The Roles of Hsp90 and Calcineurin in Antifungal Drug Resistance

by

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

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Doctor of Philosophy

Molecular Genetics
University of Toronto

2012

Abstract

Candida species are the fourth most common cause of hospital-acquired blood-stream infections in the United States with mortality rates approaching 50%. Treatment of candidiasis is hampered by the limited number of antifungal drugs whose efficacy is compromised by host toxicity, fungistatic activity, and the emergence of drug resistance. I established a new role for the essential molecular chaperone Hsp90 and the protein phosphatase calcineurin in regulating resistance to cell wall stress exerted by the echinocandins, the only new class of antifungal drug to reach the clinic in decades, in both Candida albicans and Candida glabrata, the two leading causes of candidiasis. Through reciprocal co-immunoprecipitation studies, calcineurin activation studies, and protein stability assays, I established calcineurin as the first client protein of Hsp90 in C. albicans. I found that the calcineurin downstream effector Crz1 plays only a partial role in mediating tolerance to the echinocandins in C. albicans, implicating additional downstream effectors. Complementary studies in the model organism Saccharomyces cerevisiae revealed a divergence in the requirement of Hsp90 and calcineurin in this species’ ability to tolerate echinocandins despite a conserved functional relationship
between Hsp90 and calcineurin. I also provided the first global view of mutations that accompany the evolution of fungal drug resistance in a human host. I found an association of mutations in *CDC6* and *MOH1* with echinocandin resistance in unrelated *C. glabrata* clinical isolates, genes previously not implicated in echinocandin resistance. I propose a new model by which Hsp90 and calcineurin regulate echinocandin resistance by controlling expression of the resistance determinant *FKS2*. Taken together, my research reveals new mechanisms mediating antifungal drug resistance and suggests new therapeutic strategies to save human lives.
Acknowledgments

I would first like to thank my supervisor, Dr. Leah E. Cowen, for her wonderful support and guidance over the course of my PhD. Your knowledge and wisdom molded me into the scientist that I am today. Thank you for providing me with the scientific freedom that I enjoyed over the last 5 years, it has been instrumental to my intellectual development. It was a fun adventure to start a lab with you and I have enjoyed watching it blossom into the highly productive and enriching environment that it is today. I would also like to thank my committee members, Dr. Brigitte Lavoie, Dr. Scott Gray-Owen, and Dr. Ian Crandall, for your knowledge and support. I would especially like to thank Dr. Ian Crandall who I also had the pleasure of working with as an undergraduate student. Your enthusiastic training sparked my love for science and I am incredibly grateful for that.

To the Cowen lab ladies, thank you for all of the scientific support and, almost more importantly, the laughs, the fun, the lunches, and the cakes! Fun girls <3 fungi! I would especially like to thank our Cowen lab technician, Cathy Collins. You have been such a wonderful confidante and have helped me through some of the most important landmarks in my life these past years. Your life wisdom will forever be cherished. To the wonderful friends I have made along the way, thank you for the great times during lunches, coffees, hall breaks, Friday night wine nights, and all other MoGen events. I would especially like to thank Katie McIsaac, Kelly Thickett, Jayme Salsman, and Vanessa Tran for their energy, strength, and friendship.

I would like to express my appreciation for my family for their unending love and support. To my mom, I can never express how truly grateful I am for the sacrifices that you have made to provide me with the opportunities that I have taken advantage of. I hope I have made you proud. Dad, you are my inspiration and my role model. Your accomplishments and passion for your work have been both a source of pride and motivation throughout my entire life. Finally, I would like to thank my husband and best friend Tomas Babak. Your love, insight, and encouragement have been vital to my success and happiness over these years. I cannot express how blessed I am to have you in my life. I am excited to embark on our lives together and to discover the great things the future has in store for us.
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<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACE</td>
<td>Activator of CUP1 Expression</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Ca</td>
<td>Calcium chloride</td>
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<tr>
<td>CCH</td>
<td>Calcium channel</td>
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<td>CDC</td>
<td>Cell division cycle</td>
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<td>CDR</td>
<td>Candida drug resistance</td>
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<td>CDRE</td>
<td>Calcineurin-dependent response element</td>
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<tr>
<td>CF</td>
<td>Caspofungin</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CHS</td>
<td>Chitin synthase</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and laboratory standards institute</td>
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<tr>
<td>CNA</td>
<td>Calcineurin A</td>
</tr>
<tr>
<td>CNB</td>
<td>Calcineurin subunit B</td>
</tr>
<tr>
<td>CPR</td>
<td>Cyclosporin A-sensitive rotamase</td>
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<tr>
<td>CRZ</td>
<td>Calcineurin-responsive zinc finger</td>
</tr>
<tr>
<td>CS</td>
<td>Caspofungin</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized tomography</td>
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<tr>
<td>DOT</td>
<td>Disruptor of telomeric silencing</td>
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<tr>
<td>DRE</td>
<td>Drug response element</td>
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<tr>
<td>EPA</td>
<td>Epithelial adhesin</td>
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<tr>
<td>ERG</td>
<td>Ergosterol biosynthesis</td>
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<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<td>FKS</td>
<td>FK506 sensitivity</td>
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<tr>
<td>FL</td>
<td>Fluconazole</td>
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<tr>
<td>FLO</td>
<td>Flocculation</td>
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<td>FLP</td>
<td>Site specific recombinase</td>
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<td>FPR</td>
<td>FK506-sensitive proline rotamase</td>
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<tr>
<td>GAP</td>
<td>GTP-ase activating proteins</td>
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<td>GdA</td>
<td>Geldanamycin</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GPH</td>
<td>Glycogen phosphorylase</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GRACE</td>
<td>Gene replacement and conditional expression</td>
</tr>
<tr>
<td>GSC</td>
<td>Glucan synthase of cerevisiae</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
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<td>HIS</td>
<td>Histidine</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>HOG</td>
<td>High osmolarity glycerol</td>
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<td>HPH</td>
<td>High pH</td>
</tr>
<tr>
<td>HSC</td>
<td>Heat shock constitutive</td>
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<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>HSP</td>
<td>Heat shock promotable</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
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<td>MAL</td>
<td>Maltose</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Mac1-dependent regulator</td>
</tr>
<tr>
<td>MET</td>
<td>Methionine</td>
</tr>
<tr>
<td>MF</td>
<td>Micafungin</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MID</td>
<td>Mating pheromone-induced death</td>
</tr>
<tr>
<td>MKC</td>
<td>MAP Kinase from C. albicans</td>
</tr>
<tr>
<td>MLST</td>
<td>Multilocus sequence typing</td>
</tr>
<tr>
<td>MRPL</td>
<td>Mitochondrial ribosomal protein, Large subunit</td>
</tr>
<tr>
<td>MRR</td>
<td>Multidrug resistance regulator</td>
</tr>
<tr>
<td>MRS</td>
<td>Major repeat sequence</td>
</tr>
<tr>
<td>MTL</td>
<td>Mating type-like</td>
</tr>
<tr>
<td>NAT</td>
<td>Nourseothricin</td>
</tr>
<tr>
<td>NF-AT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>ONPG</td>
<td>O-nitrophenyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>PDR</td>
<td>Pleiotropic drug resistance</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulse-field gel electrophoresis</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>RAD</td>
<td>Radicicol</td>
</tr>
<tr>
<td>RHO</td>
<td>Ras homolog</td>
</tr>
<tr>
<td>RLM</td>
<td>Resistance to lethality of MKK1P386 overexpression</td>
</tr>
<tr>
<td>ROM</td>
<td>Rhp1 multicopy suppressor</td>
</tr>
<tr>
<td>RPD3L</td>
<td>Reduced potassium dependency large subunit</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>SAP</td>
<td>Secreted aspartyl protease</td>
</tr>
<tr>
<td>SLT</td>
<td>Suppression at low temperature</td>
</tr>
<tr>
<td>SNQ</td>
<td>Sensitivity to 4-NitroQuinoline-N-oxide</td>
</tr>
<tr>
<td>SNV</td>
<td>Single nucleotide variant</td>
</tr>
<tr>
<td>SUI</td>
<td>Suppressor of initiator codon</td>
</tr>
<tr>
<td>TAC</td>
<td>Transcriptional activator of CDR genes</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>TCB</td>
<td>Three calcium and lipid binding domains</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>TPN</td>
<td>Total parenteral nutrition</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide-containing repeat</td>
</tr>
<tr>
<td>U</td>
<td>Untreated</td>
</tr>
<tr>
<td>UPC</td>
<td>Uptake control</td>
</tr>
<tr>
<td>UTR</td>
<td>Unidentified transcript</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number of tandem repeats</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast peptone dextrose</td>
</tr>
<tr>
<td>YPG</td>
<td>Yeast peptone glycerol</td>
</tr>
<tr>
<td>YPM</td>
<td>Yeast peptone maltose</td>
</tr>
<tr>
<td>YPS</td>
<td>Yapsins</td>
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Chapter 1

1 Introduction

1.1 Candida albicans

*Candida albicans* is normally a commensal yeast commonly found in diverse anatomical sites from the oral cavity to the gastrointestinal tracts of most healthy humans (Odds, Gow et al. 2001). There are no known environmental reservoirs of *C. albicans* and it has been found exclusively in association with mammalian hosts (Noble and Johnson 2007). *C. albicans* can cause disease in immune-competent patients often manifesting as infection of the skin, nails, and mucosal membranes including oral-pharyngeal, esophageal, gastrointestinal, and vaginal mucosae (Kim and Sudbery 2011). In fact, up to 75% of women may experience vulvo vaginal candidiasis at least once in their life time and some may experience recurrent infections with risk factors including antibiotic treatment, the use of oral contraceptives or reproductive hormones, and diabetes (Sobel 1997). In immunocompromised individuals such as those with inherited disease, chemotherapy patients, HIV patients, and organ transplant recipients, *C. albicans* can also cause disseminated candidiasis, a severe invasive blood-stream infection which can lead to the colonization of internal organs, with mortality rates of up to 50% (Pfaller and Diekema 2007). *C. albicans* is the causative agent of greater than 90% of mucosal Candida infections and between 40 to 70% of invasive candidiasis (Pfaller and Diekema 2010). Due to the expanding immunocompromised population, blood-stream infections caused by fungal pathogens have increased by 207% between 1979 and 2000 in the United States (Martin, Mannino et al. 2003). Invasive candidiasis is the leading cause of death due to mycotic infection as well as the fourth leading cause of hospital-acquired blood stream infections, and is estimated to cost the health care system $1 billion annually in the United States alone (Miller, Hajjeh et al. 2001; Pfaller and
Diekema 2007). *C. albicans* also has the ability to form biofilms, complex surface-associated communities containing several types of cells embedded in an extracellular matrix, on implanted medical devices such as urinary and venous catheters as well as artificial joints (Blankenship and Mitchell 2006). Biofilms are extremely resistant to antifungal therapy and once medical devices are infected by *C. albicans* biofilms, they must often be removed to avoid dissemination of the infection into the blood stream, as mortality rates approach 30% for device-associated *Candida* infection (Finkel and Mitchell 2011).

*C. albicans* is a diploid organism with eight chromosomes and a 14.3 megabase genome, with a total of 6203 predicted open reading frames, 1384 of which are characterized (Skrzypek, Arnaud et al. 2010). The ascomycete phylum includes both *C. albicans* and its relative the model yeast *Saccharomyces cerevisiae*, however, it is estimated that *C. albicans* and *S. cerevisiae* diverged between 140 – 841 million years ago (Berman and Sudbery 2002). Most *Candida* species, including *C. albicans*, exist within the *Candida* clade, which consists of related species that translate the codon CUG as serine instead of leucine, a reassignment that is thought to have occurred ~ 170 million years ago (Massey, Moura et al. 2003; Butler, Rasmussen et al. 2009). Pathogenic species within the *Candida* clade are reasoned to have coevolved certain expanded gene families and pathogenicity as they contain 21 expanded gene families that are enriched in the pathogenic species relative to the non-pathogenic species (Butler, Rasmussen et al. 2009). These gene families are known to be involved in pathogenicity and include lipases, oligopeptide transporters, and adhesins (Butler, Rasmussen et al. 2009). Cell wall gene families are also enriched in pathogens, including the Als family, which is involved in host surface adhesion, host cell invasion and iron acquisition in *C. albicans* (Phan, Myers et al. 2007; Yeater, Chandra et al. 2007; Almeida, Brunke et al. 2008). In addition, *C. albicans* has species-specific expansion of distinct gene families involved in filamentous growth, as *C. albicans* virulence is linked to its
ability to exist in multiple cellular states including yeast (blastospires), and filamentous pseudohyphal and hyphal forms (Berman and Sudbery 2002). The ability of C. albicans to switch between morphological states is an important virulence trait, as most mutants locked in either yeast or filamentous form have reduced virulence in the mouse model of invasive candidiasis, although it has been recently shown that virulence and morphogenesis can be decoupled (Lo, Kohler et al. 1997; Braun, Head et al. 2000; Bahn, Staab et al. 2003; Noble, French et al. 2010). C. albicans yeast cells are phagocytosed by macrophages, however, environmental cues within the macrophage induce a transition to hyphae, which can kill the macrophage and escape (Lo, Kohler et al. 1997). C. albicans also has expanded its gene family encoding secreted aspartyl proteases, hydrolytic enzymes involved in virulence and pathogenesis, to include 10 SAP genes, with SAP1, SAP4, SAP5, and SAP6 all located on chromosome 6 (Moran, Coleman et al. 2011).

C. albicans primarily reproduces asexually through clonal division and although once believed to be exclusively asexual, it is now known that a parasexual mating cycle exists (Forche, Alby et al. 2008). C. albicans possesses an efficient mating apparatus containing a mating-type-like (MTL) locus similar to the MAT locus in S. cerevisiae, however, mating is controlled by a process known as phenotypic switching where cells switch between ‘white’ and ‘opaque’ states (Miller and Johnson 2002). Only strains that are homozygous for either MTLa or MTLα can undergo phenotypic switching, and opaque cells are the mating-competent form with mating efficiency 10^6 times higher than white cells (Miller and Johnson 2002). In addition to traditional heterothallic mating between an a and an α cell, C. albicans has the capacity for homothallic mating, or self-fertilization whereby two opaque a or α cells mate (Alby, Schaefer et al. 2009). In S. cerevisiae, mating occurs between two haploid cells of the opposite mating type to form a heterozygous diploid which, under certain environmental conditions, undergoes
meiosis to produce 4 haploid meiotic progeny. A meiotic program has not been identified in *C. albicans* and instead the tetraploid mating product undergoes a parasexual mechanism of chromosome loss to return to a near diploid state (Forche, Alby et al. 2008). The parasexual cycle does lead to inter-chromosomal recombination and thus can generate genetic diversity, however, mating in nature is rare (Forche, Alby et al. 2008). This is exemplified in one study of 120 *C. albicans* clinical isolates of which only 12 were homozygous at the *MTL* locus and only 5 of those were able to undergo phenotypic switching to the opaque mating-competent state (Legrand, Lephart et al. 2004). Population genetic studies have also found that *C. albicans* is largely clonal with evidence for infrequent genetic exchange or recombination (Graser, Volovsek et al. 1996). Thus, the function or significance of *C. albicans* mating during commensalism or infection remains unknown.

*C. albicans* possesses a high degree of genome plasticity that can generate genetic variability. Karyotypes of *C. albicans* clinical isolates can differ dramatically due to loss of heterozygosity, aneuploidy, and gross chromosomal rearrangements (Rustchenko 2007). An interesting feature of the *C. albicans* genome is the inclusion of a major repeat sequence (MRS) in all chromosomes except chromosome 3 (Lephart, Chibana et al. 2005). The MRS accounts for approximately 3% of the total genomic DNA content, is a preferred site for chromosomal translocations, and expands and retracts resulting in karyotypic changes (Pujol, Joly et al. 1999; Chibana, Beckerman et al. 2000; Lephart, Chibana et al. 2005). Karyotypic changes could provide genetic diversity that may be beneficial to *C. albicans* in the context of commensalism and/or during infection. An example of the benefits that chromosome instability may have is the formation of isochromosome 5L during treatment with the antifungal drug fluconazole, where two copies of the left arm of chromosome 5 are in an inverted orientation flanking the chromosome 5 centromere (Selmecki, Forche et al. 2006). The resulting fluconazole-resistant
phenotype is due to increased copies of ERG11, which encodes the target of fluconazole, as well as TAC1, which encodes a transcription factor required for up-regulation of the ATP-binding cassette (ABC) transporter genes CDR1 and CDR2 (Selmecki, Forche et al. 2006). Passage of C. albicans populations through a mouse host leads to higher rates of loss of heterozygosity and chromosome rearrangements relative to populations propagated in vitro (Forche, Magee et al. 2009). Thus, C. albicans capitalizes on the remarkable plasticity of its genome to survive environmental stress.

While genomic plasticity can benefit the pathogen, it can hamper genetic analyses. During DNA transformation to delete or modify specific genes, C. albicans may acquire additional genomic changes such as alterations in chromosome copy number (Selmecki, Forche et al. 2010). C. albicans is a diploid organism thus deletion of non-essential genes requires two rounds of potentially mutagenic transformation, either chemically with lithium acetate or via electroporation. Aneuploidies that arise during transformation can have profound effects on the cell and can triplicate the target gene (Bouchonville, Forche et al. 2009). Genetic and molecular analysis in C. albicans is also hindered by its unusual codon usage, the lack of a known meiotic cycle, and lack of plasmid maintenance (Berman and Sudbery 2002). Thus, many of the tools available for study in S. cerevisiae are not useful in C. albicans and markers must often be ‘codon optimized’. Unlike S. cerevisiae, which often requires only 45 base pairs of homology for homologous recombination (Baudin, Ozier-Kalogeropoulos et al. 1993), C. albicans requires a minimum of 60 and often up to 500 base pairs of homology to drive homologous recombination. Given the plethora of tools and relative ease of working with S. cerevisiae compared to C. albicans, complementary studies are often performed in both species and can yield insight into conserved and divergent cellular processes.
Despite the challenges, *C. albicans* genetics has advanced significantly in the last few years with the development of several large-scale mutant libraries. The first two libraries both utilize transposon mutagenesis, with the first creating 217 homozygous transposon mutants (Davis, Bruno et al. 2002) and the second using Tn7 transposon mutagenesis on a larger scale to create 18,000 independent heterozygous mutants for a haploinsufficiency screen (Uhl, Biery et al. 2003). During that year, the GRACE (gene replacement and conditional expression) library for conditional expression of 1152 *C. albicans* genes, 567 deemed essential, where one allele is disrupted and the other is placed under the control of the tetracycline repressible promoter system was published, however, this library was created by Merck & Co and is currently not available for use in the scientific community (Roemer, Jiang et al. 2003). More recently, a library containing homozygous deletion mutants of 143 transcriptional regulators was created and contains two independent mutants for each gene (Homann, Dea et al. 2009); by 2010 the homozygous deletion library included mutants in 647 genes (Noble, French et al. 2010). A library containing 3633 tagged heterozygous disruption mutants was also created by another group and was used in haploinsufficiency screens to annotate genes and for drug target identification (Oh, Fung et al. 2010). In addition to the above libraries, many genetic and molecular tools have been developed for *C. albicans* including recyclable dominant drug resistance markers that can be excised for multiple rounds of transformation, which is very useful in prototrophic strains and used extensively in the works in this thesis (Shen, Guo et al. 2005). PCR-based gene deletion and epitope tagging strategies have also been established as well as regulatable gene expression systems including the tetracycline-repressible or -inducible promoters, the *MAL2* glucose-repressible promoter, and the *MET3* promoter (Noble and Johnson 2007). Genome-wide techniques, including chromatin immunoprecipitation (ChIP) and RNA
transcriptome sequencing, have been exploited successfully in *C. albicans* (Nobile, Nett et al. 2009; Tuch, Mitrovich et al. 2010).

Thus, the repertoire of sophisticated genetic and molecular tools in *C. albicans* has expanded a great deal over the past decade and will continue to do so, allowing researchers to ask more complex questions to ultimately understand the biology of this important human pathogen.

### 1.2 Candida glabrata

*C. glabrata* is now second to *C. albicans* as the most prevalent *Candida* species in the clinic due to its intrinsic tolerance and ability to quickly evolve resistance to the most widely used class of antifungals, the azoles (Fidel, Vazquez et al. 1999). As with *C. albicans*, *C. glabrata* is exclusively found associated with mammals and is part of the normal gut microbiota; it can cause both mucosal and systemic blood stream infections in immunocompromised patients, accounting for up to 20-24% of *Candida* blood stream infections in the United States, and it is considered an important emerging pathogen (Pfaller and Diekema 2004; Pfaller and Diekema 2007). Additionally, the use of broad-spectrum antibiotics including vancomycin and piperacillin-tazobactam appears to be associated with subsequent infection with *C. glabrata* over *C. albicans*, and there is an increased rate of *C. glabrata* candidiasis in elderly patients as well as an increased risk of death (Lin, Carmeli et al. 2005; Malani, Hmoud et al. 2005). Catheter-associated candiduria, infection of the urinary tract by *Candida*, can be caused by both *C. albicans* and *C. glabrata*, however, there is a specific association of fluconazole and quinolone treatment with the development *C. glabrata* candiduria (Harris, Castro et al. 1999). Although *C. glabrata* is capable of forming biofilms on medical devices, they consist of clumps of cells and are sparse relative to biofilms formed by *C. albicans* as *C. glabrata* is a non-filamentous fungus
and unable to form the dense complex three-dimensional biofilms that *C. albicans* is known for (Ramage, Mowat et al. 2009). However, in the context of a mixed infection *C. glabrata* does not compete for *C. albicans* adhesion sites and is actually able to use *C. albicans* as a stable structure to attach to (El-Azizi, Starks et al. 2004; Ramage, Mowat et al. 2009). In fact these mixed biofilms are associated with increased inflammation in the context of denture stomatitis, a form of oropharyngeal *Candida* infection afflicting wearers of oral prosthetic devices, compared to infection with either *C. albicans* or *C. glabrata* alone (Coco, Bagg et al. 2008).

*C. glabrata* is a haploid organism that is closely related to the model yeast *Saccharomyces cerevisiae* and exists within the *Saccharomyces* clade rather than the *Candida* clade to which the leading cause of candidiasis, *C. albicans*, belongs (Dujon, Sherman et al. 2004; Fitzpatrick, Logue et al. 2006; Marcet-Houben and Gabaldon 2009). Thus, it is thought that *C. glabrata* emerged as a human pathogen independently from other *Candida* species. Moreover, *C. glabrata* is the only major human pathogen in the *Saccharomyces* clade (Butler, Rasmussen et al. 2009). The *C. glabrata* genome is approximately 12.3 megabases in size, structured into 13 chromosomes, and contains 5493 open reading frames, only 178 of which are verified (Skrzypek, Arnaud et al. 2010). Compared to its relatives, *C. glabrata* literature is limited, and a PubMed literature search for the term ‘*Candida glabrata*’ retrieves 3112 journal articles compared to *C. albicans* which returns 26,381 articles and *S. cerevisiae* which returns 93,649 articles (http://www.ncbi.nlm.nih.gov/pubmed/ searched on August 21, 2011). There is a high degree of synteny between the *C. glabrata* and *S. cerevisiae* genomes, and although both organisms share a common ancestor that underwent a whole genome duplication, there appears to have been a higher rate of gene loss in *C. glabrata* resulting in lower genome redundancy (Dujon, Sherman et al. 2004). Strikingly, *C. glabrata* has lost 29 genes relative to other yeasts specifically involved in galactose, phosphate, nitrogen, and sulfur metabolism as well as
thiamine, pyridoxine, and nicotinic acid biosynthesis (Dujon, Sherman et al. 2004; Kaur, Domergue et al. 2005). It is thought that this reductive evolution may be a function of C. glabrata’s exclusive association with a mammalian host. In addition, gene families associated with pathogenicity in C. albicans including iron acquisition and host cell adhesion and invasion are absent from C. glabrata (Butler, Rasmussen et al. 2009). In fact, C. glabrata contains only 3 genes out of the 161 genes within 3 gene families that are enriched in other pathogenic Candida species (Butler, Rasmussen et al. 2009). Thus, C. glabrata must have alternative mechanisms of pathogenesis and virulence relative to C. albicans.

Two important components of C. albicans virulence are the ability to transition between yeast and hyphal forms (Sudbery, Gow et al. 2004) and the ability to secrete proteinases to degrade host tissue during invasion (Naglik, Albrecht et al. 2004), however, C. glabrata does not possess these features. Instead C. glabrata possesses a Yapsin (YPS) gene family containing 11 YPS genes encoding glycosylphosphatidylinositol (GPI)-linked aspartyl proteases that are closely related to the S. cerevisiae yapsins (Kaur, Ma et al. 2007). YPS genes play a role in cell wall integrity maintenance during stationary phase growth, as well as survival of C. glabrata within macrophages and in the mouse model of disseminated candidiasis, possibly by removing certain GPI-anchored cell wall proteins and thereby remodeling the cell surface in response to various environmental conditions within the host (Kaur, Ma et al. 2007). C. glabrata also contains an Epa family of GPI-anchored cell wall proteins, with up to 23 paralogs found in the laboratory strain BG2 (De Las Penas, Pan et al. 2003; Kaur, Domergue et al. 2005). EPA genes are located in subtelomeric regions in clusters under the control of chromatin-based transcriptional silencing and are involved in adherence to uroepithelial cells in the context of bladder colonization and the mouse model of urinary tract infections, as well as in biofilm formation in vitro (Domergue, Castano et al. 2005; Iraqui, Garcia-Sanchez et al. 2005). Although unable to form true hyphae,
*C. glabrata* is also capable of undergoing a pseudohyphal program in response to nitrogen starvation similar to *S. cerevisiae* diploid cells (Gimeno, Ljungdahl et al. 1992; Csank and Haynes 2000). These pseudohyphae are able to invade solid agar media, however, the role of this morphogenetic state in pathogenesis is unknown as pseudohyphal *C. glabrata* has not been found in the clinic (Kaur, Domergue et al. 2005).

As with *C. albicans*, *C. glabrata* seems to be largely asexual. *C. glabrata* has been classified as an asexual organism as it is an obligate haploid and mating has never been reported in the literature, although four mating types and mating type switching have been described (Brockert, Lachke et al. 2003; Lin, Chen et al. 2007; Butler 2010). When plated on media containing CuSO₄, *C. glabrata* is able to spontaneously switch at high frequency between four graded phenotypes: white, light brown, dark brown, and very dark brown. Phenotypic switching was also observed at sites of infection and specific phenotypes dominated different anatomical locations in the same host (Brockert, Lachke et al. 2003). The one available genome sequence for *C. glabrata* (strain CBS138) (Dujon, Sherman et al. 2004), revealed that the *C. glabrata* genome does contain genes encoding all of the known components required for mating in *S. cerevisiae*, including regulators of the mating type locus, pheromones, receptors, and the pheromone response pathway (Butler, Kenny et al. 2004; Muller, Hennequin et al. 2008). Further evidence for the lack of mating lies in the fact that all isolates studied thus far are haploid, and that multilocus sequence typing (MLST) as well as variable number of tandem repeat (VNTR) analysis shows a large degree of linkage disequilibrium suggesting a clonal population structure (Lin, Chen et al. 2007; Brisse, Pannier et al. 2009). One study found evidence for recombination within a *C. glabrata* population, however, it was rare and there was no evidence as to when recombination may have occurred, thus recombination may have taken place in a mating-competent ancestor (Dodgson, Pujol et al. 2005).
Given the lack of sexual reproduction, *C. glabrata* increases genetic diversity via chromosomal translocations and variation in gene copy number (Muller, Thierry et al. 2009; Polakova, Blume et al. 2009), mechanisms that are important in *C. albicans* for phenotypic diversity and resistance to the azole class of antifungal drugs (Selmecki, Forche et al. 2006; Selmecki, Gerami-Nejad et al. 2008; Selmecki, Dulmage et al. 2009). *C. glabrata* is also known to exhibit variable karyotypes from isolate to isolate, however, the *C. glabrata* genome does not contain the major repeat sequences (MRS) associated with chromosomal instability in *C. albicans* (Barchiesi, Falconi Di Francesco et al. 1999; Shin, Chae et al. 2007; Muller, Thierry et al. 2009). Interestingly, recombination within tandem arrays of repeated genes was found to contribute to karyotypic differences amongst *C. glabrata* strains and these tandem genes often encode cell wall proteins, thus this mechanism of genome variability may also be involved in pathogenicity, although further studies are required (Muller, Thierry et al. 2009). Size polymorphisms have also been found within three subtelomeric EPA genes, which are known to play a role in pathogenicity (Muller, Thierry et al. 2009). The *C. glabrata* genome contains minisatellites, which are a subclass of DNA tandem repeats that range in size from nine to fewer than 100 base pairs and may be another mechanism by which *C. glabrata* generates genome plasticity (Thierry, Bouchier et al. 2008). The *C. glabrata* genome also contains megasatellites, which have longer motifs ranging from 135 to 417 nucleotides in length and have been found in only one other species to date, *Kluyveromyces delphensis*, also within the same clade as *C. glabrata* (Kurtzman 2003; Thierry, Bouchier et al. 2008; Thierry, Dujon et al. 2010). In *S. cerevisiae*, *FLO* genes are involved in cellular adhesion and flocculation, both of which are affected by the length of a minisatellite contained within *FLO1* (Verstrepen, Jansen et al. 2005). In *C. glabrata*, there are 40 megasatellites contained in 33 genes, three of which are EPA genes, functional homologues of *FLO* genes in *S. cerevisiae*, with a bias toward subtelomeric regions
Given that minisatellite length can affect adhesion and flocculation in *S. cerevisiae*, it is reasonable to hypothesize that both mini and megasatellites may be playing a role in *C. glabrata* pathogenicity, however, a link has not yet been established.

Unlike *C. albicans* and *S. cerevisiae*, there are few reported *C. glabrata* mutant libraries in the literature to date, likely because of the relatively recent interest in this organism. Use of the bacterial transposon Tn7 for the generation of genome-wide signature-tagged insertional mutant libraries by mutagenizing fosmids containing large 40 kb fragments of genomic DNA has been reported and yielded approximately 10,000 gene replacement mutants with a broad range of phenotypes (Castano, Kaur et al. 2003). This library was used to identify the *EPA1* adhesin involved in adherence to human epithelial cells (Cormack, Ghor et al. 1999). Another study used the same insertion mutant library strategy to identify the transcription factor *ACE2* as the first virulence-associated gene described in *Candida* species as *ace2* mutants are hypervirulent in the mouse model of candidiasis due to a flocculant growth phenotype, the ability to escape into tissue from the vasculature, and an overstimulation of the innate pro-inflammatory immune response (Kamran, Calcagno et al. 2004). There is currently a consortium creating a barcoded clean deletion library for *C. glabrata* consisting of almost 600 mutants so far, however, this library has yet to be published and is not yet available for widespread use (personal communication, Karl Kuchler). *C. glabrata* is a haploid organism thus simplifying genetics considerably compared with *C. albicans* although, unlike *S. cerevisiae*, it cannot mate or undergo meiosis, thus classical genetics in this organism is currently not possible. Instead, transformation using a lithium acetate protocol or electroporation has been adapted for *C. glabrata*, however, this species is known to undergo efficient homologous and illegitimate non-homologous recombination thus gene-targeting often requires at least 500 base pairs of homology (Cormack
and Falkow 1999). In contrast to *C. albicans*, *C. glabrata* is capable of maintaining plasmids and does not require codon optimization. Thus, given its increasing medical importance and recent advances in the development of genetics and molecular tools of this organism, *C. glabrata* is poised to become a powerful system in which to study the evolution of virulence, pathogenicity, and antifungal drug resistance.

### 1.3 Azole Antifungal Drugs

Like their human hosts, fungi are eukaryotic organisms, which renders identifying drug targets that distinguish pathogen from host particularly challenging. There are only 5 classes of antifungal drugs available in the clinic: the azoles, echinocandins, polyenes, allylamines, and pyrimidines. This thesis focuses primarily on the two most widely deployed classes for the treatment of invasive fungal infections, the azoles and the echinocandins. Three out of the five classes of antifungal drugs, the azoles, polyenes, and allylamines, target a single cellular pathway responsible for the biosynthesis of ergosterol, which is the primary sterol of fungal membranes and is required for membrane fluidity and cellular signaling, and is the functional analogue of cholesterol in mammalian cells (Anderson 2005). The azoles are the most widely used class of antifungals in the clinic and consist of two subclasses: the imidazoles, which are used topically, and the triazoles, which are used to treat systemic fungal disease and encompass fluconazole, itraconazole, posaconazole, and voriconazole (Ostrosky-Zeichner, Casadevall et al. 2010). The azoles have been in clinical use for almost 30 years and target the enzyme in the ergosterol biosynthesis pathway Erg11, a cytochrome p450 enzyme called lanosterol 14α-demethylase that is involved in the later stages of ergosterol biosynthesis (Akins 2005). The azoles are five-membered nitrogen-containing heterocyclic ring compounds that enter the fungal cell via facilitated diffusion to inhibit their target, Erg11 (Cowen and Steinbach 2008; Mansfield, Oltean
et al. 2010). There are several effects of Erg11 inhibition including an overall reduction of ergosterol in the cell, which can lead to disruption of vacuolar H^+-ATPase function that is necessary for diverse cellular processes and fungal virulence (Zhang, Gamarra et al. 2010), incorporation of alternative sterols in the cell membrane, and the accumulation of a toxic sterol intermediate that intercalates into the cell membrane and disrupts membrane integrity (Akins 2005). The azoles are fungistatic against Candida species, meaning that cells are merely arrested in growth and not killed, which leaves a large surviving population of fungal cells that are subject to strong directional selection for resistance (Anderson 2005). In this thesis, drug resistance refers to the ability to withstand high concentrations of antifungals that would normally inhibit growth of that species due to specific mechanisms and drug tolerance refers to the capacity to survive and grow during drug exposure independent of changes in the minimum inhibitory concentration.

1.3.1 Azole Resistance - Efflux

The fungistatic activity of the azoles coupled with their widespread use in the clinic over the last three decades both as treatment for fungal infection and prophylactically for organ transplant and other immune-compromised patients has led to the emergence of azole resistance which is a well-documented and characterized phenomenon (Akins 2005; Anderson 2005; Cowen 2008; Cowen and Steinbach 2008). One of the most common mechanisms of azole resistance is increased cellular efflux to simply remove the drug from the cell. This mechanism of resistance is utilized across a broad range of species from medically important bacterial species, protozoan parasites, human cancer cells, and fungal species including C. albicans and C. glabrata. C. albicans possesses ATP-binding cassette (ABC) transporters encoded by CDR1 and CDR2, both of which are upregulated in clinically resistant isolates (Sanglard, Kuchler et al. 1995). In C. albicans, expression of CDR1 and CDR2 is controlled by the transcription factor Tac1, which
binds to drug response elements (DRE) located in the promoters of \textit{CDR1} and \textit{CDR2} (de Micheli, Bille et al. 2002). Gain-of-function mutations that result in hyperactivity of Tac1 resulting in the up-regulation of \textit{CDR1} and \textit{CDR2} have been identified in azole-resistant clinical isolates, however, there is often a loss of heterozygosity at this locus as the transcription factor functions as a homodimer and both alleles must possess the mutations for high-level azole resistance to occur (Coste, Turner et al. 2006). \textit{C. albicans} also possesses another multidrug transporter involved in azole resistance that is encoded by \textit{MDR1}, which belongs to the major facilitator superfamily and was the first multidrug efflux pump identified in a fungal pathogen (Morschhauser 2010). Similar to \textit{CDR1} and \textit{CDR2}-mediated azole resistance, \textit{MDR1} expression is regulated by the zinc finger transcription factor Mrr1 and gain of function mutations coupled with the loss of heterozygosity of \textit{MRR1} in the presence of fluconazole results in constitutive activity of the transcription factor which, in turn, leads to the overexpression of \textit{MDR1} and azole resistance (Morschhauser, Barker et al. 2007; Dunkel, Blass et al. 2008). However, the Mdr1 transporter is specific for fluconazole and voriconazole but not ketoconazole, itraconazole, and clotrimazole in \textit{C. albicans} (Morschhauser 2010).

Like \textit{C. albicans}, \textit{C. glabrata} also has an arsenal of efflux pumps to handle potentially harmful compounds. In \textit{C. glabrata} the zinc finger transcription factor Pdr1, of functional resemblance to Pdr1 and Pdr3 in \textit{S. cerevisiae}, activates expression of the ABC transporters \textit{CDR1} and \textit{CDR2} (\textit{PDH1}) likely through binding to pleiotropic drug response elements (PDRE) within the promoters of \textit{CDR1} and \textit{CDR2} (Vermitsky, Earhart et al. 2006). Gain of function mutations in \textit{PDR1} have been shown to increase azole resistance via overexpression of \textit{CDR1} and \textit{CDR2} (Tsai, Krol et al. 2006; Vermitsky, Earhart et al. 2006; Ferrari, Ischer et al. 2009). Analysis of 122 \textit{C. glabrata} clinical isolates yielded 58 \textit{PDR1} alleles that grouped into three major hot spot regions within \textit{PDR1} and were associated with azole resistance (Ferrari, Ischer et
al. 2009). Interestingly, gain of function mutations in *C. glabrata PDR1* are also associated with increased virulence in the mouse model of systemic infection, as represented by higher fungal burdens in kidneys and decreased survival, however, the mechanism underlying this phenomenon has yet to be elucidated (Ferrari, Ischer et al. 2009). *C. glabrata* is a petite-positive species, meaning that it is able to lose its mitochondrial function and therefore become respiration-deficient, a phenomenon often found in response to azole exposure and that results in the upregulation of *CDR1* and *CDR2* through Pdr1 as well as an increase in free ergosterol content (Sanglard, Ischer et al. 2001; Brun, Berges et al. 2004). *SNQ2* is another ABC transporter in *C. glabrata* and deletion of *SNQ2* in azole-resistant *C. glabrata* clinical isolates reduces resistance (Torelli, Posteraro et al. 2008). *SNQ2* also contains a Pdr1 binding site, which suggests that a common mechanism of regulation may exist for all three ABC transporters in *C. glabrata* (Torelli, Posteraro et al. 2008). *C. glabrata* does contain an ortholog of *C. albicans MDR1*, however, its role in drug resistance has not been characterized.

1.3.2 Azole Resistance – Drug Target Alterations

The second canonical mechanism of azole resistance is alterations in the drug target that prevent or minimize inhibition by the drug. In one study, 12 distinct amino acid substitutions in *ERG11* were identified in *C. albicans* azole-resistant clinical isolates and these mutations were clustered in three hot spot regions of the gene (Marichal, Koymans et al. 1999). Often these mutations result in reduced binding affinity of the Erg11 target enzyme to the azoles as measured by various biochemical approaches including ligand-binding assays (Lamb, Kelly et al. 1997; Kelly, Lamb et al. 1999; Lamb, Kelly et al. 2000). Overexpression of *ERG11* can also result in azole resistance; this overexpression can occur via gene amplification through formation of the i5L isochromosome in which two copies of the left side of chromosome 5, which contains genes encoding both Erg11 and the transcription factor Tac1, are inverted relative to each other around
a copy of the chromosome 5 centromere (Selmecki, Forche et al. 2006). Azole resistance was directly proportional to the number of copies of \textit{ERG11} and \textit{TAC1}, and presence of the isochromosome also contributed to the overall fitness of \textit{C. albicans} strains in the presence of azoles (Selmecki, Gerami-Nejad et al. 2008; Selmecki, Dulmage et al. 2009). Upc2 is a zinc finger transcription factor involved in the transcriptional regulation of ergosterol biosynthetic genes including \textit{ERG11}, and gain of function mutations in this transcription factor resulting in constitutive upregulation of \textit{ERG11} have been found to contribute to azole resistance in \textit{C. albicans} clinical isolates (Heilmann, Schneider et al. 2010; Hoot, Smith et al. 2011). Increased gene dosage of \textit{ERG11} (\textit{CYP51}) has also been found to play a role in azole resistance of \textit{C. glabrata} clinical isolates and, at least in one case, this was due to a duplication of the entire \textit{ERG11}-containing chromosome (Marichal, Vanden Bossche et al. 1997). Polymorphisms in \textit{ERG11} of \textit{C. glabrata} clinical isolates have been uncovered, but the functional consequence of the nucleotide changes were not investigated and further mechanisms of drug target alterations have yet to be explored in this human pathogen (Berila and Subik 2010).

1.3.3 Azole Resistance – Cellular Stress Responses

The third mechanism of azole resistance involves many cellular signaling pathways and stress responses that allow the cell to survive and handle environmental stresses imposed by the azoles. The most well studied example of this is the loss of function of the ergosterol biosynthetic gene \textit{ERG3} (Anderson 2005). When Erg11 is inhibited by the azoles there is a buildup of a toxic lanosterol intermediate, 14-methylergosta-8,24(28)-dien-3,6-diol, produced by Erg3, which destabilizes the plasma membrane and results in arrested growth. \textit{C. albicans} is able to block the accumulation of this toxic sterol through loss of function mutations in \textit{ERG3} (Kelly, Lamb et al. 1995). Loss of function of Erg3 results in the presence of an alternative sterol in lieu of ergosterol, which then allows the cell to continue to grow in the presence of the azoles and
confers cross resistance to other antifungals that target ergosterol such as the polyenes (Kelly, Lamb et al. 1996). Another pathway involved in the cell membrane stress exerted by the azoles is the cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway, as deletion of components of this pathway results in increased susceptibility of both S. cerevisiae and C. albicans to the azoles (Jain, Akula et al. 2003). Based on RNA expression analysis, theazole hypersensitivity observed in mutants defective in this pathway is due to the inability to up regulate the transporter encoded by CDR1 (Jain, Akula et al. 2003). The protein kinase C (PKC) pathway was also recently found to play a role in both tolerance and resistance to the azoles in S. cerevisiae and C. albicans (LaFayette, Collins et al. 2010). In S. cerevisiae, Pkc1 is a serine-threonine specific kinase that regulates cell wall integrity during growth, morphogenesis, and cell wall stress (Levin 2005; Fuchs and Mylonakis 2009). Pkc1 also regulates cell membrane stress exerted by the azoles, partly through the mitogen-activated protein kinase (MAPK) pathway with the terminal MAP kinase encoded by SLT2 in S. cerevisiae or MKC1 in C. albicans (LaFayette, Collins et al. 2010).

There are several other stress response pathways involved in mediating both tolerance and resistance to the azoles, but this thesis focuses mainly on the roles of calcineurin and Hsp90, each discussed in greater detail below. Calcineurin is a protein phosphatase involved in many cellular stress responses, and in fungi calcineurin plays a key role in a diverse set of processes including cell cycle progression, fungal morphogenesis, virulence, and both tolerance and resistance to antifungal drugs (Fox and Heitman 2002; Kraus and Heitman 2003; Steinbach, Reedy et al. 2007). In laboratory conditions calcineurin is not required for growth, but in stressful conditions including cell membrane stress imposed by azoles and other sources (Cruz, Goldstein et al. 2002; Onyewu, Blankenship et al. 2003), pH stress (Kullas, Martin et al. 2007), and growth in serum (Blankenship, Wormley et al. 2003; Blankenship and Heitman 2005),
calcineurin becomes essential. Genetic deletion or pharmacological inhibition of calcineurin function abrogates tolerance and thus renders \textit{C. albicans} hypersensitive to the azoles (Cruz, Goldstein et al. 2002; Sanglard, Ischer et al. 2003). Calcineurin also mediates Erg3-mediated resistance to the azoles, partially through the downstream effectors Crz1 and Hph1 in \textit{S. cerevisiae}, and partially through Crz1 in \textit{C. albicans}; there is no Hph1 in \textit{C. albicans}, suggesting that there are additional downstream effectors of calcineurin involved in azole resistance (Onyewu, Wormley et al. 2004; Cowen, Carpenter et al. 2006). In \textit{C. glabrata} calcineurin mediates tolerance to the azoles, however, Crz1 appears to have no role suggesting that calcineurin is working through a Crz1-independent pathway to enable tolerance (Miyazaki, Yamauchi et al. 2010).

Hsp90 is a molecular chaperone and is involved in both maintenance of azole resistance and the emergence of rapidly acquired Erg3-mediated azole resistance in both the model yeast \textit{S. cerevisiae} as well as in \textit{C. albicans} (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). In addition to being essential during heat stress, Hsp90’s chaperone function is required by a myriad of client proteins for the stability of their active conformations (Taipale, Jarosz et al. 2010). Hsp90’s role in azole resistance is largely to regulate calcineurin signaling as genetic or pharmacological inhibition of calcineurin phenocopies the effect of Hsp90 inhibition (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). In \textit{S. cerevisiae} calcineurin is a client protein of Hsp90 and requires it for both stability and function (Imai and Yahara 2000). Mkc1, the terminal transcription factor of the PKC pathway in \textit{C. albicans}, was also recently found to be a client protein of Hsp90 and pharmacological inhibition of Pkc1 abrogates resistance of \textit{C. albicans} clinical isolates, phenocopying inhibition of Hsp90 or calcineurin (LaFayette, Collins et al. 2010). In the greater wax moth \textit{Galleria mellonella} model of \textit{C. albicans} infection, the Hsp90 inhibitors 17-AAG and 17-DMAG that are currently in clinical development as anticancer agents
transform fluconazole from ineffective against resistant isolates to highly efficacious (Cowen, Singh et al. 2009). In addition, genetic compromise of HSP90 expression enhances the efficacy of fluconazole in the murine model of disseminated candidiasis, suggesting that Hsp90 may provide a promising new target for the development of antifungal therapeutics (Cowen, Singh et al. 2009).

1.4 Echinocandin Antifungal Drugs

The echinocandins, including micafungin, caspofungin, and anidulafungin, are the only new class of antifungal drugs to reach the clinic in decades and target the fungal cell wall, a complex structure critical for fungi but absent from mammalian cells (Denning 2003). The fungal cell wall consists of large sugars including β-1,3-D-glucans, β-1,4-D-glucans, β-1,6-D-glucans, chitin, mannans, α-glucans and glycoproteins and, as a consequence, it is structurally very rigid (Denning 2003). The echinocandins are cyclic hexapeptides, with each drug differing by their aliphatic side chains, and they non-competitively inhibit the β-1,3-D-glucan synthase catalytic subunit encoded by FKS1, FKS2, and FKS3 in S. cerevisiae, C. albicans, and C. glabrata (Perlin 2011). β-1,3-D-glucans normally serve as a scaffold, linking mannoproteins to chitin in the cell wall and inhibition of their synthesis results in fungicidal activity via cell lysis (Perlin 2007).

The glucan synthase complex has been best studied in S. cerevisiae. Deletion of FKS1 and FKS2 is synthetic lethal, and they encode alternate catalytic subunits of the glucan synthase complex as either subunit is sufficient for glucan synthase activity and viability (Mazur, Morin et al. 1995; Ram, Brekelmans et al. 1995). In S. cerevisiae, FKS1 is expressed at higher levels than other FKS genes and is regulated by the cell cycle; FKS2 expression levels are low under optimal conditions and are induced in response to calcium stress, mating pheromone, the absence of FKS1, and high temperature in a calcineurin-dependent manner (Mazur, Morin et al. 1995; Zhao,
Jung et al. 1998). Given the dependence of FKS2 expression on calcineurin, deletion of FKS1 and calcineurin results in synthetic lethality (Garrett-Engele, Moilanen et al. 1995). In addition to the catalytic subunit, the glucan synthase complex contains a regulatory subunit Rho1, a GTP-binding protein that also regulates Pkc1 of the PKC pathway (Drgonova, Drgon et al. 1996; Qadota, Python et al. 1996). The guanine-nucleotide exchange factors (GEFs) Rom1 and Rom2 and the GTPase activating proteins (GAPs) Bem2, Sac7, Bag2, and Rdi1 regulate Rho1 function (Madden and Snyder 1998). The cell wall stress sensors Mid2 and Wsc1 signal through Rom2 which then activates Rho1 to produce β-1,3-D-glucans via Fks1 and Fks2, affect actin synthesis, as well as activate Pkc1, which regulates the MAPK cascade leading to phosphorylation of the transcription factors Rlm1 and SBF, which is composed of Swi4 and Swi6 (Fuchs and Mylonakis 2009). The exact mechanism of glucan synthase inhibition by the echinocandins remains unknown. Although the echinocandins enter the cell via saturable facilitated diffusion, it is unclear as to whether cellular entry is necessary for the inhibitory activity of the drug (Paderu, Park et al. 2004).

1.4.1 Echinocandin Resistance – Efflux

The echinocandins are poor substrates for multidrug efflux transporters, which appear to play a very minimal role in echinocandin resistance, in stark contrast to the azoles where upregulation of multidrug transporters is the most common cause of clinical resistance (Perlin 2007). A survey of azole-resistant C. albicans clinical isolates found very little cross-resistance to the echinocandins, including isolates that overexpressed CDR1, CDR2, and/or MDRI (Bachmann, Patterson et al. 2002). Another study of 351 strains of fluconazole-resistant Candida species found that 99% were sensitive to caspofungin, further suggesting that the acquisition of azole resistance via mechanisms including the upregulation of efflux transporters does not result in cross-resistance to the echinocandins (Pfaller, Messer et al. 2003). One study found that
constitutive overexpression of CDR2 in both S. cerevisiae and C. albicans increased resistance to the echinocandins in the context of spotting assays on solid media containing a fixed concentration of caspofungin (Schuetzer-Muehlbauer, Willinger et al. 2003). Subsequent work has shown that although there is an increase in caspofungin tolerance of S. cerevisiae and C. albicans strains overexpressing CDR2, the effect was specific to caspofungin in the context of a solid spotting assay and the strains were in fact hypersensitive to caspofungin and micafungin in liquid antifungal susceptibility tests (Niimi, Maki et al. 2006). Together, this suggests that efflux pumps are not a major mechanism of echinocandin resistance in fungi.

1.4.2 Echinocandin Resistance – Drug Target Alterations

To date alterations in the echinocandin drug targets, Fks1 and Fks2, are the only mechanism of echinocandin resistance reported in clinical isolates of C. albicans and C. glabrata. There are two highly conserved ‘hot spot’ regions of FKS1 and FKS2 where specific resistance mutations that confer cross-resistance to all echinocandins accumulate (Balashov, Park et al. 2006; Garcia-Effron, Lee et al. 2009; Garcia-Effron, Park et al. 2009; Perlin 2011). Biochemical studies of the glucan synthase complex isolated from FKS mutants of C. albicans and C. glabrata reveal that sensitivity to the echinocandins is reduced by up to 3000-fold by specific amino acid substitutions (Park, Kelly et al. 2005; Garcia-Effron, Lee et al. 2009). In C. albicans, the S645F/P/Y mutation accounts for 46% of FKS1 mutations found in echinocandin-resistant clinical isolates and confers the greatest increase in IC₅₀, the amount of drug required to inhibit glucan synthase activity by 50% (Garcia-Effron, Park et al. 2009; Perlin 2011). In C. glabrata, the S663F/P amino acid substitution accounts for 45% of FKS1 and FKS2 mutations found in echinocandin-resistant clinical isolates and is associated with the most pronounced resistance phenotype (Garcia-Effron, Lee et al. 2009; Perlin 2011). In both C. albicans and C. glabrata these amino acid substitutions were found to decrease the catalytic capacity of the mutant glucan
synthase enzyme, and the expression of FKS1 and FKS2 was altered in mutants suggesting that expression is regulated by the function of each component (Garcia-Effron, Lee et al. 2009; Garcia-Effron, Park et al. 2009). Given the decreased catalytic capacity of mutant glucan synthase complexes it is reasonable to hypothesize that there may be a fitness cost associated with the acquisition of echinocandin resistance via mutations in the drug target.

1.4.3 Echinocandin Resistance – Cellular Stress Responses

The echinocandins cause loss of cell wall integrity and induce cell wall stress responses. Genome-wide expression profiling in response to caspofungin has been performed to determine cellular pathways involved in echinocandin tolerance in S. cerevisiae and revealed rapid activation of the PKC pathway, initiated by the cell wall sensor protein Wsc1 and executed via the terminal MAPK Slt2 (Reinoso-Martin, Schuller et al. 2003). In addition, genes involved in cell wall and membrane function including components of the PKC signaling cascade, as well as chitin, mannan, and ergosterol biosynthetic pathways were uncovered in a screen of the S. cerevisiae haploid gene deletion library to identify cellular processes affected by caspofungin (Markovich, Yekutiel et al. 2004). Caspofungin treatment also results in the upregulation of MKC1 in C. albicans, the terminal MAP kinase in the PKC pathway (Wiederhold, Kontoyiannis et al. 2005). The echinocandins are reported to have fungicidal activity against Candida species, however, C. albicans is able to grow at very high concentrations of echinocandin in vitro and this phenomenon is known as the paradoxical effect (Wiederhold 2007). Although β–glucan content in the cell wall is decreased in the presence of echinocandin, there is an increase in chitin content by as much as 898% (Stevens, Ichinomiya et al. 2006). Under normal laboratory conditions chitin synthesis is regulated by calcineurin through Crz1, the PKC pathway, and the high osmolarity glycerol (HOG) pathway (Munro, Selvaggini et al. 2007). Treatment with echinocandins activates the chitin biosynthetic genes CHS1, CHS2, and CHS8 and activation is
dependent upon calcineurin and the PKC and HOG pathways, as mutants of these pathways are unable to increase chitin synthase activity or chitin content in response to caspofungin (Walker, Munro et al. 2008). Furthermore, prior activation of the cell wall integrity pathway and chitin synthesis by pre-treatment with CaCl₂ or calcofluor white allowed *C. albicans* to survive otherwise lethal concentrations of echinocandins suggesting a potential resistance mechanism (Walker, Munro et al. 2008). In a very recent study, *C. glabrata* calcineurin mutants were found to have decreased tolerance to the echinocandin micafungin, partially through the calcineurin downstream effector Crz1 (Miyazaki, Yamauchi et al. 2010). It has also been reported that the MAP kinase Slt2 contributes to tolerance to the echinocandins in *C. glabrata* through the transcription factor Rlm1, as in *S. cerevisiae* and *C. albicans* (Miyazaki, Inamine et al. 2010).

### 1.5 Calcineurin

Calcineurin is a Ca²⁺-calmodulin-activated serine-threonine-specific protein phosphatase crucially involved in cellular stress responses and is highly conserved across all eukaryotes (Fox and Heitman 2002). In mammals, calcineurin plays a key role in neuronal development and is associated with actin and microtubule components of the cytoskeleton during neurite extension (Fox and Heitman 2002). Calcineurin is also involved in regulation of the immune response by activating the transcriptional regulator nuclear factor of activated T-cells (NF-AT) through its phosphatase activity, which subsequently induces interleukin (IL)-2 transcription and T-cell activation (Steinbach, Reedy et al. 2007). The regulation of calcineurin has been implicated in many cellular and developmental processes including, but not limited to, heart-valve morphogenesis, early vascular development, Alzheimer’s disease, pancreatic β-cell function and diabetes mellitus (Steinbach, Reedy et al. 2007). Given its role in the immune response, calcineurin has become the target of several immunosuppressive drugs including cyclosporin A,
tacrolimus (FK506) and sirolimus (rapamycin) which are used to treat several human diseases including graft-versus-host disease in transplant patients, eczema, severe asthma, refractory rheumatoid arthritis, and segmental glomerulosclerosis (Blankenship, Steinbach et al. 2003; Steinbach, Reedy et al. 2007).

Calcineurin is a heterodimer composed of a catalytic A subunit, encoded by CNA1 in C. albicans and C. glabrata, as well as a regulatory subunit B, encoded by CNB1, and the association of both subunits is required for calcineurin function (Watanabe, Perrino et al. 1996). Upon release of calcium stores in the cell through the plasma membrane Ca$^{2+}$ channel composed of Mid1 and Cch1 or the ion channel Yvc1, Ca$^{2+}$-bound calmodulin binds to the catalytic subunit of calcineurin, which displaces an autoinhibitory domain on Cna1 to reveal the active site (Fox and Heitman 2002; Cyert 2003). Cnb1 and calmodulin are structurally conserved and both bind to regulatory elements on the Cna1 subunit towards the carboxyl terminus (Stie and Fox 2008).

Cyclosporin A is a clinically approved pharmacological inhibitor of calcineurin and is a natural product of the soil fungus Tolypocladium inflatum (Hemenway and Heitman 1999). Cyclosporin A interacts with calcineurin through the immunophilin cyclophilin A in mammals, or Cpr1 in C. albicans, C. glabrata and S. cerevisiae, and sterically interferes with binding of phosphoprotein substrates when the drug-immunophilin complex is bound to calcineurin (Liu, Farmer et al. 1991). Similarly, FK506, a natural product of the bacterium Streptomyces tsukubaensis, is also approved for use in the clinic as an immunosuppressant and interacts with calcineurin through the immunophilin FKBP12, encoded by FPR1 in S. cerevisiae and C. glabrata and RBP1 in C. albicans (Hemenway and Heitman 1999). Cyclosporin A-cyclophilin A and FK506-FKBP12 are structurally unrelated complexes and bind to distinct but overlapping sites on calcineurin (Liu, Farmer et al. 1991).
In addition to their clinical value, cyclosporin A and FK506 have been instrumental in the laboratory for elucidating the role of calcineurin in diverse cellular processes in both mammalian and fungal systems. In *C. albicans*, calcineurin is required for growth in serum, which contains high calcium levels, and for virulence in the mouse model of disseminated candidiasis (Blankenship, Wormley et al. 2003). Calcineurin is also critically involved in cellular response to stress in fungi including cell membrane stress exerted by sodium dodecyl sulphate or the azoles, discussed previously, alkaline pH, pheromone response, endoplasmic reticulum stress exerted by the antifungal tunicamycin, and cation stress by Li⁺, Ca²⁺ or Na⁺ (Steinbach, Reedy et al. 2007). In *S. cerevisiae*, the transcription factor Crz1 acts downstream of calcineurin to regulate gene expression and normally exists in a phosphorylated state, however, upon activation of calcineurin by calcium-bound calmodulin, calcineurin dephosphorylates Crz1 allowing it to translocate from the cytoplasm to the nucleus to modulate gene expression (Stathopoulos and Cyert 1997; Stathopoulos-Gerontides, Guo et al. 1999). In *C. albicans*, Crz1 is also downstream of calcineurin and displays calcium- and calcineurin-dependent nuclear localization (Karababa, Valentino et al. 2006). Gene expression profiling of *C. albicans* calcineurin and *CRZ1* deletion mutants revealed very similar expression profiles with a high degree of overlap, suggesting that Crz1 is the main transcriptional regulator in the calcineurin pathway (Karababa, Valentino et al. 2006). Once in the nucleus, Crz1 binds specifically to calcineurin-dependent response elements (CDREs) within the promoter regions of calcineurin-dependent genes and is sufficient to drive Ca²⁺-induced calcineurin-dependent gene expression on its own in both *S. cerevisiae* and *C. albicans* (Stathopoulos and Cyert 1997; Karababa, Valentino et al. 2006). However, certain phenotypes displayed by calcineurin mutants, including hypersensitivity to the azoles and attenuated virulence in the mouse model of disseminated candidiasis, are not fully phenocopied
in CRZ1 null mutants suggesting that there are other downstream targets of calcineurin in fungi (Onyewu, Wormley et al. 2004; Cowen, Carpenter et al. 2006).

1.6 Hsp90

Hsp90 is essential in all eukaryotes and is a cytosolic molecular chaperone involved in the folding and transport of many meta-stable client proteins, including kinases and transcription factors involved in cellular signaling and stress responses (Picard 2002). Hsp90 is one of the most abundant proteins in the cell and has been estimated to account for approximately 1% of the total soluble protein content in the cytoplasm of human cells (Lai, Chin et al. 1984). Hsp90 is often overexpressed in cancer cells, which is correlated with poor prognosis in breast cancer (Whitesell and Lindquist 2005; Pick, Kluger et al. 2007). In cancer cells, Hsp90 is diverted from its normal chaperoning duties and instead serves as a biochemical buffer by stabilizing mutant oncoproteins and modulating tumour cell apoptosis, thereby allowing cancer cells to survive despite having accumulated detrimental mutations (Whitesell and Lindquist 2005). Thus, Hsp90 has become the target of anticancer drugs including 17-AAG and 17-DMAG, and are both derivatives of the Hsp90 inhibitor geldanamycin, currently in clinical trials (Taldone, Gozman et al. 2008).

Hsp90 has two isoforms in S. cerevisiae encoded by HSC82 and HSP82, and only one in C. albicans and C. glabrata encoded by HSP90 and HSC82, respectively. Hsp90 is primarily found in the cytoplasm and exists as a homodimer with each copy consisting of the N-terminal, middle, and C-terminal domains (Whitesell and Lindquist 2005). The N-terminal domain contains an unusual adenosine triphosphate (ATP) nucleotide-binding pocket called the Bergerat fold, which has no similarity to ATP-binding domains usually found within other kinases and chaperones (Dutta and Inouye 2000). Once bound, hydrolysis of ATP to ADP in the Bergerat
fold leads to conformational changes in the Hsp90 homodimer that are essential for chaperone function (Meyer, Prodromou et al. 2003; McLaughlin, Ventureas et al. 2004). A flexible linker region connects the middle region, which interacts with N-terminally bound ATP molecules to modulate hydrolysis and also mediates binding of Hsp90 to many of its client proteins (Meyer, Prodromou et al. 2003). Dimerization of Hsp90 occurs via its C-terminal domain that is also required for Hsp90’s ATPase activity (Whitesell and Lindquist 2005). The C-terminal domain also binds co-chaperones possessing tetratricopeptide-containing repeat (TPR)-domains, including immunophilins, which further contribute to Hsp90’s specificity (Pearl and Prodromou 2006). Geldanamycin and radicicol are structurally unrelated natural compounds that specifically inhibit Hsp90 function by mimicking the structure of adenosine diphosphate (ADP) and binding to the ATP-binding pocket of Hsp90 (Roe, Prodromou et al. 1999). Geldanamycin and radicicol actually bind with higher affinity to the ATP-binding pocket than ATP or ADP, and therefore block the ATPase activity of Hsp90, preventing the conformational changes necessary for chaperone function (Cowen 2008).

Under normal cellular conditions, chaperones function in protein folding during protein synthesis, protein shuttling across membranes, and standard protein turnover (Whitesell and Lindquist 2005). Using high throughput genomic and proteomic approaches including physical interactions, chemical-genetic interactions, and genetic interactions, it has been reported that Hsp90 interacts with 10% of the proteome in S. cerevisiae (Zhao, Davey et al. 2005). Studies in Drosophila melanogaster and Arabidopsis thaliana revealed that Hsp90 can buffer natural genetic variation within populations at the protein level, allowing mutations to accrue in signaling pathways while maintaining wild-type phenotypes (Rutherford and Lindquist 1998; Queitsch, Sangster et al. 2002). Under stressful conditions including but not limited to heat shock, Hsp90 is titrated away from its normal guardian functions by an increase in the number of
misfolded targets and, consequently, the effects of the otherwise buffered genetic polymorphisms are phenotypically revealed. Thus, Hsp90 serves as a capacitor for the storage and release of genetic variation (Rutherford and Lindquist 1998; Queitsch, Sangster et al. 2002). Hsp90 can also serve as a potentiator by immediately enabling the phenotypic effects of individual genetic variants by providing a reservoir of chaperone activity (Cowen and Lindquist 2005; Jarosz and Lindquist 2010; Taipale, Jarosz et al. 2010). During times of stress, Hsp90’s folding reservoir is depleted and the traits are lost, as is the case with Erg3-mediated azole resistance in *S. cerevisiae* and *C. albicans* where Hsp90 enables resistance through loss-of-function of *ERG3*, however, the resistance phenotype is lost when Hsp90 is compromised (Cowen and Lindquist 2005). Recent work in *S. cerevisiae* has revealed that Hsp90 broadly modulates genotypic variation, serving as both a potentiator and capacitor for ~20% of natural genetic variation (Jarosz and Lindquist 2010). Over time, traits that are initially dependent on Hsp90 can evolve independence and be expressed even when Hsp90 function is compromised. For example a series of *C. albicans* clinical isolates that evolved fluconazole resistance in a human host over two years was found to be dependent on Hsp90 and calcineurin for resistance in the early clinical isolates as pharmacological inhibition of Hsp90 or calcineurin abrogated resistance (Cowen and Lindquist 2005). However, in the later clinical isolates, Hsp90 or calcineurin inhibition had only minimal impact on fluconazole resistance suggesting that the resistance trait evolved Hsp90 independence.

### 1.7 Thesis Rationale

Previous studies in *S. cerevisiae* and *C. albicans* established that the molecular chaperone Hsp90 potentiates resistance to the azoles, which exert cell membrane stress. The echinocandins are the most recently developed class of antifungals and target the fungal cell wall. Resistance found in
clinical isolates thus far has been exclusively attributed to mutations in the echinocandin drug target, however, this class of antifungal drugs has only been in clinical use for a short period of time compared to the azoles and many resistance mechanisms remain to be discovered. *C. glabrata* is now second to *C. albicans* as the most prevalent *Candida* species in the clinic, at least in part due to its ability to quickly develop resistance to the azoles. As such, the echinocandins are commonly employed to treat *C. glabrata* infection. Treatment of fungal infection is hampered by the limited availability of antifungal drugs and by the emergence of drug resistance. Thus, it is of great importance to understand, predict, and prevent the emergence of antifungal drug resistance.

My work establishes an entirely new role for the molecular chaperone Hsp90 in regulating echinocandin resistance in the leading fungal pathogen of humans. I discovered that Hsp90 plays a key role in both tolerance and resistance to the cell wall stress exerted by the echinocandins via calcineurin in *C. albicans*. Complementary studies performed in the model yeast *S. cerevisiae* reveal a divergence in the requirement of Hsp90 and calcineurin for tolerance to the cell wall stress imposed by echinocandins, despite conserved activation of calcineurin-dependent gene expression. Through reciprocal co-immunoprecipitation studies, calcineurin activation studies, and analysis of calcineurin protein levels upon depletion of Hsp90, I establish that calcineurin is an Hsp90 client protein in *C. albicans*. I show that Crz1 plays only a partial role in mediating tolerance to the echinocandins in *C. albicans*, implicating additional downstream effectors.

My work also provides the first global analysis of mutations that accumulate during the evolution of fungal drug resistance in a human host. I characterized a series of *C. glabrata* clinical isolates that evolved echinocandin resistance over two years in a human host and
employed whole genome sequencing to identify mutations in the evolved resistant isolate relative to its sensitive counterpart. In addition to a mutation in the drug target FKS2, I report on non-synonymous mutations in 8 other genes not previously implicated in echinocandin resistance. Based on pharmacological, genetic, and gene expression analysis I propose a novel model in which Hsp90 and calcineurin regulate expression of the major resistance determinant FKS2 to mediate echinocandin resistance in *C. glabrata*.

Taken together, my research reveals new mechanisms mediating antifungal drug resistance and suggests new therapeutic strategies to save human lives.
Chapter 2

2 Hsp90 Governs Antifungal Drug Resistance of *Candida albicans* Through the Client Protein Calcineurin

This chapter has been published as:


All experiments were performed by Sheena D. Singh except Figure 2-9B, which was performed by Nicole Robbins¹ and Figure 2-10, which was performed by Aimee K. Zaas², Wiley A. Schell², and John R. Perfect²,³

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2.1 Introduction

*Candida* species have intimate yet perilous connections with their human hosts. They are commensals of the human microbiota of the gastrointestinal tract, mucous membranes, and skin. They also rank as the most common causative agents of invasive fungal infections and are responsible for a broad spectrum of disease (McNeil, Nash et al. 2001; Pfaller and Diekema 2007). For the immunocompetent individual, *Candida* infections are most often superficial in nature including thrush and vaginitis. For the immunocompromised individual, these opportunists are far more menacing, as they can disseminate and cause life-threatening systemic disease. *Candida albicans* is the most frequently encountered *Candida* species in the clinic and is the fourth most common cause of hospital acquired infectious disease with mortality rates approaching 50% (Zaoutis, Argon et al. 2005; Pfaller and Diekema 2007). The frequency of fungal infections continues to increase in pace with the growing immunocompromised patient population, including individuals undergoing chemotherapy, transplantation of solid organs or hematopoietic stem cells, as well as those infected with HIV (Wilson, Reyes et al. 2002; Enoch, Ludlam et al. 2006).

Treatment of invasive fungal infections remains notoriously challenging, due in large part to the limited availability of clinically useful antifungal drugs. Fungi are eukaryotes and share close evolutionary relationships with their human hosts (Baldauf and Palmer 1993; Wainright, Hinkle et al. 1993). This makes the identification of drug targets in fungi that do not have homologs of similar function and susceptibility to inhibition in humans a daunting task. Most antifungal drugs in clinical use target the biosynthesis or function of ergosterol, the predominant sterol of fungal membranes, or the biosynthesis of (1,3)-β-D-glucan, a critical component of the fungal cell wall (Cowen 2008; Cowen and Steinbach 2008). The azoles are the largest class of
antifungal drugs in clinical use and have been deployed for several decades. They inhibit lanosterol 14α-demethylase, blocking ergosterol biosynthesis and resulting in the accumulation of a toxic sterol intermediate that disrupts membrane integrity and results in cell membrane stress. The echinocandins are the only new class of antifungal drug to be approved for clinical use in decades and inhibit (1,3)-β-D-glucan synthase, disrupting cell wall integrity and resulting in cell wall stress.

The efficacy of antifungal drugs can be hampered by fungistatic rather than fungicidal activity, by host toxicity, and by the emergence of drug resistance. The azoles are generally fungistatic against *Candida* species and many immunocompromised patients are on long-term treatment due to persistent infections or on prophylaxis to prevent future infections. This creates favorable conditions for the evolution of drug resistance. In experimental populations and clinical isolates, resistance often emerges by multiple mechanisms (Anderson 2005; Cowen 2008; Cowen and Steinbach 2008). Resistance mechanisms that minimize the impact of the drug include overexpression of multidrug transporters or alterations of the target enzyme. Other mechanisms function to minimize drug toxicity, such as loss of function of Erg3 in the ergosterol biosynthesis pathway, which blocks the production of a toxic sterol that would otherwise accumulate when the azoles inhibit their target. Mechanisms that mitigate drug toxicity are often dependent upon cellular stress responses that are crucial for tolerance of the membrane stress exerted by azoles (Cowen 2008; Cowen and Steinbach 2008). Far less is known about resistance to echinocandins, at least in part due to their more recent approval for clinical use. The most common mechanism of echinocandin resistance is mutation of the drug target (Perlin 2007). The (1,3)-β-D-glucan synthase complex consists of a regulatory subunit, Rho1, and a catalytic subunit encoded by *FKS1*, *FKS2*, and *FKS3*. Resistance is most commonly associated with
characteristic mutations in *FKSI* that reduce sensitivity of the enzyme to inhibition by echinocandins (Balashov, Park et al. 2006; Perlin 2007; Garcia-Effron, Lee et al. 2009). While the echinocandins are thought to be fungicidal against *C. albicans*, this organism has the capacity for robust growth at high drug concentrations, known as the paradoxical effect (Wiederhold 2007). *C. albicans* may utilize multiple cellular stress response pathways to tolerate cell wall stress induced by echinocandins including upregulation of other components of the cell wall as well as responses mediated by the cell wall integrity signaling pathway (Munro, Selvaggini et al. 2007; Walker, Munro et al. 2008).

A key regulator of cellular stress responses crucial for resistance to the azoles is the molecular chaperone Hsp90. Hsp90 is an essential chaperone that regulates the form and function of many key signal transducers (Pratt and Toft 2003; Pearl and Prodromou 2006; Wandinger, Richter et al. 2008). Pharmacological inhibition of Hsp90 blocks the emergence of azole resistance in *C. albicans* and abrogates resistance of laboratory mutants and clinical isolates that evolved resistance in a human host (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). Impairing Hsp90 function converts the fungistatic azoles into a fungicidal combination and enhances the therapeutic efficacy of azoles in two metazoan models of disseminated *C. albicans* infection (Cowen, Singh et al. 2009). Hsp90’s role in the emergence and maintenance of azole resistance is conserved in the model yeast *Saccharomyces cerevisiae* (Cowen and Lindquist 2005). The key mediator of Hsp90-dependent azole resistance is calcineurin, a protein phosphatase that regulates crucial responses to environmental stress, including the membrane stress exerted by exposure to azoles (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). In both *S. cerevisiae* and *C. albicans*, compromising calcineurin phenocopies compromising Hsp90, reducing azole resistance of diverse mutants. In *S. cerevisiae*, Hsp90 interacts physically with the catalytic subunit of calcineurin keeping it stable. 
and poised for activation (Imai and Yahara 2000). High-throughput genomic and proteomic studies have mapped Hsp90 physical interactors in *S. cerevisiae* (Zhao, Davey et al. 2005), while to date not a single Hsp90 client protein has been identified in *C. albicans*.

Given Hsp90’s role in azole resistance, I postulated that this chaperone might also govern crucial responses to the cell wall stress exerted by echinocandins in *C. albicans*. I recently discovered that Hsp90 is required for the basal tolerance of *Aspergillus* species to echinocandins, which are fungistatic against *Aspergillus* species, and that Hsp90 inhibitors enhance the efficacy of echinocandins in an invertebrate model of *Aspergillus fumigatus* infection (Cowen and Lindquist 2005; Cowen, Singh et al. 2009). *A. fumigatus* is the principal causal agent of invasive aspergillosis with alarming mortality rates up to 90% that still remain at 40% with the best current treatment options (Herbrecht, Denning et al. 2002; Singh and Paterson 2005). Compromising calcineurin tracks with compromising Hsp90, enhancing the activity of echinocandins (Steinbach, Cramer et al. 2007; Fortwendel, Juvvadi et al. 2009). While initial studies did not detect a role for Hsp90 in echinocandin resistance in *C. albicans* (Cowen and Lindquist 2005), there are two lines of evidence implicating the Hsp90 client protein calcineurin in mediating responses to cell wall stress in this pathogen. First, stimulation of chitin synthesis rescues *C. albicans* from echinocandins and this stimulation is mediated via calcineurin in concert with the cell wall integrity signaling pathway and the high osmolarity glycerol signaling pathway (Munro, Selvaggini et al. 2007; Walker, Munro et al. 2008). Second, inhibition of calcineurin can block the paradoxical growth of *C. albicans* observed at elevated echinocandin concentrations (Wiederhold, Kontoyianniss et al. 2005). Whether calcineurin mediates basal tolerance to echinocandins is unclear given that in one study, deletion of calcineurin enhanced the killing activity of an echinocandin (Sanglard, Ischer et al. 2003), while in another study there
was no effect (Cruz, Goldstein et al. 2002). Thus, if Hsp90 regulates calcineurin function, then it is poised to mediate crucial cellular responses to the echinocandins.

Here, I investigated Hsp90’s role in tolerance to echinocandins in C. albicans. I found that pharmacological or genetic compromise of Hsp90 function reduced tolerance of laboratory strains to the echinocandins and created a fungicidal combination. Inhibition of Hsp90 also reduced resistance acquired by mutation in FKS1 in both laboratory-derived mutants and clinical isolates that acquired resistance in a human host. Compromising calcineurin function phenocopied compromising Hsp90 function. Consistent with calcineurin being the key mediator of Hsp90-dependent echinocandin tolerance, I established that calcineurin is an Hsp90 client protein in C. albicans. The downstream effector of calcineurin, Crz1, played a partial role in mediating calcineurin-dependent stress responses that are activated by echinocandins. Hsp90’s key role in governing crucial responses to cell wall stress exerted by echinocandins was not conserved in S. cerevisiae, emphasizing the importance of performing molecular studies in the pathogen. In a murine model of disseminated candidiasis, genetic impairment of HSP90 expression enhanced the therapeutic efficacy of an echinocandin. My findings identify the first Hsp90 client protein in C. albicans and establish an entirely new role for Hsp90 in mediating echinocandin resistance. Further, my results demonstrate that targeting Hsp90 provides a promising therapeutic strategy for the treatment of life-threatening disease.

2.2 Materials and Methods

2.2.1 Strains and Culture Conditions

Archives of C. albicans and S. cerevisiae strains were maintained at -80°C in 25% glycerol. Strains were grown in either YPD (1% yeast extract, 2% bactopeptone, 2% glucose), YPM (as YPD except with 2 % maltose), or in synthetic defined media (yeast nitrogen base, 2% glucose)
and supplemented with the required amino acids. 2% agar was added for solid media. Strains were transformed following standard protocols. Strains used in this study are listed in Table 2-1. Primers used in this study are listed in Table 2-3.

2.2.2 Strain Construction

**CaLC501**: The plasmid pLC340 was digested with *KpnI* and *SacI* to liberate the cassette to C-terminally TAP tag the native *HSP90* allele in CaLC239. For NAT resistant transformants, proper integration was verified by PCR using primers oLC313 and oLC319 as well as oLC316 and oLC319. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette.

**CaLC502**: The plasmid pLC340 was digested with *KpnI* and *SacI* to liberate the cassette to C-terminally TAP-tag the only *HSP90* allele in CaLC367. For NAT resistant transformants, proper integration was verified by PCR using primers oLC313 and oLC319 as well as oLC316 and oLC319. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette.

**CaLC590**: The plasmid pLC340 was digested with *KpnI* and *SacI* to liberate the cassette to C-terminally TAP-tag the only *HSP90* allele in CaLC367. For NAT resistant transformants, proper integration was verified by PCR using primers oLC313 and oLC319 as well as oLC316 and oLC319. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Then plasmid pLC353 was digested with *KpnI* and *SacI* to liberate the cassette to C-terminally tag *CNA1* with the 6xHISFLAG tag. For NAT resistant transformants, proper integration was verified by PCR using primers oLC342 and oLC295 as well as oLC292 and oLC343. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette.
**CaLC857:** The plasmid pLC353 was digested with *Kpn*I and *Sac*I to liberate the cassette to C-terminally tag *CNA1* with the 6xHISFLAG tag in CaLC239. For NAT resistant transformants, proper integration was verified by PCR using primers oLC342 and oLC275 as well as oLC343 and oLC274. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette.

**CaLC858:** The plasmid pLC353 was digested with *Kpn*I and *Sac*I to liberate the cassette to C-terminally tag *CNA1* with the 6xHISFLAG tag in CaLC367. For NAT resistant transformants, proper integration was verified by PCR using primers oLC342 and oLC275 as well as oLC343 and oLC274. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette.

**CaLC860:** The plasmid pLC350 was digested with *Kpn*I and *Sac*I to liberate the *CNA1* knockout cassette and was transformed into CaLC239. For NAT resistant transformants, proper integration was verified by PCR using primers oLC275 and oLC590 as well as oLC274 and oLC343. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. The plasmid pLC353 was digested with *Kpn*I and *Sac*I to liberate the cassette to C-terminally tag the other allele of *CNA1* with the 6xHISFLAG tag. For NAT resistant transformants, proper integration was verified by PCR using primers oLC342 and oLC275 as well as oLC343 and oLC274. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Presence of the deleted allele was verified by PCR using primers oLC433 and oLC436.

**CaLC861:** The plasmid pLC406 was linearized with *Eco*NI to place the *UTR2p-lacZ* construct in CAI-4. For Ura+ transformants, proper integration was verified by PCR using primers oLC661 and oLC621.
**CaLC908:** The plasmid pLC350 was digested with *KpnI* and *SacI* to liberate the *CNA1* knockout cassette and was transformed into CaLC239. For NAT resistant transformants, proper integration was verified by PCR using primers oLC275 and oLC590 as well as oLC274 and oLC343. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette.

**CaLC909:** The plasmid pLC350 was digested with *KpnI* and *SacI* to liberate the *CNA1* knockout cassette and was transformed into CaLC908 to create a *cna1* null strain. For NAT resistant transformants, proper integration was verified by PCR using primers oLC275 and oLC590 as well as oLC274 and oLC343. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Presence of the deleted allele was verified by PCR using primers oLC590 and oLC343 and absence of an intact *CNA1* allele was verified by PCR using primers oLC588 and oLC591.

**CaLC432:** The plasmid pLC329 was digested with *KpnI* and *SacI* to liberate the cassette to replace the native *HSP90* promoter with the *MAL2* promoter and was transformed into CaLC367. For NAT resistant transformants, proper integration was verified by PCR using primers oLC308 and oLC275 as well as oLC309 and oLC274. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette.

**CaLC912:** The plasmid pLC353 was digested with *KpnI* and *SacI* to liberate the cassette to C-terminally tag *CNA1* with the 6xHISFLAG tag in CaLC432. For NAT resistant transformants, proper integration was verified by PCR using primers oLC342 and oLC275 as well as oLC343 and oLC274. The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette.
**ScLC463:** To construct a \(cna1\Delta/cna2\Delta\) double mutant, the BY4742 alpha strain was mated with the BY4741 \(cna2\Delta\) deletion mutant. Diploids were sporulated in liquid medium and tetrads were dissected to obtain alpha haploid meiotic progeny. Presence of the \(cna2\Delta\) deletion was verified by PCR with primers oLC101 and oLC326. This \(cna2\Delta\) alpha strain was then mated to the BY4741 \(cna1\Delta\) deletion mutant and haploid meiotic progeny were picked as above. Presence of the \(cna2\Delta\) deletion was verified as above and deletion of \(cna1\Delta\) was verified with primers oLC101 and oLC102.

**ScLC642:** The plasmid pLC74 was linearized with Stul to place the CDRE-lacZ construct at URA3 in wild-type \(S.\ cerevisiae\) W303. For Trp+ transformants, proper integration of the construct was verified by PCR using the primers oLC870 and oLC524.

### 2.2.3 Plasmid Construction

Recombinant DNA procedures were performed according to standard protocols. Plasmids used in this study are listed in Table 2-2. Plasmids were sequenced to verify the absence of any nonsense mutations. Primers used in this study are listed in Table 2-3.

**pLC329:** The \(MAL2\) promoter was excised from plasmid pLC90 (pAU22, (Uhl, Biery et al. 2003)) by digestion with NotI and SacII and was cloned into pLC49 at NotI and SacII. ~350 base pairs of homology upstream of the \(HSP90\) promoter was PCR amplified from SC5314 genomic DNA using primers oLC294 and oLC295 and was cloned into pLC49 containing the \(MAL2\) promoter at KpnI and ApaI. ~350 base pairs of homology downstream of the HSP90 promoter was amplified from SC5314 genomic DNA with primers oLC296 and oLC297 and cloned into pLC49 containing the \(MAL2\) promoter and the upstream homology at SacII and SacI. The cassette to replace the native \(HSP90\) promoter with the \(MAL2\) promoter can be excised with KpnI and SacI.
**pLC340:** ~500 base pairs of homology downstream of *HSP90* was PCR amplified from SC5314 genomic DNA using primers oLC318 and oLC319 and cloned into pLC49 (pJK863, (Shen, Guo et al. 2005)) at *Sac*II and *Sac*I. Proper integration was verified by PCR using primers oLC274 and oLC319. ~500 base pairs of homology to the C-terminal end of *HSP90* prior to the stop codon was amplified from SC5314 genomic DNA using primers oLC313 and oLC315. The TAP tag was amplified from genomic DNA isolated from a *S. cerevisiae* strain containing the TAP tag (*HSC82-TAP*, (Ghaemmaghami, Huh et al. 2003)) using oLC316 and oLC317. A fusion PCR was performed to attach the TAP tag to the C-terminal portion of *HSP90* using oLC313 and oLC317. This fusion product was cloned into pLC49 containing the downstream homology at *Kpn*I and *Apa*I. Proper integration was verified by PCR with oLC313 and oLC275. The cassette to C-terminally TAP tag Hsp90 can be excised with *Kpn*I and *Sac*I.

**pLC350:** Homology upstream of *CNA1* before the start codon was PCR amplified from SC5314 genomic DNA using primers oLC433 and oLC434 and was cloned into pLC49 at *Kpn*I and *Apa*I. Proper integration was verified by PCR using primers oLC275 and oLC433. Homology downstream of *CNA1* after the stop codon was amplified from SC5314 genomic DNA with primers oLC435 and oLC436 and was cloned into pLC49 containing the upstream homology at *Sac*II and *Sac*I. Proper integration was verified by PCR using primers oLC274 and oLC436. The cassette to knock out *CNA1* can be excised with *Kpn*I and *Sac*I.

**pLC353:** Homology at the end of *CNA1* immediately before the stop codon was PCR amplified from SC5314 genomic DNA using primers oLC338 and oLC339, which contains the 6xHISFLAG tag within the primer. This product was cloned into pLC49 at *Kpn*I and *Apa*I and proper integration was verified by PCR using primers oLC338 and oLC275. Homology downstream of the coding region of *CNA1* was amplified from SC5314 genomic DNA using
primers oLC340 and oLC341 and cloned into pLC49 containing the upstream region of homology at SacII and SacI. Proper integration was verified by PCR using primers oLC341 and oLC274. The cassette to C-terminally 6xHISFLAG tag the catalytic subunit of calcineurin can be excised with KpnI and SacI.

**pLC406:** pLC90 (pAU22, (Uhl, Biery et al. 2003)) was digested with KpnI and XhoI to liberate the maltose promoter. ~500 base pairs of the *C. albicans UTR2* promoter was amplified from SC5314 genomic DNA with oLC616 and oLC617 and was cloned into the pLC90 backbone at KpnI and XhoI. Presence of the insert was tested by PCR with oLC616 and oLC617. This plasmid was then digested with BamHI, which is immediately after lacZ and before the MAL2 terminator. ~500 base pairs of the *UTR2* terminator was PCR amplified from SC5314 genomic DNA with oLC618 and oLC660 and was cloned into pLC90 containing the *UTR2* promoter at BamHI. Directionality of the insert was tested by PCR with oLC661 and oLC660. There is an A to G mutation at -41 and an A to G mutation at -200 but these are not within the CDRE. The *CaUTR2p-lacZ* cassette can be linearized for integration using EcoNI.

### 2.2.4 Minimum Inhibitory Concentration and Checkerboard Assays

Antifungal susceptibility was determined in flat bottom, 96-well microtiter plates (Sarstedt) using a modified broth microdilution protocol, as described (Cowen and Lindquist 2005). Minimum inhibitory concentration (MIC) tests were set up in a total volume of 0.2 ml/well with 2-fold dilutions of micafungin (MF, generously provided by Julia R. Köhler) or caspofungin (CS, generously provided by Rochelle Bagatell). Echinocandin gradients were typically from 2 µg/ml down to 0 with the following concentration steps in µg/ml: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078125, 0.00390625, 0.00195313. For gradients from 16 µg/ml down to 0, the concentration steps in µg/ml were: 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625. Cell
densities of overnight cultures were determined and dilutions were prepared such that \( \sim 10^3 \) cells were inoculated into each well. Geldanamycin (GdA, A.G. Scientific, Inc.) and radicicol (RAD, A.G. Scientific, Inc.) were used to inhibit Hsp90 at the indicated concentrations, and cyclosporin A (CsA, CalBiochem) and FK506 (A.G. Scientific, Inc.) were used to inhibit calcineurin at the indicated concentrations. Checkerboard assays were set up in a total volume of 0.2 ml/well with 2-fold dilutions of MF across the x-axis of the plate and 2-fold dilutions of either GdA or CsA across the y-axis of the plate. Plates were inoculated as with MIC tests. Dimethyl sulfoxide (DMSO, Sigma Aldrich Co.) was the vehicle for GdA, RAD, CsA, and FK506. Sterile water was the vehicle for MF and CS. Plates were incubated in the dark at 30°C for the time period indicated, at which point plates were sealed and re-suspended by agitation. 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 on at least two occasions. MIC data was quantitatively displayed with color using the program Java TreeView 1.1.3 (http://jtreeview.sourceforge.net).

2.2.5 Spotting Assays

Strains were grown overnight to saturation in YPD and cell concentrations were standardized based on optical density. Five-fold dilutions (from \( \sim 1 \times 10^6 \) cells/ml) were spotted onto indicated media using a spotter (Frogger, V&P Scientific, Inc). Plates were photographed after 2 days in the dark at 30°C. All spottings were done in duplicate on at least two separate occasions.

2.2.6 β-Galactosidase Assays

*C. albicans* cultures were grown overnight in YPD at 30°C with or without 10 µM CsA, 5 µg/ml FK506, 5 µM GdA, or 5 µM RAD. Cells were diluted to OD\(_{600}\) of 0.5 and grown at 25°C for 2h, at which point they were treated with MF, FL, or CaCl\(_2\), as indicated. *S. cerevisiae* cultures were
grown overnight in synthetic defined medium containing ammonium chloride at 30°C with 1 µg/mL FK506 or 5 µM GdA, as indicated. Cells were diluted to OD$_{600}$ of 0.3 and treated with 0.2 M CaCl$_2$, FK506, or GdA, as indicated. Cells were grown for 3 hours at 25°C. Protein was extracted as described (Stathopoulos and Cyert 1997; Withee, Mulholland et al. 1997), and protein concentrations were determined by Bradford analysis. β-galactosidase activity was measured using the substrate ONPG (O-nitrophenyl-β-D-galactopyranoside, Sigma Aldrich Co.), as described (Stathopoulos and Cyert 1997). β-galactosidase activity is given in units of nanomoles ONPG converted per minute per milligram of protein (Miller Units). Statistical significance was evaluated using GraphPad Prism 4.0.

2.2.7 Immunoprecipitation

Yeast cultures were grown overnight in YPD at 30°C. Cells were diluted to OD$_{600}$ of 0.2 in 40 ml and grown to mid-log phase. Cells were washed with sterile H$_2$O and resuspended in 500 µl of lysis buffer containing 20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl$_2$ and 20% glycerol, with one protease inhibitor cocktail tablet (complete, EDTA-free tablet, Roche Diagnostics) per 10 ml, 1 mM PMSF (EMD Chemicals) and 20 mM sodium molybdate (Sigma Aldrich Co.) added fresh before use. Cells were transferred to a 2 mL screw-cap tube and the tube was filled, alternating with glass beads and additional lysis buffer until the beads were just below the meniscus at the top of the tube to reduce foaming during bead beating. Cells were disrupted by bead beating twice for 4 minutes with a 10 minute break on ice between cycles. Lysates were recovered by piercing a hole in the bottom of each tube, placing each tube in a larger 14 ml tube, and centrifuging at 1308 x g for three 5-minute cycles, recovering the lysates at each interval. Total collected lysates were cleared by centrifugation at 20817 x g for 10 minutes at 4°C and protein concentrations were determined by Bradford analysis.
Anti-FLAG immunoprecipitations were done by diluting protein samples to 1 mg/ml in tris-buffered saline with 20 mM sodium molybdate and incubating with anti-FLAG M2 affinity agarose (Sigma Aldrich Co.) that was washed twice with tris-buffered saline prior to use, as per the manufacturer’s specifications, at 4°C overnight. Unbound material was removed by three washes with 1 ml tris-buffered saline and protein was eluted by boiling the sample in one volume of 2X sample buffer.

Anti-IgG immunoprecipitations were done by diluting protein samples to 1 mg/ml in lysis buffer with 0.2% Tween 20 and incubating with rabbit IgG agarose (Sigma Aldrich Co.) that was washed three times with lysis buffer prior to use, at 4°C overnight. Unbound material was removed by washing six times with 1 ml lysis buffer with 0.1% tween and protein was eluted by boiling the sample in one volume of 2X sample buffer.

2.2.8 Immune Blot Analysis
Yeast cultures were grown to mid-log phase, protein was extracted as above, and protein concentrations were determined by Bradford analysis. Protein samples were mixed with one-fifth volume of 6X sample buffer, were boiled for 5 minutes, and then separated on a 10% SDS-PAGE gel. Protein was electrotransferred to PVDF membrane (Bio-Rad Laboratories, Inc.) and blocked with 5% skim milk in phosphate buffered saline with 0.1% tween. Blots were hybridized with antibody against CaHsp90 (1:10000 dilution, (Burt, Daly et al. 2003)), histone H3 (1:3000 dilution; Abcam ab1791), FLAG (1:10000, Sigma Aldrich Co.), Hsc82/Hsp82 (1:5000, (Borkovich, Farrelly et al. 1989)), or TAP (1:5000, Open Biosystems).

2.2.9 Murine Model of C. albicans Infection
Inoculum was prepared as described for injection of 100 µL of a 2 x 10^6 CFU/mL suspension (Cowen, Singh et al. 2009). Inoculum concentrations were verified by cell counts and CFU
measurements. Male CD1 mice (Charles River Laboratories) age 8 weeks (weight 30-34 g) were infected via the tail vein. For infection with the wild type, the sample sizes were \( n = 6 \) mice for the untreated group and \( n = 5 \) mice for the MF treatment group. For the \( \text{tetO-}H\text{SP90/hsp90}\Delta \) strain the sample sizes were \( n = 7 \) mice for the untreated group and \( n = 8 \) for the MF treatment group. An initial dose finding experiment was performed to determine a concentration of MF that would have negligible effect on fungal burden of mice infected with the wild type; a dose of 2 mg/kg MF (Astellas Pharma, Inc; Deerfield, IL) delivered intraperitoneally at one-hour post infection and then daily resulted in clearance of the fungal burden (data not shown), while a dose of 0.2 mg/kg had no significant effect and was chosen as the dose for this study. Mice were observed three times daily for signs of illness and weighed daily. At day 4 following injection, mice were sacrificed by \( \text{CO}_2 \) asphyxiation and the left kidney was removed aseptically, homogenized in PBS and serial dilutions plated for determination of kidney fungal burden, as described (Cowen, Singh et al. 2009). CFU values were expressed as CFU/g of tissue, log-transformed and compared using an ANOVA with post-hoc testing of significance between groups (GraphPad Prism 4.0). Murine work was performed under a protocol approved by the Institutional Animal Use and Care Committee at Duke University Medical Center.

2.2.10 E-tests

Resistance of \( C. \text{ albicans} \) strains to CS was determined with Etest strips (AB Biodisk) on RPMI solid medium. \( \sim 10^5 \) cells were plated prior to application of a test strip. Plates were photographed after 48 hours at 30°C in the dark.
2.3 Results

2.3.1 Hsp90 plays a crucial role in echinocandin tolerance of *Candida albicans*.

To determine the impact of compromising Hsp90 function on tolerance to echinocandins, I first used two structurally unrelated inhibitors geldanamycin (GdA) or radicicol (RAD) that bind with high affinity to Hsp90’s unusual adenosine triphosphate (ATP) binding pocket and inhibit ATP-dependent chaperone function (Whitesell, Mimnaugh et al. 1994; Roe, Prodromou et al. 1999). I used concentrations that abrogate resistance to azoles, but have no impact on growth on their own (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006; Cowen, Singh et al. 2009). The impact of Hsp90 inhibitors on tolerance to the widely used echinocandin micafungin (MF) was evaluated using an antifungal susceptibility test that measures growth across a gradient of MF concentrations relative to a MF-free growth control. Both strains tested showed robust tolerance to MF (Figure 2-1A). Inhibition of Hsp90 with GdA or RAD dramatically enhanced sensitivity to MF in either synthetic defined medium (Figure 2-1A) or in rich medium (Figure 2-2A). Comparable effects were observed with another widely used echinocandin, caspofungin (CS, Figure 2-2B). The same trends were observed when a dilution series of cells was spotted on solid medium with a fixed concentration of MF; concentrations of Hsp90 inhibitors that had no impact on growth on their own enhanced susceptibility to MF (Figure 2-1B). Notably, while synergy of Hsp90 inhibitors with MF was observed in both liquid and solid media (Figure 2-1), the synergy with CS was restricted to liquid medium (data not shown). This explains why the synergy between Hsp90 inhibitors and echinocandins was not detected in a previous study, which used CS on solid medium [21]. The basis for the different responses with MF and CS on solid medium is unclear and the response with a third echinocandin, anidulafungin, remains to be determined. Strains were more sensitive to echinocandins in a medium used for clinical
susceptibility testing (RPMI, Figure 2-2C and D), however, compromising Hsp90 or calcineurin function further enhanced the sensitivity (Figure 2-2C).

Next, I exploited genetic regulation of Hsp90 to validate the impact of compromising Hsp90 function on echinocandin tolerance. Deletion of one HSP90 allele had negligible effect on MF tolerance (Figure 2-1C and D). Replacing the native HSP90 promoter of the heterozygote with a tetracycline-repressible promoter has no effect on basal Hsp90 levels in the absence of tetracycline at 30°C, but blocks induction of HSP90 in response to stress such as elevated temperature of 37°C or exposure to antifungal drugs (Cowen, Singh et al. 2009). Even in the absence of tetracycline, compromising HSP90 expression in the tetO-HSP90/hsp90Δ strain resulted in hypersensitivity to MF in both liquid and solid media (Figure 2-1C and D). While the tetO-HSP90/hsp90Δ strain also had a reduced growth rate, Hsp90 inhibitors at concentrations that have no effect on growth on their own dramatically enhanced echinocandin sensitivity ruling out the possibility that the hypersensitivity is simply due to reduced growth rate (Figure 2-1B). Restoring a wild-type HSP90 allele to the tetO-HSP90/hsp90Δ strain complemented both the reduced growth rate and the hypersensitivity to MF. Thus, pharmacological and genetic studies establish that Hsp90 enables tolerance to echinocandins.
Figure 2-1. Hsp90 plays a crucial role in echinocandin tolerance of Candida albicans.

(A) Pharmacological inhibition of Hsp90 with GdA or RAD reduces MF tolerance of C. albicans laboratory strains in an MIC assay. Assays were done in synthetic defined medium at 30°C for
72 hours. Optical densities were averaged for duplicate measurements and normalized relative to MF-free controls (see colour bar). (B) Pharmacological inhibition of Hsp90 reduces MF tolerance on solid rich medium (YPD). Cells were spotted in fivefold dilutions (from $1 \times 10^6$ cells/ml) onto plates with a fixed concentration of MF (30 ng/ml), GdA, or RAD, as indicated, and were photographed after 48 hours in the dark at 30°C. (C) Genetic compromise of Hsp90 expression reduces MF tolerance in an MIC assay. The assay was performed and analyzed as in part A. (D) Genetic compromise of Hsp90 expression reduces MF tolerance on solid rich medium (YPD). The assay was performed and analyzed as in part B.
Figure 2-2. Hsp90 plays a crucial role in echinocandin tolerance of *Candida albicans*.

(A) Pharmacological inhibition of Hsp90 with geldanamycin (GdA) or radicicol (RAD) reduces micafungin (MF) tolerance of *C. albicans* laboratory strains in an MIC assay. Assays were done in rich medium (YPD) at 30°C for 72 hours. Optical densities were averaged for duplicate measurements and normalized relative to MF-free controls (see colour bar).

(B)
Pharmacological inhibition of Hsp90 with GdA or inhibition of calcineurin with cyclosporine A (CsA) reduces caspofungin (CS) tolerance of *C. albicans* laboratory strains in an MIC assay. Assays were done in rich medium (YPD) at 30°C for 72 hours. Data was analyzed as in part A. (C) Pharmacological inhibition of Hsp90 with GdA or pharmacological inhibition of calcineurin with CsA reduces CS tolerance of *C. albicans* laboratory strains in an MIC assay. Assays were done in RPMI at 30°C for 72 hours. Data was analyzed as in part A. (D) *C. albicans* laboratory strains are susceptible to CS in an E-test. Resistance of standard *C. albicans* laboratory strains to CS is shown on RPMI solid medium. CS test strips (Etest, AB Biodisk) produced a gradient of drug concentration, highest at the top. Plates were incubated at 30°C for 48 hours.
2.3.2 Compromising calcineurin function phenocopies compromising Hsp90 function

It is now well established that a key mediator of Hsp90-dependent azole resistance is calcineurin, a protein phosphatase that regulates numerous responses to membrane stress in *C. albicans* (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006; Cowen 2008). If Hsp90 governs crucial cellular responses to echinocandins via calcineurin, then inhibition of calcineurin should phenocopy Hsp90 inhibition.

I initially compromised calcineurin function pharmacologically using two structurally unrelated inhibitors cyclosporin A (CsA) and FK506 that inhibit calcineurin by distinct mechanisms (Hemenway and Heitman 1999). CsA binds to Cpr1, a peptidyl-prolyl cis-trans isomerase (cyclophilin A), forming a drug-protein complex that blocks calcineurin function. FK506 forms a different drug-protein complex that binds to the structurally unrelated peptidyl-prolyl cis-trans isomerase FKBP12 to block calcineurin function. I used concentrations of CsA and FK506 that had no impact on growth on their own but that abrogate azole resistance (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). Inhibition of calcineurin with either CsA or FK506 abolished MF tolerance of *C. albicans* (Figure 2-3).

Next, I abolished calcineurin function genetically by either deleting the gene encoding the catalytic subunit of calcineurin, *CNA1*, or by deleting the gene encoding the regulatory subunit of calcineurin required for its activation, *CNB1*. In both cases, loss of calcineurin function abrogated MF tolerance (Figure 2-3). Reconstituting a wild-type allele of *CNB1* restored tolerance. Thus, impairing calcineurin function recapitulates the effects of impairing Hsp90, reducing echinocandin tolerance of *C. albicans*. 
Figure 2-3. Compromising calcineurin function phenocopies compromising Hsp90 function. Deletion of either the catalytic subunit of calcineurin, CNA1, or the regulatory subunit of calcineurin, CNB1, abrogates MF tolerance. Pharmacological inhibition of calcineurin with either CsA or FK506 also abrogates MF tolerance. The assay was performed and analyzed as in Figure 2-1.
2.3.3 Inhibition of Hsp90 or calcineurin creates a fungicidal combination with MF

The echinocandins are generally fungicidal against yeast species such as *C. albicans* (Perlin 2007). However, *C. albicans* is able to grow vigorously at intermediate echinocandin concentrations in laboratory growth conditions (Figures 2-1 and 2-2 and Figure 2-3). My previous assays did not resolve whether inhibition of Hsp90 or calcineurin results in a complete block in fungal growth in the presence of echinocandins or whether it creates a fungicidal condition.

To determine if compromising Hsp90 or calcineurin function is fungistatic or fungicidal in the presence of echinocandins, I used tandem assays with an antifungal susceptibility test followed by spotting onto rich medium without any inhibitors. The common approach to address cidality by measuring colony forming units (CFU) in a culture exposed to treatment over time worked well for azoles (Cowen, Singh et al. 2009), but was not accurate for echinocandins. Exposure of *C. albicans* to MF caused severe clumping such that large aggregates of cells were not separable, rendering CFU counts inaccurate (data not shown). A strain with wild-type or heterozygous HSP90 levels was able to grow on rich medium following exposure to all concentrations of MF tested (Figure 2-4, left panel). Genetic compromise of HSP90 expression in the tetO-HSP90/hsp90Δ strain or pharmacological inhibition of Hsp90 with GdA was cidal in combination with any dose of MF tested; no cells were able to grow on the rich medium following exposure to the treatments (Figure 2-4). Comparable effects were seen with genetic or pharmacological compromise of calcineurin function (Figure 2-4). Thus, Hsp90 and calcineurin regulate crucial cellular responses for surviving the cell wall stress exerted by the echinocandins.
Figure 2-4. Inhibition of Hsp90 or calcineurin creates a fungicidal combination with MF. An MIC assay with four-fold MF dilutions was performed in YPD with or without either the Hsp90 inhibitor GdA or the calcineurin inhibitor CsA and incubated for 72 hours at 30°C. Cells from the MIC assay were spotted onto solid YPD medium and incubated at 30°C for 48 hours.
2.3.4 Calcineurin is an Hsp90 client protein in *C. albicans*

Compromising calcineurin pharmacologically or genetically phenocopies compromising Hsp90 suggesting a functional relationship between these regulators. Genetic studies established that calcineurin is a key mediator of Hsp90-dependent azole resistance (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). In *S. cerevisiae*, Hsp90 physically interacts with the catalytic subunit of calcineurin keeping it stable and poised for activation (Imai and Yahara 2000). High-throughput studies have mapped Hsp90 physical interactors in *S. cerevisiae* (Zhao, Davey et al. 2005), while to date not a single Hsp90 client protein has been characterized in *C. albicans*.

In order to determine if Hsp90 and calcineurin physically interact in *C. albicans*, I engineered strains harboring epitope-tagged proteins for co-immunoprecipitation. I tagged the catalytic subunit of calcineurin, Cna1, at the C-terminus using a 6X-histidine and FLAG epitope tag that has been used successfully for purification of the *C. albicans* septin complex (Kaneko, Umeyama et al. 2004). The Cna1-His-FLAG protein is functional and sufficient to mediate the canonical calcineurin-dependent response to calcium stress (Figure 2-5A). Immunoprecipitation with anti-FLAG agarose co-purified both FLAG-tagged Cna1 and wild-type Hsp90 (Figure 2-6A). For the control strain lacking the tagged CNA1 allele, Hsp90 was present in the input but was not immunoprecipitated. To further validate the physical interaction between Hsp90 and calcineurin, I performed the reciprocal co-immunoprecipitation using the same tagged allele of calcineurin in addition to an *HSP90* allele tagged at the C-terminus with a tandem affinity purification (TAP) tag which consists of a calmodulin binding peptide, a TEV cleavage site and two IgG binding domains of *Staphylococcus aureus* protein A that has been used with great success in *S. cerevisiae* (Ghaemmaghami, Huh et al. 2003). The Hsp90-TAP protein is functional and able to support growth and all essential Hsp90 functions (Figure 2-5B). Immunoprecipitation with IgG sepharose for the TAP tag, co-purifies both Hsp90-TAP and
Cna1-His-FLAG (Figure 2-6B). For the control strain lacking Hsp90-TAP, the tagged allele of calcineurin was present in the input but was not immunoprecipitated. Thus, reciprocal co-immunoprecipitation demonstrates physical interaction between Hsp90 and calcineurin in *C. albicans*.

If calcineurin is an Hsp90 client protein, then one would expect that inhibition of Hsp90 function would compromise calcineurin activation. To determine if this is indeed the case, I used a well-established reporter system that exploits the calcineurin downstream effector Crz1. Crz1 is a transcription factor that is dephosphorylated by calcineurin in response to calcineurin activation by calcium (Stathopoulos-Gerontides, Guo et al. 1999; Karababa, Valentino et al. 2006; Roy, Li et al. 2007). Dephosphorylated Crz1 translocates to the nucleus and drives expression of genes containing calcineurin-dependent response elements (CDREs) in their promoters. I used a strain harboring a construct with the *UTR2* promoter, which contains a CDRE element and is regulated by calcineurin (Karababa, Valentino et al. 2006), fused to *lacZ* and integrated at the *UTR2* locus (Shapiro, Uppuluri et al. 2009). In *S. cerevisiae*, a similar reporter that contains four tandem copies of CDRE and a *CYC1* minimal promoter driving *lacZ* has been used extensively (Stathopoulos-Gerontides, Guo et al. 1999). As expected, exposure of cells containing the *UTR2-lacZ* reporter to calcium chloride resulted in activation of calcineurin relative to the untreated control (*P* < 0.001, ANOVA, Bonferroni’s Multiple Comparison Test Figure 2-6C). Inhibition of calcineurin with CsA caused a dramatic reduction of calcineurin activation (*P* < 0.001). Inhibition of Hsp90 with GdA or RAD was as effective in blocking calcineurin activation as CsA (Figure 2-6C).

A hallmark of Hsp90 client proteins is that they are destabilized and degraded upon compromising Hsp90 function. To determine if calcineurin levels are reduced upon genetic
reduction of Hsp90, I turned to a strain with its only HSP90 allele regulated by the MAL2 repressible promoter. In this system, HSP90 expression is induced by maltose and repressed by glucose (Figure 2-6D). The MAL2 promoter does not drive as strong expression as the native HSP90 promoter, thus even when fully induced in maltose, the MAL2p-HSP90/hsp90∆ strain had a modest reduction of Hsp90 levels relative to a heterozygote with its only HSP90 allele under the control of the native promoter (Figure 2-6D). Growth of cells in an equal mixture of glucose and maltose as the carbon source resulted in a dramatic reduction of Hsp90 levels (Figure 2-6D). Under these conditions, the MAL2p-HSP90/hsp90∆ strain has reduced growth rate and reaches approximately half the stationary phase cell density as a wild-type strain (Shapiro, Uppuluri et al. 2009). This genetic depletion of Hsp90 was accompanied by a dramatic reduction of calcineurin levels as measured by immunoblot hybridization with an anti-FLAG antibody to detect the Cna1-His-FLAG protein (Figure 2-6D). Hybridization with an anti-H3 antibody confirmed comparable amounts of protein were loaded for all strains. Taken together, these results support the model that calcineurin is a client protein in C. albicans.
Figure 2-5. Tagged alleles of *Candida albicans* CNA1 and HSP90 are functional.

(A) The *C. albicans* HIS-FLAG tagged allele of CNA1 is functional. Cells were spotted in five-fold dilutions (from 1 x 10^6 cells/ml) onto solid rich medium with or without CaCl2 to assess calcineurin function. The mutant lacking the regulatory subunit of calcineurin required for its activation, Cnb1, is hypersensitive to calcium stress. The strain with its only allele encoding the catalytic subunit of calcineurin C-terminally HIS-FLAG tagged shows no increase in sensitivity to calcium stress, consistent with functionality of the tagged allele. Plates were photographed after 48 hours in the dark at 30°C. (B) The *C. albicans* TAP-tagged allele of HSP90 is functional. Cells were spotted as in part A onto solid rich medium to assess function of Hsp90-TAP. Since Hsp90 is essential, the equivalent growth of the strain with its only HSP90 allele TAP tagged compared to the untagged counterpart indicates functionality of the tagged allele. Plates were photographed after 48 hours in the dark at 30°C.
Calcineurin is an Hsp90 client protein in *C. albicans*. (A) Hsp90 and calcineurin physically interact as measured by co-immunoprecipitation of Hsp90 with Cna1-HisFLAG. Immunoprecipitation of HisFLAG-tagged Cna1 with anti-FLAG M2 affinity agarose, co-purifies Hsp90. Hsp90 was not immunoprecipitated by anti-FLAG M2 affinity agarose in control cells harboring untagged Cna1. (B) Hsp90 and calcineurin physically interact as measured by the reciprocal co-immunoprecipitation of Cna1-HisFLAG with Hsp90-TAP. Immunoprecipitation of Hsp90-TAP with IgG agarose, co-purifies Cna1-HisFLAG. Cna1-HisFLAG was not immunoprecipitated by IgG agarose in control cells harboring untagged Hsp90. (C) Calcineurin activation is blocked by pharmacological inhibition of Hsp90. A strain harboring a *UTR2p-lacZ* construct was incubated in rich medium with no treatment (U) or with 0.2 M CaCl$_2$ (Ca) to activate calcineurin. The impact of the calcineurin inhibitor CsA (10 µM), or the Hsp90 inhibitors GdA (5 µM) or RAD (5 µM) on calcineurin activation was determined by
measurement of β-galactosidase activity. Data are means ± standard deviations for triplicate samples. (D) Genetic reduction of Hsp90 levels results in depletion of calcineurin. All strains shown in this panel have one allele of Cna1-HisFLAG in addition to the indicated genotype. Even when fully induced in the maltose, expression of Hsp90 from the MAL2 promoter is not as strong as from the native promoter, while glucose results in further reduction of Hsp90 expression. This reduction of Hsp90 levels is accompanied by depletion of calcineurin. Top two panels, immune blot analysis of Hsp90 levels relative to the histone H3 loading control (5 µg protein loaded per well). Bottom two panels, immune blot analysis of Cna1-HisFLAG relative to the histone H3 loading control (50 µg protein loaded per well).
2.3.5 Azoles and echinocandins activate calcineurin-dependent stress responses.

Due to the important role of calcineurin in mediating crucial responses to the stress exerted by exposure to azoles and echinocandins (Cruz, Goldstein et al. 2002; Sanglard, Ischer et al. 2003; Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006), I postulated that these drugs would cause activation of calcineurin. I used the UTR2p-lacZ reporter to monitor calcineurin activation in response to concentrations of theazole antifungal drug fluconazole (FL) and the echinocandin MF that each cause modest inhibition of growth. Preliminary studies revealed maximum activation of calcineurin occurred at different time points in response to the different drugs (data not shown). Exposure to MF for 8 hours caused significant activation of calcineurin (Figure 2-7A, \( P < 0.001 \), ANOVA, Bonferroni’s Multiple Comparison Test). Pharmacological inhibition of calcineurin or Hsp90 blocked MF-induced calcineurin activation \( (P < 0.001) \). Treatment conditions were optimized such that all cultures underwent comparable growth with equivalent protein yields. Exposure to FL for 24 hours also led to significant calcineurin activation (Figure 2-7B, \( P < 0.001 \), ANOVA, Bonferroni’s Multiple Comparison Test). Inhibition of calcineurin or Hsp90 blocked FL-induced calcineurin activation \( (P < 0.001) \). Thus, both echinocandins and azoles activate calcineurin-dependent stress responses mediated via the transcription factor Crz1 and inhibition of Hsp90 blocks these responses.
Figure 2-7. Echinocandins and azoles activate calcineurin-dependent stress responses. (A) The echinocandin MF activates calcineurin. A strain harboring a *UTR2p-lacZ* construct was incubated in rich medium without treatment (U) or with 30 ng/ml MF in combination with the calcineurin inhibitor CsA (10 µM) or the Hsp90 inhibitor RAD (5 µM), as indicated, for 8 hours. Data are means ± standard deviations for triplicate samples. (B) The azole fluconazole (FL) activates calcineurin in an Hsp90-dependent manner. A strain harboring a *UTR2p-lacZ* construct was incubated in rich medium without treatment (U) or with 16 µg/ml FL in combination with the calcineurin inhibitor CsA (10 µM) or the Hsp90 inhibitor RAD (5 µM), as indicated, for 24 hours.
2.3.6 The calcineurin-dependent transcription factor Crz1 plays a partial role in echinocandin tolerance.

Crz1 is the key mediator of calcineurin-dependent transcriptional responses (Yoshimoto, Saltsman et al. 2002; Karababa, Valentino et al. 2006) and is implicated in tolerance to azoles in both *S. cerevisiae* and *C. albicans* (Onyewu, Wormley et al. 2004; Cowen, Carpenter et al. 2006). While deletion of calcineurin causes a complete loss of azole tolerance, deletion of *CRZ1* causes only a partial reduction in both species. To determine if Crz1 is also an important effector of calcineurin-dependent echinocandin tolerance, I compared the phenotypic consequences of deletion of *CRZ1* with deletion of the catalytic subunit of calcineurin, *CNA1*. Mutants with homozygous deletion of *CNA1* were hypersensitive to MF in both liquid and solid assays (Figures 2-3 and 2-8). Two independent *crz1* null mutants demonstrated partial loss of MF tolerance, but were not as sensitive as the *cna1* mutants (Figure 2-8). Reconstitution of a wild-type *CRZ1* allele restored MF tolerance. Thus, Crz1 is a key mediator of calcineurin-dependent echinocandin tolerance, but other calcineurin downstream effectors affecting this trait remain to be identified.
Figure 2-8. The calcineurin-dependent transcription factor Crz1 plays a partial role in echinocandin tolerance. (A) Homozygous deletion of CRZ1 partially reduces tolerance to MF in an MIC assay. The assay was performed and analyzed as in Figure 1A. (B) Homozygous deletion of CRZ1 partially reduces tolerance to MF on solid rich medium (YPD). Complementation with a wild-type CRZ1 allele restores tolerance. The assay was performed and analyzed as in Figure 2-1B.
2.3.7 Clinical relevance of Hsp90 and calcineurin-mediated echinocandin resistance.

To determine if Hsp90 and calcineurin are involved in bona fide echinocandin resistance arising due to mutations in the target Fks1 I tested for synergy between inhibitors of Hsp90 (GdA) or calcineurin (CsA) and the echinocandin MF. I utilized a checkerboard format to explore a range of concentrations of each inhibitor to more accurately define the thresholds of synergy. For a standard laboratory strain, SC5314, potent synergy was observed such that very low concentrations of either GdA or CsA were sufficient to abrogate MF tolerance (Figure 2-9). Next, I tested an echinocandin resistant mutant that was selected in vitro in the SC5314 background by plating on a high concentration of the echinocandin caspofungin (CS) and contained the common Fks1 mutation F641S (Balashov, Park et al. 2006). For this laboratory derived Fks1 mutant, C42, synergy was observed; GdA or CsA reduced MF resistance, though not to the same extent as for SC5314 (Figure 2-9). To determine if the synergy between GdA or CsA and MF was conserved in an isolate that evolved echinocandin resistance in a human host, I tested a clinical isolate harboring the same F641S Fks1 mutation (DPL15, generously provided by D. S. Perlin). Comparable synergy between GdA and MF was observed for both the clinical and laboratory-derived Fks1 mutants, however, the synergy between CsA and MF was more potent against the clinical isolate (Figure 2-9). Interestingly, these synergies were not observed for all echinocandin resistant clinical isolates tested, even those harboring the identical FKS1 mutation; of the 14 FKS1 mutants tested, synergy was observed for 8 (data not shown). These results suggest that Hsp90 and calcineurin enable cellular stress responses required for clinically relevant echinocandin resistance.
Figure 2-9. Hsp90 and calcineurin mediate echinocandin resistance of isolates that acquired Fks1 mutations during selection in vitro or in a human host. Pharmacological inhibition of Hsp90 or calcineurin reduces MF tolerance of a laboratory strain (SC5314), a laboratory derived Fks1 F641S mutant (C42), and a clinical isolate harboring the same Fks1 mutation (DPL15). Checkerboards were performed in synthetic defined medium and incubated at 30°C for 72 hours. Data were analyzed as in Figure 2-1A.
2.3.8 Genetic compromise of HSP90 expression enhanced the therapeutic efficacy of micafungin in a murine model of disseminated C. albicans infection.

To determine if impairing Hsp90 function holds therapeutic potential in combination with an echinocandin, I turned to a well-established murine model in which fungal inoculum is delivered by tail vein injection and progresses from the bloodstream to deep-seated infection of major organs such as the kidney (Cowen, Singh et al. 2009; Shapiro, Uppuluri et al. 2009). Due to toxicity of currently available Hsp90 inhibitors that do not distinguish pathogen from host in the context of an acute fungal infection (Cowen, Singh et al. 2009), genetic regulation of HSP90 was used to test this hypothesis in an in vivo system. We compared kidney fungal burden of mice infected with either a strain with wild-type HSP90 levels or a strain with its only HSP90 allele expressed under the tetO promoter. In the absence of tetracycline, the tetO-HSP90/hsp90\(\Delta\) strain has HSP90 levels comparable to a heterozygote but HSP90 expression from the tetO promoter cannot be upregulated in response to host temperatures or drug stress (Cowen, Singh et al. 2009). Mice infected with the tetO-HSP90/hsp90\(\Delta\) strain demonstrated significantly reduced kidney fungal burden relative to those infected with a strain expressing wild-type HSP90 levels (\(P < 0.05\), ANOVA, Bonferroni’s Multiple Comparison Test, Figure 2-10). Treatment of mice with a dose of MF that had negligible effect on mice infected with the strain with wild-type HSP90 levels resulted in a significant reduction in fungal burden for mice infected with the tetO-HSP90/hsp90\(\Delta\) strain (\(P < 0.001\), ANOVA, Bonferroni’s Multiple Comparison Test, Figure 2-10). Thus, genetic compromise of HSP90 expression enhances the efficacy of MF in a murine model.
Figure 2-10. Genetic compromise of *C. albicans* HSP90 renders micafungin (MF) more efficacious in a murine model of disseminated disease. CD1 mice were infected with an inoculum of 100 µl of 2 x 10^6 colony forming units (CFU)/ml of a strain expressing wild-type HSP90 levels or a strain with its only HSP90 allele regulated by tetO. MF was administered at 0.2 mg/kg intraperitoneally at one-hour post infection and then daily, as indicated. One asterisk indicates *P* < 0.05; two asterisks indicate *P* < 0.001 (ANOVA, Bonferroni’s Multiple Comparison Test).
2.3.9 Divergence of Hsp90 and calcineurin’s role in echinocandin tolerance in *Saccharomyces cerevisiae*

Given that calcineurin is the key mediator of Hsp90-dependent resistance to azoles in both *S. cerevisiae* and *C. albicans*, I postulated that these key regulators of cellular signaling might also mediate tolerance to echinocandins in both species. Consistent with previous findings (Imai and Yahara 2000), I confirmed that Hsp90 and calcineurin physically interact in *S. cerevisiae* (Figure 2-11A), as they do in *C. albicans* (Figure 2-6). To monitor calcineurin activation in *S. cerevisiae*, a reporter system was used similar to that used for *C. albicans*. Cells contained an integrated plasmid with four tandem copies of CDRE and a *CYC1* minimal promoter driving *lacZ* (Stathopoulos-Gerontides, Guo et al. 1999). As expected for an Hsp90 client protein, calcineurin activation was blocked upon pharmacological inhibition of Hsp90 (Figure 2-11B, $P < 0.001$, ANOVA, Bonferroni’s Multiple Comparison Test). FL activated calcineurin in *S. cerevisiae* (Figure 2-11C, $P < 0.0001$, $t$-test), as it did with *C. albicans* (Figure 2-7B), consistent with the key role for both regulators in azole tolerance. MF also activated calcineurin in *S. cerevisiae* (Figure 2-11C, $P < 0.0001$) as it did in *C. albicans* (Figure 2-7A).

Despite activation of calcineurin by MF in *S. cerevisiae*, compromise of calcineurin or Hsp90 function had negligible effect on MF tolerance. Neither deletion of the gene encoding the regulatory subunit Cnb1 nor deletion of the redundant genes encoding the catalytic subunit Cna1 and Cna2 reduced MF tolerance (Figure 2-11D). Consistent with this result, pharmacological inhibition of calcineurin with CsA had no impact on MF tolerance. A strain with genetically reduced Hsp90 levels (Lo90 (Cowen and Lindquist 2005)) had a modest reduction in tolerance, however, a concentration the Hsp90 inhibitor GdA that abrogates azole resistance had no effect on MF tolerance (Figure 2-11D). This suggests that the slight reduction in MF tolerance of the Lo90 strain may be due to a reduced growth rate rather than compromise of Hsp90 function.
Thus, while the functional relationship between Hsp90 and calcineurin is conserved between *C. albicans* and *S. cerevisiae*, as is the activation of calcineurin in response to drug stress, these regulators play a crucial role in cellular responses to echinocandins in the pathogenic yeast but not in the model yeast.
Figure 2-11. Divergence of Hsp90 and calcineurin’s role in echinocandin tolerance in S. cerevisiae. (A) Hsp90 and calcineurin physically interact in S. cerevisiae as measured by co-immunoprecipitation of Hsp90 (encoded by HSC82 and HSP82) with Cna1-TAP. Immunoprecipitation of TAP-tagged Cna1 with IgG agarose co-purifies Hsp90. Hsp90 was not immunoprecipitated by IgG agarose in control cells harboring untagged Cna1. (B) Calcineurin activation is blocked by pharmacological inhibition of Hsp90. A strain harboring a CDRE-lacZ construct was incubated in synthetic defined medium with no treatment (U) or with 0.2 M CaCl₂ (Ca) to activate calcineurin. The impact of the calcineurin inhibitor FK506 (1 µg/ml) or the Hsp90 inhibitor GdA (5 µM) on calcineurin activation was determined by measurement of β-galactosidase activity. Data are means ± standard deviations for triplicate samples. (C) The echinocandin MF and the azole FL activate calcineurin. A strain harboring a CDRE-lacZ construct was incubated in synthetic defined medium without treatment (U), with 30 ng/ml MF for 8 hours, or with 16 µg/ml FL for 24 hours. Data are means ± standard deviations for triplicate samples. (D) Compromising calcineurin or Hsp90 has minimal effect on tolerance to
MF in *S. cerevisiae* in an MIC assay. The effects of CsA are not due to inhibition of calcineurin given that genetic compromise of calcineurin by deletion of the regulatory subunit encoded by *CNB1* or by deletion of the catalytic subunit encoded by *CNA1* and *CNA2* has no impact on MF tolerance. The assay was performed in synthetic defined medium at 25°C and was analyzed as in Figure 2-1A.
2.4 Discussion

My results establish a new role for Hsp90 in echinocandin resistance in the pathogenic yeast *C. albicans*. Hsp90 regulates crucial cellular responses to the cell wall stress exerted by echinocandins such that compromising Hsp90 function reduces echinocandin tolerance of laboratory strains and resistance of clinical isolates (Figures 2-1 and 2-9). In a murine model of disseminated *C. albicans* infection, genetic compromise of *HSP90* enhances the efficacy of an echinocandin (Figure 2-10). I demonstrate that calcineurin is an Hsp90 client protein (Figure 2-6): calcineurin physically interacts with Hsp90; calcineurin activation is blocked upon impairment of Hsp90 function; and calcineurin levels are depleted upon genetic reduction of Hsp90. My findings implicate calcineurin as the key mediator of Hsp90-dependent echinocandin resistance. Exposure to azoles and echinocandins activates calcineurin-dependent stress responses (Figure 2-7) and the downstream effector Crz1 plays a partial role in echinocandin tolerance (Figure 2-8). In addition to defining a novel mechanism of resistance to the only new class of antifungal drugs to reach the clinic in decades, these results provide the first characterization of an Hsp90 client protein in *C. albicans*.

The requirement for Hsp90 and calcineurin in mediating crucial cellular responses to the echinocandins in *C. albicans* but not in *S. cerevisiae* (Figures 2-1, 2-3, and 2-11) stands in contrast to the conserved role for both regulators in cellular responses to azoles in both species. It is intriguing that calcineurin is activated in response to echinocandin stress in *S. cerevisiae* yet the functional consequence of deleting calcineurin is negligible for this trait (Figure 2-11). Activation of signaling molecules does not always predict functional consequences of their deletion under equivalent conditions. For example, Mkc1, the mitogen activated protein kinase (MAPK) in the PKC pathway, is activated by hydrogen peroxide but is not required for survival
under this condition (Navarro-Garcia, Eisman et al. 2005). My results suggest that there may be other redundant pathways operating in parallel with Hsp90 and calcineurin in *S. cerevisiae*. The protein kinase C (PKC) cell wall integrity pathway has a well-established function in mediating tolerance to echinocandins in *S. cerevisiae* (Reinoso-Martin, Schuller et al. 2003; Markovich, Yekutiel et al. 2004). In *C. albicans*, the PKC pathway is activated under diverse stress conditions (Navarro-Garcia, Eisman et al. 2005) and works in concert with calcineurin and the high osmolarity glycerol pathway to regulate chitin synthesis, which can enhance tolerance to echinocandins (Munro, Selvaggini et al. 2007; Walker, Munro et al. 2008). There may be considerable interaction between PKC signaling, calcineurin, and Hsp90. In *S. cerevisiae*, expression of one of the two partially redundant genes encoding the essential (1,3)-β-D-glucan synthase activity, *FKS2*, is regulated by both PKC signaling and calcineurin (Stathopoulos and Cyert 1997; Zhao, Jung et al. 1998). In *S. cerevisiae*, Hsp90 may also interact with PKC signaling by chaperoning PKC (Gould, Kannan et al. 2009) and the MAPK Slt2 (Millson, Truman et al. 2005; Truman, Millson et al. 2007).

Stress response signaling and canonical resistance mechanisms are intimately connected in defining a resistance phenotype. Compromising Hsp90 or calcineurin blocks the stress responses crucial for basal tolerance of strains that were not previously exposed to echinocandins (Figures 2-1, 2-3, and 2-4). There is heterogeneity in the phenotypic consequences of compromising these cellular regulators in strains that acquired resistance by mutation in the drug target Fks1 (Figure 2-9). For some isolates, resistance is not affected (data not shown), while for others resistance is reduced, though not to the extent of a sensitive strain (Figure 2-9). This suggests that Hsp90 is not required to enable the phenotypic consequences of the mutant Fks1 protein. Rather, in many of the Fks1 mutants, Hsp90 and calcineurin-dependent stress responses
contribute to the overall resistance phenotypes. Notably, the calcineurin inhibitor was more effective than the Hsp90 inhibitor at reducing MF resistance of some clinical isolates (Figure 2-9 and data not shown); this may be due to additional effects of CsA on targets distinct from calcineurin. The accumulation of mutations that reduce the dependence of resistance on Hsp90 is reminiscent of the evolution of azole resistance from Hsp90-dependence towards Hsp90-independence observed in isolates that evolved azole resistance in a human host (Cowen and Lindquist 2005).

Hsp90 chaperones many cellular regulators in addition to calcineurin. High-throughput genomic and proteomic studies suggest that Hsp90 may interact with up to 10% of the S. cerevisiae proteome (Zhao, Davey et al. 2005). Thus, Hsp90 is poised to regulate responses to antifungal drugs via other signal transduction pathways governing cellular stress responses. That Hsp90 regulates cellular responses to antifungal drugs targeting both the cell membrane and the cell wall via calcineurin emphasizes the importance of calcineurin as regulator of cellular stress responses. Cases of discordance between the phenotypic effects of compromising Hsp90 versus compromising calcineurin may reflect the relative importance of other Hsp90 client proteins in a particular trait or may reflect specificity of the agents used to inhibit these regulators (Dudgeon, Zhang et al. 2008).

My results suggest that targeting Hsp90 may provide a powerful therapeutic strategy in the treatment of fungal infectious disease. In vitro, compromising Hsp90 function enhances the efficacy of echinocandins against isolates that evolved resistance in a human host and against isolates not previously exposed to echinocandins (Figure 2-1, 2-4, and 2-9). In a murine model of disseminated candidiasis, genetic impairment of HSP90 expression enhances the efficacy of an echinocandin (Figure 2-10). These findings add a new dimension to combinatorial
therapeutic strategies for the treatment of *C. albicans* infections. My previous work established that genetic reduction of Hsp90 levels enhances the efficacy of fluconazole in a murine model of disseminated *C. albicans* infection (Cowen, Singh et al. 2009) and that further genetic depletion of *C. albicans* Hsp90 results in complete clearance of an infection in the murine model (Shapiro, Uppuluri et al. 2009). These studies establish firm proof-of-principle of Hsp90 as a therapeutic target. Current Hsp90 inhibitors that are well-tolerated in humans as anti-cancer agents exhibit toxicity in the mouse model in the context of an acute fungal infection (Cowen, Singh et al. 2009). However, in an invertebrate model of fungal pathogenesis, these pharmacological inhibitors of Hsp90 function enhance the efficacy of the two most widely deployed classes of antifungal drugs, azoles and echinocandins, against the two leading fungal pathogens of humans, *Candida albicans* and *Aspergillus fumigatus* (Cowen, Singh et al. 2009). Thus, compromising Hsp90 has broad therapeutic potential in combinatorial therapeutic regimens against fungal infections.

Further support for targeting Hsp90 in antifungal therapy emerges from a recombinant antibody against the *C. albicans* chaperone. This recombinant antibody had therapeutic benefits in a clinical trial in combination with amphotericin B, which targets ergosterol (Pachl, Svoboda et al. 2006). This antibody also demonstrated synergy with the echinocandin caspofungin in a murine model (Hodgetts, Nooney et al. 2008). The mechanism by which this antibody works, however, is unclear as the antibody is unlikely to be able to cross the fungal cell wall and access the cytosol of intact fungal cells, where Hsp90 regulates calcineurin-dependent signaling governing drug resistance. The antibody may work by influencing host immune responses to the pathogen. Consistent with this thinking, heat-shock proteins are immunodominant antigens for the recognition of many pathogens and play a central role in mediating both innate and adaptive immune responses (Srivastava 2002; Stewart and Young 2004).
Hsp90 has taken center stage as a therapeutic target for diverse diseases including cancer and neurodegeneration. My findings suggest that Hsp90 may provide a much-needed target for life-threatening fungal infectious disease. Inhibitors of Hsp90 and calcineurin both have potent anti-malarial activity, thus extending their impact to the protozoan parasite *Plasmodium falciparum* (Kumar, Musiyenko et al. 2005). Compromising host Hsp90 function in the context of an acute fungal infection is not well tolerated (Cowen, Singh et al. 2009). Perhaps in a related manner, the utility of calcineurin inhibitors in antifungal therapy has been complicated by their immunosuppressive effects (Steinbach, Reedy et al. 2007). Thus, the challenge in successfully exploiting this strategy lies in developing fungal selective inhibitors of Hsp90 or in targeting fungal specific components of the Hsp90 chaperone machine. My findings may point to broader paradigm of targeting fungal stress response pathways in the treatment of life-threatening fungal infectious disease.
Table 2-1 Strains used in this study

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<th>Strain Name</th>
<th>Genotype</th>
<th>Source</th>
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<td>(Jones, Federspiel et al. 2004)</td>
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<td>(Blankenship and Heitman 2005)</td>
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<td>As SN95, HIS1/his1::tetR-FRT</td>
<td>This Study</td>
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hsp90::CdHIS1/HSP90

hsp90::CdHIS1/MAL2p-HSP90

hsp90::CdHIS1/tetO-HSP90

hsp90::CdHIS1/HSP90-TAP-FRT
CalC587  
\textit{crz1Δ/crz1Δ}  
(Onyewu, Wormley et al. 2004)

CalC589  
\textit{crz1Δ/crz1Δ + CRZ1}  
(Onyewu, Wormley et al. 2004)

CalC590  
As SN95, \textit{HIS1/his1::tet-R-FRT}  
This study

\textit{CNA1/CNA1-HISFLAG-FRT hsp90::CdHIS1/HSP90-TAP-FRT}

CalC857  
As SN95, \textit{CNA1/CNA1-HISFLAG-FRT}  
This study

CalC858  
As SN95, \textit{CNA1/CNA1-HISFLAG-FRT}  
This study

\textit{HIS1/his1::tet-R-FRT hsp90::CdHIS1/HSP90}

CalC860  
As SN95, \textit{cna1Δ/CNA1-HISFLAG-FRT}  
This study

CalC861 (CA14)  
\textit{ura3::imm434/ura3::imm434}  
(Shapiro, Uppuluri et al. 2009)

\textit{UTR2-UTR2p-lacZ-URA3}

CalC908  
As SN95, \textit{CNA1/cna1::FRT}  
This study

CalC909  
As SN95, \textit{cna1::FRT/cna1::FRT}  
This study

CalC912  
As SN95, \textit{HIS1/his1::tet-R-FRT}  
This study

\textit{hsp90::CdHIS1/MAL2p-HSP90 CNA1/CNA1-HISFLAG-FRT}
CaLC946  As SN95, HIS1/his1::tet-R-FRT  (Cowen, Singh et al. 2009)

HSP90/FRT-tetO-HSP90

ScLC151 (BY4741)  his3Δ leu2Δ met15Δ ura3Δ  (Winzeler, Shoemaker et al. 1999; Giaever, Chu et al. 2002)

ScLC400 (Lo90)  can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1  (Cowen and Lindquist 2005)

   ade2-1 hsc82::KAN; hsp82::KAN; LEP-HSP82-HIS3, pRS304 (TRP1)

ScLC402  can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1  (Cowen and Lindquist 2005)

   ade2-1, pRS304 (TRP1), pRS303 (HIS3)

ScLC408  can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1  (Cowen and Lindquist 2005)

   ade2-1 cnb1::KAN, pRS304 (TRP1), pRS303 (HIS3)

ScLC463  his3Δ leu2Δ met15Δ ura3Δ cna1::KAN  This study

   cna2::KAN

ScLC642  can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-11  This study

   ade2-1 CDRE-lacZ-URA3-TRP
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<td>ScLC863</td>
<td><em>his3Δ leu2Δ met15Δ ura3Δ CNA1-TAP-HIS3MX6</em></td>
<td>(Ghaemmaghami, Huh et al. 2003)</td>
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<tr>
<td>C42 (CaLC986)</td>
<td>As SC5314, <em>FKSI F641S</em></td>
<td>(Balashov, Park et al. 2006)</td>
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<td>DPL15 (CaLC990)</td>
<td>Clinical isolate, <em>FKSI F641S</em></td>
<td>David Perlin</td>
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Table 2-2 Plasmids used in this study

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<td><em>FLP-CaNAT</em>, ampR</td>
<td>(Shen, Guo et al. 2005)</td>
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<td>pLC74</td>
<td><em>CYC1p-CDRE-lacZ</em></td>
<td>(Stathopoulos and Cyert 1997)</td>
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<td>pLC329</td>
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<td><em>CaHSP90-TAP</em>, ampR, NAT (pLC49)</td>
<td>This study</td>
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<td>pLC350</td>
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AACCCGGGGATCCGACCATCAAACCTCT-
         TCCATAGC

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         GCTATGGGAAGAGTTGGATG-
         GTCGACGGATCCCCGGG

oLC317  TAP + 558R - ApaI
         TTGCGGGCCCTCAGTATGATTCCGCTCTCT-
         ACTTTAG

oLC318  CaHsp90 + 2125F-SacII
         TCCCCGGGACACCAGAGGGCTACAGTT

oLC319  CaHsp90 + 2467R – SacI
         CGAGCTCTCTCTATGTGTATG

oLC326  CNA2-A (YML057W)
         GCCCGAGACAAATGAGAAAATGTC

oLC338  CaCNA1 + 1431F – KpnI
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         TGG

oLC339  6x HIS-FLAG &
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         CaCNA1 + 1827R – ApaI
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         GACTTTGAGATAATCTTTCTTA

oLC340  CaCNA1 + 1831F - SacII
         TCCCCGCGGACTTTCTTTTGCCCTGTTT

oLC341  CaCNA1 + 2180R - SacI
         CGAGCTCGTAAAACGTGTGTAATCAAATG
oLC342  CaCNA1 + 1294F  TTTAGTGAAGCTGAAATAGG
oLC343  CaCNA1 + 2355R  AAGATCTGAGATCTTCTGCC
oLC433  CaCNA – 433F-KpnI  GGGGTACCGACGAAATAGTTAATAAAGC
oLC434  CaCNA + 3R-ApaI  TTGCGGGCCCATGATGATAATGGGAGCC
oLC435  CaCNA+1828F-SacII  TCCCCCGGGTAAAACCTTTTCTTTCGCCCTG
oLC436  CaCNA+2184R-SacI  CGAGCTCGGTGTTGAAACGTGGTAATCA
oLC524  CYC1-467F – NotI  ATAAGAATGCGGCGCCTCCGTTGAGA
                     CGACATCG
oLC588  6xHISFLAG-CaCNA  TCCCCCGGGATGCACCACCACCAACCACCA-
                     +4F-SacII  CGGTGTTGATTATAAAGATGATGATGATAA-
                     ATCAGGAAATACTGTTCAACG
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oLC591  CaCNA+764-R  AATTGAGGTGATAATCCTCC
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oLC617  CaUTR2-1R-XhoI  GCTCGAGAAACAATAGTAGTAATAGTATCG
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Chapter 3

3 Global Analysis of the Evolution and Mechanism of Echinocandin Resistance in a Series of Candida glabrata Clinical Isolates

Most experiments were performed by Sheena D. Singh. Clinical isolates, case report, clinical MICs (Table 3-1) and karyotype data (Figure 3-1) provided by Susan Poutanen¹, Genome-wide sequencing and analysis performed by Tomas Babak².

¹Toronto Medical Laboratories and Mount Sinai Hospital, Toronto, Ontario, Canada

²Merck Research Laboratories, Boston, Massachusetts, USA
3.1 Introduction

The emergence of drug resistance is an evolutionary process with a profound impact on human health. The widespread deployment of antimicrobial agents in medicine and agriculture exerts strong selection for organisms with enhanced capacity to survive and reproduce in the presence of drug, which has led to the rapid emergence of drug resistance in diverse pathogen populations (Levin, Lipsitch et al. 1999; Anderson 2005; Antonovics, Abbate et al. 2007; Cowen 2008). The evolution of drug resistance compromises the efficacy of drugs that we depend on critically for a myriad of therapeutic interventions, and has striking economic consequences. The annual “evolution bill” in the United States alone exceeds $33 billion to cover treatment of patients with drug-resistant infections, additional pesticides required to manage resistant pests, and loss of crops to resistant pests (Palumbi 2001). The emergence of drug resistance in fungal pathogens is of particular concern given the increasing incidence of invasive fungal infections, and the limited number of antifungal drugs. Fungi can cause life-threatening infectious disease in immunocompromised hosts, as well as in healthy humans, and the incidence of fungal bloodstream infections has increased by 207% in recent decades (Martin, Mannino et al. 2003; Pfäffer and Diekema 2007; Finkel and Mitchell 2011). Fungi are eukaryotes and share close evolutionary relationships with their human hosts, which limit the number of drug targets that can be exploited to selectively kill the fungal pathogen with minimal host toxicity (Anderson 2005; Cowen 2008). Even with current treatment options, mortality rates due to invasive fungal infections can reach 50-90% depending on the pathogen and patient population (Pfäffer and Diekema 2007; Finkel and Mitchell 2011), demanding new strategies to prevent the evolution of drug resistance and enhance the efficacy of antifungal drugs.
The evolution of drug resistance is contingent on genetic variability, the ultimate source of which is mutation. One of the most fundamental questions of central importance to predicting and preventing the evolution of drug resistance is what mutations accompany the evolution of drug resistance in the human host. Developments in sequencing technology (Mardis 2008; Kircher and Kelso 2010; Nowrousian 2010) now enable this question to be addressed on a genome-wide scale to reveal the identity of mutations that either confer drug resistance in a clinically relevant context or that modify the fitness consequences of resistance mutations. Whole genome sequencing has been applied to bacterial systems and has revealed principles underpinning the evolution and transmission of drug-resistant pathogens (Harris, Feil et al. 2010), risk factors for the evolution of drug resistance (Ford, Lin et al. 2011), and population dynamics during the evolution of drug resistance in vitro (Lee, Molla et al. 2010). In fungal systems, changes in genome-wide gene expression and chromosomal alterations that accompany the evolution of drug resistance have been monitored in experimental populations that evolved resistance in vitro (Cowen, Nantel et al. 2002; Selmeci, Dulmage et al. 2009), and targeted sequence and expression analysis of specific genes has been implemented to identify mechanisms of resistance that evolve in the human host (Cowen, Anderson et al. 2002; Anderson 2005). However, a global approach to mapping mutations that underpin the evolution of fungal drug resistance has yet to be achieved.

*Candida glabrata* is one of the leading fungal pathogens of humans and provides a particularly powerful system for studying the evolution of drug resistance in a human host. *Candida* species are the fourth most common cause of hospital acquired blood-stream infections and are the most prevalent cause of invasive fungal infection worldwide, with mortality rates approaching 50% (Zaoutis, Argon et al. 2005; Pfaller and Diekema 2007). *C. glabrata* is now second to *C. albicans* as the most prevalent *Candida* species in the clinic (Fidel, Vazquez et al.
1999; Pfaller and Diekema 2007; Finkel and Mitchell 2011). This due in part to both intrinsic and rapidly acquired resistance of *C. glabrata* to the azoles, which are the most widely used class of antifungal drugs and inhibit the biosynthesis of the key sterol in fungal cell membranes, ergosterol (Shapiro, Uppuluri et al. 2009). As a consequence, the echinocandins are the front line therapeutic agent for *C. glabrata* infections (Fidel, Vazquez et al. 1999). *C. glabrata* is closely related to the model yeast *Saccharomyces cerevisiae* and is placed within the *Saccharomyces* clade rather than the *Candida* clade to which the leading cause of candidiasis, *C. albicans*, belongs (Dujon, Sherman et al. 2004; Fitzpatrick, Logue et al. 2006; Marcet-Houben and Gabaldon 2009). Thus, it is thought that *C. glabrata* emerged as a human pathogen independently from other *Candida* species. Notably, gene families associated with pathogenicity in *C. albicans* including iron acquisition and host cell adhesion and invasion are absent from *C. glabrata* (Butler, Rasmussen et al. 2009). *C. glabrata* is an obligate haploid and mating has never been reported, although four mating types and mating type switching have been described (Brockert, Lachke et al. 2003; Lin, Chen et al. 2007; Butler 2010). To increase genetic diversity *C. glabrata* undergoes chromosomal translocations and variation in gene copy number (Muller, Thierry et al. 2009; Polakova, Blume et al. 2009), mechanisms that contribute to *C. albicans* resistance to the azoles (Selmecki, Forche et al. 2006; Selmecki, Gerami-Nejad et al. 2008; Selmecki, Dulmage et al. 2009). As *C. glabrata* is haploid, analysis of genome sequence is simpler than in diploids such as *C. albicans*, where mitotic recombination and gene conversion can inflate the number of polymorphisms that accrue and obscure the signal of those functionally associated with drug resistance or adaptation to the host.

Of the two main classes of antifungal drugs in clinical use for the treatment of systemic infections, mechanisms of resistance remain the most enigmatic for echinocandins. The echinocandins are the only novel class of antifungal to reach the clinic in decades and target the
biosynthesis of the key fungal cell wall component, 1,3-β-D-glucan (Cowen 2008; Cowen and Steinbach 2008; Shapiro, Robbins et al. 2011). The 1,3-β-D-glucan synthases are encoded by FKS1, FKS2, and FKS3 in S. cerevisiae, C. glabrata, and C. albicans, and require a regulatory subunit encoded by RHO1 for activity (Perlin 2007; Cowen 2008). It is thought that the echinocandins bind to and inhibit the Fks protein, however, the exact mechanism of inhibition remains unknown (Perlin 2007). Although the echinocandins have been in clinical use only since 2001, there have been numerous reports of C. glabrata echinocandin resistance in patients (Katiyar, Pfaller et al. 2006; Cleary, Garcia-Effron et al. 2008; Garcia-Effron, Lee et al. 2009; Pfeiffer, Garcia-Effron et al. 2010). Thus far, the only echinocandin resistance mechanism described is mutation in the drug target, Fks, particularly in highly conserved hot spot regions (Balashov, Park et al. 2006; Perlin 2007; Garcia-Effron, Lee et al. 2009; Garcia-Effron, Park et al. 2009; Pfeiffer, Garcia-Effron et al. 2010). Such mutations can reduce echinocandin sensitivity of 1,3-β-D-glucan synthase by 2 to 3 log orders relative to the wild-type enzyme (Garcia-Effron, Lee et al. 2009). There is strong evidence for additional resistance mechanisms that remain to be described given that many echinocandin resistant isolates lack Fks mutations (Castanheira, Woosley et al. 2010; Pfeiffer, Garcia-Effron et al. 2010), and that isolates with identical Fks mutations have different resistance phenotypes with distinct responses to cellular perturbations (Singh, Robbins et al. 2009). Even with azoles, for which resistance mechanisms have been studied for decades, new resistance mechanisms and modulators of resistance continue to be discovered expanding the repertoire of strategies employed by fungi to survive drug exposure to include mutation in the drug target, overexpression of multidrug-efflux transporters, metabolic alterations that minimize drug toxicity, and modulation of complex stress response signaling pathways (Cowen 2008; Cowen and Steinbach 2008; Shapiro, Robbins et al. 2011).
Beyond mapping mutations that confer resistance, there is pressing clinical need to elucidate strategies to block the evolution of drug resistance and abrogate resistance once it has evolved. One of the most well studied examples of a protein that governs the emergence and maintenance of fungal drug resistance is the molecular chaperone Hsp90. Hsp90 regulates the folding and function of diverse client proteins, including many signal transducers (Wandinger, Richter et al. 2008; Taipale, Jarosz et al. 2010). In *C. albicans*, compromise of Hsp90 function reduces basal tolerance and resistance of clinical isolates to both the azoles and the echinocandins (Cowen and Lindquist 2005; Cowen, Singh et al. 2009; Singh, Robbins et al. 2009). Hsp90 enables crucial responses to drug-induced stress by orchestrating signaling through the protein phosphatase calcineurin and the protein kinase C (PKC) cell wall integrity signaling cascade (Singh, Robbins et al. 2009; LaFayette, Collins et al. 2010). Hsp90 stabilizes the catalytic subunit of calcineurin and the terminal mitogen-activated protein kinase (MAPK) in the Pkc1 cell wall integrity pathway. Genetic or pharmacological compromise of Hsp90 function can enhance the efficacy of antifungals against *C. albicans* in multiple metazoan models of infection (Cowen, Singh et al. 2009; Singh, Robbins et al. 2009). Notably, Hsp90’s role in governing cellular responses to azoles in *C. albicans* is conserved in *S. cerevisiae* (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). In contrast, Hsp90 and calcineurin play a key role in crucial cellular responses to echinocandins in *C. albicans*, but not in *S. cerevisiae* (Singh, Robbins et al. 2009). Whether Hsp90 influences drug resistance in *C. glabrata* remains entirely unknown. In *C. glabrata*, both calcineurin and PKC signaling have been implicated in basal tolerance to echinocandins (Miyazaki, Inamine et al. 2010; Miyazaki, Yamauchi et al. 2010), though the role of Hsp90 remains unknown as does the impact of any of these regulators on *bona fide* echinocandin resistance.
Here, I provide the first global analysis of mutations accompanying the evolution of fungal drug resistance in a human host. I report on a series of *C. glabrata* isolates that evolved echinocandin resistance in a patient undergoing treatment with the echinocandin caspofungin for recurring bloodstream *C. glabrata* candidemia over a 10-month period. Whole genome sequencing revealed that a mutation occurred in the drug target, Fks2, accompanying a major increase in resistance, as well as 8 other non-synonymous mutations in 8 genes not previously implicated in echinocandin resistance. The *FKS2* mutation was sufficient to confer echinocandin resistance in a sensitive laboratory strain, however, the mutant allele also imparted a growth defect in clinically relevant conditions. Analysis of 10 independent *C. glabrata* clinical isolates recovered at different time points from separate patients in distinct geographical locations revealed an association between Fks2-mediated resistance and mutations in *MOH1* and *CDC6*. To uncover mechanisms that abrogate echinocandin resistance I turned to Hsp90 and found that pharmacological inhibition of Hsp90 both reduced basal tolerance of *C. glabrata* and abrogated resistance of clinical isolates. Compromising calcineurin function either pharmacologically or genetically phenocopied compromising Hsp90 function. I propose a model in which Hsp90 and calcineurin mediate echinocandin resistance in *C. glabrata* by regulating expression of the resistance determinant *FKS2*, consistent with my findings that caspofungin induced both calcineurin and *FKS2* expression and that increased *FKS2* expression was dependent upon calcineurin. Furthermore, I report that one of the clinical isolates in the series is a petite mutant due to its morphology and the inability to respire; although the petite phenotype was not intrinsically involved in echinocandin resistance, it imparted resistance to the combination of echinocandins and Hsp90 inhibitors. Thus, my results provide the first global view of mutations that accompany the evolution of fungal drug resistance in a human host, establish novel
resistance mutations, and reveal a new molecular mechanism of echinocandin resistance, with broad therapeutic potential.
3.2 Materials and Methods

3.2.1 Strains and Culture Conditions

Archives of *C. glabrata* strains were maintained at -80°C in 25% glycerol. Strains were grown in either YPD (1% yeast extract, 2% bactopeptone, 2% glucose), YPG (1% yeast extract, 2% bactopeptone, 2% glycerol), synthetic defined medium (0.67% yeast nitrogen base, 2% glucose) supplemented with required amino acids, or RPMI medium 1640 (Gibco, 3.5% MOPS, 2% glucose, pH 7.0). 2% agar was added for solid media. Strains used in this study are listed in Table 3-3.

3.2.2 Strain Construction

*CgLC1550, 1551, 1552*

To knock-out *CgCNB1* in *C. glabrata*, a split-marker PCR method using pLC540 was employed. pLC540 contains ~400 bp of homology to *CgCNB1* flanking an *HPH1* cassette with FRT sites for recycling of the marker. Primers oLC1208/1388 were used to amplify the first half of this cassette (1581 bp) and oLC1389/1211 were used to amplify the second half of the cassette. The first and second halves have 200 bp of overlapping sequence in the middle of Hph1 such that transformation with equal amounts of the two halves require three separate recombination events to get HygBR, reducing the amount of illegitimate recombination. 20 µg of DNA was used in the transformation, cells were harvested for standard LiOAC transformation at OD600 = 0.4, with 10% DMSO added right before heat shock. Upstream integration was confirmed using primers oLC1212/1216 (690 bp) and downstream integration was confirmed using oLC1213/1217 (685 bp). Lack of a WT allele was confirmed with oLC1214/1215 (485 bp if a WT allele is still present). Three separate transformants were archived and used in subsequent experiments.

The CgFKS2 T1987C mutation was introduced in CgLC1272 by transformation via lithium acetate with 10 µg of the synthetic oligonucleotide oLC1900, a 90-mer containing the T1987C mutation in the center as well as a silent mutation G1980A to track resistant mutants that arise due to homologous recombination. Transformants were selected for on YPD agar with 1 µg/ml caspofungin. The CgFKS2 hot spot 1 region of the transformants was amplified with oLC1344/1345 and sequenced with oLC1344 to verify both the presence of the CgFKS2 T1987C mutation as well as the silent G1980A mutation to differentiate between homologous recombinants versus spontaneous mutants. Four sequence-verified transformants were archived and used in subsequent experiments.

3.2.3 Plasmid Construction

Recombinant DNA procedures were performed according to standard protocols. Plasmids used in this study are listed in Table 3-2. Plasmids were sequenced to verify the absence of any nonsense mutations. Primers used in this study are listed in Table 3-3.

pLC540

Homology upstream of CgCNB1 was amplified from genomic DNA with oLC1208/oLC1209 and cloned into pLC530 at SacI and NotI. Homology downstream of CgCNB1 was amplified from genomic DNA with primers oLC1210/oLC1211 and cloned into pLC530 containing the upstream homology at SalI and KpnI. The presence of the inserts was tested by PCR with oLC1208/oLC1216 and oLC1211/oLC1217.
3.2.4 Whole Genome Sequencing

Genomic DNA was extracted from clinical isolate A and F, and sequencing libraries were prepared using the Illumina genomic DNA library preparation kit according to the manufacturers recommendations (Illumina, CA) with several modifications. In brief, DNA was sheared by sonication to an average fragment length of 200 base pairs. Illumina adapters were blunt-end ligated and libraries were amplified by PCR and purified using Ampure (Agencourt) beads at a DNA:bead ratio of 1:0.9. Each sample was sequenced together in a single lane on an Illumina Genome Analyzer II platform, yielding 5.1 and 3.8 million 76 base pair single-end reads for isolate A and isolate G, respectively, resulting in 22 to 30x genome coverage. Reads were aligned using SOAP2 (PMID: 19497933) against the reference genome sequence of CBS138 (Sherman, Martin et al. 2009). Single nucleotide variants were identified using a machine learning approach as described previously (Babak, Deveale et al. 2008). All non-synonymous mutations identified were validated independently using Sanger sequencing.

3.2.5 Mutation Mapping

The 9 non-synonymous mutations identified by whole genome sequencing were mapped across clinical isolates B, C, D, E, and F using Sanger sequencing. CgFKS2 was amplified using oLC1344/1345, CgDOT6 with oLC1559/1560, CgMOH1 with oLC1561/1562, CgGPH1 with oLC1563/1564, CgMRPL11 with oLC1565/1566, CgCDC6 with oLC1567/1568, CgCDC55 with oLC1569/1570, CgSUI2 with oLC1571/1572, and CgTCB1/2 with oLC1573/1574. CgFKS2 was sequenced with oLC1344, CgDOT6 with oLC1559, CgMOH1 with oLC1561, CgGPH1 with oLC1563, CgMRPL11 with oLC1565, CgCDC6 with oLC1567, CgCDC55 with oLC1569, CgSUI2 with oLC1571, and CgTCB1/2 with oLC1573.
3.2.6 Minimum Inhibitory Concentration Assays

Antifungal susceptibility was determined in flat bottom, 96-well microtiter plates (Sarstedt) using a modified broth microdilution protocol, a described (Cowen and Lindquist 2005). Minimum inhibitor concentration (MIC) tests were set up in a total volume of 0.2 ml/well with 2-fold dilutions of caspofungin (CF, generously provided by Rochelle Bagatell). Echinocandin gradients were from 16 µg/ml down to 0 with the following concentration steps in µg/ml: 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0. Cell densities of overnight cultures were determined and dilutions were prepared such that ~10^3 cells were inoculated into each well. Geldanamycin (GdA, A.G. Scientific, Inc.) and radicicol (RAD, A.G. Scientific, Inc.) were used to inhibit Hsp90 at the indicated concentrations, and cyclosporin A (CsA, CalBiochem) and FK506 (A.G. Scientific, Inc.) were used to inhibit calcineurin at the indicated concentrations. Dimethyl sulfoxide (DMSO, Sigma Aldrich Co.) was the vehicle for GdA, RAD, CsA, and FK506. Sterile water was the vehicle for CF. Plates were incubated in the dark at 30˚C for the time period indicated, at which point plates were sealed and re-suspended by agitation. 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 on at least two occasions. MIC data was quantitatively displayed with colour using the program Java TreeView 1.1.3 (http://jtreeview.sourceforge.net).

3.2.7 Quantitative Reverse Transcription PCR (qRT-PCR)

To measure gene expression changes in response to caspofungin treatment in C. glabrata, cells were grown overnight in YPD at 30˚C. Cells were diluted to OD_600 of 0.2 in SD and grown for 2 hours in duplicate for each strain at 25˚C. After 2 hours of growth 120 ng/ml CF was added to one of the two duplicate cultures and left to grow for one additional hour at 25˚C. Cells were centrifuged and pellets were frozen at -80˚C immediately. RNA was isolated using the QIAGEN
RNase-free DNase (QIAGEN), and cDNA synthesis was performed using the AffinityScript cDNA synthesis kit (Stratagene). PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich Co) with the following cycling conditions: 94°C for 2 minutes, 94°C for 15 seconds, 60°C for 1 minute, 72°C for 1 minute, for 40 cycles. All reactions were performed in triplicate, using primers for the following genes: *CgACT1* (oLC1500/1501), *CgCNB1* (oLC1502/1503), and *CgFKS2* (oLC1498/1499). Data were analyzed using iQ5 Optical System Software Version 2.0 (Bio-Rad Laboratories, Inc). Statistical significance was evaluated using GraphPad Prism 5.0.

3.2.8 Growth Curves

Growth kinetics were measured in *C. glabrata* strains by inoculating cells from an overnight culture grown in YPD at 30°C to an OD$_{600}$ of 0.0625 in 100 µl of RPMI with 2% glucose in flat bottom, 96-well microtiter plates (Sarstedt). Cells were grown in a Tecan GENios microplate reader (Tecan Systems Inc., San Jose, USA) at 37°C with orbital shaking. Optical density measurements (OD$_{600}$) were taken every 15 minutes for 48 hours. Statistical significance was evaluated using GraphPad Prism 5.0.

3.2.9 Generation of *C. glabrata* Petite Mutants

*C. glabrata* strains were inoculated from solid YPD medium to liquid YPD medium containing 10 µg/ml ethidium bromide (EtBr). The culture was grown overnight, shaking at 30°C in the dark. Approximately 100 cells were plated on YPD agar to isolate single colonies. After 2 days of incubation at 30°C, single colonies were tested for growth on YPD agar and YP-glycerol agar. Colonies able to grow on glucose as the sole carbon source but not on glycerol as the sole carbon source were selected.
3.3 Results

3.3.1 The Evolution of Echinocandin Resistance in \textit{C. glabrata} in a human host.

While numerous echinocandin resistant isolates have been recovered from patients (Katiyar, Pfaller et al. 2006; Cleary, Garcia-Effron et al. 2008; Garcia-Effron, Lee et al. 2009; Pfeiffer, Garcia-Effron et al. 2010), in most cases, there has not been adequate sampling over the course of drug treatment to identify related fungal lineages with which to study the evolution of drug resistance in the human host. The most detailed sampling includes a clinical isolate pre-caspofungin treatment and two isolates taken serially post-caspofungin treatment, however, the mechanism by which resistance was acquired was not investigated (Hernandez, Lopez-Ribot et al. 2004). In contrast, series of clinical isolates recovered over time from patients undergoing treatment with azoles have proven instrumental for dissecting mechanisms of azole resistance in \textit{C. albicans} (White 1997; Cowen and Lindquist 2005; Selmecki, Dulmage et al. 2009; Hoot, Smith et al. 2011). The more detailed sampling and analysis of the evolution of resistance to azoles likely reflects their much more lengthy use in the clinic.

Reported here are a series of \textit{C. glabrata} isolates recovered over a 10-month period from a 43-year old female patient with Crohn’s disease who suffered from recurrent \textit{C. glabrata} candidemia and underwent multiple rounds of caspofungin treatment before ultimately succumbing to the infection. A case report on the details of the patient history and therapeutic interventions is provided as follows: A 43-year old female had four episodes of \textit{Candida glabrata} candidemia between April 2004 – October 2005. Her past medical history was significant for severe fistulizing Crohn’s disease diagnosed at the age of 9 years. She had been on total parenteral nutrition (TPN) since 2003 for short gut syndrome due to numerous small bowel resections over the 30-year course of her disease.
April 2004: The patient was admitted to hospital with generalized malaise, fever, and
Clostridium difficile associated diarrhea (CDAD). Admission blood cultures demonstrated C.
glabrata candidemia with evidence of pulmonary nodules on thorax computerized tomography
(CT). No evidence of endophthalmitis was noted on ophthalmologic exam. Her two central
venous catheters were removed and caspofungin was started at 70 mg od x 1 then 50 mg od
intravenously with clinical response. Cultures of the catheter tip and a repeat set of blood
cultures were negative. After 3 weeks of treatment, she was discharged with plans to continue
caspofungin until resolution of the pulmonary nodule on repeat thorax CT. An outpatient
echocardiogram was also ordered. The patient was non-adherent with outpatient follow-up but
had three sets of negative blood cultures drawn while off caspofungin therapy during emergency
department visits 6 months after discharge.

January 2005: The patient presented to the hospital with a 1 week history of fatigue, nausea,
vomiting and diarrhea, and was diagnosed with TPN-associated cholestasis. Three out of four
sets of blood cultures drawn at admission grew C. glabrata. Eye exams revealed no evidence of
endophthalmitis. Her central venous catheter was removed and she was restarted on caspofungin
at the same dose. Four sets of repeat blood cultures were negative. She continued to have
intermittent fevers and one week after admission was transferred to the intensive care unit with
septic shock and intubated for hypoxic respiratory distress. A thorax CT demonstrated bilateral
airspace consolidation consistent with acute respiratory distress syndrome and an abdominal CT
demonstrated a fistulous tract to a 5 cm x 0.3 cm anterior abdominal wall collection. The patient
was treated with piperacillin-tazobactam with an excellent clinical response. Piperacillin-
tazobactam was ordered to continue until the collection resolved. Caspofungin was stopped two
weeks after the last positive culture for C. glabrata.
February 2005: One week after the caspofungin was stopped while continuing on piperacillin-tazobactam, the patient presented with recrudescence of fever and abdominal pain. 5 out of 5 sets of blood cultures grew *C. glabrata*. Eye exam and transthoracic echocardiogram revealed no evidence of endophthalmitis or endocarditis. Her central venous catheter was removed and the catheter tip was positive for *Candida* species, not *C. albicans*. Caspofungin was restarted at the same dose with good clinical response. Two sets of repeat blood cultures were negative. Caspofungin was continued for a total of 4 weeks and the patient was discharged. The patient was not adherent to outpatient follow-up appointments but multiple repeat blood cultures drawn at the TPN clinic over the following six months off caspofungin were negative.

October 2005: The patient presented to the emergency department with a lower gastrointestinal bleed and shock and was immediately transferred to the intensive care unit. She was empirically started on piperacillin-tazobactam and fluconazole for presumed intra-abdominal sepsis but died 3 days after admission. Three sets of ante-mortem blood cultures grew *C. glabrata*.

The 7 isolates analyzed are labeled alphabetically in the order in which they were recovered such that isolate A was recovered prior to caspofungin treatment and isolate G was recovered 10 months after recurrent infection and several rounds of caspofungin treatment. The isolates were determined to be the same strain based on molecular typing analysis including pulsed-field gel electrophoresis (PFGE) - karyotype analysis (Figure 3-1), as well as restriction enzyme PFGE using *Sfi*I (data not shown). Antifungal susceptibility of the 7 isolates in the series was determined for caspofungin, as well as for numerous azoles (fluconazole, ketoconazole, itraconazole, and voriconazole) and for amphotericin B, which binds to ergosterol and disrupts membrane integrity (Shapiro, Robbins et al. 2011), using broth microdilution with RPMI 1640 and following the standard CLSI M27-A2 protocol (Table 3-1). The major trend observed was
an increase in caspofungin resistance over the course of treatment. There were only minimal changes in susceptibility to the other antifungal drugs tested, with the exception of an increase in resistance to fluconazole that peaked at isolate F and returned to intermediate levels at isolate G. Since the patient was not treated with azoles, the changes in fluconazole susceptibility may be due to mutations that arose in the lineage due to genetic drift rather than selection, or may reflect additional phenotypic consequences of mutations associated with echinocandin resistance, mutations associated with adaptation to the bloodstream, or mutations that were not directly selected for but simply hitch-hiked along with mutations under selection in this predominantly clonal system.
Figure 3-1. *C. glabrata* clinical isolates serially isolated from a patient are of the same lineage.

Pulsed-field gel electrophoresis (PFGE) karyotype analysis reveals that isolates A through G, inclusive, are related and likely of the same lineage. All samples were run on the same gel and the picture was cropped to order the isolates. Lanes ‘M’ contain a marker, lanes 1 and 2 are of two control *C. glabrata* strains and lanes A through G are of isolate A through isolate G, in the same order.
Table 3-1. *In Vitro* susceptibility of *C. glabrata* clinical isolates to clinically relevant antifungal drugs.

Antifungal minimum inhibitory concentrations (MICs) for serially isolated *C. glabrata* clinical isolates were determined using broth microdilution with RPMI 1640 broth for amphotericin, fluconazole, ketoconazole, itraconazole, voriconazole, and caspofungin following Clinical and Laboratory Standards Institute document M27-A2. MIC endpoints were read after 24 hours of incubation at 35°C for caspofungin and after 48 hours of incubation for all other drugs. Complete inhibition was used to determine amphotericin endpoints; 50% inhibition (compared to a drug-free growth control) was used for caspofungin and 80% inhibition was used for other drugs.

<table>
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<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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<tr>
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<tr>
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<td>8</td>
<td>8</td>
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<td>&gt;16</td>
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Whole Genome Sequencing Reveals 9 Non-Synonymous Mutations between Early Clinical Isolate A and Late Clinical Isolate G.

The diversity of resistance mutations that emerge during the evolution of a fungal pathogen in its human host can be addressed for the first time using next-generation sequencing technology and the series of *C. glabrata* clinical isolates I describe here. As a haploid, genome analysis of *C. glabrata* is simplified relative to diploids such as *C. albicans*, where mitotic recombination and gene conversion can amplify the number of polymorphisms observed between early and late isolates and thereby hinder functional analysis of mutations conferring resistance. Further, *C. glabrata* shares a recent common ancestor with the model yeast *S. cerevisiae* with a large number of orthologues between the two species, facilitating bioinformatic analysis (Dujon, Sherman et al. 2004; Marcet-Houben and Gabaldon 2009). Given the clinical importance of echinocandin resistance, and that to date resistance has only been attributed to mutations in the echinocandin target, with many isolates harboring additional resistance determinants that remain to be discovered (Balashov, Park et al. 2006; Perlin 2007; Garcia-Effron, Lee et al. 2009; Garcia-Effron, Park et al. 2009; Castanheira, Woosley et al. 2010; Pfeiffer, Garcia-Effron et al. 2010), I sought to identify the mutations that accompany the evolution of echinocandin resistance in the human host on a genome-wide scale.

Whole genome sequencing of the *C. glabrata* isolate recovered prior to caspofungin treatment (isolate A) and the last isolate recovered after multiple rounds of treatment (isolate G) was performed using the Illumina Genome Analyzer II platform. 5.1 and 3.8 million 76 base pair single-end reads for isolate A and isolate G, was obtained respectively, resulting in 22 to 30 x genome coverage. Reads were aligned against the reference genome sequence of CBS138 (Sherman, Martin et al. 2009). Single nucleotide variants were identified using a machine
learning approach. A total of 45,797 single nucleotide variants were identified between late clinical isolate G and CBS138. Of these single nucleotide variants, 39,146 had sufficient sequencing depth in isolate A to be reliably called. Overall, 26 single nucleotide variants were uncovered between isolate A and G, with only 17 of these within open reading frames and only 9 resulting in non-synonymous changes (Table 3-2). All 9 non-synonymous changes were verified and then mapped across isolates B to F using Sanger sequencing to determine when each mutation arose in the series (Figure 3-2). Genes are named based on homology to *S. cerevisiae* genes (Sherman, Martin et al. 2009).

Mutations in *MOH1*, *GPH1*, *CDC6*, and *TCB1/2* accompanied the first small increase in resistance in the series at isolate C (Figure 3-2). The function of *MOH1* in *S. cerevisiae* is largely unknown except that it is essential for survival in stationary phase (Ashrafi, Farazi et al. 1998; Martinez, Roy et al. 2004), and it was found to genetically interact with Hsp90 in a genome-wide chemical-genetic screen (McClellan, Xia et al. 2007). Expression of *C. albicans MOH1* is induced by alpha pheromone in filament-inducing Spider medium, by weak acid stress via Mnl1, and in biofilm conditions (Bennett and Johnson 2006; Ramsdale, Selway et al. 2008; Bonhomme, Chauvel et al. 2011). *GPH1* is a Glycogen PHosphorylase regulated by the high osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway in *S. cerevisiae* (Hwang, Tugendreich et al. 1989; Sunnarborg, Miller et al. 2001), and is induced upon fluconazole treatment in *C. albicans* (Copping, Barelle et al. 2005). *CDC6* is involved in onset of S Phase in DNA replication initiation by forming and maintaining the pre-replicative complex and serving as a loading factor for the Mcm2-7 proteins onto chromatin (Cocker, Piatti et al. 1996; Detweiler and Li 1997). *TCB1* and *TCB2* contain both calcium and lipid binding domains and appear to be involved in membrane trafficking in *S. cerevisiae* (Huh, Falvo et al. 2003;
Creutz, Snyder et al. 2004). None of these genes have been previously implicated in echinocandin resistance.

Additional mutations emerged accompanying the subsequent major increase in echinocandin resistance, and in the last isolate of the series. Mutations in FKS2/GSC2 (Glucan Synthetase Cerevisiae), DOT6 (Disruptor of Telomeric Silencing), MRPL11 (Mitochondrial Ribosomal Protein), and SUI2 (SUppresor of Initiator codon) accompanied the largest increase in echinocandin resistance at isolate D (Figure 3-2). Of these genes, only FKS2 has been implicated in echinocandin resistance. FKS2 encodes the catalytic subunit of 1,3-β-D-glucan synthase, the target of the echinocandins, and the Fks2 S663P mutation identified is in mutational hot spot 1 and has been found in other echinocandin-resistant C. glabrata clinical isolates (Garcia-Effron, Lee et al. 2009; Pfeiffer, Garcia-Effron et al. 2010; Zimbeck, Iqbal et al. 2010). DOT6 is a subunit of the RPD3L histone deacetylase complex in S. cerevisiae and is involved in both pseudohyphal morphogenesis as well as silencing at telomeres (Lorenz and Heitman 1998; Singer, Kahana et al. 1998; Zhu, Byers et al. 2009). MRPL11 is a mitochondrial protein and part of the large ribosomal subunit (Bui, Jarosch et al. 1997; Kitakawa, Graack et al. 1997). SUI2 plays a role in translation initiation and encodes the alpha subunit of the translation initiation factor eIF2 in S. cerevisiae (Laurino, Thompson et al. 1999). Finally, a non-synonymous mutation occurred in CDC55 in the latest clinical isolate G, despite no further increase in echinocandin resistance (Figure 3-2). CDC55 encodes the regulatory B subunit of protein phosphatase 2A and has a number of functions including a role in spindle assembly during meiosis, mitotic exit, pseudohyphal morphogenesis, and chromosome disjunction (Healy, Zolnierowicz et al. 1991; Wang and Burke 1997; Queralt, Lehane et al. 2006; Bizzari and Marston 2011).
Thus, in addition to a mutation in the known echinocandin target, whole genome sequencing revealed the acquisition of 8 additional non-synonymous mutations in 8 genes not previously implicated in echinocandin resistance or adaptation to host conditions during the evolution of echinocandin resistance in a human host. The genome sequence analysis further confirms clonality of the lineage given the very limited number of single nucleotide variants genome-wide compared to large number observed between the late clinical isolate G and the reference genome CBS138. Further, that each of the mutations identified persisted throughout the lineage once it emerged suggests that there may have been strong selective sweeps in the population such that polymorphisms rapidly reached near fixation.
Whole genome sequencing reveals 9 non-synonymous mutations between early clinical isolate A and late clinical isolate G.

Whole genome sequencing was performed on clinical isolate A and G using the Illumina GAII platform. All high-confidence single nucleotide variants (SNVs) resulting in non-synonymous changes between the early clinical isolate A and late clinical isolate G are listed. Genome coverage of 22 to 30x was obtained with a total of 45 797 SNVs identified between isolate G and the reference CSB138 strain (Sherman, Martin et al. 2009). Shown are the 17 SNVs found within open reading frames. *Conservation score is the one-base Phastcons score representing level of evolutionary conservation in yeast alignments (S. cerevisiae, S. paradoxus, S. mikatae, S. kudriavzevii, S. bayanus, S. castelli, and S. kluveri; Siepel et al. 2005). Score shown is for the homologous base in S. cerevisiae when aligned to C. glabrata by BLAST (0=no conservation, 1=very high conservation).
Figure 3-2. Non-synonymous mutations mapped across the *C. glabrata* clinical isolate series.

*C. glabrata* clinical isolates display a step-wise increase in caspofungin (CF) resistance in an MIC assay. Isolates are in the same order as they were recovered from the patient. Assays were performed in RPMI media with 2% glucose at 30°C for 72 hours. Optical densities were averaged for duplicate measurements and normalized relative to CF-free controls (see colour bar). The nine non-synonymous mutations uncovered in isolate G compared to isolate A using whole genome sequencing (marked in red) were mapped across isolates B to F using Sanger sequencing. Mutations in *MOH1, GPH1, CDC6*, and *TCB1/2* occur between isolates B and C, which correspond to a small increase in echinocandin resistance, outlined in blue boxes. Mutations in *DOT6, MRPL11, FKS2*, and *SUI2* correspond to a sharp increase in echinocandin resistance, outlined in black boxes.
3.3.3 The Fks2 S663P Mutation Confers Echinocandin Resistance and Imparts a Fitness Deficit in a *C. glabrata* Clinical Laboratory Strain.

To determine which of the mutations identified by whole genome sequencing contributes to echinocandin resistance I first turned to the most likely candidate resistance gene, *FKS2*. The *FKS2* mutation that emerged in isolate D and was maintained throughout the rest of the series (T1987C) results in substitution of a serine to proline at amino acid 663 in the target of the echinocandins. This Fks2 S663P mutation has been previously associated with high levels of caspofungin resistance in *C. glabrata* clinical isolates (Garcia-Effron, Lee et al. 2009; Pfeiffer, Garcia-Effron et al. 2010). *In vitro* biochemical studies established that this mutant Fks2 enzyme displays reduced sensitivity to inhibition by echinocandins, as indicated by a higher kinetic inhibition parameter (*IC*$_{50}$), as well as decreased catalytic capacity, as indicated by reduced enzyme velocity (*V*$_{max}$), compared to the wild-type enzyme; notably the binding affinity (*K*$_m$) of the mutant enzyme for echinocandins remains unchanged (Garcia-Effron, Lee et al. 2009). While there is an association of Fks2 mutations with resistance, and biochemical data support the resistance mechanism, it has never been conclusively demonstrated that such mutations are sufficient to confer echinocandin resistance. To test whether the Fks2 S663P mutation is sufficient to confer echinocandin resistance, I introduced the T1987C mutation into the sensitive laboratory strain BG2 using a strategy involving single-stranded DNA containing the mutation and a silent marker, followed by selection of transformants on medium containing caspofungin. I then assessed resistance of four sequence-verified transformants that harbored both the T1987C and silent mutation via minimum inhibitory concentration (MIC) assays. The transformants displayed resistance similar to clinical isolate G (Figure 3-3A). Thus I conclude that the Fks2 S663P mutation is sufficient to confer echinocandin resistance in a sensitive laboratory strain of *C. glabrata*. 
Given that specific Fks amino acid substitutions that decrease sensitivity of the 1,3-β-D-glucan synthase enzyme to echinocandins also reduce the enzyme catalytic capacity, the Fks2 S663P mutation may confer a fitness cost in terms of reduced growth rate in the absence of the drug (Garcia-Effron, Lee et al. 2009; Garcia-Effron, Park et al. 2009). To determine if the Fks2 mutations compromises fitness, I monitored growth kinetics of the lab strain BG2 and the progeny harboring the Fks2 S663P substitution. In clinically relevant conditions, RPMI at 37°C, I found that strains harbouring the Fks2 S663P substitution displayed significantly reduced growth relative to the parental wild-type laboratory strain BG2 ($P<0.01$, three technical replicates, area under the curve followed by ANOVA, Bonferroni’s multiple comparison test, Figure 3-3B, left panel). Furthermore, I observed a significant growth defect between isolate A and isolate D ($P<0.001$, ANOVA, Bonferroni’s multiple comparison test), which is rescued to some extent in isolate G (Figure 3-3B, right panel). Thus, the FKS2 mutation that emerged in clinical isolate D is sufficient to confer high level of caspofungin resistance equivalent to that observed in the late isolate G, however, it is also associated with a fitness cost in terms of reduced growth rate in the absence of the drug.
Figure 3-3. The Fks2 S663P mutation is sufficient for echinocandin resistance in a C. glabrata laboratory strain and imparts a growth defect in the absence of echinocandin.

(A) The FKS2 T1987C (Fks2 S663P) mutation uncovered in caspofungin-resistant clinical isolate G was introduced into a sensitive laboratory C. glabrata strain BG2 via single-stranded DNA containing a silent marker. Resistance profiles of three independent sequence-verified transformants were tested via MIC. MIC assays were performed in Figure 1 and plotted graphically as caspofungin concentration (µg/ml) versus optical density at 600 nm.  

(B) Growth
kinetics of the BG2 laboratory strain of *C. glabrata* and three independent transformants harboring a mutant Fks2 S663P allele (left panel) and of *C. glabrata* clinical isolate A, C, D, and G (right panel). Growth curves were performed in RPMI at 37°C with orbital shaking, measurements taken every 15 minutes for 48 hours.
3.3.4 Mutations in MOH1 and CDC6 are Associated with Fks2-Mediated Echinocandin Resistance.

Despite the fact that the FKS2 T1987C mutation was sufficient to impart the full level of caspofungin resistance of isolate G on an otherwise susceptible laboratory strain (Figure 3-3A), there is evidence for additional mutations affecting resistance in the evolved lineage. The initial small increase in echinocandin resistance observed at isolate C (Figure 3-2) occurred prior to the FKS2 mutation and thus other mutations identified at this early transition may confer the small increase in resistance. Further, given the fitness cost imposed by the Fks2 substitution it is possible that mutations that accumulated early in the series may create a genetic background that minimizes the deleterious effects of the FKS2 mutation, or mutations that accumulated later in the series may reflect compensatory evolution and mitigate the fitness defect imparted by the FKS2 mutation (Figure 3-3B).

Next, I addressed whether there was an association between the Fks2 S663P amino acid substitution and mutations in any of the 8 additional genes found to harbor mutations in my whole genome sequence analysis. To do so, I obtained 10 additional unrelated C. glabrata clinical isolates harboring the Fks2 S663P mutation (Zimbeck, Iqbal et al. 2010), and sequenced across the 8 genomic regions that were mutated in clinical isolate G via Sanger sequencing. Strikingly, I discovered non-synonymous mutations in MOH1 in two out of the 10 clinical isolates sequenced and non-synonymous mutations in CDC6 in 7 out of 10 of the clinical isolates (Table 3-3). Thus I establish two genes, MOH1 and CDC6 as associated with Fks2-mediated echinocandin resistance in a broad range of C. glabrata clinical isolates.
<table>
<thead>
<tr>
<th>Strain</th>
<th>MOH1</th>
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<th>CDC6</th>
<th>TCB1/2</th>
<th>FKS2</th>
<th>DOT6</th>
<th>MRPL11</th>
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<td>T579C C585G</td>
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Table 3-3. Mutations in MOH1 and CDC6 are associated with FKS2-mediated echinocandin resistance in C. glabrata clinical isolates.

Genomic regions harboring the 8 non-synonymous mutations uncovered in isolate G relative to isolate A were sequenced by Sanger sequencing in 10 unrelated C. glabrata echinocandin-resistant clinical isolates containing the resistance mutation FKS2 T1987C / Fks2 S663P. Synonymous mutations are in black font; non-synonymous mutations are in red with amino acid mutations listed in parentheses. Sequences were compared to both the reference CBS138 sequence as well as a control sample sequenced from the laboratory isolate BG2.
3.3.5 Hsp90 Plays a Critical Role in Echinocandin Resistance of *C. glabrata*.

Given the importance of echinocandins in the therapeutic front line against *C. glabrata* infections and the acute problem imposed by the evolution of echinocandin resistance, there is pressing need to discover strategies to abrogate drug resistance. I focused on Hsp90 due to its critical role in enabling basal tolerance and acquired drug resistance in pathogenic fungi such as *C. albicans* and the most lethal mould *Aspergillus fumigatus* (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006; Cowen, Singh et al. 2009; Singh, Robbins et al. 2009). In the context of the echinocandins, *C. albicans* Hsp90 regulates crucial cellular responses to survive echinocandin exposure by stabilizing the catalytic subunit of the protein phosphatase calcineurin, while in *S. cerevisiae* Hsp90 and calcineurin do not modulate echinocandin susceptibility (Singh, Robbins et al. 2009). To date, no studies have examined the consequences of pharmacological or genetic compromise of Hsp90 function in *C. glabrata*, and thus whether this pathogen shows resistance circuitry more akin to the pathogen *C. albicans* or its closer relative *S. cerevisiae* remains unknown.

I first took a pharmacological approach to determine if inhibition of Hsp90 abrogates echinocandin resistance of *C. glabrata*. I monitored growth across a gradient of the widely used echinocandin caspofungin relative to a drug-free growth control in the presence or absence of the two structurally unrelated Hsp90 inhibitors, geldanamycin and radicicol, that bind to the adenosine triphosphate (ATP) binding pocket of Hsp90 and thereby compromise ATP-dependent chaperone function (Whitesell, Mimnaugh et al. 1994; Roe, Prodromou et al. 1999). In the absence of geldanamycin there was a small increase in caspofungin resistance between isolate B and isolate C and a large increase in resistance between isolate C and isolate D (Figures 3-2 and 3-4A). Pharmacological inhibition of Hsp90 with geldanamycin or radicicol decreased tolerance
of the early clinical isolates A, B, and C, and abrogated resistance of the late clinical isolates D, E, and G (Figure 3-4A). Notably, isolate F was refractory to the synergy between caspofungin and Hsp90 inhibitors, as discussed in more detail below (see Figure 3-7). Inhibition of Hsp90 also reduced caspofungin resistance of the laboratory strain harboring the FKS2 T1987C mutation, confirming Hsp90’s crucial role in Fks2-mediated echinocandin resistance in C. glabrata (Figure 3-4B). Synergy between caspofungin and geldanamycin was also observed in RPMI, a medium used for clinical susceptibility testing (data not shown). Thus, inhibition of Hsp90 provides the first and much-needed strategy to abrogate echinocandin resistance of C. glabrata.
Hsp90 plays a critical role in echinocandin resistance of *Candida glabrata* clinical isolates. (A) Pharmacological inhibition of Hsp90 with geldanamycin (GdA) or radicicol (RAD) reduces caspofungin (CF) resistance of *C. glabrata* clinical isolates in an MIC assay. Isolates are in the
same order as they were recovered from a patient who was on caspofungin treatment where isolate A was recovered pre-treatment and isolate G was recovered post-mortem. Assays were done in synthetic defined medium at 30°C for 72 hours. Data was analyzed as in Figure 1. (B) Resistance of the transformants harboring the T1987C FKS2 mutant allele is reduced upon inhibition of Hsp90 with geldanamycin (GdA) or inhibition of calcineurin with cyclosporin A (CsA). Assays were performed as in part (A) and data was analyzed as in Figure 1.
3.3.6 Compromising Calcineurin Function Abrogates Echinocandin Resistance of *C. glabrata*.

Hsp90 enables resistance to both the azoles and echinocandins in large part via the protein phosphatase calcineurin in *C. albicans* (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006; Cowen 2008; Singh, Robbins et al. 2009). Hsp90 stabilizes the catalytic subunit of calcineurin in both *S. cerevisiae* and *C. albicans* (Imai and Yahara 2000; Singh, Robbins et al. 2009), thereby enabling calcineurin-dependent responses to drug-induced cellular stress (Steinbach, Reedy et al. 2007; Cowen 2008). While calcineurin has been implicated in basal tolerance to echinocandins in *C. glabrata* (Miyazaki, Yamauchi et al. 2010), whether calcineurin affects *bona fide* resistance remains unknown.

First, I took a pharmacological approach to determine whether calcineurin is a key mediator of Hsp90-dependent echinocandin resistance in *C. glabrata*. I assessed growth across a gradient of caspofungin concentrations in the presence or absence of two structurally unrelated calcineurin inhibitors, cyclosporin A and FK506. Cyclosporin A and FK506 inhibit calcineurin function in different ways. Cyclosporin A binds to Cpr1 (cyclophilin A), a peptidyl-prolyl cis-trans isomerase, and it is this drug-protein complex that inhibits calcineurin function (Hemenway and Heitman 1999). FK506 binds a structurally unrelated peptidyl-prolyl cis-trans isomerase,FKBP12, and this distinct drug-protein complex also inhibits calcineurin function (Hemenway and Heitman 1999). I used concentrations of each pharmacological calcineurin inhibitor that abolished echinocandin resistance in *C. albicans* but did not inhibit growth on their own (Singh, Robbins et al. 2009). I found that inhibition of calcineurin with cyclosporin A or FK506 abolished caspofungin resistance of the late resistant *C. glabrata* clinical isolate G (Figure 3-5).

Next, I corroborated my pharmacological findings genetically by deletion of the regulatory subunit of calcineurin, encoded by *CNB1*, that is required for calcineurin function. I
confirmed that CNBI transcript was undetectable in the cnb1Δ mutant (Figure 3-6A). Loss of calcineurin function abrogated resistance of clinical isolate G in three independent mutants (Figure 3-5). Thus, calcineurin is a key mediator of Hsp90-dependent echinocandin resistance in C. glabrata, providing the first circuitry that governs echinocandin resistance in this pathogen.
Figure 3-5. Compromising calcineurin function abrogates echinocandin resistance of *C. glabrata* clinical isolates.

Deletion of the regulatory subunit of calcineurin, *CNB1*, required for calcineurin function abrogates CF resistance in late resistant clinical isolates. Pharmacological inhibition of calcineurin with either cyclosporin A (CsA) or FK506 also abrogates CF resistance. The assay was performed and analyzed as in Figure 3-4.
3.3.7 Caspofungin Induces FKS2 Expression in a Calcineurin-Dependent Manner in C. glabrata

The specific mechanism by which calcineurin governs echinocandin resistance remains unknown in any system. In *S. cerevisiae* FKS2 expression is induced during high temperature growth via calcineurin, and deletion of both *FKS1* and *FKS2* is synthetic lethal (Inoue, Takewaki et al. 1995; Mazur, Morin et al. 1995; Zhao, Jung et al. 1998; Lesage, Sdicu et al. 2004). Based on these findings, I propose a model in which calcineurin regulates echinocandin resistance in *C. glabrata* by controlling expression of the resistance determinant *FKS2*. In this case, inhibition of calcineurin would compromise expression of the echinocandin-resistant 1,3-β-D-glucan synthase thereby abrogating resistance. Calcineurin would also reduce basal tolerance of sensitive strains by reducing *FKS2* expression and thus decreasing the cellular pool of 1,3-β-D-glucan synthase, thereby enhancing sensitivity to a given concentration of echinocandin.

To test this model, I used quantitative RT-PCR to measure transcript levels of *CNB1*, encoding the regulatory subunit of calcineurin, and *FKS2*, encoding the catalytic subunit of 1,3-β-D-glucan synthase. Transcript levels of isolate G or its derivative in which *CNB1* was deleted were monitored after growth in rich medium for one hour with or without caspofungin treatment. I found that caspofungin induced expression of both *CNB1* (*P < 0.001*, ANOVA, Bonferroni’s Multiple Comparison Test, Figure 3-6A) and *FKS2* (*P < 0.001*, Figure 3-6B). Importantly, deletion of *CNB1* blocked caspofungin-induced upregulation of *FKS2* (*P < 0.001*, Figure 3-6B). Taken together, these results establish that *CNB1* and *FKS2* are induced upon echinocandin exposure, and that induction of the resistance determinant *FKS2* is critically dependent on calcineurin. This provides a novel mechanism by which calcineurin regulates echinocandin resistance in pathogenic fungi.
Figure 3-6. Caspofungin induces *FKS2* expression in a calcineurin-dependent manner in *C. glabrata*.

(A) Caspofungin induces calcineurin expression in the late clinical isolate G. Transcript levels of the regulatory subunit of calcineurin, *CNB1*, was measured by quantitative RT-PCR after growth in rich medium at 30°C for one hour without antifungal (- CF) or in the presence of caspofungin at 120 ng/ml (+ CF), as indicated. Transcript levels are normalized relative to *ACT1*. Expression is relative to the untreated sample, which was set to 1. Data are means ± standard deviation for
triplicate samples. Three asterisks indicate $P < 0.001$ (ANOVA, Bonferroni’s Multiple Comparison Test). (B) Caspofungin induces expression of resistance determinant $FKS2$ in the late clinical isolate $G$ in a calcineurin-dependent manner. Transcript levels of $FKS2$ was measured by quantitative RT-PCR after growth in conditions listed in (A). Transcript levels are normalized relative to $ACT1$. Expression is relative to the untreated sample, which was set to 1. Data are means ± standard deviation for triplicate samples. Three asterisks indicate $P < 0.001$ (ANOVA, Bonferroni’s Multiple Comparison Test).
3.3.8 *C. glabrata* Petite Mutants are not Intrinsically Resistant to Echinocandins but are Refractory to the Synergy between Caspofungin and Inhibitors of Hsp90 or Calcineurin.

Respiratory deficient mutants with loss of mitochondrial function, referred to as petite mutants, are associated with azole resistance in *S. cerevisiae*, *C. albicans*, and *C. glabrata* (Kontoyiannis 2000; Sanglard, Ischer et al. 2001; Brun, Berges et al. 2004; Cheng, Clancy et al. 2007; Ferrari, Sanguinetti et al. 2011). To date, there have been no reports of the petite phenotype contributing to echinocandin resistance, although *C. glabrata* is able to produce petite mutants at high frequency *in vitro* as well as *in vivo* (Bouchara, Zouhair et al. 2000; Sanglard, Ischer et al. 2001; Brun, Berges et al. 2004). Isolate F in my *C. glabrata* series was isolated from the patient at the same time point as isolate E, however, it was morphologically distinct and thus archived separately. When cultured on rich medium containing dextrose as the carbon source, this isolate had a reduced growth rate relative to the other isolates and produced smaller more transparent colonies (Figure 3-7A), despite possessing the same karyotype (Figure 3-1) and complement of polymorphisms in the nuclear genome as isolate E (Figure 3-2). When cultured on rich medium containing glycerol as the carbon source, which is non-fermentable, isolate F was unable to grow (Figure 3-7A). These results suggests that isolate F is a petite mutant as it behaves morphologically as a petite and is unable to respire based on failure to grow on glycerol. Notably, isolate F had a distinct echinocandin resistance phenotype from the other resistant isolates in the series in that its resistance was maintained even in the presence of the Hsp90 inhibitors geldanamycin or radicicol (Figure 3-4), or the calcineurin inhibitor cyclosporin A (Figure 3-7).

To determine if the petite phenotype is intrinsically involved in echinocandin resistance of *C. glabrata* clinical isolates, petite mutants were generated from clinical isolate A and their
resistance profiles were tested via MIC assays (Figure 3-7B). If the petite phenotype is sufficient to impart echinocandin resistance, then petite mutants generated from isolate A should acquire resistance, however, they do not. This suggests that the petite phenotype is not intrinsically involved in *C. glabrata* echinocandin resistance.

To determine if petite mutants of *C. glabrata* are intrinsically refractory to the synergy between echinocandins and inhibitors of Hsp90 or calcineurin, petite mutants were generated from late clinical isolate G and their resistance profiles were tested via MIC assays (Figure 3-7C). The petite mutants generated from isolate G are indeed resistant to the combination of caspofungin and geldanamycin or cyclosporin A, suggesting that they either no longer require calcineurin or Hsp90 for *FKS2*-mediated resistance, or that they are simply resistant to geldanamycin and cyclosporin A. Notably, petite mutants are known to up-regulate multidrug efflux transporters (Brun, Berges et al. 2004), which may confer resistance to these pharmacological inhibitors by their increased efflux from the cell. To distinguish between these two possibilities, petite mutants were generated from the isolate G derivative in which calcineurin function was genetically compromised due to deletion of *CNB1*. If petite mutants no longer require calcineurin for echinocandin resistance, then deletion of *CNB1* should have no impact on resistance, but this was not the case (Figure 3-7D). Petite mutants generated from isolate G *cnb1Δ* mutants were no longer resistant to caspofungin, suggesting that petite mutants are able to bypass the effects of cyclosporin A, and likely geldanamycin, potentially due to up-regulation of efflux pumps.
Clinical isolate F is a petite mutant and is refractory to the synergy between caspofungin and Hsp90 or calcineurin inhibitors.

(A) Clinical isolate F is a petite mutant. Each clinical isolate from the *C. glabrata* echinocandin resistant series was streaked onto rich yeast extract-peptone-dextrose (YPD) agar medium or yeast extract-peptone-glycerol (YPG) agar medium which have glucose or glycerol as the sole carbon source, respectively. Isolate F produces small, transparent colonies on YPD and is unable to grow on YPG. Petite mutants have lost their mitochondrial function, are unable to respire, and
therefore unable to utilize non-fermentable carbon sources (Brun, Berges et al. 2004). Isolates were patched onto the plates and photographed after incubation in the dark at 30°C for 24h. (B) The petite phenotype is non intrinsically involved in caspofungin resistance of *C. glabrata* clinical isolates. Petite mutants were generated from early clinical isolate A by growth in 10 µg/ml ethidium bromide for 48 hours in the dark and were identified based on morphology and inability to grow on YPG. Caspofungin (CF) resistance was then compared to resistant petite clinical isolate F in an MIC assay along with inhibition of Hsp90 with 20 µM geldanamycin (GdA) or inhibition of calcineurin with 10 µM cyclosporin A (CsA). (C) Caspofungin resistant petite mutants of *C. glabrata* are refractory to the synergy between caspofungin and Hsp90 or calcineurin inhibition. Petite mutants were generated from late clinical isolate G as in part (B). Caspofungin (CF) resistance was then compared to resistant petite clinical isolate F in an MIC assay with or without pharmacological inhibition of Hsp90 with 20 µM geldanamycin (GdA) or pharmacological inhibition of calcineurin with 10 µM cyclosporin A (CsA). (D) Caspofungin resistance of petite mutants of *C. glabrata* is dependent on calcineurin. Petite mutants were generated from the late clinical isolate G *cnb1Δ* deletion mutant as in part (B). Synergy between caspofungin (CF) and Hsp90 inhibition with GdA (20 µM) or calcineurin inhibition with CsA (10 µM) was tested. MIC assays were performed and analyzed as in Figure 3-4.
3.4 Discussion

My results provide the first global view of mutations that accompany the evolution of fungal drug resistance in a human host, establish novel echinocandin resistance mutations, and reveal a new molecular mechanism of echinocandin resistance, with broad therapeutic potential. I characterized *C. glabrata* bloodstream isolates that evolved increased resistance to the echinocandin caspofungin over a 10-month period during which the patient underwent multiple rounds of caspofungin treatment for recurrent candidemia (Figure 3-1, and Table 3-1). Whole genome sequencing of the susceptible isolate recovered prior to drug treatment and the last resistant isolate, revealed that 9 non-synonymous mutations accumulated during evolution in the human host (Table 3-2 and Figure 3-2). A mutation in *FKS2*, encoding the drug target, accompanied the largest increase in echinocandin resistance in the lineage; this mutation was sufficient to confer echinocandin resistance in a sensitive *C. glabrata* laboratory strain, but was associated with a fitness cost in terms of reduced growth rate in the absence of drug (Figure 3-3). The 8 additional mutations in genes not previously implicated in echinocandin resistance (Table 3-2) may include novel resistance determinants as well as mutations that mitigate the cost of the *FKS2* mutation. Consistent with these possibilities, non-synonymous mutations in 2 out of the 8 genes, *MOH1* and *CDC6*, are associated with *FKS2*-mediated echinocandin resistance in additional unrelated echinocandin-resistant clinical isolates (Table 3-3). Further, I establish that Hsp90 governs both basal tolerance to the echinocandins and *bona fide* resistance of clinical isolates and that calcineurin is a key mediator of Hsp90-dependent resistance (Figures 3-4 and 3-5). Calcineurin in turn regulates echinocandin resistance by controlling expression of the resistance determinant *FKS2* (Figures 3-5 and 3-6), providing a novel mechanism by which calcineurin regulates echinocandin resistance in pathogenic fungi.
The whole genome sequence analysis yields powerful insights into the evolutionary dynamics of adaptation in the host, as well as novel mutations associated with resistance or with ameliorating the fitness cost of resistance. Beyond mutations in the drug target, mechanisms of echinocandin resistance remained enigmatic. In the *C. glabrata* series, mutations in 4 genes not previously associated with echinocandin resistance (*MOH1*, *GPH1*, *CDC6*, and *TCB1/2*) accompanied an early and small increase in echinocandin resistance (Figure 3-2). Mutations in these genes could confer the small increase in resistance, or could create a genetic background in which the FKS2 mutation is less detrimental; in the latter case, the mutation would have to confer a fitness benefit on its own in order to be selected for in advance of the FKS2 mutation, or it could be selectively neutral and fixed by genetic drift. That mutations in *MOH1* and *CDC6* are associated with FKS2-mediated echinocandin resistance in unrelated clinical isolates supports a possible role for these genes in modulating resistance phenotypes (Table 3-3). Mutations in 3 additional genes not previously implicated in echinocandin resistance (*DOT6*, *MRPL11*, and *SUI2*) coincided with the FKS2 mutation, and a last mutation in *CDC55* arose in the last isolate, without any associated change in resistance. Given that FKS2 is sufficient for the full resistance phenotype of the late clinical isolate, it is likely that these other mutations are unrelated to echinocandin resistance or that they mitigate the fitness cost of the FKS2 mutation. It is notable that multiple mutations accumulated at the two major transitions in resistance. This is consistent with strong selection favoring the rapid accumulation of mutations in the lineage. That each of the mutations identified persisted throughout the entire lineage is consistent with the occurrence of selective sweeps, such that each mutation rose to near fixation in the lineage. Selective sweeps in response to drug selection are also observed in experimental populations of *C. albicans* during the evolution of azole resistance *in vitro* (Cowen, Sanglard et al. 2000).
The fate of drug-resistant mutants in nature depends on their fitness relative to drug-sensitive counterparts. While resistance mutations are expected to confer a fitness benefit in the presence of the drug, they may also confer a cost in terms of reduced fitness in the absence of the drug. This model is consistent with the impact of the FKS2 T1987C mutation observed in the C. glabrata lineage. This mutation confers a major increase in growth in the presence of echinocandin (Figure 3-4A), but also confers reduced growth in the absence of the drug (Figure 3-3B). The deleterious impact on fitness is likely due to the reduced catalytic capacity commonly observed among 1,3-β-D-glucan synthase enzymes that acquire amino acid substitutions that reduce their sensitivity to echinocandins (Garcia-Effron, Lee et al. 2009). C. glabrata may upregulate FKS2 expression to compensate for its decreased catalytic capacity (Garcia-Effron, Lee et al. 2009), or may acquire additional mutations that mitigate the cost of the resistance mutation. The fitness effects of antibiotic resistance mutations have been studied extensively in bacteria, where most resistance mechanisms are associated with a fitness cost that manifests in reduced growth rate (Andersson and Hughes 2010). In the vast majority of cases, the fitness cost is mitigated by the acquisition of compensatory mutations (Levin, Perrot et al. 2000; Andersson and Hughes 2010; Schulz zur Wiesch, Engelstadter et al. 2010; Tanaka and Valckenborgh 2011). Consistent with these patterns, any cost of resistance in experimental populations of C. albicans that evolved azole resistance in vitro was mitigated with further evolution (Cowen, Kohn et al. 2001), as many changes in gene expression observed in the less fit, resistant population were restored to the ancestral state (Cowen, Nantel et al. 2002).

Morphological variants that emerge in an evolutionary lineage can reveal important features of mechanisms of drug resistance or drug synergy. Isolate F is a petite mutant based on morphology and inability to grow on a non-fermentable carbon source (Figure 3-7A). Such respiratory deficient mutants with loss of mitochondrial function are associated with azole
resistance in *S. cerevisiae*, *C. albicans*, and *C. glabrata* (Kontoyiannis 2000; Sanglard, Ischer et al. 2001; Brun, Berges et al. 2004; Cheng, Clancy et al. 2007; Ferrari, Sanguinetti et al. 2011). The azole resistance of petites is attributed to increased expression of multidrug transporters of the ATP binding cassette family (Sanglard, Ischer et al. 2001; Brun, Berges et al. 2004; Cheng, Clancy et al. 2007). The petite phenotype has not been previously linked to echinocandin resistance to date, consistent with the limited evidence that multidrug transporters are involved in resistance to this drug class (Bachmann, Patterson et al. 2002; Schuetzer-Muehlbauer, Willinger et al. 2003; Niimi, Maki et al. 2006; Posteraro, Sanguinetti et al. 2006). Indeed, induction of petite mutants in the early *C. glabrata* clinical isolate A does not confer echinocandin resistance, confirming that petite mutants are not intrinsically resistant to echinocandins (Figure 3-7B). A striking feature of the isolate F is that its echinocandin resistance phenotype is recalcitrant to the impact of the Hsp90 inhibitor geldanamycin or calcineurin inhibitor cyclosporin A, unlike that of all other isolates in the series (Figure 3-7C). Induction of petite mutants in late clinical isolate G confirms that the petite phenotype is intrinsically recalcitrant to the impact of geldanamycin or cyclosporin A (Figure 3-7C). Genetic compromise of calcineurin function in petite mutants abrogates echinocandin resistance, suggesting that petites are simply resistant to cyclosporin A (Figure 3-7D), and likely geldanamycin; this may be attributable to overexpression of multidrug transporters in petites that remove these inhibitors from the cell. Whether the original isolate F petite mutant arose during evolution in the human host or during sampling remains unknown, although *C. glabrata* petite mutants are certainly relevant for *in vivo* infections given that they have increased virulence, and increased fitness in mouse models (Ferrari, Sanguinetti et al. 2011).

My results further establish Hsp90 and calcineurin as the first regulators of *bona fide* echinocandin resistance in *C. glabrata*, and reveal that resistance circuitry has been rewired over
evolutionary time. The molecular chaperone Hsp90 and its client protein calcineurin govern basal tolerance and resistance to both the azoles and the echinocandins in *C. albicans* (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006; Singh, Robbins et al. 2009). The roles of Hsp90 and calcineurin in azole resistance are conserved in *S. cerevisiae* (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). However, compromise of Hsp90 or calcineurin function does not alter echinocandin susceptibility in *S. cerevisiae* (Singh, Robbins et al. 2009). Here, I find that Hsp90 and calcineurin are required for basal tolerance to echinocandins in *C. glabrata* as well as for resistance that evolves in a human host (Figures 3-4 and 3-5), suggesting that despite the closer evolutionary relationship of *C. glabrata* to *S. cerevisiae*, its cellular circuitry governing resistance to drugs that target the cell wall shares more similarity to that of its more distant pathogenic relative, *C. albicans*. Notably, signaling pathways governing cell wall integrity have been rewired between *C. albicans* and *S. cerevisiae* (Blankenship, Fanning et al. 2010). The cell wall is essential for fungal viability and is an elaborate structure, components of which are recognized by vigilant immune cells in the human host (Netea, Brown et al. 2008). As commensals and opportunistic pathogens, *C. glabrata* and *C. albicans* are likely to harbor circuitry governing cell wall architecture that is subject to strong selection in response to host immune system challenge.

This work establishes that targeting Hsp90 or calcineurin has broad therapeutic potential for infections caused by one of the leading fungal pathogens of humans, and complements the expanding repertoire of therapeutic applications for inhibitors of Hsp90 and calcineurin in the treatment of infectious disease. Inhibition of Hsp90 or calcineurin transforms echinocandins from ineffective to highly efficacious against echinocandin-resistant *C. glabrata* (Figures 3-4 and 3-5). Notably, a human recombinant antibody against Hsp90 also has synergistic activity with echinocandins against *C. glabrata* in a mouse model (Hodgetts, Nooney et al. 2008), though the
mechanism by which this antibody works remains entirely unknown as it unlikely to enter intact fungal cells to influence function of the cytosolic Hsp90 chaperone. Genetic compromise of Hsp90 function enhances the efficacy of azoles and echinocandins in a mouse model of systemic C. albicans infection (Cowen, Singh et al. 2009; Singh, Robbins et al. 2009). Genetic or pharmacological compromise of Hsp90 also transforms fluconazole from ineffective to highly efficacious against C. albicans biofilms in a mammalian model of biofilm infection (Shapiro, Robbins et al. 2011). Beyond Candida species, inhibition of Hsp90 also enhances antifungal efficacy against the most lethal mould, A. fumigatus, in a metazoan model of infection (Cowen, Singh et al. 2009). Consistent with the functional relationship between Hsp90 and calcineurin, calcineurin inhibitors also have therapeutic potential and are synergistic with azoles against C. albicans endocarditis, keratitis, and biofilms in mammalian models (Marchetti, Moreillon et al. 2003; Onyewu, Afshari et al. 2006; Uppuluri, Nett et al. 2008). Beyond their utility in the treatment of fungal infections, Hsp90 and calcineurin are promising targets for treating infections caused by protozoan parasites including Plasmodium falciparum and Trypanosoma evansi (Kumar, Musiyenko et al. 2005; Pallavi, Roy et al. 2010; Shahinas, Liang et al. 2010). Supporting their clinical relevance, Hsp90 inhibitors have advanced in clinical trials for the treatment of cancer and other diseases (Kim, Alarcon et al. 2009; Prodromou 2009; Trepel, Mollapour et al. 2010), and calcineurin inhibitors are widely used in the clinic as immunosuppressants (Steinbach, Reedy et al. 2007). Given the potential for toxicity upon inhibition of key cellular regulators in the host during infection (Cowen, Singh et al. 2009), the challenge for further development of Hsp90 and calcineurin as therapeutic targets for infectious disease lies in developing pathogen-selective inhibitors or drugs that target pathogen-specific components of the cellular circuitry governing drug resistance and virulence.
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Table 3-5 Plasmids used in this study

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Chapter 4

4 General Discussion and Future Directions

4.1 General Discussion

This work establishes a novel role for the molecular chaperone Hsp90 and the protein phosphatase calcineurin in echinocandin resistance in the leading causes of candidiasis, *C. albicans* and *C. glabrata*. Previous work established the roles of Hsp90 and calcineurin in resistance to the cell membrane stress exerted by the most widely used class of antifungals, the azoles (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006; Cowen, Singh et al. 2009). However, very little work had been done elucidating molecular mechanisms of resistance to the echinocandins, the newest class of antifungal to reach the clinic. Work in *C. albicans* and *C. glabrata* found that the protein kinase C pathway is involved in mediating tolerance to the echinocandins; however, only mutations in the drug target have ever been implicated in *bona fide* echinocandin resistance. My work reveals that Hsp90 plays a critical role in both tolerance and resistance to the cell wall stress exerted by the echinocandins, largely through calcineurin in both medically important pathogens.

In *C. albicans*, both genetic and pharmacological compromise of Hsp90 or calcineurin function results in potent synergy with the echinocandins in minimum inhibitory concentration (MIC) assays, checkerboard assays, and tandem assays with an MIC followed by spotting onto rich medium without any inhibitors, Figures 2-1, 2-2, 2-3, and 2-4. I also establish calcineurin as the first client protein of Hsp90 identified in *C. albicans* by showing a physical interaction between the proteins through reciprocal co-immunoprecipitation assays and dependence of calcineurin on Hsp90 for both its activity and stability, Figures 2-6, and 2-7. The downstream
effector of calcineurin, Crz1, plays only a partial role in echinocandin tolerance suggesting that there are other players downstream of calcineurin involved in echinocandin tolerance, Figure 2-8. I also found Hsp90 and calcineurin to be involved in mediating true echinocandin resistance in both clinical and laboratory-derived isolates of *C. albicans* and genetic compromise of *HSP90* rendered micafungin much more efficacious in the mouse model of candidiasis, providing clinical relevance to the findings, Figures 2-9 and 2-10.

Complementary studies were performed in the model organism *S. cerevisiae* and physical interaction between Hsp90 and calcineurin was confirmed via co-immunoprecipitation assays, Figure 2-11. I found that while both the azole fluconazole (FL) and the echinocandin micafungin (MF) activate calcineurin-dependent stress responses, compromise of calcineurin or Hsp90 function had negligible effects on MF tolerance, Figure 2-11. Thus, while the functional relationship between Hsp90 and calcineurin is conserved between *C. albicans* and *S. cerevisiae*, as is the activation of calcineurin in response to drug stress, these regulators play a crucial role in cellular responses to the echinocandins in the pathogenic yeast but not the model yeast.

Given the divergence between Hsp90 and calcineurin’s role in echinocandin tolerance between *C. albicans* and *S. cerevisiae*, I turned to the second leading cause of candidiasis and a pathogen much more closely related to *S. cerevisiae*, *C. glabrata*. Through collaboration with researchers at the University of Toronto Health Network, I obtained a series of *C. glabrata* clinical isolates recovered from a single patient on echinocandin treatment that were collected over the course of 10 months. The clinical isolates in this series had been karyotyped and were found to be the same strain that evolved echinocandin resistance *in vivo* over time, Figure 3-1. Through next generation sequencing, I provide the first genome-wide view of mutations that accompany the evolution of fungal drug resistance in a human host. In addition to a known
resistance mutation, 8 other mutations that had never been associated with echinocandin resistance are uncovered, Figure 3-2. I found that the mutation in the known drug target FKS2 was sufficient for high-level resistance, but it was associated with a fitness deficit, Figure 3-3. Of the 8 additional mutations identified, mutations in CDC6 and MOH1 were also found in unrelated echinocandin-resistant clinical isolates harboring the Fks2 S663P mutation. These genes may be involved in compensatory mechanisms that alleviate the fitness cost associated with the mutant allele of FKS2, or they may be involved in adaptation to the host as all specimens were isolated from human sources. I establish a role for Hsp90 and calcineurin in mediating both echinocandin tolerance and bona fide echinocandin resistance in C. glabrata and propose a new model by which Hsp90 and calcineurin regulate echinocandin resistance by controlling expression of the resistance determinant, FKS2. I also find that, in contrast to azole resistance, the petite phenotype is not intrinsically involved in echinocandin resistance in C. glabrata but does render echinocandin resistance phenotypes recalcitrant to the effects of inhibitors of Hsp90 or calcineurin.

Previous to these works, only mutations in the echinocandin targets, FKS1 and FKS2, had been implicated in clinical resistance to the echinocandins. Given that biochemical analysis of mutant FKS alleles in both C. albicans and C. glabrata reveal reduced enzyme capacity, it is reasonable to hypothesize that there would be an associated fitness cost to harboring such mutations even when the gene is non-essential. This is evident in Figure 3-3 where a C. glabrata laboratory strain harboring the Fks2 S663P mutation has a growth defect relative to the parental strain. In C. albicans, FKS1 mutant strains display attenuated virulence in both the Drosophila invertebrate model of infection as well as in the context of a competitive mixed infection in the mouse model of disseminated candidiasis (Ben-Ami, Garcia-Effron et al. 2011). It is thought that these associated fitness costs may limit the spread of echinocandin-resistant strains in the C.
*albicans* population given their competitive disadvantage in the absence of echinocandin compared to their sensitive counterparts. However, in the context of echinocandin treatment the mutant strains would have an advantage and, if exposed to the drug for long enough periods of time, may evolve other compensatory mechanisms that ameliorate the fitness cost of the drug target mutations. For example, in bacteria resistance mutations that first arise often have an associated fitness cost that is alleviated with further evolution by the acquisition of compensatory mutations (Levin, Perrot et al. 2000; Maisnier-Patin and Andersson 2004). Over time as echinocandin use expands and as research beyond drug target alterations is further explored, other resistance mechanisms will undoubtedly be uncovered.

### 4.2 Future Directions

To uncover novel mechanisms of echinocandin resistance that may already exist in the clinic it would be beneficial to sequence the genomes of additional sets of matched clinical isolates of *C. glabrata* and *C. albicans* where both a sensitive early clinical isolate and late isolate that evolved echinocandin resistance in a patient exist. Currently only mutations in the *FKS* targets are the focus of attention in all reported cases of clinically resistant *C. albicans* and *C. glabrata* isolates, even to the point where only the hot spot regions of *FKS1* and *FKS2* are being analyzed. This restricted scope of analysis has led to many echinocandin-resistant isolates lacking any known mechanism of resistance (Castanheira, Woosley et al. 2010). For example, the minimum inhibitory concentration assay protocol used in the clinic categorizes *C. glabrata* clinical isolate C as clinically resistant, however, the Fks2 S663P mutation does not arise until isolate D (Figure 3-2). Thus this isolate lacks any known resistance mechanism. I think it is important to understand on a more global level the cellular processes that are altered in response to
echinocandins in order to not only predict resistance, but to perhaps identify novel fungal-specific pathways that may prove to be attractive therapeutic targets.

To distinguish between mutations that arise due to drug exposure versus those involved in adaptation to the human host, it would be useful to experimentally evolve resistance to the echinocandins. This would involve monitoring artificial *C. glabrata* or *C. albicans* populations as they are propagated through serial transfer between batch cultures for many generations in various concentrations of echinocandin, as has already been done in the context of azole resistance in *S. cerevisiae* and *C. albicans* (Cowen, Sanglard et al. 2000; Cowen, Kohn et al. 2001; Cowen, Nantel et al. 2002; Anderson, Sirjusingh et al. 2003). In these experiments the mechanisms that were acquired under selection for azole resistance in both *S. cerevisiae* and *C. albicans* were consistent with those observed in populations that evolved in the human host (Cowen, Nantel et al. 2002; Anderson, Sirjusingh et al. 2003). Further, I think it is would be most useful to perform such experiments with *C. glabrata* as it is already inherently refractory to the azoles and, consequently, echinocandins have become the front-line treatment for this species. It is also a haploid organism, which simplifies bioinformatics analysis, and requires no codon optimization such that experiments to determine the functional consequence of specific mutations could be carried out in *S. cerevisiae*. To determine the functional consequence of mutations that arise either during *in vivo* or *in vitro* selection for echinocandin resistance I would perform allele swap experiments where I would express the mutant allele, and wild type as a control, in the parental sensitive strain and perform minimum inhibitory concentration assays or kinetic growth curves to determine whether the mutation contributes to resistance, to fitness in the context of growth, or to both. This strategy would work if the mutations that arise were dominant. If they were not, then it would be necessary to knock out the wild-type allele in the
sensitive ancestral strain using fusion PCR combined with a split-marker approach detailed earlier.

In addition to non-synonymous mutations, it would be useful to determine global changes in gene expression in resistant strains versus ancestral sensitive strains via microarray profiling or RNA sequencing (RNA-seq). In fact, it would be more effective to perform RNA-seq from the outset given that the cost of this technology is rapidly falling and paired-end sequencing yields all the genomic data that genome sequencing yields, is better at resolving gene fusions that may result from genomic rearrangements, and provides quantitative gene expression data (Ozsolak and Milos 2011). For example, in experimental populations of *C. albicans* evolved in the presence of fluconazole, genome-wide expression profiling of over 5,000 open reading frames revealed that 301 had significantly modified expression relative to the sensitive ancestral strain, and there were distinct profiles depending on the mechanism of resistance that was acquired (Cowen, Nantel et al. 2002). Additionally, there were distinct expression profiles for early and late-stage evolution of azole resistance, and eventually the experimental populations converged on two adaptive solutions that likely represent the most common pathways for the evolution of fluconazole resistance (Cowen, Nantel et al. 2002). Using a similar approach it would be possible to determine what common pathways are taken for the experimental evolution of echinocandin resistance and therefore predict how the evolution of drug resistance may occur in the clinic. Thus experimental evolution of echinocandin resistance may provide insight into what mechanisms of resistance will likely appear in the clinic as echinocandin use becomes more widespread.

The architecture of the stress response signaling pathways that influence echinocandin resistance remains another promising area for future investigation. My work establishes a role for
calcineurin in mediating echinocandin resistance in *C. albicans* partially through the downstream transcription factor Crz1, Figure 2-8. Calcineurin also mediates echinocandin resistance in *C. glabrata*, Figures 3-5 and 3-6, and although the role of Crz1 was not assessed, a study investigating the role of Crz1 in antifungal tolerance found only a partial role in *C. glabrata* (Miyazaki, Yamauchi et al. 2010). Calcineurin is also crucial for azole resistance in *S. cerevisiae* and *C. albicans*, however, only partially through Crz1 (Onyewu, Wormley et al. 2004; Cowen, Carpenter et al. 2006). In *C. glabrata*, genetic compromise of calcineurin function results in hypersensitivity to fluconazole relative to a parental wild-type strain, but genetic compromise of *CRZ1* yields no observable phenotype (Miyazaki, Yamauchi et al. 2010).

Together these data suggest that there are additional downstream targets of calcineurin that mediate antifungal drug resistance and that have yet to be identified. A study that performed microarray expression profiling of calcineurin and *CRZ1* null mutants in response to calcium stress reported that 9 genes were identified that were strictly calcineurin-dependent in their expression profiles in response to calcium and unchanged in the *CRZ1* null mutant, but the identity of the 9 genes was not specified (Karababa, Valentino et al. 2006). To determine what additional targets calcineurin may affect the expression of, I would repeat this experiment with a wild-type strain, a calcineurin null mutant, and a *CRZ1* null mutant with and without calcium and also in the presence of an azole or an echinocandin in order to identify genes that are differentially expressed in response to these stressors that are calcineurin-dependent but not *CRZ1*-dependent. If there are any overlapping genes, I would genetically compromise them both on their own and in the context of a *CRZ1* null strain to determine whether they play a role themselves in antifungal tolerance and whether the effects of compromising calcineurin function are fully phenocopied when both *CRZ1* and the discovered gene are genetically compromised together. The phenotypic results of a minimum inhibitory concentration assay with these strains
would allow me to determine whether the uncovered genes are upstream of CRZ1 or if they function in a separate pathway.

Deciphering the specific mechanism of glucan synthase inhibition by the echinocandins remains another priority for the field. Specifically, it is unclear as to whether echinocandins must be transported into the cell to exert their effect. Biochemical kinetic experiments using labeled and unlabeled caspofungin revealed that echinocandins are moved into the cell via energy-independent saturable high affinity and low affinity transporters (Paderu, Park et al. 2004). It has been proposed that the aliphatic tails of the echinocandins may intercalate the lipid bilayer and inhibit glucan synthase activity at the plasma membrane from the extracellular face based on topology models as to where resistance mutations in the drug target occur (Perlin 2011). To begin understanding how echinocandins interact with the glucan synthase complex it will also be necessary to solve the crystal structure of the enzyme bound to the drug.

Taken together, the work presented in this thesis sheds light not only on the evolution of drug resistance in medically important human pathogens, but also suggests promising therapeutic targets that may save human lives.
References


