antifungal drug resistance. My doctoral research focuses on two cellular stress response pathways that are essential for drug resistance and the morphological transition in *C. albicans*. First, I uncover the regulatory circuitry through which the transcription factor Cas5 mediates cell wall stress responses, and establish Cas5 as a novel regulator of resistance to the echinocandin caspofungin. This represents the first example of transcriptional regulation as a mechanism of echinocandin resistance. Second, I reveal a novel and essential role for the protein kinase Pkc1 in regulating morphogenesis of *C. albicans*. I demonstrate that Pkc1 functions downstream of Rho1 in a
signaling pathway that operates in parallel with the Ras1-PKA pathway. This is only the second pathway identified in C. albicans that has the capacity to integrate multiple filament-inducing cues and transduce the signals necessary for the transition from yeast to filamentous growth. Together, this research highlights the central role of cellular stress circuitry in drug resistance and morphogenesis, and uncovers attractive targets for the development of novel antifungal drugs, suggesting new avenues for combination therapies with current antifungal agents.
Acknowledgments

The work of this thesis would not have been possible without a number of individuals.

I would like to first thank my supervisor Dr. Leah Cowen for her wisdom, guidance and continual support throughout my graduate career. Over the past six years, you have been the mentor and role model I needed as a young scientist. Your trust and words of encouragement have given me the confidence I was lacking, and your enthusiasm towards science has been a constant source of motivation. You have been incredibly supportive both intellectually and emotionally, and I am forever grateful for your mentorship.

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I would like to thank my family for their unconditional love and unwavering support. Mom and dad, thank you for all the sacrifices you have made in order to build a life for our family here in Canada. You made it possible for me to pursue a graduate degree and discover my potential. Thank you to my brother Jinhao for the endless entertainments.

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Table of Contents

Acknowledgments........................................................................................................ iv
Table of Contents.......................................................................................................... vi
List of Tables .................................................................................................................. x
List of Figures ............................................................................................................... xi
List of Abbreviations ..................................................................................................... xiv
Chapter 1 Introduction ................................................................................................. 1
  1 Introduction ............................................................................................................... 2
    1.1 The human fungal pathogen Candida albicans .................................................... 2
    1.2 Antifungal drugs .................................................................................................. 4
      1.2.1 Major classes of antifungal drugs ................................................................. 5
      1.2.2 Mechanism of action of echinocandins ........................................................ 6
      1.2.3 Mechanisms of echinocandin resistance ...................................................... 8
      1.2.4 Approaches to elucidating mechanisms of drug resistance ......................... 14
    1.3 Morphogenesis in C. albicans ............................................................................ 17
      1.3.1 Morphogenesis and virulence ................................................................. 17
      1.3.2 Morphotypes in C. albicans ........................................................................ 18
      1.3.3 Regulation of morphogenesis ....................................................................... 22
    1.4 Thesis rationale ................................................................................................... 26
Chapter 2 Dephosphorylation of the Transcription Factor Cas5 Modulates Cell CycleProgression and Antifungal Drug Resistance ........................................................................ 28
  2 Dephosphorylation of the Transcription Factor Cas5 Modulates Cell Cycle Progressionand Antifungal Drug Resistance .............................................................................. 28
    2.1 Introduction ........................................................................................................ 28
    2.2 Materials and Methods ..................................................................................... 30
      2.2.1 Strains and culture conditions ....................................................................... 30
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.2</td>
<td>Strain construction</td>
<td>33</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Plasmids</td>
<td>40</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Plasmid construction</td>
<td>40</td>
</tr>
<tr>
<td>2.2.5</td>
<td>Oligonucleotides</td>
<td>43</td>
</tr>
<tr>
<td>2.2.6</td>
<td>Experimental growth conditions</td>
<td>47</td>
</tr>
<tr>
<td>2.2.7</td>
<td>Minimum Inhibitory Concentration (MIC) Assay</td>
<td>48</td>
</tr>
<tr>
<td>2.2.8</td>
<td>Indirect Immunofluorescence</td>
<td>48</td>
</tr>
<tr>
<td>2.2.9</td>
<td>Western blot analysis</td>
<td>49</td>
</tr>
<tr>
<td>2.2.10</td>
<td>2-Dimensional gel electrophoresis</td>
<td>49</td>
</tr>
<tr>
<td>2.2.11</td>
<td>Phosphoshift assay</td>
<td>50</td>
</tr>
<tr>
<td>2.2.12</td>
<td>qRT-PCR</td>
<td>51</td>
</tr>
<tr>
<td>2.2.13</td>
<td>Affinity purification (AP) and LC-MS analysis</td>
<td>51</td>
</tr>
<tr>
<td>2.2.14</td>
<td>Co-Immunoprecipitations</td>
<td>52</td>
</tr>
<tr>
<td>2.2.15</td>
<td>Staining</td>
<td>53</td>
</tr>
<tr>
<td>2.2.16</td>
<td>General Imaging Techniques</td>
<td>53</td>
</tr>
<tr>
<td>2.2.17</td>
<td>Selection Experiment</td>
<td>53</td>
</tr>
<tr>
<td>2.3</td>
<td>Results</td>
<td>53</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Cas5 is activated by dephosphorylation in response to cell wall stress</td>
<td>53</td>
</tr>
<tr>
<td>2.3.2</td>
<td>A conserved serine residue in the Cas5 DNA binding domain is critical for caspofungin tolerance</td>
<td>58</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Glc7 dephosphorylates Cas5 in response to cell wall stress</td>
<td>62</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Cas5 physically interacts with Swi4/Swi6 to regulate caspofungin-responsive genes</td>
<td>65</td>
</tr>
<tr>
<td>2.3.5</td>
<td>Cas5 is required for proper morphogenesis and cell cycle progression</td>
<td>67</td>
</tr>
<tr>
<td>2.3.6</td>
<td>Cas5 regulates bona fide caspofungin resistance independently of cell cycle progression</td>
<td>71</td>
</tr>
<tr>
<td>2.4</td>
<td>Discussion</td>
<td>74</td>
</tr>
</tbody>
</table>
Chapter 3 Signaling through Lrg1, Rho1 and Pkc1 Governs Candida albicans Morphogenesis in Response to Diverse Cues ........................................................................78

3 Signaling through Lrg1, Rho1 and Pkc1 Governs Candida albicans Morphogenesis in Response to Diverse Cues ........................................................................79

3.1 Introduction ........................................................................................................79

3.2 Materials and methods ......................................................................................81

3.2.1 Strains and culture conditions .......................................................................81

3.2.2 Strain construction .........................................................................................83

3.2.3 Plasmids .........................................................................................................86

3.2.4 Plasmid construction .......................................................................................87

3.2.5 Oligonucleotides ...........................................................................................88

3.2.6 Experimental growth conditions ...................................................................91

3.2.7 Arrayed Morphology Screen .........................................................................92

3.2.8 qRT-PCR .........................................................................................................92

3.2.9 Minimum Inhibitory Concentration Assay .....................................................93

3.2.10 Active Ras1 pull-down ..................................................................................93

3.2.11 Selection Experiment ....................................................................................93

3.2.12 Whole Genome Sequencing .........................................................................94

3.2.13 Spotting Assay ............................................................................................95

3.3 Results ................................................................................................................95

3.3.1 Lrg1 is a repressor of filamentation ...............................................................95

3.3.2 Activation of Rho1 promotes filamentation ....................................................97

3.3.3 Pkc1 is a master regulator of morphogenesis .................................................100

3.3.4 Pkc1 kinase activity is important for the initiation and maintenance of filamentation .........................................................................................................................103

3.3.5 Pkc1 regulates filamentation independent of the MAP kinases in the cell wall integrity pathway ..............................................................105

3.3.6 Pkc1 signaling operates in parallel to Ras1 signaling to enable filamentation 107
3.3.7  Pkc1 may also regulate filamentation via actin. ...........................................110

3.4  Discussion ................................................................................................................115

Chapter 4 Conclusion, General Discussion, and Future Directions ................................120

4  Conclusion, General Discussion, and Future Directions ........................................120

4.1  Conclusion .................................................................................................................120

4.2  General Discussion ....................................................................................................120

4.2.1  Drug resistance – the ongoing challenge in the treatment of infectious diseases 120

4.2.2  Morphogenesis – the capacity to adapt to host environment by shapeshifting...126

4.3  Future Directions .....................................................................................................129

References .......................................................................................................................133
List of Tables

Table 2.1 Strains used in this study. ................................................................. 30

Table 2.2 Plasmids used in this study .............................................................. 40

Table 2.3 Oligonucleotides used in this study ............................................... 43

Table 3.1 Strains used in this study. ................................................................. 81

Table 3.2 Plasmids used in this study .............................................................. 86

Table 3.3 Oligonucleotides used in this study ............................................... 88
List of Figures

Figure 1.1 Mechanism of action of the major classes of antifungal drugs. ........................................ 7

Figure 1.2 Mechanisms of echinocandin resistance. ........................................................................... 9

Figure 1.3 Overview of approaches to elucidating antifungal drug resistance. ................................. 14

Figure 2.1 Cas5 initiates cell wall stress response by upregulating the expression of cell wall genes upon change in its post-translational modification. ......................................................... 56

Figure 2.2 HA-tagged allele of CAS5 is functional. .............................................................................. 57

Figure 2.3 Cas5 shows a change in post-translational modification upon caspofungin or calcofluor white treatment. ........................................................................................................ 57

Figure 2.4 ATP analogue 1-NA-PP1 specifically inhibits Pkc1 kinase activity................................. 58

Figure 2.5 The phosphorylation sites identified by mass spectrometry are not sufficient for Cas5 function. ........................................................................................................................................ 59

Figure 2.6 The S769E mutation in the Cas5 DNA binding domain phenocopies CAS5 deletion. 62

Figure 2.7 GLC7 is depleted upon doxycycline treatment. ................................................................. 63

Figure 2.8 Cas5 activation in response to caspofungin is associated with dephosphorylation by Glc7........................................................................................................................................ 64

Figure 2.9 Cas5 regulates caspofungin tolerance in part through interaction with the components of the SBF complex Swi4 and Swi6. ....................................................................................... 66

Figure 2.10 The TAP tagged Swi4 and Swi6 are functional. ............................................................... 67

Figure 2.11 Cas5 plays a similar role as Swi4 and Swi6 in hyphal development and G1/S transition. ........................................................................................................................................ 68

Figure 2.12 Cas5 is a key regulator of cell cycle progression .............................................................. 71
Figure 2.13 Cas5 regulates bona fide caspofungin resistance independent its role in cell cycle. 72
Figure 2.14 Cas5 regulates caspofungin resistance independent of FKS1. 73
Figure 2.15 Mutation that confers caspofungin-resistance independent of Cas5 does not restore proper cell cycle progression. 74
Figure 2.16 Schematic diagram of Cas5 regulation. 75
Figure 3.1 Lrg1 is a negative regulator of filamentation. 96
Figure 3.2 Lrg1 is not required of cell wall stress response. 97
Figure 3.3 RHO1 is depleted upon doxycycline treatment. 98
Figure 3.4 Activated Rho1 promotes filamentation. 99
Figure 3.5 Pck1 is required for filamentous growth induced by deletion of LRG1. 100
Figure 3.6 Pck1 is required for filamentation in response to diverse cues. 102
Figure 3.7 The Pck1M850G gatekeeper mutant is sensitive to high temperature or to inhibition by ATP analogue 1-NA-PP1. 104
Figure 3.8 Pck1 kinase activity is critical for filamentation. 105
Figure 3.9 The MAP kinase cascade downstream of Pck1 is not required for filamentous growth. 106
Figure 3.10 Pck1 does not function upstream of the Ras-PKA pathway. 109
Figure 3.11 Pck1 is not required for Ras1 activation. 109
Figure 3.12 The cAMP signaling is required for Pck1-dependent filamentous growth. 110
Figure 3.13 The HWP1p-NAT reporter construct enables cells expressing the filament-specific transcript HWP1 to grow on NAT. 111
Figure 3.14 The NAT-resistant isolates have restored capacity to filament. 113
Figure 3.15 Destabilization of actin blocks filamentation in the \textit{lrG1Δ/lrg1Δ} mutant............. 114

Figure 3.16 Model depicting Pkc1-dependent morphogenetic regulation in \textit{C. albicans}. ........ 116
List of Abbreviations

Hsp90  Heat shock protein 90
MAPK  Mitogen-activated protein kinase
17-AAG  17-N-allylamino-17-demethoxygeldanamycin
17-DMAG  17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin
DNA  Deoxyribonucleic acid
LOPAC  Library of Pharmacologically Active Compounds
GUT  Gastrointestinally induced transition'
HIV  Human immunodeficiency virus
AIDS  Acquired immunodeficiency syndrome
DTPA  Diethylenetriaminepentaacetic acid
cAMP  Cyclic adenosine monophosphate
PKA  Protein kinase A
GTP  Guanosine-5'-triphosphate
GAP  GTPase activating protein
GEF  Guanine nucleotide exchange factor
APSES  Asm1p, Phd1p, Sok2p, Efg1p and StuAp
bHLH  Basic helix-loop-helix
bZip  Basic leucine zipper domain
MADS  MCM1, AGAMOUS, DEFICIENS, and SRF
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBF</td>
<td>SCB binding factor</td>
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<td>SCB</td>
<td>Swi4/6 cell cycle box</td>
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<td>NAT</td>
<td>Nourseothricin</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>FLP</td>
<td>Flippase</td>
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<tr>
<td>FRT</td>
<td>Flp recombination target</td>
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<tr>
<td>HIS</td>
<td>Histidine</td>
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<tr>
<td>ARG</td>
<td>Arginine</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>YPD</td>
<td>Yeast peptone dextrose</td>
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<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<td>TAP</td>
<td>Tandem affinity purification</td>
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<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>AMP</td>
<td>Ampicillin</td>
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<tr>
<td>TAr</td>
<td>Trans-activation response</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>SDM</td>
<td>Site directed mutagenesis</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>CF</td>
<td>Caspofungin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline/tween</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>PMP</td>
<td>Protein metallo phosphatases</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>AP</td>
<td>Affinity purification</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography mass spectrometry/mass spectrometry</td>
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<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>LFQ</td>
<td>Label-free quantification</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy-number variation</td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithm of odds score</td>
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<tr>
<td>1-NA-PP1</td>
<td>1-Naphthyl-PP1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>MBF</td>
<td>MCB binding factor</td>
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<tr>
<td>MSB</td>
<td>MluI cell cycle box</td>
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<tr>
<td>RSC</td>
<td>Remodel the structure of chromatin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>NBCS</td>
<td>Newborn calf serum</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>GdA</td>
<td>Geldanamycin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>Phosphatidylinositol-(4,5)-bisphosphate</td>
</tr>
<tr>
<td>PI(3,4,5)P</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

Section 1.1 and 1.2 of this chapter are adapted from:


1Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada
1 Introduction

1.1 The human fungal pathogen *Candida albicans*

The devastating effects of fungal infections on human health worldwide remain largely unappreciated. Pathogenic fungi infect billions of people every year, with over 1.5 million of these infections resulting in death (G. D. Brown et al., 2012). In fact, fungi kill as many people annually as do malaria or tuberculosis (G. D. Brown et al., 2012). Fungi are generally opportunistic pathogens, preying on individuals with compromised immune systems including those with HIV/AIDS, those receiving immunosuppressive drugs for organ transplantation, and those undergoing cancer treatment. As the number of severely immunocompromised individuals increases, so does the incidence of invasive fungal infections. In recent years, the occurrence of systemic fungal infections has increased by 207% (Pfaller & Diekema, 2010).

Among the most deadly fungal pathogens are *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, with *C. albicans* reigning as the most prevalent invasive fungal pathogen of humans (Pfaller & Diekema, 2010). *C. albicans* is a natural member of the human microbiota; however, it is capable of causing severe systemic infections in immunocompromised individuals, with mortality rates that approach 40% (Shapiro, Robbins, & Cowen, 2011). Other *Candida* species, such as *C. glabrata* and *C. parapsilosis* are also common causes of invasive mycoses (Pfaller & Diekema, 2010). *C. neoformans* is an opportunistic pathogen, with immunocompromised individuals acquiring infections from environmental sources (X. Lin & Heitman, 2006). *C. neoformans* is the third most common cause of central nervous system complications in AIDS patients (X. Lin & Heitman, 2006). Over 600,000 deaths are attributable to the one million new cases of cryptococcal meningitis that occur every year (B. J. Park et al., 2009). Finally, the filamentous mould *A. fumigatus* is the most common cause of invasive aspergillosis, with mortality rates of 40 to 90% (S. J. Lin, Schranz, & Teutsch, 2001). Inhalation of *A. fumigatus* conidia can also lead to allergic bronchopulmonary aspergillosis in patients with pulmonary disorders (S. J. Lin et al., 2001).

*C. albicans* is a remarkably versatile commensal that thrives in diverse host niches. In a healthy individual, *C. albicans* can asymptptomatically colonize anatomic sites such as the skin, oral cavity, vaginal and gastrointestinal tract, which are highly disparate and can vary substantially...
in temperature, oxygen level, pH, nutrient availability and microflora. While the physiological
temperature in the human body is typically maintained at 37°C, the oral cavity is kept between
34°C to 36°C (Marcotte & Lavoie, 1998) and the skin is only at 32°C (Enfert & Hube, 2007). In
regions of the gastrointestinal tract, \textit{C. albicans} has to tolerate hypoxic conditions (Grahl,
Shepardson, Chung, & Cramer, 2012) in addition to pH that can range from pH 2 in the stomach
to pH 6 – pH 7.4 in the intestine (Fallingborg, 1999). The pH also varies from slightly alkaline
(pH 7.4) in the blood and tissues to mildly acidic (pH 4) in the vagina (Mayer, Wilson, & Hube,
2013). For most fungi, glucose is the preferred source of nutrient; however, sugar availability is
limited on the mucosal or skin surfaces, where the pathogen must turn to alternative carbon
sources such as amino and organic acids (Ene, Cheng, Netea, & Brown, 2013; Mayer et al.,
2013).

Although \textit{Candida} species can be acquired from exogenous sources, most \textit{Candida} infection
arises from endogenous flora (Bendinelli, Friedman, & Murphy, 1993). In its commensal state,
\textit{C. albicans} exists in a dynamic equilibrium with the host immune defenses, the native microbial
flora, as well as other local environmental conditions (Dalle et al., 2010; Jouault et al., 2009).
Upon perturbation in the host that shifts this equilibrium, conditions can become favorable for
the fungi to proliferate, resulting in excess fungal burden with the potential to cause localized
infection as well as hematogenous dissemination (Calderone, 2002; Casadevall & Pirofski, 2007;
Dalle et al., 2010). Individuals with intact immunity and mucosal barriers rely on components of
the innate immune system, such as neutrophils and macrophages, to mediate the resistance to
tissue invasion and dissemination of \textit{C. albicans} (Calderone, 2002). However, cancer patients
receiving antineoplastic therapy are often immunosuppressed; in addition, the antineoplastic
agents damage the GI tract, further pre-disposing the host to systemic candidiasis (Calderone,
2002). Hence, the growing population of immunocompromised individuals is particularly
susceptible to opportunistic fungal infections.

As a successful fungal pathogen, \textit{C. albicans} has evolved a number of strategies to survive the
defensive responses mounted by the host during infection. One mechanism of host defense
against infection is fever, which can elevate the body temperature to over 40°C (Leach, Tyc,
Brown, & Klipp, 2012). However, a recent study has shown that 10-minute heat shock can in
fact increase fungal virulence in multiple model systems, including the wax moth \textit{Galleria}
mellonella and zebrafish (Leach et al., 2016), suggesting that \textit{C. albicans} may use host febrile
response as a cue to transition from a commensal state to a pathogenic state. During infection, *C. albicans* is also challenged with a number of host immune response mediated by phagocytes such as macrophages and neutrophils (Ashman et al., 2004). Macrophages restrict fungal growth and recruit additional immune cells, while neutrophils actively kill the invading fungal cells. Remarkably, *C. albicans* has the capacity to evade immune surveillance and escape engulfment by phagocytes. Since the fungal cell wall makes the initial contact with the host innate immune cells, *C. albicans* can effectively alter its cell wall properties to avoid detection. This is best demonstrated by the masking of the cell wall β-glucan, which prevents fungal recognition by the β-glucan receptor Dectin-1, expressed on immune cells such as macrophages and dendritic cells (Wheeler, Kombe, Agarwala, & Fink, 2008). *C. albicans* is also a polymorphic fungus that can transition from yeast to different filamentous forms, such as hyphae and pseudohyphae (A. J. Brown, Argimon, & Gow, 2007; P. E. Sudbery, 2011). Filamentation allows *C. albicans* to initiate dissemination by invading the epithelial barrier and enables yeast cells engulfed by immune cells to escape by active penetration (Cheng, Joosten, Kullberg, & Netea, 2012). More recently, *C. albicans* has been shown to induce macrophage lysis via pyroptosis, an inflammatory host programmed cell death (Krysan, Sutterwala, & Wellington, 2014). It has been proposed that pyroptosis is activated by *C. albicans* during the early phase of infection, and is mediated by exposure of glycosylated proteins on the fungal cell surface, while hyphal protrusion occurs during later phase of infection (O'Meara et al., 2015; Uwamahoro et al., 2014). Since pyroptosis is typically activated to deprive intracellular pathogens of nutrients and protective niche, it is still unclear whether this process ultimately benefits the host or *C. albicans* (Krysan et al., 2014).

### 1.2 Antifungal drugs

Estimated mortality rates for invasive fungal infections range from 20 to 95%, depending on the pathogen and patient population (G. D. Brown et al., 2012), despite currently available treatment options. Since fungi are eukaryotes like their human hosts, there are a limited number of drug targets that can be exploited to selectively kill the pathogen with minimal host toxicity. As such, new classes of antifungal drugs have not reached the clinic since the late 1990’s. Most antifungals in clinical use target the cell membrane sterol ergosterol, or its biosynthesis, or the cell wall linker molecule 1,3-β-D-glucan. Resistance to the available classes of antifungals has emerged as a severe problem, with fungal infections becoming increasingly difficult to treat.
Recently, the Centers for Disease Control and Prevention listed azole-resistant *Candida* as a serious threat to human health, at the same threat level as methicillin-resistant *Staphylococcus aureus* (MRSA), causing approximately 46,000 infections annually among hospitalized patients (CDC, 2013). Fungal species may have intrinsic resistance to a specific antifungal drug or class, which will be referred to as tolerance from hereon; fungi could also readily evolve resistance upon exposure to the antifungal drug (Cowen & Steinbach, 2008; Perea & Patterson, 2002). Resistance in fungal pathogens can lead to therapeutic failures and poor clinical outcome for patients suffering from life-threatening fungal infections.

### 1.2.1 Major classes of antifungal drugs

Currently, there are three major classes of antifungals in clinical use for the treatment of fungal infections in humans: the polyenes, the azoles, and the echinocandins (Figure 1.1). The polyenes include amphotericin B and nystatin, and were the first class of antifungal to reach the clinic over 50 years ago (Mohr, Johnson, Cooper, Lewis, & Ostrosky-Zeichner, 2008). These amphipathic molecules bind to the fungal cell membrane sterol ergosterol and extract it from the lipid bilayer (T. M. Anderson et al., 2014). Polyenes have a broad spectrum of activity against species of *Candida*, *Cryptococcus*, and *Aspergillus*, but suffer from considerable host toxicity problems (Fanos & Cataldi, 2000). The most widely used antifungals are the azoles, including fluconazole, voriconazole, and posaconazole. Azoles function by inhibiting lanosterol demethylase, an enzyme involved in the biosynthesis of ergosterol; this leads to depletion of ergosterol and accumulation of a toxic sterol intermediate, thereby disrupting the integrity of fungal cell membranes (Shapiro et al., 2011). The azoles are generally fungistatic against yeasts and fungicidal against molds (Shapiro et al., 2011). The newest class of antifungal to reach the clinic is the echinocandins, including anidulafungin, micafungin and caspofungin. They impair integrity of the fungal cell wall by inhibiting synthesis of a structural polysaccharide, 1,3-β-D-glucan (Denning, 2003). Echinocandins generally have fungicidal activity against *Candida* species, and fungistatic activity against *Aspergillus* species, though they are clinically ineffective against *Cryptococci* (Shapiro et al., 2011).

Beyond the three major classes of antifungals, there are a limited number of antifungals in clinical use or in development. Flucytosine is the only antifungal in the pyrimidine class that is approved for clinical use (Dismukes, 2000). It targets DNA synthesis, but its antifungal activity
is restricted to *Candida* species and *C. neoformans* (Odds, Brown, & Gow, 2003). Due to the rapid emergence of resistance to flucytosine, it is commonly used only in combination with amphotericin B, particularly in the treatment of cryptococcal meningitis (Mohr et al., 2008). Antifungals in development include sordarins and nikkomycin Z. Sordarins inhibit fungal elongation factor 2 thereby blocking protein biosynthesis, with the derivative FR290581 in clinical development (Ostrosky-Zeichner, Casadevall, Galgiani, Odds, & Rex, 2010). Nikkomycin Z inhibits cell wall biosynthesis by targeting chitin synthases, with Phase I clinical trials underway (Ostrosky-Zeichner et al., 2010).

### 1.2.2 Mechanism of action of echinocandins

The echinocandins are large semisynthetic lipopeptides that inhibit synthesis of the fungal cell wall, a dynamic structure that is absent from mammals but is critical for fungal growth and survival (Denning, 2003). The echinocandins target the catalytic subunit of the membrane-bound 1,3-β-D-glucan synthase, Fks1, which catalyzes the synthesis of 1,3-β-D-glucan (Denning, 2003; Taft, Stark, & Selitrennikoff, 1988). Polymers of 1,3-β-D-glucan serve as a scaffold to which other cell wall components are covalently attached (Bowman & Free, 2006). Exposure to echinocandins causes swelling and lysis at sites of active glucan synthesis, leading to cell death (Bowman & Free, 2006). With impressive safety and efficacy profiles, the echinocandins have become the recommended alternative to the standard regimes of antifungal agents for treating refractory *Candida* infections (Deresinski & Stevens, 2003; Ostrosky-Zeichner et al., 2010).
Figure 1.1 Mechanism of action of the major classes of antifungal drugs.

(A) Azoles inhibit the ergosterol biosynthesis enzyme lanosterol demethylase encoded by ERG11 in *Candida albicans* and *Cryptococcus neoformans*, or by cyp51A and cyp51B in *Aspergillus fumigatus*; azoles inhibit ergosterol production and cause accumulation of a toxic sterol.
sterol generated by Erg3, leading to cell membrane stress. The colored circles represent intermediates in ergosterol biosynthesis. (B) Polyenes bind to ergosterol creating drug–lipid complexes that intercalate into the fungal cell membrane forming a channel that spans the membrane; polyenes cause leakage of cellular ions, destroying the proton gradient and causing osmotic cellular lysis. (C) Echinocandins inhibit (1,3)-β-D-glucan synthase, which is encoded by FKS1 in C. albicans, C. neoformans and A. fumigatus, and by FKS1 and FKS2 in Candida glabrata and Saccharomyces cerevisiae; (1,3)-β-d-glucan is a key structural component of the fungal cell wall, and inhibition of its synthesis causes loss of cell wall integrity and cell wall stress. Adapted with permission from (J. L. Xie, Polvi, Shekhar-Guturja, & Cowen, 2014).

1.2.3 Mechanisms of echinocandin resistance

The therapeutic efficacy of echinocandins and other antifungals can be compromised by the natural tolerance of fungal pathogens and by the rapid emergence of drug resistance. C. albicans can mount cellular stress responses that enhance tolerance to antifungals, thereby allowing cells to survive otherwise lethal concentrations of echinocandins (Walker, Gow, & Munro, 2010). A number of stress response pathways implicated in echinocandin tolerance in the model yeast Saccharomyces cerevisiae have also been shown to modulate bona fide echinocandin resistance that emerges in clinical isolates of C. albicans (Robbins et al., 2011; S. D. Singh et al., 2009). Echinocandin resistance most often associated with clinical failure is due to mutations in the drug target Fks1 (Ben-Ami et al., 2011; S. Park et al., 2005).

1.2.3.1 Drug target alteration

Alterations of the target of the echinocandins, specifically the catalytic Fks subunits responsible for the synthesis of 1,3-β-D-glucan, is the prevailing cause of acquired echinocandin resistance identified to date (Figure 1.2). Single nucleotide substitutions in two hot spot regions of FKS1 are often implicated in echinocandin resistance (Perlin, 2007a). The first region corresponds to amino acids 641 to 648, with the most frequent substitution being at Ser645 (Balashov, Park, & Perlin, 2006). Analysis of 85 caspofungin-resistant C. albicans clinical isolates identified 93% with mutations at Ser645 (Balashov et al., 2006). The second hot spot was identified in S. cerevisiae and includes amino acids 1345 to 1365 (S. Park et al., 2005). A mutation in this region was identified in an echinocandin-resistant C. krusei isolate (S. Park et al., 2005). Mutations in the hot spot region 1 of both FKS1 and FKS2 have been identified in bloodstream isolates of C. glabrata (Zimbeck et al., 2010), and a recent global analysis of mutations accompanying the evolution of echinocandin-resistance in C. glabrata revealed mutations in FKS2 and other genes (Singh-Babak et al., 2012). Polymorphisms in these hot spot regions of other Candida species
(such as *C. parapsilosis*) may contribute to their intrinsic resistance to echinocandins (Perlin, 2007a). Finally, a third hot spot region from position 690 to 700 has been identified in *S. cerevisiae* and *C. glabrata* (M. E. Johnson, Katiyar, & Edlind, 2011). Substitutions in this region have a distinct impact on resistance to different echinocandins, and may give insight into the mechanism by which echinocandins interact with Fks1 (M. E. Johnson et al., 2011).

Echinocandin resistance due to *FKSI* mutations is also associated with constitutive upregulation of cell wall chitin (Walker, Gow, & Munro, 2013). In *C. albicans*, exposure to echinocandins upregulates chitin production via protein kinase C (PKC), HOG, and calcineurin signaling, and reduces echinocandin efficacy, emphasizing the central role of cellular stress response circuitry in drug resistance (H. Lee, Khanal Lamichhane, Garraffo, Kwon-Chung, & Chang, 2012; Munro et al., 2007; Walker et al., 2008).

**Figure 1.2 Mechanisms of echinocandin resistance.**
Mutations in *FKSI*, which encodes the (1,3)-β-D-glucan synthase catalytic subunit, are the most prevalent mechanism of echinocandin resistance. Resistance phenotypes are modulated by cellular stress response pathways. Bullet points describe resistance mechanisms for *C. albicans* and *Aspergillus fumigatus*. Echinocandin resistance due to *FKSI* mutations is also associated with constitutive upregulation of cell wall chitin (Walker, Gow, & Munro, 2013). In *C. albicans*, exposure to echinocandins upregulates chitin production via protein kinase C (PKC), HOG, and calcineurin signaling, and reduces echinocandin efficacy, emphasizing the central role of cellular stress response circuitry in drug resistance (H. Lee, Khanal Lamichhane, Garraffo, Kwon-Chung, & Chang, 2012; Munro et al., 2007; Walker et al., 2008).
and *A. fumigatus*. Dimmed images indicate mechanisms that do not play a key role in resistance. Adapted with permission from (J. L. Xie et al., 2014).

1.2.3.2 Modulation of cellular stress response

Adaptation to the cellular stress imposed by antifungal drugs is often contingent upon cellular stress response circuitry. Like all unfavorable environmental conditions, exposure to antifungals activates stress response pathways that confer immediate cellular protection and promote survival. Since stress responses in *C. albicans* enhance antifungal tolerance and enable drug resistance, the tactical targeting of specific stress response pathways in combination with antifungal agents should be a viable strategy to enhance the efficacy of the existing antifungals and suppress the emergence of antifungal drug resistance. Further, it is critical to identify stress response pathways that are not well conserved in mammals in order to devise new strategies to improve the effectiveness of antifungals such as the echinocandins.

1.2.3.2.1 Molecular chaperone Hsp90

One of the key regulators of cellular stress responses is the heat shock protein 90 (Hsp90), which stabilizes diverse regulators of cellular signaling (Cowen, 2009). It enables basal tolerance and resistance acquired by diverse mutations in *C. albicans*, *C. glabrata*, and *A. fumigatus* (Cowen & Lindquist, 2005; Cowen et al., 2009a; Singh-Babak et al., 2012; S. D. Singh et al., 2009). Hsp90 is an essential and ubiquitous molecular chaperone that orchestrates crucial cellular stress response by assisting in the folding and maturation of metastable client proteins (Picard, 2002; Pratt & Toft, 2003; Young, Moarefi, & Hartl, 2001). Key Hsp90 client proteins for echinocandin tolerance and resistance in *C. albicans* are the protein phosphatase calcineurin and the terminal mitogen-activated protein kinase (MAPK) of the cell wall integrity pathway, Mkc1 (Cowen & Lindquist, 2005; LaFayette et al., 2010). Regulators of Hsp90 function, such as the Hsp90 co-chaperone Sgt1, also have a major impact on echinocandin resistance (Shapiro, Zaas, Betancourt-Quiroz, Perfect, & Cowen, 2012). Compromising Hsp90 function enhances antifungal activity in multiple models of fungal pathogenesis (Cowen et al., 2009a; S. D. Singh et al., 2009), suggesting profound therapeutic benefits of combination therapy.

In recent years, the therapeutic potential of Hsp90 as a novel antifungal drug target in combination with existing antifungal agents has been explored against a number of fungal pathogens, including *C. albicans*, *C. neoformans*, *A. fumigatus*, and the dermatophyte
*Trichophyton rubrum* (Cordeiro Rde et al., 2016; Cowen, Carpenter, Matangkasombut, Fink, & Lindquist, 2006; Cowen & Lindquist, 2005; Cowen et al., 2009a; Jacob et al., 2015; Lamoth, Juvvadi, & Steinbach, 2016). Hsp90 is required for the evolution of resistance to azoles in *C. albicans* and the intrinsic resistance to echinocandins in *A. fumigatus* (Cowen & Lindquist, 2005). Hsp90 inhibitors currently in clinical development, 17-AAG and 17-DMAG, have been shown to improve the efficacy of fluconazole from fungistatic to fungicidal against *C. albicans*, and to enhance caspofungin efficacy against *A. fumigatus* in the host-model system *Galleria mellonella* (Cowen et al., 2009a). 17-AAG is also effective against *T. rubrum* in combination with the itraconazole or micafungin (Jacob et al., 2015). The high conservation of Hsp90 between yeast species allows Hsp90 inhibitors to be effective against diverse fungal pathogens, but the high conservation of Hsp90 from yeast to human limits its therapeutic potential due to host toxicity, highlighting the importance of developing fungal-selective Hsp90 inhibitors based on structural differences (Veri & Cowen, 2014).

While Hsp90 inhibitors that are well tolerated in humans greatly improve the effectiveness of existing antifungals, including echinocandins, adverse effects have been noted when they are used in the context of disseminated *C. albicans* infections in mice, emphasizing the need to identify additional drug targets not conserved in the mammalian host (Cowen et al., 2009b). One possibility is to exploit regulators of Hsp90 function or effectors downstream of Hsp90 for combination therapy (Veri & Cowen, 2014). The functional properties and client specificity of Hsp90 are modulated by a number of co-chaperones and post-translational modifications (Veri & Cowen, 2014). Sgt1 is a co-chaperone that recruits clients to Hsp90 and bridges their interactions in yeast, human and plants (Bansal, Abdulle, & Kitagawa, 2004; Y. T. Lee et al., 2004; Shapiro, Zaas, et al., 2012; Takahashi, Casais, Ichimura, & Shirasu, 2003). Similar to Hsp90, Sgt1 is required for antifungal tolerance and resistance to the azoles and echinocandins in *C. albicans* (Shapiro, Zaas, et al., 2012). Although Sgt1 is found in yeast and human, the low level of conservation between yeast and human Sgt1 makes it an attractive therapeutic target (Shapiro, Zaas, et al., 2012). Hsp90 function is also regulated by phosphorylation and acetylation (Diezmann, Michaut, Shapiro, Bader, & Cowen, 2012; Lamoth et al., 2014; Robbins, Leach, & Cowen, 2012). In particular, hyperacetylation of Hsp90 by the histone deacetylase inhibitor trichostatin A has been shown to alter Hsp90 chaperone activity (Bali et al., 2005) and phenocopies Hsp90 inhibition in abrogating azole resistance in *C. albicans* and echinocandin
resistance in *A. fumigatus* (Lamoth et al., 2014; Robbins et al., 2012). Although trichostatin A is not an ideal drug due to its genotoxic effects in human cells (Kemp, Ghosh, Liu, & Leffak, 2005; Olaharski et al., 2006), chemical modulation of Hsp90 post-translational modification represents an alternative therapeutic strategy to compromise Hsp90 function.

1.2.3.2.2 **Calcineurin signaling pathway**

Hsp90 regulates responses to cell wall and cell membrane stress by stabilizing a number of clients, including the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (Cruz et al., 2002; S. D. Singh et al., 2009). In *S. cerevisiae*, the transcription factor Crz1 is the most well characterized effector downstream of calcineurin. Upon activation, calcineurin dephosphorylates Crz1, which translocates into the nucleus to promote the expression of calcineurin-dependent transcripts (Stathopoulos-Gerontides, Guo, & Cyert, 1999). However, Crz1 only plays a partial role in mediating calcineurin-dependent cell wall stress response in *C. albicans*, suggesting that additional effectors downstream of calcineurin remain to be identified (S. D. Singh et al., 2009). Although the inhibition of calcineurin with FK506 or cyclosporin A abolishes caspofungin resistance in *C. albicans*, the therapeutic application of calcineurin inhibitors as antifungal agents is hindered by their immunosuppressive effects in human (S. D. Singh et al., 2009).

1.2.3.2.3 **Cell wall integrity pathway**

The protein kinase Pkc1 plays a key role in maintenance of a functional cell wall and repair of cell wall damage. In *S. cerevisiae*, Pkc1 physically interacts with the small GTPase Rho1, which is the regulatory subunit of the 1,3-β-D-glucan synthase (Kamada et al., 1996). Rho1 receives a cell wall integrity signal from a family of cell surface receptors, including Wsc1 and Wsc2, and transduces the signal by activating Pkc1 (Levin, 2005). The cell wall integrity signal is further amplified by a MAP kinase cascade downstream of Pkc1, consisting of Bck1, Mkk2, and the Hsp90 client Mkc1 in *C. albicans* (LaFayette et al., 2010). In *S. cerevisiae*, the transcriptional regulation of cell wall integrity genes is mediated by the transcription factors Rlm1 and the SBF complex Swi4/Swi6. However, Rlm1 has limited roles in cell wall stress response in *C. albicans* (Bruno et al., 2006). Given that the circuitry downstream of Pkc1 has been found to be rewired considerably between *C. albicans* and *S. cerevisiae* in terms of membrane stress response (LaFayette et al., 2010), the regulation of cell wall stress response is also likely to have diverged between the two fungal species.
Since Pkc1 regulates cell wall and cell membrane stress responses, it represents a promising antifungal drug target, particularly for combination therapy with the azoles and echinocandins. High throughput screening of synthetic and natural products against *C. albicans* Pkc1 has led to the discovery of cercosporamide, a fungal-selective Pkc1 inhibitor (Sussman et al., 2004). Cercosporamide is effective against *C. albicans* in combination with echinocandins and azoles (LaFayette et al., 2010; Sussman et al., 2004). It has also been evaluated in preclinical studies for its anticancer activity through selective inhibition of human Mnk kinase (Konicek et al., 2011). Although Mnk is required for the phosphorylation of the translation initiation factor eIF4E in malignant transformation (Konicek et al., 2011), Mnk function is not required for normal cell growth or development (Ueda, Watanabe-Fukunaga, Fukuyama, Nagata, & Fukunaga, 2004). Therefore, cercosporamide could be developed as a potent antifungal agent with limited toxicity to the host.

### 1.2.3.2.4 Transcription factor Cas5

Cas5 is a zinc finger transcription factor that regulates tolerance to both theazole fluconazole and the echinocandin caspofungin (Bruno et al., 2006; Vasicek et al., 2014). It is required for the expression of cell wall integrity genes in response to cell wall stress in *C. albicans* (Bruno et al., 2006). It lacks a distinct ortholog in *S. cerevisiae*, but acts as a functional equivalent of Rlm1 (Bruno et al., 2006). Interestingly, a number of cell wall genes regulated by Cas5 in response to cell wall stress are also calcineurin-dependent, suggesting that the rewired transcriptional network downstream of Pkc1-mediated cell wall integrity pathway and the calcineurin signaling pathway may converge on Cas5 (Bruno et al., 2006).

Despite the limited number of drugs in clinical use that target transcription factors, there is considerable interest in developing antifungals that target transcription factors by blocking dimerization, interfering with DNA binding, or disrupting the interaction with regulatory proteins (Bahn, 2015; Nishikawa et al., 2016). Transcription factors are particularly attractive as antifungal drug targets because they are typically not well conserved between fungi and their eukaryotic host (Bahn, 2015). There is now strong evidence to suggest that transcription factors can be chemically modulated, and growing interest in the structural and functional characterization of transcription factors important for fungal virulence (Bahn, 2015; Nishikawa et al., 2016). Ultimately, understanding the dynamic regulation of fungal-specific transcriptional
regulators such as Cas5 and elucidating the transcriptional circuitry it controls would reveal new strategies to enhance the therapeutic efficacy of the few available antifungal drugs.

1.2.4 Approaches to elucidating mechanisms of drug resistance

Drug resistance is a complex trait involving multiple mechanisms. Our current understanding of mechanisms controlling antifungal drug resistance has been informed through complementary approaches to determine differences between resistant isolates and their susceptible counterparts. Much of the early work on drug resistance focused on the model yeast *S. cerevisiae* and the pathogenic yeast *C. albicans*, given the availability of genetic tools (Figure 1.3).

![Figure 1.3 Overview of approaches to elucidating antifungal drug resistance.](image)

Adapted with permission from (J. L. Xie et al., 2014).

1.2.4.1 Genetic screens to identify resistance determinants

Forward and reverse genetics provide powerful approaches for analyzing diverse traits, including drug resistance. The basic principle underlying both approaches is to explore the genetic basis of a mutant phenotype. Forward genetics was one of the earliest approaches used to identify drug resistance mutations, based on selection of drug-resistant mutants. Resistance mutations are often
further characterized in *S. cerevisiae* by classical genetic means such as genetic crosses, complementation and dominance tests. Notably, genes encoding the drug target for rapamycin and the echinocandins were both identified based on analysis of spontaneous resistant mutants of *S. cerevisiae* (Douglas, Marrinan, Li, & Kurtz, 1994; Heitman, Movva, & Hall, 1991). Identification of recessive resistance mutations in *S. cerevisiae* can be facilitated by expression of barcoded plasmids with each wild-type gene to identify the gene that complements the resistance phenotype by pooled analysis of fitness (Ho et al., 2009). Functional genomic approaches have also been developed to identify drug resistance mutations based on simultaneous screening of high complexity randomly mutagenized libraries of ~90% of *S. cerevisiae* genes (Z. Huang et al., 2013). Recent advances in the whole-genome sequencing technologies coupled with forward genetics offer exciting opportunities to study resistance mechanisms that evolve *in vitro* and *in vivo*, facilitating the identification of clinically relevant resistance mutations (Hill, Ammar, Torti, Nislow, & Cowen, 2013; S. D. Singh et al., 2009).

Over the last decade, the availability of diverse fungal genome sequences has enabled the development of reverse genetic approaches. Reverse genetics involves targeting genes of interest for disruption and assessing the phenotypic consequences. The construction of mutant libraries has enabled large-scale systematic screening (Bruno & Mitchell, 2004; Kaur, Castano, & Cormack, 2004; O. W. Liu et al., 2008; Noble & Johnson, 2005; Roemer et al., 2003). Mutants have been screened for drug susceptibility phenotypes, leading to the identification of genes involved in resistance to caspofungin (Lesage et al., 2004), amphotericin B (Z. Huang et al., 2013), and fluconazole (Kaur et al., 2004). A complementary strategy involves libraries of pharmacological compounds that can be screened to identify molecules that abrogate drug resistance. For example, screening the LOPAC\textsuperscript{1280} Navigator library revealed that inhibition of protein kinase C abrogates azole resistance of *C. albicans*, and further genetic analysis implicated the Pkc1-dependent cell wall integrity pathway in modulating resistance to drug-induced membrane stress (LaFayette et al., 2010). Genetic approaches such as haploinsufficiency profiling can reveal targets of drugs that modulate drug resistance, based on the principle that reducing dosage of the drug target confers hypersensitivity to that drug (Giaever et al., 1999). Integrating additional genome-wide gene dosage assays provides enhanced power to identify drug targets and modulators of resistance phenotypes (Hoon et al., 2008).
1.2.4.2 Biochemical analysis of resistance mechanisms

Discovery of a drug target and mode of action paves the way for biochemical analyses of drug action and cellular responses. For example, polyenes and azoles target ergosterol or its biosynthesis. Analysis of the membrane lipid composition of \textit{C. albicans} mutants that are resistant to both azoles and polyenes implicated the absence of ergosterol and accumulation of methylated sterol as a cause for resistance (Hitchcock, Barrett-Bee, & Russell, 1987). Analysis of the intracellular accumulation of azole demonstrated reduced azole accumulation in azole-resistant isolates than in their susceptible counterparts (Sanglard et al., 1995); gene expression analysis later demonstrated that overexpression of drug efflux transporters is a key mechanism of azole resistance (Venkateswarlu, Denning, Manning, & Kelly, 1995). Further, analysis of drug-target interaction in an azole-resistant mutant implicated decreased interaction between azole and Erg11 as a cause of resistance (Vanden Bossche et al., 1990). In the context of echinocandins, which inhibit cell wall synthesis, analysis of cell wall composition in response to echinocandin exposure revealed that increased chitin level confers resistance to echinocandins (K. K. Lee et al., 2012; Walker et al., 2008).

1.2.4.3 Global analysis of transcripts, proteins, and lipids in drug response and resistance

Technological advances have provided powerful tools for systems level analyses, enabling resistance circuitry to be dissected with unprecedented resolution. Gene expression analysis in \textit{S. cerevisiae} using microarrays revealed rapid upregulation of cell wall genes in response to caspofungin (Reinoso-Martin, Schuller, Schuetzer-Muehlbauer, & Kuchler, 2003). Proteomic analysis in \textit{C. albicans} using two-dimensional gel electrophoresis coupled to mass spectrometry (2-DE-MS) identified proteins upregulated in response to ketoconazole, amphotericin B, and caspofungin, and those that are part of the adaptive response to all three antifungals (Hoehamer, Cummings, Hilliard, & Rogers, 2010). Comparative lipidomics revealed quantitative and qualitative changes in lipid classes in azole-resistant \textit{C. albicans} clinical isolates relative to their susceptible counterparts (A. Singh, Yadav, & Prasad, 2012). Analysis of metabolite profiles of \textit{Candida} strains revealed changes in key metabolic pathways in response to caspofungin, amphotericin B, and voriconazole (Coen et al., 2006). Integration of transcriptional, proteomic, lipidomic, and metabolomic data is poised to reveal systems level regulatory networks controlling drug response and resistance (Baginsky, Hennig, Zimmermann, & Gruissem, 2010;
Elucidating drug resistance circuitry is important not only for identifying drug targets and deciphering drug mode of action, but it will ultimately guide the development of strategies to prevent the emergence of drug resistance and improve efficacy of existing antifungal drugs.

1.3 Morphogenesis in *C. albicans*

Among the estimated 99,000 described fungal species, only several hundred are pathogenic to humans (Ainsworth, Kirk, Bisby, & CABI Bioscience., 2008; Kohler, Casadevall, & Perfect, 2015). Remarkably, the majority of the systemic fungal infections, such as candidiasis, histoplasmosis, and aspergillosis, are caused by fungi that are able to take on distinct morphological forms depending on intrinsic developmental cues or extrinsic environmental cues, including temperature, nutrient availability, and CO$_2$ level (Gauthier, 2015; L. S. Wilson et al., 2002). Histoplasmosis and aspergillosis are responsible for over 200,000 life threatening infections every year, and candidiasis accounts for over 80% of systemic fungal infection in the United States and over 200,000 death worldwide (G. D. Brown et al., 2012; L. S. Wilson et al., 2002). Hence, morphogenesis represents a key virulence factor in fungal pathogenesis and provides a novel therapeutic target.

1.3.1 Morphogenesis and virulence

The association between morphogenesis and fungal virulence was established based on the observation that morphological transitions are often required for host adaptation and pathogenicity (Gauthier, 2015; X. Lin, Alspaugh, Liu, & Harris, 2015; Wang & Lin, 2012). Thermally dimorphic fungi such as *Histoplasma capsulatum* grow as chain of filaments (mycelia) in the soil (22 – 25°C) and generate infectious spores (conidia) that can be inhaled by mammalian hosts (Gauthier, 2015). Inside the host (37°C), conidia germinate into yeast to facilitate intracellular replication and systemic dissemination (X. Lin et al., 2015). *A. fumigatus* is a filamentous fungus and undergoes several morphological transitions throughout its life cycle, including conidiophore formation, conidiation, germination, and hyphae formation (Shapiro et al., 2011). The conidiophore is a specialized hyphae that produces asexual conidia for airborne dispersal (Kohler et al., 2015). The metabolically inactive conidia can withstand harsh environmental stresses, such as fluctuation in temperature and osmolarity (Kohler et al., 2015). Conidia are readily inhaled into human lungs, where conditions are favorable for germination
and hyphae formation, particularly in individuals with a compromised immune system (Shapiro et al., 2011). Proliferating hyphae have the capacity to damage the lung epithelia and enter the vascular system for dissemination to perfused organs, causing invasive aspergillosis (Kohler et al., 2015; Shapiro et al., 2011).

*C. albicans* is unique in the variety of morphological states it can manifest. Depending on the transcriptional output, *C. albicans* can exist as one of the four phenotypically distinct cell types, including white, opaque, GUT, and gray cells (Pande, Chen, & Noble, 2013; Slutsky et al., 1987; Tao et al., 2014). Since the cell identity significantly affects the nature of interactions between *C. albicans* and the host, it has been proposed that phenotypic switching among the cell types has evolved to facilitate the transition between commensal and pathogenic states (Gow, 2013; Lohse & Johnson, 2009; Pande et al., 2013). Independent of these cell types, *C. albicans* can also grow as ovoid yeast or morphologically distinct filamentous forms, including pseudohyphae and hyphae (P. E. Sudbery, 2011). This morphological transition is triggered by diverse environmental cues from the host, such as elevated temperature, serum, and CO$_2$ (P. E. Sudbery, 2011). The current paradigm maintains that the yeast form of *C. albicans* promotes surface adhesion and bloodstream dissemination during early stages of infection, while the filamentous forms are involved in tissue invasion and immune cell escape (Gow, van de Veerdonk, Brown, & Netea, 2012; Grubb et al., 2009; Saville, Lazzell, Monteagudo, & Lopez-Ribot, 2003).

### 1.3.2 Morphotypes in *C. albicans*

#### 1.3.2.1 Phenotypic switching

White-opaque switching is a heritable but reversible transition between two phenotypically distinct cell types, the white and opaque cells (Slutsky et al., 1987). Standard laboratory strains are white cells, which adopt ovoid shape that resembles the budding yeasts and form domed colonies (Slutsky et al., 1987; Whiteway & Bachewich, 2007). Opaque cells are elliptically shaped and form flattened colonies that stain dark pink with the vital dye phloxine B (J. M. Anderson & Soll, 1987; Slutsky et al., 1987). In addition to a clear distinction between their physical attributes, the white and opaque cells exhibit differential gene expression, metabolic preference, mating competency, and host adaptation (Lan et al., 2002; Miller & Johnson, 2002; Soll, 2009). The frequency of switching between white and opaque cells is strongly dependent on host cues, including temperature, oxygen and CO$_2$ level, and available carbon source (G. Huang,
Srikantha, Sahni, Yi, & Soll, 2009; G. Huang et al., 2010; Ramirez-Zavala, Reuss, Park, Ohlsen, & Morschhauser, 2008; Rikkerink, Magee, & Magee, 1988; Slutsky et al., 1987).

The transcriptional program that defines cell identity in *C. albicans* is highly regulated. To date, Wor1, Wor2, Wor3, Czf1, Efg1, Ahr1, and Ssn6 are seven transcription factors that have been shown to regulate the switch via coordinated gene expression (Hernday et al., 2016). Wor1, or White-Opaque Regulator 1, is the master regulator of white-opaque switching, which functions in conjunction with Wor2 and Czf1 (G. Huang et al., 2006). Wor1 is only expressed in opaque cells and its expression is critical for the establishment and maintenance of the opaque state (Guan & Liu, 2015; G. Huang et al., 2006). Efg1 is a white-phase specific regulator of phenotypic switching (Sonneborn, Tebarth, & Ernst, 1999). Efg1 antagonizes Wor1 via Wor2, and its overexpression drives switching to the white state while deletion locks the cell in the opaque state (Zordan, Miller, Galgoczy, Tuch, & Johnson, 2007).

White-opaque switching plays a crucial role in mating. Similar to *S. cerevisiae*, mating in *C. albicans* occurs when an a/a cell meets an α/α cell. However, studies have shown that only *C. albicans* opaque cells are capable of mating (Bennett & Johnson, 2005). The fact that white-opaque switching in *C. albicans* is transcriptionally regulated by homeodomain proteins a1 and a2 from the mating locus, such that switching is repressed in strains that are heterozygous at the mating locus, further supports the notion that white-opaque switching and mating are intimately coupled (Miller & Johnson, 2002). Interestingly, genes that are differentially expressed between white and opaque cells are not restricted to mating; there is also a number of genes involved in adhesion and metabolism, suggesting that the two cell types may represent distinct modes of host adaptation (Morschhauser, 2010; Sasse, Hasenberg, Weyler, Gunzer, & Morschhauser, 2013).

Recent studies have identified additional cell types that lend support to the notion that phenotypic switching in *C. albicans* is initiated as an adaptive response to the host environment. Previous work has established that white cells are, in general, more stable than opaque cells, and optimized for systemic infection; opaque cells are more stable at a lower temperature of 25°C, and thus better suited for the colonization and infection of the skin (Lohse & Johnson, 2009; Morschhauser, 2010). Over two decades after the initial discovery of white and opaque cells, GUT (gastrointestinally induced transition) cells and gray cells were characterized as novel cell identities adopted by *C. albicans* to occupy specific host niches (Pande et al., 2013; Tao et al.,
The GUT cells exhibit an intermediate phenotype compared to white and opaque cells, and are thought to promote commensalism in the gastrointestinal tract (Pande et al., 2013). The gray cells were isolated from genital tract, and are distinct from other cell types in morphology, expression profile, and virulence (Tao et al., 2014). Therefore, phenotypic switching is an important trait that enables *C. albicans* to thrive as an opportunistic pathogen.

### 1.3.2.2 Morphological transition

*C. albicans* can grow as yeast, hyphae, or pseudohyphae (P. Sudbery, Gow, & Berman, 2004). The ability to transition from yeast to filamentous growth is a defining characteristic that was initially used to differentiate *C. albicans* from other yeasts as a rapid diagnostic test (Taschdjian, Burchall, & Kozinn, 1960). In its unicellular yeast form, *C. albicans* is virtually indistinguishable from *S. cerevisiae* and reproduces asexually by budding. However, it is also prone to morphological changes triggered by host cues, rapidly transitioning from yeast to filamentous forms of pseudohyphae or hyphae. Pseudohyphae and hyphae are distinct morphological states that can be differentiated based on the size and shape of the filaments, position of the septa and nuclei, as well as branching patterns (P. Sudbery et al., 2004). Pseudohyphae are often considered an intermediate state between yeast and hyphae (P. Sudbery et al., 2004). Pseudohyphae prominently feature constrictions at the sites of septation, similar to chains of elongated yeast cells, while hyphae are characterized by parallel walls with no obvious constriction at their septa (P. Sudbery et al., 2004). In pseudohyphae, the first septal junction and first round of nuclear division take place at the mother-bud neck, similar to a yeast cell prior to cytokinesis (P. Sudbery et al., 2004). By contrast, the first septin ring in hyphae forms within the germ tube and nucleus migrates away from the mother cell to divide in the germ tube, (P. Sudbery et al., 2004). These observations reveal substantial differences in cell cycle regulation and progression between the two morphological states. Hence, it is important to distinguish pseudohyphae from hyphae in the process of unraveling the biology of *C. albicans* morphogenesis.

Pseudohyphae and hyphae are often triggered by different environmental cues. *C. albicans* typically grow as yeast at 30°C or pH 4 and as pseudohyphae or hyphae at elevated temperature (above 35°C), depending on the nature of additional cue(s) (P. Sudbery et al., 2004). For example, pH 6 induces pseudohyphae growth at 35°C while pH 7 induces hyphae growth at 37°C.
(P. Sudbery et al., 2004). Interestingly, although both hyphae and pseudohyphae are found in vivo (Guarner & Brandt, 2011), many of the conditions that mimic host environments lead to hyphae formation at 37°C, including as serum, nitrogen-limiting Lee’s medium, and carbon-limiting Spider medium (P. Sudbery et al., 2004; P. E. Sudbery, 2011), suggesting the possibility that hyphae and pseudohyphae have distinct roles during infection.

Although hyphae and pseudohyphae are invasive and have the capacity to penetrate tissues, filamentation and virulence are not strictly coupled. Many studies have established that filamentation in is an important virulence trait for C. albicans (Carlisle et al., 2009; Lo et al., 1997; Moyes et al., 2016; Saville et al., 2003; P. E. Sudbery, 2011). For example, mutants unable to execute the morphological transition sustain reduced virulence in a mouse model of infection (Lo et al., 1997), and inhibiting filamentation has therapeutic potential against candidiasis (Saville et al., 2006). In addition, in a genetically engineered strain that is locked in the yeast state and severely attenuated for virulence, re-activation of the morphological transition at various times post-infection restores virulence (Saville et al., 2003). More recently, Candidalysin, a cytolytic peptide toxin highly expressed by C. albicans during hyphal growth, has been shown to play a crucial role in epithelial damage and immune recognition of hyphae (Moyes et al., 2016). Despite the strong association between morphogenesis and virulence, a systematic screen has identified a few mutants that have reduced virulence without an accompanying defect in morphogenesis, suggesting that there are additional virulence traits that are distinct from morphogenesis (Noble, French, Kohn, Chen, & Johnson, 2010). Consistent with this idea, it has recently been shown that C. albicans mutants that are unable to undergo filamentous growth can still escape from macrophages by inducing pyroptosis (O'Meara et al., 2015), demonstrating that morphogenesis is not crucial for immune evasion. The complex relationship between morphology and virulence suggests that further studies are necessary to define the key role for morphogenesis in the infection process.

Given that morphogenesis in C. albicans can be modulated by diverse cues, it has emerged as an attractive target for the development of antifungal therapies (Jacobsen et al., 2012; Shareck & Belhumeur, 2011). In fact, current antifungals that target ergosterol or its biosynthesis are known to have a profound impact on C. albicans morphogenesis; in addition to disrupting cellular membrane integrity, the azole fluconazole and the polyene amphotericin B effectively suppress C. albicans filamentation in response to serum (O'Meara et al., 2015). However, a proof-of-
principle study in mice has shown that although inhibition of the morphogenesis program post-infection improved host survival, the *C. albicans* yeast cells remain viable, suggesting that filamentation inhibitors may be more effective in combination with conventional antifungals (Saville et al., 2006). More recently, a number of small molecules that act synergistically with azoles and echinocandins have been shown to activate *C. albicans* morphogenesis in the absence of other cues. For example, the metal chelator diethylenetriaminepentaacetic acid (DTPA) and the Hsp90 inhibitor geldanamycin both abrogate resistance to the echinocandin caspofungin and induce filamentous growth at 30°C, a temperature that does not generally support filamentation in *C. albicans* (Polvi et al., 2016; Shapiro et al., 2009; S. D. Singh et al., 2009). Hence, a comprehensive understanding of the positive and negative regulation of the morphogenetic program in *C. albicans* may facilitate the identification of new antifungal drug targets.

### 1.3.3 Regulation of morphogenesis

There is growing interest in mapping the regulatory circuitry that controls the *C. albicans* morphological transition and dissecting the molecular mechanism involved given the profound impact of morphogenesis on virulence. It is now widely appreciated that the switch from yeast to filamentous growth in *C. albicans* begins with sensing of a morphogen, followed by relaying a signal to transcription factors, which in turn activate a complex transcriptional program, leading to the initiation and maintenance of polarized growth. Given the extent of pathway redundancy in the regulation of *C. albicans* morphogenesis (A. J. Brown et al., 2007; Osiewacz, 2002; P. E. Sudbery, 2011), components of the regulatory network that are crucial for filamentation represent the ideal targets for therapeutic intervention.

#### 1.3.3.1 Sensing environmental cues

*C. albicans* morphogenesis is triggered by a multitude of environmental cues, many of which are a direct reflection of host conditions. One of the first reported filament-inducing cues is serum (Reynolds & Braude, 1956; Taschdjian et al., 1960). At 37°C, serum induces the formation of germ tubes that eventually becomes hyphae (P. Sudbery et al., 2004). Bacterial peptidoglycans were recently identified as the key component of serum that is sensed by *C. albicans* (Xu et al., 2008). Upon exposure to serum, muramyl dipeptides interact with the adenylate cyclase Cyr1 and enhance the production of cAMP, a key signaling molecule in *C. albicans* morphogenesis (Xu et al., 2008). However, since Cyr1 functions intracellularly, it is unclear how muramyl
dipeptides are transported into the cell. Although Ras1, a small GTPase operating upstream of Cyr1, is also required for filamentation in response to serum and muramyl dipeptides, additional sensors remain to be identified.

The observation that elevated temperature increases the efficacy of many cues that induce morphogenesis (A. J. Brown et al., 2007; P. Sudbery et al., 2004; P. E. Sudbery, 2011) suggests that temperature plays a key role in C. albicans morphogenesis. At 30°C, a mixture of yeast and pseudohyphae is observed in response to serum (Kadosh & Johnson, 2005). In contrast, at 37°C, serum rapidly induces the formation of hyphae in 100% of the cell population (Kadosh & Johnson, 2005). Even in the absence of other inducing cues, a further increase in temperature to 39°C is sufficient to induce morphogenesis (Shapiro, Sellam, et al., 2012), emphasizing the importance of temperature sensing in the activation of the morphogenetic program. In S. cerevisiae, Wsc1 and Mid2 are two cell wall sensors that are critical for growth at elevated temperature (Straede & Heinisch, 2007). While Wsc1 is present in C. albicans, its role in temperature-dependent growth remains elusive. The molecular chaperone Hsp90 is another key regulator of C. albicans temperature-dependent morphogenesis (Shapiro et al., 2009; Shapiro, Zaas, et al., 2012). It physically interacts with co-chaperone Sgt1 to repress the activity of the adenyl cyclase Cyr1, preventing filamentation via the activation of the cAMP signaling.

Although not all environmental stresses stimulate morphological transitions, many of the filament-inducing cues are associated with stress in host. The prime example is the engulfment of C. albicans by macrophages; in response to phagocytosis, yeast cells undergo robust filamentation and escape containment by the host immune cells (Miramon, Kasper, & Hube, 2013; Uwamahoro et al., 2014). The specific cue(s) that induce the C. albicans morphogenesis program inside phagocytes remain enigmatic. One potential cue is pH, which induces filamentation when shifted from acidic (pH 4.5) to alkaline (pH 6.5) (A. J. Brown et al., 2007). While the acidic pH in the phagolysosome inhibits filamentous growth, a recent study has shown that C. albicans alkalinizes the phagosome to promote the morphological transition and immune escape (Vylkova & Lorenz, 2014). The morphological transition is also triggered by nutrient deprivation as exemplified by carbon starvation in Spider medium (which contains mannitol as a carbon source), nitrogen starvation in Lee’s medium (which contains a mixture of amino acids as a nitrogen source) (P. E. Sudbery, 2011), and hypoxic conditions (Doedt et al., 2004). Nitrogen
starvation on a solid surface is sensed by the transmembrane ammonium permease Mep2 and activates the morphogenetic program via Ras1 (A. J. Brown et al., 2007).

### 1.3.3.2 Signal transduction pathways

Despite the vast number of cues that trigger the morphogenetic program, many of the signals converge on the adenylyl cyclase Cyr1 in the cAMP-dependent signaling pathway (P. E. Sudbery, 2011). In *C. albicans*, Cyr1 is not essential for yeast growth but is indispensable for yeast-to-hyphal transition (Rocha et al., 2001). Although it functions downstream of Ras1, Cyr1 can integrate morphogenetic signals in both Ras1-dependent and Ras1-independent manners (P. E. Sudbery, 2011). Cyr1 has been shown to sense CO$_2$ via direct interaction with HCO$_3^-$ and serum via direct interaction with the muramyl dipeptides (Klengel et al., 2005; Xu et al., 2008). It is also well established that activation of Ras1 by the guanine exchange factor Cdc25 stimulates the adenylyl cyclase activity of Cyr1 to generate cAMP (Harcus, Nantel, Marcil, Rigby, & Whiteway, 2004; Leberer et al., 2001; Piispanen et al., 2011). Elevated intracellular cAMP levels activate protein kinase A (PKA) by binding to its regulatory subunit, encoded by *BCY1*, and releasing the catalytic subunits Tpk1 and Tpk2 to activate the transcription factor Efg1 (Cloutier et al., 2003). Although Efg1 is the terminal transcription factor downstream of the PKA (Bockmuhl & Ernst, 2001; Sonneborn et al., 2000; Stoldt, Sonneborn, Leuker, & Ernst, 1997; P. E. Sudbery, 2011), it is required for filamentation in response to some but not all cues that activate cAMP-PKA signaling (Polvi et al., 2016; Shapiro et al., 2009), suggesting that PKA has additional effectors that remain to be identified.

Another small GTPase important for morphogenesis is Cdc42. Both Cdc42 and its guanine exchange factor (GEF) Cdc24 are necessary for hyphae formation in response to serum in liquid and Spider medium on solid (Bassilana, Blyth, & Arkowitz, 2003; Ushinsky et al., 2002; VandenBerg, Ibrahim, Edwards, Toenjes, & Johnson, 2004). In *S. cerevisiae*, the MAP kinase cascade downstream of Cdc42 is required for pseudohyphae formation (A. J. Brown et al., 2007). Given that the Cdc42/Cdc24 GTPase module is highly conserved between *S. cerevisiae* and *C. albicans*, the pathway in *C. albicans* has been largely defined by studies in *S. cerevisiae* such that Cdc42 is thought to activate the protein kinase Cst20 upstream of the MAPKKK Ste11, MAPKK Hst7, MAPK Cek1, and the transcription factor Cph1 (A. J. Brown et al., 2007). Notably, the MAP kinase signaling pathway is only found to be required for filamentous growth on solid
Spider medium but not serum (Kohler & Fink, 1996; Leberer et al., 1996), suggesting that Cdc42 also regulates filamentation independent of the MAP kinase cascade. Another effector downstream of Cdc42 in *S. cerevisiae* is the kinase Cla4. Deletion of *CLA4* results in severe defect in hyphal formation in response to a number of cues, including serum and Spider medium (Leberer et al., 1997), implicating Cla4 as a mediator of Cdc42 function in morphogenetic regulation. In addition, Cdc42 is required for Efg1-dependent gene expression during morphogenesis (Bassilana & Arkowitz, 2006), suggesting that there is crosstalk between the Ras1 and Cdc42 signaling pathways that converge on the activation of Efg1.

Efg1 and its homolog Efh1 are members of the APSES family proteins and play an important role in governing the transcriptional program during morphogenesis (Stoldt et al., 1997; Zhao et al., 2015). The APSES family proteins are unique to fungi (Zhao et al., 2015). They belong to the class of basic helix-loop-helix (bHLH) transcription factors, and regulate cellular differentiation in diverse fungal species (Zhao et al., 2015). In *C. albicans*, Efg1 and Efh1 are the only two APSES proteins identified thus far (Doedt et al., 2004). Although they regulate overlapping sets of genes involved in morphogenesis, Efg1 remains the central regulator of filamentation, modulating the expression of 238 genes during morphogenesis (Doedt et al., 2004). Efg1 is required for filamentous growth in response to serum, CO₂, neutral pH and N-acetyl-D-glucosamine in liquid medium, and Spider medium on solid (P. E. Sudbery, 2011). Surprisingly, Efg1 is dispensable for filamentous growth in response to cues that do not require a concurrent increase in temperature, including Hsp90 inhibition by geldanamycin and cell cycle inhibition by hydroxyurea (Bachewich, Thomas, & Whiteway, 2003; Shapiro et al., 2009). This raises the possibility that Efg1 could to respond to high temperature as a filament-inducing cue, possibly in conjunction with the heat shock type-transcription factors Sfl1 and Sfl2 (Znaidi, Nesseir, Chauvel, Rossignol, & d'Enfert, 2013).

Efg1 positively regulates morphological transitions via interaction with other transcription factors, transcriptional regulation of target genes, and modulation of nucleosome positioning. In *C. albicans*, Efg1 physically interacts with the transcription factor Flo8, which is required for filamentation and regulates the expression of a subset of Efg1-dependent genes (Cao et al., 2006). Flo8 functions downstream of the cAMP signaling pathway, essential for pseudohyphal formation in *S. cerevisiae*. Together with another bHLH transcription factor, Cph2, Efg1 regulates the expression of *TECI*, which encodes a morphogenetic regulator that is
predominantly expressed during hyphal growth (Lane, Zhou, Pan, Dai, & Liu, 2001; Schweizer, Rupp, Taylor, Rollinghoff, & Schroppel, 2000). Lastly, Efg1 has been shown to recruit the histone acetyltransferase complex to reduce nucleosome occupancy and promote the transcriptional activation of hyphal gene expression (Lu et al., 2008).

Morphogenesis in *C. albicans* is also coordinately regulated by a number of transcriptional repressors, including Nrg1, Tup1 and Rfg1 (Kadosh & Johnson, 2005). As negative regulators of filamentous growth, they repress the expression of genes associated with filamentous growth, and maintain *C. albicans* in yeast state in the absence of filament-inducing cues. Once the morphogenetic program is triggered, the transcriptional repression is relieved to facilitate the morphological transition (Kadosh & Johnson, 2005). As such, deletion of *NRG1*, *TUP1*, or *RFG1* results in constitutive filamentation and that is not affected by farnesol, a quorum sensing molecule produced by *C. albicans* to inhibit cAMP signaling pathway and the morphological transition. Based on transcriptional profiling, Nrg1 functions mostly through Tup1 while Rfg1 functions partially through Tup1 (Kadosh & Johnson, 2005). A less well characterized repressor of filamentation is the bHLH transcription factor Rbf1; deletion of *RBF1* also leads to constitutive filamentation in the absence of any filament-inducing cue (Aoki, Ishii, Watanabe, Yoshihara, & Arisawa, 1998; Khamooshi, Sikorski, Sun, Calderone, & Li, 2014), although the mechanisms involved remains elusive.

### 1.4 Thesis rationale

*C. albicans* thrives as an opportunistic fungal pathogen, despite the tremendous stress exerted by antifungal therapy and host responses during infection. The adaptive capacity of *C. albicans* to acquire drug resistance and transition between multiple morphological states is contingent upon its ability to mount robust stress responses, which allow the pathogen to both survive drug treatment and evade host immune responses. Given that stress response pathways are essential to *C. albicans* for adaptation and survival in the host, they have emerged as attractive therapeutic targets that could extend the lifespan of existing antifungal drugs. The aim of the research presented in this thesis is to dissect novel cellular circuitry that governs responses to antifungal treatment and that orchestrates the key virulence trait of morphogenesis.

Although a number of important regulators of cellular stress response have been implicated in drug tolerance and resistance in *C. albicans*, very few are fungal-specific. This applies to the
molecular chaperone Hsp90, protein kinase Pkc1, and protein phosphatase calcineurin
(LaFayette et al., 2010; S. D. Singh et al., 2009). The transcription factor Cas5 is a regulator of
stress response that is unique to fungi. Despite its crucial role in mediating tolerance to both
azoles and echinocandins (Bruno et al., 2006; Vasicek et al., 2014), very little is known about its
regulation and its relationship with existing stress response networks. The research presented
here seeks to: first, determine the mode of regulation that activates Cas5 through biochemical
analysis; second, identify Cas5 interacting partners using a targeted approach combined with an
unbiased approach; and third, elucidate the mechanism of Cas5-dependent drug resistance in a
systematic manner (Chapter 2). This study also revealed a novel and unexpected role for Cas5 in
the regulation of morphogenesis and cell cycle progression.

Another aspect of this research focuses on establishing key modulators of cellular stress response
in the regulation of *C. albicans* morphogenesis. To date, the cAMP signaling cascade remains to
be the central pathway that integrates multiple filament-inducing cues. An initial finding from
my work demonstrated that Pkc1 and components of the cell wall integrity pathway are required
for filamentous growth in response to diverse environmental cues (Chapter 3). Although
regulators of cell wall integrity have been implicated in hyphal formation (Corvest, Bogliolo,
Follette, Arkowitz, & Bassilana, 2013), the underlying mechanism remains elusive. In order to
identify effectors downstream of Pkc1 that mediate the morphological transition, I designed a
reporter system that enables selection for mutations that restore filamentation in nonfilamentous
mutants and used whole genome sequencing to determine the underlying mechanism. This work
defined novel regulatory circuitry essential for *C. albicans* morphogenesis that functions in
concert with the canonical cAMP signaling pathway.

Overall, this work provides novel insights into two cellular stress response pathways in *C.
albicans*. In addition to extensive characterization of Cas5 regulation, it provides the first
evidence for Cas5 as a regulator of morphogenesis and cell cycle. This work also reveals a novel
role for Pkc1 in filamentous growth, highlighting its potential as a therapeutic target. This
research complements and enhances our understanding of cellular stress response pathways, and
addresses their therapeutic potential as targets for antifungal drug development.
Chapter 2
Dephosphorylation of the Transcription Factor Cas5 Modulates Cell Cycle Progression and Antifungal Drug Resistance

2 Dephosphorylation of the Transcription Factor Cas5 Modulates Cell Cycle Progression and Antifungal Drug Resistance

2.1 Introduction

The fungal kingdom includes diverse pathogens that have a devastating impact on human health. One of the leading causative agents of fungal infections in humans is *Candida albicans* (Falgas, Roussos, & Vardakas, 2010; Pfaller & Diekema, 2007). It is an opportunistic fungal pathogen that exists as a commensal on the skin and mucosal surfaces of the respiratory and digestive tract of up to 60% of healthy individuals (Sanchez-Vargas et al., 2005). However, a decline in immunity or an imbalance in the host microbiome can result in *C. albicans* overgrowth, leading to diverse pathologies such as oral thrush, vaginal candidiasis, or life-threatening bloodstream infections that are associated with mortality rates of ~40% (Southern, Horbul, Maher, & Davis, 2008) (J. Kim & Sudbery, 2011). *C. albicans* thrives as a human pathogen in part due to its ability to evade host immunity by switching between a yeast and filamentous form, as well as due to its adaptive capacity to withstand the hostile host environment by activating robust stress responses (Mayer et al., 2013). The emerging paradigm is that stress response pathways in *C. albicans* are not only critical for adaptation to restrictive host conditions, such as changes in temperature, osmolarity, and oxidative state, but that they also play a key role in fungal virulence and drug resistance (Calderone RA, 2012; Cannon et al., 2007; Chamilos et al., 2009; LaFayette et al., 2010; S. D. Singh et al., 2009).

There is a very limited arsenal of antifungal drugs, and the emergence of resistance to these agents is on the rise, becoming a major impediment to the treatment of fungal infections (CDC, 2013; Mishra et al., 2007; Ruggiero & Topal, 2014). A poignant example of this problem is that resistance has already emerged in the clinic to the echinocandins, which are the only new class of antifungal to be approved in recent decades (Marco, Pfaller, Messer, & Jones, 1998; Walker et al., 2010). The echinocandins target the biosynthesis of β-1,3-glucan in the fungal cell wall via
non-competitive inhibition of the glucan synthase, Fks1, thereby compromising cell wall integrity. The most common mechanism of resistance to the echinocandins is through hot-spot mutations in the drug target gene \textit{FKS1} (S. Park et al., 2005; Perlin, 2007b). Resistance phenotypes are also modulated by fungal cellular stress responses, which enhance antifungal tolerance and enable the emergence of drug resistance (Cannon et al., 2007; LaFayette et al., 2010; S. D. Singh et al., 2009). Targeting core hubs in cellular circuitry that control responses to stress, such as the molecular chaperone Hsp90 or protein phosphatase calcineurin, has emerged as a powerful strategy to enhance antifungal activity against diverse fungi, including those that have evolved drug resistance in the human host (S. D. Singh et al., 2009; Walker et al., 2010).

In the host, \textit{C. albicans} mobilizes a diverse array of environmental stress response programs through the action of transcription factors. For example, in response to elevated temperature, such as that associated with fever, the heat shock factor Hsf1 is activated to protect against misfolded proteins by modulating the expression of key molecular chaperones (Nicholls, Leach, Priest, & Brown, 2009; Zapater, Clotet, Escote, & Posas, 2005). In response to reactive oxygen species produced by the host immune cells, the bZip transcription factor Cap1 is activated to protect against oxidative stress by upregulating antioxidant genes (Komalapriya et al., 2015). In response to membrane stress caused by azoles, a major class of antifungal drugs that targets ergosterol biosynthesis, the protein phosphatase calcineurin activates the zinc finger transcription factor Crz1, leading to the induction of calcineurin-dependent stress response genes (Santos & de Larrinoa, 2005; Stathopoulos-Gerontides et al., 1999). Another classic example is the cell wall stress-dependent activation of the MADS-box transcription factor Rlm1 by the cell wall integrity MAP kinase Mpk1/Slt2 in the model yeast \textit{S. cerevisiae} (Jung, Sobering, Romeo, & Levin, 2002). Although Rlm1 is the main transcriptional regulator of cell wall stress response in \textit{S. cerevisiae}, it does not have a conserved function in \textit{C. albicans} (Bruno et al., 2006). The \textit{C. albicans} zinc finger transcription factor Cas5, which lacks an ortholog in \textit{S. cerevisiae}, has been identified as a key transcriptional regulator of cellular responses to cell wall stress, such as that induced by echinocandins (Bruno et al., 2006), however, the mechanism by which Cas5 is regulated in response to cell wall stress remains enigmatic.

Here, I have uncovered the dynamic regulation of Cas5 in response to the cell wall stress and explored the mechanisms by which Cas5 mediate echinocandin resistance. I also described an unexpected yet crucial role for Cas5 in governing cell cycle progression. Specifically, I
discovered that the type I protein phosphatase Glc7 dephosphorylates Cas5. This change in post-translational modification activates Cas5 in response to cell wall stress, including exposure to echinocandins, thereby inducing a transcriptional program to mitigate cell wall stress. Cas5 physically interacts with the SBF complex members Swi4 and Swi6 to co-regulate the expression of genes involved in cell wall biosynthesis, as well as cell cycle-dependent G1/S genes. Thus, my work illuminates a novel mechanism through which cell wall remodeling and cell cycle progression are coupled, and highlights new strategies to abrogate drug resistance of a leading human fungal pathogen.

2.2 Materials and Methods

2.2.1 Strains and culture conditions

All *C. albicans* strains were archived in 25% glycerol and stored at -80°C. Strains were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) at 30°C. All cultures were grown shaking at 200 rpm. 2% agar was added for solid media. Strains used in this study is listed in Table 2.1.

**Table 2.1 Strains used in this study.**

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaLC191 (DAY185)</td>
<td>pARG4::URA3::arg4::hisG/arg4::hisG pHIS1::his1::his1/his1::hisG</td>
<td>(D. Davis, Edwards, Mitchell, &amp; Ibrahim, 2000)</td>
</tr>
<tr>
<td>CaLC1349</td>
<td>DAY185 cas5::ARG4/cas5::URA3</td>
<td>(Bruno et al., 2006)</td>
</tr>
<tr>
<td>CaLC1350</td>
<td>DAY185 cas5::ARG4/cas5::URA3 pCAS5::HIS1::his1::hisG/his1::hisG</td>
<td>(Bruno et al., 2006)</td>
</tr>
<tr>
<td>CaLC239 (SN95)</td>
<td>arg4Δ/arg4Δ his1Δ/his1Δ URA3/ura3Δ::imm434 IRO1/iro1::imm434</td>
<td>(Noble &amp; Johnson, 2005)</td>
</tr>
<tr>
<td>CaLC2034</td>
<td>SN95 cas5::FRT/CAS5</td>
<td>This study</td>
</tr>
<tr>
<td>CaLC2056</td>
<td>SN95 cas5::FRT/cas5::FRT</td>
<td>This study</td>
</tr>
<tr>
<td>CaLC1255</td>
<td>CaLC206 pkc1::FRT/pkc1::FRT</td>
<td>(LaFayette et al., 2010)</td>
</tr>
<tr>
<td>CaLC1256</td>
<td>CaLC206 PKC1-FRT/pkc1::FRT</td>
<td>(LaFayette et al., 2010)</td>
</tr>
<tr>
<td>Slot</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>CaLC3076</td>
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iro1/iro1::imm434 his1::hisG/his1::hisG  
| CaLC4285 | DAY286 CAS5-HA-HIS1/CAS5                                                     | This study                                          |
| CaLC192 (BWP17) | ura3::imm434/ura3::imm434  
iro1/iro1::imm434 his1::hisG/his1::hisG  
| CaLC4182 | BWP17 cbk1::Tn7-UAU1/cbk1::Tn7-URA3                                         | (Blankenship, Fanning, Hamaker, & Mitchell, 2010)  |
| CaLC4514 | CaLC4182 CAS5-HA-HIS1/CAS5 | This study |
| CaLC3676 | SN95 GLC7/glc7::FRT | This study |
| CaLC3932 | SN95 CaTAR-FRT-tetO-GLC7/glc7::FRT | This study |
| CaLC3952 | CaLC3932 CAS5-HA-HIS1/CAS5 | This study |
| CaLC2087 | SN95 FKS1/FKS1 T1917C;T1922C | [6] |
| CaLC3857 | CaLC2087 CAS5/cas5::FRT | This study |
| CaLC3908 | CaLC2087 cas5::FRT/cas5::FRT | This study |
| CaLC3909 | CaLC2087 cas5::FRT/cas5::FRT | This study |
| CaLC4249 | CaLC3908 tetO-FKS1/FKS1 A | This study |
| CaLC4250 | CaLC3908 tetO-FKS1/FKS1 B | This study |
| CaLC4007 | CaLC3908 CF-R | This study |
| CaLC4010 | CaLC3909 CF-R | This study |
| CaLC3672 | CaLC2056 CAS5S462A/S464A/S472A/S476A-HA-HIS/cas5::FRT | This study |
| CaLC3673 | CaLC2056 CAS5S462A/S464A/S472A/S476A-HA-HIS/cas5::FRT | This study |
| CaLC3693 | CaLC2056 CAS5S462E/S464E/S472E/S476E-HA-HIS/cas5::FRT | This study |
| CaLC3694 | CaLC2056 CAS5S462E/S464E/S472E/S476E-HA-HIS/cas5::FRT | This study |
| CaLC3695 | CaLC2056 CAS5S462E/S464E/S472E/S476E-HA-HIS/cas5::FRT | This study |
| CaLC2148 | SN95 cna1::FRT/cna1::FRT CAS5-HA-HIS/CAS5 | This study |
| CaLC4471 | SN95 SWI4-TAP-ARG4/swi4::FRT | This study |
| CaLC4499 | SN95 SWI6-TAP-ARG4/swi6::FRT | This study |
2.2.2 Strain construction

**CaLC2034:** To generate a CAS5 heterozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser, Michel, & Staib, 1999) was PCR amplified using primers oLC2017 and oLC2018 (4366 bp) and transformed into CaLC239 (SN95). NAT resistant transformants were PCR tested with oLC275 + oLC2034 (673 bp) for upstream integration and oLC274 + oLC2035 (1158 bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC2056:** To generate a CAS5 homozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC2017 and oLC2018 (4366 bp) and transformed into CaLC2034. NAT resistant transformants were PCR tested with oLC275 + oLC2034 (673 bp) for upstream integration and oLC274 + oLC2035 (1158 bp) for downstream integration. The presence of deleted allele was verified by amplification with primer pairs oLC2034 + oLC2035 (deleted allele - 1421 bp, WT allele - 3888 bp), and absence of wild type allele was verified by amplification with primer pairs oLC2047 + oLC2048 (230 bp). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3076:** To introduce the M850G mutation into PKC1, the PKC1 complementation vector carrying the M850G mutation was released from pLC770 with BssHII and transformed into CaLC1255. NAT resistant transformants were PCR tested with oLC275 + oLC1042 (660 bp) for upstream integration and oLC274 + oLC1030 (555 bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3113:** To C-terminally HA tag CAS5 in a mutant heterozygous for CAS5, the HA-HIS1 cassette was PCR amplified from pLC575 (Lavoie, Sellam, Askew, Nantel, & Whiteway, 2008) using primers oLC2161 and oLC2162 (1724 bp) and transformed into CaLC2034. HIS prototrophic transformants were PCR tested with C2163 + oLC2029 (462 bp) for upstream integration and oLC2164 + oLC1645 (902 bp) for downstream integration.

**CaLC3151:** To C-terminally HA tag CAS5 in a mutant heterozygous for CAS5, the CAS5-HA-HIS cassette was released from pLC790 with ApaI and transformed into CaLC2034. HIS prototrophic
transformants were PCR tested with C2163 + oLC2029 (462 bp) for upstream integration and oLC2164 + oLC1645 (902 bp) for downstream integration. This strain shares the same genotype as CaLC3113 but was generated as a control for strains made using plasmid with pLC790 as the backbone, such pLC791 and CaLC800.

**CaLC2213**: To C-terminally HA tag **CAS5** in SN95, the **HA-HIS1** cassette was PCR amplified from pLC575 (Lavoie et al., 2008) using primers oLC2161 and oLC2162 (1724 bp) and transformed into CaLC239 (SN95). HIS prototrophic transformants were PCR tested with C2163 + oLC2029 (462 bp) for upstream integration and oLC2164 + oLC1645 (902 bp) for downstream integration.

**CaLC3044**: To C-terminally HA tag the both alleles of **CAS5**, the **HA-ARG4** cassette was PCR amplified from pLC576 (Lavoie et al., 2008) using primers oLC2161 and oLC2162 (2468 bp) and transformed into CaLC2213. HIS and ARG prototrophic transformants were PCR tested with C2163 + oLC2029 (462 bp) for upstream integration and oLC2164 + oLC1645 (902 bp) for downstream integration.

**CaLC3209**: To introduce a mutant allele of **CAS5** carrying the S769E mutation, the **CAS5^{S769E}-HA-HIS1** cassette was released from pLC791 with ApaI and transformed into CaLC2034. HIS prototrophic transformants were PCR tested with oLC3052 + oLC2029 (927 bp) for upstream integration and oLC2164 + oLC1645 (906 bp) for downstream integration. The absence of WT was verified with oLC3052 + oLC2164 (WT - 925 bp). The S769E mutation was sequence verified with oLC2029.

**CaLC3189**: To introduce a mutant allele of **CAS5** carrying the S769A mutation, the **CAS5^{S769A}-HA-HIS** cassette was released from pLC800 with ApaI and transformed into CaLC2034. HIS prototrophic transformants were PCR tested with oLC3052 + oLC2029 (927 bp) for upstream integration and oLC2164 + oLC1645 (906 bp) for downstream integration. The absence of WT was verified with oLC3052 + oLC2164 (WT - 925 bp). The S769A mutation was sequence verified with oLC2029.

**CaLC3679**: To generate a **SWI4** heterozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC3472 and oLC3473 (4366 bp) and transformed into CaLC239 (SN95). NAT resistant transformants were PCR tested
with oLC275 + oLC3474 (1196 bp) for upstream integration and oLC274 + oLC3427 (566 bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4036:** To generate a *SWI4* homozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC3472 and oLC3473 (4366 bp) and transformed into CaLC3679. NAT resistant transformants were PCR tested with oLC275 + oLC3474 (1196 bp) for upstream integration and oLC274 + oLC3427 (566 bp) for downstream integration. The absence of WT allele was verified with oLC3426 + oLC3427 (WT - 668 bp) and the presence of deleted allele was verified by oLC3473 and oLC3427 (1593 bp). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3680:** To generate a *SWI6* heterozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC3641 and oLC3642 (4366 bp) and transformed into CaLC239 (SN95). NAT resistant transformants were PCR tested with oLC275 + oLC3645 (654 bp) for upstream integration and oLC274 + oLC3654 (671 bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4330:** To generate a *SWI6* homozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC3641 and oLC3642 (4366 bp) and transformed into CaLC3680. NAT resistant transformants were PCR tested with oLC275 + oLC3645 (654 bp) for upstream integration and oLC274 + oLC3654 (671 bp) for downstream integration. The absence of WT allele and the presence of deleted allele was verified with oLC3645 + oLC3431 (3290 bp for WT allele and 1243 bp for deleted allele). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4019:** To generate a *NDT80* heterozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC4036 and oLC4037 and transformed into CaLC239 (SN95). NAT resistant transformants were PCR tested with oLC4049 + oLC275 (628 bp) for upstream integration and oLC274 + oLC4018 (630 bp) for
downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4319**: To generate a NDT80 homozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC4036 and oLC4037 and transformed into CaLC4019. NAT resistant transformants were PCR tested with oLC4049 + oLC275 (628 bp) for upstream integration and oLC274 + oLC4018 (630 bp) for downstream integration. The absence of the WT allele and presence of deleted allele was verified with oLC4049 + oLC4018 (2364 bp for WT allele and 840 bp for deleted allele). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3391**: To C-terminally TAP tag Swi4, the TAP-ARG4 cassette was PCR amplified from pLC573 (Lavoie et al., 2008) using primers oLC3424 and oLC3425 (2468 bp) and transformed into CaLC239. ARG prototrophic transformants were PCR tested with oLC3426 + oLC1593 (591 bp) for upstream integration and oLC1594 + oLC3427 (762 bp) for downstream integration.

**CaLC3393**: To C-terminally TAP tag Swi6, the TAP-ARG4 cassette was PCR amplified from pLC573 (Lavoie et al., 2008) using primers oLC3428 and oLC3429 (2468 bp) and transformed into CaLC239. ARG prototrophic transformants were PCR tested with oLC3430 + oLC1593 (740 bp) for upstream integration and oLC1594 + oLC3431 (1051 bp) for downstream integration.

**CaLC3395**: To C-terminally TAP tag Swi4 in a strain with HA tagged Cas5, the TAP-ARG4 cassette was PCR amplified from pLC573 (Lavoie et al., 2008) using primers oLC3424 and oLC3425 (2468 bp) and transformed into CaLC3151. HIS and ARG prototrophic transformants were PCR tested with oLC3426 + oLC1593 (591 bp) for upstream integration and oLC1594 + oLC3427 (762 bp) for downstream integration.

**CaLC3398**: To C-terminally TAP tag Swi6 in a strain with HA tagged Cas5, the TAP-ARG4 cassette was PCR amplified from pLC573 (Lavoie et al., 2008) using primers oLC3428 and oLC3429 (2468 bp) and transformed into CaLC239. HIS and ARG prototrophic transformants were PCR tested with oLC3430 + oLC1593 (740 bp) for upstream integration and oLC1594 + oLC3431 (1051 bp) for downstream integration.
**CaLC4285:** To C-terminally HA tag **CAS5** in DAY286, the **HA-HIS1** cassette was PCR amplified from pLC575 (Lavoie et al., 2008) using primers oLC2161 and oLC2162 (1724 bp) and transformed into CaLC1900 (DAY286). HIS prototrophic transformants were PCR tested with oLC2163 + oLC2164 (2198 bp for tagged allele and 458 bp for untagged allele).

**CaLC4514:** To C-terminally HA tag **CAS5** in the **cbk1** transposon insertion mutant, the **HA-HIS1** cassette was PCR amplified from pLC575 (Lavoie et al., 2008) using primers oLC2161 and oLC2162 (1724 bp) and transformed into CaLC1900 (DAY286). HIS prototrophic transformants were PCR tested with C2163 + oLC2029 (462 bp) for upstream integration and oLC2164 + oLC1645 (902 bp) for downstream integration.

**CaLC3676:** To generate a **GLC7** heterozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC3490 and oLC3491 (4366 bp) and transformed into CaLC239 (SN95). NAT resistant transformants were PCR tested with oLC275 + oLC3558 (640 bp) for upstream integration and oLC274 + oLC3561 (639 bp) for downstream integration. The **SAP2** promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3932:** To regulate the expression of **GLC7**, the tetracycline-repressible transactivator, the tetO promoter, and the NAT flipper cassette were PCR amplified from pLC605 (Leach & Cowen, 2014) using primers oLC3793 and oLC3492 (4957 bp) and transformed into CaLC3676. NAT resistant transformants were PCR tested with oLC3794 + oLC534 (344 bp) for upstream integration and oLC274 + oLC3495 (1047 bp) for downstream integration. The absence of WT **GLC7** promoter is verified with oLC3794 + oLC3495 (685 bp for WT promoter) and the presence of a deleted allele is verified with oLC3794 + oLC3494 (1100 bp). The **SAP2** promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3952:** To C-terminally HA tag **CAS5** in the **GLC7** repressible strain, the **HA-HIS1** cassette was PCR amplified from pLC575 (Lavoie et al., 2008) using primers oLC2161 and oLC2162 (1724 bp) and transformed into CaLC3932. HIS prototrophic transformants were PCR tested with oLC2163 + oLC2164 (2198 bp for tagged allele and 458 bp for untagged allele).

**CaLC3857:** To generate a **CAS5** heterozygous deletion mutant in a strain harboring the **F641S** mutation in **FKS1**, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR
amplified using primers oLC2017 and oLC2018 (4366 bp) and transformed into CaLC239 (SN95). NAT resistant transformants were PCR tested with oLC275 + oLC2034 (673 bp) for upstream integration and oLC274 + oLC2035 (1158 bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3908/CaLC3909:** To generate a CAS5 homozygous deletion mutant in a strain harboring the *F641S* mutation in *FKS1*, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC2017 and oLC2018 (4366 bp) and transformed into CaLC2034. NAT resistant transformants were PCR tested with oLC275 + oLC2034 (673 bp) for upstream integration and oLC274 + oLC2035 (1158 bp) for downstream integration. Absence of WT allele was confirmed with oLC2034 + oLC2164 (3201 for the WT allele, 651 bp for the deleted allele, and 4961 bp for the allele replaced by NAT). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4249/CaLC4250:** To regulate the expression of *FKS1* in a mutant lacking CAS5, the tetracycline-repressible transactivator, the tetO promoter, and the NAT flipper cassette were PCR amplified from pLC605 (Leach & Cowen, 2014) using primers oLC3952 and oLC3953 (4957 bp) and transformed into CaLC2056. NAT resistant transformants were PCR tested with oLC3955 + oLC534 (497 bp) for upstream integration and oLC3956 + oLC300 (1018 bp) for downstream integration. The absence of WT GLC7 promoter is verified with oLC3794 + oLC3495 (685 bp for WT promoter) and the presence of a deleted allele is verified with oLC3794 + oLC3494 (1100 bp). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4007:** To select for spontaneous mutations that confer resistance to caspofungin, CaLC3908 was spread on YPD plates supplemented with 1 µg/ml of caspofungin at 2 x 10^7 cells. Resistant colonies were patched on YPD plate supplemented with 1 µg/ml of caspofungin and the resistance phenotype was confirmed by MIC.

**CaLC4010:** To select for spontaneous mutations that confer resistance to caspofungin, CaLC3908 was spread on YPD plates supplemented with 1 µg/ml of caspofungin at 2 x 10^7 cells. Resistant colonies were patched on YPD plate supplemented with 1 µg/ml of caspofungin and the resistance phenotype was confirmed by MIC.
CaLC3693/CaLC3694/CaLC3695: To introduce a mutant allele of CAS5 carrying S462E, S464E, S472E, and S476E mutations, the CAS5S462E/S464E/S472E/S476E-HA-HIS cassette was released from pLC857 with SacII and transformed into CaLC2034. HIS prototrophic transformants were PCR tested with oLC3052 + oLC2029 (927 bp) for upstream integration and oLC2164 + oLC1645 (906 bp) for downstream integration. The absence of untagged WT CAS5 allele was verified with oLC3052 + oLC2164 (WT - 925 bp). The S462A/S464A/S472A/S476A mutations were sequence verified with oLC3371.

CaLC3672/CaLC3673: To introduce a mutant allele of CAS5 carrying S462A, S464A, S472A, and S476A mutations, the CAS5S462A/S464A/S472A/S476A-HA-HIS cassette was released from pLC858 with SacII and transformed into CaLC2034. His prototrophic transformants were PCR tested with oLC3052 + oLC2029 (927 bp) for upstream integration and oLC2164 + oLC1645 (906 bp) for downstream integration. The absence of untagged WT CAS5 allele was verified with oLC3052 + oLC2164 (WT - 925 bp). The S462A/S464A/S472A/S476A mutations were sequence verified with oLC3371.

CaLC2148: To C-terminally HA tag CAS5 in a mutant lacking CNA1, the HA-HIS cassette was PCR amplified from pLC575 (Lavoie et al., 2008) using primers oLC2161 and oLC2162 (1724 bp) and transformed into CaLC909 (S. D. Singh et al., 2009). His prototrophic transformants were PCR tested with C2163 + oLC2029 (462 bp) for upstream integration and oLC2164 + oLC1645 (902 bp) for downstream integration.

CaLC4471: To C-terminally TAP tag Swi4 in a mutant heterozygous for SWI4, the TAP-ARG4 cassette was PCR amplified from pLC573 (Lavoie et al., 2008) using primers oLC3424 and oLC3425 (2468 bp) and transformed into CaLC3151. ARG prototrophic transformants were PCR tested with oLC3426 + oLC3427 (3458 bp for the presence of tagged allele and 668 bp for the absence of WT allele).

CaLC4499: To C-terminally TAP tag Swi6 in a mutant heterozygous for SWI6, the TAP-ARG4 cassette was PCR amplified from pLC573 (Lavoie et al., 2008) using primers oLC3428 and oLC3429 (2468 bp) and transformed into CaLC239. HIS prototrophic transformants were PCR tested with oLC3430 + oLC1593 (740 bp) for upstream integration and oLC3430 + oLC3431 (3896 bp for the presence of tagged allele and 1106 bp for the absence of WT allele).
2.2.3 Plasmids

All bacterial strains carrying plasmids were archived in 33% glycerol and stored at -80°C. Strains were grown in LB broth at 37°C. 2% agar was added for solid media. Plasmids used in this study are listed in Table 2.2. The absence of nonsense mutations on the plasmid was verified by sequencing. Primers used in this study are listed in Table 2.3.

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2.2.4 Plasmid construction

Cloning procedures were performed following standard protocols.
**pLC706**: This is a construct to complement WT CaPKC1 in C. albicans. Homology downstream of PKC1 was amplified from SC5314 genomic DNA with primers oLC1029/oLC1030 (bp) and cloned into pLC49 at NotI and SacII. The presence of the inserts were tested by PCR with oLC274/oLC1030 (538 bp) and oLC1029/oLC244 (473 bp). Downstream homology was sequenced verified with oLC244 and oLC274. PKC1 with a promoter region and some of the terminator region was amplified from SC5314 genomic DNA with oLC1027/oLC1028 (4138 bp) and cloned into pLC49 containing the downstream homology of PKC1 at ApaI. The presence of the inserts were tested by PCR with oLC243/oLC956 (942 bp) and oLC275/oLC1042 (605 bp). Upstream homology and ORF was sequence verified with oLC243, 1027, 1035, 1036, 1037, 1038, 1039, 1040, 1041 and downstream homology was re-verified with oLC244. The reconstitution construct can be liberated by digestion with BsshII.

**pLC770**: This is a construct to complement mutant CaPKC1 carrying the M850G mutation in C. albicans. This plasmid is based on pLC706 but harbors a mutation in Pkc1 (M850G) that will not affect the kinase activity but render the kinase susceptible to 1-NA-PP1. This mutation was introduced by site-directed mutagenesis with primers oLC2998 and oLC2999. The clone was sequence verified with the following primers: oLC243, oLC1027, oLC1035, oLC1036, oLC1037, oLC1038, oLC1039, oLC1040, and oLC1041. The reconstitution construct can be liberated by digestion with BsshII.

**pLC790**: This is a construct to C-terminally HA-tag Cas5 in C. albicans. The CAS5 DNA binding domain, HA and HIS marker were amplified from CaLC3113 genomic DNA with primers oLC3092/oLC3052 (2834 bp) and cloned into pLC49 at ApaI site. The ligation mixture was transformed into TOP10 cells because no colonies appeared with DH5alpha. Cells were plated on LB + amp + NAT and incubated for 48 hours at room temperature. The amp and NAT resistant colonies were minipreped (with overnight at 25°C) and sent for sequencing with oLC2029. Sequence verified plasmid was retransformed into TOP10 and a single colony was cultured for miniprep and archive. The plasmid was sequence-verified once more with oLC2029. This construct can be liberated by digestion with ApaI and transformed into Candida albicans.

**pLC791**: This plasmid is based on pLC706 but harbors a phosphomimetic mutation in Cas5 (S769E). This mutation was introduced by site-directed mutagenesis with primers oLC2986 and oLC2987 (10 minutes extension, 18 cycles, used 50 ng of plasmid DNA for SDM PCR, and
transformed 1 ul of DpnI digested product into TOP10, all cells were plated). The clone was sequence verified with oLC2029. This construct can be liberated by digestion with ApaI and transformed into *Candida albicans*.

**pLC800:** This plasmid is based on pLC790 but harbors a phosphomimetic mutation in Cas5 (S772E) that may stabilize Cas5 in *C. albicans* mutant lacking Pkc1. This mutation was introduced by site-directed mutagenesis with primers oLC2990 and oLC2991 (10 minutes extension, 18 cycles, used 50 ng of plasmid DNA for SDM PCR, and transformed 1 ul of DpnI digested product into TOP10, all cells were plated). The plasmid was sequence verified with oLC2029. This construct can be liberated by digestion with ApaI and transformed into *Candida albicans*.

**pLC818:** This is a construct to C-terminally HA-tag CaCas5 in *C. albicans*. CAS5 orf, HA and HIS were amplified from CaLC3113 genomic DNA (4665 bp) with primers oLC3365/oLC3366 and cloned into pLC49 at SacII site. The ligation mixture was transformed into TOP10 cells because no colonies appeared with DH5alpha. Cells were plated on LB + amp + NAT and incubated for 48 hours at room temperature. The amp and NAT resistant colonies were minipreped (with overnight at 25°C) and sent for sequencing with oLC2029/oLC244/oLC3371. Sequence verified plasmid was retransformed into TOP10 and a streak of colonies was cultured for miniprep and archive. The plasmid was sequence-verified once more with oLC2029/oLC244/oLC3371. This construct can be liberated by digestion with SacII and transformed into *Candida albicans*.

**pLC828:** This plasmid is based on pLC818 but harbors a phosphomimetic mutation in Cas5 (S472E/S476E). This mutation was introduced by site-directed mutagenesis with primers oLC3410 and oLC3411 (12 minutes extension, 18 cycles, used 50 ng of plasmid DNA for SDM PCR, and transformed 5 ul of DpnI digested product into TOP10, all cells were plated). The plasmid was sequence verified with oLC3371. This construct can be liberated by digestion with SacII and transformed into *Candida albicans*.

**pLC857:** This plasmid is based on pLC828 but harbors additional phosphomimetic mutations in Cas5 (S462E/S464E). This mutation was introduced by site-directed mutagenesis with primers oLC3416 and oLC3417 (12 minutes extension, 18 cycles, used 50 ng of plasmid DNA for SDM PCR, and transformed 5 ul of DpnI digested product into TOP10, all cells were plated). The
plasmid was sequence verified with oLC3371. This construct can be liberated by digestion with SacII and transformed into *Candida albicans*.

**pLC833**: This plasmid is based on pLC818 but harbors a phosphomimetic mutation in Cas5 (S472A/S476A). This mutation was introduced by site-directed mutagenesis with primers oLC3412 and oLC3413 (12 minutes extension, 18 cycles, used 50 ng of plasmid DNA for SDM PCR, and transformed 5 ul of DpnI digested product into TOP10, all cells were plated). The clone was sequence verified with oLC3371. This construct can be liberated by digestion with SacII and transformed into *Candida albicans*.

**pLC858**: This plasmid is based on pLC833 but harbors additional phosphodeficient mutations in Cas5 (S462A/S464A). This mutation was introduced by site-directed mutagenesis with primers oLC3418 and oLC3419 (12 minutes extension, 18 cycles, used 50 ng of plasmid DNA for SDM PCR, and transformed 5 ul of DpnI digested product into TOP10, all cells were plated). The plasmid was sequence verified with oLC3371. This construct can be liberated by digestion with SacII and transformed into *Candida albicans*.

### 2.2.5 Oligonucleotides

**Table 2.3 Oligonucleotides used in this study.**

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**Note:** The sequences are likely DNA or RNA sequences, and may represent regions of interest for genetic analysis or cloning. The table format provides a structured way to display these sequences for easier reference and comparison.
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### 2.2.6 Experimental growth conditions

To assess the effects of caspofungin and calcofluor white treatment, overnight cultures were diluted to OD$_{600}$ of 0.15 in YPD for 3 hrs and either left untreated or treated with 125 ng/ml of caspofungin or 100 µg/ml of calcofluor white for 1 hour or 2 hours, as specified. Caspofungin was generously provided by Rochelle Bagatell and was diluted to 100 µg/ml stock. Calcofluor white (Fluorescent Brightener 28, Sigma-Aldrich) was formulated in water as a 10 mg/ml stock. To inhibit Pkc1<sup>M850G</sup>, 1-NA-PP1 (Cayman Chemical, CAS #221243-82-9) was formulated in DMSO as 5 mM stock and used at a final concentration of 5 µM.

To deplete GLC7, strains were grown overnight at 30°C in YPD. Stationary phase cultures were split, adjusted to an OD$_{600}$ of 0.15 where one culture was treated with 0.02 µg/ml of doxycycline, while the other was left untreated. Cells were grown for 16 hours and were split again, adjusted to an OD$_{600}$ of 0.15 and grown for 3 hours. One culture was subsequently treated with 125 ng/ml of caspofungin for 1 hour or 2 hours, as specified, while the other was left untreated. Doxycycline (DOX, BD Biosciences #631311) was formulated in a 20 mg/ml stock in water and used at a final concentration of 0.02 µg/ml or 1 µg/ml as specified.
2.2.7 Minimum Inhibitory Concentration (MIC) Assay

Antifungal susceptibility was performed in flat bottom, 96-well microtitre plates (Sarstedt # 83.3924) using a broth microdilution protocol described in (Jinglin L. Xie, Singh-Babak, & Cowen, 2012). Minimum inhibitory concentration (MIC) assays were set up in 2-fold serial dilutions of caspofungin or calcofluor white in a final volume of 200 µl per well. Caspofungin gradients were diluted either from 125 ng/ml down to 0.12 ng/ml or 2000 ng/ml to 3.9 ng/ml. Where applicable, doxycycline was added to a final concentration of 0.1 µg/ml. Cell densities of overnight cultures were determined by measuring 100 µl of the culture in the 96-well plate in duplicates and dilutions were prepared such that ~10^3 cells were inoculated into each well. Calcofluor white gradients were diluted from 250 µg/ml down to 0.24 µg/ml. Plates were incubated in the dark at 30°C for 48 hours, at which point the absorbance was determined at 600 nm using a spectrophotometer (Molecular Devices) and was corrected for background from the corresponding medium. Each strain was tested in duplicate in three biological replicates. MIC data was quantitatively displayed with color using Java TreeView 1.1.3 (http://jtreeview.sourceforge.net).

2.2.8 Indirect Immunofluorescence

To obtain cells for immunofluorescence, 5 – 10 ml of subculture was grown to mid exponential phase (OD_{600} between 0.5 and 0.8) and fixed with 5% formaldehyde for 3 – 4 hours (varies depending on the strain) at 30°C. Cells were harvested at 3000 rpm for 5 minutes and washed once with 5 ml S-Buffer (50 mM HEPES pH 7.5, 1.2 M Sorbitol) before resuspended in 1ml S-Buffer. To induce spheroplast formation, 10 µl of 1M DTT, 30 µl of gluosulase and 40 µl of 2.5 mg/ml zymolase were added to the cells and the mix was incubated for 30 minutes at 37°C. The extent of spheroplasting is monitored under the microscope. The poly lysine coated slides were prepared by adding 15 µl of 0.1% poly Lys per well in the slides and set aside for 15 minute. Wash the wells three times with phosphate buffered saline (PBS). The fixed cells were centrifuged for 1 minute at 5000 rpm and gently resuspended in 1 ml S-buffer. Following the addition of 0.1% Triton X-100, the mix was incubated for 5 minutes on rocker. The cells were centrifuged for 1 minute at 5000 rpm and resuspended in 1 ml S-Buffer. To adhere the cells, 20 µl of cell suspension was added on to poly Lys coated well and incubated it for 15 min. The cells were washed three times with PBST and the wells were blocked with 20 µl PBS/BSA for 5 – 10 minute. Anti-HA antibody diluted 1:300 in 20 µl was added and incubated for 3 – 4 hours or
overnight in a humid chamber. The cells were washed 4 times in PBST. Anti-mouse IgG-Cy3 diluted 1:500 in PBST was added to the wells and incubated for 1 – 2 hours. The cell were washed 4 times with PBST and once with only PBS. 20 µl of 1 mg/ml DAPI diluted 1:1000 in PBS was added to the wells and incubated for 5 minutes. The cells were washed 4 times with PBS. The cells were left to dry in the dark for 30 minutes at room temperature. The mounting media was added and cells were viewed under the microscope. The slides can be stored in -20°C.

2.2.9 Western blot analysis

Protein was extracted by pelleting cells at OD$_{600}$ of 0.8 and the pellet was resuspended in 2x sample buffer (one-third volume of 6x sample buffer containing 0.35 M Tris-HCl, 10% (w/w) SDS, 36% glycerol, 5% β-mercaptoethanol, and 0.012% bromophenol blue). The samples were boiled for 5 minutes at 95°C. The cell debris was pelleted and the supernatant can be stored in the sample buffer at -20°C or separated on a 6% SDS-PAGE gel to observe changes in Cas5 band size. Resolved proteins were electrotransferred to PVDF membrane (Bio-Rad Laboratories, Inc.) and blocked with 5% skim milk in PBS with 0.2% tween-20 at room temperature for 1 hour. Blots were hybridized with antibody against HA (1:5000 dilution; Roche Diagnostics), TAP (1:5,000; Open Biosystems), or alpha-tubulin (1:1000; AbD Serotec, MCA78G) overnight at 4°C.

2.2.10 2-Dimensional gel electrophoresis

Protein was extracted by pelleting 50 ml of culture at 4,000rpm for 5 minutes and the pellet was washed with 1 - 1.5mls of H$_2$O and transfer to a 1.5 ml eppendorf tube. Cells were spun for 5 minutes at 4,000 rpm and washed 2 times with 1 - 1.5mls H$_2$O. The washed pellet was frozen at -80°C. Depending on the pellet size, cells were resuspended in 200 – 400 µls of lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 15 mM ethylene glycol tetraacetic acid, 1% Triton 100-X, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 60 mM β-glycerophosphate, EDTA-free protease inhibitor mix (11836170001), and Phosphatase Inhibitor Cocktail #78420 Thermo Scientific) (Gutierrez-Escribano et al., 2011). Glass beads were added until all liquid has been submerged under the beads and cells were disrupted with mini-bead beater for 15 x 30 seconds with 1 minute on ice in between beatings. The sample was centrifuged for 10 minutes at 14,000 rpm and the supernatant was transferred to a new 1.5 ml eppendorf tube. The lysate was centrifuged for 5 min at 4,000 rpm and the supernatant was transferred to a fresh 1.5 ml eppendorf tube.
Subsequently, a methanol:chloroform extraction was performed and the protein-precipitate was dried in a speedvac for no more than 5 minutes and re-solubilized in 131uls of BioRad Rehydration/Sample Buffer (#1632106). 5 μl of the sample was added to 1 ml of 1x Bio-rad Protein Assay Dye Reagent Concentrate (diluted in ddH2O) (#500-0006) and protein concentration is determined by measuring absorbance. Approximately 169 μg of protein (125ul) was loaded into 7 cm IPG strip (BioRad, Ready Strip IPG strips #163-2000). Rehydration/Sample Buffer was used to adjust protein concentration and subsequent steps were performed per instruction manual for Biorad ReadyPrep 2-D Starter Kit Instructional Manual #163-2105. The first dimension (isoelectric separation) ran for approximately 5 hours before frozen at -80°C. The second dimension (size/charge separation) ran for 10 minutes at 65 mv and 40 minutes at 175 mv. The proteins were transferred to a nitrocellulose membrane #9004-70-0 for 2hrs at 0.2 amps. The blot was blocked in 10 – 15ml of 5% non-fat dry milk (a. Blotting-Grade Blocker #170-6404) in PBST for 1 hour shaking. The blot was probed with ant-HA antibody diluted 1:5,000 in 10 ml – 15 ml of 5% nonfat dry milk in PBST overnight at 4°C shaking. The blots were washed 3 times with 15 ml – 20 ml of PBST for 10-15mins and imaged using Amersham ECL Western blotting detection reagents (Amersham ECL Prime # 45-002-401).

2.2.11 Phosphoshift assay

Protein was extracted by pelleting cells for 5 minute at 3,000 rpm and 4°C. The pellet was washed in twice with ice-cold 1xPBS and resuspended in 225 μl of lysis buffer (100 mM Tris-HCl pH 8, 1 mM DTT, 10% glycerol) and 25 μl of 10x protease inhibitor cocktail (Tris pH 10, pepstatin A, DTT, and Roche cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail #11836170001). 250 μl of acid-washed glass beads were added to the lysate and cells were disrupted with a mini-bead beater for 6 x 30 seconds with 1 minute on ice in between beatings. Protein concentration was determined by Bradford assay. For phosphatase treatment, 150 μg of protein was diluted to 39 μl with the lysis buffer and 10x protease inhibitor cocktail. The protein sample was mixed with 1 μl of lambda phosphatase (NEB, # P0753S), 5 μl of NEBuffer for PMP, and 5 μl of MnCl2. The untreated sample was prepared the same way except with 1 μl of lysis buffer. The two samples were incubated at 30°C for 30 minutes and subsequently mixed with 6x sample buffer (0.35 M Tris-HCl, 10% (w/v) SDS, 36% glycerol, 5% β-mercaptoethanol, and 0.012% bromophenol blue) for resolution on 6% SDS-PAGE.
2.2.12 qRT-PCR

To prepare samples for RNA extraction, 10 ml of subcultures were harvested by centrifugation at 3000 rpm for 5 minutes. The pellet was flash-frozen and stored at -80°C overnight. RNA was isolated using the Qiagen RNeasy kit and cDNA was generated using the AffinityScript cDNA synthesis kit (Stratagene). qRT-PCR was carried out using the Fast SYBR Green Master Mix (Thermo Fisher Scientific) in 384-well plate with the following cycle conditions: 95°C for 10 minutes, repeat 95°C for 10 seconds, 60°C for 30 seconds for 40 cycles. The melt curve was completed with the following cycle conditions: 95°C for 10 seconds and 65°C for 5 seconds with an increase of 0.5°C per cycle up to 95°C. All reactions were done in triplicate. Data were analyzed in the Bio-Rad CFX manager 3.1.

2.2.13 Affinity purification (AP) and LC-MS analysis

For AP and LC-MS/MS analysis, clarified whole-cell lysates (10 mg protein/sample) were extracted with immobilized anti-HA beads (Pierce HA-Tag IP/Co-IP kit (Thermo Fisher Scientific, PI23610)). After 3 hours incubation, the beads were washed 3 times with lysis buffer and twice with HPLC water. The proteins bound on beads were eluted with 150 µl of 0.15 % of trifluoroacetic acid, neutralized to 100 mM NH₄HCO₃, digested with trypsin. The tryptic peptides were purified by 200 µl C18 stage tips (Thermo Scientific, Rockford, IL USA) and analyzed by Q-Exactive LC-MS/MS (Tong, Taylor, Peterman, Prakash, & Moran, 2009).

The tryptic peptides from anti-HA IP complexes were separated on a 50-cm Easy-Spray column with a 75-µm inner diameter packed with 2 µm C18 resin (Thermo Scientific, Odense Denmark). The peptides were eluted over 120 minutes (250 nl/minute) using a 0 to 40% acetonitrile gradient in 0.1% formic acid with an EASY nLC 1000 chromatography system operating at 50 ºC (Thermo-Fisher Scientific). The LC was coupled to a Q Exactive mass spectrometer (Michalski et al., 2011) by using a nano-ESI source (Thermo Fisher Scientific). Mass spectra were acquired in a data-dependent mode with an automatic switch between a full scan and up to 10 data-dependent MS/MS scans. Target value for the full scan MS spectra was 1e6 with a maximum injection time of 120 ms and a resolution of 70,000 at m/z 400. The ion target value for MS/MS was set to 1,000,000 with a maximum injection time of 120 ms and a resolution of 17,500 at m/z 400. The first mass for the MS/MS was set to 140 m/z and the normalized collision energy was set to 27. Unassigned, as well as charge states 1, and greater than 5 were ignored for MS/MS
selection. Repeat sequencing of peptides was kept to a minimum by dynamic exclusion of sequenced peptides for 12 seconds (Deeb, D'Souza, Cox, Schmidt-Supprian, & Mann, 2012).

Acquired raw files were analyzed by using MaxQuant software (Cox & Mann, 2008) (version 1.3.0.5) for quantification, and X! Tandem (The GPM, thegpm.org; version CYCLONE; 2010.12.01.1) and Scaffold (version Scaffold_3.4.3, Proteome Software Inc., Portland, OR) for further validation. The default search parameters were used as describe by Deeb et al. (Deeb et al., 2012). The search included cysteine carboxamidomethylation as a fixed modification, N-terminal acetylation, methionine oxidation as variable modifications. The second peptide identification option in Andromeda was enabled. For statistical evaluation of the data obtained, the posterior error probability and false discovery rate were used. The false discovery rate was determined by searching a reverse database. A false discovery rate of 0.01 for proteins and peptides was permitted. Two miscleavages were allowed, and a minimum of seven amino acids per identified peptide were required. Peptide identification was based on a search with an initial mass deviation of the precursor ion of up to 6 ppm, and the allowed fragment mass deviation was set to 20 ppm. To match identifications across different replicates and adjacent fractions, the “match between runs” option in MaxQuant was enabled within a time window of 2 minutes. For the determination of protein levels, at least two unmodified peptides were required for LFQ calculation.

2.2.14 Co-Immunoprecipitations

Cultures were grown to mid exponential phase and harvested at 3000 rpm for 5 minutes. The cells were washed with sterile H₂O and resuspended in 1 ml of lysis buffer (20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl and 20% glycerol, with one protease inhibitor cocktail per 50 ml (complete, EDTA-free tablet, Roche Diagnostics, Indianapolis, IN, USA) and 1 mM PMSF (EMD Chemicals, Gibbstown, NJ, USA). Cells were lysed by bead beating twice for 4 minutes with 7 minutes on ice between cycles. Lysates were recovered by piercing a hole in the bottom of each tube, placing each tube in a 14 ml conical tube, and centrifuged at 1300xg for three 5-minute cycle, recovering the supernatant after each stage. The combined lysate was cleared by centrifugation at 21,000xg for 10 minutes at 4°C. The protein concentrations were determined using the Bradford assay (Bradford, 1976). Anti-HA immunoprecipitations were done using
Pierce HA-Tag IP/Co-IP kit (Thermo Fisher Scientific, PI23610) as per manufacturer’s instructions.

2.2.15 Staining

Cultures were grown to mid exponential phase and 1 ml of the cells was centrifuged at 14,000 rpm for 1 minute. The supernatant was removed and the cells were washed with PBS. To stain for chitin, calcofluor white was added to a final concentration of 1 µg/ml in a final volume of 100 µl. The cells were incubated in the dark for 15 minutes and were gently vortexted every 3-4 minutes. Cells were washed with PBS and 2 µl of the cells were deposited on a cover slide. To stain for nuclei, the cells were heat fixed on the slide for 1 minute at 75°C and 0.5 µl of 1 mg/ml DAPI (Fluka, Sigma-Aldrich) was added to the cells for viewing under the microscope.

2.2.16 General Imaging Techniques

All imaging was performed using Differential Interface Contrast (DIC) microscopy on a Zeiss Imager M1 upright microscope and AxioCam Mrm with AxioVision 4.7 software. For fluorescence microscopy, an X-cite series 120 light source with 4’,6-diamidino-2-phenylindole (DAPI) hybrid, and ET HQ tetramethylrhodamine isothiocyanate/DsRED filter sets from Chroma Technology (Bellows Falls, VT) was used. Calcofluor white and DAPI were viewed under the DAPI hybrid filter and HA-tagged Cas5 was viewed under the Texas Red filter. To facilitate nuclei counting, 12 Z stacks were taken for each image, where each slice is 0.3 um.

2.2.17 Selection Experiment

To select for caspofungin resistant colonies, 2 x 10⁷ cells from an overnight culture was plated on YPD plates supplemented with 1 µg/ml of caspofungin for 3 days at 30°C. Resistant colonies were patched on YPD plates supplemented with 1 µg/ml caspofungin and resistance phenotype was confirmed by MIC.

2.3 Results

2.3.1 Cas5 is activated by dephosphorylation in response to cell wall stress.

Cas5 was originally identified in a genetic screen for transcription factor mutants that are hypersensitive to the echinocandin caspofungin (Bruno et al., 2006). To determine whether Cas5
plays a broader role in cell wall stress response, I evaluated susceptibility of a cas5Δ/cas5Δ mutant to calcofluor white, a dye that interferes with cell wall assembly by binding to chitins. The cas5Δ/cas5Δ mutant was hypersensitive to caspofungin and calcofluor white, and the hypersensitivity phenotypes were complemented by the re-introduction of a wild-type allele of CAS5 (Figure 2.1A), suggesting that Cas5 is a core regulator of cell wall stress responses.

Next, I assessed whether Cas5 localization or expression was modulated in response to cell wall stress. I monitored the subcellular localization of Cas5 by indirect immunofluorescence in a strain in which both alleles of CAS5 were HA-tagged at the C-terminus. The Cas5-HA protein was functional and sufficient to confer wild-type tolerance to caspofungin (Figure 2.2). Following one hour of treatment with 125 ng/ml caspofungin, Cas5 was nuclear localized, as visualized by immunostaining with monoclonal antibodies against the HA epitope (Figure 2.1B). Cas5 localization was difficult to assess in the absence of stress due to the low expression levels. I confirmed that CAS5 transcript and protein levels were induced in response to cell wall stress by quantitative RT-PCR and western blot analysis (Figure 2.1C and Figure 2.1D). Notably, the Cas5 protein demonstrated a downward bandshift in response to caspofungin and calcofluor white, suggesting a change in post-translational modification (Figure 2.1D and Figure 2.3). I observed the increase in Cas5 protein and the downward bandshift not only upon cell wall stress exerted by caspofungin, but also upon genetic perturbation of cell wall integrity signaling. To impair cell wall integrity signaling, I engineered a strain in which I could specifically inhibit kinase activity of the master regulator Pkc1; in this strain, the only allele of PKC1 carries the M850G gatekeeper mutation, which renders the kinase susceptible to inhibition by ATP analog 1-Naphthyl-PP1 (1-NA-PP1) without affecting its kinase activity in the absence of the inhibitor (Gould et al., 2011). In the presence of 1-NA-PP1, the gatekeeper mutant was hypersensitive to caspofungin, similar to a pkc1Δ/pkc1Δ mutant (Figure 2.4). Inhibition of Pkc1 kinase activity had a similar impact on Cas5 level and mobility as caspofungin treatment (Figure 2.1D). Thus, Cas5 is induced in response to external and intrinsic cell wall stress, and may be activated by a change in post-translational modification to mobilize transcriptional responses to cell wall stress.

Given that dephosphorylation of transcription factors is a common mechanism by which they can be activated, I further examined the post-translational modification on Cas5 and the effects on
activation of gene expression in response to cell wall stress. To examine the post-translational modification on Cas5 at higher resolution, a two-dimensional gel electrophoresis coupled to western blotting was performed. In the absence of stress, Cas5-HA resolves into a heterogeneous population of differentially charged species (Figure 2.1E). In response to caspofungin, Cas5 collapses into a less electronegative species, a change that is consistent with protein dephosphorylation. To confirm that Cas5 is indeed phosphorylated, I performed a phosphoshift assay. Following phosphatase treatment, Cas5 ran as a faster migrating band, consistent with it being a phosphoprotein (Figure 2.1F). To determine if this modification of Cas5 is coupled to activation of gene expression, I monitored transcript levels of two Cas5-dependent caspofungin-responsive cell wall genes, ECM331 and PGA13 (Bruno et al., 2006). I found that ECM331 and PGA13 transcript levels are highly upregulated in wild-type cells, but this upregulation is partially blocked by deletion of CAS5 (Figure 2.1G). My results suggest that Cas5 is activated by dephosphorylation in a manner that is exquisitely responsive to cell wall stress induced by environmental or genetic perturbation.
Figure 2.1 Cas5 initiates cell wall stress response by upregulating the expression of cell wall genes upon change in its post-translational modification.

(A) A mutant lacking Cas5 is sensitive to cell wall stress caused by caspofungin or calcofluor white. The drug susceptibility assay was analyzed after 48 hours of growth at 30°C. (B) Cas5 localizes to the nucleus in response to caspofungin. Both alleles of CAS5 were HA-tagged in the
wild-type reference strain SN95. Cells were subcultured in rich media to log phase and treated with 120 ng/ml of caspofungin for 2 hours. The cells were fixed with 4% formaldehyde and Cas5 (red) is detected by indirect immunofluorescence using anti-HA antibody followed by anti-mouse antibody. Nuclei (green) were visualized by DAPI staining. (C) CAS5 expression is induced by caspofungin. SN95 was subcultured to log phase and treated without or with 125 ng/ml of caspofungin for 1 hour. cDNA was prepared from total RNA for qRT-PCR. The transcript level of CAS5 was monitored and normalized to GPD1. (D) Cas5 shows a change in post-translational modification upon Pkc1 inhibition. One allele of CA55 was C-terminally HA-tagged in SN95 and a mutant in which the only allele of PKC1 carries the M850G gatekeeper mutation. Cells were subcultures in rich media with or without 5 uM 1-NA-PP1 for 4 hours to reach log phase. Western blot was performed as described previously. (E) Cas5 is dephosphorylated upon caspofungin treatment. SN95 was subcultured in rich media to log phase and subsequently treated with 125 ng/ml of caspofungin for two hours. Cell lysates were subjected to two-dimensional gel electrophoresis and probed with anti-HA antibodies. (F) Cas5 is phosphorylated in the absence of stress. SN95 carrying HA-tagged Cas5 was subcultured to log phase and proteins were extracted under non-denaturing conditions. The lysate was subsequently treated without or with lambda phosphatase. (G) Cas5 is required for the upregulation of cell wall gene expression in response to caspofungin. SN95 and cas5∆/cas5∆ were subcultured to log phase and treated without or with 125 ng/ml of caspofungin for 1 hour. cDNA was prepared from total RNA for qRT-PCR. The transcript level of ECM331 and PGA13 was monitored and normalized to GPD1. The treatment conditions were compared using a Tukey's multiple comparisons test in GraphPad Prism (**** P<0.0001, ** P<0.01).

![Figure 2.2 HA-tagged allele of CAS5 is functional.](image)

The drug susceptibility assay was analyzed after 48 hours of growth at 30°C.

![Figure 2.3 Cas5 shows a change in post-translational modification upon caspofungin or calcofluor white treatment.](image)

Cells were subcultured in rich media for 3 hours to log phase and subsequently treated with 125 ng/ml of caspofungin or 50 ug/ml of calcofluor white for 1 hour. Total proteins were resolved by SDS-PAGE and the blot was hybridized with α-HA to monitor Cas5 status.
Figure 2.4 ATP analogue 1-NA-PP1 specifically inhibits Pkc1 kinase activity.

The caspofungin susceptibility assay was set up in the presence of 0.05% DMSO or 5 μM 1-NA-PP19 and was analyzed after 48 hours of growth at 30°C.

2.3.2 A conserved serine residue in the Cas5 DNA binding domain is critical for caspofungin tolerance.

To map phosphorylation sites on Cas5, I performed immunoprecipitation coupled with mass spectrometry with HA-tagged Cas5. Phosphorylation was detected at serine residues S464 and S476 (Figure 2.5A). However, even when all four serines in the cluster (S462, S464, S472 and S476) were mutagenized to either glutamic acid, to mimic constitutively a phosphorylated state, or alanine, to mimic a constitutively unphosphorylated state, the resulting phosphomutants displayed wild-type tolerance to caspofungin (Figure 2.5B), with no appreciable difference in band shift upon caspofungin treatment (Figure 2.5C). This suggests that the sites identified by mass spectrometry were not sufficient to control Cas5 function.
Figure 2.5 The phosphorylation sites identified by mass spectrometry are not sufficient for Cas5 function.

(A) A schematic showing the phosphorylated serine residues identified in Cas5 by the mass spectrometry analysis. (B) Both phosphomimetic and phosphodeficient mutations of the serine residues on Cas5 did not affect caspofungin tolerance. The mutant alleles of CAS5 were introduced into a cas5Δ/cas5Δ mutant individually as the only allele of CAS5 in this strain. The drug susceptibility assay was analyzed after 48 hours of growth at 30°C. (C) The phosphomimetic and phosphodeficient mutations of the serine residues on Cas5 does not affect band shift associated with the activation of cell wall stress response. Cells were subcultured in rich media for 3 hours to reach log phase and subsequently treated with 125 ng/ml of caspofungin for 2 hours. The Western blot was performed as described previously.

To identify other potential phosphorylation sites on Cas5, I analyzed Cas5 amino acid sequence using NetPhos and followed up on S769, a highly conserved serine residue in one of the zinc finger domains (Figure 2.6A). It has one of the highest phosphorylation site prediction scores of 0.973 (with 1 being the highest possible score), and is found in Cas5 homologs from 7 Candida species based on protein sequence alignments in Candida Genome Database (CGD) (Blom, Sicheritz-Ponten, Gupta, Gammeltoft, & Brunak, 2004; Inglis et al., 2012). To assess the functional significance of this residue, I engineered strains in which one allele of CAS5 was deleted and the other was replaced with a wild-type allele, the phosphomimetic S769E allele, or
the phosphodeficient S769A allele. Introduction of the phosphomimetic S769E allele as the only source of CAS5 phenocopied homozygous deletion of CAS5 in terms of hypersensitivity to caspofungin, and introduction of the phosphodeficient S769A allele conferred a wild-type phenotype (Figure 2.6B). Consistent with this, the S769E mutation blocked the upregulation of ECM331 and PGA13 in response to caspofungin, while the S769A mutation did not (Figure 2.6C). Notably, these mutations did not affect Cas5 induction or dephosphorylation in response to caspofungin (Figure 2.6D), suggesting that perturbation of Cas5 function does not cause an intrinsic cell wall defect nor does it perturb the capacity to sense cell wall stress. This also suggests that additional phosphorylation sites remain to be identified. Together, my data support a model in which Cas5 is activated by dephosphorylation in response to cell wall stress to mobilize crucial transcriptional responses.
A. Cas5 (821 aa)

<table>
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<th>Motif</th>
<th>Score</th>
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<td>RHLKHSSE</td>
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<td>77%</td>
</tr>
<tr>
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<td>RHLKHSSE</td>
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<td>85%</td>
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<td>Candida albicans</td>
<td>S-769</td>
<td>RHLKHSSE</td>
<td>0.980</td>
<td>100%</td>
</tr>
</tbody>
</table>

B. SN95 (WT)  
cas5Δ/cas5Δ  
CASS-HA/cas5Δ  
CASS^{5769E}-HA/cas5Δ  
CASS^{5769A}-HA/cas5Δ

C. ECM331

- Fold Change in Response to Caspofungin

D. CASS  
CASS^{5769E}  
CASS^{5769A}

α-HA  
Caspofungin: - + - + - +
Figure 2.6 The S769E mutation in the Cas5 DNA binding domain phenocopies CAS5 deletion.

(A) Schematics showing the position of S769 in the Cas5 zinc finger domain and the alignment of Cas5 orthologs in related fungal species. The alignment was performed by CGD and the phosphorylation site prediction score was performed by NetPhos. (B) The phosphomimetic mutation S769E in Cas5 confers hypersensitivity to caspofungin. The mutant alleles of CAS5 were introduced into a cas5∆/cas5∆ mutant individually as the only allele of CAS5 in this strain. The drug susceptibility assay was conducted in YPD at 30°C and incubated for 48 hours. (C) The S769E mutation renders Cas5 transcriptionally inactive upon caspofungin treatment. Cells were subcultured in rich media for 3 hours to reach log phase and subsequently treated with 125 ng/ml of caspofungin for 1 hour. The qRT-PCR was performed as described previously. The treatment conditions were compared using a Tukey's multiple comparisons test in GraphPad Prism (**** P<0.0001, * P<0.05). (D) The phosphomutation in Cas5 does not affect band shift associated with the activation of cell wall stress response. The culture condition is the same as described previously except the caspofungin treatment was 2 hours. The Western blot was performed as described previously.

2.3.3 Glc7 dephosphorylates Cas5 in response to cell wall stress.

In order to identify the phosphatase that activates Cas5 by dephosphorylation, I leveraged insights into regulation of transcription factors similar to Cas5 in S. cerevisiae. A BLASTp search of the Cas5 protein sequence against the S. cerevisiae protein database identified Mig1, Msn4, Mig2, and Msn2 as top hits (Gish & States, 1993). Mig1, Msn4 and Msn2 are known to be dephosphorylated by the type I protein phosphatase Glc7 (Lenssen et al., 2005; Schuller, 2003). Since Glc7 has been shown to play a role in cell wall maintenance S. cerevisiae (Andrews & Stark, 2000), I asked whether Glc7 is also required for cell wall stress response in C. albicans. To this end, I engineered a strain in which I could achieve transcriptional repression of GLC7, by deleting one allele of GLC7 and replacing the native promoter of the remaining allele with a tetracycline-repressible (tetO) promoter; I confirmed that the expression of GLC7 is repressed in the presence of the tetracycline analog doxycycline by qRT-PCR (Figure 2.7). I confirmed that Glc7 enables cell wall stress responses in C. albicans, as depletion of GLC7 with 1 μg/ml of doxycycline conferred hypersensitivity to caspofungin (Figure 2.8A). Next, I examined the effect of GLC7 depletion on Cas5 expression and function. Doxycycline-mediated transcriptional repression of GLC7 did not block upregulation of Cas5 in response to caspofungin, but did block the downward bandshift (Figure 2.8B). I validated that Glc7 was required for the mobility shift of Cas5 by running another SDS-PAGE gel with equivalent Cas5 levels loaded per sample (Figure 2.8C). Depletion of GLC7 also blocked the upregulation of Cas5-dependent caspofungin-responsive genes (Figure 2.8D). My results strongly suggest that Glc7 activates
Cas5 by dephosphorylation in response to cell wall stress, thereby enabling transcriptional upregulation of cell wall genes.

**Figure 2.7 GLC7 is depleted upon doxycycline treatment.**

The overnights were subcultured for 24 hours in the presence or absence of 0.02 μg/ml of doxycycline. The strains were subcultured again in the same conditions for 3 hours and subsequently treated with or without caspofungin for 1 hour. The qRT-PCR was performed as described previously. The transcript levels of *GLC7* was monitored and normalized to *GPD1*. 
Figure 2.8 Cas5 activation in response to caspofungin is associated with dephosphorylation by Glc7.

(A) Depletion of GLC7 confers sensitivity to caspofungin. Genetic compromise of GLC7 expression is achieved in a strain where the only allele of GLC7 is expressed under the control of a doxycycline-repressible promoter (tetO). The caspofungin susceptibility assay was set up in the
presence or absence of 1 μg/ml of doxycycline and was analyzed after 48 hours of growth at 30°C. (B) Upregulation of Cas5 expression does not depend on Glc7. One allele of CAS5 is C-terminally HA-tagged in a WT and a tetO-GLC7/glc7Δ mutant. The overnights were subcultured for 24 hours in the presence or absence of 0.02 μg/ml of doxycycline. The strains were subcultured again in the same conditions for 3 hours and subsequently treated with or without caspofungin for 2 hours. The Western blot was performed as described previously. (C) The change in post-translational modification on Cas5 is absent upon GLC7 depletion. The Western blot was performed as described in B except caspofungin treated samples were diluted 5-fold to achieve equal loading. (C) Dephosphorylation of Cas5 is required for its transcriptional activity in response to caspofungin. The culture condition is the same as described previously except the caspofungin treatment was 1 hour. The qRT-PCR was performed as described previously. The treatment conditions were compared using a Tukey's multiple comparisons test in GraphPad Prism (**** P<0.0001, *** P<0.001).

2.3.4 Cas5 physically interacts with Swi4/Swi6 to regulate caspofungin-responsive genes.

To identify physical interactors of Cas5, I leveraged the results from my immunoprecipitation coupled to mass spectrometry analysis (Table 2.1). The only interaction that passed a stringent statistical analysis using Significance Analysis of Interactome (SAINT) (Choi et al., 2011) with a probability threshold of 0.9 was Swi4, which forms the SBF complex together with Swi6. To determine if the SBF complex plays a role in cell wall stress responses, I generated swi4Δ/swi4Δ and swi6Δ/swi6Δ homozygous deletion mutants. Strains lacking Swi4 or Swi6 were both hypersensitive to caspofungin, though not to the same extent as the strain lacking Cas5 (Figure 2.9A). To validate the interaction between Cas5 and the SBF complex, I individually tagged Swi4 or Swi6 at the C-terminus with a tandem affinity purification (TAP) tag in a strain harboring Cas5-HA to enable co-immunoprecipitation experiments. The Swi4-TAP and Swi6-TAP proteins are functional, and sufficient to mediate wild-type tolerance to caspofungin (Figure 2.10). Immunoprecipitation of Cas5 with anti-HA agarose co-purified both Swi4 and Swi6 (Figure 2.9B). To determine if Cas5 and the SBF complex co-regulate caspofungin-responsive genes, I monitored the expression of ECM331 and PGA13 in mutants lacking Swi4, Swi6, or Cas5. In all three mutants, upregulation of these cell wall genes in response to caspofungin was blocked (Figure 2.9C). Since deletion of CAS5 causes a greater magnitude of hypersensitivity to caspofungin than deletion of SWI4 or SWI6, Cas5 may have additional targets independent of the SBF complex. Together, my results suggest that Cas5 regulates caspofungin tolerance in part via physical interaction with the SBF complex to enable transcriptional responses to cell wall stress.
Figure 2.9 Cas5 regulates caspofungin tolerance in part through interaction with the components of the SBF complex Swi4 and Swi6.

(A) Swi4 and Swi6 play a role in regulating cell wall stress. The drug susceptibility assay was conducted with caspofungin in YPD at 30°C and incubated for 48 hours. (B) Swi4 and Swi6 co-purify with Cas5. C-terminally HA-tagged Cas5 was immunoprecipitated with HA beads. The input and IP samples were resolved by SDS-PAGE and the blot was hybridized with α-TAP to monitor Swi4 and Swi6 co-purification and α-HA to confirm Cas5 pull down. (C) Cas5 and Swi4/Swi6 are required for the upregulation of caspofungin-responsive genes. Cells were subcultured in rich media for 3 hours to reach log phase and subsequently treated with 125 ng/ml of caspofungin for 1 hour. qRT-PCR was performed as described previously. The treatment
conditions were compared using a Tukey’s multiple comparisons test in GraphPad Prism (** P<0.0001).

Figure 2.10 The TAP tagged Swi4 and Swi6 are functional.

The drug susceptibility assay was conducted with caspofungin and analyzed after 48 hours of growth at 30°C.

2.3.5 Cas5 is required for proper morphogenesis and cell cycle progression.

Swi4 and Swi6 regulate G1/S phase progression in both \textit{S. cerevisiae} and \textit{C. albicans} (Hussein et al., 2011; Koch, Schleif\;er, Ammerer, & Nasmyth, 1996). In \textit{C. albicans}, a phenotype typically associated with cell cycle mutants is polarized growth (Berman, 2006; O’Meara et al., 2015). For example, \textit{swi4Δ/swi4Δ} and \textit{swi6Δ/swi6Δ} mutants undergo polarized growth in the absence of filament-inducing cues (Hussein et al., 2011). To determine if the \textit{cas5Δ/cas5Δ} mutant exhibits such cell cycle defects, the mutant was diluted into fresh rich medium (YPD) and grown for six hours. I found that the mutant lacking Cas5 was flocculant and formed pseudohyphae in the absence of filament-inducing cues (Figure 2.11A). To assess the extent of the filamentation, I monitored the expression of \textit{HWP1} and \textit{IHD1}, which are filament-specific transcripts that are upregulated in a mutant lacking both Swi4 and Swi6 (Hussein et al., 2011). Similar to previous findings for a \textit{swi4Δ/swi4Δ swi6Δ/swi6Δ} double mutant, \textit{HWP1} and \textit{IHD1} transcript levels were highly upregulated in the \textit{cas5Δ/cas5Δ} mutant (Figure 2.11B). However, unlike \textit{SWI4}, the
expression of CAS5 itself is not cell-cycle regulated (Cote, Hogues, & Whiteway, 2009). To determine whether Cas5 is instead important for the expression of Swi4/Swi6-dependent G1/S genes, I monitored the transcript levels of PCL2 and PGA6 in the transcription factor mutants (Hussein et al., 2011). Similar to the swi4Δ/swi4Δ and swi6Δ/swi6Δ mutants, the cas5Δ/cas5Δ mutant had reduced PCL2 and PGA6 transcript levels (Figure 2.11C). My data suggests that Cas5 co-regulates G1/S transition with the SBF complex.

Figure 2.11 Cas5 plays a similar role as Swi4 and Swi6 in hyphal development and G1/S transition.
(A) Deletion of CAS5 results in pseudohyphal growth in the absence of filament-inducing cue. Cells were subcultures in rich media for 4 hours to reach log phase and DIC images were taken at 100X. The scale bar represents 10 μm. (B) Hyphal-specific genes are highly upregulated in mutant lacking Cas5 and Swi4/Swi6. Cells were subcultured in rich media for 4 hours to reach log phase and the transcript levels of *HWPI* and *IHD1* were monitored and normalized to *GPD1*. qRT-PCR was performed as described previously. (C) Cas5 and Swi4/Swi6 are required for the expression of G1/S regulated genes. Cells were subcultured in rich media for 4 hours to reach log phase and qRT-PCR was performed as described previously. The transcript levels of *PCL2* and *PGA6* were monitored and normalized to *GPD1*.

I further investigated the role of Cas5 in cell cycle progression by monitoring the number of nuclei per cell in mutant and wild-type strains. I stained wild-type and *cas5δ/cas5Δ* mutant cells with DAPI for DNA content and calcofluor white for chitin (**Figure 2.12A**). At log phase, approximately 45% of the mutant cells lacking Cas5 were multinucleated, an indication of severe cell cycle defect (**Figure 2.12B**). However, it is unclear whether the multinucleated phenotype is due to multiple rounds of nuclear division during a single cell cycle, multiple rounds of cell cycle without cytokinesis, or spontaneous nuclear fragmentation. Notably, less than 10% of mutant cells lacking either Swi4 or Swi6 showed a similar defect, suggesting that Cas5 has additional roles in cell cycle regulation independent of the SBF complex.
Figure 2.12 Cas5 is a key regulator of cell cycle progression

(A) Deletion of CAS5 results in a multinucleated phenotype. Each strain was subcultured in rich media to log phase and cells were stained with DAPI for nuclei and calcofluor white for chitin. For each image, 12 z-stack slices were taken at 0.3 μm each. The histogram shows the proportion of cells with different number of nuclei. The red arrows point to the nuclei. (B) Mutant lacking Cas5 exhibits severe cell cycle defect. The number of nuclei is counted in at least 120 cells for each strain in 3 biological replicates. The nuclei counts for each strain are averaged and compared to that of the WT using student’s t-test in GraphPad Prism. Statistical significance determined using the Holm-Sidak method, with alpha=5.000% (* P<0.05).

2.3.6 Cas5 regulates *bona fide* caspofungin resistance independently of cell cycle progression.

The most common mechanism of resistance to caspofungin is through mutation in the drug target gene, *FKS1*. To determine if Cas5 is also involved in caspofungin resistance, I deleted CAS5 from a caspofungin-resistant strain carrying the Fks1^F641S^ substitution. Homozygous deletion of CAS5 abrogated resistance conferred by the *FKS1* point mutation, suggesting that Cas5 is a key regulator of caspofungin resistance (**Figure 2.13A**). To determine if Cas5 regulates Fks1-dependent caspofungin resistance by upregulating the expression of the resistance determinant, I performed qRT-PCR to monitor the expression of *FKS1* in the Fks1^F641S^ mutant, with and without both alleles of CAS5 deleted. I found that *FKS1* expression was not upregulated in the caspofungin-resistant Fks1^F641S^ mutant despite Cas5 activation, suggesting that Cas5 regulates Fks1-dependent caspofungin resistance independent of *FKS1* expression (**Figure 2.14**).
Figure 2.13 Cas5 regulates bona fide caspofungin resistance independent its role in cell cycle.

(A) Loss of Cas5 abrogates Fks1-dependent caspofungin resistance. The drug susceptibility assay was conducted with caspofungin in YPD at 30°C and incubated for 48 hours. (B) Mutants from the caspofungin selection experiment show robust resistance to caspofungin, with minimal effect on resistance to calcofluor white. P for parent, R for resistant isolate. The drug susceptibility assay was conducted with caspofungin or calcofluor white in YPD at 30°C and analyzed after 48 hours.
Figure 2.14 Cas5 regulates caspofungin resistance independent of FKS1.

(A) FKS1 is not upregulated in the Fks1^{F641S} mutant. Cells were subcultured in rich media for 3 hours to reach log phase and subsequently treated without or with 125 ng/ml of caspofungin for 1 hour. The qRT-PCR was performed as described previously. The transcript levels of FKS1 were monitored and normalized to GPD1. (B) Cas5 is activated in the Fks1^{F641S} mutant in response to caspofungin. Cells were subcultured in rich media for 3 hours to reach log phase and subsequently treated without or with 125 ng/ml of caspofungin for 2 hour. The Western blot was performed as described previously.

To determine the mechanisms through which Cas5 regulates caspofungin resistance, I performed a selection experiment to identify mutations that could restore resistance of the Fks1^{F641S} mutant lacking Cas5. I plated cells on solid medium with a high concentration of caspofungin (1 µg/ml), and characterized the resistance phenotype of three independent mutants (Figure 2.13B). The mutants exhibited a strong increase in resistance to caspofungin, and a minor increase in resistance to calcofluor white. Whole genome sequencing of the parents and the resistant mutants were performed in order to identify the resistance determinants and the analysis is currently in progress.

To further define the relationship between cell wall stress response and cell cycle progression, I assayed the caspofungin-resistant isolates from my selection experiment for cell cycle defects by staining DNA with DAPI and chitin with calcofluor white. I found that the suppressor mutations restored caspofungin resistance without rescuing the cell cycle defects, characterized by the multinucleated phenotype (Figure 2.15), suggesting that Cas5 regulates drug resistance and cell cycle progression via distinct mechanisms.
Figure 2.15 Mutation that confers caspofungin-resistance independent of Cas5 does not restore proper cell cycle progression.

Each strain was subcultured in rich media to log phase and cells were stained with DAPI for nuclei and calcofluor white for chitin. For each image, 12 z-stack slices were taken at 0.3 μm each. The number of nuclei is counted in at least 120 cells.

2.4 Discussion

I have uncovered a dual role for Cas5 in regulating cell wall stress responses and cell cycle progression in the fungal pathogen *C. albicans* (Figure 2.16) Cas5 transcriptionally regulates cellular responses to both external and intrinsic cell wall stress, such that compromising Cas5 function reduces caspofungin tolerance of laboratory strains and resistance of clinical isolates (Figures 2.1 and 2.13). I demonstrate that in response to cell wall stress, Cas5 is nuclear-localized and activated by Glc7-mediated dephosphorylation (Figures 2.1 and 2.8), whereby mutation locking Cas5 in the constitutively phosphorylated form or genetic depletion of the phosphatase blocks Cas5-dependent transcriptional responses to cell wall stress. I find that Cas5 regulates caspofungin-responsive cell wall genes in part through interaction with Swi4 and Swi6 (Figure 2.9). Further, I show that Cas5 and Swi4/Swi6 also co-regulate morphogenesis and G1/S transition (Figure 2.11). My findings implicate Cas5 as a key regulator of cell wall stress response and cell cycle progression, and suggest a novel mechanism by which cell wall state and cell cycle are co-regulated.
In response to cell wall stress, Cas5 is desphosphorylated by Glc7 and upregulates the expression of caspofungin-responsive cell wall genes. Cas5 transcriptionally regulates cell wall stress and cell cycle progression via distinct mechanisms, in part through interaction with Swi4 and Swi6.

Coordination of sensing and responding to cell wall stress is crucial for all fungi, as the fungal cell wall is a dynamic structure that serves as a core permeability barrier that is required for osmotic stability and cellular integrity. Although Cas5 is not broadly conserved in the fungal kingdom, its activation is regulated by a conserved protein phosphatase with a core role in cell wall stress responses. Cas5 is activated in response to cell wall stress induced by either cell wall perturbing agents, such as the echinocandin antifungal drugs, or by genetic perturbation, such as inhibition of cell wall integrity signaling via Pkc1 (Figure 2.1). Cas5 is activated by dephosphorylation mediated by Glc7 (Figure 2.8), which has 91% protein identity with the *S. cerevisiae* ortholog. In *S. cerevisiae*, a mutant carrying a temperature sensitive *glc7* allele exhibits a sorbitol-remediable lysis defect at the restrictive temperature, a consequence of cell wall defect (Andrews & Stark, 2000). Glc7 is also required for cell wall maintenance in *C. albicans*, as depletion of *GLC7* causes hypersensitivity to caspofungin (Figure 2.8). Glc7 substrates in *S. cerevisiae* include three proteins with sequence similarity to Cas5: Mig1, Msn2, and Msn4. Msn2 and Msn4 are transcription factors that are required for general stress response in *S. cerevisiae*, but not in *C. albicans* (Nicholls et al., 2004; Sadeh, Movshovich, Volokh, Gheber, & Aharoni, 2011). This suggests that Cas5 may serve as a core downstream effector of Glc7 that modulates general stress responses in *C. albicans*. Consistent with this possibility, Cas5 controls responses not only to cell wall stress, but also to cell membrane stress exerted by the azole antifungal drugs, which inhibit biosynthesis of the membrane sterol ergosterol (Vasicek et al., 2014). The kinase that phosphorylates Cas5 remains enigmatic, but inhibition of kinase
activity of Pkc1, the master regulator of the cell wall integrity pathway (Figure 2.1D), does not block Cas5 phosphorylation implicating alternate or additional kinases.

Cell wall stress can originate not only through environmental or genetic perturbations, but also through changes coupled to cell cycle progression in budding yeasts, as cell wall remodeling and biosynthesis are required to enable the emergence of the growing daughter bud. Consistent with the finding that Glc7 is required for cell cycle progression (Hisamoto, Sugimoto, & Matsumoto, 1994), I discovered that deletion of CAS5 causes severe cell cycle defects in addition to hypersensitivity to cell wall stressors (Figure 2.1 and 2.11). Cas5 regulates the cell cycle in part via interaction with the SBF complex, including Swi4 and Swi6. Cas5, Swi4, and Swi6 are each required for induction of cell wall genes and G1/S phase genes, and accumulate multinucleated cells. Deletion of CAS5 causes a more severe defect in cell wall stress responses and cell cycle progression than deletion of SWI4 and SWI6 (Figure 2.9 and 2.11), suggesting that Cas5 has additional roles in these processes that are independent of the SBF complex. In S. cerevisiae, cell cycle genes regulated by the SBF complex are typically involved in budding and membrane/cell-wall biosynthesis, whereas the MBF complex, composed of Mbp1 and Swi6, is responsible for the expression of genes related to DNA replication and repair (Iyer et al., 2001). In C. albicans, Mbp1 has only a minor role in cell cycle regulation (Hussein et al., 2011). Thus, Cas5 may be core to the rewiring of circuitry governing cell cycle regulation and cell wall stress response over evolutionary time.

Independent of its cell cycle functions, Cas5 also plays a crucial role in caspofungin resistance. Although the deletion of CAS5 completely abrogates caspofungin resistance conferred by the Fks1F641S point mutation, the resistance phenotype can be rescued without restoring proper cell cycle control, suggesting that different mechanisms are involved. Since Cas5 regulates the expression of cell wall genes in response to cell wall stress and cell cycle genes in the absence of stress, its targets could be affected by changes in nucleosome positioning and identified by genome-wide nucleosome profiling experiments. Nonetheless, my results highlight transcriptional regulation as a key mechanism of drug resistance.

Targeting core regulators of cellular circuitry that are crucial for cellular stress responses and cell cycle progression may provide a powerful strategy for antifungal drug development, given that the emergence of drug resistance outpaces the discovery of new antifungal drugs. This strategy
expands the potential chemical diversity of therapeutic agents, as the cognate inhibitors are rarely identified by classical screening methods. It also opens up the opportunity for combination therapy, which is widely used in the treatment of infections caused by bacteria, protozoan parasites, and viruses, but has been less well developed for fungal pathogens (Cowen, 2013). Promising examples of this approach include targeting the molecular chaperone Hsp90 or its downstream effector the protein phosphatase calcineurin, which are required for drug resistance and virulence of diverse fungal pathogens (Cowen, 2013; S. D. Singh et al., 2009). Given that both of these core cellular regulators are highly conserved in humans and have essential functions in mobilizing immune responses, the challenge in exploiting the therapeutic utility of these targets for antifungal drug development hinges on the development of fungal-selective inhibitors (Cowen et al., 2009b). Since Cas5 lacks an identifiable ortholog in humans (Bruno et al., 2006) but is required for drug resistance and virulence of *C. albicans*, it provides an attractive target for antifungal drug development. There is growing support for the feasibility of chemical modulation of transcription factors based on blocking transcription factor dimerization, DNA binding, or the interaction with regulatory proteins, which creates important opportunities to target the transcriptional circuitry controlling virulence traits for antifungal drug development (Bahn, 2015; Nishikawa et al., 2016).
Chapter 3
Signaling through Lrg1, Rho1 and Pkc1 Governs *Candida albicans* Morphogenesis in Response to Diverse Cues

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3 Signaling through Lrg1, Rho1 and Pkc1 Governs *Candida albicans* Morphogenesis in Response to Diverse Cues

3.1 Introduction

Fungi are ubiquitous in the environment, and are among the most pervasive opportunistic pathogens of humans. One of the leading fungal pathogens of humans is *Candida albicans*, which is a common constituent of the human mucosal microbiota, and an opportunistic pathogen. In most individuals, it is found asymptptomatically in the oral cavity, gastrointestinal tract and genital area (MacCallum, 2010; Naglik & Moyes, 2011). However, *C. albicans* can be pathogenic in patients with compromised immunity and cause superficial oral and vaginal infections. If it enters the bloodstream or reaches vital organs, the infection can be fatal, with mortality rates approaching 40% (Pfaller & Diekema, 2007). Fundamental to *C. albicans* pathogenesis is its ability to transition between yeast and filamentous forms, including hyphae and pseudohyphae. Mutants unable to undergo morphological transitions typically have reduced virulence (Braun, Head, Wang, & Johnson, 2000; Braun, Kadosh, & Johnson, 2001; Lo et al., 1997). During the early stages of infection, *C. albicans* is disseminated in the yeast form (Saville et al., 2003). Filamentation is important for tissue penetration and escape from host immune cells (Saville et al., 2003). The genetic circuitry controlling morphogenetic transitions provides a rich source of virulence factors that expands the potential chemical diversity in therapeutics, as these would be typically overlooked by conventional screening strategies.

The transition from yeast to filamentous growth in *C. albicans* is triggered by diverse host relevant cues, including serum, nutrient limitation, alkaline pH, and elevated temperature (Shapiro et al., 2011; P. E. Sudbery, 2011). This process is governed by a complex network of signaling pathways, many of which are involved in the positive regulation of filamentation (Shapiro et al., 2011; P. E. Sudbery, 2011). For example, the Ras1-PKA pathway plays a central role in transducing filament-inducing cues through the activation of the transcription factor Efg1. Mitogen-activated protein (MAP) kinases upstream of the transcription factor Cph1, including Ste11, Hst7, and Cek1/Cek2, have also been implicated in filamentous growth. Many of the filament-inducing cues also require a concomitant increase in temperature to relieve repression of morphogenetic signaling mediated by the molecular chaperone Hsp90 (Shapiro et al., 2009).
Additionally, morphogenesis in *C. albicans* is negatively regulated by a number of transcription factors, including Sfl1, Tup1, Nrg1, and Rfg1 (Braun & Johnson, 1997; Braun et al., 2001; Kadosh & Johnson, 2005; Y. Li, Su, Mao, Cao, & Chen, 2007). The complexity of the regulatory network controlling morphogenesis is only beginning to be appreciated.

*C. albicans* coordinates morphogenesis in response to filament-inducing cues by establishing cellular asymmetry and initiating polarized growth through changes in the actin cytoskeleton (Drubin & Nelson, 1996). During hyphal formation, actin accumulates at the site of the incipient germ tube and polymerizes towards the hyphal tip to form filamentous-actin (F-actin) (Hazan & Liu, 2002). The nucleation of actin filaments depends on the formins Bni1 and Bnr1, which are regulated by the Rho family GTPases Cdc42 and Rho1 (Dong, Pruyne, & Bretscher, 2003; Evangelista et al., 1997; Kohno et al., 1996). In the model yeast *Saccharomyces cerevisiae*, Bni1 has been shown to bind the activated form of Cdc42 and Rho1 (Evangelista et al., 1997; Kohno et al., 1996). In *C. albicans*, both Cdc42 and Rho1 localize to the hyphal tip during polarized growth (Corvest et al., 2013). In addition, Cdc42 and its guanine exchange factor (GEF) Cdc24 are both required for hyphal maintenance. While Rho1 has been implicated in morphogenesis, its mechanism of action remains unknown (Corvest et al., 2013).

Cellular morphogenesis in *C. albicans* is also contingent upon extensive remodeling of the cell wall structure and composition. This highly dynamic process involves loosening of the cell wall by digestive enzymes such as glucanases and chitinases, and directing cell wall synthesis towards a specific region on the cell surface (Levin, 2005). It is well established that Rho1 is a master regulator of the cell wall integrity signaling (Levin, 2011). It is the regulatory subunit of β-1,3-D-glucan synthase, and directly controls cell wall biosynthesis via the binding and activation of the catalytic subunits, Fks1 and Fks2 (Qadota et al., 1996). Rho1 is also responsible for orchestrating changes in the cell wall in response to various forms of cell wall stress by activating the Pkc1-dependent MAP kinase cascade that includes Bck1, Mkk2 and Mkc1 (Levin, 2005). Like other small GTPases, Rho1 cycles between an active GTP-bound state and an inactive GDP-bound state. It is activated by GEFs, such as Rom2, and inactivated by GTPase-activating proteins (GAPs), such as Lrg1 (Perez & Rincon, 2010). In its active form, Rho1 signals through interaction with downstream effectors such as Pkc1 (Perez & Rincon, 2010). Despite the critical role of Rho1 in cell wall biogenesis, none of its regulators or effectors have been implicated in the *C. albicans* morphogenesis.
Here, I screened a collection of 1,248 *C. albicans* homozygous transposon insertion mutants to identify novel negative regulators of filamentation. I identified the Rho1 GTPase activating protein (GAP) Lrg1, which negatively regulates Rho1 activity. Deletion of *LRG1* or introduction of a *RHO1* mutation that locks Rho1 in a constitutively active, GTP-bound state leads to filamentation in the absence of filament-inducing cues. Deletion of the Rho1 downstream effector *PKC1* or introduction of a *RHO1* mutation that locks Rho1 in constitutively inactive, GDP-bound state results in a defect in filamentation. I found that Pkc1 is not required to sense filament-inducing cues, but rather its kinase activity is critical for the initiation of filamentous growth. I discovered that Pkc1 regulates filamentation independent of the canonical MAP kinase cascade, and functions in parallel with the Ras1-PKA pathway. Thus, a new role has been established for signaling through Lrg1, Rho1, and Pkc1 in controlling *C. albicans* morphogenesis in response to diverse cues, demonstrating a novel link between cell wall remodeling and cellular morphogenesis.

### 3.2 Materials and methods

#### 3.2.1 Strains and culture conditions

All *C. albicans* strains were archived in 25% glycerol and stored at -80°C. Strains were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) at 30°C. All cultures were grown shaking at 200 rpm. 2% agar was added for solid media. Strains used in this study is listed in Table 3.1.

**Table 3.1 Strains used in this study.**

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<td>(R. B. Wilson et al., 1999)</td>
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<td>(Enloe, Diamond, &amp; Mitchell, 2000)</td>
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<td>Genetic Modification</td>
<td>Reference</td>
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3.2.2 Strain construction

**CaLC2872:** To generate a *RHO1* heterozygous deletion mutant in a strain carrying the transactivator, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC2836 and oLC2837 (4366 bp) and transformed into CaLC206. NAT resistant transformants were PCR tested with oLC2840 + oLC275 (654 bp) for upstream integration and oLC274 + oLC2843 (515 bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3935:** To regulate the expression of *RHO1*, the tetracycline-repressible transactivator, the tetO promoter, and the NAT flipper cassette were PCR amplified from pLC605 (Leach & Cowen, 2014) using primers oLC2838 and oLC2860 (4957 bp) and transformed into CaLC2872. NAT resistant transformants were PCR tested with oLC2862 + oLC534 (522 bp) for upstream integration and oLC274 + oLC2842 (1223 bp) for downstream integration. The absence of WT *RHO1* promoter is verified with oLC2862 + oLC2842 (847 bp for WT promoter) and the presence of a deleted *RHO1* allele is verified with oLC2862 + oLC2843 (760 bp). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4501:** To introduce the Q67L mutation into *RHO1*, the *RHO1* complementation vector carrying the Q67L mutation was released from pLC772 with BssHII and transformed into CaLC3935. NAT resistant transformants were PCR tested with oLC275 + oLC2841 (1076 bp) for upstream integration and oLC274 + oLC2868 (483 bp) for downstream integration. The presence of WT *RHO1* promoter is verified oLC2864 + oLC2843 (1159 bp for WT promoter; 562 bp for the absence of WT promoter) and the presence of a tetO-*RHO1* allele is verified with oLC2862 + oLC534 (522 bp). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4502:** To introduce the T23N mutation into *RHO1*, the *RHO1* complementation vector carrying the T23N mutation was released from pLC771 with BssHII and transformed into CaLC3935. NAT resistant transformants were PCR tested with oLC275 + oLC2841 (1076 bp) for upstream integration and oLC274 + oLC2868 (483 bp) for downstream integration. The
presence of WT RHO1 promoter is verified oLC2864 + oLC2843 (1159 bp for WT promoter; 562 bp for the absence of WT promoter) and the presence of a tetO-RHO1 allele is verified with oLC2862 + oLC534 (522 bp). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC3076:** To introduce the M850G mutation into PKC1, the PKC1 complementation vector carrying the M850G mutation was released from pLC770 with BssHII and transformed into CaLC1255. NAT resistant transformants were PCR tested with oLC275 + oLC1042 (660 bp) for upstream integration and oLC274 + oLC1030 (555 bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4000:** To generate a LRG1 heterozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC3976 and oLC3977 (4366 bp) and transformed into CaLC239. NAT resistant transformants were PCR tested with oLC3978 + oLC275 (bp) for upstream integration and oLC274 + oLC3979 (bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4090:** To generate a LRG1 homozygous deletion mutant, the NAT flipper cassette (pLC49) (Morschhauser et al., 1999) was PCR amplified using primers oLC3976 and oLC3977 (4366 bp) and transformed into CaLC239. NAT resistant transformants were PCR tested with oLC3972 + oLC275 (bp) for upstream integration and oLC274 + oLC3973 (bp) for downstream integration. The absence of WT LRG1 allele was verified with oLC3980 + oLC3981 (bp). The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.

**CaLC4375:** To generate a PKC1 heterozygous deletion mutant in a strain lacking LRG1, the PKC1 deletion construct was released from pLC470 (LaFayette et al., 2010) using BssHII and transformed into CaLC4090. NAT resistant transformants were PCR tested with oLC950 + oLC275 (850 bp) for upstream integration and oLC274 + oLC951 (730 bp) for downstream integration. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT flipper cassette.
CaLC3869: To introduce the G13V mutation into \textit{RAS1} and N-terminal tag Ras1 with GFP in SN95, the GFP-Ras1 construct carrying the G13V mutation was released from pLC379 (Shapiro et al., 2009) with Sac1 and Kpn1 and transformed into CaLC1255. NAT resistant transformants were PCR tested with oLC275 + oLC554 (465 bp) for upstream integration and oLC559 and oLC563 (1246 bp) for downstream integration. The integrated \textit{RAS1} mutant allele is PCR amplified with oLC274 + oLC563 (~2 kb) and presence of the G13V mutation was sequenced with oLC562.

CaLC3844/CaLC3845: To introduce the G13V mutation into \textit{RAS1} and N-terminal tag Ras1 with GFP in a strain lacking \textit{PKC1}, the GFP-Ras1 construct carrying the G13V mutation was released from pLC379 (Shapiro et al., 2009) with Sac1 and Kpn1 and transformed into CaLC1255. NAT resistant transformants were PCR tested with oLC275 + oLC554 (465 bp) for upstream integration and oLC559 and oLC563 (1246 bp) for downstream integration. The integrated \textit{RAS1} mutant allele is PCR amplified with oLC274 + oLC563 (~2 kb) and presence of the G13V mutation was sequenced with oLC562.

CaLC3861: To tag the NAT cassette with a HIS marker, the HIS cassette was amplified from pLC575 (Lavoie et al., 2008) with oLC3847 and oLC3848 (bp) and transformed into CaLC3844. HIS prototrophic transformants were PCR tested with oLC3849 + oLC2029 (566 bp) for upstream integration and oLC1645 + oLC3850 (812 bp) for downstream integration.

CaLC3900/CaLC3901: To drive the expression of NAT with \textit{HWP1} promoter, the NAT-HIS cassette was amplified from the genomic DNA of CaLC3861 with oLC3875 and oLC3876 (~5 kb). The PCR product was cleaned up by spin column purification and used as a template for amplification with oLC3851 and oLC3852 (2357 bp). The HWP1p-NAT-HIS construct was transformed into CaLC239. HIS prototrophic transformants were PCR tested with oLC3887 + oLC2029 (981 bp) for upstream integration and oLC1645 + oLC3855 (1016 bp) for downstream integration.

CaLC3898: To drive the expression of NAT with \textit{HWP1} promoter in a mutant lacking \textit{PKC1}, the NAT-HIS cassette was amplified from the genomic DNA of CaLC3861 with oLC3875 and oLC3876 (~5 kb). The PCR product was cleaned up by spin column purification and used as a template for amplification with oLC3851 and oLC3852 (2357 bp). The HWP1p-NAT-HIS construct was transformed into CaLC1255. HIS prototrophic transformants were PCR tested with
oLC3887 + oLC2029 (981 bp) for upstream integration and oLC1645 + oLC3855 (1016 bp) for downstream integration.

**CaLC3962/CaLC4252**: To select for suppressor mutation that rescues filamentous growth in a mutant lacking *PKC1*, CaLC3898 was subcultured for 3 hours at 37°C in YPD + 10% NBCS and 2 x10^6 cells were plated on YPD+10%NBCS+NAT. The plates were incubated at 37°C for 5 days and NAT resistant colonies were patched on YPD plate because the colonies grew slowly. The rescue of filamentation was tested by growth at 37°C in YPD + 10% NBCS + 1M sorbitol. Sorbitol was supplemented to stabilize cell wall defect caused by *PKC1* deletion.

### 3.2.3 Plasmids

All bacterial strains carrying plasmids were archived in 33% glycerol and stored at -80°C. Strains were grown in LB broth at 37°C. 2% agar was added for solid media. Plasmids used in this study are listed in Table 3.2. The absence of nonsense mutations on the plasmid was verified by sequencing. Primers used in this study are listed in Table 3.3.

**Table 3.2 Plasmids used in this study**

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<td>pLC575</td>
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<tr>
<td>pLC765</td>
<td>pLC49 <em>CaRHO1, ampR, NAT</em></td>
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</table>
3.2.4 Plasmid construction

Cloning procedures were performed following standard protocols.

**pLC706:** This is a construct to complement WT *CaPKC1* in *C. albicans*. Homology downstream of *PKC1* was amplified from SC5314 genomic DNA with primers oLC1029/oLC1030 (bp) and cloned into pLC49 at NotI and SacII. The presence of the inserts were tested by PCR with oLC274/oLC1030 (538 bp) and oLC1029/oLC244 (473 bp). Downstream homology was sequenced verified with oLC244 and oLC274. PKC1 with a promoter region and some of the terminator region was amplified from SC5314 genomic DNA with oLC1027/oLC1028 (4138 bp) and cloned into pLC49 containing the downstream homology of *PKC1* at ApaI. The presence of the inserts were tested by PCR with oLC243/oLC956 (942 bp) and oLC275/oLC1042 (605 bp). Upstream homology and ORF was sequence verified with oLC243, 1027, 1035, 1036, 1037, 1038, 1039, 1040, 1041 and downstream homology was re-verified with oLC244. The reconstitution construct can be liberated by digestion with BsshII.

**pLC770:** This is a construct to complement mutant *CaPKC1* carrying the M850G mutation in *C. albicans*. This plasmid is based on pLC706 but harbors a mutation in Pkc1 (M850G) that will not affect the kinase activity but render the kinase susceptible to 1-NA-PP1. This mutation was introduced by site-directed mutagenesis with primers oLC2998 and oLC2999. The clone was sequence verified with the following primers: oLC243, oLC1027, oLC1035, oLC1036, oLC1037, oLC1038, oLC1039, oLC1040, and oLC1041. The reconstitution construct can be liberated by digestion with BsshII.

**pLC765:** This is a construct to complement WT *CaRHO1* in *C. albicans*. Homology downstream of *RHO1* was amplified from SC5314 genomic DNA with primers oLC2867/oLC2868 (351 bp) and cloned into pLC49 at NotI and SacII. The presence of the inserts were tested by PCR with oLC274/oLC244 (550 bp). Downstream homology was sequenced verified with oLC274. RHO1 with a promoter region and some of the terminator region was amplified from SC5314 genomic DNA with oLC2862/oLC2866 (1557 bp) and
cloned into pLC49 containing the downstream homology of \textit{RHO1} at ApaI. The presence of the inserts were tested by PCR with oLC243/oLC2863 (296 bp) and oLC275/oLC2865 (356 bp). Upstream homology and ORF was sequence verified with oLC243 and oLC275. The reconstitution construct can be liberated by digestion with BsshII.

\textbf{pLC771}: This is a construct to complement mutant \textit{CaRHO1} carrying the T23N mutation in \textit{C. albicans}. This is based on pLC765 but harbors a mutation in Rho1 (T23N). This mutation was introduced by site-directed mutagenesis with primers oLC2994 and oLC2995. The clone was sequence verified with oLC2864. The reconstitution construct can be liberated by digestion with BsshII.

\textbf{pLC772}: This is a construct to complement mutant \textit{CaRHO1} carrying the Q67L mutation in \textit{C. albicans}. This is based on pLC765 but harbors a mutation in Rho1 (Q67L). This mutation was introduced by site-directed mutagenesis with primers oLC2996 and oLC2997. The clone was sequence verified with oLC2864. The reconstitution construct can be liberated by digestion with BsshII.

3.2.5 Oligonucleotides

\textbf{Table 3.3 Oligonucleotides used in this study.}

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| oLC1457 | IHD1+614-R | GCAGCATTTAGTAGCGTCAGA |
| oLC1645 | HIS-F | ACAAAACCTACTAATATCAGAT |
| oLC2029 | CaHA-R | ggcgaggtattggatgttc |
| oLC244 | M13-F | GTAAAACGACGGCCAG |
| oLC274 | pJK863down-F | CTGTCAAGGAGGGTATTCTGG |
| oLC275 | pJK863up-R | AAAGTCAAAAGTTCCAAGGGG |
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| oLC2837 | CaRHO1+667R M13F | TTTTTCCTTTTTTCTTTGTCTTCTTCTTTGTCTTTTTTAGTTGTGTTCTCATGTAATACGACGGCCAG |
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Note: The primer sequences are given in the context of their respective genes and positions. The sequences are aligned to show the corresponding regions of DNA or RNA.
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### 3.2.6 Experimental growth conditions

To deplete *RHO1*, strains were grown overnight at 30°C in YPD. Stationary phase cultures were split, adjusted to an OD<sub>600</sub> of 0.1 where one culture was treated with 0.05 ug/ml of doxycycline, while the other was left untreated. Cells were grown for 16 hours and were split again. The cells were adjusted to an OD<sub>600</sub> of 0.2 and grown for 6 hrs.
To assess mutant phenotype in response to filament-inducing cues by microscopy, cells were subcultured for 6 hours at 37°C in YPD only, YPD with 10% v/v heat-inactivated newborn calf serum (NBCS, Gibco #26010-066), RPMI medium (Gibco #11875-093), Spider medium (1% mannitol, 1% nutrient broth and 0.2% K$_2$HPO$_4$), or 6 hours at 30°C in YPD with 10 μM geldanamycin. To block filamentation, cytochalasin A (Bioshop # CYT555.5) was used at a final concentration of 5 μg/ml and farnesol (Sigma Aldrich # 277541) was used at a final concentration of 200 μM.

To monitor expression of hyphal genes in response to filament-inducing cues by qRT-PCR, cells were subcultured in YPD at 30°C or YPD with 10% NBCS at an OD$_{600}$ of 0.2 and grown for 3.5 hours.

3.2.7 Arrayed Morphology Screen

The Mitchell library (Blankenship et al., 2010; Nobile et al., 2006) was screened by microscopy in 96-well format. Overnight cultures were set up by transferring colonies from solid media with multichannel pipet and grown statically in 200 μl YPD in 96-well plates at 30°C. Approximately 0.5 ul of the cells were then transferred via 96-well pinner into rich medium (YPD) and incubated at 30°C for 8 hours under static conditions. Images of potential hits were captured on a Zeiss Axio Observer.Z1 (Carl Zeiss) using 10x and 40x magnifications. The mutants were scored for degree of filamentation on a scale from 0 (yeast), 1 (chained yeast), 2 (short filaments) or 3 (long filaments). To validate the phenotypes, overnight cultures were set up for the strains of interest in YPD at 30°C and grown shaking at 200 rpm. Images were captured using DIC microscopy on a Zeiss Imager M1 upright microscope and AxioCam Mrm with AxioVision 4.7 software at 100x magnification.

3.2.8 qRT-PCR

To prepare samples for RNA extraction, 10 ml of subcultures were harvested by centrifugation at 3000 rpm for 5 min. The pellet was flash-frozen and stored at -80°C overnight. RNA was isolated using the Qiagen RNeasy kit and cDNA was generated using the AffinityScript cDNA synthesis kit (Stratagene). qRT-PCR was carried out using the Fast SYBR Green Master Mix (Thermo Fisher Scientific) in 384-well plate with the following cycle conditions: 95°C for 10 min, repeat 95°C for 10 sec, 60°C for 30 sec for 40 cycles. The melt curve was completed with
the following cycle conditions: 95°C for 10 sec and 65°C for 5 seconds with an increase of 0.5°C per cycle up to 95°C. All reactions were done in triplicate. Data were analyzed in the Bio-Rad CFX manager 3.1.

3.2.9 Minimum Inhibitory Concentration Assay

Antifungal susceptibility was performed in flat bottom, 96-well microtitre plates (Sarstedt) using a broth microdilution protocol described in [1]. Minimum inhibitory concentration (MIC) assays were set up in 2-fold serial dilutions of caspofungin or calcofluor white in a final volume of 200 ul per well. Caspofungin gradient was from 125 ng/ml to 0.12 ng/ml. Calcofluor white gradient was from 250 μg/ml down to 3.9 μg/ml. Where applicable, DMSO was added to a final concentration of 0.05% and 1-NA-PP1 was added to a final concentration of 5 μM. Cell densities of overnight cultures were determined by measuring 100 ul of the culture in the 96-well plate in duplicates and dilutions were prepared such that ~10^3 cells were inoculated into each well. Plates were incubated in the dark at 30°C or 37°C for 48 hours, at which point the plates were sealed with tape and agitated to suspend the re-suspend the cells. Absorbance was determined at 600 nm using a spectrophotometer (Molecular Devices) and was corrected for background from the corresponding medium. Each strain was tested in duplicate in two biological replicates. MIC data was quantitatively displayed with color using the program Java TreeView 1.1.3 (http://jtreeview.sourceforge.net).

3.2.10 Active Ras1 pull-down

Active GTP-bound Ras1 was isolated using the Active Ras Pull-Down and Detection Kit (Pierce) following protocol as described previously (Grahl et al., 2015).

3.2.11 Selection Experiment

Non-filamentous strain carrying the HWP1p-NAT-HIS construct was subcultured in 10 ml YPD for 3 hours and the entire subculture is plated on YPD+10%NBS+NAT at 37°C. NAT resistant colonies were patched on YPD plate and rescued filamentation phenotype was confirmed in YPD+10%NBS+1M sorbitol.
3.2.12 Whole Genome Sequencing

Genomic DNA was isolated using the MasterPure™ Yeast DNA Purification Kit (MPY80200) and visualized on a 0.7% agarose gel after 100 minutes of electrophoresis at 70V. Genomic DNA was quantified using picogreen (ThermoFisher Scientific Inc. catalog code: P7589). Library preparation was performed using the standard Illumina indices and Nextera XT library preparation kit for paired end sequencing. Prepared libraries were sequenced using 126 bp reads on an Illumina HiSeq2500 in high output mode on a PE126 flow cell. Illumina adapters were trimmed from raw sequence FASTQ files using Trimmomatic (version 0.36) (Bolger, Lohse, & Usadel, 2014) for the standard nextera adapters. All reads were mapped to SC5314 assembly 21 (Candida Genome Database Assembly 21, FASTA downloaded on May 11, 2016) using the Bowtie2 alignment tool (version 2-2.2.9) (Langmead & Salzberg, 2012). PCR or optical duplicates were marked prior to SNP calling to reduce their influence on downstream analyses using the Picard tools MarkDuplicates function (Picard Tools - By Broad Institute. (n.d.). Retrieved May 26, 2016, from http://broadinstitute.github.io/picard/). To reduce false positive SNP calls insertions and deletions were realigned against the reference genome using the GATK RealignerTargetCreator, and IndelRealigner (GATK version 3.5) (McKenna et al., 2010). Coverage was also determined using GATK (version 3.5) using the DepthOfCoverage function (McKenna et al., 2010).

SNP calling and CNV analysis: SNPs were identified using MuTect1 (version 1.1.4) (Cibulskis et al., 2013) with read alignments to the SC5314 reference assembly 21. Quality SNPs were identified based on their logarithm of odds score (LOD). SNPs with a LOD score of below 15 were neglected. SNPs were manually verified using the integrated genome viewer (IGV version 2.3.72) (Robinson et al., 2011). All SNPs that passed the initial filtration were then analyzed for amino acid changes if the SNP fell within an ORF. SNPs occurring in non-coding regions also had the nearest genes flanking the SNP reported. Copy number variation was analyzed using the YMAP tool (http://lovelace.cs.umn.edu/Ymap/) (Abbey et al., 2014). Trimmed FASTQ files were uploaded to YMAP for each sample, and mapped to the reference C_albicans_SC5314_vA21-s02-m09-r07. GC-content bias and chromosome-end bias corrections were both applied to each samples prior to visualization.
3.2.13 Spotting Assay

Strains were grown overnight in YPD media at 30°C. 5 µl of cells were spotted on YPD, YPD + NAT, YPD + 1M sorbitol + 10% serum + NAT. Plates were incubated at 30°C or 37°C for 5 days and digitally imaged.

3.3 Results

3.3.1 Lrg1 is a repressor of filamentation.

I screened a homozygous transposon insertion mutant library to identify mutants that filament in the absence of filament-inducing cues (Blankenship et al., 2010; Nobile et al., 2006). I scored mutants based on degree of filamentous growth in YPD at 30°C and identified 3 negative regulators of filamentation: Tup1, Sfl1, and Lrg1 (Figure 3.1). Both Tup1 and Sfl1 have been characterized extensively as transcriptional repressors of hyphal growth (Braun & Johnson, 1997; Y. Li et al., 2007). In contrast, Lrg1 is largely uncharacterized in C. albicans. Its ortholog in S. cerevisiae is a Rho GTPase activator with roles in cell wall integrity (Lorberg, Schmitz, Jacoby, & Heinisch, 2001; Watanabe, Abe, & Ohya, 2001) and cell separation (Svarovsky & Palecek, 2005). However, Lrg1 is not required for cell wall stress responses in C. albicans; unlike a pkc1Δ/pkc1Δ mutant, the lrg1Δ/lrg1Δ mutant is not hypersensitive to the cell wall biosynthesis inhibitor caspofungin (Figure 3.2). Since Rho1 and its downstream effectors in the cell wall integrity pathway have never been implicated in morphogenesis, I mutated components in this pathway and assessed the impact on filamentous growth.
Figure 3.1 Lrg1 is a negative regulator of filamentation.

Strains were subcultured to log phase in YPD at 30°C for 4 hours and cells were imaged by DIC microscopy. The scale bar indicates 10 μm.
Figure 3.2 Lrg1 is not required of cell wall stress response.

The drug susceptibility assay was conducted with caspofungin in YPD at 30°C and incubated for 48 hours.

3.3.2 Activation of Rho1 promotes filamentation.

To determine if Rho1 modulates morphogenesis, I generated a strain in which I deleted one allele of RHO1 and placed the only remaining allele under the control of the tetO doxycycline-repressible promoter. In the absence of doxycycline, RHO1 was overexpressed in the tetO-RHO1/rho1Δ strain (Figure 3.3), but this did not alter cellular morphology in the absence of filament-inducing cues or in the presence of 10% serum (Figure 3.4). Doxycycline-mediated transcriptional repression of RHO1 with 0.05 µg/ml (Figure 3.3) also had no impact on morphology in these conditions (Figure 3.4). This low concentration of doxycycline has been optimized to enable transcriptional repression of target genes, while allowing the study of essential genes (Smith, Csank, Reyes, Ghannoum, & Berlin, 2002). Since Rho1 is known to cycle between an active GTP-bound state and an inactive GDP-bound state, I replaced the deleted RHO1 allele in the tetO-RHO1/rho1Δ strain with either RHO1Q67L, which mimics a constitutively GTP-bound form of Rho1, or RHO1T23N, which mimics a constitutively GDP-bound form of Rho1. A study in S. cerevisiae initially identified Q68L and T24N as substitutions that lock Rho1 in the active GTP-bound state and the inactive GDP-bound state, respectively (Nonaka et al., 1995). The two residues are conserved in C. albicans as Q67 and T23. Strikingly, introduction of the RHO1Q67L allele causes filamentation in the absence of any filament-inducing cue, with or without the addition of doxycycline to transcriptionally repress expression of the wild-type allele (Figure 2). In contrast, introduction of the RHO1T23N allele causes truncated filaments in response to serum, upon doxycycline-mediated transcriptional repression of the
wild-type allele (Figure 3.4). My results suggest that Rho1 in its active state promotes filamentation.

![RHO1 Graph]

**Figure 3.3** *RHO1* is depleted upon doxycycline treatment.

The overnights were subcultured for 24 hours in the presence or absence of 0.05 μg/ml of doxycycline. The strains were subcultured again in the same conditions for 4. cDNA was prepared from total RNA for qRT-PCR. The transcript level of *RHO1* was monitored and normalized to *GPD1*. Data are plotted as means ± SD for triplicate samples and are representative of two independent experiments.
Figure 3.4 Activated Rho1 promotes filamentation.

Overnight cultures were subcultured for 24 hours in the presence or absence of 0.05 μg/ml of doxycycline to achieve transcriptional repression of the wild-type allele of RHO1 that is under the control of the tetO promoter. Strains were subcultured in YPD at 30°C or YPD + 10% serum at 37°C, with or without 0.05 μg/ml of doxycycline, for 4 hours. Cells were imaged by DIC microscopy. The scale bar indicates 10 μm.
3.3.3 Pkc1 is a master regulator of morphogenesis.

The most well characterized effector downstream of Rho1 is the protein kinase Pkc1. Since deletion of *LRG1* is expected to lock Rho1 in its active form, I deleted one allele of *PKC1* in a wild-type strain and an *lrg1Δ*/*lrg1Δ* mutant to determine if signaling through Lrg1-Rho1-Pkc1 mediates filamentous growth. Deletion of one allele of *PKC1* had no impact on morphogenesis in the wild-type strain (Figure 3.5). In contrast, deletion of one allele of *PKC1* completely blocked the filamentous growth induced by *LRG1* deletion but not that induced by serum (Figure 3.5).

![Figure 3.5 Pkc1 is required for filamentous growth induced by deletion of *LRG1*.](image)

Strains were subcultured to log phase in YPD at 30°C or YPD + 10% serum at 37°C for 4 hours. Cells were imaged by DIC microscopy. The scale bar indicates 10 μm.
To further explore the role of Pkc1 in morphogenesis, I characterized a homozygous deletion mutant. Since the inactive form of Rho1 does not support full filamentation, I predicted that a mutant lacking Pkc1 would be blocked in filamentous growth. I found that the \textit{pkc1Δ/pkc1Δ} mutant was indeed unable to filament in response to various filament-inducing cues, including RPMI medium, and carbon-limiting Spider medium (\textbf{Figure 3.6A}). Filamentation was rescued by complementation with a wild-type allele of \textit{PKC1} (\textbf{Figure 3.6A}). Further, I performed qRT-PCR to monitor the expression of hyphal-specific genes \textit{HWP1} and \textit{IHD1} in the wild-type strain, \textit{pkc1Δ/pkc1Δ} mutant, and complemented strain grown in 10% serum at 37°C. Consistent with the morphology observed by microscopy, deletion of \textit{PKC1} blocks the upregulation of \textit{HWP1} and \textit{IHD1} (\textbf{Figure 3.6B}). Together, my results suggest that Rho1-mediated activation of Pkc1 is required for morphogenesis in response to diverse cues.
Figure 3.6 Pkc1 is required for filamentation in response to diverse cues.
(A) Homozygous deletion of *PKC1* blocks filamentation. Strains were subcultured to log phase in the specified conditions for 4 hours. Cells were imaged by DIC microscopy. The scale bar indicates 10 μm. (B) Deletion of *PKC1* blocks the upregulation of filament-specific transcripts *HWP1* and *IHD1*. Strains were subcultured to log phase in YPD at 30°C or YPD + 10% serum at 37°C, and the transcript levels of *HWP1* and *IHD1* was monitored by qRT-PCR and normalized to *GPD1*. Data are plotted as means ± SD for triplicate samples and are representative of two independent experiments.

3.3.4 Pkc1 kinase activity is important for the initiation and maintenance of filamentation.

In order to more precisely define the role of Pkc1 in morphogenesis, I tested whether Pkc1 kinase activity is required for the initiation of filamentation. To do so, I constructed a strain in which the *pkc1Δ/pkc1Δ* mutant is complemented with *PKC1<sup>M850G</sup>*, a gatekeeper allele that renders the kinase susceptible to inhibition by the ATP analog 1-Naphthyl-PP1 (1-NA-PP1) without affecting its kinase activity in the absence of the inhibitor (Gould et al., 2011). I validated that this allele functions as expected, by demonstrating that 1-NA-PP1 treatment phenocopies deletion of *PKC1* in conferring hypersensitivity to calcofluor white in the *pkc1Δ/pkc1Δ* mutant complemented with *PKC1<sup>M850G</sup>* allele, but has no impact on the strain harboring wild-type *PKC1* (Figure 3.7). This strain allowed me to interrogate the impact of Pkc1 kinase activity on filamentation. Since the Pkc1 gatekeeper strain is temperature-sensitive (Figure 3.7), I focused on the Hsp90 inhibitor geldanamycin as a cue that induces filamentation at 30°C. As controls, I showed that treatment with 1-NA-PP1 does not induce filamentous growth, and that treatment with geldanamycin alone induces filamentation in the *pkc1Δ/pkc1Δ* mutant complemented with either a wild-type *PKC1* allele or the *PKC1<sup>M850G</sup>* allele, but not in the strain lacking *PKC1* (Figure 3.8). Importantly, I found that 1-NA-PP1 completely blocked filamentation induced by geldanamycin in the *pkc1Δ/pkc1Δ* mutant complemented with the *PKC1<sup>M850G</sup>* allele, but did not affect filamentation of the *pkc1Δ/pkc1Δ* mutant complemented with a wild-type *PKC1* allele (Figure 3.8). My results demonstrate that Pkc1 kinase activity is critical for filamentous growth.
Figure 3.7 The Pkc1M850G gatekeeper mutant is sensitive to high temperature or to inhibition by ATP analogue 1-NA-PP1.

The calcofluor white susceptibility assay was set up in the presence of 0.05% DMSO or 5 μM 1-NA-PP1, and was analyzed after 48 hours of growth at 30°C or 37°C.
Figure 3.8 Pkc1 kinase activity is critical for filamentation.

Strains were subcultured for 4 hours with either 10 μM of the Hsp90 inhibitor geldanamycin, 5 μM of the ATP analog 1-NA-PP1 that inhibits the gatekeeper allele, or both. Cells were imaged by DIC microscopy. The scale bar indicates 10 μm.

3.3.5 Pkc1 regulates filamentation independent of the MAP kinases in the cell wall integrity pathway.

The canonical MAPK kinase cascade downstream of Pkc1 includes Bck1, Mkk2 and Mkc1. Strikingly, deletion of BCK1 or MKC1 did not impair filamentation in response to serum or
geldanamycin, suggesting that additional targets downstream of Pkc1 remain to be identified (Figure 3.9).

Figure 3.9 The MAP kinase cascade downstream of Pkc1 is not required for filamentous growth.

Strains were subcultured for 4 hours in YPD at 30°C or YPD + 10% serum at 37°C or YPD + 10 μM geldanamycin (GdA). Cells were imaged by DIC microscopy. The scale bar indicates 10 μm.
3.3.6 Pkc1 signaling operates in parallel to Ras1 signaling to enable filamentation.

The role of Pkc1 as a positive regulator of morphogenesis in response to diverse cues is reminiscent of components of the Ras1-protein kinase A (PKA) signaling cascade (Feng, Summers, Guo, & Fink, 1999; Shapiro et al., 2009). To test for a genetic interaction between Ras1 and Pkc1, I introduced a dominant active $\text{RAS1}^{\text{V13}}$ allele into the $\text{pkc1}^{\Delta}/\text{pkc1}^{\Delta}$ mutant. The dominant $\text{RAS1}$ mutation is known to enhance filamentation under permissive conditions without affecting the yeast morphology in rich medium at 30°C (Feng et al., 1999; Leberer et al., 2001).

If Pkc1 functions downstream of the Ras-PKA pathway and is required to execute polarized growth programs in response to signals transduced via Ras1-PKA signaling, then the $\text{RAS1}^{\text{V13}}$ allele should not be able to rescue filamentation in the $\text{pkc1}^{\Delta}/\text{pkc1}^{\Delta}$ mutant. If, on the other hand, Pkc1 is required for sensing cues and functions upstream of the Ras-PKA pathway, then I would expect that the $\text{RAS1}^{\text{V13}}$ allele would rescue filamentation in the $\text{pkc1}^{\Delta}/\text{pkc1}^{\Delta}$ mutant. Instead, I found that the hyperactivation of the Ras1-PKA pathway in a mutant lacking Pkc1 minimally restored filamentation in response to 10% serum at 37°C in liquid or on solid rich medium (Figure 3.10). The $\text{RAS1}^{\text{V13}}$ allele also minimally restored the expression of $\text{HWP1}$ in the $\text{pkc1}^{\Delta}/\text{pkc1}^{\Delta}$ mutant when cultured in rich medium with 10% serum at 37°C (Figure 3.10B).

These results suggest that Pkc1 does not act directly upstream Ras1-PKA pathway to regulating morphogenesis.
Figure 3.10 Pkc1 does not function upstream of the Ras-PKA pathway.

(A) Hyperactivation of Ras1 minially rescues filamentation in mutant lacking Pkc1. Strains were subcultured for 4 hours in YPD at 30°C or YPD + 10% serum at 37°C. Cells were imaged by DIC microscopy. The scale bar indicates 10 μm. (B) Hyperactivation of Ras1 partially restores HWP1 transcript level in a mutant lacking Pkc1. Strains were subcultured to log phase in YPD at 30°C or YPD + 10% serum at 37°C. The transcript levels of HWP1 and IHD1 in YPD + 10% serum at 37°C were first normalized to GPD1 and then normalized to the level in YPD at 30°C. Data are plotted as means ± SD for triplicate samples and are representative of two independent experiments.

To corroborate these findings, GTP-Ras1 was monitored as a reporter for Ras1 activation. I found that deletion of PKC1 led to hyperactivated Ras1 in response to 10% serum at 37°C, indicating that Pkc1 is not required for Ras1 activation (Figure 3.11). In addition, deletion of LRG1 did not hyperactivate Ras1 despite causing constitutive filamentous growth (Figure 3.11). Taken together, my results suggest that Lrg1 negatively regulates filamentation through Rho1 and Pkc1, independent of Ras1 signaling.

![Figure 3.11 Pkc1 is not required for Ras1 activation.](image)

Figure 3.11 Pkc1 is not required for Ras1 activation.

Strains were grown in YPD at 30°C or YPD + 10% serum at 37°C for 3.5 hours. The total Ras1 protein and GTP-Ras1 fraction were resolved by SDS-PAGE gel. The GTP-Ras1/total Ras1 ratio is shown as a percentage.

To determine whether the adenylyl cyclase Cyr1 is required for filamentation caused by Lrg1-dependent activation of Pkc1, I used the quorum sensing molecule farnesol to inhibit cAMP signaling (Davis-Hanna, Piispanen, Stateva, & Hogan, 2008; Lindsay, Deveau, Piispanen, & Hogan, 2012). Strikingly, farnesol completely blocked filamentous growth associated with LRG1.
deletion (Figure 3.12). Together, my results indicate that Pkc1 functions in parallel with the Ras1 but upstream of Cyr1 in transducing filament-inducing cues.

![WT, 37°C](image1) ![lrgΔ/lrg1Δ, 30°C](image2)

**Figure 3.12** The cAMP signaling is required for Pkc1-dependent filamentous growth.

Strains were grown in YPD at 30°C, YPD at 37°C, or YPD + 200 μM farnesol for 3.5 hours. Cells were imaged by DIC microscopy. The scale bar indicates 10 μm.

3.3.7 Pkc1 may also regulate filamentation via actin.

In order to identify the circuitry through which Pkc1 controls filamentation, I aimed to identify mutations that could restore filamentation in a pkc1Δ/pkc1Δ mutant. One strategy that has been used to restore filamentation in a non-filamentous mutant is passage through macrophages (Wartenberg et al., 2014). To facilitate the selection process, I generated a reporter system by replacing one allele of *HWP1* with a nourseothricin (NAT) resistance marker, such that the expression of NAT is under the control of the endogenous *HWP1* promoter. Since *HWP1* is highly upregulated in filaments compared to yeast cells, only strains that could filament in response to filament-inducing conditions would be able to grow on plates with a high concentration of NAT. To test this system, I introduced the *HWP1p-NAT-HIS* construct into both the wild type and the pkc1Δ/pkc1Δ mutant. As expected, the wild-type strain harboring the reporter shows robust growth on NAT at 39°C, an elevated temperature that induces filamentous
growth in the absence of additional cues (Shapiro, Sellam, et al., 2012), whereas no growth was observed for the pkc1Δ:pkc1Δ mutant. This provides a tractable system to enable selection for filamentous growth (Figure 3.13).

Figure 3.13 The HWP1p-NAT reporter construct enables cells expressing the filament-specific transcript HWP1 to grow on NAT.

Strains grown in YPD overnight were spotted on YPD and YPD + NAT plates. The plates were incubated at 30°C or 37°C for 5 days and digitally imaged.

For the selection experiment, I plated the pkc1Δ:pkc1Δ mutant harboring the reporter on solid rich medium supplemented with 10% serum and 250 µg/ml NAT. To confirm that the NAT-resistant isolates could filament in liquid medium, I subcultured the strains in rich medium...
supplemented with 10% serum and 1M sorbitol. The *pck1Δ/pck1Δ* mutant is unable to filament even in the presence of osmotic stabilizer, while the NAT-resistant isolates showed filamentous growth similar to that of the wild type (*Figure 3.14*). To identify candidate mutations that rescued the filamentous growth of the *pck1Δ/pck1Δ* mutant, whole genome sequencing was performed.
Figure 3.14 The NAT-resistant isolates have restored capacity to filament.
Strains were subcultured for 4 hours in YPD at 30°C or YPD + 10% serum + 1M sorbitol at 37°C. Cells were imaged by DIC microscopy. The scale bar indicates 10 μm. Gene carrying the suppressor mutation is labeled in red.

The two \( pkc1\Delta/pkc1\Delta \) mutants with rescued filamentous growth had nonsynonymous mutations in \( ORF19.7149 \), which encodes a putative GTPase inhibitor with a predicted role in endocytosis, and \( ORF19.1689 \), which encodes a putative phosphatidate phosphatase with a predicted role in reorganization of the actin cytoskeleton (Blom et al., 2004; Inglis et al., 2012). Given that both mutations were heterozygous, they are likely dominant in nature and affect functions downstream of Pkc1. Interestingly, both gene products are implicated in actin-related processes. Since actin mutants in \( S.\ cerevisiae \) exhibit defects in filamentous growth (Cali, Doyle, Botstein, & Fink, 1998) and inhibition of actin polymerization with actin destabilizers cytochalasin A or latrunculin A blocks germ tube formation (Wolyniak & Sundstrom, 2007), I asked whether actin function is also required for Pkc1-dependent filamentation. To this end, I tested the effect of cytochalasin A on filamentous growth mediated by \( LRG1 \) deletion and found that disrupting the actin cytoskeleton also blocks the formation of pseudohyphae in the \( lrg1\Delta/lrg1\Delta \) mutant (Figure 3.15), suggesting that Pkc1 may regulate filamentation by modulating actin function.

![Figure 3.15 Destabilization of actin blocks filamentation in the \( lrg1\Delta/lrg1\Delta \) mutant.](image)
Strains were subcultured to log phase in YPD at 30°C or 37°C for 3.5 hours in the presence or absence of 5 μg/ml of cytochalasin A.

3.4 Discussion

Morphogenetic transitions underpin virulence of diverse fungal pathogens. My work has identified a novel core regulatory pathway that controls morphogenesis of a leading human fungal pathogen in response to diverse host-relevant cues (Figure 3.16). Leveraging a genetic screen, I identified the Rho1 GAP Lrg1 as a repressor of filamentation in C. albicans (Figure 3.1). In the absence of filament-inducing cues, Lrg1 inactivates Rho1 by stimulating its GTPase activity. Locking Rho1 in the active GTP-bound form by introducing a T23N substitution or deleting LRG1 leads to constitutive filamentation in the absence of cues (Figures 3.1 and 3.4). Reciprocally, locking Rho1 in the inactive GDP-bound form by introducing the Q67L substitution impairs filamentation (Figure 3.4). Rho1 controls filamentation through the protein kinase Pkc1 (Figure 3.5), but in a manner that is independent of the canonical MAP kinase cascade (Figures 3.8). In addition, Pkc1 functions in parallel with the small GTPase Ras1 and may co-regulate cAMP signaling via Cyr1 (Figure 3.9). Lastly, mutations in genes involved in actin-related processes rescued the filamentation defect in mutant lacking Pkc1. My results illuminate a new role for signaling through Lrg1, Rho1, and Pkc1 in orchestrating morphogenetic programs in response to diverse cues, possibly via actin, and establish a novel link between cell wall remodeling and cellular morphogenesis.
Figure 3.16 Model depicting Pkc1-dependent morphogenetic regulation in *C. albicans*.

Fungi utilize complex circuitry to control cellular morphology. For *C. albicans*, the number of positive regulators of morphogenesis that enable filamentation in response to cues vastly exceeds the number of negative regulators that promote yeast form growth in the absence of inducing cues (O'Meara et al., 2015). Lrg1 is distinct among the repressors of filamentation that have been identified to date. Only a handful of repressors of filamentation have being well characterized, many of which are DNA binding proteins, such as Rfg1 (Khalaf & Zitomer, 2001), Sfl1 (Bauer & Wendland, 2007), Nrg1 (Murad et al., 2001), and Tup1 (Braun & Johnson, 1997). Other than transcriptional repressors, cell cycle proteins feature as the most prominent repressors of filamentation (O'Meara et al., 2015). In contrast, Lrg1 is a Rho1 GAP, which negatively
regulates Rho1 function by stimulating its GTPase activity, thereby converting Rho1 from an active, GTP-bound state to an inactive, GDP-bound state. My results suggest that Lrg1 represses filamentous growth by downregulating Rho1 activity. In *S. cerevisiae*, Lrg1 functions upstream of the Pkc1-dependent cell wall integrity pathway and specializes in the negative regulation of 1,3-beta-glucan synthesis via Rho1 (Lorberg et al., 2001; Watanabe et al., 2001). In *C. albicans*, Lrg1 functions upstream of Pkc1 in negatively regulating filamentation via Rho1 but does not seem to affect glucan synthesis (Figure 3.2 and 3.5), suggesting that the Rho1 regulation has been rewired between *S. cerevisiae* and *C. albicans*.

Pkc1 is a central hub in cellular circuitry and is fundamental to coordinating sensing environmental cues and orchestrating cellular responses via the cell wall integrity pathway. My discovery that Pkc1 is required for *C. albicans* filamentation in response to diverse cues, but that the MAP kinase cascade is dispensable, implicates alternative downstream effectors. In *S. cerevisiae*, Pkc1 controls multiple cellular processes in addition to the downstream MAP kinase cascade, including activation of the DNA integrity checkpoint (Soriano-Carot, Quilis, Bano, & Igual, 2014), microtubule functions (Hosotani, Koyama, Uchino, Miyakawa, & Tsuchiya, 2001), glycerol metabolism (Brandao et al., 2002; Gomes et al., 2005), as well as organization of the actin cytoskeleton (Chai, Hsu, Du, & Laurent, 2002). In *C. albicans*, the impact of these processes on filamentation may suggest clues as to mechanisms through which Pkc1 affects morphogenesis. In *C. albicans*, inhibition of DNA replication with hydroxyurea or disruption of microtubules with nocodazole triggers polarized growth (Shareck & Belhumeur, 2011), and thus alterations in these processes are unlikely to account for the filamentation defect observed upon loss of Pkc1 kinase activity. In contrast, the destabilization of actin cables blocks filamentous growth (Wolyniak & Sundstrom, 2007). Further, dominant mutations in ORF19.7149 and ORF19.1689, two genes with known roles in actin-related processes, rescued the filamentation defect associated with loss of Pkc1, suggesting that Pkc1 may enable filamentation by controlling remodeling of the actin cytoskeleton.

The central importance of morphogenesis for fungal nutrient foraging, virulence, and immune evasion, may underpin the elaboration of complex morphogenetic circuitry. It is striking that despite many pathways that operate in parallel, there is little functional redundancy at levels above transcriptional regulation. For example, the classic central pathway that governs *C. albicans* morphogenesis is the Ras1-PKA cascade (Hogan & Sundstrom, 2009; P. E. Sudbery,
Deletion of genes encoding positive regulators of the pathway, such as the small GTPase Ras1 or the adenylyl cyclase Cyr1, blocks filamentation in response to diverse cues. The Lrg1-Rho1-Pkc1 pathway is also required for filamentation in response to diverse cues, highlighting its key role in morphogenesis. These pathways also share a number of similar features. For example, both Rho1 and Ras1 are small GTPases whose function in filamentation is negatively regulated by their respective GAPs, Lrg1 and Ira2, and facilitated by their respective downstream kinases, Pkc1 and PKA. Although it has been well established that PKA signals through the transcription factor Efg1, downstream targets of Pkc1 in *C. albicans* distinct from the MAP kinase cascade remain elusive. Since Pkc1 and Ras1 function in parallel and upstream of Cyr1, Pkc1 may be regulating Cyr1 activity via phosphorylation of Cyr1 or the highly phosphorylated Cyr1-associated protein Srv2 (Willger et al., 2015). Notably, Pkc1 has been shown to phosphorylate Bni1 in *S. cerevisiae* (Kono, Saeki, Yoshida, Tanaka, & Pellman, 2012). Bni1 and Bnr1 are the two formins that participate in the nucleation of actin (Pruyne et al., 2002). Genetic compromise of both formins in *C. albicans* has been shown to block filamentation in response to serum (C. R. Li et al., 2005), providing a possible functional connection between Pkc1 and actin dynamics.

Pkc1 regulates diverse fungal cellular processes and is emerging as a promising drug target for the development of antifungal therapy. In addition to morphogenesis, Pkc1 also plays a key role in mediating drug resistance in *C. albicans* (LaFayette et al., 2010). In fact, a number of Pkc1 inhibitors were identified in a screen for small molecules that abrogate azole resistance of a *C. albicans* clinical isolate (LaFayette et al., 2010), suggesting that targeting Pkc1 holds great therapeutic potential in combination with existing antifungal drugs. Kinases provide tractable targets for pharmacological inhibition and a number of PKC inhibitors have advanced in clinical trials for a number of diseases, including oncology, congestive heart failure, bipolar disorder, and diabetic retinopathy (Mochly-Rosen, Das, & Grimes, 2012). However, targeting mammalian PKC is complicated by the existence of eight isoforms, encoded by seven closely related genes (Mochly-Rosen et al., 2012). Although fungal Pkc1 is biochemically and functionally distinct from the mammalian PKCs, the catalytic domain is highly conserved between yeast and human (Paravicini et al., 1996). However, it is possible to specifically target Pkc1 in fungi, as demonstrated by the discovery of cercosporamide, a natural product identified in a screen for inhibitor selective against *C. albicans* Pkc1 (Paravicini et al., 1996). Therefore, Pkc1 is a major...
virulence factor in *C. albicans* with significant role in the regulation of morphogenesis and drug resistance, and represents a feasible target for the development of novel antifungal drugs.
Chapter 4  
Conclusion, General Discussion, and Future Directions

4 Conclusion, General Discussion, and Future Directions

4.1 Conclusion

Collectively, the work presented here provides novel insights into molecular mechanisms that govern drug resistance and morphogenesis in *C. albicans*. I have established that the transcription factor Cas5 orchestrates a key transcriptional program required for echinocandin tolerance and resistance (Chapter 2). Cas5 is activated by Glc7-dependent dephosphorylation and modulates cellular stress response and cell cycle progression in part via interaction with Swi4 and Swi6, components of SBF complex. My work is the first to identify transcriptional activation as a mechanism of echinocandin resistance, and reveals a novel role for Cas5 in cell cycle regulation. I have also discovered a novel link between Pkc1 signaling and cellular morphogenesis in *C. albicans* (Chapter 3). Upon receiving the filament-inducing cue, GTP-bound Rho1 activates Pkc1 and transduces the morphogenesis signal via a pathway that is independent of the canonical MAP kinase cascade downstream of Pkc1, possibly through regulation of actin biology. This is one of the few pathways that functions in parallel with Ras1 in promoting filamentous growth in response to diverse cues. Together, this research reveals novel mechanisms of drug resistance and morphogenesis, and emphasizes the therapeutic potential of targeting key regulators of cellular stress response in the development of much needed antifungal drugs.

4.2 General Discussion

4.2.1 Drug resistance – the ongoing challenge in the treatment of infectious diseases

During the course of antimicrobial therapy, drug resistance is often the main cause of treatment failure. The U.S. Centers for Disease Control and Prevention (CDC) has estimated that antibiotic resistance is responsible for more than 23,000 deaths each year in the United States, costing $20 billion in healthcare and $35 billion in loss of productivity (CDC, 2013). Penicillin, the very first antibiotic, was discovered by Sir Alexander Fleming in 1928 (Fleming, 2001). An enzyme that
inactivates penicillin was reported in 1940; as such, a mechanism of penicillin resistance was characterized even before penicillin was widely used as a therapeutic (Abraham & Chain, 1988). Ever since, the emergence of antibiotic resistance has quickly outpaced the development of new drugs. Oxazolidinone, the newest class of antibiotics, was discovered in 1987 (Shaw & Barbachyn, 2011). By binding to the 23S rRNA of the 50S ribosomal subunit, oxazolidinones interfere with the initiation of protein translation, arresting bacterial growth (Shaw & Barbachyn, 2011). It is effective against a number of gram-positive bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (Tsiodras et al., 2001). The oxazolidinones linezolid was first approved for clinical use in 2000, and the first case of clinically resistant *S. aureus* was subsequently reported in 2001 (French, 2003; Tsiodras et al., 2001). Later studies showed that a number of mutations in the genes encoding 23S rRNA can confer resistance to linezolid (Shaw & Barbachyn, 2011), an example of target alteration as a mechanism of drug resistance. The discovery of antibiotics inaugurated a new era for the treatment of bacterial infections; however, the future of antibiotics faces uncertainty due to the declining effectiveness of antibiotics associated with increasing prevalence of resistance, substantiating the need for innovative approaches in the development of effective therapeutic strategies.

Given that no new class of antibiotics has been discovered in almost thirty years, alternative strategies have been explored to enhance the potency of the existing drugs. In recent years, combination therapy has emerged as a promising regimen that could significantly improve the clinical outcome of multidrug resistant bacterial infections. The value of multidrug therapy is best exemplified by the treatment of tuberculosis, for which fixed-dose combinations of rifampicin, isoniazid, pyrazinamide, and ethambutol were recommended by the World Health Organization (Blomberg, Spinaci, Fourie, & Laing, 2001). In addition to tuberculosis, the clinical use of antibiotic cocktails has been recommended for the treatment of infections caused by *Pseudomonas aeruginosa* and MRSA (C. Liu et al., 2011; Mandell et al., 2007; Tamma, Cosgrove, & Maragakis, 2012). Another strategy focuses on the identification of the optimal drug combination such that resistance to one antibiotic confer sensitivity to the other, a phenomenon known as collateral sensitivity (Imamovic & Sommer, 2013; Pal, Papp, & Lazar, 2015). In one study, *E. coli* isolates were evolved in parallel to become resistant to 23 different clinically relevant antibiotics, and 74% of the resistant isolates were sensitive towards at least
one antibiotics (Imamovic & Sommer, 2013). Similar results were obtained with pathogenic *E. coli* isolates (Imamovic & Sommer, 2013), demonstrating that systematic search for drug pairs with collateral sensitivity could have great therapeutic potential. In addition to traditional combination therapy, sequential treatments with two antibiotics at sublethal doses have also been shown to clear bacteria (Fuentes-Hernandez et al., 2015). Sequential treatments are particularly effective against *Helicobacter pylori*, resulting in eradication of *H. pylori* from a staggering 91% of the treated patients (Y. S. Kim et al., 2011; Vaira, Zullo, Hassan, Fiorini, & Vakil, 2009), suggesting that sequential treatments provide another strategy to combat the emergence of drug resistance.

Drug resistance in fungi is also becoming a major concern in the clinic due to the growing population of immunocompromised individuals that is susceptible to fungal infection and the limited number of treatment options (Perea & Patterson, 2002; Pfaller & Diekema, 2010; Roemer & Krysan, 2014). One of the main challenges in the ongoing effort to develop novel antifungals is the identification of drug targets that are not well conserved between fungi and their closely related eukaryotic host. As such, structures unique to fungi have been the primary targets of the existing antifungal drugs. The azoles and polyenes both target the fungal sterol ergosterol or its biosynthesis. Ergosterol serves the same function in the fungal cell membrane as cholesterol in mammalian cell membrane. The azoles inhibit lanosterol demethylase, encoded by *ERG11* in *C. albicans*, one of the biosynthetic enzymes involved in the production of ergosterol. Although biosynthesis of mammalian cholesterol is also affected by azoles, a much higher dose of the drug is required to achieve similar inhibition as in fungi (Ghannoum & Rice, 1999). The polyenes bind and remove ergosterol from the fungal cell membrane, leading to cell death (T. M. Anderson et al., 2014). However, polyenes exhibit similar activity against cholesterol in the mammalian cell membrane, leading to host toxicity (T. M. Anderson et al., 2014). In contrast, the echinocandins target the production of 1,3-β-D-glucan, a key linker molecule in the fungal cell wall, via non-competitive inhibition of the catalytic subunit of the 1,3-β-D-glucan synthase Fks1. Since mammalian cells lack cell wall, the echinocandins are broadly effective against many pathogenic fungi, including *C. albicans*, while maintaining low toxicity to the host (Denning, 2002). Despite the clinical success, the echinocandins remain to be the only class of antifungals to have reach the clinic in decades (Denning, 2002), and their utility is limited to intravenous administration with no clinical efficacy against *Cryptococcus* species. Furthermore,
clinically acquired resistance to the echinocandins has already been reported, with the most common mechanism being mutations in the drug target gene, FKS1. The current lack of new antifungal drugs and the rapid emergence of drug resistance further emphasize the urgent need for novel antifungal therapeutics.

Similar to the problem with antibiotic resistance, one promising strategy that can overcome antifungal drug resistance and facilitate new drug development is to pursue combinatorial therapy. Although resistance to antifungal combinations in *C. albicans* can arise *in vitro*, the mutations acquired are often associated with fitness costs that would likely minimize the evolution of resistance to drug combinations *in vivo* (Hill, O’Meara, & Cowen, 2015). A number of studies have explored the efficacy of drug combinations in the treatment of fungal infections with clinically approved antifungals, many of which have no additive benefits when used in combination (M. D. Johnson, MacDougall, Ostrosky-Zeichner, Perfect, & Rex, 2004). Nonetheless, synergy with existing antifungals is a useful parameter in the search for novel drug targets and compounds. For example, a screen of clinically approved drugs in combination with azoles or echinocandins can lead to the identification of new antifungals with known pharmacokinetics and safety profiles (Robbins et al., 2015). Drug repurposing is not a new concept (Strittmatter, 2014). One prominent example is azidothymidine (AZT), which was initially developed as an anticancer agent; although its activity against tumor cells was limited, it was highly effective against HIV and subsequently approved as an anti-HIV agent (Strittmatter, 2014). Alternatively, new compounds can be designed to specifically target signaling pathways that facilitate the development of antifungal drug resistance. There is strong momentum in exploring this approach, particularly in the context of *C. albicans*.

A number of the key regulators of cellular stress responses have been shown to play a role in the evolution of antifungal drug resistance. In *C. albicans*, the essential and ubiquitous molecular chaperone Hsp90 enhances drug tolerance and enables drug resistance by stabilizing important signal transducers, including calcineurin and Mkc1 (Cowen & Lindquist, 2005; LaFayette et al., 2010; S. D. Singh et al., 2009). Genetic depletion or pharmacological inhibition of Hsp90 improves the efficacy of azoles and impedes the evolution of azole resistance (Cowen & Lindquist, 2005; LaFayette et al., 2010). Similarly, compromising the function of Hsp90 or its client calcineurin reduces intrinsic tolerance to echinocandins and abrogates clinically acquired echinocandin resistance (S. D. Singh et al., 2009). Mkc1 is another Hsp90 client protein and it
functions downstream of Pkc1 in the cell wall integrity pathway (LaFayette et al., 2010). Deletion of PKC1 or inhibition of the kinase phenocopies Hsp90 inhibition and attenuates clinically relevant azole resistance (LaFayette et al., 2010). One mechanism by which Hsp90 clients regulate echinocandin resistance is through synthesis of cell wall chitin. In response to echinocandin, Pkc1, calcineurin, and the MAP kinase Hog1 upregulate the expression of chitin synthases genes, thereby increasing chitin production and reducing echinocandin efficacy (Walker et al., 2008). However, it is still unclear which transcription factors orchestrate the transcriptional response that mediates drug tolerance and resistance, particularly in the context of echinocandins.

The current understanding of transcriptional regulation that promotes drug resistance has been largely focused on azoles (Sanglard, Coste, & Ferrari, 2009). One of the main mechanisms of azole resistance in the clinic is through overexpression of genes encoding multi-drug transporters CDR1, CDR2, or MDR1 (Lohberger, Coste, & Sanglard, 2014). The transcription factor Tac1 controls the expression of CDR1 and CDR2, while Mrr1 controls the expression of Mdr1 (Coste, Karababa, Ischer, Bille, & Sanglard, 2004; Lohberger et al., 2014; Morschhauser et al., 2007). Gain-of-function mutations in TAC1 cause upregulation of the expression of CDR1 and CDR2 and confer azole resistance in clinical isolates (Morio et al., 2013), and deletion of TAC1 abolishes the expression of CDR1 and CDR2 as well as associated drug resistance phenotypes (Sanglard et al., 2009), highlighting the role of this transcription factor in the regulation of azole resistance. Although one study showed that overexpression of CDR2 confers low levels of resistance to caspofungin (Schuetzer-Muehlbauer et al., 2003), other studies suggested that there is no correlation between the expression of multi-drug transporters and caspofungin resistance (Bachmann, Patterson, & Lopez-Ribot, 2002; Niimi et al., 2006). A transporter that mediates the uptake of caspofungin also remains to be identified (Paderu, Park, & Perlin, 2004). These data suggest that upregulating the expression of drug pumps is not likely a central mechanism of echinocandin resistance. Another mechanism of azole resistance is through upregulation of the drug target Erg11 via the transcription factor Upc2 (Marichal et al., 1997; Perea et al., 2001). Gain-of-function mutations in UPC2 result in upregulation of ERG11 expression and a corresponding increase in azole resistance (Dunkel et al., 2008). However, intrinsic overexpression of the echinocandin drug target Fks1 has not been associated with echinocandin
resistance (Sanglard et al., 2009), raising the question of whether it is possible to promote echinocandin resistance via transcriptional regulation.

Although transcriptional profiling analyses have shed light on the genes that are differentially regulated in response to echinocandins, only a few transcription factors have been implicated in their regulation, and none have been implicated in echinocandin resistance (Bruno et al., 2006; T. T. Liu et al., 2005; Rauceo et al., 2008). For example, the bZIP transcription factor Sko1 is required for caspofungin tolerance in *C. albicans* (Rauceo et al., 2008). Sko1 is required for the expression of cell wall biogenesis genes *CRH11, MNN2*, and *SKN1*, and its own expression is indirectly regulated by the PAS kinase Psk1 (Rauceo et al., 2008). However, there is no clear evidence to suggest that Sko1 directly regulates the expression of caspofungin-responsive genes or that Sko1 plays a role in caspofungin resistance. Another example is the MADS-box transcription factor Rlm1, which functions downstream of the cell wall integrity pathway in *S. cerevisiae*. Although Rlm1 is required for the expression of the cell wall gene *PGA13* in response to caspofungin in *C. albicans*, deletion of *RLM1* has limited impact on caspofungin tolerance in both fungal species (Chamilos et al., 2009; Reinoso-Martin et al., 2003). Instead, the zinc finger transcription factor Cas5 has been considered as the major transcriptional regulator of caspofungin tolerance in *C. albicans* (Bruno et al., 2006). While Cas5 has been shown to govern the expression of a core set of caspofungin-responsive cell wall genes, including *ECM331* and *PGA13*, it has remained unclear how Cas5 is regulated and whether it is also important for caspofungin resistance.

The work presented in this thesis provides the first evidence for a functional relationship between transcriptional regulation and echinocandin resistance. This study demonstrated that Cas5 is not only required for caspofungin tolerance, it is also necessary for Fks1-dependent caspofungin resistance. In response to caspofungin, Cas5 is activated by Glc7-mediated dephosphorylation. Cas5 physically interacts with Swi4 and Swi6 to co-regulate the expression of caspofungin-responsive genes, implicating Swi4 and Swi6 as novel regulator of caspofungin tolerance in *C. albicans*. This work demonstrated that transcription factors such as Cas5 can significantly impact drug resistance and serve as promising targets for the development of novel antifungal therapies.
4.2.2 Morphogenesis – the capacity to adapt to host environment by shapeshifting

Recent advances in the understanding of *C. albicans* biology have linked multiple cellular processes to morphogenesis signaling. A recent study has established a novel connection between mitochondrial function and the yeast-to-hyphal transition (Grahl et al., 2015; Guedouari et al., 2014). During the filamentation process, *C. albicans* has been shown to activate the TCA cycle and increase mitochondrial respiration (Guedouari et al., 2014). In addition, genetic compromise of the mitochondrial electron transport chain or chemical perturbation of mitochondrial activity with bacterial toxin pyocyanin both resulted in reduced Ras1 activation and decreased filamentation in response to N-acetylglucosamine at 37°C (Grahl et al., 2015), suggesting that the metabolic need for filamentous growth is integrated into the morphogenetic program. Aside from respiration, nutritional availability also affects filamentation. In response to nitrogen starvation on solid surfaces, the ammonium permease Mep2 is required to sense the nitrogen limitation and mediate filamentous growth via Cph1-dependent MAP kinase signaling and the cAMP-dependent PKA signaling in a Ras1-dependent fashion (Biswa & Morschhauser, 2005). Furthermore, high concentrations of ammonium can suppress filamentation (Biswa & Morschhauser, 2005), indicating that ammonia could signal the repression of filamentous growth as the preferred nitrogen source for yeast growth. *C. albicans* has also been shown to stimulate its own filamentation by releasing ammonia and alkalinizing the otherwise acidic environment in phagosomes (Vylkova & Lorenz, 2014). This shift in pH activates the Rim101-dependent signaling pathway and drives morphogenetic change via the transcription factor Efg1 (El Barkani et al., 2000). Notably, Efg1 is not part of the pH response pathway and does not regulate pH-dependent gene expression (El Barkani et al., 2000). These studies demonstrate that various physiological events feed into the regulatory network that controls morphogenesis in *C. albicans*, influencing the morphological transition.

Integration of morphogenesis signals involves the activation of concerted transcriptional responses that would promote the drastic changes in cellular morphology associated with filamentous growth. A large number of transcription factors positively regulate the expression of filament-specific genes such as *HWP1* (Sharkey, McNemar, Saporito-Irwin, Sypherd, & Fonzi, 1999), *HYR1* (Bailey, Feldmann, Bovey, Gow, & Brown, 1996), *ECE1* (Birse, Irwin, Fonzi, & Sypherd, 1993) and *ALS3* (Argimon et al., 2007). In the absence of filament-inducing cues, their
expression is repressed by negative regulators such as Tup1, Nrg1, and Rfg1 (Kadosh & Johnson, 2005). While these genes encode either filament-specific cell wall components or adhesion factors, they are not absolutely required for filamentation (A. J. Brown et al., 2007). As such, their role in morphological transition remains elusive. One filament-specific gene whose expression is required for filamentation is the G1 cyclin-related protein Hgc1 (Zheng, Wang, & Wang, 2004). It is transcriptionally regulated by the filament-specific transcription factor Ume6 and functions downstream of the small GTPase Cdc42 (Carlisle & Kadosh, 2010). However, the role of Hgc1 in morphogenesis is restricted to the extension of hyphal filaments (Carlisle & Kadosh, 2010), suggesting that further studies are necessary to identify genes that are activated by the signal transduction pathways and that play a key role in filamentous growth.

The morphological transition is completed in two stages: initiation and maintenance of filamentous growth. The initiation phase is characterized by the establishment of cell polarity and bud elongation, while the maintenance phase is characterized by sustained polarized growth. Central to these processes are structural organization and cellular functions of the actin cytoskeleton (P. E. Sudbery, 2011). Actin polymerization is required for cell polarity and hyphal growth in a number of fungi, including C. albicans, A. fumigatus, the mold Neurospora crassa and the plant pathogenic fungus Ustilago maydis (Epp et al., 2010; Steinberg, 2007). In C. albicans, depolymerization of actin filaments by sequestering the monomeric actin subunits with cytochalasin A or latrunculin A blocks the induction of HWP1 expression and hyphal formation (Wolyniak & Sundstrom, 2007). Notably, bud formation and germ tube formation could still be observed occasionally even in the presence of the actin destabilizers (Wolyniak & Sundstrom, 2007), suggesting that cell polarity can be determined but not established without proper actin functions. In mouse embryonic fibroblasts, the small GTPases RhoA, Cdc42, and Rac1 play important roles in membrane extension during cellular protrusion by controlling the actin cytoskeleton dynamics (Machacek et al., 2009). It was proposed that RhoA initiates actin polymerization at the site of polarized growth through the activation of formins, which nucleate the assembly of actin monomers; subsequently, Cdc42 and Rac1 are activated sequentially to reinforce and stabilize the membrane protrusion (Machacek et al., 2009). The functional homolog of RhoA in yeast is Rho1 (Qadota, Anraku, Botstein, & Ohya, 1994); its GTPase activity is stimulated by GEFs such as Rom2 and inactivated by GAPs such as Lrg1. In S. cerevisiae, Rho1 functions through multiple downstream effectors, including the catalytic
subunit of the glucan synthase Fks1 (Mazur & Baginsky, 1996), protein kinase Pkc1, formin family protein Bni1, and the transcription factor Skn7 (Alberts, Bouquin, Johnston, & Treisman, 1998). Despite the physical interaction between Rho1 and Bni1 (Fujiwara et al., 1998), the actin polarization defects associated with a mutant lacking Rho1 is only suppressed by the overexpression of PKC1, suggesting that Rho1 drives the polarization of actin through Pkc1 (Helliwell, Schmidt, Ohya, & Hall, 1998). Given that Rho1 is required for actin polarization and is localized to germ tube tips during morphogenesis in C. albicans (Corvest et al., 2013), Rho1 is likely to be a key player in mediating morphological transitions. While Rho1 is necessary for invasive growth on solid surfaces (Corvest et al., 2013), its signaling pathway has not been broadly implicated in morphogenesis.

The work presented in this thesis provides the first evidence that Rho1 and its downstream effector Pkc1 regulate the morphogenetic transition, possibly via actin remodeling in C. albicans. While the activation of Rho1 triggers filamentous growth, the inactivation of Rho1 signaling causes defects in morphogenesis in response to diverse filament-inducing cues. Downstream of Rho1, Pkc1 has been identified and characterized as a master regulator of filamentation, and its kinase activity is crucial for the initiation of the morphological transition from yeast to filamentous growth. Pkc1 does not regulate filamentation via the canonical MAP kinase cascade mediated by Mkc1; its genetic interaction with proteins involved in actin-related processes implicate Pkc1 as an upstream regulator of actin function. Not surprisingly, the Rho1-Pkc1 signaling pathway functions in parallel with the existing Ras1-PKA signaling pathway; both pathways need to be activated in order to achieve proper a morphological transition in response to inducing cues. Together, this research extends our current understanding of signaling transduction required to enable C. albicans filamentous growth.

Similar to other fungal-specific processes, regulation of morphological transition provides a rich source of targets for the development of antifungal therapeutics. Alternative to the targeted approach presented in this work, which led to the identification and characterization of novel morphogenetic regulators, an unbiased approach can be taken to identify small molecules that modulate filamentation in high-throughput chemical screens. To date, several small molecules have been shown to inhibit filamentation (Shareck & Belhumeur, 2011), including the quorum-sensing (QS) molecule farnesol (Davis-Hanna et al., 2008), redox toxin pyocyanin (Grahl et al., 2015), and histone deacetylase inhibitor trichostatin A (Hnisz, Majer, Frohner, Komnenovic, &
Kuchler, 2010). Recently, a study has identified filastatin as an inhibitor of filamentation in a chemical screen for compounds that prevent *C. albicans* adhesion to polystyrene and to human cells with minimal host toxicity (Fazly et al., 2013). Additional biochemical and genetic experiments are required to understand the mechanism of action (Fazly et al., 2013). One caveat to small molecule screens for morphogenesis inhibitors is to determine the ideal filament-inducing condition for screening. This can be addressed by testing candidate compounds for inhibitory effects under multiple filament-inducing conditions. Additionally, a chemical genetic screen can be performed using a strain that is constitutively filamentous, such as a mutant lacking Nrg1 or Tup1. Given that the core transcriptional program governing morphogenesis is de-repressed in these mutants, small molecules that can inhibit their constitutive filamentous phenotype are likely to be effective under other filament-inducing conditions. Given that mutants locked in either the yeast or filamentous forms are attenuated in virulence (Cleary et al., 2016; Saville et al., 2003), it is also possible to screen for compounds that induce constitutive filamentation. Small molecules known to activate the morphogenesis program include the Hsp90 inhibitor geldanamycin (Shapiro et al., 2009) and the metal chelator DTPA (Polvi et al., 2016). While regulators of the morphological transition can provide attract antifungal drug targets, the therapeutic potential of filamentation modulators is maximized when employed as adjuvants to existing antifungals (Saville et al., 2006; Shapiro et al., 2009; S. D. Singh et al., 2009).

### 4.3 Future Directions

Research in this thesis project provides novel mechanistic insight into signaling pathways that regulate drug resistance and morphogenesis in *C. albicans*, and identifies novel targets for the development of antifungal therapeutics. I discovered that Cas5 (Chapter 2) and Pkc1 (Chapter 3) can both modulate drug resistance and morphogenesis. Although each regulator can control both biological processes, it is unclear whether they control drug resistance and morphogenesis through distinct mechanisms. Additional experiments can further address the relationship between drug resistance and morphogenesis by: 1) identifying mutated gene that rescued caspofungin resistance of an *Fk1*\(^{F642S}\) mutant lacking Cas5 and evaluating its capacity to restore normal yeast growth; 2) determining whether the mutations that restored filamentation in a mutant lacking Pkc1 can rescue tolerance to cell wall stress; and 3) assessing whether any of these mutations can restore virulence in an animal model of *Candida* infection. Previous studies have shown that Cas5 is required for virulence in a mouse model of hematogenous candidiasis.
and a *Toll* mutant fly model of invasive candidiasis (Chamilos et al., 2009), and that Pkc1 is required for virulence in a murine model of systemic disease (LaFayette et al., 2010). An ideal drug target would regulate two virulence traits via distinct mechanisms, such that mutation(s) needed to restore both in the presence of an inhibitor might be more difficult to evolve.

Work presented in Chapter 2 identified Cas5 as a novel regulator of drug resistance in *C. albicans*. The outstanding question is whether Cas5 regulates caspofungin tolerance and resistance via the same mechanism. This can be addressed by: 1) assessing whether mutation that restored caspofungin resistance could also restore caspofungin tolerance in a mutant lacking Cas5 but without the *FKSI* resistance mutation; and 2) assessing whether depletion of *GLC7* could abrogate caspofungin resistance in a mutant harboring Fks1<sup>F641S</sup>. Given that the mutation that rescued caspofungin resistance also confers an increase in tolerance to calcofluor white (Figure 2.13) and that the mechanism of action differs between the two cell wall perturbing agents, the mechanism that mediates caspofungin resistance may also enhance caspofungin tolerance.

Perhaps one of the more puzzling observations is that all of the Cas5-dependent genes tested require Swi4 and Swi6 for expression, yet the SBF complex is not required for cell wall stress response, cell cycle regulation, or morphogenesis to the same extend as Cas5 (Figure 2.9A and 2.12). The discordance between transcriptional regulation mediated by these three regulators and the severity of mutant phenotypes suggested that either Cas5 regulates a set of genes independent of Swi4 and Swi6, or that Cas5 has regulatory functions that are not restricted to transcriptional control. Based on transcriptional profiling analyses of a *swi4Δ/swi4Δ swi6Δ/swi6Δ* double mutant (Hussein et al., 2011) and a *cas5Δ/cas5Δ* single mutant (Vasicek et al., 2014) under standard laboratory conditions, the overlap between their transcriptional profiles is minimal, suggesting that Cas5 could be interacting with other transcription factors that confer specificity to Cas5-dependent transcriptional regulation. Although no additional Cas5 interactors were identified in this study, it is possible that Cas5 interacts with different transcription factor depending on the conditions. For example, Cas5 may interact with cell cycle regulator(s) during a specific phase of cell cycle, and interact with cell wall regulator(s) in response to cell wall stress. These interactions could potentially be identified through condition-specific immunoprecipitation coupled with mass spectrometry experiments.
Work presented in Chapter 3 revealed a novel role for Lrg1, Rho1, and Pkc1 in morphogenesis. In an attempt to identify suppressor mutations that can rescue filamentation in a non-filamentous mutant, I designed a reporter system based on the expression of the filament-specific transcript \textit{HWP1}, using growth on NAT as a read out for filamentous growth. This system circumvents the need to screen for mutants by morphology and allows for rapid selection experiments. This would also provide a useful tool for chemical genetic screens for inhibitors of filamentation. The introduction of an \textit{HWP1p-NAT} reporter construct in a wild-type background can facilitate small molecule screens in the presence of NAT, which would kill any cells that are not filamenting and pinpoint the small molecule that blocks filamentous growth. Future studies could engineer an additional reporter system based on the expression of yeast-specific transcript such as \textit{YWP1}, which would allow for the selection of mutants that are unable to undergo morphological transition in response to filament-inducing cues.

Interestingly, selection for the restoration of filamentation in a nonfilamentatous \textit{pkc1Δ/pkc1Δ} mutant uncovered mutations in \textit{ORF19.7149} and \textit{ORF19.1689}, which encode proteins involved in actin-related processes. Since Pkc1 kinase activity is required for filamentation (Figure 3.8) and it is known to phosphorylate the formin Bni1 in \textit{S. cerevisiae} (Kono et al., 2012), phosphoshift assays could be performed to assess whether Bni1 is also phosphorylated in \textit{C. albicans} under filament-inducing conditions, and whether this requires Pkc1. To further investigate the actin-related function of Orf19.7149 and Orf19.1689 in filamentation, one could assess whether cytochalasin A inhibits the morphological transition in the selected mutants, and whether the mutations in \textit{ORF19.7149} and \textit{ORF19.1689} can confer resistance to cytochalasin A.

Another question of considerable interest is related to the mechanism of activation of Rho1-Pkc1 signaling. Since activation of the morphogenesis program through the Rho1-Pkc1 pathway requires Ras1-PKA signaling and vice versa, they are likely activated by different mechanisms. While inactivation of Lrg1 induces pseudohyphae formation, it remains unclear whether the filament-inducing cues signal through the Rho1 GAP. As such, various Rho1 GAPs and GEFs as well as cell wall receptors such as Wsc1 should be tested for roles in filamentation. One potential activator of Rho1-Pkc1 signaling is phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2], which forms a steep gradient that is crucial for filamentous growth (Vernay, Schaub, Guillas, Bassilana, & Arkowitz, 2012). PI(4,5)P2 is a phospholipid that can be converted to key secondary messenger phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P] by phosphoinositide 3-kinase...
(PI3K) (Strahl & Thorner, 2007). In *S. cerevisiae*, PI(4,5)P2 generated at the plasma membrane is essential for the localization of Rho1 GEF Rom2 to the sites of polarized growth (Audhya & Emr, 2002; Manning, Padmanabha, & Snyder, 1997). More recently, studies in *C. albicans* have shown that the rapid redistribution of PI(4,5)P2 in response to caspofungin (Badrane et al., 2012) could be important for Pkc1-dependent Mkcl activation (Badrane, Nguyen, & Clancy, 2016). Thus, probing the relationship between PI(4,5)P2 and Rom2-dependent Rho1 activation in response to filament-inducing cues could provide a mechanistic link for the activation of Rho1-Pkc1 signaling in the context of morphogenetic regulation.

Through the characterization of novel signaling pathways, work in this thesis advances our understanding of regulatory circuitries that control drug resistance and morphogenesis. The future directions outlined above indicate that further investigation of the Cas5-dependent regulation of drug resistance and Pkc1-dependent regulation of morphogenesis could reveal additional targets for the development of novel antifungal therapeutics.


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