

Sexual Reproduction and Signal Transduction in the *Candida* Species Complex

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Molecular Genetics and Microbiology in the Graduate School
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ABSTRACT

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Abstract

Although the majority of the population carries *Candida spp* as normal components of their microbiota, these species are important human pathogens that have the ability to cause disease under conditions of immunosuppression or altered host defenses. The spectrum of disease caused by these species ranges from cutaneous infections of the skin, mouth, esophagus and vagina, to life-threatening systemic disease. Despite increases in drug resistance, the antifungal armamentarium has changed little over the past decade. Thus, increasing our understanding of the life cycles of these organisms, not only how they propagate themselves but also how genetic diversity is created within the population, is of considerable import. Additionally, expanding our knowledge of key signal transduction cascades that are important for cell survival and response to stress will aid in developing new antifungal therapies and strategies.

This thesis addresses both of these key areas of fungal pathogenesis. In the first chapter, we use genome comparisons between parasexual, asexual, and sexual species of pathogenic *Candida* as a first approximation to answer the question of whether examining genome content alone can allow us to understand why species have a particular life cycle. We start by examining the structure of the mating type locus (*MAT*) of two sexual species *C. lusitaniae* and *C. guilliermondii*. Interestingly, both species are missing either one or two (respectively) canonical transcription factors, suggesting that the control of sexual identity and meiosis in these organisms has been significantly reconfigured. Mutational analysis of the retained transcription factors is used to understand how sexual identity and meiosis are controlled in these species. Secondly, based on the observation that these species are missing many key genes involved in mating and meiosis, we use meiotic mapping, *SPO11* mutant analysis, and

comparative genome hybridization to demonstrate that these species are indeed meiotic, but that the meiosis that occurs often generates aneuploid and diploid progeny.

In the second and third chapters we examine the calcineurin signaling pathway, which is crucial for mediating tolerance to cellular stresses, including cations, azole antifungals, and passage through the host bloodstream. First, we show that clinical use of calcineurin inhibitors in combination with azole antifungals does not result in resistance to the combination, suggesting that if non-immunosuppressive analogs could be further developed this combinatorial strategy may have great clinical efficacy. Second, we use previous studies of the calcineurin signaling pathway in *S. cerevisiae* to direct a candidate gene approach for elucidating other components of this pathway in *C. albicans*. Specifically, we identify homologs of the *RCN1*, *MID1*, and *CCH1* genes, and use a combination of phenotypic assays and heterologous expression studies to understand the roles of these proteins in *C. albicans*. Although the mutant strains share some phenotypic properties with calcineurin deletion strains, none completely recapitulate a calcineurin mutant.

In the last chapter, we examine the plausibility of targeting the homoserine dehydrogenase (Hom6) protein in *C. albicans* and *C. glabrata* as a novel antifungal strategy. Studies in *S. cerevisiae* have demonstrated synthetic lethality between *hom6* and *fpr1*, the gene encoding the FKBP12 prolyl-isomerase that is the binding target of the immunosuppressant FK506. This synthetic lethality is due to the accumulation of a toxic intermediate in the methionine and threonine biosynthetic pathway as a result of deletion of *hom6* and inhibition of FKBP12. We deleted *HOM6* from both *C. albicans* and the more highly drug-resistant species *C. glabrata*. Studies suggest that regulation of the threonine and methionine biosynthetic pathway in *C. albicans* differs such that the synthetic lethality between *hom6* and FKBP12 inhibition no longer exists or *C. albicans* is

not sensitive to the intermediate. However, in *C. glabrata* preliminary analysis suggests that, similar to *S. cerevisiae*, mutation of *hom6* and inhibition of FKBP12 can result in cell death.

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1. Introduction

1.1 *Candida* species as human pathogens

Although the majority of the population carries *Candida spp.* as normal components of their mucosal microbiota, these commensal organisms have the ability to cause serious cutaneous and systemic disease. In fact, *Candida* species are the most common causative agents of invasive fungal infections. *Candida spp.* cause a broad spectrum of disease ranging from mucocutaneous infections such as thrush and vaginitis in both immunocompetent and immunocompromised individuals, to life-threatening systemic infections in immunocompromised patients (Edwards 1991). Risk factors for developing candidiasis include indwelling catheters, immunosuppression due to organ transplantation, cancer, chemotherapy, or HIV / AIDS, and the use of broad-spectrum antibiotics (Odds 1988; Edwards 1991; Beck-Sague and Jarvis 1993; Wenzel 1995). Interestingly, immune status seems to influence the spectrum of disease with systemic infections common to neutropenic patients, but a predilection for oroesophageal candidiasis in AIDS patients. Additionally, a subset of women are subject to recurrent vulvovaginal candidiasis, the pathology of which appears to involve an overactive immune response. Historically, *Candida albicans* has accounted for the majority of candidal infections; however, following the introduction of fluconazole numerous species have increased in prevalence including *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* (Nguyen, Peacock et al. 1996; Husain, Tollemar et al. 2003).

1.2 The calcineurin signaling pathway

The calcineurin inhibitors, FK506 and CsA, were initially isolated for their potent immunosuppressive properties (Borel 1976; Goto, Kino et al. 1991) and subsequently became cornerstones of therapy in solid organ transplantation. More recent evidence has

suggested a role for these drugs, and non-immunosuppressive analogs, as novel antifungal therapeutics (Blankenship, Steinbach et al. 2003; Steinbach, Reedy et al. 2007). The target of these drugs, calcineurin, is a serine-threonine-specific protein phosphatase that is conserved from yeasts to humans and is crucial for mediating cellular stress responses. Functional calcineurin consists of two subunits, a catalytic A subunit and a regulatory B subunit, both of which are required for proper functioning of the protein (Hemenway and Heitman 1999). When Ca^{2+} is influxed into the cytosol from either intracellular or extracellular stores, calcineurin is bound by Ca^{2+} -calmodulin causing a conformational change that relieves repression of the catalytic site by an autoinhibitory domain (Hemenway and Heitman 1999). In mammalian T-cells, activated calcineurin dephosphorylates the nuclear factor of activated T-cells (NF-AT), allowing translocation of the cytoplasmic component of this transcription factor to the nucleus where it activates cytokine production and T-cell proliferation (Clipstone and Crabtree 1992; Clipstone and Crabtree 1993; Cardenas and Heitman 1995). FK506 and CsA enter cells and bind to the immunophilins FKBP12 and cyclophilin, respectively, (Ho, Clipstone et al. 1996) and the resulting drug-protein complexes bind calcineurin preventing T-cell activation and suppressing host immune responses (Fruman, Klee et al. 1992; Clipstone, Fiorentino et al. 1994; Heitman, Cardenas et al. 1994; Cardenas, Muir et al. 1995; Cardenas, Zhu et al. 1995). In addition to their ability to inhibit mammalian calcineurin, FK506-FKBP12 and CsA-cyclophilin have been shown to inhibit calcineurin function in three fungal species: *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus* (Heitman, Cardenas et al. 1994; Odom, Del Poeta et al. 1997; Odom, Muir et al. 1997; Cruz, Del Poeta et al. 2000; Fox, Cruz et al. 2001; Cruz, Goldstein et al. 2002; Kontoyiannis, Lewis et al. 2003; Steinbach, Schell et al. 2004; Steinbach, Cramer et al. 2007).

1.3 Calcineurin inhibitors act synergistically with ergosterol biosynthesis inhibitors

Interest in the calcineurin signaling pathway of *Candida* was first piqued by a series of *in vitro* studies demonstrating the ability of calcineurin inhibitors to augment the efficacy of current antifungal therapies. In the clinical setting fluconazole is a commonly used antifungal agent for the treatment and prevention of candidal infections. The azole antifungals, including fluconazole, itraconazole, voriconazole and ketoconazole, target the enzyme lanosterol 14 α -demethylase that is required for ergosterol biosynthesis (Vanden Bosche 1985; Vanden Bosche, Willemsens et al. 1987).

However, azoles are fungistatic drugs that inhibit the growth of, but do not kill *Candida spp.* and resistance to these drugs is common, particularly among *C. glabrata* and *C. krusei* (Rex, Rinaldi et al. 1995; Arias, Arevalo et al. 1996; Pfaller, Jones et al. 1998; Pfaller, Jones et al. 1998; Akins 2005). A screen of multiple commercially available drugs in combination with fluconazole demonstrated the ability of the calcineurin inhibitor cyclosporine A to enhance the action of fluconazole towards *C. albicans in vitro* (Marchetti, Moreillon et al. 2000; Marchetti, Moreillon et al. 2003). Subsequently, FK506 was also shown to act synergistically with fluconazole (Cruz, Goldstein et al. 2002). Three additional experiments demonstrated that it was indeed the inhibition of calcineurin by these compounds that was responsible for the synergism (Cruz, Goldstein et al. 2002). Firstly, deletion of the FK506 binding protein FKBP12 abolished the synergism of FK506 with fluconazole. Secondly, a dominant FK506-resistant calcineurin mutant unable to bind the FK506-FKBP12 complex was also resistant to the synergism (Cruz, Goldstein et al. 2002). Thirdly, calcineurin deletion mutants were found to be viable and azole-hypersensitive.

The synergistic activity of calcineurin inhibitors was not limited to fluconazole, being also seen with other azoles (Cruz, Goldstein et al. 2002). Furthermore, drugs targeting different steps in the ergosterol biosynthesis pathway, such as terbinafine and fenpropimorph, were also shown to act synergistically with calcineurin inhibitors (Onyewu, Blankenship et al. 2003). Although calcineurin inhibitors have no intrinsic effect on the survival of *Candida spp.* under non-stress condition, in combination with ergosterol biosynthesis inhibitors, calcineurin inhibitors were able to convert these normally fungistatic compounds into potent fungicidal drugs (Marchetti, Moreillon et al. 2000; Cruz, Goldstein et al. 2002; Onyewu, Blankenship et al. 2003). Interestingly, this effect was not limited to *C. albicans*, but other more resistant species of *Candida*, including *C. glabrata* and *C. krusei*, were also susceptible to the drug combinations (Cruz, Goldstein et al. 2002; Onyewu, Blankenship et al. 2003; Reedy, Husain et al. 2006). The immunosuppressive properties of the calcineurin inhibitors could limit their clinical utility in combination therapy. However, this azole synergism was also shown with a non-immunosuppressive analog of FK506, L-685,818 (Becker, Rotonda et al. 1993; Rotonda, Burbaum et al. 1993; Odom, Del Poeta et al. 1997; Cruz, Goldstein et al. 2002; Onyewu, Blankenship et al. 2003), which has potential for clinical use to inhibit fungal calcineurin without the side effect of also suppressing the human immune system.

1.4 The calcineurin pathway of *Candida albicans*

C. albicans calcineurin consists of two subunits: a catalytic subunit A (Cna1/Cmp1) and a regulatory B subunit (Cnb1). Deletion of either subunit abrogates calcineurin activity (Cruz, Goldstein et al. 2002; Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003). Independently, three groups deleted either *CNA1/CMP1* or *CNB1* and observed similar phenotypes for the calcineurin mutants. Deletion of calcineurin resulted in cells that were sensitive to

multiple stress conditions, including cell membrane stress (SDS, azoles), cation stress (Li, Ca, Na), high pH, and ER stress (tunicamycin) (Cruz, Goldstein et al. 2002; Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003). Despite an initial report that calcineurin may affect filamentation of *Candida albicans*, this phenotype has only been observed in one of multiple deletion mutants constructed (Sanglard, Ischer et al. 2003; Bader, Schroppel et al. 2006).

Deletion of calcineurin A or calcineurin B resulted in avirulence in a murine tail vein model of systemic candidiasis with all mice infected with the deletion strain surviving (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003). Histology of the organs at the termination of the experiment revealed nearly complete clearing of the kidneys with only scarce small foci of infection (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003). However, the mechanistic explanation for the avirulence of these mutants was initially uncertain, since calcineurin mutants were not defective for factors commonly associated with virulence in *C. albicans*, including adherence, filamentation, the ability to injure host cells, and growth at high temperature (Cutler 1991; Filler, Swerdloff et al. 1995; Lo, Kohler et al. 1997; Mitchell 1998; Sundstrom 1999; Calderone and Fonzi 2001; Berman and Sudbery 2002; Felk, Kretschmar et al. 2002; Sundstrom, Balish et al. 2002; Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003; Naglik, Albrecht et al. 2004; Bader, Schroppel et al. 2006). However, subsequent experiments suggested that *C. albicans* avirulence may be attributable to a sensitivity towards serum (Blankenship, Wormley et al. 2003; Blankenship and Heitman 2005). While wild-type *Candida* possesses the ability to grow robustly in serum (FBS, human, murine, ovine), calcineurin mutants are killed, thus the deletion strains may not survive transit through the bloodstream to disseminate and embed in target organs (Blankenship and Heitman 2005). Fractionation of serum to determine the lethal component showed

that it was a small molecule (<3kD), and that it was not a protein, peptide, or lipid moiety. Using chelation and add back experiments calcium was identified as the toxic component of serum (Blankenship and Heitman 2005).

Current work focuses on elucidating other components of the calcineurin signaling pathway in *C. albicans*. The homologs of known *S. cerevisiae* components were identified in *C. albicans*, including the downstream transcription factor Crz1 (Onyewu, Wormley et al. 2004; Santos and de Larrinoa 2005; Karababa, Valentino et al. 2006). In *S. cerevisiae*, Crz1 mediates calcineurin-dependent transcription (Matheos, Kingsbury et al. 1997; Stathopoulos and Cyert 1997; Stathopoulos-Gerontides, Guo et al. 1999; Mendizabal, Pascual-Ahuir et al. 2001; Polizotto and Cyert 2001; Boustany and Cyert 2002; Yoshimoto, Saltsman et al. 2002; Cyert 2003). In response to calcium or under stress conditions calcineurin dephosphorylates cytoplasmic Crz1 allowing it to translocate to the nucleus and activate the transcription of calcineurin-dependent genes involved in cell wall maintenance and ion homeostasis, such as *FKS2* and *PMC1* (Matheos, Kingsbury et al. 1997; Stathopoulos and Cyert 1997; Stathopoulos-Gerontides, Guo et al. 1999; Mendizabal, Pascual-Ahuir et al. 2001; Polizotto and Cyert 2001; Boustany and Cyert 2002; Yoshimoto, Saltsman et al. 2002; Cyert 2003). Extensive work has demonstrated that *Candida albicans* Crz1 indeed functions downstream of calcineurin (Onyewu, Wormley et al. 2004; Santos and de Larrinoa 2005; Karababa, Valentino et al. 2006). Crz1 was shown to shuttle from the cytoplasm to the nucleus in a calcium- and calcineurin-dependent manner (Santos and de Larrinoa 2005; Karababa, Valentino et al. 2006). Furthermore, microarray studies examining the transcriptional response to calcium in calcineurin and *crz1* deletion strains found 69 and 65 genes, respectively, that were differentially regulated. Of those genes identified, 60 were common to both proteins (Karababa, Valentino et al. 2006), suggesting that Crz1 is likely the primary transcription factor target of calcineurin in response to calcium stimulation in *C. albicans*.

However, the phenotypes of a *crz1* mutant strain do not completely recapitulate those of a calcineurin mutant. Although *crz1* deletion strains are sensitive to membrane and cation stresses they have an intermediate phenotype compared with calcineurin mutants that can be further augmented by the addition of a calcineurin inhibitor (Onyewu, Wormley et al. 2004; Santos and de Larrinoa 2005; Karababa, Valentino et al. 2006). Furthermore, *crz1* mutants are not sensitive to serum, and show only a modest decrease in virulence (Onyewu, Wormley et al. 2004; Santos and de Larrinoa 2005; Karababa, Valentino et al. 2006). Thus other, as yet unidentified, downstream effectors of calcineurin play important roles in mediating these phenotypes.

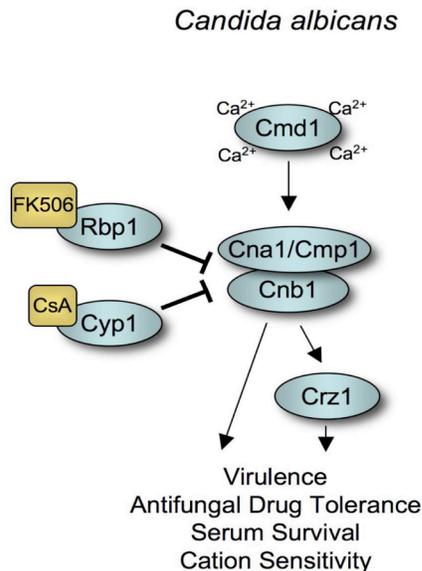


Figure 1: Calcineurin signaling pathways of *C. albicans*

One of the genes highly induced in a calcineurin-, Crz1-dependent manner is *UTR2* encoding a member of the CRH family of GPI-dependent cell wall proteins (Karababa, Valentino et al. 2006; Pardini, De Groot et al. 2006). The role of the CRH family (*UTR2*, *CRH11*, and *CRH12*) with respect to calcineurin related phenotypes was examined through the isolation of single and multiple deletion mutants (Pardini, De Groot et al. 2006). Although these mutants shared some calcineurin related phenotypes,

such as sensitivity to cell membrane stress and a virulence defect in mice, deletion of these genes did not recapitulate the complete phenotypic profile of calcineurin mutants (Pardini, De Groot et al. 2006).

Interestingly, the influence of calcineurin on the virulence potential of *C. albicans* appears to be host niche specific. Initial studies demonstrated that calcineurin mutants were avirulent in murine models of disseminated candidiasis (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003), but subsequent studies demonstrated no role for calcineurin in vaginal or pulmonary models of candidiasis (Bader, Schroppel et al. 2006). However, a murine keratitis model demonstrated that calcineurin mutants establish less robust infections than wild type cells and have accelerated disease resolution (Onyewu, Afshari et al. 2006). This raises important questions regarding the medical application of therapy with calcineurin inhibitors. Calcineurin inhibitors may have the intrinsic ability to interfere with the natural progression of disseminated candidiasis and ocular infections, but treatment with a calcineurin inhibitor alone may not be appropriate for vaginal or pulmonary infections. Instead, the clinical utility of calcineurin inhibitors in these settings may be in combination therapy with azoles (or other antifungals). A proof of principle, *in vivo* rat model of endocarditis demonstrated superiority of combination high dose fluconazole and cyclosporine A over monotherapy with either fluconazole, Amphotericin B, or cyclosporine A at treating both primary vegetative lesions and kidney lesions formed by hematogenous dissemination (Marchetti, Entenza et al. 2000). Similarly, the combination of fluconazole and cyclosporine A demonstrated *in vivo* synergy in a murine keratitis model, resulting in faster resolution of infection than treatment with fluconazole alone (Onyewu, Afshari et al. 2006). The current use of calcineurin inhibitors to augment antifungal therapy is hindered by their immunosuppressive effects, which likely outweigh the antifungal properties. However, recent studies suggest that

other mechanisms exist to inhibit calcineurin activity. Hsp90 is a molecular chaperone recently shown to facilitate the evolution of rapid resistance to fluconazole in *S. cerevisiae* and *C. albicans* (Cowen and Lindquist 2005; Cowen, Carpenter et al. 2006). Furthermore, it was found that calcineurin, a client protein of Hsp90, was the key effector of Hsp90 mediated azole resistance. Hsp90 inhibitors, such as geldanamycin, are commercially available anticancer agents that inhibit Hsp90 by binding its ATP binding pocket resulting in degradation of the client proteins (Roe, Prodromou et al. 1999). Initial *in vitro* experiments have demonstrated that Hsp90 inhibitors, like calcineurin inhibitors, can act synergistically with azole antifungals (Cowen and Lindquist 2005; Heitman 2005; Cowen, Carpenter et al. 2006). Thus, there is great clinical potential for harnessing the Hsp90-calcineurin pathway as a means of augmenting current antifungal treatment strategies.

1.5 Mating and life cycle of *Candida* species

The genus *Candida* affords an excellent opportunity to study the evolution of sexual reproduction, as this genus contains closely related asexual and sexual species. Historically, *Candida* described a group of yeasts that lacked sexual cycles and ascospore production, propagated through asexual budding, and formed pseudo- and/or true hyphae. However, some of the 151 *Candida* species were subsequently found to represent the anamorphic form of a teleomorphic species, and thus are capable of sexual reproduction and sporulation, either alone or when mated with cells of a compatible mating type. Many of the species within this clade are human pathogens, and thus understanding the life cycle of these organisms and developing robust genetic systems for the study of these species is of considerable import. The best-studied to date are the human pathogens *C. albicans* and *C. glabrata*. However, neither of these species is as yet known to possess a complete meiotic sexual cycle.

Candida lusitaniae and *Candida guilliermondii* are two members of this genus that are exciting prospects for further study. Firstly, both possess complete sexual cycles. Secondly, they are opportunistic human pathogens. And lastly, the genomes of *C. lusitaniae* and *C. guilliermondii* have been sequenced, which will facilitate robust comparative genomic studies. These organisms are attractive candidates for the development of classical genetic systems for the study of mating, meiosis, sporulation, and virulence in the *Candida* species complex. Furthermore, comparisons between the sexual and asexual *Candida* species will help elucidate the mechanisms and evolutionary timing of the loss or modification of sexual reproduction within this complex genus and provide insights into the evolution of signaling cascades with dual roles in mating and virulence.

Table 1: *Candida* species with known teleomorphs

Anamorph	Teleomorph	Sexual Type
<i>Candida bimbundalis</i>	<i>Pichia americana</i>	Heterothallic
<i>Candida ciferrii</i>	<i>Stephanoascus ciferrii</i>	Heterothallic
<i>Candida edax</i>	<i>Stephanoascus smithiae</i>	Heterothallic
<i>Candida famata</i>	<i>Debaryomyces hansenii</i>	Homothallic
<i>Candida guilliermondii</i> var. <i>guilliermondii</i>	<i>Pichia guilliermondii</i>	Heterothallic
<i>Candida guilliermondii</i> var. <i>membranaefaciens</i>	<i>Pichia ohmeri</i>	Heterothallic
<i>Candida kefyri</i>	<i>Kluyveromyces marxianus</i>	Homothallic
<i>Candida krusei</i>	<i>Issatchenkia orientalis</i>	Heterothallic
<i>Candida lambica</i>	<i>Pichia fermentans</i>	Homothallic
<i>Candida lipolytica</i>	<i>Yarrowia lipolytica</i>	Heterothallic
<i>Candida lusitaniae</i>	<i>Clavispora lusitaniae</i>	Heterothallic
<i>Candida norvegensis</i>	<i>Pichia norvegensis</i>	Homothallic
<i>Candida pelliculosa</i>	<i>Pichia anomala</i>	Heterothallic
<i>Candida pintolopesii</i>	<i>Arxiozyma telluris</i>	Homothallic
<i>Candida pulcherrima</i>	<i>Metschnikowia pulcherrima</i>	Homothallic
<i>Candida sorbosa</i>	<i>Issatchenkia occidentalis</i>	Heterothallic
<i>Candida utilis</i>	<i>Pichia jadinii</i>	Homothallic
<i>Candida valida</i>	<i>Pichia membranifaciens</i>	Heter/Homothallic

1.6 Evolutionary relationships of the *Candida* species complex

Because species belonging to the genus *Candida* were classified originally quite broadly (based primarily upon their vegetative growth as budding yeast, ability to produce pseudohyphae or true hyphae, and lack of a sexual cycle), the resulting genus is polyphyletic. Multiple phylogenies have been constructed containing either some or all

of the medically relevant *Candida* species. Most published phylogenies are based on single genes or regions such as the large or small ribosomal subunits (Barns, Lane et al. 1991; Lott, Kuykendall et al. 1993; Cai, Roberts et al. 1996; Kurtzman and Robnett 1998), topoisomerase II (Kato, Ozeki et al. 2001), cytochrome b (Yokoyama, Biswas et al. 2000) or the actin 1 gene (Daniel, Sorrell et al. 2001). The most robust analysis containing all of the pathogenic *Candida* species, including the sexual species *C. lusitaniae* and *C. guilliermondii*, employed 6 nuclear genes (4 protein encoding genes: *EF2*, *ACT1*, *RPB1*, and *RPB2*, and the 18S and the 26S rDNA genes) to construct a phylogeny (Diezmann, Cox et al. 2004). These analyses have shown that *C. guilliermondii* and *C. lusitaniae* are closely related to *C. albicans*. In addition to these medically relevant *Candida* species, there are other known *Candida* species that possess sexual cycles (Table 1) (Kwon-Chung and Bennett 1992; Kurtzman 1994; Hazen 1995; Barnett, Payne et al. 2000; Daniel, Sorrell et al. 2001; Kurtzman, Robnett et al. 2005).

Another interesting character of the *Candida* genus is the evolution of CUG codon reassignment. This non-universal codon usage was first recognized in the asexual species *Candida cylindracea* (Kawaguchi, Honda et al. 1989; Yokogawa, Suzuki et al. 1992). Subsequently, many *Candida* species were shown to translate CUG codons as serine rather than leucine. The tRNA responsible for this transition arose from a serine tRNA approximately 270 million years ago, prior to the divergence of the *Candida* and *Saccharomyces* lineages (Santos, Keith et al. 1993; Suzuki, Ueda et al. 1994; Ueda, Suzuki et al. 1994; Santos and Tuite 1995; Sugita and Nakase 1999; Massey, Moura et al. 2003). The “ambiguous intermediate theory” suggests that the common ancestor could decode CUG as either serine or leucine, and that codon reassignment occurred only after divergence approximately 170 million years ago (Tuite and Santos 1996; Santos, Cheeseman et al. 1999; Massey, Moura et al. 2003). This theory is supported by evidence that some *Candida* species are still able to decode CUG as either serine or

leucine, such as *Candida zeylanoides* (Suzuki, Ueda et al. 1997). Both *C. guilliermondii* and *C. lusitaniae*, like *C. albicans*, decode CUG as serine; however, *C. krusei* translates this codon as leucine similarly to *C. glabrata* and *S. cerevisiae* (Pesole, Lotti et al. 1995; Young, Lorenz et al. 2000).

1.7 *Candida lusitaniae* (teleomorph *Clavispora lusitaniae*)

C. lusitaniae was first described in 1970 by van Uden and do Carmo-Sousa, who isolated the organism from the gastrointestinal tract of animals (van Uden and Buckley 1970). Of the two sexual species discussed in this introduction, the sexual lifecycle has been most well-studied for this species. The teleomorphic form of *Candida lusitaniae*, *Clavispora lusitaniae*, was described by Rodrigues de Miranda in 1979 after noting mating between previously collected strains and a newly isolated sample from citrus peel juice (Kurtzman and Fell 1998; Lachance and Phaff 1998). *C. lusitaniae* can be isolated from human and animal samples, as well as from environmental sources such as decaying trees and fruit (Kwon-Chung and Bennett 1992; Barnett, Payne et al. 2000). In the clinic, *C. lusitaniae* accounts for ~1% of candidemia, but is notable for its propensity to develop resistance to amphotericin B (Ahearn and McGlohn 1984; Merz 1984; Blinkhorn, Adelstein et al. 1989; Merz, Khazan et al. 1992; Yoon, Vazquez et al. 1999).

C. lusitaniae is an experimentally tractable haploid organism. The genome sequence completed by the Broad Institute reveals a genome size of 16 Mb arranged into eight chromosomes (*Candida*; Logue, Wong et al. 2005). CHEF gel analyses of multiple isolates observed six to eight distinguishable chromosomal bands (Vazquez, Beckley et al. 1993; King, Rhine-Chalberg et al. 1995). The haploid nature of the genome makes *C. lusitaniae* a more attractive species for manipulation than the diploid *C. albicans*. Techniques for transformation and gene disruption by homologous recombination have

already been established for *C. lusitaniae* (Young, Lorenz et al. 2000; Young, Hull et al. 2003; Francois, Chapeland-Leclerc et al. 2004).

Mating between strains of *C. lusitaniae* is consistent with a heterothallic species containing a single mating type determining locus. Historically, the two mating types in this biallelic system were designated h⁺ and h⁻, but were subsequently referred to as **a** and α , respectively (Kurtzman and Fell 1998; Lachance and Phaff 1998). Original studies suggested that the ratio of α to **a** cells in the environment was skewed 6:1 (Gargeya, Pruitt et al. 1990). However, a larger study of 76 clinical isolates from 60 patients demonstrated an equal distribution of mating types, and also revealed no correlation between mating type and severity of disease or site of isolation (Gargeya, Pruitt et al. 1990; François, Noël et al. 2001). In this larger study, all isolates of *C. lusitaniae* tested were capable of mating (Rodrigues de Miranda 1979; Francois, Noel et al. 2001).

Identification of *C. lusitaniae* in the clinical setting is usually accomplished based on carbon assimilation profiles, most notably the ability to assimilate L-rhamnose, and to ferment cellobiose (Ramani, Gromadzki et al. 1998; Michel-Nguyen, Favel et al. 2000). However, assimilation profiles cannot reliably type all strains. Due to the robustness of mating, it was proposed that ability to mate with tester strains could be used as a technique for positively identifying *C. lusitaniae* isolates (François, Noël et al. 2001; Noël, Favel et al. 2005). As a proof of principle, five strains that were indistinguishable as either *C. lusitaniae* or *C. pulcherrirria* using standard clinical carbon assimilation profiles were positively identified as *C. lusitaniae* based upon mating preference (Noël, Favel et al. 2005). However, other studies have suggested that the efficiency of sporulation can vary among isolates (Gargeya, Pruitt et al. 1990; Lachance, Daniel et al. 2003).

Although *C. lusitaniae* is regarded as heterothallic, there is a single report of a strain capable of producing spores in the absence of a mating partner, either through

isogamous conjugation or bud-parent cell conjugation (Lachance, Daniel et al. 2003). Sequencing of a portion of the large ribosomal subunit demonstrated that this strain was similar to other isolates of *C. lusitaniae* (Lachance, Daniel et al. 2003); however, no further studies have been conducted with this strain. One possibility is that this isolate could be a diploid; however, there have been no recorded accounts of diploids being isolated either in nature or in the laboratory as products of mating events. Presumably, diploids formed by mating rapidly undergo meiosis and thus only haploid cells have been isolated from matings and the environment. Interestingly, sequence analysis of the D1/D2 domain of the large-subunit rDNA revealed the presence of significant polymorphisms within an interbreeding population of *C. lusitaniae* (Lachance, Daniel et al. 2003). There was no association between these polymorphisms and mating type, suggesting that there is recombination and sexual reproduction within the natural population.

Multiple types of media are capable of inducing mating including dilute potato dextrose agar (PDA), 1% malt extract media, sodium acetate, yeast carbon base, V8, and SLAD (Young, Lorenz et al. 2000; François, Noël et al. 2001). Effective matings are supported by low concentrations of ammonium and require a solid support, as increasing the concentration of ammonium sulfate or incubating cells in liquid media blocked mating (Lachance, Nair et al. 1994; Young, Lorenz et al. 2000; François, Noël et al. 2001). Additionally, the efficiency of conjugation and ascospore formation is optimal at temperatures between 18 and 28°C (François, Noël et al. 2001). Within 24 hours of co-incubating cells of opposite mating type, conjugating cells can be observed. Scanning and transmission electron microscopy revealed that the conjugation tube bridging a mating **a** and α cells averages 1 μm in length (François, Noël et al. 2001).

At the point of cell fusion, the conjugation tube contains a central septal perforation through which nuclear transfer presumably occurs. The nucleus of one parent

traverses the conjugation tube, undergoes karyogamy, and meiosis then occurs inside the mating partner cell. The parent that donates a nucleus is referred to as the “head cell” and remains grossly unchanged throughout the process of meiosis and ascospore formation. The other parent, the nucleus acceptor, becomes the ascus. Labeling studies suggest that the nuclear transfer is highly polarized with one parental strain in a mating serving primarily as the nucleus donor (François, Noël et al. 2001). Based upon the small set of strains tested there was no definitive linkage between the mating type of the parent and whether the cell served as a nuclear donor or acceptor (François, Noël et al. 2001). However, a more robust analysis using multiple labeling techniques is necessary to establish this point rigorously. After 48 hours of incubation, one to four clavate (majority are dyads and some monads), echinulate ascospores are formed sometimes containing a small oil droplet (Rodrigues de Miranda 1979; Gargeya, Pruitt et al. 1990; François, Noël et al. 2001). Ascospores are readily released upon maturation and often agglutinate; empty asci and free ascospores can be readily observed at 72 hours (Rodrigues de Miranda 1979; François, Noël et al. 2001).

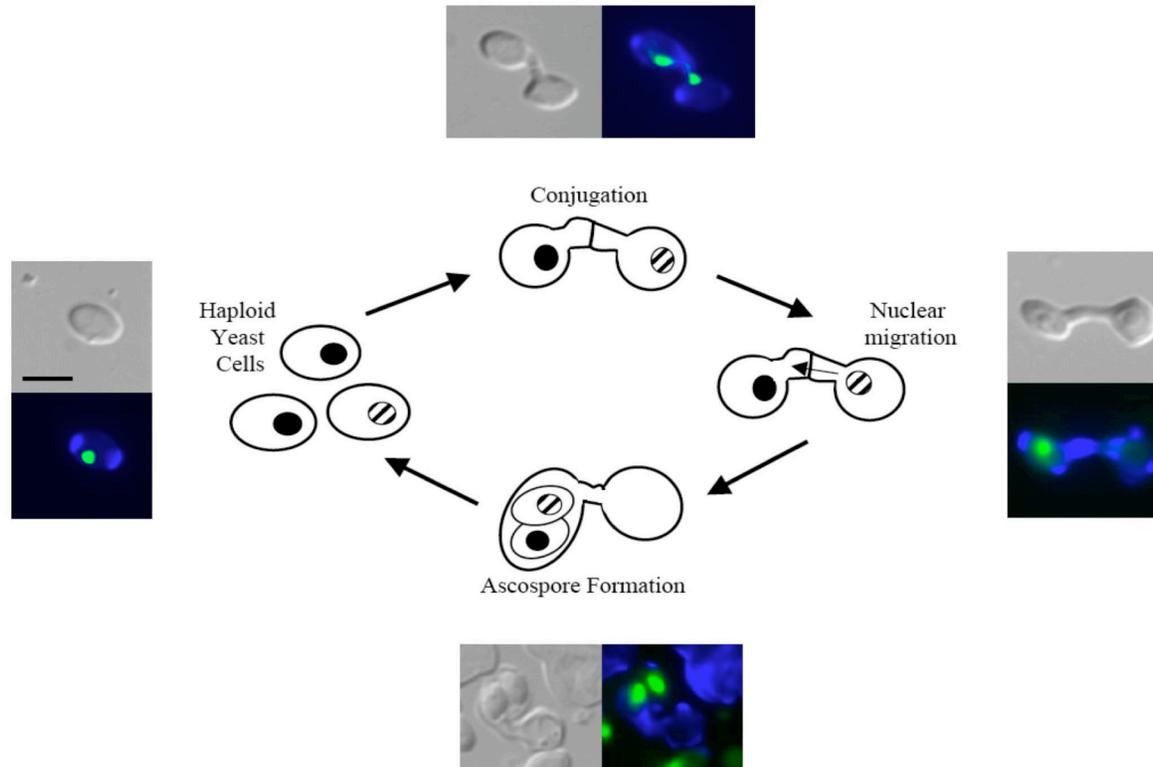


Figure 2: Life cycle of *Candida lusitanae*

Mating of *C. lusitanae* on dilute Potato Dextrose Agar (PDA) with cells stained with calcofluor white and sytox green. Cells of opposite mating type (denoted by differently shaded nuclei) were mixed together on a solid surface under nutrient limiting conditions. At 24 hours, cellular conjugation is observed. By 48 hours, nuclear transfer has occurred and at 72 hours ascospore formation can be observed. While one to four ascospores have been reported to form per asci, the vast majority of asci contain 2 ascospores (72 to 81% dyads, the rest are monads with a single spore). By 96 hours (not shown), matings contain many disrupted asci from which the ascospores have been liberated.

An initial study to elucidate the signaling pathways controlling mating in *C. lusitaniae* focused on homologs of the *S. cerevisiae* MAP kinase pheromone response cascade. In *S. cerevisiae* the MAP kinase pheromone response cascade regulates both mating and filamentation (pseudohyphae) (Liu, Styles et al. 1993). Mutation of the Ste12 transcription factor results in loss of mating ability (Hartwell 1980), and a filamentation defect in *S. cerevisiae* (Liu, Styles et al. 1993). The *C. albicans* homolog of Ste12 is Cph1, the deletion of which reduced pseudohyphal filamentation in response to certain environmental stimuli (Liu, Kohler et al. 1994) and also blocked mating between homozygous **a** and α strains (Magee, Legrand et al. 2002). On rich media, *C. lusitaniae* propagates via budding, but can form pseudohyphae and hyphae under conditions of nutrient deprivation on V8, filament, or SLAD media (Rodrigues de Miranda 1979; Kurtzman and Fell 1998). Interestingly, the *C. lusitaniae* Ste12 homolog, Cls12, is required for mating, but dispensable for filamentation under the conditions studied (Young, Lorenz et al. 2000). The divergent functions of this conserved transcription factor demonstrate how studying both sexual and asexual species within the genus *Candida* provides interesting insights into the evolution of signal transduction cascades involved in mating and filamentation, but also in other pathways important for pathogenesis.

1.8 *Candida guilliermondii*

Candida guilliermondii is a haploid heterothallic yeast whose teleomorphic form is *Pichia guilliermondii*. The anamorph has been cultured from a variety of ecological niches, including human clinical specimens, insects, fruit, and decaying matter (Kwon-Chung and Bennett 1992; Barnett, Payne et al. 2000). Depending upon the geographical location, *C. guilliermondii* has been identified as the causative agent in 2% to 10% of all candidal bloodstream infections (Sandven 2000; Krcmery and Barnes 2002). Therefore, most studies of this fungus have been oriented toward topics such as clinical

epidemiology and antifungal susceptibility, and relatively little work has been done to characterize the sexual cycle and the pathways involved in sexual reproduction, meiosis, and sporulation. Vegetative yeast cells are ellipsoidal or ovoid, reproduce via budding, and can form pseudohyphae, but not true hyphae (Barnett, Payne et al. 2000).

Early studies employing pulsed-field gel electrophoresis suggest that, unlike *C. albicans*, *C. guilliermondii* is a haploid yeast with a genome size of approximately 12 Mb (Doi, Homma et al. 1992). The number of chromosomal bands observed has ranged from six to eight (Magee and Magee 1987; Doi, Homma et al. 1992; Bai 1996). The Broad Institute recently released a 12X first draft of the *C. guilliermondii* genome organized into nine supercontigs which confirms the estimated genome size (Lorenz, Wells et al. 2004).

The sexual cycle of *C. guilliermondii* was first identified in 1952 by Wickerham and Burton, after recognizing that some yeasts previously classified to non-ascospore forming genera actually represented the anamorphic form of a sexual species (Wickerham and Burton 1954). The teleomorph *Pichia guilliermondii* was formally described in 1966 (Wickerham 1966) and subsequent DNA complementarity tests confirmed the teleomorph-anamorph relationship (Kurtzman 1992). The initial matings were carried out on malt extract sporulation media at 25⁰C for 6 to 14 days. Positive mating mixtures were characterized by the presence of conjugating cells and ascospore formation. The rate of sporulation is relatively low; the most efficiently sporulating pair produced only 4% ascospores (Wickerham 1966). In an attempt to increase ascospore formation, germinated ascospores were backcrossed with the parental mating competent strains with the aim of producing a strain with a higher mating efficiency; however, this was unsuccessful (Wickerham and Burton 1954). The asci are formed from two conjugated cells and contain one or two hat-shaped spores. Upon maturation the asci rupture releasing the ascospores, which become swollen and refractile (Wickerham and Burton 1954; Wickerham 1966).

Recently, some cryptic species were identified within the *C. guilliermondii* clade (Bai 1996; San Millán, Wu et al. 1997; Bai, Liang et al. 2000; Vaughan-Martini, Kurtzman et al. 2005). Notably *Candida fermentati* and *Candida carpophila*, although phenotypically indistinguishable from *C. guilliermondii*, were shown to be genetically different on the basis of DNA reassociation and electrophoretic karyotyping and thus were described as separate species (Vaughan-Martini, Kurtzman et al. 2005). Additionally, it was noted that strains of *Candida fermentati* were capable of ascospore formation when mixed together, and this teleomorphic form was designated *Pichia caribbica* (Vaughan-Martini, Kurtzman et al. 2005). Thus one possibility for the low rate of ascospore formation described previously within the *C. guilliermondii* clade could be the presence of cryptic species within this group that are incapable of mating.

Although low efficiency sexual reproduction has been reported in the laboratory, it is unclear what role sexual reproduction plays in the environment. MLST analysis of 32 strains of *C. guilliermondii* isolated from Ontario (Canada), China, and the Philippines suggest that the population is primarily clonal (Lan and Xu 2006). However, due to the small sample size and the limited molecular variation among the isolates, sexual reproduction among natural populations could not be excluded. To further address this question, a larger population and the identification of more markers for typing of strains will be required.

1.9 The evolution/ structure of the mating type locus in the *Candida* species complex

The most well-studied species in the *Candida* genus are *Candida albicans* and *Candida glabrata*. *C. glabrata* is more closely related to *S. cerevisiae* than to *C. albicans*, which is reflected in the organization of its mating type locus. The structure of the *MTL* locus of *C. glabrata* is similar to that in *S. cerevisiae* in that *C. glabrata* contains three

mating type like loci, containing either **a** or α specific information (Srikantha, Lachke et al. 2003). Mating has never been observed in *C. glabrata*. *C. albicans* was also thought to lack a sexual cycle; however, in 1999 the mating type like locus (*MTL*) of *C. albicans* was identified (Hull and Johnson 1999). The majority of isolates were found to be *MTL* heterozygotes possessing both **a** and α information rendering them incapable of mating. Strains engineered to possess only **a** or α information, and naturally occurring **a/a** or α/α isolates (approx. 3 - 10% of clinical samples) are capable of mating (Hull, Raisner et al. 2000; Magee and Magee 2000; Lockhart, Pujol et al. 2002; Legrand, Lephart et al. 2004). These strains can switch from the white to the opaque (mating efficient) cell type and can subsequently undergo shmooing and cell fusion (Lockhart, Pujol et al. 2002; Miller and Johnson 2002; Lachke, Lockhart et al. 2003; Lockhart, Daniels et al. 2003; Soll, Lockhart et al. 2003; Legrand, Lephart et al. 2004). The product of mating is a tetraploid **a/a/ α/α** yeast cell that can be induced to undergo a parasexual cycle of chromosome loss *in vitro* to return to a diploid state (Bennett and Johnson 2003).

The *MTL* locus alleles of *C. albicans* contain genes encoding homologs of the transcription factors **a1**, $\alpha1$ and $\alpha2$, the primary regulators of cell type in *S. cerevisiae*. The *MTLa* allele of *C. albicans* also contains the HMG-box transcriptional regulator, **a2**, which is not present in *S. cerevisiae* (Hull and Johnson 1999; Johnson 2003; Bennett and Johnson 2005). Additionally, both *MTL* loci contain three extra genes encoding: poly (A) polymerase (*PAP1*), phosphatidylinositol-4 kinase (*PIK1*), and an oxysterol binding protein (*OBP1*). The **a** and α alleles of these genes share approximately 60% identity (Hull and Johnson 1999; Johnson 2003). The function of the transcriptional regulators also differs between *S. cerevisiae* and *C. albicans*. In haploid *S. cerevisiae* an **a**-type cell is the default cell type, whereas specification as an α cell requires $\alpha1$ to turn on α specific genes, and $\alpha2$ to repress **a** specific genes. After mating, formation of an **a1/ $\alpha2$** dimer represses haploid specific genes, and promotes meiosis. The transcriptional networks

have been reconfigured in *C. albicans*, such that $\alpha 2$ activates α -specific genes and $\alpha 1$ activates α -specific genes, and the $\alpha 1 / \alpha 2$ dimer represses mating by inhibiting the white to opaque transition (Miller and Johnson 2002; Johnson 2003). Thus, *C. albicans* differs from *S. cerevisiae* in several crucial ways including the structure of the mating type loci, and control of both haploid and diploid specific gene expression.

Recently, the *MTLa* locus of *Candida parapsilosis*, an asexual species closely related to *C. albicans*, was sequenced (Logue, Wong et al. 2005). The structure of the *C. parapsilosis MTLa* locus is similar to the *C. albicans MTLa* locus, with conserved syntenic relationships among the genes contained in the locus. However, sequence analysis revealed that the $\alpha 1$ gene is a pseudogene containing four stop codons (Logue, Wong et al. 2005). Although hybridization studies suggested that *C. parapsilosis* contains α information as well, the locus could not be identified (Logue, Wong et al. 2005).

The majority of work with *Candida* has focused on the human pathogens, *C. albicans* and *C. glabrata*. *C. albicans* is the most prevalent of the *Candida* species isolated from patient specimens and *C. glabrata* has increased in incidence, but more importantly can be highly resistant to azole antifungals rendering treatment more difficult. Although, neither *C. albicans* nor *C. glabrata* are known to have a complete meiotic sexual cycle, several of the *Candida* species that infect humans are sexually competent, mate, and sporulate, and have been presumed to undergo meiosis. These include *C. lusitaniae* (of interest clinically due to a propensity to develop resistance to amphotericin B) and *C. guilliermondii*. Understanding the life cycle of these *Candida* species is important in developing a complete understanding of these pathogenic fungi. Because *C. albicans* is the most common clinically isolated species, the majority of effort has been focused on understanding this yeast. However, the lack of a complete meiotic sexual cycle has limited the use of classical genetics in *C. albicans*. In addition, heterologous expression experiments are complicated by the alternative genetic code of *C. albicans* where the CUG

codon encodes serine rather than leucine. Thus, developing sexual models in species that are more closely related to *C. albicans* may provide interesting information regarding the evolution of the signaling pathways that govern sexual reproduction, and also provide useful systems for heterologous expression studies and for comparison with *C. albicans*.

Investigations of the signal transduction pathways involved in mating and potentially meiosis / sporulation in *C. albicans* have relied heavily upon the established paradigms in *S. cerevisiae*. This reliance upon *S. cerevisiae* was natural, as it is the most closely related ascomycete to *C. albicans* in which the pathways regulating sexual development have been extensively studied. Thus, to determine whether *C. albicans* can undergo meiosis (or altered forms of meiosis) and to identify the changes that potentially rendered this fungus defective in sporulation, attempts were made to find homologs of the crucial signaling components from *S. cerevisiae* in *C. albicans* (Tzung, Williams et al. 2001). These studies have demonstrated that not all of the meiosis and sporulation genes are present in *C. albicans*, but the implications of this are still unknown. A potentially more robust comparison will be between *C. albicans* and other closely related *Candida* species capable of sexual reproduction, including meiosis and sporulation.

1.10 Overview of this work

This thesis focuses on several topics crucial for our understanding of fungal life cycles and pathogenesis. In Chapter 2, we report the identification and characterization of the *MAT* loci from the sexual species *C. lusitaniae* and *C. guilliermondii*. We show that these *MAT* loci are missing key transcription factors that are required for the repression of haploid specific genes (*C. albicans*, *S. cerevisiae*) and entry into meiosis (*S. cerevisiae*) (Tsong, Tuch et al. 2007). Using mutant analysis we disrupted the retained

transcription factor genes contained in the *C. lusitaniae* MAT locus (**a1**, **a2**, and α 1) and demonstrate that, similarly to *C. albicans*, **a2** and α 1 control cell identity and are required for mating (Tsong, Tuch et al. 2007). Interestingly, we show that despite lacking its canonical partner, α 2, the **a1** transcription factor regulates meiosis and sporulation suggesting that transcriptional circuitry has been significantly rewired. Additionally, we apply a combination of meiotic mapping, *SPO11* mutant analysis, and CGH analysis to demonstrate that, despite lacking many key meiotic genes, *C. lusitaniae* is a meiotic organism. This surprising finding leads to a reevaluation of the key components necessary for meiosis, and suggests that alternative mechanisms may exist in these species. Additionally, it challenges the notion that *C. albicans* lacks meiosis based upon the production of aneuploid progeny via the reported parasexual cycle.

In Chapter 3, we analyzed invasive and non-invasive clinical isolates of *Candida spp.* from liver transplant recipients receiving FK506. We demonstrate that despite exposure to FK506 and fluconazole, none of the isolates developed resistance to the combination, suggesting that the combination of calcineurin inhibitor and azole antifungals could have clinical efficacy if non-immunosuppressant analogs of calcineurin inhibitors could be further developed to circumvent the immunosuppressive effects of calcineurin inhibition.

In Chapter 4, we use a candidate gene approach to identify components of the calcineurin signaling pathway. We identified homologs of Rcn1, Cch1, and Mid1 and use mutational analysis to determine the role that these genes play in mediating tolerance to numerous stresses including cations, serum, and azole drugs. Although disruption mutants share some phenotypes with calcineurin mutants, none completely recapitulate the calcineurin mutant phenotype.

Finally, in Chapter 5 we study the role of Hom6 in *C. albicans* and *C. glabrata*, based on studies in *S. cerevisiae* that demonstrated a synthetic lethality between *hom6*

and *fpr1* (Arevalo-Rodriguez, Pan et al. 2004), the gene encoding FKBP12 a prolyl-isomerase that is the binding target of the immunosuppressant FK506. Synthetic lethality results from the buildup of a toxic intermediate in the methionine and threonine biosynthetic pathway as a result of deletion of *Hom6* and mutation or inhibition of FKBP12 (Arevalo-Rodriguez, Pan et al. 2004). To understand whether inhibition of *hom6* and FKBP12 could be used as a new antifungal therapeutic strategy, we deleted *HOM6* from both *C. albicans* and the more highly drug-resistant species *C. glabrata*. Studies suggest that regulation of the threonine and methionine biosynthetic pathway in *C. albicans* differs such that the synthetic lethality between *hom6* and FKBP12 inhibition no longer exists. However, in *C. glabrata* preliminary analysis suggests that, similarly to *S. cerevisiae*, *hom6* and inhibition of FKBP12 can result in cell death.

2. Mechanistic plasticity of sexual reproduction and meiosis in the *Candida* pathogenic species complex

2.1 Introduction

Candida spp. commonly infect humans to cause both mucocutaneous and life-threatening systemic infections. The majority of *Candida* species define a monophyletic clade whose evolution was punctuated by a genetic code reconfiguration ~100 mya. Historically, all *Candida* species were asexual, yet several species are known to mate and some even produce spores. *Candida albicans*, the most common fungus infecting humans, is an obligate diploid with an unusual parasexual cycle involving mating, recombination, and genome reduction but with no recognized meiosis (Hull and Johnson 1999; Hull, Raisner et al. 2000; Magee and Magee 2000; Bennett and Johnson 2003; Johnson 2003; Forche, Alby et al. 2008). Others (*Candida lusitanae*, *Candida guilliermondii*) are haploid and their mating produces spores, suggestive of complete meiotic sexual cycles (Gargeya, Pruitt et al. 1990; Kurtzman and Fell 2000; Young, Lorenz et al. 2000; Francois, Noel et al. 2001; Noel, Favel et al. 2005). The emerging pathogen *C. lusitanae* has a defined sexual cycle presumed to include meiosis based on its ability to produce spores (Kurtzman and Fell 2000; Francois, Noel et al. 2001).

To gain insight into parasexual, sexual, or asexual life cycles, multiple members of the genus *Candida* were sequenced at the Broad Institute (*Candida; Candida*). In contrast to the hypothesis that sexual species might retain meiotic homologs lost in parasexual species, the sexual species lack the same meiotic homologs as *C. albicans* and, in addition, have lost dozens of additional key meiotic genes (Geraldine Butler, pers. comm.). Comparative genomic analysis reveals these species lack multiple key meiotic components, including the recombinase Dmc1 and co-factors (Mei5/Sae3), proteins necessary for synaptonemal complex structure/function (Zip1-Zip4/Hop1), and the crossover interference pathway (Msh4/5) (Butler submitted).

Here we elucidated the structure and functions of the mating-type locus (*MAT*) and encoded products, and established *C. lusitaniae* undergoes a complete sexual cycle including meiosis. The *MAT*-encoded **a2** HMG and $\alpha 1$ α -domain factors specify **a**- and α -cell identity, whereas the **a1** homeodomain protein drives meiosis and sporulation and functions without its canonical heterodimeric partner, $\alpha 2$. Despite the apparent loss of meiotic genes, *C. lusitaniae* undergoes meiosis during sexual reproduction involving diploid intermediates, frequent *SPO11*-dependent recombination, and whole genome reduction generating haploid progeny. The majority of meiotic progeny are euploid, but approximately one-third are diploid / aneuploid and likely arise via precocious sister chromatid segregation and meiotic nondisjunction.

In summary, the cell identity and meiotic pathways have been substantially rewired, and meiotic generation of aneuploidy may expand genetic diversity (Torres, Sokolsky et al. 2007). These findings inform our understanding of sexual reproduction in pathogenic microbes and the evolutionary plasticity of the meiotic machinery, with implications for the generation and consequences of aneuploidy in biology and medicine.

2.2 Materials and Methods

2.2.1 Strains

All strains are described in Table 2. All strains are derived from strain ATCC 38533, an environmental isolate (*MATa*), or strain ATCC 42720 (*MAT α*), a clinical isolate (Broad sequence strain). Two or three independent disruption mutants of the **a1** (JLR806, JLR808, JLR809), **a2** (JLR760, JLR763), and $\alpha 1$ (JLR741, JLR755) transcription factor genes were generated and phenotypically analyzed. Three independent *spo11* strains were constructed, two in the *MAT α* background (JLR843, JLR834), and one in the *MATa* background (JLR805). Strains were confirmed by PCR and Southern blot

analysis. All strains were routinely grown on YPD (1% yeast extract, 2% BactoPeptone, 2% glucose, 2% bacto-agar (DIFCO)) medium at 30°C unless otherwise indicated.

Table 2: Strains used in Chapter 2

Strain	Genotype	Parent strain	Source/Reference
CL16	<i>MATa ura3</i>	ATCC 38533	Young, Lorenz et al. 2000
ATCC 42720	<i>MATα</i>		ATCC
CL143	<i>MATa</i>	congenic with CL16	unpublished
NRRL Y-2075	<i>MATα</i>		(Wickerham and Burton 1954; Wickerham 1966)
NRRL Y-2076	<i>MATa</i>		(Wickerham and Burton 1954; Wickerham 1966)
JLR610	<i>MATα leu2::SAT1 chx^R</i>	ATCC 42720	This study
JLR741	<i>MATα α1::SAT1</i>	ATCC 42720	This study
JLR755	<i>MATα α1::SAT1</i>	ATCC 42720	This study
JLR760	<i>MATa a2::URA3</i>	CL16	This study
JLR763	<i>MATa a2::URA3</i>	CL16	This study
JLR806	<i>MATa a1::SAT1 ura3</i>	CL16	This study
JLR808	<i>MATa a1::SAT1 ura3</i>	CL16	This study
JLR809	<i>MATa a1::SAT1 ura3</i>	CL16	This study
JLR854	<i>MATα leu2::SAT1 ura3 chx^R</i>	JLR610	This study
JLR805	<i>MATa spo11::SAT1 ura3</i>	CL16	This study
JLR834	<i>MATα spo11::URA3 leu2 chx^R</i>	JLR854	This study
JLR843	<i>MATα spo11::URA3 leu2 chx^R</i>	JLR854	This study

2.2.2 Matings/Genetic Crosses

Strains of opposite mating type were co-cultured on dilute Potato Dextrose Agar (1:10 dilution PDA, 14.5 g/L Bactoagar (Difco)) at room temperature. Overnight cultures were grown in YPD at 30°C, washed with PBS, and equivalent cell counts were mixed together and then spotted onto dilute PDA media. For the isolation of individual recombinant progeny, cells were scraped from plates, resuspended in water, serially diluted and replated onto selective media, either Yeast Nitrogen Base (YNB; DIFCO) or Synthetic Dextrose lacking leucine (SD-leu) with 10 µg/ml cycloheximide (Sigma). To

quantitate mating efficiency, after 72 hours co-incubation on dilute PDA, matings were serially diluted to selective media and mating efficiency was calculated (Miller and Johnson 2002). Briefly, each mating was plated onto media to differentially select for the parental strains. For instance, in a *URA3 leu2* × *ura3 LEU2*, the matings were plated onto SD-ura and SD-leu to estimate the number of each parental strain in the mating mixture (these numbers will also include some progeny that are prototrophic for leucine or uracil as well). Matings were also plated to media that would only support the growth of recombinant *URA3 LEU2* progeny, but not either parental strain (YNB). The number of recombinant progeny were counted and multiplied by 4 since we were selecting for only one-fourth of the progeny based upon independent assortment of chromosomes (the possible progeny genotypes are *URA3 leu2*, *URA3 LEU2*, *ura3 LEU2*, or *ura3 leu2*). Thus, to calculate the efficiency of mating and progeny formation the following formula was used: $(4 \times \# \text{ recombinant progeny}) / (\text{limiting parent} + \text{recombinants})$. While, some recombinants will be counted along with the parents in the denominator, these will only account for 1 to 4% of the total number.

2.2.3 Annotation and isolation of *MAT* loci

Genes from the characterized *C. albicans* *MTL* locus were used to BLAST search the *C. lusitaniae* and *C. guilliermondii* genome databases (Broad Institute) and homologs were identified based on best reciprocal BLAST matches. Mating-competent strains of *C. lusitaniae* (strain CL143) and *C. guilliermondii* (NRRL Y-2075) were used to isolate the opposite *MAT* allele. To isolate the opposite *MAT* allele exact primers were designed to the *MAT* flanking genes *HIP1/MAS2* and *RCY1*, while degenerate primers were designed for *PAP1*. A fragment of *PAP1* was amplified for both species using degenerate primers. For *C. guilliermondii*, exact primers to *PAP1* were then used in combination with primers to *HIP1* and *RCY1* to generate overlapping PCR fragments spanning the *MAT* locus. The

C. lusitaniae *MATa* allele was further amplified in three overlapping segments. The first fragment was amplified using exact primers to *PAP1* and *MAS2*, the second using an exact primer to *PAP1* and a degenerate primer to *PIK1*. Following sequencing of the second region, exact primers were designed to *OBP1* and used in combination with *RCY1* specific primers to amplify the right border of the *MATa* locus. Multiple independent PCR fragments were sequenced to generate at least 3X coverage across the *MAT* locus. *PAP1*, *PIK1*, and *OBP1* were annotated based upon homology and ORF annotation. The structure of the $\alpha 1$, *a2*, and *a1* genes were determined by 5' and 3' RACE (GeneRacer™; Invitrogen) to determine the intronic structure, stop, and start sites for these genes.

2.2.4 Progeny mapping

An RFLP map was generated by sequencing multiple 3 kb regions spaced across chromosomes 1, 5, and 6. Target regions were amplified by PCR from strains CL16 and JLR610, and three independent PCR reactions were used to generate at least 3X sequence coverage for each region and restriction maps were generated using MacVector® software. DNA was isolated from single colonies of each progeny strain. To determine the *MAT* configuration, a common primer that anneals to both *MAT* loci was used in conjunction with a *MATa* or *MAT α* specific primer. For RFLP typing, each locus was PCR amplified, digested, and identified as deriving from either parent *a* (0), parent alpha (1), or both *a* / alpha (2). The progeny set was generated by mating strains CL16 x JLR610 on dilute PDA for 96 hours, resuspending the mating in water, and plating various dilutions onto YNB media to select for recombinant prototrophic progeny. 94 independent colonies were selected and streaked for single colonies that were used for subsequent analysis. Recombination frequencies were calculated for each pair of loci. An independent mating was performed similarly on Yeast Carbon Base medium (YCB;

Difco) and 94 progeny were analyzed and showed similar patterns of recombination, aneuploidy, and diploidy to the cross discussed in this chapter (data not shown).

For *spo11* crosses, 78 progeny were isolated from each bilateral and unilateral mutant cross and typed for the *MAT* locus and all RFLP markers on Chromosome 6, and for one RFLP marker each on Chromosomes 1 and 5 to establish independent assortment of chromosomes.

2.2.5 CGH analysis

Unique 70-mer primers were designed to cover every ORF in the *C. lusitaniae* genome using the ArrayOligoSelector software (Bozdech, Llinas et al. 2003; Bozdech, Zhu et al. 2003). The input genomic sequence used for primer design was from the *Candida lusitaniae* genome database and the *MATa* allele sequence generated in this work. The program was run using the commands line:

```
./Pick70_script1_contig 2candida_genes 2candida_contigs 70 yes blast  
./Pick70_script2 45.0 70 1
```

Only one orf (CLUG_05253) could not be assigned a unique primer. To create a low-density CGH array we selected 93 primers (in addition to 3 *MAT* specific primers to $\alpha 1$, $\alpha 2$, and $\alpha 1$) that were evenly spaced across all 8 chromosomes (~1 primer every 124 kb), with at least two primers per chromosome arm. The oligos were synthesized by Operon and spotted onto glass slides at the Duke University DNA Microarray Facility. Three primers were excluded from the analysis due to a failure to hybridize to one or both parental strains. Genomic DNA from progeny strains was hybridized against strain JLR610 (reference strain to which the array was designed) and the average \log_2 ratios from 4 duplicate spots per array and at least 3 hybridizations (at least 12 spots total) were plotted for genome analysis. Primers are listed in Table 3.

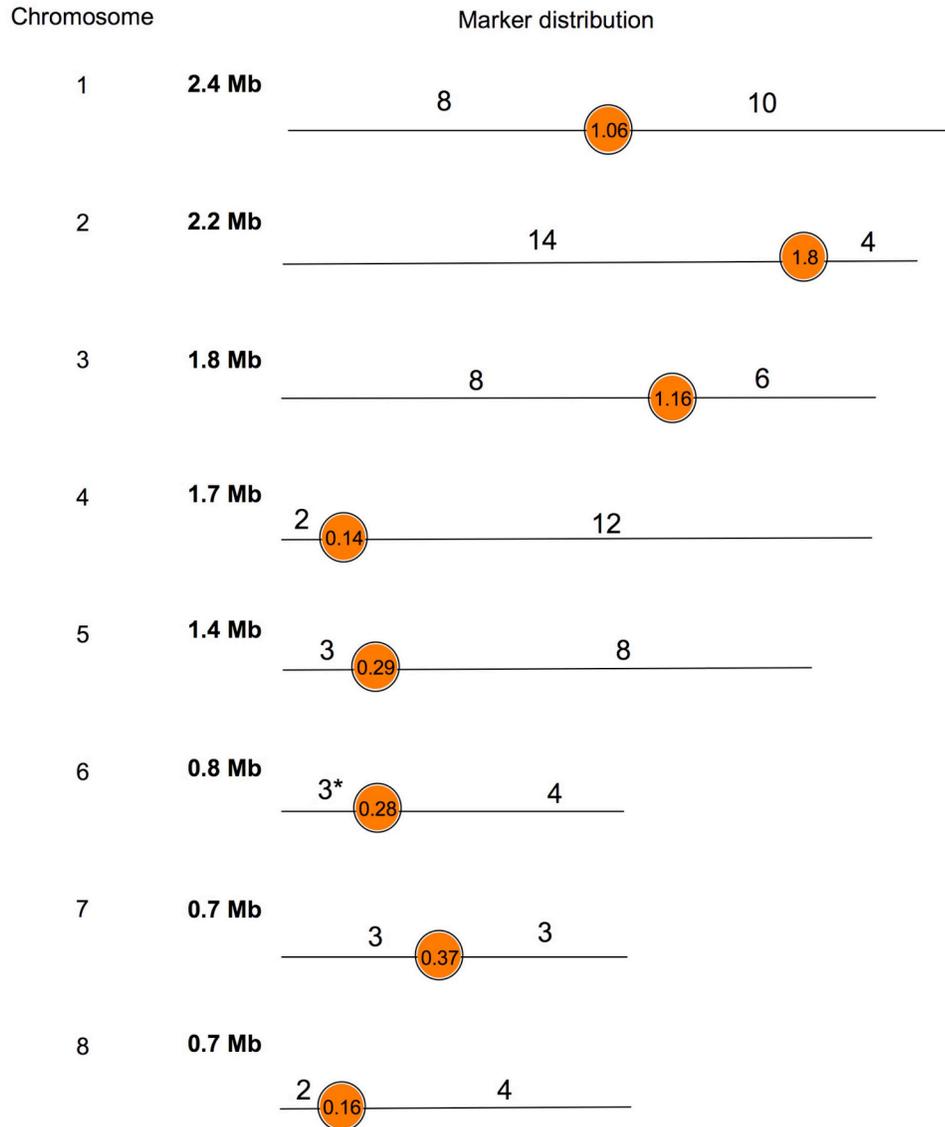


Figure 3: Chromosomal distribution of markers in CGH array

The eight chromosomes of *C. lusitaniae* are diagrammed above. The approximate size of each chromosome as determined by supercontig length from the *C. lusitaniae* genome database is indicated to the left. The orange circles indicate the putative centromere positions and the distance in Mb from the beginning of the contig. The number above each chromosome arm indicates the number of probes designed to cover that arm. The short arm of chromosome 6 contains the *MAT* locus (indicated by an asterisk); for this arm a total of 5 probes were actually generated; however, 3 of these probes are to the *MAT* specific genes *a1*, *a2*, and $\alpha 1$ which belong to essentially the same genomic locus for the purpose of the array. Thus, probes were designed against a total of 3 independent loci for this chromosomal arm (the *MAT* locus, and 2 other independent loci).

Genomic DNA was sonicated to generate ~500 bp fragments and purified with a QiaQuick PCR purification kit (QIAGEN). Five micrograms of DNA was labeled with Cy-3 dUTP or Cy-5 dUTP (Amersham/GE) using the Random Primer/Reaction Buffer mix (Bioprime Array CGH Genomic Labeling System, Invitrogen). Hybridization conditions were as described previously (Kraus, Boily et al. 2004) except the slides used were the custom designed and spotted arrays described above. After hybridization, arrays were scanned with a GenePix 4000B scanner (Axon Instruments) and analyzed using GenePix Pro version 4.0 and BRB Array Tools (developed by R. Simon and A. Peng Lam at the National Cancer Institute; <http://linus.nci.nih.gov/BRB-ArrayTools.html>).

2.2.6 FACS analysis

FACS analysis was performed as previously described (Lin, Hull et al. 2005). Cells were harvested from YPD medium, washed in PBS buffer, and fixed with 1 ml ice cold 70% EtOH overnight at 4°C. Fixed cells were washed with 1 ml NS buffer (10 mM Tris-HCl [pH 7.6], 250 mM sucrose, 1 mM EDTA [pH 8.0], 1 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM ZnCl₂) and then resuspended in 0.2 ml NS buffer containing propidium iodide (10 mg/ml) and RNaseA (1 mg/ml) at 4°C overnight. 50 µl of stained cells were diluted into 2 ml of 50 mM Tris-HCl [pH 8.0] and sonicated for 1 min. Flow cytometry was performed on 10,000 cells and analyzed on the FL1 channel with a Becton-Dickinson FACScan.

2.2.7 Gene disruption

Gene disruptions cassettes were generated by overlap PCR and contained either the *C. albicans* *SAT1* gene (amplified from plasmid pSFS2A (Reuss, Vik et al. 2004) conferring resistance to nourseothricin (NAT) or *URA3* (amplified from plasmid pAG64)

flanked by 800 to 1000 bp of gene specific sequence. The strains were transformed with 1-5 µg of DNA by a modified electroporation protocol (Reuss, Vik et al. 2004). Briefly, cells were grown to $OD_{600}=1.5$, resuspended in 0.1 M LiOAc, TE, 0.01 M DTT and incubated at room temperature for 1 hour. Cells were washed with H₂O and resuspended in 1 M sorbitol. 40 µl of cells were used per reaction. Cells were allowed to recover in YPD for 4 hours at 30°C before plating on selective media; either Synthetic Dextrose medium lacking uracil (SD-ura) or on YPD + 100 µg/ml NAT (YPD NAT).

Due to relatively short intergenic regions between genes of interest and flanking genes, only a portion of the orf was deleted by the marker insertion. The *a1* gene was disrupted using the *SAT1* gene flanked to replace residues +161 to +537 (which includes the entire second exon and homeodomain) generating strains JLR806, JLR808, and JLR809. The $\alpha 1$ gene was disrupted with the *SAT1* gene to replace residues +87 to +194 generating strains JLR741 and JLR755. The *a2* gene was disrupted using *URA3* to replace residues +115 to +540 generating strains JLR760 and JLR761 used in this study. The *SPO11* gene was disrupted using *SAT1* to generate strain *MATa* strain JLR805 and with *URA3* to generate strains *MAT α* JLR834 and JLR843. In each case, the marker replaced residues +461 to +528 (which encompasses part of the conserved hydrolase domain). All primers are listed in Table 4.

2.2.8 Nucleic acid extraction and manipulation

For northern blots, strains were either incubated alone or co-cultured on dilute Potato dextrose agar or YPD for 24 hours. Cells were harvested from the agar plates and RNA was extracted using the RiboPure™-Yeast kit (Ambion). RNA was resolved on 1.2% agarose / formaldehyde gels and transferred to Zeta-probe membranes. The membranes were probed with [³²P]dCTP labeled DNA PCR fragments. Hybridization was performed using Ultrahyb (Ambion) according to the manufacturer's instructions.

To identify the structure of the $a1$, $a2$, and $\alpha1$ genes, RNA extracted from a cross between CL16 and JLR610 was used as a template for cDNA synthesis. 5' and 3' RACE was performed using the GeneRacer kit (Invitrogen).

For CGH analysis, genomic DNA was prepared by growing 50 ml YPD cultures overnight at 30°C with shaking. The cells were washed three times with H₂O and harvested by centrifugation at 4,000g for 8 min. The cell pellet was frozen at -80°C, lyophilized overnight, and stored at -20°C. Genomic DNA extraction was performed using the CTAB protocol as described previously (Pitkin, Panaccione et al. 1996) and DNA quality was assessed by agarose gel electrophoresis.

2.2.9 Dyad dissection

Diploid a/α progeny 33 was sporulated on dilute potato dextrose agar at room temperature for 24 hours. Cells were scraped from the plate, resuspended in water and spotted dilutely onto a YPD plate for microdissection. Asci with formed ascospores were identified and the entire contents of the ascus was microdissected to a discrete location on a YPD plate. After 48 hours at 30°C, any colonies formed were streak purified onto YPD plates and incubated at 30°C to isolate single colonies. Thirty-two single colonies were isolated for each ascus and were plated onto YPD, SD-ura, SD-leu, 5-FOA, and YPD + 10 µg/ml cycloheximide to determine whether isolates of multiple genotypes could be isolated from a single ascus. Two strains of each genotype found in an ascus were subjected to FACS analysis to determine ploidy.

2.2.10 DNA replication inhibition

Diploid a/α progeny 33 (self sporulating) was grown overnight in YPD at 30°C and washed twice in H₂O. Cells were spotted onto dilute potato dextrose agar plates with or without 100 mM hydroxyurea (HU) (Sigma) and incubated at room temperature.

Cells were collected at the 0 time point and fixed in ice cold 70% ETOH at 4°C for 6 hours. At 24 hours, a time point at which the majority of wild-type diploid cells have sporulated, cells were scraped from the sporulation plates and either fixed in ice cold 70% ETOH or respotted onto dilute potato dextrose agar without HU to determine whether the cells could recover and sporulate post-HU treatment. After 24 hours on the recovery sporulation medium, cells were again scraped from the plates and fixed in ice cold 70% ETOH. At least 800 cells from each sample were microscopically examined for morphology and sporulation.

2.3 Results

2.3.1 Annotation of sequenced *MAT* alleles

First, the mating-type (*MAT*) locus of *C. lusitaniae* and *C. guilliermondii* was identified. In fungi, the *MAT* locus is a specialized region of the genome that confers sexual identity, specifying cells as α , **a**, or α/\mathbf{a} . Both species are known to be heterothallic (outcrossing) with two opposite mating-types (Reedy and Heitman 2007). *MAT* was identified by BLAST searches of the *C. lusitaniae* and *C. guilliermondii* genomes (*Candida*; *Candida*) using genes of the *C. albicans* *MTL* locus encoding the α -domain factor $\alpha 1$, polyA polymerase *Pap1 α* , phosphatidylinositol-4 kinase *Pik1 α* , and oxysterol binding protein *Obp1 α* . The *C. lusitaniae* sequenced strain is *MAT α* and homologs of four of five genes corresponding to the *C. albicans* *MTL α* locus were identified, organized into a contiguous syntenic locus. Notably, the $\alpha 2$ gene, which encodes a homeodomain cell identity factor, was missing, similar to *C. parapsilosis* (Logue, Wong et al. 2005). The *C. guilliermondii* sequenced strain contains a *MAT \mathbf{a}* allele with four genes that share identity and synteny with four of the five *C. albicans* *MTL \mathbf{a}* genes (**a2** HMG domain factor, *PAP1 \mathbf{a}* , *PIK1 \mathbf{a}* , *OBP1 \mathbf{a}*). Notably, the **a1** gene, which also encodes a

homeodomain factor, was missing. Synteny between *MAT* and flanking regions is largely conserved, except on the left *C. lusitaniae* *MAT* border where a translocation occurred.

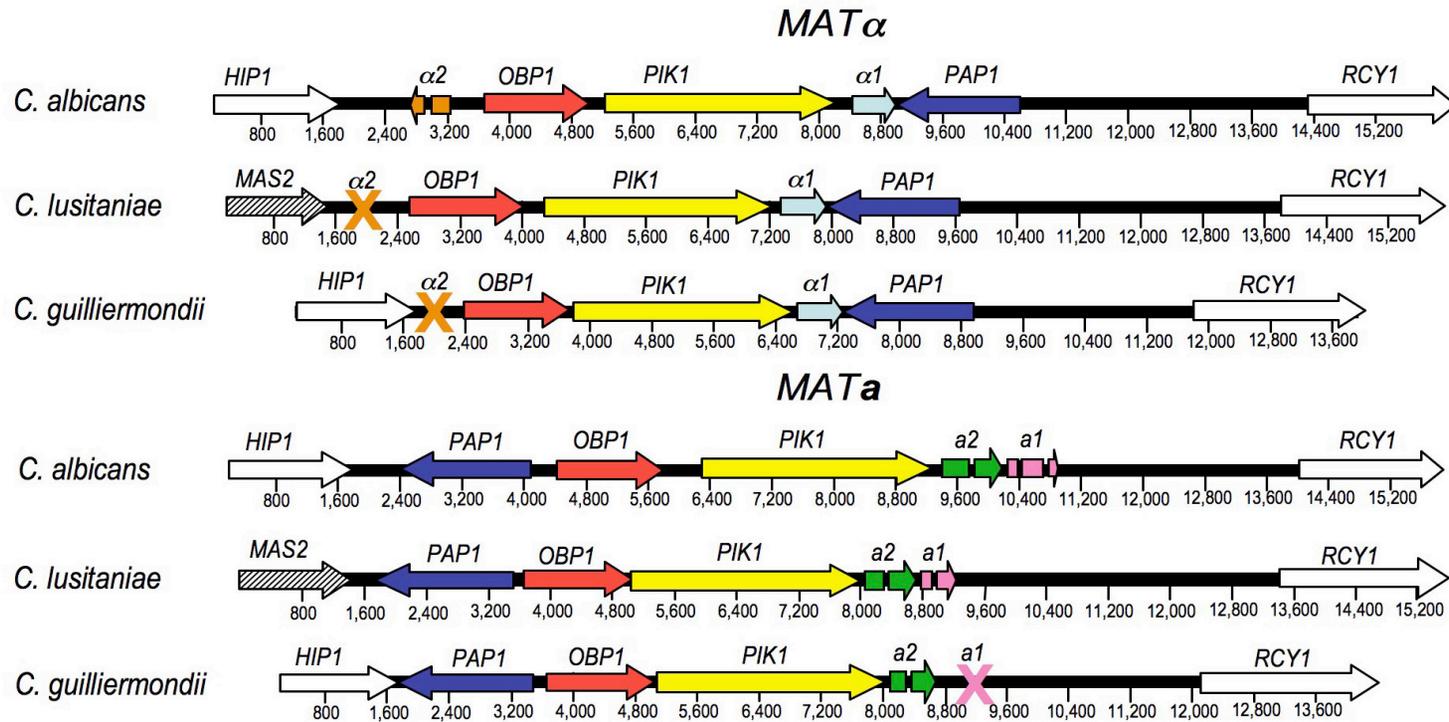


Figure 4: Mating (*MAT*) loci of *C. lusitanae* and *C. guilliermondii* are syntenic with *C. albicans*

Alignment of the *MAT* loci from the sexual species *C. lusitanae* and *C. guilliermondii* with that of the parasexual species *C. albicans*. The colored genes are contained within the *MAT* locus (*PAP1*, *OBP1*, *PIK1*, *a1*, *a2*, and $\alpha 1$), while the white/striped genes (*HIP1*, *MAS2*, and *RCY1*) flank the locus. The intron and exon structure of the *C. guilliermondii* and *C. lusitanae* *a1* and *a2* genes are shown. Crosses denote the loss of either the *a1* or $\alpha 2$ transcription factor genes. The *C. lusitanae* *MAT α* allele is 7741 bp and the *MATa* allele is 7389 bp. The *C. guilliermondii* *MAT α* allele is 9228 bp and the *MATa* allele is 9534 bp.

2.3.2 Isolation of *MAT* from an opposite mating-type partner

The opposite *MAT* alleles were isolated from fertile strains and sequenced. Interestingly, the *MAT* α alleles both lack the α 2 homeodomain gene. *C. guilliermondii* *MAT***a** also lacks the **a**1 homeodomain gene whereas this gene is retained and expressed from *C. lusitaniae* *MAT***a** based on RACE analysis. The *C. lusitaniae* *MAT***a** locus spans 7389 bp, while the *MAT* α locus spans 7741 bp. In *C. guilliermondii*, the *MAT***a** allele encompasses 9534 bp and the *MAT* α allele 9228 bp. The extent of the *MAT* loci was determined by alignment of the opposite *MAT* alleles; the beginning of the *MAT* locus was considered to be the point at which nucleotide homology between the two strains deteriorated and the end of the locus was the point at which nucleotide identity was reestablished. Consistent with these loci being *MAT* alleles and areas of low recombination, the three non-transcription factor genes contained within the locus shared significantly less nucleotide identity (60 - 80%) than genes flanking the locus (99-100%). A large percentage of the *MAT* locus from an additional fertile *MAT***a** *C. guilliermondii* strain was sequenced, particularly covering the **a**2 region where **a**1 would also likely reside if present. The sequence was largely similar to the strain sequenced by the Broad Institute and importantly there was no **a**1 gene present, suggesting this is not a strain specific phenomenon.

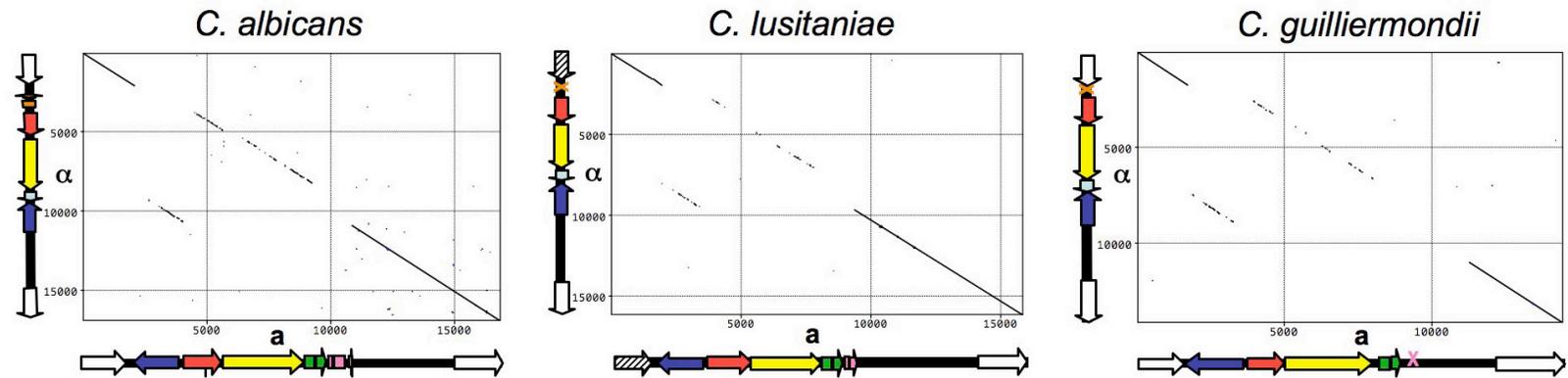


Figure 5: Gene rearrangement and reduced synteny within the *MAT* locus

Pustell DNA scoring matrices (created with MacVector[®]) demonstrating the gene rearrangement and sequence divergence between the *MAT* locus alleles of a single species. Gene identity flanking the locus is 97 – 99%, while genes within the locus share 60 – 80% identity. The *MAT* α allele is plotted on the y-axis and the *MAT**a* allele is plotted on the x-axis for each species.

To determine the intron/exon structure of the **a1**, **a2**, and $\alpha 1$ transcription factors 5' and 3' RACE was performed using cDNA from a mating mixture. Interestingly, in all species where a homolog of **a1** has been identified (including *S. cerevisiae* and *C. albicans*) it contains two well-conserved introns. Although the first intron is present in *C. lusitaniae*, the second intron is absent as determined by RACE and RT-PCR, possibly lost by gene conversion (Stajich and Dietrich 2006). In *S. cerevisiae* and *C. albicans*, **a1** and $\alpha 2$ form a heterodimer that represses haploid specific genes and, in *S. cerevisiae*, **a1** / $\alpha 2$ promotes meiotic entry (Johnson 2003; Tsong, Tuch et al. 2007). The absence of one or both components of this canonical heterodimeric cell identity determinant indicates transcriptional circuits controlling meiotic entry and haploid-specific gene repression were either lost or rewired as these sexual species diverged from the last common ancestor, possibly reflecting a transient nature of their diploid state or loss of or altered meiosis.

2.3.3 a2 and $\alpha 1$ are required for mating and haploid-specific gene expression

We hypothesized that **a2** and $\alpha 1$ may function in *C. lusitaniae* similarly to *C. albicans* where they are required for mating and the expression of haploid specific genes (Tsong, Tuch et al. 2007). In *C. lusitaniae*, **a** and α cells mate when co-cultured and nutrient limited. Mating involves pheromone production and sensing to trigger conjugation tube formation, cell and nuclear fusion, and the formation of spore-containing asci (Gargeya, Pruitt et al. 1990; Young, Lorenz et al. 2000; Francois, Noel et al. 2001). Functional roles of the **a2** and $\alpha 1$ transcription factors were assigned by genetic and phenotypic analyses of independent disruption mutants isolated for each gene. Mating was assessed by microscopy, assays that monitor complementation and

segregation of mutations during mating, and gene expression studies. Disruption of the $\alpha 1$ or **a2** genes abolished mating, and no mating structures (conjugation tubes, zygotes, asci, or spores) were observed. In patch and quantitative mating assays, wild-type **a** x wild-type α crosses yielded abundant haploid recombinant products (~14%), whereas mating efficiency was dramatically reduced (>400,000 fold) in **a2** or $\alpha 1$ mutant crosses.

Moreover, expression of the *STE2* gene (encoding the α -pheromone receptor) is weakly induced by nutrient limitation in an **a2**-dependent fashion, and co-incubation with an opposite mating-type partner leads to marked induction of *STE2* and *STE3* in wild-type cells, but not in $\alpha 1$ (*STE3*) or **a2** mutant cells (*STE2*). Therefore, **a2** and $\alpha 1$ control cell-type specific gene expression and specify **a**- or α -cell fate, analogous to *C. albicans*.

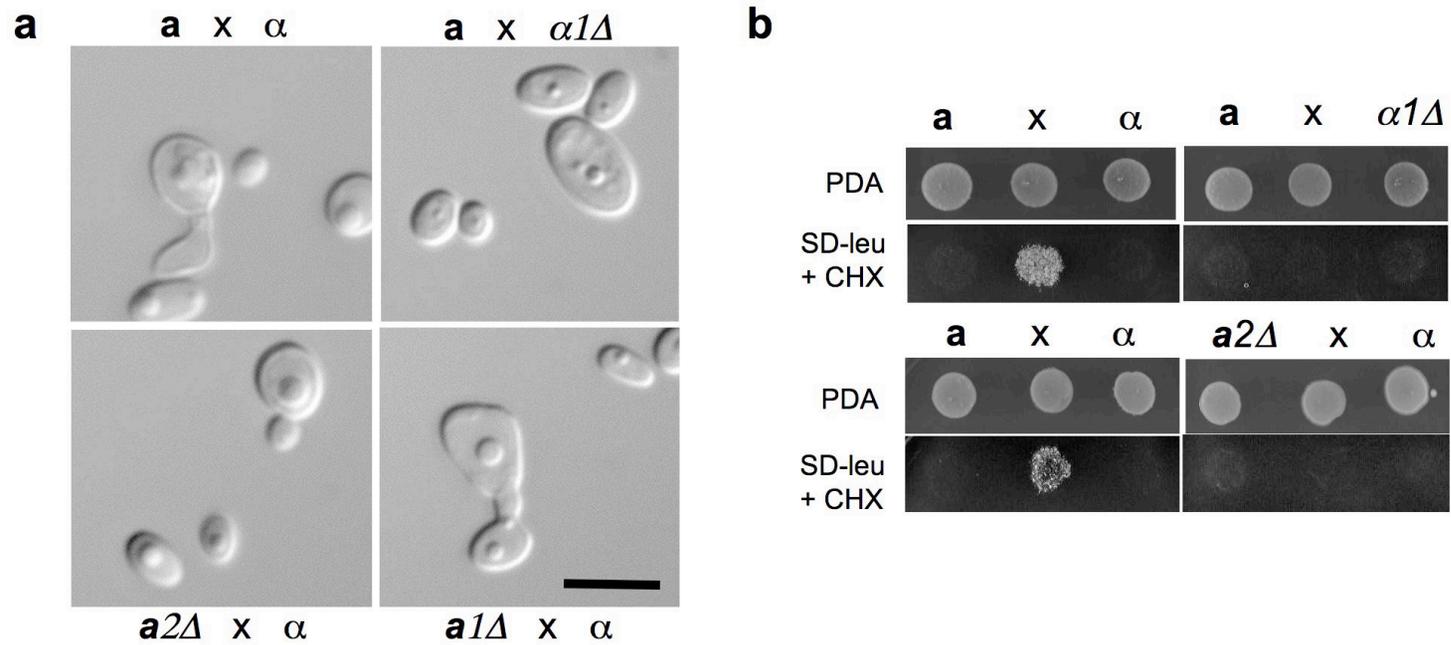


Figure 6: $\alpha 1$ and $a 2$ are required for mating

a, Representative images of *C. lusitaniae* matings at 24 hours showing a conjugated cell pair in the wild-type mating. In crosses with $\alpha 1$ or $a 2$ mutants, cells continue to bud as though an opposite mating type partner is not present, and no conjugated cells were observed. Scale bar, 10 μm . **b**, Plate matings with each parent alone flanking a mating mixture in the center. After 96 hours, the matings were replica plated to media to select for recombinant progeny. No recombinant progeny were formed in either mutant mating.

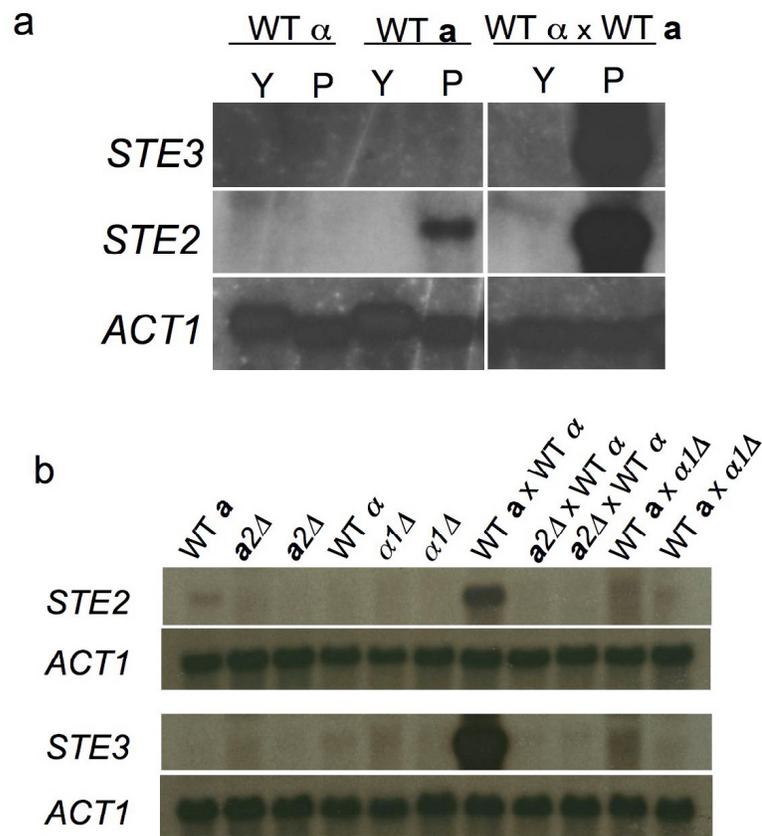


Figure 7: α 1 and a2 are required for haploid specific gene expression

a, Wild-type cells (2406 *MATa*, ATCC 42720 *MAT α*) were incubated alone or co-cultured onto YPD (Y) or dilute Potato Dextrose Agar (P) for 24 hours at room temperature. Cells were harvested, RNA extracted, and 15 μ g total RNA was run on a denaturing agarose gel. Expression of the haploid specific pheromone receptor genes, *STE2* and *STE3*, was increased during co-culture of cells on PDA mating media, but not on the rich media YPD, suggesting that a nutrient deprivation signal is required for haploid specific gene expression and mating. **b**, Northern blot showing the expression of the *C. lusitaniae* *STE3* homolog (**a** pheromone receptor) and *STE2* homolog (α pheromone receptor). All strains were grown on dilute PDA for 24 hours prior to harvesting and RNA purification. Two independent **a2** Δ and α 1 Δ mutants are shown. The expression of both pheromone receptors is induced during mating; however, no induction is seen in **a2** Δ x WT α or WT**a** x α 1 Δ matings.

2.3.4 *a1* is required for meiosis and sporulation

In both *S. cerevisiae* and *C. albicans*, the only known role of *a1* is to function in a heterodimeric complex with $\alpha 2$ to repress haploid specific genes, prevent white to opaque switching (*C. albicans*), and to promote entry into meiosis by the repression of the meiotic repressor *RME1* (*S. cerevisiae*) (Tsong, Tuch et al. 2007). Since the *C. lusitaniae* homolog of *a1* has been retained in the genome and is expressed, we were interested to know what function this protein may play in the absence of $\alpha 2$. Three independent *a1* Δ disruption mutants were constructed using the *SAT1* cassette. In contrast to *a2* Δ and $\alpha 1$ Δ mutant strains, *a1* Δ mutant cells form conjugation tubes and fuse with wild-type efficiently early in mating. However, as mating progresses key differences become apparent. In the *a1* Δ x WT α matings, conjugated cells continue to shmoo and eventually lose their recognizable shape, and no sporulating cells or asci are observed (0 of 800 cells examined). These findings suggest that *a1* is required to promote meiosis and sporulation.

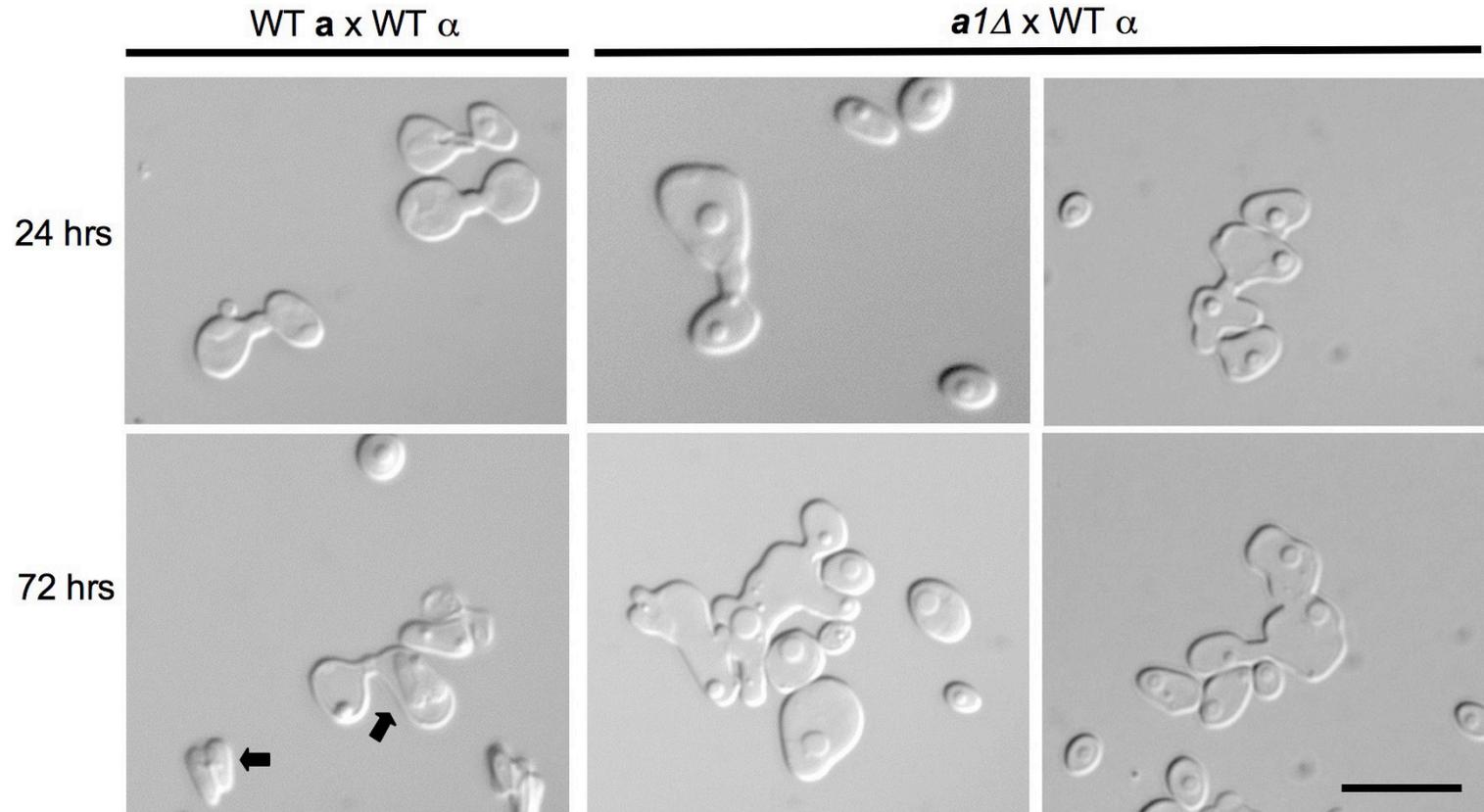


Figure 8: Morphology of *a1Δ* mutant cell matings

Strains were incubated on dilute PDA for either 24 or 48 hours at room temperature. WT *a* x WT *α* crosses show conjugated cells at 24 hours, and sporulating asci and liberated spores by 72 hours. Although *a1Δ* x WT *α* matings appear to conjugate normally at 24 hours, no sporulating asci were observed at 72 hours (0/ 800 cells). Instead, the *a1Δ* cells appear to continue to shmoo after conjugation and eventually lose their shape forming atypical enlarged cells. Scale bar, 10 μ M.

To determine whether recombinant progeny can be formed from $a1\Delta \times WT$ matings, three independent mutant strains ($MATa\ a1::SAT1\ ura3$) were crossed with a $WT\alpha$ tester strain ($MAT\alpha\ leu2\ chx^R$). Consistent with the hypothesis that $a1$ is dispensible for cell-cell fusion, but required for meiosis and sporulation, when prototrophic fusion progeny from $a1\Delta \times$ wild-type matings ($MATa\ a1::SAT\ ura3 \times MAT\alpha\ leu2\ chx^R$) are selected on minimal media ($Ura^+ Leu^+$) the efficiency of diploid progeny formation is similar to wild-type (8.6 – 11.3%); however, when haploid progeny are selected using the recessive cycloheximide resistance marker ($LEU2\ chx^R$), the efficiency of progeny formation is significantly reduced (0.017 – 0.028%).

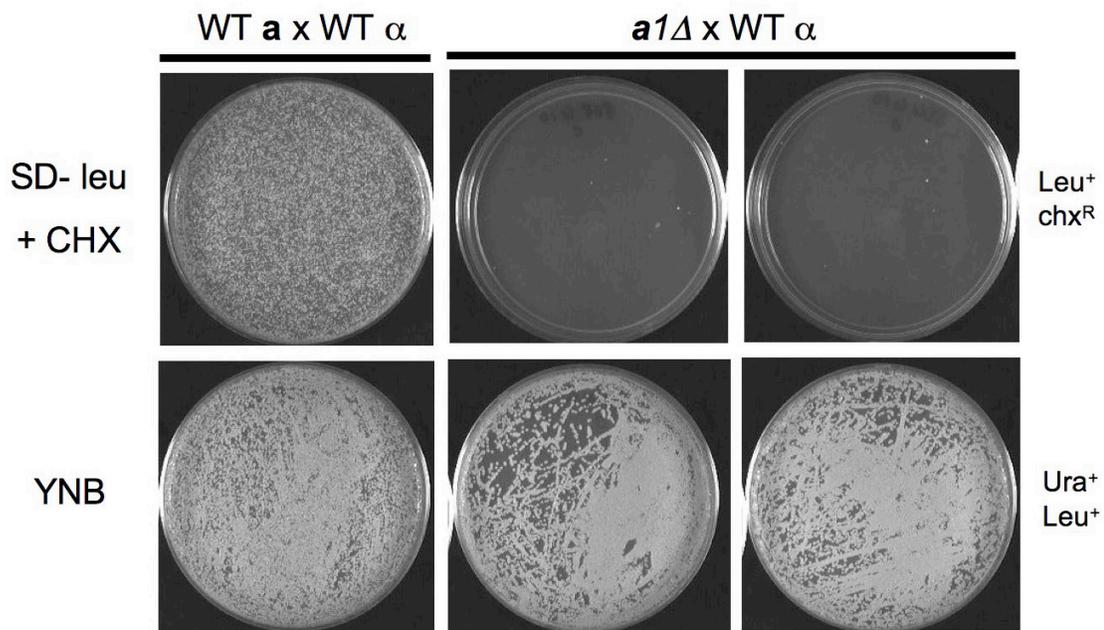


Figure 9: Selection of $a1\Delta$ mutant x WT progeny

WT mating (CL16 $MATa\ ura3 \times JLR610\ MAT\alpha\ leu2\ chx^R$) or 2 independent $a1\Delta \times WT\ \alpha$ matings (JLR806 or JLR 808 $MATa\ a1\Delta\ ura3 \times JLR610\ MAT\alpha\ leu2\ chx^R$) were incubated on PDA for 72 hours and then plated to selective media that selected for either prototrophic progeny ($URA3\ LEU2$) or recombinant progeny possessing the recessive cycloheximide resistance marker ($LEU2\ chx^R$). While WT crosses produced equal numbers of progeny under both conditions, the $a1\Delta$ crosses produced significantly less $LEU2\ chx^R$ progeny suggesting that $a1\Delta$ cells are capable of fusion, but not sporulation.

To generate protrophic progeny, only cell-cell fusion is necessary; however, to form chx^R progeny, loss of the wildtype gene which confers cycloheximide sensitivity is required, either through fusion followed by chromosome loss, or by generating haploid recombinant progeny via meiosis. Our results suggest that the $a1\Delta$ mutants are defective in the latter processes. Consistent with the idea that the progeny recovered are diploid, these strains were able to acquire 5-FOA and cycloheximide resistance at a higher frequency than wild-type strains, likely through the ability to lose the chromosome conferring sensitivity ($URA3$, CHX^S). Interestingly, both $a1$ and an α -specific component are necessary for sporulation; diploid a/α strains sporulate whereas a/a , and α/α diploid strains do not. This reveals a novel, $\alpha2$ -independent role for $a1$ in *C. lusitaniae*, in contrast to *S. cerevisiae* in which $a1$ only functions with $\alpha2$ (Johnson 2003).

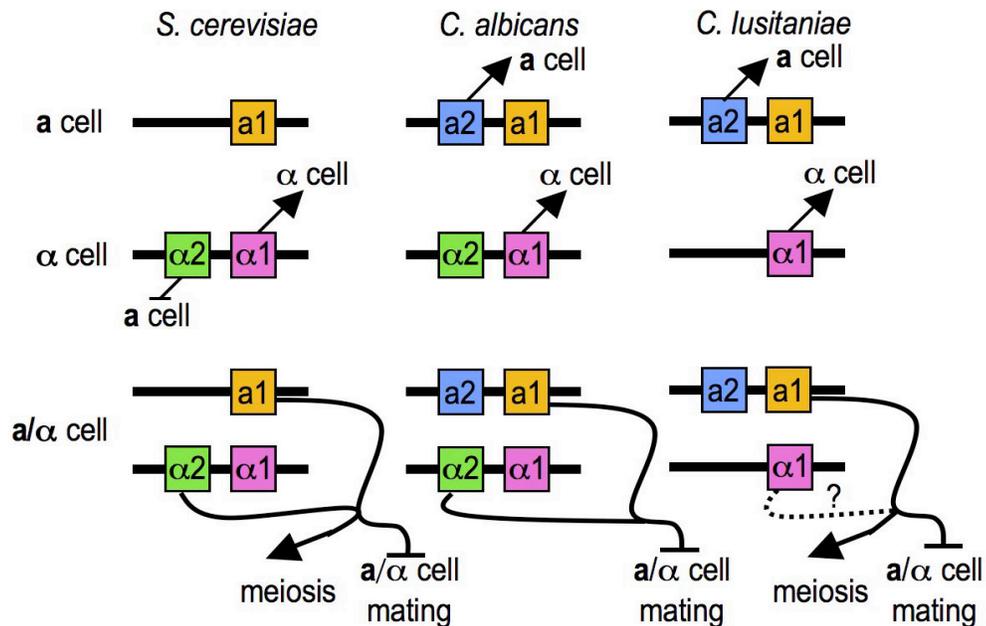


Figure 10: Model for the control of haploid specific genes and cell identity

In both *C. albicans* and *C. lusitaniae* $a2$ controls a cell identity, the default cell-state in *S. cerevisiae*. In all three species, $\alpha1$ controls alpha cell identity, and in *S. cerevisiae* $\alpha2$ functions to repress a cell specific genes. In both *S. cerevisiae* and *C. albicans*, the $a1/\alpha2$

heterodimer functions to repress haploid specific genes and additionally to promote entry into meiosis in *S. cerevisiae*. In *C. lusitaniae*, **a1** lacks its canonical partner, and is necessary for sporulation and meiosis but not conjugation. Heterozygosity at the *MAT* locus appears necessary for sporulation, suggesting a *MAT* α locus component, such as $\alpha 1$, also plays a role in sporulation and meiosis. Figure adapted from (Tsong, Tuch et al. 2006).

2.3.5 *C. lusitaniae* and *C. guilliermondii* are missing homologs of many genes crucial for meiosis and sporulation

Additional analyses completed during the Broad Institute *Candida* species comparative genomics project raised further questions regarding whether meiosis could occur in *C. lusitaniae*. *C. albicans* is thought to lack several important meiotic genes, and this could explain why *C. albicans* undergoes a parasexual cycle with no evidence for meiosis (Tzung, Williams et al. 2001; Bennett and Johnson 2003; Jones, Federspiel et al. 2004). However, comparative genome analysis revealed the sexual species *C. lusitaniae* and *C. guilliermondii* also lack the same meiotic gene homologs missing in *C. albicans* and, in addition, also lack clear homologs of numerous additional key meiotic components (Butler submitted). The presumption that *C. lusitaniae* undergoes meiosis has been based on the ability of this species to mate and form spores. Marker assortment observed in previous genetic crosses could result from independent chromosomal segregation rather than meiotic recombination, as these loci lie on different chromosomes (Young, Lorenz et al. 2000). Additionally, *C. lusitaniae* generally produces asci with only two (dyads) rather than four spores (tetrads) (Lachance and Phaff 1998; Kurtzman and Fell 2000). Mutants of *S. cerevisiae* that produce dyads are known (Klapholz and Esposito 1980; Neiman 2005); however, meiosis typically yields four products.

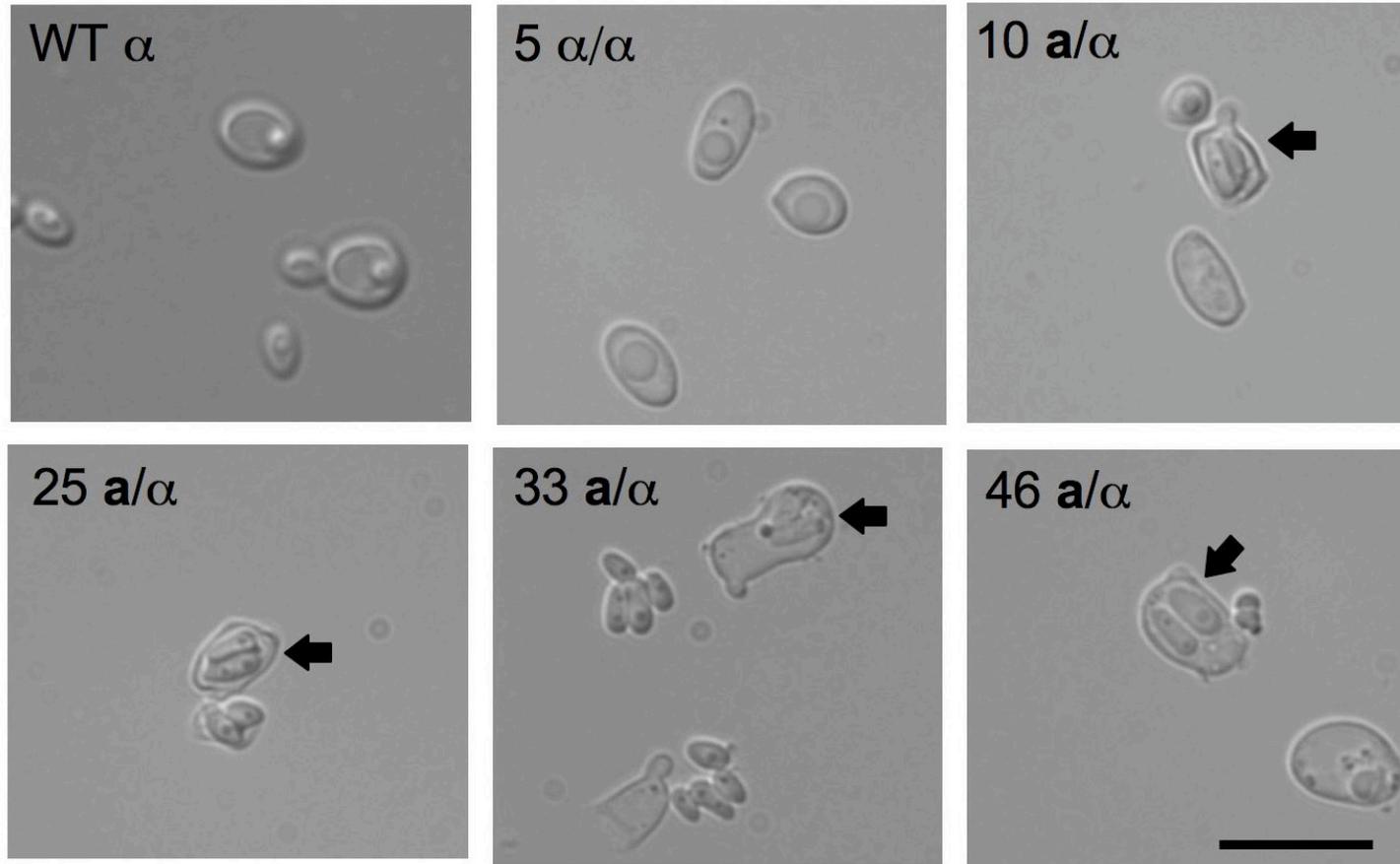


Figure 11: Sporulation of a/α diploid cells

Individual strains were incubated on dilute PDA for 48 hours at room temperature. Shown are representative images. Neither a haploid WT $MAT\alpha$ strain (JLR610), nor homozygous a/a or α/α cells (such as progeny 5 $MAT\alpha/\alpha$) sporulated. Numbers refer to the progeny identification number. However, all diploid strains that were $MATa/\alpha$ were capable of sporulation when incubated alone. Asci containing spores are indicated by arrows. Scale bar, 10 μm .

2.3.6 Frequency of recombination in F1 progeny is consistent with meiosis

To establish whether *C. lusitaniae* undergoes meiosis, a restriction fragment length polymorphism (RFLP) map was developed for three of the eight chromosomes (1, 5, and 6 (*MAT*)) and used to determine recombination frequency following genetic crosses. To generate the RFLP map, 3 kb regions spaced across chromosomes 1, 5, and 6 were amplified by PCR and sequenced. An F1 progeny set was generated by crossing a *MAT* α *leu2 chx^R* and a *MAT***a** *ura3* strain on dilute potato dextrose agar, and 94 Ura⁺ Leu⁺ progeny were selected. DNA was isolated from all progeny and was subjected to RFLP analysis. Additionally, the progeny were tested for cycloheximide sensitivity. Both *MAT* and *chx^{R/S}* should segregate independently in these progeny; thus, an additional confirmation that the strains selected were progeny and not parental contamination is that 53.7% of the progeny were *MAT***a** (36 / 67) and 46.3% were *MAT* α (31/67). 50.7% of the progeny were non-parental *MAT***a** *chx^R* or *MAT* α *CHX^S*, while 49.3% were parental *MAT***a** *CHX^S* or *MAT* α *chx^R*. The majority of progeny (67 / 94, or ~70%) mated as **a** or α and inherited only one parental allele at each locus. A high level of recombination was apparent. Based upon recombination frequencies calculated for each region, the average centiMorgan values (1 cM= 1% recombination) were 4 to 16 kb / cM (Chr 1: 8.85 - 15.7 kb / cM, Chr 5: 3.89 - 4.78 kb / cM, and Chr 6: 3.92 - 7.63 kb / cM), well within the range of known meiotic recombination frequency for other fungi, including *S. cerevisiae* (~2.7 kb / cM), *Cryptococcus neoformans* (~13.2 kb / cM), and *Aspergillus nidulans* (~43 kb / cM). Thus, recombination occurs frequently during *C. lusitaniae* sexual reproduction, consistent with meiosis. Analysis of a second F1 progeny set yielded similar results (data not shown).

		JLR610 CL16		F1 Progeny															
		(P1)	(P2)																
				1	3	6	11	12	30	55	58	19	7	5	33				
		H	H	H	H	H	H	H	H	H	H	H	H	D	D				
CHR ^a	Dist ^b	chx ^R	chx ^S	chx ^R	chx ^S	chx ^S	chx ^S	chx ^R	chx ^R	chx ^S	chx ^S	% Rec ^f	kb / cM ^g	% <i>spo11</i> ^h					
1	400	1	0	0	1	0	1	0	1	1	1	0	2	0	2	11.3	8.85	< 1.6	
1	500	1	0	1	1	0	0	0	1	1	0	0	2	0	2				
1	600	1	0	1	1	0	0	0	0	1	0	1	2	2	2				
1	1106 ^c	1	0	0	1	0	1	1	0	1	0	0	2	2	2	41.8	11.34		
5	500	1	0	1	1	1	1	0	1	0	1	1	0	2	2	20.9	4.78	< 1.6	
5	600	1	0	1	1	1	1	1	1	0	1	1	1	2	2				
5	629 ^d	1	0	1	1	1	1	1	1	1	1	1	1	2	2	7.46	3.89		
6	13	1	0	1	1	1	0	1	0	1	1	2	1	2	2	37.6	4.79	< 1.6	
6	193	1	0	0	0	0	0	1	1	1	1	2	0	1	2				
6	MAT ^e	α	a	a	a	a	a	α	α	a	α	aα	a	α	a	5.1	3.92	< 1.6	
6	303 ^c	1	0	0	0	0	0	1	1	1	0	2	0	1	2	13.1	7.63	< 1.6	
6	703	1	0	1	0	1	1	0	1	1	0	2	0	1	2	56.7	7.05	< 1.6	

Figure 12: RFLP typing and frequency of recombination in progeny

Parental strains JLR610 (P1) *MATα leu2 chx^R* and CL16 (P2) *MATa ura3* were co-cultured on dilute PDA for 96 hours and recombinant prototrophic progeny were selected on YNB medium. Shown above is a random sampling of progeny. Strains were FACS analyzed and found to be either haploid (H) or diploid (D) as indicated. Sensitivity to cycloheximide (chx) was also tested and strains were scored as either resistant (chx^R) or sensitive (chx^S). Shading indicates regions of recombination. See Figure 12 for all 94 F1 progeny data. ^aCHR, chromosome number. ^bDist, chromosomal position of locus. Distance in kb from the beginning of the appropriate chromosomal contig sequence provided at the *C. lusitaniae* genome database. ^c, centromere proximal markers. Chromosome 1, 1106 is 40 kb from the centromere, Chromosome 6, 303 is 20 kb from the centromere. ^d*URA3* locus, since all progeny analyzed are prototrophic, all inherited the *URA3* locus from parental strain JLR610 (P1). ^e*MAT* locus, 203 kb. ^f% Rec, frequency of recombination between the two indicated loci. ^gkb/cM, average kb per centiMorgan (1 cM= 1% recombination) calculated from the frequency of recombination between the indicated loci. ^h% *spo11*, frequency of recombination in *spo11 x spo11* crosses. Progeny were typed for every marker on chromosome 6 to determine recombination frequencies, but only one marker each for chromosomes 1 and 5 was scored to establish independent assortment of chromosomes.

Further analysis provides evidence these progeny are euploid, haploid isolates. First, FACS analysis of representative isolates revealed haploid (or near haploid) DNA content. Second, because RFLP mapping provides limited information about only a subset of chromosomes, a low-density 70-mer array was generated containing 96 probes spanning all 8 chromosomes for comparative genome hybridization analysis (CGH). By CGH, the parental **a** and α strains are euploid and representative presumptive haploid isolates were also found to be euploid, containing one copy of each of the eight parental chromosomes. Thus, during sexual reproduction haploid strains fuse to produce a transient diploid zygote intermediate, and complete genome reduction then occurs producing haploid recombinant spores.

2.3.7 Disruption of the meiotic specific recombinase *SPO11* decreases efficiency of progeny formation and abolishes recombination

Mutation of *SPO11* impaired sporulation and abolished recombination, further supporting the hypothesis that meiotic recombination occurs during *C. lusitaniae* sexual reproduction. *SPO11* encodes a meiosis-specific topoisomerase homolog highly conserved throughout eukaryotes that causes DNA double strand breaks that provoke meiotic recombination (Klapholz, Waddell et al. 1985; Keeney 2001). The *SPO11* gene was identified in the *C. lusitaniae* genome by BLAST search and homology to two conserved domains: a DNA binding domain and a catalytic hydrolase domain. In bilateral *spo11* x *spo11* mutant crosses, the production of spores and meiotic progeny was considerably reduced and no recombination was observed in germinated spore products compared with wild-type (**a** x α) or wild-type x *spo11* unilateral crosses. Wild-type x wild-type crosses yielded ~4 to 16% spores, whereas *spo11* x *spo11* strains

had an ~15- to 80-fold reduction (0.23 to 0.31%). This analysis was repeated with an independently derived *spo11* x *spo11* mutant pair with similar results (data not shown).

Three independent lines of evidence support the conclusion that meiosis occurs during *C. lusitaniae* sexual reproduction: 1) recombination is frequent, on par with meiosis in other organisms, 2) 70% of F1 progeny are euploid and haploid (based on RFLP, FACS, CGH) and therefore complete genome duplication and reduction occurs, and 3) recombination is Spo11-dependent.

2.3.8 *C. lusitaniae* crosses generate a large number of diploid/aneuploid progeny

Although ~70% of the F1 progeny are haploid, the remaining progeny (27/94, or ~30%) inherited both parental alleles at one or more loci. By FACS analysis, one-fourth are haploid/aneuploid (6/27), and three-fourths are diploid (21/27).

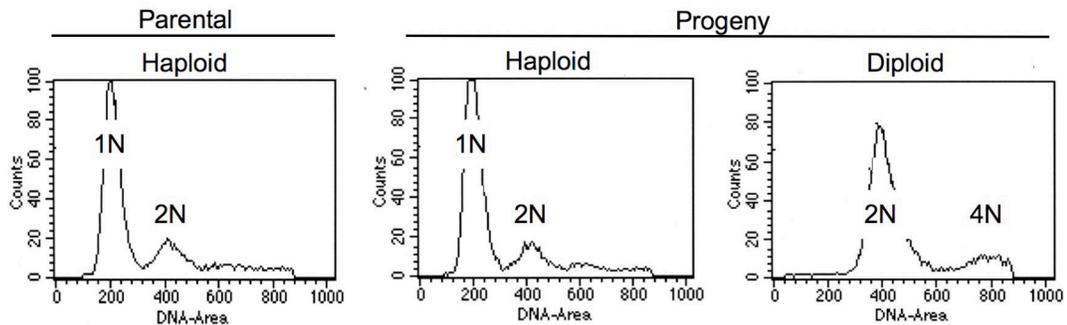


Figure 14: FACS analysis of progeny

Representative FACS plots of parental and progeny strains. The FACS plot of parental α strain JLR610 is shown. Of the 27 progeny typing as α and α for at least one RFLP loci, 6 strains were haploid by FACS (representative strain, progeny p2 shown), and 21 were diploid by FACS analysis (representative strain, progeny p33 shown).

The haploid/aneuploid progeny could contain one or a few extra chromosomes (i.e. 1N+1) as all markers typed as heterozygous along one of three chromosomes analyzed

(except for one strain, progeny 39, which typed as heterozygous at only the *MAT* locus). Based on RFLP or CGH analysis, 1N+1 aneuploid progeny containing an extra copy of chromosomes 1, 5, 6, or 7 were observed. These aneuploid progeny could arise via concerted chromosome loss by a parasexual process from a diploid intermediate, or from precocious sister chromatid segregation or meiotic nondisjunction. Given that the majority of progeny were haploid and produced by meiosis, it seems less likely a concomitant parasexual process occurs (analysis of centromere segregation supports this conclusion, see below). Recombination frequency in these aneuploid segregants was similar to isolates that had undergone a complete genome reduction, consistent with meiotic reduction. Moreover, the aneuploid progeny observed were all 1N+1, and no examples of higher order aneuploidy (other than diploid) were observed, consistent with concerted meiotic loss of 7 of 8 parental chromosomes. Finally, analysis of chromosomal configuration in 1N+1 aneuploid isolates revealed heterozygosity at centromere linked proximal markers on Chromosomes 1 and 6, consistent with either precocious sister segregation or meiosis I nondisjunction.

The final progeny class (21/94, 22%) are diploid, based on RFLP, FACS, and CGH analysis. One of the diploid strains appears to be a possible chromosome 7 aneuploid by CGH (we did not test for this chromosome by RFLP) and is a possible 2N+1 aneuploid. Mating could produce diploids via several routes. First, cell-cell fusion during mating produces diploid zygotes that might be recovered before meiosis and sporulation occur. Second, diploids could be products of meiosis involving only one reductive division, similar to *S. cerevisiae spo12,13* mutants (Klapholz and Esposito 1980). Third, diploids could be products of F1 spore-spore matings. Seven diploid progeny were found to be capable of sporulation on dilute PDA when plated by themselves, and all were found to be a/α *MAT* heterozygous but homozygous at some other loci and could have resulted from either meiosis or subsequent inter-progeny

mating of haploid meiotic products. Finally, diploids could be the direct product of meiosis. The remaining 14 diploid progeny are incapable of sporulation by themselves and are *MAT* homozygous (**a/a**, α/α), and thus are unlikely the result of **a**- α progeny spore matings, suggesting these are meiotic products. All 14 appear to have undergone meiotic recombination based on the observation of homozygous and heterozygous parental marker configurations. Because all 14 are homozygous at the Chromosome 6 centromere-proximal marker, the most parsimonious explanation is that they result from meiosis II non-disjunction or a failure to undergo meiosis II.

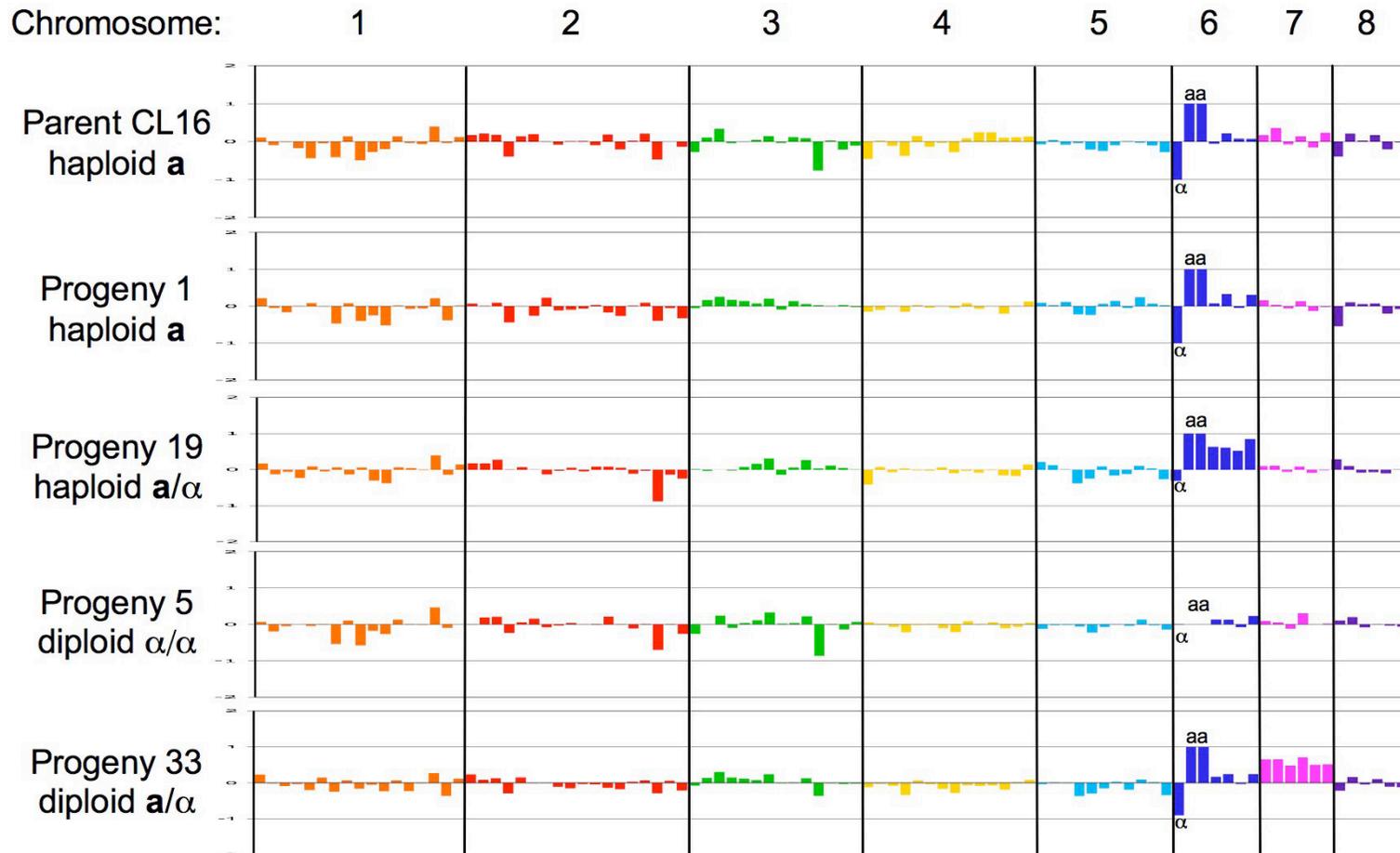


Figure 15: CGH analysis of progeny

CGH analysis plots of \log_2 ratio of medians for each experimental strain versus parental α strain JLR610 for which the array was designed. The 8 chromosomes are plotted in order and separated by thick black lines. For the mating-type specific primers, if only one strain hybridized to a particular probe the \log_2 ratio was set as -1 ($\alpha 1$) or 1 (**a1** or **a2**). Traditional CGH analysis plots a scanning window average of several genes; however, due to the low-density nature of this array each probe is plotted individually. To determine if a strain appeared aneuploid, we looked for a global increase in \log_2 ratio for the majority of probes along a chromosome. Shown from top to bottom are parental strain CL16, haploid progeny p1, aneuploid progeny p19, diploid progeny p5, diploid progeny p33. α denotes the $\alpha 1$ probe, and **aa** denotes the **a1** and **a2** probes. Any strains whose genome content is an exact multiple of the control strains JLR610 will appear isogenic (ie. strains that are 1N, 2N, 3N, etc would appear isogenic), thus diploid p5 appears isogenic with control strain JLR610; however, it is known that this strain is diploid by FACS analysis.

2.3.9 Microdissection of asci reveals both haploid and diploid progeny

To determine the nature of the spores (whether diploids are products of meiosis) and provide insight into why two rather than four spores are produced, dissection of individual spores from a single ascus would be the ideal approach. However, due to the small size of the cells and spores of *C. lusitaniae*, this has proved technically challenging. As a first step to address this question, the contents of intact asci from a sporulated diploid cell (*URA3/ura3 LEU2/leu2 CHX^s/chx^R*) were micromanipulated to a YPD plate. Once the dyad ascus germinated, 32 single colonies were isolated and typed by plating on SD-ura, SD-leu, 5-FOA, and YPD + CHX to determine if more than one cell type was present per ascus. Two single colonies of each spore type were further analyzed from each ascus by RFLP and FACS analysis to determine the nature of the spores. One obvious concern with this method is that any fitness difference between spores from a single ascus could result in an unequal ratio of cells in the initial colony, thus biasing against the detection of more than one spore type. However, given the inability to micromanipulate the individual spores away from each other, this method allows an estimation of spore germination frequency and their genetic nature. 68.3% gave rise to

colonies (28/41). Given that the majority of asci are dyads, ~44% of the spores germinated. Single colonies from twenty-three of the asci were further analyzed. 18 asci contained a single cell type, 4 contained two cell types, and 1 had three cell types, in accord with ratios one would predict for dyads with two distinct cell types with an ~44% germination rate. Two of each cell type were chosen if possible (one cell type only had 1 strain), and when examined by FACS analysis 4/57 were found to be diploid. Thus, both diploid and haploid progeny can be isolated from asci.

2.3.10 DNA replication is required for sporulation of diploids

Previous studies document that haploid parental strains mate to produce diploid zygote intermediates in which DNA replication occurs on mating/sporulation media, based on appearance of 2N/4N ploidy by FACS (Young, Lorenz et al. 2000). To further address the question of whether DNA replication is required prior to meiosis, we tested the ability of the self-sporulating diploid strain progeny p33 to sporulate in the presence and absence of hydroxyurea. At 24 hours, the yeasts incubated on dilute potato dextrose agar alone sporulated robustly (~80% of cells sporulating or disrupted), while cells that were incubated on dilute PDA containing hydroxyurea failed to sporulate (0 of 800 cells examined). To ensure that the failure to sporulate was not due to a loss of viability, cells were scraped from the dilute PDA medium containing hydroxyurea and were replated on dilute PDA alone. After 24 hours of incubation, robust sporulation could be observed in these samples, suggesting that DNA replication is required for sporulation.

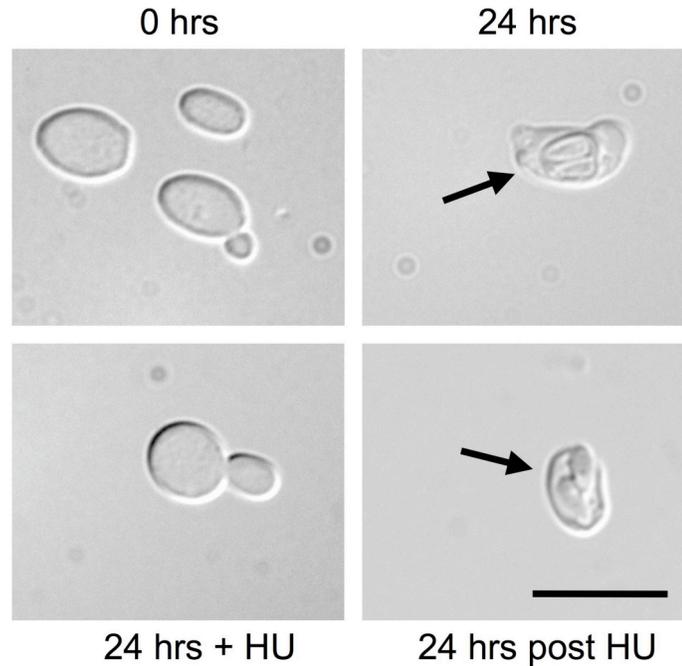


Figure 16: Treatment with hydroxyurea blocks sporulation

The self-sporulating diploid progeny strain p33 was incubated on dilute potato dextrose agar plates in the presence or absence of 100 mM hydroxyurea (HU) at room temperature for 24 hours. Cells from the plates containing hydroxyurea were then transferred to fresh dilute PDA plates without HU and allowed to recover for 24 hours. At all time points, cells were collected, fixed, and examined microscopically for the presence of sporulating cells. Arrows indicate spores. Shown above are representative images from each time point. Scale bar, 10 μ M.

2.4 Discussion

We conclude that meiosis occurs during mating of *C. lusitaniae*, despite the absence of many key meiotic components. Even with a limited meiotic component repertoire, ~70% euploid, haploid progeny are faithfully produced. Previous studies document that haploid parental strains mate to produce diploid zygote intermediates in which DNA replication occurs on mating/ sporulation media, based on appearance of 2N/4N ploidy by FACS (Young, Lorenz et al. 2000). Incubation of the \mathbf{a}/α progeny progeny p33 on PDA plus hydroxyurea resulted in no sporulation, providing additional evidence that replication precedes meiosis and sporulation. Our studies provide

evidence that Spo11 acts to provoke meiotic DNA recombination. The absence of Dmc1 and its conserved co-factors (Mei5/Sae3) suggests DNA DSB repair is mediated by Rad51-dependent mechanisms, similar to *U. maydis*, *C. elegans*, and *Drosophila* (Villeneuve and Hillers 2001; Donaldson and Saville 2008). Genome reduction to euploid haploid progeny occurs faithfully in the majority of progeny in the absence of conserved synaptonemal complex proteins, possibly analogous to *S. pombe* in which LinE elements promote chromosomal pairing and segregation (Lorenz, Wells et al. 2004; Loidl 2006). At the same time, sexual reproduction of *C. lusitaniae* produces a surprisingly high level of aneuploid (~6%) and diploid (~22%) progeny via a spectrum of meiotic errors, possibly as a consequence of the loss of conserved meiotic components. These aneuploid/diploid isolates can be stably propagated on rich media (YPD), and testing of F1 progeny (1N, 1N+1, 2N) has not revealed obvious differences in susceptibility to fluconazole or amphotericin B.

Taken together, our findings challenge the notion that genomic inspection alone can provide insights into whether meiosis can occur, and illustrate the mechanistic and evolutionary plasticity of this process central to genetic exchange and sexual reproduction. The level of aneuploidy produced during *C. lusitaniae* mating is similar to that observed during human oogenesis (estimates range from 7 – 35%), which leads to miscarriage and birth defects associated with trisomy, and in tumor cells leading to metastasis (Hunt and Hassold 2008). Aneuploidy is deleterious in these situations; however, aneuploidy effects in *C. lusitaniae* are less clear. Aneuploidy could generate diversity that is beneficial, deleterious, or neutral, acting over short or long evolutionary time spans. Aneuploidy in *S. cerevisiae* triggers common phenotypic/genotypic responses (Torres, Sokolsky et al. 2007), which may be adaptive, or maladaptive, depending on environment.

Our findings have implications on the sexual nature of *C. albicans*. The frequent recovery of aneuploid progeny from *C. albicans* matings has fueled arguments that this process is parasexual and lacks meiosis (Bennett and Johnson 2003; Johnson 2003; Forche, Alby et al. 2008). However, in light of the findings presented here, the life cycle of *C. albicans* may require further scrutiny. Given that *C. lusitaniae* undergoes meiosis yet lacks more than a dozen key meiotic genes retained in *C. albicans*, and aneuploidy is frequently generated during sexual reproduction, *C. albicans* sex might involve an unrecognized form of meiosis. Spo11-dependent recombination does occur during tetraploid-diploid reduction (Forche, Alby et al. 2008), suggesting the *C. albicans* parasexual cycle might turn out to be sexual. Finally, there are parallels with the recently discovered genetic exchange akin to sex in the protozoan parasite *Giardia* (Poxleitner, Carpenter et al. 2008). During encystment, the two paired nuclei fuse (without cell fusion), key meiotic homologs (Spo11, Dmc1) are expressed, and genetic exchange and genome reduction occurs (Poxleitner, Carpenter et al. 2008), illustrating the plasticity of sexual reproduction in microbial pathogens, and its potential impact on virulence and population structures beyond the fungi.

2.4.1 A comparison of mating in *C. lusitaniae* and *C. albicans*

The biology of mating in *C. lusitaniae* and *C. albicans* has several key differences. Although both processes involve a doubling of DNA content and a subsequent reduction, in *C. lusitaniae* this involves a haploid to diploid to haploid transition, whereas *C. albicans* is an obligate diploid and thus it transitions from diploid to tetraploid to diploid (Bennett and Johnson 2003; Johnson 2003; Bennett and Johnson 2005). Additionally, the tetraploid fusion intermediate of *C. albicans* is relatively stable and can be propagated in the lab, whereas the true diploid fusion state of *C. lusitaniae*

appears to be transient and short-lived, based on the fact that we have yet to isolate diploid progeny that appear to be true fusion products.

As mentioned previously, *C. albicans* is required to undergo phenotypic switching from the white to opaque cell type prior to becoming mating competent. Morphologically, white cells are generally round with a smooth surface, whereas opaque cells are elongated with a rough pimply surface. The cell types can also be distinguished by pigment uptake on media containing phyloxine B. The majority of clinical and environmental isolates of *C. albicans* are white cells and are heterozygous at the *MTL*. To become opaque, white cells must be homozygous at *MTL*, either through a process of chromosome loss, or gene conversion. Conversion to the white form increases the mating efficient of cells 1000-fold when mating to a white cell, or 1,000,000 fold if both partners are opaque (Miller and Johnson 2002; Johnson 2003; Soll 2004). *C. lusitaniae* is not known to undergo a phenotypic switching process to become mating competent. Almost all clinical and environmental strains analyzed thus far are mating competent (Gargeya, Pruitt et al. 1990; François, Noël et al. 2001). *C. lusitaniae* does, however, show phenotypic switching on media containing copper. In the presence of copper *C. lusitaniae* strains exhibit pigmentation ranging from white to light brown to dark brown. Although some correlation between pigmentation and Amphotericin B resistance has been noted, there is no reported correlation between colony color and mating ability (Miller, Dick et al. 2006).

During mating of *C. lusitaniae* two cells of opposite mating type form conjugation tubes that subsequently fuse together. After conjugation tube fusion, the nucleus from one of the parental cells (the “head cell”) is transferred through a central septal perforation where the conjugation tubes fused. The “head cell” which donates its nucleus remains relatively unchanged, however the nucleus of the acceptor cell is the site of karyogamy and meiosis and eventually becomes the ascus and site of spore formation

(François, Noël et al. 2001). Nuclear transfer appears to be highly polarized; labeling studies suggest that one parent generally serves as the nuclear donor. However, there is no definitive linkage between mating-type and whether a cell serves as the nuclear donor or acceptor (François, Noël et al. 2001).

One question raised from the studies presented here is whether *C. albicans* could undergo a meiotic reduction of chromosomes. The sexual cycles of both *C. albicans* and *C. lusitaniae* produce a high proportion of aneuploid progeny (1N+1 for *C. lusitaniae*, 2N+1 for *C. albicans*) (Bennett and Johnson 2003). Given the low level of recombination observed in *C. albicans*, and the absence of numerous meiotic components the presence of aneuploidy was thought to indicate a parasexual process; however, in *C. lusitaniae* meiotic mapping, and *SPO11* dependent recombination provide support for meiosis even in the presence of a high frequency of aneuploidy. Unlike *C. lusitaniae* which forms spores, *C. albicans* has never been observed to form spores. One possibility is that a meiosis in *C. albicans* results in only one 'viable' nucleus that may not require packaging into a spore form, and *C. albicans* may have evolved to eschew sporulation as a means to avoid detection of antigenic spore proteins by the immune system.

2.4.2 Transcriptional rewiring: $a1/\alpha$ control of meiosis and sporulation and a role for $a1$ independent of $\alpha2$

In both *S. cerevisiae* and *C. albicans*, the $a1$ and $\alpha2$ homeodomain transcription factors form a heterodimer that represses haploid specific gene expression, blocks white-opaque switching (*C. albicans*), and promotes entry into meiosis (*S. cerevisiae*) (Johnson 2003; Tsong, Tuch et al. 2007). Interestingly both sexual species, *C. guilliermondii* and *C. lusitaniae*, lack either one or both components of this canonical heterodimer suggesting that the transcriptional circuits controlling these processes are either absent or have been

significantly reconfigured. There are several hypotheses for how these processes could be controlled in the absence of the $\mathbf{a1}/\alpha2$ heterodimer. If the genetic programs of mating and meiosis are intimately linked and occur in rapid succession, perhaps it is not necessary to repress haploid specific gene function if the diploid state is transient, unstable, and fleeting. Unlike *C. albicans*, *C. lusitaniae* does not need to undergo phenotypic switching to become mating competent, and thus $\mathbf{a1}/\alpha2$ is not required in this capacity. In *S. cerevisiae*, $\mathbf{a1}/\alpha2$ represses the repressor of meiosis (*RME1*) allowing expression of the master regulator Ime1, and subsequent induction of Ime2 (Mitchell and Herskowitz 1986; Vershon and Pierce 2000). *C. guilliermondii* has a possible *RME1* homolog (PGUG_00281.1) based on reciprocal BLAST analysis; however, *C. lusitaniae* lacks a clear *RME1* homolog. Additionally, although both species have candidate *IME2* genes (CLUG_00015.1, PGUG_00045.1) they lack *IME1*. In the absence of *RME1* the repressive action of the $\mathbf{a1}/\alpha2$ heterodimer may not be required, and instead meiotic control could be through a direct induction of *IME2*. Notably, in *S. cerevisiae*, overexpression of Ime2 promotes meiosis in the absence of Ime1 (Mitchell, Driscoll et al. 1990).

Although in *S. cerevisiae* α cells $\alpha2$ can function independently of $\mathbf{a1}$ by partnering with Mcm1 to repress the expression of \mathbf{a} -cell specific genes, there is no known function of $\mathbf{a1}$ independent of $\alpha2$ (Tsong, Tuch et al. 2007). Interestingly, *C. lusitaniae* has retained the $\mathbf{a1}$ gene despite lacking $\alpha2$, thus raising the question of $\mathbf{a1}$ functions in this species. Based upon our observations that $\mathbf{a1}\Delta$ strains are sporulation defective, it appears that $\mathbf{a1}$ has acquired new $\alpha2$ independent roles in *C. lusitaniae*.

If, as our studies suggest, $\mathbf{a1}$ is required for meiosis and sporulation, is it acting independently or does it require a partner? Similar to *S. cerevisiae*, a nutrient signal is required for meiosis as wild-type cells or diploids do not mate or sporulate under rich nutrient conditions (Smith and Mitchell 1989). Thus, signals from both nutrient sensing

and the mating pathways must converge to promote meiosis. Additionally, analysis of diploid strain sporulation provides evidence that a component from the *MAT* α locus is also required. When incubated on mating media only *MAT* **a**/ α heterozygous diploids sporulated, whereas **a/a** or α/α diploids failed to sporulate. In the absence of α 2, the transcription factor α 1 is the most likely candidate to promote sporulation in concert with **a**1. Hypotheses for how these genes could function together include: 1) **a**1 and α 1 directly interact, 2) **a**1 and α 2 coordinately regulate a subset of genes required for meiosis and sporulation by converging on common target promoters, or 3) **a**1 and α 2 act sequentially, each activating (or repressing) elements of a common regulatory circuit.

2.4.3 Dyad formation by *C. lusitaniae*

Meiosis is classically envisioned in eukaryotes as a process that forms haploid gametes from a diploid precursor. Typically this involves a single round of DNA replication followed by two cycles of nuclear division. In the first division, meiosis I, homologous chromosomes separate to opposite spindle poles. This is followed by a reductional division, meiosis II, during which sister chromatids segregate producing haploid nuclei that are subsequently packaged into gametes (mammals) or spores (yeast). In ascomycetous yeast, the recombinant progeny are termed spores and are contained within an ascus. Interestingly, although *C. lusitaniae* has been reported to form one to four spores per ascus (Lachance and Phaff 1998), in our studies the vast majority (72 – 80%) formed dyads with two clavate ascospores, while the remainder formed monads with a single spore.

In *S. cerevisiae* several genetic mutations result in the formation of two-spored asci, the most prominent of which is *spo12*, *spo13*. Cells that are deficient for Spo12 and Spo13 undergo only one meiotic division during which sister chromatids segregate from each other (Klapholz and Esposito 1980). These mutants produce a two-spored ascus

in which both spores are diploid rather than haploid (Neiman 2005). Thus, as *C. lusitaniae* is missing both *SPO12* and *SPO13* homologs one possibility is that the spores produced are diploid and reduce to a haploid state following germination through a parasexual process. However, there are several lines of evidence against this hypothesis. Firstly, micromanipulation of asci from a sporulated diploid cell produced both haploid and diploid progeny and the majority were haploid. Secondly, the majority of progeny from matings appear to be haploid, euploid progeny whereas a parasexual chromosome reduction would be thought to yield both a higher proportion of aneuploidies and more complex aneuploidy than $1N+1$, which was not observed here. Thirdly, in a *spo12,13* mutant centromere linked markers would be expected to be heterozygous in the progeny. However, for the centromere-linked marker on chromosome 6 we observe the opposite and most diploid strains are homozygous for this marker.

The number of spores produced in *S. cerevisiae* can also be regulated by nutrient availability and flux through the glyoxylate pathway (Nickas, Diamond et al. 2004; Neiman 2005). Growth under limiting acetate conditions results in the formation of asci containing two non-sister spores (non-sister dyads; NSD). The formation of NSD is due to the differential marking of mother and daughter spindle pole bodies in meiosis II so that only the daughter spindle pole bodies are capable of recruiting the components necessary for spore formation (Nickas, Diamond et al. 2004; Neiman 2005). Therefore, after meiosis II only one haploid nucleus from each spindle is packaged into a spore resulting in a two spored ascus. The spores formed are non-sisters meaning that they did not result from the separation of sister chromatids.

Typing the spores for centromeric markers allows the classification of spores as being sisters (SD) or non-sisters (NSD) because SDs share centromeric markers whereas NSDs differ at centromeric markers. In *S. cerevisiae*, the coupling of spindle pole body marking and spore formation with nutrient status is thought to be a means of controlling

precious resources when nutrients are lacking in the environment. Essentially, it is a mechanism to ensure some spores are produced under less than optimal conditions, and that those spores produced are as genetically diverse as possible. Interestingly, *C. lusitaniae* is missing homologs of three key genes (*MPC54*, *SPO21*, and *MPC74*) that are necessary to mark spindle poles and recruit spore membrane proteins (Nickas, Diamond et al. 2004; Neiman 2005). One possibility to consider is that the formation of two spores is a strategic response to a lack of fidelity in meiosis. If the meioses of *C. lusitaniae* are not always faithful cells may regulate the number of spores formed in an effort to conserve energy and resources that would be expended in packaging non-viable nuclei due to chromosomal number aberrations. Whether this process occurs in a similar manner to *S. cerevisiae* and is influenced by the nutritional status of the cell remains to be explored.

2.4.4 The sexual species lack synaptonemal complexes

In order for chromosomes to recombine and segregate properly during meiosis a mechanism by which chromosomes are correctly oriented and paired is necessary. In eukaryotes, this function is generally fulfilled by the synaptonemal complex (SC). Cohesion between sister chromatids during meiosis I ensures that sister chromatids segregate together on the meiotic spindle and that only homologous chromosomes are separated during the first division. The proper alignment and positioning of chromosomes is also required for genetic recombination. Generally, at least one recombination event is thought to be required per homologous chromosome pair during meiosis. The formation of chiasmata creates tension between homologs that can be sensed by the spindle apparatus to ensure proper chromosomal attachment and subsequent segregation. Although the SC is ubiquitous among eukaryotes, there are some notable examples of organisms that lack SCs including *Schizosaccharomyces pombe*,

Aspergillus nidulans, and likely *Ustilago maydis*. Although *S. pombe* lacks SCs they form Linear elements (LinEs) that serve a similar function in chromosome pairing (Lorenz, Wells et al. 2004; Loidl 2006). Although *C. lusitaniae* contains a homolog of the meiotic cohesin Rec8, it is missing many genes encoding key components of the SC including Zip1, Red1, Hop1, Mek1, Zip2, and Zip3. It is possible that *C. lusitaniae* could form structures similar to *S. pombe* LinEs. Although Hop1 and Mek1 are involved in LinE formation, they are not essential; however, the Rec10 protein with homology to Red1 is required for LinE function, and is missing in *C. lusitaniae* (Lorenz, Wells et al. 2004; Loidl 2006). In the absence of SC or LinE formation, missegregation of chromosomes can occur, generating aneuploid progeny.

The haploid aneuploid progeny that we observed are generally heterozygous for every RFLP marker examined along the aneuploid chromosome. This indicates that the two chromosomes are non-sisters and were inherited from each parent. Two types of meiotic errors could explain this phenomenon: meiosis I non-disjunction or precocious sister segregation. In meiosis I non-disjunction, homologous chromosomes fail to separate and segregate to the same spindle pole, such that during meiosis II each product formed from this nucleus contains one chromosome copy from each parent. In precocious sister segregation, cohesion between one pair of sister chromatids is disrupted, resulting in one sister chromatid co-migrating with the homologous chromosome. At the conclusion of meiosis I, one spindle pole body will have inherited the 2 sister chromatids from one homolog in addition to a sister chromatid from the other homolog, while the second spindle pole body will have only a single chromatid. In the absence of SCs, *C. lusitaniae* appears to have adapted a mechanism of ensuring faithful partitioning of chromosomes for the majority of meiotic events; however, a fraction of meioses appear to go awry generating aneuploid or diploid progeny, perhaps

due to a failure to properly synapse homologous chromosomes and maintain chromatid cohesion.

2.4.5 *C. lusitaniae* and *C. guilliermondii* likely lack *DMC1* mediated meiotic recombination

Most eukaryotic species possess two related homologs of the prokaryotic recA recombinase, Dmc1 and Rad51. In *S. cerevisiae*, Rad51 has been shown to function in both meiosis and mitosis, while Dmc1 is a meiosis-specific recombinase that is involved in homologous recombination and double strand break repair (Symington 2002; Krogh and Symington 2004; Tsubouchi and Roeder 2004). Interestingly, both *C. guilliermondii* and *C. lusitaniae* have a Rad51, but not a Dmc1 homolog suggesting that recombination might be mediated by only a Rad51-dependent pathway in these species. Additionally, these species are also lacking homologs of Mei5 and Sae3 which are co-factors of Dmc1 in *S. cerevisiae* (Tsubouchi and Roeder 2004). Although the majority of eukaryotic species studied to date possess both Rad51 and Dmc1, *Drosophila*, *Caenorhabditis elegans* and *U. maydis* are three notable examples of organisms that lack Dmc1 and have only a Rad51-dependent meiotic recombination pathway (Symington 2002). It is likely that *C. guilliermondii* and *C. lusitaniae* also only use a Rad51-dependent recombination pathway for meiosis.

2.4.6 Small intergenic regions

Interestingly, we found *C. lusitaniae* to have relatively short intergenic regions flanking the genes analyzed. For instance, the stop codon of *SPO11* is 80 bp from the start of a flanking gene with homology to an inorganic pyrophosphatase, suggesting that some portion of the *SPO11* gene itself may contain promoter sequence for the flanking gene, or promoters are unusually short in this species. For this reason, the majority of

gene disruptions that we generated were not complete ORF replacements, but rather smaller deletions coupled with insertion of a selectable marker gene. While this phenomenon was true for all four genes (**a1**, **a2**, $\alpha 1$, and *SPO11*) studied in this paper, we did not investigate whether this was a genome-wide phenomenon.

2.4.7 Candidate centromeric regions

For our RFLP analysis and design of the CGH arrays, we were interested to know the position of centromeres for each chromosome. We based our assumption as to the location of the centromeres on personal communication from G. Butler and M. Logue, who performed an analysis of AT and GC content across the chromosomes and found one area of increased AT content per chromosome consistent with a centromeric region. For the design of the CGH array, we used these regions to define the two chromosomal arms and ensured that we had a least 2 probes per chromosome arm. For the RFLP analysis we have two markers that are relatively close to the putative centromeric regions: chromosome 6, marker 303 (~20 kb from the centromere) and chromosome 1, marker 1106 (~40 kb from the centromere).

Table 3: CGH array primers

Name	Sequence
CL1_00004	ATATCTCAATTTTGAAGGTAACGAGTTTGGTCACCCTGAGTGGTTGGACTTCCCCAGAGAAGGAAATGGG
CL1_00077	TCATTGATTTACTACGCAAGCTTTTTGTGATAAACCCAAGGGAGCGAATCACAGCGCTAGAGGCGCTTGA
CL1_00156	AAAACATACATGCGGACAGTACAAGAAAACAACCCTGCAAAGAACCACAAGGCGCCCTATAGTGCTGCATT
CL1_00223	TCGCGGCTTGGTGCCTCGCAATTTAAATGGCAGGCTTGTCTGAGTTGCCACGTATCTTTGAAGAGATG
CL1_00306	ACGATTTATTTGTTAGTCCCAACTCGAGACTTCATGGTGTGGGGCGCGCCTTGATCGAATTTGTCTATGG
CL1_00380	AACTTCCGTTTGGACGGAAGATTGGCGATTCTTACTGGCGGCTCCGGCGGAATGAGCCACGTGGTCTCGC
CL1_00446	ATCGTCTTGCCTACTTTTGACAGCGAGGAGTTCATCAAGTTGATTGAGGAGTTTTTGCCTTTGGAACAGG
CL1c_00520	AAGAAAAGATCATTGATCCTTTTCAGTAGACAGGGCACGGGTCCAAAAGCAGCGAAGAGAAGCAGAAATG
CL1c_00525	ACAGAGGCTCCGAAATTTCAAATCGCCATTTTCTATGGAGACCACGAAAACCAGGACAATACCATCCTC
CL1_00595	TCGACCTACTGTTTTGCCATAACATCAATGGCGTGGGACTAATGAACCTGGTCAATGCTTCTATTTCGCA
CL1_00675	AATTTGGGCAGCTTCCCTTCGTTTGAGTTGCGTCACAAGCATCCACGGCTCTTTATTGTTACTGTGCAT
CL1_00745	TTGGTGGAAATGCAACGTGGGTTGGGATATAATAGAGAGTCTCGCCCGAGAAATACTGTTTCCGATTGA
CL1_00817	AGATCAACGACAACATCAAACCATGGTTGGCTCCTACTGACACGAGATTAAGACCTGACCAACGAGCAAT
CL1_00833	CTTTTTAAGTAGAATATTTGCCAGCGCGGTGAAAACAGATCCCAGACTTTTGTAGTCCAAAGGAGACGAT
CL1_00964	GTAGGAAGGTTATGGCCATTGGCCGTGTGGCACGTATGTATCAAGTTTTGAGAGAGGAATCTGAGAATGT
CL1_01037	ATATTTCAAGCCTTCTACTAATAAACTTGGCGCATCTCCTCTGACTGCAACCACTATAGCGCATGCGACC

CL1_01113	GTTACTTCTTTGTCTCGATCCGGTAAACCGCCTTCGCTGTCTCGTATCTGGATAATGCTTGGATCTCCA
CL1_01207	GGGTCTCGGGCTTATCAACTTTGGAATGTCAGCGATTACCGCATGTATGTTAACTTACGTCATAGAGTGT
CL2_01211	TATCAATCTAGACCCATATTTGAGAACAAGGGGCATTCTGACAGGAACGGTGAGGTGGCAATCATTTC
CL2_01277	AGTGTTCACATTCATAATCTACAGCGAGAAGCAGAATTGGCTTCAATGCCACCAGCAACGATCGAGGAG
CL2_01344	TCCAATGGGGTAAATGCAATACAAGTGAGTGCCTCACAAACAGAGCGAAAAAGGTTTAGTGAGACCA
CL2_01413	TTGTTGGGTCTGCGTGTACACCACGTCCATTGACATTTGGTCTGCCGGCTGTATTTTTGCAGAGATGT
CL2_01479	TTCAGGATGCCTTGAAGCCTGTTTTCGAGGAATTGTACGAGTCTGTTAAGAACGGTCTGAGACCCAGA
CL2_01556	AGTTCAACTCGACTGATAGAATCCTTCAAAGAAACACAAACCAAGCGGGAGTTACCGATTCTCGTGCAC
CL2_01631	ACAGCAGTTAGGCGATTTGAATATCAAAATCCCAGACATTGAAAAAATTTGGCGGTGATCCGCCTTTTG
CL2_01702	TGGCTCTAAAATGACGGGTACCTTCCAGGGTATCTTCCAACCAAGAGGTACTCACAACAATGATACTGGT
CL2_01777	GTATGACAATCTCAACATTTTTACAGTGGACTCCGATTTCCCGAGTGCAGCTGACTCTGTTCTATGATG
CL2_01841	CTACTCCAAGGACAAAAAGTCGGTGAGGGAACATATGCTGTCGTCTATGTCGGTAAGCAGATTTCCACT
CL2_01901	ACTGAGTTCGGTGACTGGTTATTGGCTGAGTCCAAGGAGTCTAAGGAAGACTTGCCTTCTGACATTGATA
CL2_01967	ATCGTCAAGCGTGAGTTGTTGTTGGCTCGTATTTCTTGTGGGCCCTGAGTACTCCAGGAGTTGATTG
CL2_02033	TTGCCAAGATTGCATTGGGACTTTGCGACAATCTTTAACCAACGTGATTCGGGCATGGAACACGGCCTA
CL2c_02100	GCCTATAGCGTGTGTTCTTGGTCTCAACTCATTATTCCAATTACAACCTTTACCTGTTACGGTAAGCGGT
CL2c_02108	GAGTTATATCAAGAGGTTGATTACCCGTACGTTCTTGTTCGGGTTTTGGCTAACGGGTGTTACAACATCC
CL2_02178	TCTGGACGTTGCTGCTGTGGCGTCTTTGTTGAAGAGAATCTTCTGATAATTCGTTTACTTCAGCGAG
CL2_02238	CTTATCACCAAGTCAACGGGGGTCAAAGTTGGCAAGAGATTTTGTATCTACAACGATAACGAAACGGGGA
CL2_02292	ACATTACATTAAGCTTCGTCTATGCCGAAAAAGATGGCTCCAACGGCTTCCCGCTGATTTGGTCACTTA
CL2_02306	CCGGCAGGGCCAATTTGTCCTTTGAAGTTATCTGTCACTGCTAAAAAGGTATGCTCTGAACACGAGCATA
CL3_02306	CCGGCAGGGCCAATTTGTCCTTTGAAGTTATCTGTCACTGCTAAAAAGGTATGCTCTGAACACGAGCATA
CL3_02389	AGTATAAGGACTCGAATATCGATTGATTTTGTGGCTGTGCCGGAATGGCTGGGCTAGATGACAAGCT
CL3_02472	TAAAAGAAGCAAGCATGCACCGGCAGAGCCAGTTTTAAGCGTCTGTGCCAAAAATCAGAGAAATTCCT
CL3_02567	CAAAGATTGAGAGACTGTGGGCCAAGAAGTTTCCAGCGACGAGGAGTAGGAAAAACATTGCGAACAAT
CL3_02650	TTCCACCAATCTAAAAGTACTCCTGAAGACAGCAGGCGCATAACCGCTGTGAGTGAACACTTGAGGAT
CL3_02719	ATAACCTCTCAGACTTCTTGCACCAGAACCGTATCTCATGAAGGAAAAACACTCTCCACCGATCAACGA
CL3_02783	TTGACCGTTACCCAGACGAGCCTCCAGCAGGTGTGGTTTTGCCTCCAGGCTCCAACACTACAACCCACT
CL3c_02870	GATTGACTCTAGTCTTTTATGCATTGCAAGTTGCCCTTCCAATTGGTCTGTACAATATGGGGCATCTG
CL3c_02875	AGATCAGGATATGATTGACAGTTTTAGGCAACTGAGGGAAGTCGAGGATGACCAAGGTATAGAAACGGGA
CL3_02935	TATATGTGCATGCGTACCAGTCTATGTGTGGAACCTTGTGTGTCCAAGAGAATTGGAGCTTTTCGGGTT
CL3_02999	CATTGTGTACGGTAGCACAGAGATTGTTACGGCTAAGGAGTTTTAAGCGAGTTTCTGAACTTGGGAAG
CL3_03063	CCGTACATTAACGAGCATTTCATCACTGCATGGTTCGAGACAGAAAAATTTCAAGATTTGGGACGCCATG
CL3_03125	TGTTGAAGGGTCTATCCCAGGTATCAAGAAGAGAGTGCACCTTGAGAAAGTCTTGTACGTTGACAC
CL3_03193	TACTGATAATGGAGTGGCGCGTCTCTCATTGCCATTCAACGTTTTGGTCTTGGATTTTACAACAA
CL4_03200	TACCAGAGAATGCGGATGCGAGTGGACAGTGCCTTCAGCCAAGCTTTTCTGACCTCGGCAAAAGATT
CL4c_03259	AGCCAAATACGCCACCCCAACAATATGTCGGAGTACAGAAATCTGAACATAAAAAAGCATTGAGCCAAGG
CL4c_03266	TATATCAGTCTACCTTTTTTGAACCTCCGTTTTAACAGCGTCAGTTTGAACCGTGTGTGAATGCC
CL4_03338	ATCTTGAACAAGTCCGACTCCTACAAGGACAGGACCATCAAGTTCCACGACTACCACAAGGCACGTTTTT
CL4_03412	CAGCAGCTAGTTGGAATTTACAAGGTGAACAACCGCACGAAAAGCATTTCGCTCCAAAACAGGGAAATTGA
CL4_03488	AGAGGGAACAAGCCAGGACCTTCGGATCTAATGAAGAGATTGGTAAATCTGCAATGGCAGTGCCAAT
CL4_03563	ATCTCCAGTCACAGAGCAAACATGAAATTTGAGAACCGCAAGCAAAGGCTAATTTGAAAGGCTCG
CL4_03654	CATACTTGGTTGCTGACCATGGCGAACAAGAAAAGGGTGTGCCGATCAAGAGAACGAGGTAATGAATTT
CL4_03729	CAAGAACCCCGCATTAGTCTCATTATGATGTGCTTTTTGAAATCACCGTGGTTAACCGGTACAGAGAT
CL4_03809	AAACTACAGGTACGATTATGTGTTGACTGGACCTTGTATAAGTTTCAGCAGGAAAGGCAGAGGGAGCAG
CL4_03890	CGTATGATCGTTCGGTTGATTGGTGGGCTTTCGGTGTGCTCATGTTTCAAATGTTATTATGCCAAAGTCC
CL4_03965	ATACTACGGTTTACTGAGGAATCTTCTACACTGTCTTGTTCGGTGTGCCAGAGCTTTCGGTGTCTTG
CL4_04030	GCTCATGAGCTGGGAAAAACAACTTATCTATTTTGTGCGTGTGGTCTGGCCACATGGATCTGCAAATCA
CL4_04099	CATTGGATCCTTATGTGCGTTATAGGGGAGCGTTTTGACAGGGACGTTCTCTAGGAGAGATTGGTATTTA
CL5_04105	ACTTGGCCTTTATCAAAGACCCGATGGATACTCGGTGGAGATCATTCAAACAACATTTTCCACGACTG
CL5_04167	TTGTACATGGGCAAGCTGTCCAAGTCGCCTGAAACGAGATACTTTCGGTCCGAGTTGAAGTGTCTCATTG
CL5c_04238	ACCATGAGGATTATGATGTCATAAGCATTGCCGACGGGGAAGAAACCAAAGTACTATGAGGTTTTGTC
CL5c_04244	AGTACGGTGTCCAGTTCCTGCTTCTCTACTGCTTTGTCTTCTACGATGGTTACAGATCGGCTCAATT
CL5_04321	TCCATTGTTGGTGTCAATTTGTTGCGACTACTACTACGTGAGAAGAGGTTACTTGAAGCTCACCCACTTGT
CL5_04405	GCAGAGTTGGTGAAGGATGATGTTTTCCATACAGACTTGAATGGGTTTTGAGAGACGACGTTCAAATCC
CL5_04482	TATTAAGTCCAGAGGTCAGTACCCGGAACCACTTTCTTGACCCGTATGGATAGCAACTTTTCTGTACGT
CL5_04566	CCTTGGGTGCTCTCGCAGGGTACTTTTTTCAATGAAGTTGAATCATGCCGGAGAGAGTGATTAATTTGGA

CL5_04672	TCATAAGGGGTGATCTTGGCAGGTTACACGCTGTTTCTATAGAAGGGCTGACCGATCAGGTATTCTTGAA
CL5_04757	ACTATTGGCTTGCTCCTTGTGGGATACACATTAGCTGCCAGTATCACGTCATTTGCTCTTGATTGGCAG
CL5_04834	TACCACTCACGTTAAACAGCTTGGGACCCACAGAAGACGGATACCACTTCTCTACTACTCCAAATAT
CL6_04839	TATTGATAACACCAGCCATTGGATTCTTTACGAGGGACCCAAGCGCAAAAAGTTGGCGGACACGTTTTT
CL6_04923_alpha1	CCATCAATGCACTTGAAGCAGGTATTGGACGAGTTACATACTGGCGCTCCTCTGAAATTAAGTTGCAAG
CL6_a1	TTACCTCGGAAGCTAAACTAAATTAAGAACTATTCTACTTAGCAAACACTAACCTCGAAAAGCGGAAAT
CL6_a2	TTCTGCATCTAGATCAAAGCTAGTATCGTGTATATGGAAAACCAAGACTTCACAATTTGAAAAGTATTTT
CL6c_04965	AACTACAGTCTGTTCCACCAGCAGATGTCGTTAAGACCAGAATTATGAATGCCAAGGGAACGAGTGGCGGT
CL6c_04971	CGGGGTGAAGGCCCTACGGAAGTCGTATAGCAATTCATTACCAAATCACAGAAGATGCCGTATCAAATTTG
CL6_05054	AAAATACCAATCGGGCAGCAACAGAAACGACAAGGTGCTCATCTTTGCGTTGTACAAGAAAGAGGCTCTG
CL6_05143	ACAGGCGAAGACGCTGAAACCGCCGAGAAAAAGTTGTTAATGGTCAATCATCTCAAGGTATTCTCACAG
CL6_05233	TGGAAATGATTCCATGATTCTTTGGACTCTCTGTGACCTGGGACTTAGGTTCCGGCCAAACAGTGTAT
CL7_05235	TCAATAATAGGGGCCGACTTATCGTTGCCAGCTTCTGTCAAAGAAGTTACTCATCGACTGGAGAAATGG
CL7_05325	ACTTTTTGGGATACGCATGGATCGTATGTGGCGGAATCTGTATTATCTTGTCGTTGGTCTTGTGACCGC
CL7c_05416	CATCATCACGATTGCTTTAATCTGGTCTCTTACAGGTTCCACTTCACATGGATGGGGTTGGAAGTTTAC
CL7c_05424	AAGTGCGGGAGCAAAATGACGATCATGAAGTTGTTACGTTAGTGTGCAGCATGTGTGCTCAAGTCATAGG
CL7_05511	AAGCATGCGTATGAACAACCATTTATTTCCGGCGAAGATGCCCACTTTGTGGATTCCAAAAGACCCTATGG
CL7_05591	GCCAGATTTCCAAGATTGGGCTGGTGTGTTGATTGGTAATTTGCGTCTCTTGCTTTGTGTATCCCAGCTT
CL8_05603	AAGTACTGCTCCAATTCCTATGTCTGCTTCCCATTACTGTCTCCAAGTCTTCAACAGCTGAGTGAATCG
CL8c_05666	TGATTGCCCAATTTATTTGAACCTGAGTTCATGACGGCAGTTCCCTTCTGTCGATCCTTCCACCAACGCA
CL8c_05673	TAAACGCTTCCACGAGCTAACTGTGCTTTACAAAAACTTACGCTCTTTTTGCGGGCCGTGATCAAATCC
CL8_05755	ACTAACCAGTTCACGACCTAAGAAAAAGTAAAGAAAATGGACTTGGGCGCCTTTTTGGCCGACGACT
CL8_05850	ATTGTTGCTACCCTCGGTAATCTGGTTTTACGCGTTTGTAGGGCCTGCTCTTGACGCTTGAGAAAAC
CL8_05939	ACACTGAGTATATGGCTGCTGACCCTGAGACCCTTTACTGAATTCACCTGATTTGAAATGGAAGT

Table 4: Primers used in Chapter 2

Meiotic Mapping Primers		
JLR1089/JOHE19306	GTCACCGAATCATCCACATGAG	Chromosome 1: 400 kb
JLR1098/JOHE19315	CACACAATTGTACAAGACAGAGC	cut HindIII, KpnI
JLR1099/JOHE19316	CAGTCTTCAATGTTTCATGGAAC	Chromosome 1: 500 kb
JLR1108/JOHE19325	CCTTGACGTGTGCATCACATG	cut XhoI, BglII
JLR1109/JOHE19326	GTATCGTAACCTTCCATGATG	Chromosome 1: 600 kb
JLR1118/JOHE19335	CACATCTGCAGTTGAAGGTAC	cut HhaI
JLR1166/JOHE19839	ACCTCTCATTATGATTCTCG	Chromosome 1: 1006 kb
JLR1174/JOHE19847	CTCAGGTATCACGAGGAGGC	cut EcoRV
JLR1047/JOHE19263	CATGAGCACCAGACGAATATTTT	Chromosome 5: 500 kb
JLR1056/JOHE19272	AGGCTTGGTTGCCTGAGGTC	cut BamHI
JLR1057/JOHE19273	CAGGAGTACCAGACTCGATC	Chromosome 5: 600 kb
JLR1066/JOHE19282	ACAGCTCTTGAAGATGCCTC	cut BamHI, Sall
JLR727/JOHE18866	GTGCTGAATAGGCTTGGAAAG	Chromosome 6: 13 kb
JLR737/JOHE18876	GTGGTGTATAAACGTATCTAC	cut XbaI
JLR866/JOHE18942	CCACATCCAAGTTGGAATTGC	Chromosome 6: 193 kb
JLR873/JOHE18949	ATGCAGAGACACCATGTCAATG	cut XhoI
JLR242/JOHE16130	CATCAGGCTGACACTACGCTT	Common MAT primer
JLR271/JOHE16476	CCCTACAGAAGATCCGTAAT	MATa allele
JLR510/JOHE18101	CTCGTGCCGAAACCTACAGTT	MATa allele
JLR903/JOHE19058	TGGTTTTCCGAAACACTTCTC	Chromosome 6: 303 kb
JLR913/JOHE19067	CGACCTTATCTACACGTAATC	cut EcoRV
JLR844/JOHE18920	CTAAGCAGCTCAGTCAGGTTTC	Chromosome 6: 703 kb
JLR854/JOHE18930	GAATTTGCCTGCTGGCAAGATC	cut HindIII
General Primers		
JLR427/JOHE17951	ATGAGCAMTMARACDTAYGG	PAP1 degenerate primer

JLR432/JOHE17956	GAGTYKMGAAAKHYTTCACRTA	PAP1 degenerate primer
JLR565/JOHE18276	GATCAGAAAGGAGACCAATTGCTG	<i>C. guilliermondii</i> HIP1
JLR558/JOHE18269	AAACTGGGCAGACCTCTTTC	<i>C. guilliermondii</i> PAP1
JLR556/JOHE18267	CAAAGTGACAGCAACGAATCTTG	<i>C. guilliermondii</i> PAP1
JLR406/JOHE17625	GATGGCACAACCTCAATCGAAT	<i>C. guilliermondii</i> RCY1
JLR240/JOHE16128	CACCTTGAATGTGCTCTC	<i>C. lusitaniae</i> MAS2
JLR506/JOHE18107	GGTATCTCAATAGATTTGATTTTCG	<i>C. lusitaniae</i> PAP1
JLR511/JOHE18112	AACTGTAGGTTTCGGCAGGAG	<i>C. lusitaniae</i> PAP1
JLR553/JOHE18229	CCHAYACTKCKKGAGARTTWG	PIK1 degenerate primer
JLR676/JOHE18495	CTCTATCAAATCAACAGACTC	<i>C. lusitaniae</i> OBP1
JLR247/JOHE16135	GCGAGTACATGGACTGTTG	<i>C. lusitaniae</i> RCY1
JLR717/JOHE18857	GTGCACAATAGCGAACCCTG	SPO11 5' flank
JLR718/JOHE18858	CTCTAGTTTTGACGCTCGACATTTTATGATGACGTCACCAACCACCG ACAAGT	SPO11 5' flank overlap with SAT1
JLR1134/JOHE19351	GACCTGCAGCGTACGAAGCTTCAGCTG ACGTCACCAACCACGACAAGT	SPO11 5' flank overlap with URA3
JLR1135/JOHE19352	ACTTGTCCGGTGGTGGTACGCTCAGCTGAAGCTTCGTACGCTGCAG GTC	SPO11 URA3
JLR1136/JOHE19353	GATCAATTTAACACCTGTTGCG GCATAGGCCACTAGTGGATCTG	SPO11 URA3
JLR719/JOHE18859	ACTTGTCCGGTGGTGGTACGCTCATATAAAATGTCGAGCGTCAAAA CTAGAG	SPO11 SAT1
JLR720/JOHE18860	GATCAATTTAACACCTGTTGCGTTCCTGCAGGACCACCTTTGATTGT AAATAG	SPO11 SAT1
JLR1137/JOHE19354	CAGATCCACTAGTGGCCTATGC CGCAACAGGTGTTAAATTGATC	SPO11 3' flank overlap with URA3
JLR721/JOHE18861	CTATTTACAATCAAAGGTGGTCTGCAGGAACGCAACAGGTGTTAAA TTGATC	SPO11 3' flank overlap with SAT1
JLR722/JOHE18862	TCGGTCTCACCTTCGTTCAAC	SPO11 3' flank
JLR963/JOHE19157	GAGATGATTTGCAGCAGGAAGC	a2 5' flank
JLR964/JOHE19158	GACCTGCAGCGTACGAAGCTTCAGCTGGCCATGATGAATTGGTTTC GAGATC	a2 5' flank overlap with URA3
JLR965/JOHE19159	GATCTCGAAACCAATTCATCATGGCCAGCTGAAGCTTCGTACGCTGC AGGTC	a2 URA3
JLR966/JOHE19160	CAACTTGAAGGCCAAAGAAGTACTC GCATAGGCCACTAGTGGATCTG	a2 URA3
JLR967/JOHE19161	CAGATCCACTAGTGGCCTATGC GAGTACTTCTTTGGCCTCAAGTTG	a2 3' flank overlap with URA3
JLR968/JOHE19162	CCTGGTGCTTGAACGTATGC	a2 3' flank
JLR976/JOHE19170	CAGTGCAAACACAGGTCTAG	α 1 5' flank
JLR982/JOHE19176	CTCTAGTTTTGACGCTCGACATTTTATGATGCGTCCAATACCTGCTTC AAGTGC	α 1 5' flank overlap with SAT1
JLR983/JOHE19177	GCACTTGAAGCAGGTATTGGACGCATCATAAAATGTCGAGCGTCAAA ACTAGAG	α 1 SAT1
JLR984/JOHE19178	GAATAATAGGTGCGAAAGCAATGTTCTGCAGGACCACCTTTGATT GTAATAG	α 1 SAT1
JLR985/JOHE19179	CTATTTACAATCAAAGGTGGTCTGCAGGAACATTGCCTTTTCGCACC TATTATTC	α 1 3' flank overlap with SAT1
JLR981/JOHE19175	TTACTAGTTCAACTCAGAAGG	α 1 3' flank
JLR1138/JOHE19443	CTTCGTGGAATCAGTCCTCATTG	a1 5' flank
JLR1144/JOHE19444	CTCTAGTTTTGACGCTCGACATTTTATGATGAAGATGATGAACTATACG TACATG	a1 5' flank overlap with SAT1
JLR1145/JOHE19445	CATGTACGTATAGTTCATCATCTTCATCATAAAATGTCGAGCGTCAAA ACTAGAG	a1 SAT1
JLR1146/JOHE19446	GCACTGTACATAGACTTATTGAATTCCTGCAGGACCACCTTTGATTG TAAATAG	a1 SAT1
JLR1147/JOHE19447	CTATTTACAATCAAAGGTGGTCTGCAGGAATTCAATAAGTCTATGTA CAGTGC	a1 3' flank overlap with SAT1
JLR1143/JOHE19448	GACACCTCTACCTTCGTTG	a1 3' flank

3. Immunotherapy with FK506 does not select for resistance to calcineurin inhibitors in *Candida albicans* isolates from liver transplant patients

3.1 Introduction

Invasive fungal infections are a significant complication of organ transplantation occurring in up to 42% of liver transplant recipients (Castaldo, Stratta et al. 1991; Collins, Samore et al. 1994; Singh 1997; Singh 1998; Singh 2003; Singh 2003). *Candida* species are the causative agent in 62% to 91% of invasive fungal infections after liver transplantation (Singh 2003). Manipulation of the gastrointestinal tract during surgery allows translocation of endogenous organisms across the intestinal epithelium resulting in a unique susceptibility to invasive candidiasis with most infections occurring within the first month post-transplantation. Although *Candida albicans* is the predominant species with fluconazole therapy, up to one-third of isolates are non-*albicans* *Candida* including *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (Fortun, Lopez-San Roman et al. 1997; Husain, Tollemar et al. 2003). Although the incidence of fungal disease in liver transplantation has declined largely due to advancements in surgical techniques (Singh, Wagener et al. 2002), the high associated mortality (25% to 67%) (Singh, Gayowski et al. 1997; Gayowski, Marino et al. 1998; Singh 1998; Singh, Wagener et al. 2002; Singh 2003) highlights the continued need to understand the pathogenesis of these infections and to develop new treatment strategies.

Two mainstay immunosuppressants in liver transplantation, Tacrolimus (FK506) and Cyclosporine A (CsA), inhibit the Ca^{2+} /-calmodulin-dependent protein phosphatase calcineurin that is required for T-cell activation in response to antigen presentation (Clipstone and Crabtree 1992; Fruman, Klee et al. 1992; O'Keefe, Tamura et al. 1992; Clipstone and Crabtree 1993). CsA and FK506 enter the cell, bind to the

immunophilins cyclophilin A and FKBP12, respectively, and the resulting drug-protein complexes bind calcineurin (Cardenas, Muir et al. 1995; Hemenway and Heitman 1999) preventing T-cell proliferation and suppressing immune responses involved in transplant rejection (Clipstone and Crabtree 1993; Cruz, Fox et al. 2001; Blankenship, Wormley et al. 2003; Blankenship and Heitman 2005).

In addition to their roles in human immunotherapy, these drugs also inhibit calcineurin function in several human fungal pathogens including *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (Odom, Muir et al. 1997; Cruz, Fox et al. 2001; Fox, Cruz et al. 2001; Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003; Steinbach, Schell et al. 2004). In *C. albicans*, inhibition of calcineurin results in enhanced susceptibility to azole antifungal drugs, susceptibility to cation stresses (including Ca^{2+} , Na^{+} , and Li^{+}), decreased survival in serum, and avirulence in a murine systemic candidiasis model (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003). Thus, calcineurin inhibitors could have two potential roles in antifungal therapy, either through use in a combination with azole antifungals, or via an intrinsic ability to decrease serum survival. A previous proof of principal study used a rat endocarditis model to show that the combination of fluconazole and cyclosporine A was more effective than either drug alone at treating both primary heart vegetative lesions and kidney lesions formed via hematogenous dissemination (Marchetti, Entenza et al. 2000), suggesting that combination therapy could be effective in an *in vivo* setting. Although liver transplant patients receive a calcineurin inhibitor, which previous studies suggest could protect against invasive candidiasis (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Blankenship and Heitman 2005), a substantial proportion of patients still develop disease. Therefore, we investigated whether *Candida* isolates from patients

immunosuppressed with FK506 exhibited altered susceptibility to these drugs with respect to azole tolerance, serum survival, and Ca²⁺ stress.

3.2 Materials and Methods

3.2.1 Strains and Media

All strains were routinely propagated on YPD (1% yeast extract, 2% bacto peptone, 2% glucose, 2% bacto-agar (DIFCO)) medium at 30⁰C unless otherwise indicated. Clinical isolates were streaked for single colonies prior to analysis and were stored as frozen stock in 15% glycerol at -80⁰C. Species identity of strains was established using API carbohydrate assimilation strips (bioMérieux) according to the manufacturers instructions, and also by chromatogenic typing with CHROMagar™ Candida (Hardy diagnostics). Serum solid media consisted of 50% Certified Fetal Bovine Serum (FBS; Gibco), and 2% bacto-agar.

3.2.2 Fluconazole susceptibility testing

The minimum inhibitory concentrations 80 (MIC₈₀) for all strains were determined using E-test strips (AB Biodisk) and confirmed by NCCLS microdilution testing (National Committee for Clinical Laboratory Standards 1997). For the serial dilution spot assay strains were grown overnight in YPD at 30⁰C, washed twice with PBS, and normalized to 1 x 10⁷ cells/ml. 1:10 serial dilutions were spotted onto YPD, YPD containing fluconazole (Diflucan®, Pfizer) with or without FK506 (Prograf, Astellas Pharma US, Inc). Cells were grown at 30⁰C and monitored for growth at 24 and 48 hours.

3.2.3 Serum sensitivity assays

Solid plate assays were performed by streaking single colonies from fresh YPD plates onto 50% FBS plates and incubating at 30°C for 24 to 48 hours. For liquid assays strains were grown overnight in YPD at 30°C, washed twice with PBS, and inoculated into 100% FBS with or without 1 µg FK506 at 2000 cells/ml. Cultures were incubated at 30°C for 24 hours. Since human body temperature is 37°C, incubating at this temperature would have been ideal, however wild-type *C. albicans* forms robust hyphae under these conditions, thus accurate cell counts are difficult to obtain. Since the temperature does not affect the interaction between serum and FK506 (i.e. calcineurin mutants lose viability at both 30°C and 37°C), we chose to incubate our cells at 30°C so that we could accurately measure fold population changes. Appropriate serial dilutions of the cultures were plated onto YPD for CFU (colony forming unit) counts at 0 and 24 hours. The fold population change was determined by dividing the CFU's at 24 hours by the CFU's at 0 hours. Calcium studies were performed in a similar manner except strains were inoculated into PBS with or without 1 µg /ml FK506 and 0, 2, 6, or 10 mM CaCl₂.

3.3 Results

3.3.1 Species identification and clinical characterization of isolates

Twenty-four *Candida* isolates were collected from 22 liver transplant recipients receiving Tacrolimus. The median duration of immunosuppression prior to isolate collection was 26 days and ranged from 5 days to 11 years. The species identity of each strain was established using API carbohydrate assimilation strips (bioMérieux) (Land, Harrison et al. 1979) and CHROMagar™ *Candida* (Hardy Diagnostics). Isolates consisted of 18 *Candida albicans*, 3 *Candida glabrata*, 2 *Candida parapsilosis*, and 1 *Candida*

tropicalis (Table 1). The strains were further classified as either invasive or colonizing. Invasive candidiasis was defined as isolation of *Candida* from at least one blood culture or from normally sterile body fluids either intraoperatively or by percutaneous needle aspiration in patients with signs and symptoms indicative of infection (Land, Harrison et al. 1979). Isolation of *Candida* from non-sterile samples in patients who did not fulfill the above criteria, and for which antifungal therapy was not employed as treatment, was considered to represent colonization. Eleven strains were considered invasive, while 13 were classified as colonizing (Land, Harrison et al. 1979).

Table 5: Phenotypes of clinical isolates

ISOLATE ¹	SOURCE ²	SPECIES ³	Antifungal Prophylaxis	Fluconazole MIC ⁴	Fluconazole + FK506 susceptibility ⁵	Serum + FK506 susceptibility ⁶
PAT1 ISO1	eye	<i>C. albicans</i>	None	2.0 -3.0	S	S
PAT2 ISO1	peritoneal fluid	<i>C. albicans</i>	None	0.25	S	S
PAT3 ISO1	peritoneal fluid	<i>C. albicans</i>	None	0.25 - 0.38	S	S
PAT4 ISO1	peritoneal fluid	<i>C. albicans</i>	None	0.25 - 0.38	S	S
PAT5 ISO1	blood	<i>C. albicans</i>	None	0.25 - 0.38	S	S
PAT6 ISO1	hematoma	<i>C. albicans</i>	None	0.75	S	S
PAT7 ISO1	BAL	<i>C. albicans</i>	None	0.38	S	S
PAT8 ISO1	sputum	<i>C. albicans</i>	None	1.5 - 2.0	S	S
PAT9 ISO1	BAL	<i>C. albicans</i>	Fluconazole	0.25	S	S
PAT9 ISO2	urine	<i>C. albicans</i>	Fluconazole	0.5	S	S
PAT9 ISO3	sputum	<i>C. albicans</i>	Fluconazole	0.38 - 0.50	S	S
PAT10 ISO1	urine	<i>C. albicans</i>	None	0.38 - 0.50	S	S
PAT11 ISO1	BAL	<i>C. albicans</i>	Fluconazole	1.5 - 2.0	S	S
PAT12 ISO1	BAL	<i>C. albicans</i>	None	1.5	S	S
PAT12 ISO2	sputum	<i>C. albicans</i>	None	1.5 - 2.0	S	S
PAT13 ISO1	urine	<i>C. albicans</i>	Voriconazole	0.25 - 0.50	S	S
PAT14 ISO1	blood	<i>C. albicans</i>	Fluconazole	0.25 - 0.38	S	S
PAT15 ISO1	pleural fluid	<i>C. albicans</i>	Fluconazole	0.38 - 0.50	S	S
PAT16 ISO1	peritoneal fluid	<i>C. tropicalis</i>	None	0.25 - 0.38	S	S
PAT17 ISO1	urine	<i>C. parapsilosis</i>	None	1.5 - 2.0	S	S
PAT18 ISO1	BAL	<i>C. glabrata</i>	Voriconazole	> 256	S	R
PAT19 ISO1	urine	<i>C. glabrata</i>	Voriconazole	> 256	S	R
PAT20 ISO1	blood	<i>C. parapsilosis</i>	Fluconazole	0.75 - 1.5	S	S
PAT21 ISO1	blood	<i>C. glabrata</i>	Fluconazole	32 - 48	S	S

¹Strains in bold are invasive isolates.

²BAL, broncheal alveolar lavage; eye, endophthalmitis.

³Species was determined by API testing.

⁴Fluconazole MICs were determined by E-Test; resistance MIC $\geq 64 \mu\text{g} / \text{ml}$, sensitive-dose dependent MIC = 16 – 32 $\mu\text{g} / \text{ml}$, sensitive MIC $\leq 8 \mu\text{g} / \text{ml}$

⁵S, susceptible; R, resistant

⁶Susceptibility as measured in liquid culture. S, sensitive (fold population change <1); R, resistant (fold population change >1)

3.3.2 All clinical isolates are sensitive to the combination of FK506 and fluconazole

In vitro fluconazole MIC testing was performed in triplicate for all isolates using E-test strips (AB Biodisk) on RPMI medium, and confirmed by NCCLS microdilution

testing (National Committee for Clinical Laboratory Standards 1997). Only two isolates, both *Candida glabrata*, were resistant to fluconazole (MIC > 64 µg/ml). All other isolates were susceptible to fluconazole, except *C. glabrata* isolate PAT21 ISO1 which was sensitive-dose dependent. All three *C. glabrata* isolates with decreased susceptibility to fluconazole were collected from patients that had received an azole as antifungal prophylaxis; however, azole prophylaxis was not always associated with fluconazole resistance.

Previous studies with *Candida* have shown that treatment with a calcineurin inhibitor results in enhanced susceptibility to azole antifungals (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003). Eleven of the strains were from patients that had received prophylactic treatment with an azole concurrently with their FK506 immunosuppressive therapy. The average duration of azole therapy was 86 days, and ranged from 3 to 397 days. We tested whether the clinical isolates were susceptible to the combination of fluconazole and FK506 or CsA (data not shown) by serial spot dilution assays. All strains were counted with a hemocytometer, normalized to an initial concentration of 1×10^7 cells/ml, and 1:10 serial dilutions were spotted onto YPD or YPD containing 10 µg/ml fluconazole (Diflucan[®], Pfizer) with or without 1 µg of FK506 (Prograf, Astellas Pharma US, Inc) per ml. All plates were incubated at 30°C for 24 to 48 hours and observed for growth. Controls included *C. albicans* strains SC5314 (wild-type strain), a homozygous calcineurin B deletion mutant (JRB64, *cnb1/cnb1*), and an *rbp1/rbp1* deletion mutant (YAG171) that lacks FKBP12 and is therefore insensitive to FK506. Due to their resistance to fluconazole alone, the *C. glabrata* isolates were also tested on solid medium containing 256 µg per ml fluconazole with and without 1 µg per ml FK506. At the higher concentration of fluconazole all three *C. glabrata* isolates demonstrated susceptibility to the combination of fluconazole and FK506. Therefore, despite previous exposure to

FK506 there was no selection for resistance to the combination of calcineurin inhibitors and fluconazole in any of the isolates.

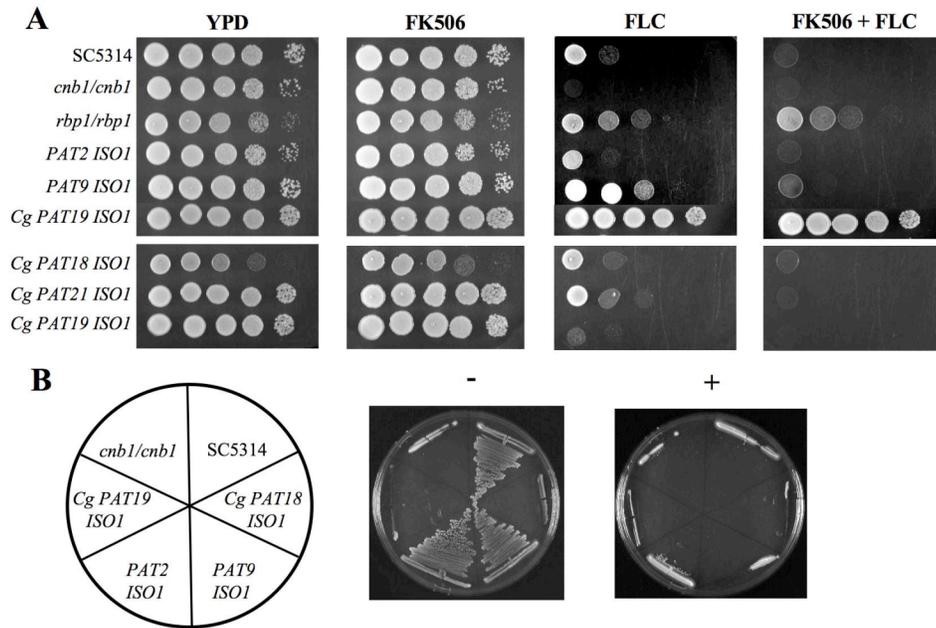


Figure 17: Fluconazole and serum susceptibility of invasive and non-invasive clinical isolates

(A) Isolates were grown for 48 hours at 30°C on YPD medium alone, or containing FK506 (1 µg per ml), Fluconazole (10 µg per ml, upper panels; 256 µg per ml, lower panels), or both drugs. Control strains SC5314 (WT), *cnb1/cnb1* (calcineurin deletion mutant), and *rbp1/rbp1* (FKBP12 deletion mutant) were spotted along with representative clinical isolates. (B) Isolates were streaked on 50% FBS medium alone (-) or containing 1 µg per ml FK506 (+) and grown for 48 hours at 30°C. *C. glabrata* isolates have reduced growth compared with *C. albicans* strains on solid serum media. All strains failed to grow in the presence of FK506 and serum.

3.3.3 *C. glabrata* isolates have decreased growth in serum in the presence of FK506, but unlike *C. albicans* do not lose viability

Calcineurin is also required for *Candida albicans* survival in serum (Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003). Wild-type *C. albicans* strains grow robustly in Fetal Bovine Serum (FBS) at 30°C, but lose viability under the same

conditions when CsA or FK506 are added. Therefore, it is possible that calcineurin inhibitors alone would exert an intrinsic antifungal activity *in vivo* when present in serum, which would hinder hematogenous dissemination. We hypothesized that successfully invading isolates may have a decreased susceptibility to the combination of calcineurin inhibitors and serum. We measured the fold population change of all isolates after 24 hours growth in FBS (Gibco certified FBS) with or without 1 μg per ml FK506. Cells were grown overnight in YPD at 30°C, washed twice in PBS, inoculated into FBS at a concentration of approximately 2000 cells per ml, and incubated for 24 hours at 30°C. Dilutions were plated onto YPD plates for CFU counts at 0 and 24 hours. The fold population change was determined by dividing the CFU's at 24 hours by the CFU's at 0 hours. Strains were also streaked onto serum plates (50% FBS, dH₂O, and 2% bacto agar) with or without 1 $\mu\text{g}/\text{ml}$ FK506. All *C. albicans* strains were able to grow robustly in liquid serum culture undergoing a 10,000 to 100,000-fold increase in population. By contrast, the *C. glabrata* and *C. parapsilosis* strains underwent more limited expansion in liquid serum on the order of 10 to 100 fold ($p = 0.0005$, $p = 0.0045$ respectively). In accordance, the *C. glabrata* isolates did not exhibit visible growth on solid serum medium as judged by the failure to form colonies. All isolates demonstrated a reduced growth in serum containing FK506. Only two isolates, *C. glabrata* PAT18 ISO1 and PAT19 ISO1, were resistant to the combination. Although these strains have a reduced growth rate in the presence of FK506, the fold population change was still greater than or approximately 1 indicating continued growth rather than a reduction in viability (fold population change <1) as seen with all other isolates. Interestingly, invasive and colonizing isolates did not differ in their ability to proliferate in serum, and invasive isolates did not show selection for resistance to the combination of serum and calcineurin inhibitor.

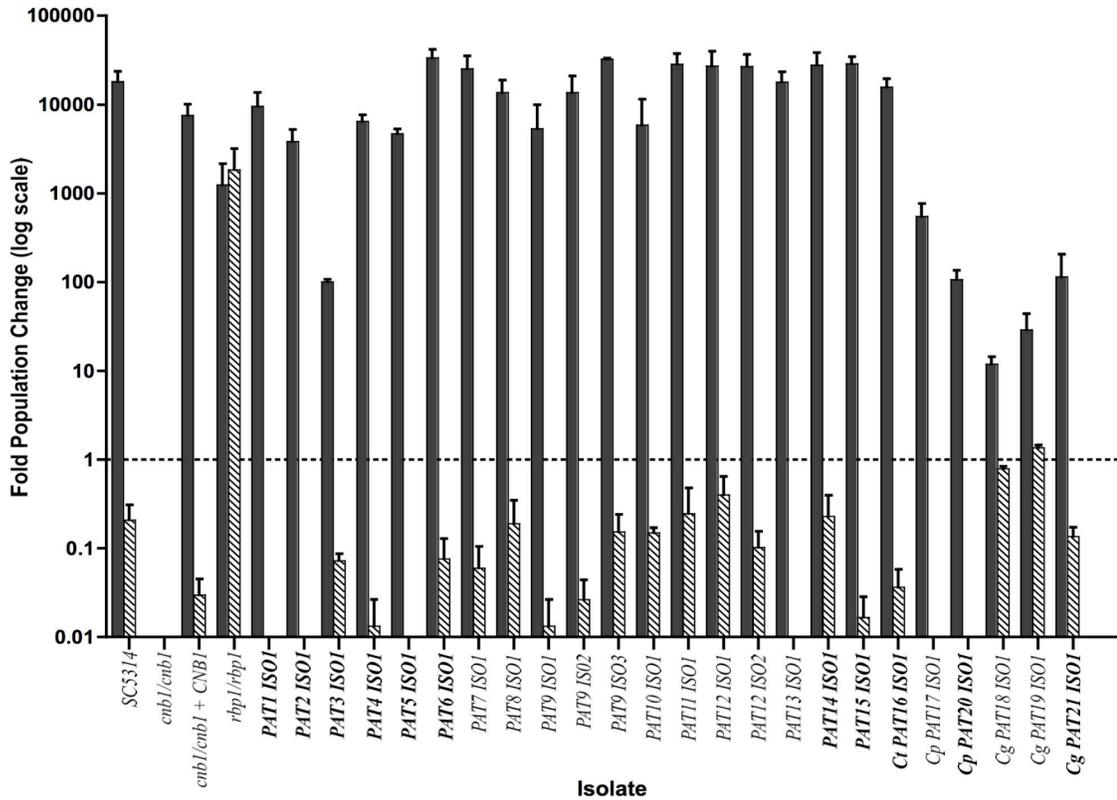


Figure 18: Clinical *Candida* isolates are not resistant to the combination of serum and FK506

Fold population change after 24 hours growth in liquid FBS in the presence (diagonal hatched bars) or absence (solid bars) of 1 µg per ml of FK506. Invasive isolates are in bold. Error bars represent the standard error calculated from three independent experiments.

3.3.4 Both invasive and non-invasive isolates respond similarly to the combination of calcium and FK506

Recent studies have indicated that calcium is the component within serum that is toxic to *C. albicans* calcineurin mutants (Blankenship and Heitman 2005). Fetal Bovine Serum contains approximately 3.5 to 4.0 mM calcium, a concentration at which calcineurin mutants are unable to survive (Blankenship and Heitman 2005). Although there was no difference in susceptibility to serum and FK506 between the invasive and

colonizing isolates, we determined whether any exhibit altered calcium susceptibility (Fig. 3). The clinical isolates were prepared as in the serum assay, and inoculated into PBS with or without 1 μ g per ml FK506 and 0, 2, 6, or 10 mM CaCl₂ (Sigma). Cultures were incubated at 30°C and CFU counts determined at 0 and 24 hours. For each concentration of calcium tested we compared the fold population change of the FK506 treated and untreated samples. Overall, there was no difference in the calcium susceptibility patterns of invasive and colonizing isolates. In the presence of FK506 all of the *C. albicans* isolates had a significant reduction in viability at the 6 mM and 10 mM calcium concentrations ($p < 0.05$, two-tailed, t-test). Additionally, at 2 mM calcium 7 out of the 18 *C. albicans* strains had a significantly reduced viability ($p < 0.05$); the remaining strains showed a modest decrease that was not statistically significant. The *C. glabrata* isolates demonstrated an increase in viability as the concentration of calcium in the media increased (Fold population change in 0 mM or 2 mM calcium versus 10 mM calcium, $p < 0.05$). This is similar to *S. cerevisiae*, where inhibition of calcineurin results in decreased survival in low calcium environments (Cunningham and Fink 1994).

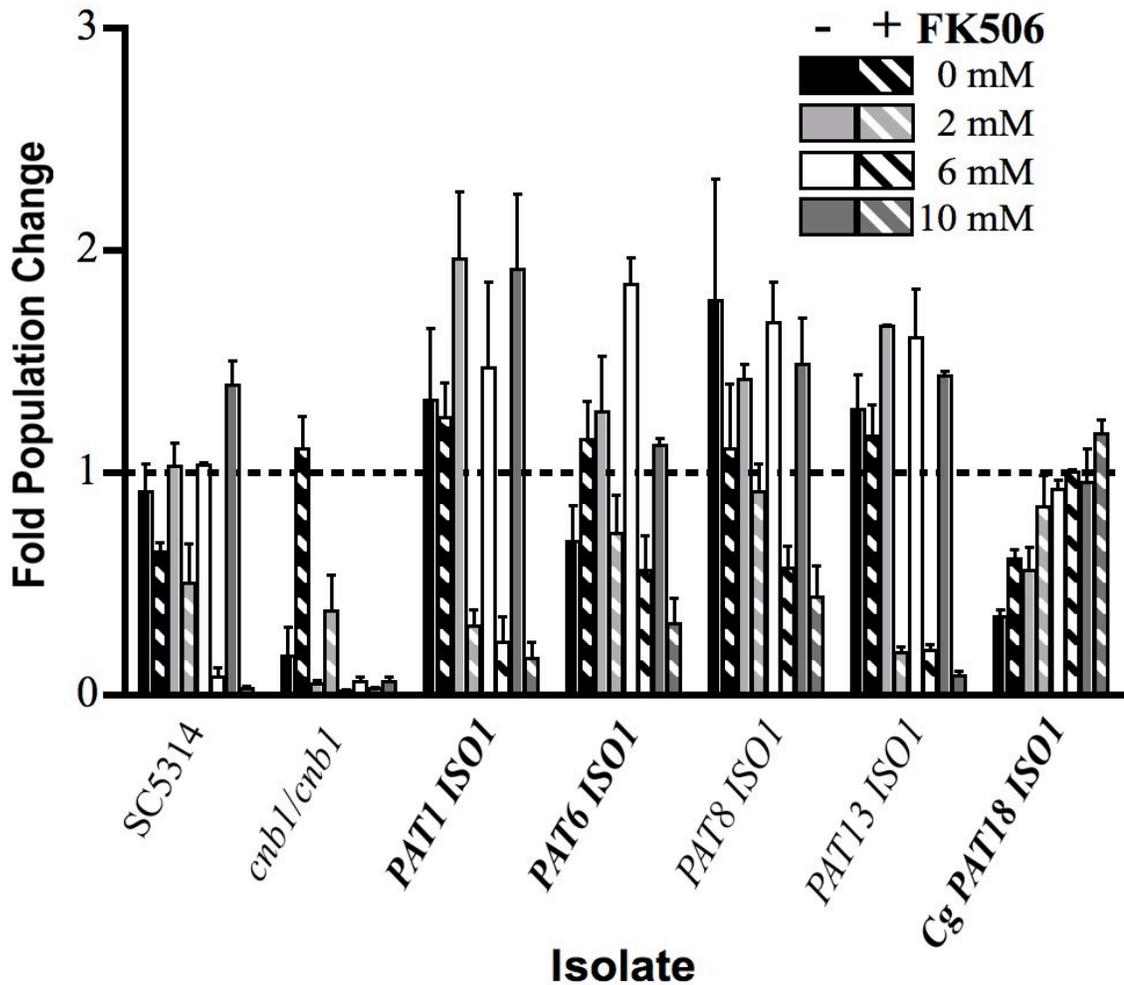


Figure 19: Calcium susceptibility profile of representative invasive and non-invasive isolates

Fold population change was determined after 24 hours incubation in PBS containing 0, 2, 6, or 10 mM CaCl₂ alone (solid bars) or with 1 μg per ml FK506 (diagonal striped bars). All *C. albicans* isolates had significantly decreased viability in the presence of FK506 at 6 mM and 10 mM ($p < 0.05$, two-tailed, T-test). PAT6 ISO1 and PAT13 ISO1 also had a significant reduction at 2mM ($p < 0.05$). Invasive isolates are in bold. Error bars represent the standard error calculated from three independent experiments.

3.4 Discussion

The purpose of this study was to determine if FK506 used for immunosuppression could exert enough antifungal activity in vivo in combination with fluconazole or serum to select for resistant isolates. Analysis of isolates from liver transplant recipients did not reveal any differences in responsiveness to FK506 combined with serum, fluconazole, or calcium.

Previous studies have shown that, in combination with fluconazole, FK506 has an MIC \leq 40 ng/ml (Cruz, Fox et al. 2001). FK506 therapeutic blood levels clinically can range from 5 – 20 ng / ml (trough level) to 68.5 ± 30 ng/ml (peak level). Although the levels obtained in the blood could be high enough to exert an effect, the local tissue concentrations that cells are exposed to in vivo is not known. One hypothesis as to why resistance towards calcineurin inhibitors may not develop in invasive isolates is that the local concentration of drug to which fungal cells are exposed is less than that needed to exert antifungal action. This could be a result of a lower therapeutic dose than optimal for antifungal activity with serum or fluconazole, sequestration of drug through binding to plasma proteins, or alternatively *Candida* may be sheltered from drug exposure by residing within tissues or within cells where drug concentrations may be lower. The use of current clinical formulations of calcineurin inhibitors to augment antifungal therapy is hindered by their immunosuppressive effects, which likely outweigh the antifungal properties. Thus, the development of non-immunosuppressive analogs that retain the ability to target fungal calcineurin could have greater potential as therapeutic drugs. Alternatively, combination of calcineurin inhibitors with other agents that augment their intrinsic antifungal activity may be a viable therapeutic approach.

Chapter modified from published paper: Reedy, et al. 2005 *Antimicrob Agents Chemother.*

4. Elucidating the *Candida albicans* calcineurin signaling cascade controlling stress response and virulence

4.1 Introduction

Calcineurin is a calcium, calmodulin dependent serine threonine protein phosphatase that is highly conserved from yeast to humans and mediates many important cellular processes (Hemenway and Heitman 1999). In mammalian cells, calcineurin is involved in cardiac muscle differentiation (Chin, Olson et al. 1998; Kramer, Fresu et al. 2003; Parsons, Millay et al. 2007), memory (Mansuy, Mayford et al. 1998; Weitlauf and Winder 2001), T-cell activation (Clipstone and Crabtree 1992), and apoptosis (Shibasaki and McKeon 1995; Krebs 1998; Wang, Pathan et al. 1999; Saito, Hiroi et al. 2000). The immunosuppressants FK506 and Cyclosporin A (CsA) exert their effect by entering cells and binding to an immunophilin protein partner (FKBP12 for FK506, and Cyclophilin A for CsA) (Cardenas, Hemenway et al. 1994; Clipstone, Fiorentino et al. 1994; Cardenas, Muir et al. 1995; Ho, Clipstone et al. 1996). This protein-drug complex subsequently binds calcineurin inhibiting its function. In human T-cells, calcineurin activates a transcription factor (NF-AT), which promotes the expression of cytokines and T-cell proliferation (Crabtree 1999). Due to the highly conserved nature of calcineurin, it was subsequently found that FK506 and CsA can also inhibit not only mammalian calcineurin, but also fungal calcineurin (Blankenship, Steinbach et al. 2003; Steinbach, Reedy et al. 2007).

Candida spp. are normal components of the human microbiota; however, under conditions of immunosuppression or altered host defenses these commensals have the ability to cause serious cutaneous and systemic disease (Odds 1988). Diseases due to *Candida spp.* have a variety of clinical manifestations, including mucocutaneous infections of the mouth (thrush), esophagus, and vagina to life-threatening systemic infections, where *Candida spp.* enter the bloodstream and disseminate throughout the

body embedding themselves in target organs (Edwards 1991). Although *C. albicans* has historically accounted for the majority of candidal infections, following the introduction of the antifungal fluconazole numerous other species have increased in prevalence including *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (Merz, Karp et al. 1986; Hazen 1995; Nguyen, Peacock et al. 1996; Pfaller, Jones et al. 1998; Krcmery and Barnes 2002). With the exception of the candins, voriconazole, posaconazole, and lipid formulations of Amphotericin B, the antifungal armamentarium has grown little in the past decade, despite rising rates of resistance, and the fact that the majority of commercially available antifungals are fungistatic rather than fungicidal. Thus, there is need for new strategies and therapeutics to combat fungal infections.

Interestingly, the combination of calcineurin inhibitors and the normally fungistatic antifungal fluconazole resulted in potent killing of *C. albicans*, as well as other more drug resistant species such as *C. glabrata* (Cruz, Goldstein et al. 2002; Onyewu, Blankenship et al. 2003). Additionally, *C. albicans* calcineurin mutants are avirulent in murine systemic models of infections (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003), and had faster rates of disease resolution in murine keratitis models (Onyewu, Afshari et al. 2006). The avirulence of *C. albicans* calcineurin mutants in systemic disease is attributable to the inability of these strains to survive the calcium stress imposed by serum and thus they are unable to survive transit through the bloodstream (Blankenship and Heitman 2005). However, the direct role of calcineurin in *C. albicans* virulence appears to be host niche-specific as there was no virulence defect seen in either a vaginal or a pulmonary model of infection (Bader, Schroppel et al. 2006). Thus, calcineurin inhibitors have two potential mechanisms of action in the clinic: 1) as single agents in cases of disseminated disease or ocular infections to directly impair survival of the yeasts, or 2) as combination therapy to enhance the efficacy of current antifungal therapies. However, the immunosuppressive nature of these drugs limits their

use in systemic therapy, thus we were interested in further characterizing the calcineurin signaling cascade to learn more about this important stress response pathway, and also to elucidate other components that could serve as alternative drug targets that would circumvent the immunosuppressive effects of inhibiting calcineurin.

C. albicans calcineurin is required for cells to survive stresses such as high cations (Li^+ , Na^+ , and Ca^{2+}), antifungal drug treatment (azoles), and survival in the host bloodstream (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003; Blankenship and Heitman 2005). Based upon homology with *S. cerevisiae* the downstream transcription factor Crz1 (Cyert 2003) was previously identified, and shown to shuttle into the nucleus in a calcineurin dependent manner (Karababa, Valentino et al. 2006). However, phenotypic analysis of *crz1/crz1* strains only partially recapitulated a calcineurin mutant phenotype. Although the *crz1/crz1* strains were sensitive to cations and membrane stresses, they had an intermediate phenotype compared with calcineurin mutants (Onyewu, Wormley et al. 2004; Santos and de Larrinoa 2005; Karababa, Valentino et al. 2006). Microarray studies have suggested that Crz1 is the primary mediator of the calcineurin-dependent transcriptional response (Karababa, Valentino et al. 2006). As a first step towards elucidating other potential genes in the *C. albicans* calcineurin pathway, we decided to take a candidate gene approach based upon what is known about the signaling pathway in *S. cerevisiae*.

Few proteins are known that are direct binding partners of calcineurin; these include calmodulin, transcription factors (Crz1, *C. albicans*; Crz1 / Tcn1 *S. cerevisiae*; NF-AT, mammalian cells), and the RCAN family of proteins (Klee, Crouch et al. 1979; Beals, Clipstone et al. 1997; Matheos, Kingsbury et al. 1997; Stathopoulos and Cyert 1997; Cyert 2003; Hilioti and Cunningham 2003; Onyewu, Wormley et al. 2004; Santos and de Larrinoa 2005; Karababa, Valentino et al. 2006; Davies, Ermak et al. 2007). Members of the RCAN family have been identified in species such as *S. cerevisiae* (Rcn1),

C. neoformans (Cbp1), and humans (DSCR1 /MCIP1) based upon a conserved FLSPPxSP domain (Gorlach, Fox et al. 2000; Strippoli, Lenzi et al. 2000; Hilioti and Cunningham 2003; Davies, Ermak et al. 2007). The function of these proteins has been best explored in *S. cerevisiae* where they were shown to have both positive and negative effects on calcineurin function. Rcn1 binds calcineurin and inhibits its function. However, upon phosphorylation by a GSK3 kinase Rcn1 is degraded thereby relieving calcineurin inhibition (Hilioti, Gallagher et al. 2004). In *S. cerevisiae*, Rcn1 expression is induced in a calcineurin-dependent manner, and the phosphorylated protein is itself a substrate for calcineurin (Gorlach, Fox et al. 2000; Hilioti and Cunningham 2003; Hilioti, Gallagher et al. 2004; Kishi, Ikeda et al. 2007). Overexpression of RCAN family members, or their calcineurin binding domain inhibits calcineurin function in both *S. cerevisiae* and in mammalian cells (Fuentes, Genesca et al. 2000; Gorlach, Fox et al. 2000; Vega, Yang et al. 2002; Hilioti and Cunningham 2003; Hilioti, Gallagher et al. 2004). Thus, RCANs serve as important controllers of the calcineurin cascade that could potentially be manipulated to therapeutically inhibit calcineurin function.

Another key aspect of calcineurin signaling is regulation of cellular calcium homeostasis and signaling. Direct targets of calcineurin include multiple calcium channels (Vcx1, Mid1 /Cch1) (Cunningham and Fink 1996; Bonilla, Nastase et al. 2002). In *S. cerevisiae*, endoplasmic reticulum stress activates the Mpk1 pathway, which activates a plasma membrane calcium channel composed of Cch1 and Mid1 (Bonilla, Nastase et al. 2002; Bonilla and Cunningham 2003). Activation of the channel results in calcium influx and activation of calcineurin, which subsequently feedback inhibits the channel through dephosphorylation (Cunningham and Fink 1994). Thus, Mid1 and Cch1 may be important proteins for controlling calcineurin activation. Previous studies in *C. albicans* characterized the role of Mid1 and Cch1 in galvano- and thigmo-tropism. Deletion of either or both calcium channels significantly decreased calcium accumulation.

However, the mutants differed in their response to the various stimuli; Cch1 appears to play a larger role in hyphal orientation in response to electric fields, while loss of Mid1 had a greater impact on hyphal tip reorientation in response to physical contract (Brand, Shanks et al. 2007). Interestingly, calcineurin was required for the reorientation of hyphae in an electric field, but not involved in thigmotropism; however, Crz1 was required for both processes (Brand, Shanks et al. 2007).

In this study, we identified the *C. albicans* homologs of Rcn1, Mid1, and Cch1 and created disruption mutants of these genes. Overexpression of *C. albicans RCN1* in *S. cerevisiae* inhibits calcineurin function. However, similarly to studies in *S. cerevisiae* and *C. neoformans rcn1/rcn1*, *mid1/mid1* and *cch1/cch1* strains share some phenotypes with calcineurin mutants, but do not completely recapitulate the phenotypes of a calcineurin mutant strain.

4.2 Materials and Methods

4.2.1 Strains and Media

All strains were routinely propagated on YPD (1% yeast extract, 2% bacto peptone, 2% dextrose, and 2% bacto agar (DIFCO)). YPD + 300 mM CaCl₂ media was made similarly to YPD except that the media was adjusted to pH 5 prior to autoclaving. The CaCl₂ solution was sterilized separately and the two solutions were mixed after removal from the autoclave. All strains used in this study are listed in Table 6.

4.2.2 Gene disruptions

All deletion strains were created in the SC5314 background. For disruption of *RCN1* two ~500 bp fragments with homology to the 5' promoter and 3' terminator of *RCN1* were PCR amplified, and cloned into plasmid pSFS2A (Reuss, Vik et al. 2004)

with KpnI/XhoI, and NotI/SacI, respectively, generating plasmid pJLR1. Plasmid pJLR1 was digested with KpnI/SacI and the gel purified disruption cassette consisting of the *SAT1* flipper cassette surrounded by ~500 bp of homology flanking *RCN1*. SC5314 was transformed with approximately 1 µg of DNA as previously described (Reuss, Vik et al. 2004). For all deletion strains, at least 3 independent transformations were performed at each step of disruption and an independent transformant was selected from each transformation for further analysis. Nourseothricin resistant isolates were selected for on YPD + 200 µg/ml NAT (Werner). Correct integrants (*RCN1/rcn1::SAT1*) were confirmed by colony PCR and then by Southern blot. At least 3 independent strains that had correctly integrated the disruption cassette were grown overnight in YPM (1% yeast extract, 2% bacto peptone, 2% maltose, and 2% Bactoagar (Difco)) at 30°C and then plated onto YPD + 25 µg/ml NAT. Small colonies which represented those that had flipped out the *SAT1* cassette (*RCN1/rcn1::frit*) were selected and confirmed by Southern blot. Three independent transformants were selected to undergo a second round of transformation to disrupt the remaining *RCN1* allele.

The *RCN1* complementation cassette to reintroduce the *RCN1* gene at the native locus was generated by PCR amplifying the full length *RCN1* gene plus ~500 bp of promoter sequence and ~300 bp of terminator, cloned into plasmid pJLR-RCN3 (containing ~500 bp of homology to the 3' terminator of *RCN1* cloned into the NotI/SacI site of pSFS2A) and sequenced and no extraneous mutations were found. Because the two alleles of *RCN1* differ in *C. albicans*, plasmids containing each allele were selected (pJLR35 and pJLR37) and used for reconstitution. Similarly to above, a KpnI/SacI gel purified fragment was used for transformation.

The *rcn1/rcn1 crz1/crz1* strains were isolated by disrupting *CRZ1* in strains JLR36.3, JLR37.1 and JLR38.1. The *CRZ1* disruption cassette was obtained by amplifying the *SAT1* flipper cassette using long primers with ~90 bp of homology to the region

flanking *CRZ1*. PCRs were carried out using the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 68°C for 4 minutes, and a final extension of 68°C for 15 min. All PCRs were performed using exTaq polymerase (Takara), and 5% DMSO. An ~4.2 kb PCR fragment was gel purified and 1 µg was used for transformation as discussed above.

The *CCH1* disruption cassette was generated by PCR amplifying two ~500 bp fragments consisting of 5' promoter and 3' terminator of *CCH1*, and cloned into plasmid pSFS2A with KpnI/XhoI, and NotI/SacI, respectively yielding plasmid pJLR3. The procedure followed for disruption was the same as used for *RCN1*. Three independent transformants were confirmed by Southern blot.

The *MID1* disruption cassette was produced by amplifying the *SAT1* flipper cassette using long primers with ~90 bp of homology to the region flanking *MID1* in a similar manner to the disruption of *CRZ1*. Three independent transformants were selected and confirmed by Southern blot. The *MID1* complementation cassette was engineered by PCR amplifying the full *MID1* ORF with ~500 bp of promoter and ~300 bp of terminator. This PCR fragment was cloned into plasmid pJLR-CCH3 (containing ~500 bp 3' *MID1* flanking homology cloned into the NotI/SacI site of pSFS2A) using KpnI/XhoI. Complementation was carried out as discussed above for *RCN1*.

The *mid1/mid1 cch1/cch1* strain was created by disrupting *CCH1* in the *mid1/mid1* strains JLR248, JLR253, and JLR255 using the disruption cassette for *CCH1* described above. All primers used for strain creation are listed in Table 7.

4.2.3 *In vitro* stress testing

The minimum inhibitory concentrations 80 (MIC₈₀) for all strains was determined using E-test strips (AB Biodisk). For the serial dilution spot assays, strains were grown overnight in YPD at 30°C, washed twice with PBS, and normalized to 1 × 10⁷ cells/ml.

1:10 serial dilutions were spotted onto YPD, YPD containing fluconazole (Diflucan[®], Pfizer), FK506 (Prograf, Astellas Pharma US, Inc) or cations. Cells were grown at 30⁰C and monitored for growth at 24 and 48 hours.

For liquid serum assays, strains were grown overnight in YPD at 30⁰C, washed twice with PBS, and inoculated into 100% FBS with or without 1 µg FK506 at 2000 cells/ml. Cultures were incubated at 30⁰C for 24 hours. Since human body temperature is 37⁰C, incubating at this temperature would have been ideal; however, wild-type *C. albicans* forms robust hyphae under these conditions, thus accurate cell counts are difficult to obtain. Because the temperature does not affect the interaction between serum and FK506 (i.e. calcineurin mutants lose viability at both 30⁰C and 37⁰C), cells were incubated at 30⁰C so that fold population changes could be accurately measured. Appropriate serial dilutions of the cultures were plated onto YPD for CFU (colony forming unit) counts at 0 and 24 hours. The fold population change was determined by dividing the CFU's at 24 hours by the CFU's at 0 hours.

4.2.4 Heterologous complementation in *S. cerevisiae*

The *C. albicans* and *S. cerevisiae* homologs of *RCN1* were PCR amplified and subcloned into the pCR2.1 TOPO TA vector (Invitrogen) and sequenced. The *RCN1* genes were released by cutting with PvuII and XbaI, gel purified, and cloned into the PvuII/XbaI site of plasmid pYES placing them under the control of the *S. cerevisiae* *GAL1* promoter. Plasmids were confirmed by digestion and sequencing to have the desired structure. *S. cerevisiae* strains K661 (*vcx1*), K605 (*pmc1*), and K665 (*vcx1 pmc1*) (Cunningham and Fink 1994; Cunningham and Fink 1996; Hilioti and Cunningham 2003) were transformed with either pYES alone, or plasmids containing either the *C. albicans* or *S. cerevisiae* *GAL1-RCN1*. Cells were grown to an OD₆₀₀=0.5 to 0.7, washed with 1 mL 0.1 M LiOAc TE, then resuspended in 0.1 M LiOAc TE. 50 µl of cells were mixed with

2.5 μ l salmon sperm (11 μ g/ml) (Sigma), 1 μ l plasmid, and 300 μ l 40% PEG 0.1 M LiOAc. The mixture was incubated at 30°C for 30 minutes, and then heat shocked at 42°C for 15 minutes. Cells were then washed with H₂O and plated on SD-ura to select positive transformants. To test for complementation strains carrying pYES alone, *GAL1-ScRCN1*, or *GAL1-CaRCN1* were streaked onto YPD pH 5, YP galactose pH 5, YPD pH 5 with 0.3 M CaCl₂, and YP galactose pH 5 with 0.3 M CaCl₂. The experiment was performed in duplicate with two different *GAL1-RCN1* plasmids for each species.

4.2.5 Virulence assays

For the disseminated candidiasis model, 5×10^6 cells of wild-type (SC5314), two independent *rcn1/rcn1* (JLR36.3 and JLR37.1), and two *rcn1/rcn1* + *RCN1* strains (JLR180 and JLR323) were injected into the lateral tail vein. Five outbred ICR mice (NCI) were infected per group. There was no significant difference in the survival between the wild-type and either the mutant or reconstituted strains. Log rank statistical analysis of the survival data was performed using the PRISM 4.02 program (GraphPad Software, San Diego, Calif.)

The oropharyngeal candidiasis model was performed as previously described in collaboration with Scott Filler (Park, Myers et al. 2005). Seven mice were infected per group. Both histology and colony forming units (CFU's) per gram tissue were used to evaluate virulence. There was no significant difference between the wild-type SC5314 and *rcn1/rcn1* mutant or between the wild-type DAY185 and the *cnb1/cnb1* mutant.

4.3 Results

4.3.1 Identification of *C. albicans* RCN1 homolog

The RCAN (Regulator of Calcineurin) family of proteins are calcineurin binding proteins that modulate calcineurin activity in *S. cerevisiae* (RCN1), *C. neoformans* (CBP1), and mammalian cells (*DSCR1/MCIP1*) (Gorlach, Fox et al. 2000; Strippoli, Lenzi et al. 2000; Strippoli, Lenzi et al. 2000; Hilioti and Cunningham 2003; Davies, Ermak et al. 2007). A member of this family in *C. albicans*, Rcn1, was identified based upon the conserved KxFLSPPxSPP domain (Hilioti and Cunningham 2003). With the exception of this conserved domain the RCAN family members generally share little homology.

H	N	F	L	I	S	P	P	G	S	P	P	E	G	W	<i>Cryptococcus neoformans</i>
K	M	F	L	I	S	P	P	A	S	P	P	P	E	F	<i>Saccharomyces cerevisiae</i>
K	N	W	L	I	S	P	P	G	S	P	P	V	G	W	<i>Schizosaccharomyces pombe</i>
-	-	F	L	I	S	P	P	S	S	P	P	V	S	W	<i>Homo sapiens</i>
K	R	F	L	I	S	P	P	A	S	P	H	S	E	W	<i>C. albicans</i> orf 19.7770
K	R	F	L	I	S	P	P	A	S	P	H	S	E	W	<i>C. albicans</i> orf 19.123
K		F	L	I	S	P	P		S	P	P			W	

Figure 20: Conserved domain of RCAN family members

ClustalW alignment of the conserved domain found in all RCAN family members (created with MacVector™). Shown are *C. neoformans* Cbp1, *S. cerevisiae* Rcn1, *S. pombe* SPAC13G6.15c, *H. sapiens* DSCR1, and both alleles of *C. albicans* Rcn1.

In *S. cerevisiae*, Rcn1 was identified in a screen for proteins that inhibited calcineurin function when overexpressed (Hilioti and Cunningham 2003). Subsequent studies have shown that the transcription of *RCN1* in *S. cerevisiae* is induced by calcineurin and that Rcn1 has both positive and negative effects on calcineurin function depending on the cellular concentration and phosphorylation state of the protein (Hilioti, Gallagher et al. 2004; Kishi, Ikeda et al. 2007). The two alleles of *RCN1* in *C.*

albicans wild-type strain SC5314 were found to differ by ten amino acids, including 3 amino acid changes and an insert of 7 amino acids in Rcn1-1.



Figure 21: Alignment of *C. albicans* RCN1 alleles

Alignment of the two alleles of *RCN1* in *C. albicans* strain SC5314 (orf 19.7770 and orf 19.123) (MacVector™)

Three independent *rcn1/rcn1* disruption mutants were created using the *SAT1* flipper cassette method described previously. For complementation experiments, a wild-type allele of *RCN1* was reintroduced at the native locus in all three independent mutants. Two of the mutants were complemented with allele *RCN1-2* and one was complemented with allele *RCN1-1*. Both alleles were capable of restoring wild-type sensitivities, and thus there was no observed functional difference between the two alleles.

4.3.2 *rcn1/rcn1* strains are sensitive to LiCl, SDS, and fluconazole

Calcineurin mutants are exquisitely sensitive to cellular stress imposed by cell wall and cell membrane perturbing compounds, as well as various cations (Ca²⁺, Li⁺, and Na⁺) (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003; Sanglard, Ischer et al. 2003). The susceptibility of calcineurin mutants is of particular interest in the clinical setting because calcineurin mutant strains, or wild-type strains in the presence of calcineurin inhibitory drugs (FK506 or Cyclosporin A) are killed by the normally

fungistatic azole antifungal drugs (Cruz, Goldstein et al. 2002; Onyewu, Blankenship et al. 2003). Thus, we were interested in whether *rcn1/rcn1* mutants would have similar sensitivities as calcineurin mutant strains. The susceptibility of *rcn1/rcn1* strains to multiple stresses was tested including: 0.3 M CaCl₂, 0.3 M LiCl, 1.8 M NaCl, 0.05% SDS (sodium dodecylsulfate), and various concentrations of the azole antifungal, fluconazole. *C. albicans rcn1/rcn1* deletion strains were more susceptible to LiCl, SDS, and fluconazole than the wild-type strain (SC5314), but are less sensitive than *cnb1/cnb1* or *cna1/cna1* calcineurin mutant strains. In contrast to calcineurin mutant strains, *rcn1/rcn1* mutants are not hypersensitive to calcium or serum. This suggests that the *RCN1* gene may be involved in the execution of some calcineurin dependent functions, but is not required for all calcineurin functions. Similar results have been found in studies of *RCN1* homologs in both *S. cerevisiae* and *C. neoformans*. Deletion of *rcn1* in *S. cerevisiae* resulted in a modest sensitivity to Li⁺ that was less severe than the calcineurin mutant phenotype (Gorlach, Fox et al. 2000; Hilioti and Cunningham 2003). Likewise, *C. neoformans cbp1* mutants were still able to grow at 37°C whereas calcineurin mutants are inviable at this temperature. However, although to a lesser degree than calcineurin mutants, deletion of *cbp1* resulted in reduced virulence in a murine model of cryptococcal infection (Gorlach, Fox et al. 2000).

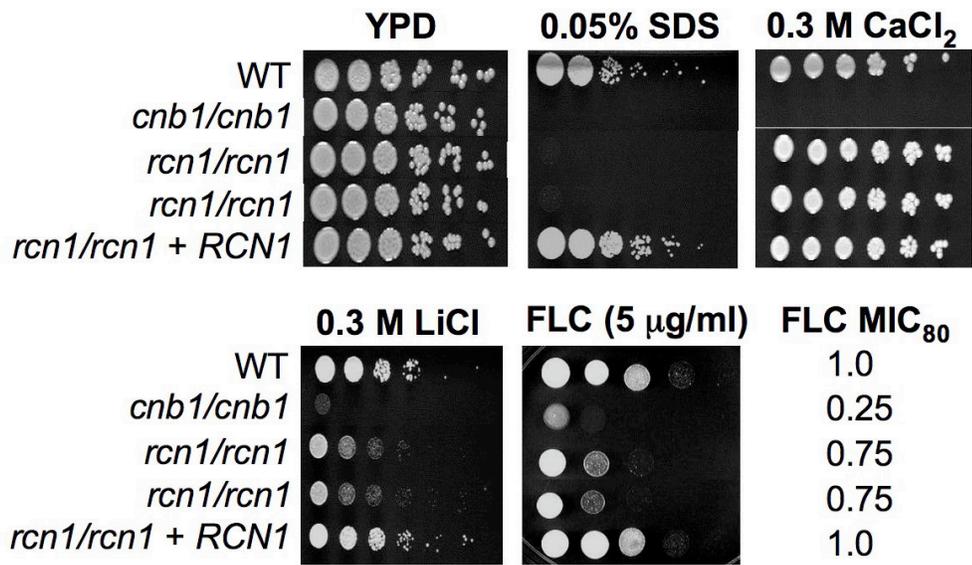


Figure 22: *rcn1/rcn1* strains are sensitive to SDS, Li⁺, and fluconazole

Serial dilutions of strains SC5314 (wildtype), JLR36.3 and JLR 37.1 (*rcn1/rcn1*) and JLR180 (*rcn1/rcn1+ RCN1*) were spotted onto solid YPD media containing the designated salt or drug concentration and incubated at 30°C for 48 hours. Fluconazole MICs were determined by E-TEST.

4.3.3 Neither *rcn1/rcn1* nor *cnb1/cnb1* strains have a virulence defect in an oropharyngeal candidiasis model

The calcineurin pathway has also been of considerable clinical interest due to the avirulence of calcineurin mutant strains in murine models of disseminated candidiasis (Bader, Bodendorfer et al. 2003; Blankenship, Wormley et al. 2003). This avirulence is due to the inability of *C. albicans* calcineurin mutants to withstand the calcium stress imposed by serum, and thus they are unable to survive transit through the bloodstream to disseminate throughout the body (Blankenship and Heitman 2005). The virulence of *rcn1/rcn1* strains was tested in a murine disseminated candidiasis model, and was found to be similar to wild-type *C. albicans*.

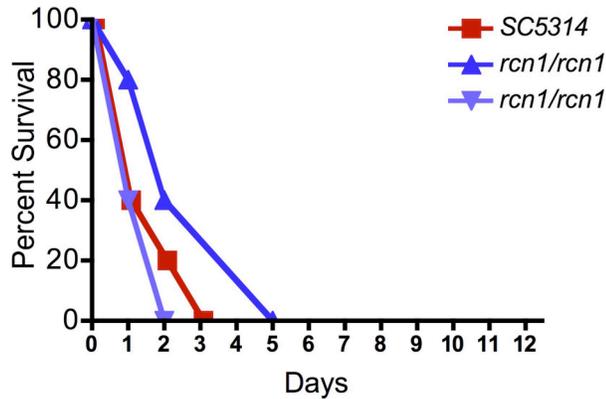


Figure 23: Virulence of *rcn1/rcn1* in a murine disseminated candidiasis model

An inoculum of 5×10^6 cells of wildtype SC5314, two independent *rcn1/rcn1* (JLR36.1 and JLR37.1), and two *rcn1/rcn1 + RCN1* (JLR180, JLR323) strains was injected into the lateral tail vein. Five outbred ICR mice were infected per group. There was no significant difference in the survival between the wildtype and either the mutant or complemented strains. Shown above are only the wildtype and deletion mutant strains.

Interestingly, further studies have demonstrated that the virulence defect of calcineurin mutant strains is host niche-specific. In contrast to disseminated models of candidiasis, calcineurin mutants exhibit wild-type virulence in both pulmonary and vaginal models of infection (Bader, Schroppel et al. 2006). However, a murine keratitis model demonstrated faster resolution of infection in animals infected with *cnb1/cnb1* mutant strain or in those animals treated with both a calcineurin inhibitor and antifungal compared with either drug alone (Onyewu, Afshari et al. 2006). Because oropharyngeal candidiasis is a major manifestation of *Candida* infection particularly in HIV / AIDS patients (Sweet 1997; Fidel 2006), we tested the virulence of both *cnb1/cnb1* and *rcn1/rcn1* strains in this host-niche. Animals were infected with either wild-type, *cnb1/cnb1*, *cnb1/cnb1 + CNB1*, *rcn1/rcn1*, or *rcn1/rcn1 + RCN1* strains. Both histology and log CFU / g tissue were evaluated and no difference in virulence was seen between the wild-type and mutant strains, suggesting that neither calcineurin nor Rcn1 are required for virulence during oropharyngeal infections.

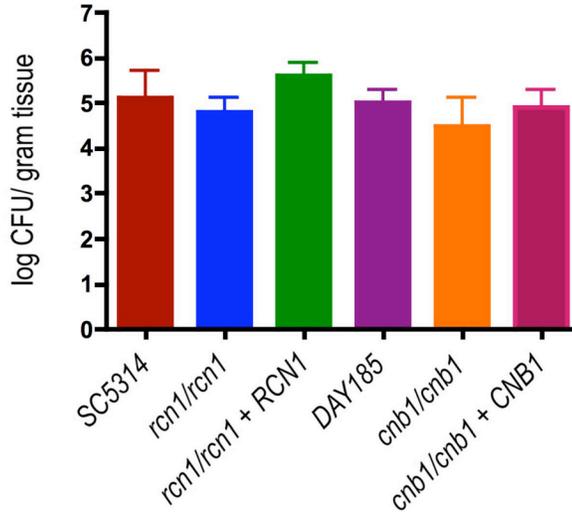


Figure 24: Virulence in a murine oropharyngeal candidiasis model

The oropharyngeal candidiasis infections were performed according to (Park, Myers et al. 2005). 7 mice were infected per group. There was no significant difference between the wild-type SC5314 and *rcn1/rcn1* mutant or between the wild-type DAY185 and *cnb1/cnb1* mutant.

4.3.4 Overexpression of *C. albicans* RCN1 can inhibit *S. cerevisiae* calcineurin function

To determine whether *C. albicans* Rcn1 is capable of interacting with calcineurin, RCN1 was expressed heterologously in *S. cerevisiae* to test whether it functions similarly to *S. cerevisiae* RCN1. In *S. cerevisiae*, RCN1 was originally identified in a screen for genes that, when overexpressed, inhibit calcineurin function and thus promote survival of a *pmc1* strain in the presence of high Ca²⁺ (Hilioti and Cunningham 2003). In *S. cerevisiae*, calcineurin plays a key role in regulating intracellular compartmentalization of Ca²⁺ (Cunningham and Fink 1994). When activated by the presence of exogenous extracellular Ca²⁺, calcineurin is activated and promotes the expression of the gene encoding Pmc1, a vacuolar Ca²⁺ pump, promoting influx of calcium into the vacuole. However, calcineurin also simultaneously inhibits the function of another vacuolar

calcium pump, *Vcx1*, through a post-translational modification. The activity of either *Pmc1* or *Vcx1* is required to redistribute intracellular Ca^{2+} and survive high calcium stress conditions. In the presence of a functional calcineurin protein, deletion of *PMC1* is fatal to cells in a high calcium environment; however, if calcineurin function is inhibited then *Vcx1* is activated and cells survive calcium stress (Cunningham and Fink 1994; Hilioti and Cunningham 2003). Thus, if overexpression of *RCN1* is capable of inhibiting calcineurin function it will be able to rescue *pmc1* strains in the presence of calcium.

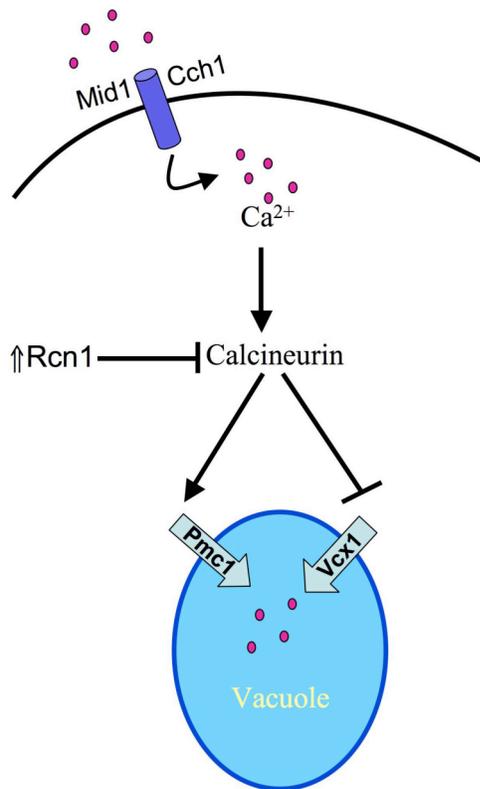


Figure 25: Calcineurin control of calcium compartmentalization in *S. cerevisiae*

Model of calcineurin regulation of intracellular calcium distribution. In response to calcium calcineurin inhibits the expression of *PMC1* and post-translationally inhibits *Vcx1*. In the presence of increased expression of *RCN1*, calcineurin function is inhibited, relieving the calcineurin mediated inhibition of *Vcx1*.

Plasmids were generated allowing expression of both the *S. cerevisiae* and *C. albicans* *RCN1* genes under the control of the inducible *S. cerevisiae* *GAL1* promoter. These plasmids were transformed into *vcx1*, *pmc1*, or *pmc1 vcx1* *S. cerevisiae* strains. As expected, overexpression of *S. cerevisiae* *RCN1* was able to rescue *pmc1* strains in the presence of 300 mM calcium. The *C. albicans* *RCN1* homolog was also able to promote the survival of *pmc1* strains in a high calcium environment, suggesting that *C. albicans* *RCN1* is capable of interacting with and inhibiting *S. cerevisiae* calcineurin. Due to the highly conserved nature of calcineurin, *CaRcn1* may interact similarly with *C. albicans* calcineurin.

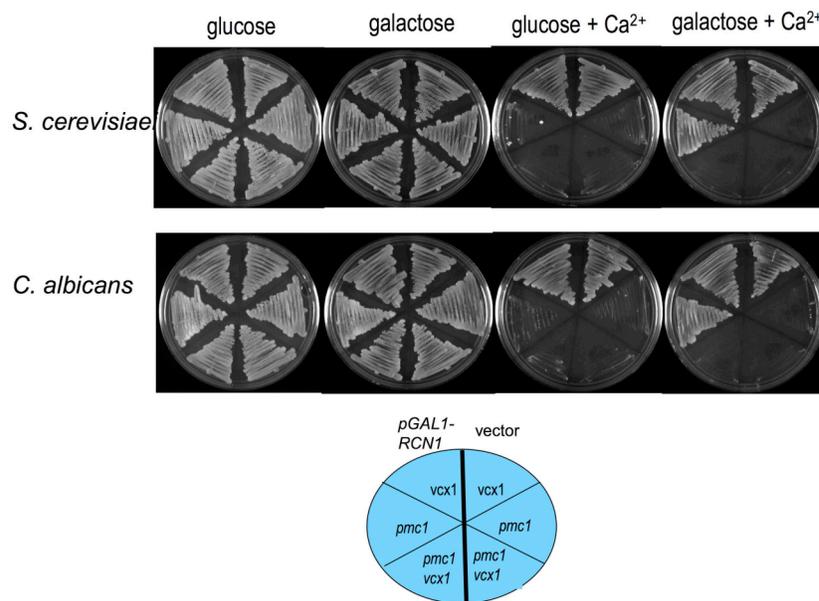


Figure 26: Overexpression of *CaRCN1* rescues the viability of *pmc1* strains

The *C. albicans* or *S. cerevisiae* *RCN1* gene was cloned into plasmid pYES for expression in *S. cerevisiae* under the control of the *GAL1* promoter. The plasmids were sequence confirmed and then strains K665 (*pmc1::TRP1 vcx1Δ*), K605 (*pmc1::TRP1*), and K661 (*vcx1*) were transformed. Under conditions of high calcium, calcineurin promotes the expression of *PMC1* and inhibits *Vcx1* function. *pmc1* strains are unable to grow at high calcium levels unless calcineurin inhibition of *Vcx1* is abolished. Overexpression of either the *CaRCN1* or *ScRCN1* gene allows growth of *pmc1* strains in high calcium suggesting that calcineurin function is inhibited, whereas presence of the vector alone did not effect the growth of *pmc1* strains. Strains were plated on solid YPD medium alone or containing 0.3 M CaCl_2 . The carbon source was either 2% glucose or 2% galactose, as indicated.

4.3.5 *rcn1/rcn1 crz1/crz1* double mutants have slightly increased serum, Li⁺, and SDS sensitivity compared with either single mutant alone

Thus far, in *C. albicans*, only the Crz1 protein has been shown to act downstream of calcineurin (Onyewu, Wormley et al. 2004; Santos and de Larrinoa 2005; Karababa, Valentino et al. 2006). Similar to *rcn1/rcn1* strains, *crz1/crz1* strains share a subset of cation and drug sensitivities with calcineurin mutant strains, but fail to entirely mimic the sensitivity profile of calcineurin deletion strains. As a protein phosphatase calcineurin likely has multiple downstream effectors and thus multiple deletion mutants may need to be combined to completely recapitulate the calcineurin mutant phenotype. Thus, we tested whether *rcn1/rcn1 crz1/crz1* strains would be more sensitive than either single mutant. Multiple independent double mutant strains were isolated and tested for sensitivity to a variety of stress conditions. The double mutants were found to be more sensitive to Li⁺ and SDS than either single mutant. Additionally, the double mutants had significantly reduced growth in serum compared with either single mutant, however unlike calcineurin mutants, which are killed in serum, the *rcn1/rcn1 crz1/crz1* mutants remain viable.

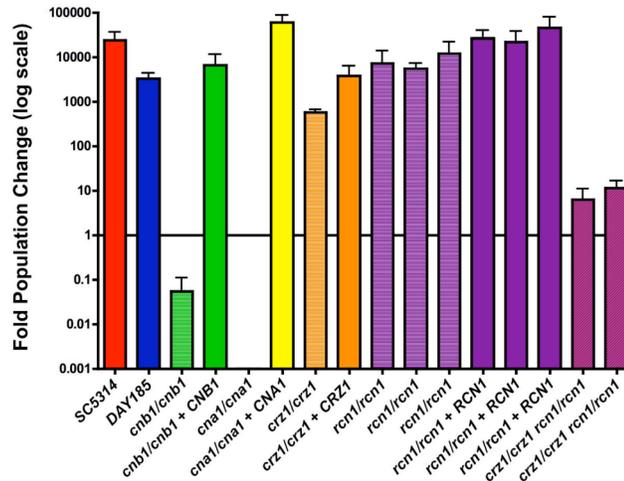


Figure 27: *rcn1/rcn1 crz1/crz1* double mutants have increased serum sensitivity compared to either single mutant alone

Strains were grown for 24 hours in Fetal Bovine Serum at 30°C. Appropriate dilutions from each culture were plated onto YPD at 0 and 24 hours and then incubated at 30°C overnight. Fold population change was determined by dividing CFUs at 24 hours by CFUs at 0 hours. Error bars represent the standard error from two independent experiments. SC5314 and DAY185 served as the wild-type controls.

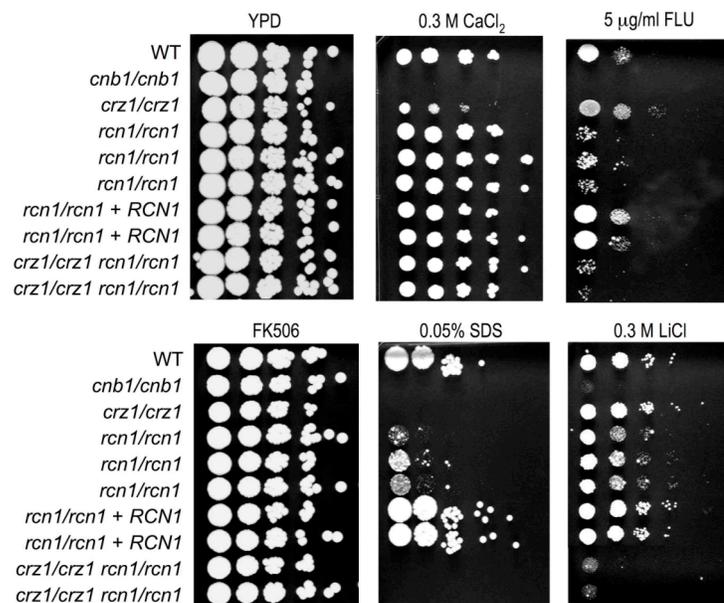


Figure 28: Phenotypic analysis of *rcn1/rcn1 crz1/crz1* strains

Wild-type strain (SC5314), *rcn1/rcn1* mutants (JLR36.3, JLR37.1, and JLR38.1), *rcn1/rcn1 + RCN1* complemented strains (JLR180, and JLR 323), and *rcn1/rcn1 crz1/crz1* double mutants (JLR548 and JLR578) were serially diluted, and spotted onto solid YPD

media containing the designated salt or drug concentration, and incubated at 30°C for 48 hours. The double mutants have a more severe phenotype than the single mutants on LiCl and fluconazole (FLU).

4.3.6 Identification of *C. albicans* MID1 and CCH1

The *C. albicans* *MID1* and *CCH1* genes were identified based upon best hit reciprocal BLAST searches using the *S. cerevisiae* homologs. In *S. cerevisiae*, Mid1 and Cch1 form a calcium channel complex that promotes entry of calcium into cells in response to various stresses, including exposure to mating pheromone, endoplasmic reticulum stress, and cations (Iida, Nakamura et al. 1994; Fischer, Schnell et al. 1997; Bonilla, Nastase et al. 2002; Bonilla and Cunningham 2003; Peiter, Fischer et al. 2005). In response to ER stress, the calcium channels are activated by the Mpk1 pathway and are necessary for calcineurin activation and cell survival (Bonilla and Cunningham 2003). Activated calcineurin dephosphorylates Mid1, resulting in feedback inhibition of the channel and preventing further influx of calcium (Bonilla, Nastase et al. 2002). Therefore, the phenotypes of *mid1* and *cch1* cells in response to ER stress are similar to those of a calcineurin mutant. Consistent with the hypothesis that Mid1 and Cch1 form a complex, both the single and double mutants of *S. cerevisiae* have the same phenotype; however, in other species, such as *C. neoformans*, some *CCH1* independent functions have been observed (Liu, Du et al. 2006). Previous analysis in *C. albicans* of the role of calcium signaling in thigmotropism and galvanotropism reported that while Mid1 played a larger role in thigmotropism, Cch1 was more important for galvanotropism, suggesting that the proteins may have some differential function (Brand, Shanks et al. 2007). Recent studies examining the genes regulated in a calcineurin- and Crz1-dependent manner found that *CCH1* is upregulated by activation of either calcineurin or Crz1 (Karababa, Valentino et al. 2006). Here both the *MID1* and the *CCH1* gene were deleted

with the *SAT1* flipper cassette in the wildtype SC5314 background, and *mid1/mid1 cch1/cch1* double mutants generated.

4.3.7 *mid1/mid1* and *cch1/cch1* strains are sensitive to LiCl and SDS

The susceptibility of *mid1/mid1* and *cch1/cch1* strains was tested in response to cations (Ca^{2+} , Na^+ , and Li^+), SDS, and fluconazole. Both single mutants were sensitive to LiCl and SDS, although not to the same extent as calcineurin mutants. Although neither mutant was sensitive to high Ca^{2+} , the *cch1/cch1* deletion strains were sensitive to low calcium environments (The *mid1/mid1* strains were not tested). The fluconazole sensitivity of both single mutants was tested using E-test strips, and demonstrated modestly increased susceptibility ($\text{MIC}_{80} = 0.5 - 0.75$) compared with wild-type ($\text{MIC}_{80} = 1.0 - 1.5$). In all cases the *mid1/mid1 cch1/cch1* double mutant behaved similarly to the single mutants, suggesting that at least for the phenotypes studied, neither protein has independent functions.

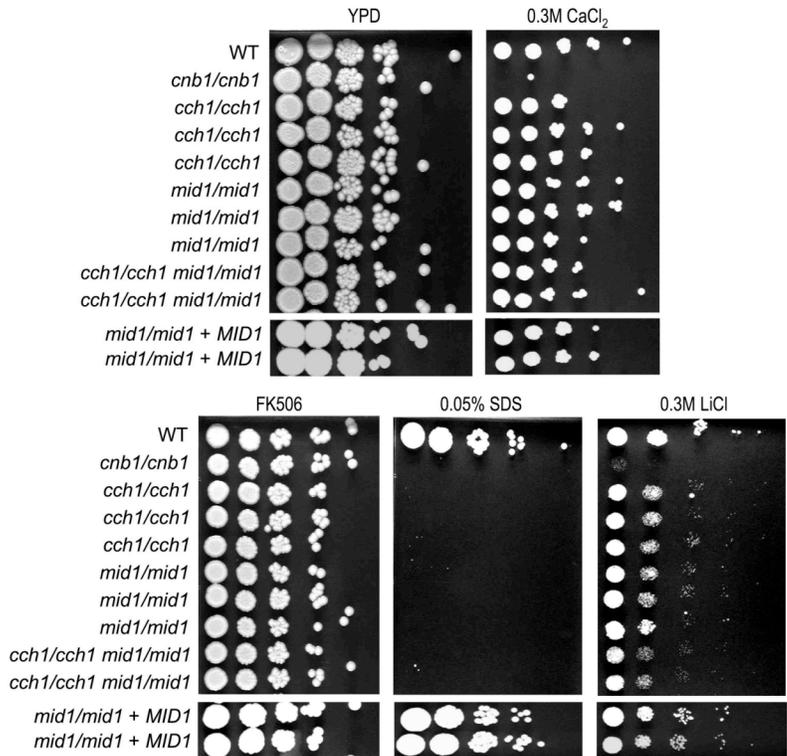


Figure 29: *mid1/mid1* and *cch1/cch1* are sensitive to Li and SDS

Serial dilutions were spotted onto solid YPD media containing the designated salt or drug concentration and incubated at 30°C for 48 hours.

4.4 Discussion

The calcineurin signaling pathway is a key mediator of stress responses in *C. albicans* and has clinical potential as a therapeutic target to enhance the efficacy of the current antifungal armamentarium. Here we have identified additional components of the calcineurin signaling pathway using a candidate gene approach based upon knowledge of the pathway in other organisms. Heterologous expression experiments suggest that *Rcn1* is capable of inhibiting calcineurin function, similar to the *S. cerevisiae* homolog. Similarly to *S. cerevisiae*, an *rcn1/rcn1* strain shares some similar sensitivities with calcineurin deletion strains; however, they have only an intermediate phenotype and do not completely recapitulate a calcineurin mutant phenotype. Microarray studies

suggest that Crz1 may be solely responsible for the calcineurin dependent transcriptional response (Karababa, Valentino et al. 2006); however, calcineurin likely has other downstream effectors that are direct phosphorylation targets. Thus, to completely recapitulate a calcineurin mutant phenotype it is likely that multiple simultaneous gene deletions will be required. The double *crz1/crz1 rcn1/rcn1* mutant had enhanced sensitivity to several stresses suggesting that although both proteins interact with calcineurin they may be influencing different downstream effects. Further studies are needed to establish whether Rcn1 is directly interacting with calcineurin in *C. albicans* in a manner similar to *S. cerevisiae*. The high level of conservation of calcineurin suggests that the relationship between Rcn1 and calcineurin may be similar in both species.

Several lines of evidence suggest that *MID1* and *CCH1* may play a role in calcineurin signaling. Studies in *S. cerevisiae* have shown that in response to endoplasmic reticulum stress the *MID1/CCH1* calcium channel is activated and the resulting influx of calcium activates calcineurin signaling (Bonilla, Nastase et al. 2002; Bonilla and Cunningham 2003). Studies on thigmotropism and galvanotropism showed that Mid1 and Cch1 are required for hyphal reorientation (respectively) and that calcineurin was required for the galvanotropism, but not the thigmotropism response. However, Crz1 was required for both processes, suggesting that Crz1 could have calcineurin independent functions (Brand, Shanks et al. 2007). Additionally, microarray studies in *C. albicans* have shown that calcineurin regulates expression of the *CCH1* gene (Karababa, Valentino et al. 2006). Deletion analysis of *CCH1* and *MID1* suggests that these genes play a role in tolerance to membrane stress and tolerance to fluconazole, although they have an intermediate phenotype compared with calcineurin mutants. Further studies examining the role that these proteins have in activating calcineurin signaling will be required, including studying the activation of Crz1 in these mutants. For the phenotypes analyzed in this paper, both single and double mutants had similar

phenotypes suggesting that the two genes act as a complex as in *S. cerevisiae* or act in a linear pathway and thus there is no additive effect.

The therapeutic potential of calcineurin inhibitors in combination therapy to enhance the efficacy of current antifungals makes furthering our understanding of this signaling pathway of considerable import. Prior to this study only one direct target of calcineurin, Crz1, had been extensively studied in *C. albicans*. The role of Rcn1 is of interest in *C. albicans* as peptides corresponding to the calcineurin binding segment of human RCAN family members have also been shown to inhibit calcineurin function, and could potentially be used as starting points to develop novel therapeutics.

Table 6: Strains used in Chapter 4

Strain	Genotype	Background	Source
SC5314			(Gillum, Tsay et al. 1984)
DAY185	<i>URA3/ura3Δ::λimm434</i> <i>HIS1/his1::hisG</i> <i>ARG4/arg4::hisG</i>	BWP17	(Davis, Edwards et al. 2000)
JRB64	<i>ura3Δ::λimm434/ura3Δ::λimm434</i> <i>his1::hisG::HIS1/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i> <i>cnb1::UAU1/cnb1::URA3</i>	BWP17	(Blankenship, Wormley et al. 2003)
MCC85	<i>ura3Δ::λimm434/ura3Δ::λimm434</i> <i>his1::hisG::CNB1-HIS1/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i> <i>cnb1::UAU1/cnb1::URA3</i>	BWP17	(Blankenship, Wormley et al. 2003)
SCCMP1M4	<i>cmp1::frt/cmp1::frt</i>	SC5314	(Bader, Schröppel et al. 2006)
SCCMP1M2	<i>cmp1::frt/cmp1::frt + CMP1</i>	SC5314	(Bader, Schröppel et al. 2006)
OCC1.1	<i>ura3Δ::λimm434/ura3Δ::λimm434</i> <i>his1::hisG::HIS1/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i> <i>crz1::UAU1/crz1::URA3</i>	BWP17	(Onyewu, Wormley et al. 2004)
OCC7	<i>ura3Δ::λimm434/ura3Δ::λimm434</i> <i>his1::hisG::CRZ1-HIS1/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i> <i>crz1::UAU1/crz1::URA3</i>	BWP17	(Onyewu, Wormley et al. 2004)
JLR36.3	<i>rcn1::frt/rcn1::frt</i>	SC5314	this study
JLR37.1	<i>rcn1::frt/rcn1::frt</i>	SC5314	this study

JLR180	<i>rcn1::frt/rcn1::frt + RCN1</i>	SC5314	this study
JLR323	<i>rcn1::frt/rcn1::frt + RCN1</i>	SC5314	this study
JLR248	<i>mid1::frt/mid1::frt</i>	SC5314	this study
JLR253	<i>mid1::frt/mid1::frt</i>	SC5314	this study
JLR255	<i>mid1::frt/mid1::frt</i>	SC5314	this study
JLR284	<i>mid1::frt/mid1::frt + MID1</i>	SC5314	this study
JLR301	<i>mid1::frt/mid1::frt + MID1</i>	SC5314	this study
JLR48	<i>cch1::frt/cch1::frt</i>	SC5314	this study
JLR50	<i>cch1::frt/cch1::frt</i>	SC5314	this study
JLR265	<i>cch1::frt/cch1::frt</i>	SC5314	this study
JLR548	<i>rcn1/rcn1 crz1/crz1</i>	SC5314	this study
JLR578	<i>rcn1/rcn1 crz1/crz1</i>	SC5314	this study
JLR521	<i>mid1/mid1 cch1/cch1</i>	SC5314	this study
JLR519	<i>mid1/mid1 cch1/cch1</i>	SC5314	this study
K661	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3-1 vcx1Δ</i>	W303-1A	(Cunningham, Fink 1996)
K605	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3-1 pmc1::TRP1</i>	W303-1A	(Cunningham, Fink 1994)
K665	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3-1 pmc1::TRP1 vcx1Δ</i>	W303-1A	(Cunningham, Fink 1996)

Table 7: Primers used in Chapter 4

Name	Sequence	Notes
JLR121	AGTCAACGTAGGTACCATGAACCTTAGAT AATTACG	<i>RCN1</i> 5' flank KpnI
JLR120	TCAACTTCTCGAGCATCAATATTGGTAAT GATTAATG	<i>RCN1</i> 5' flank XhoI
JLR118	TCAATCATTGCGGCCGCAAGATCATCGAT GTAATATGC	<i>RCN1</i> 3' flank NotI
JLR119	TACTTACAAGAGCTCGTCTTGGATAGAAC ATAAAATAC	<i>RCN1</i> 3' flank SacI
JLR130/JOHE14337	TCAACTTCTCGAGGTCTTGGATAGAACAT AAAATAC	<i>RCN1</i> complementation 3' XhoI
JLR126	AGTCAACGTAGGTACCCATACACTTGCTT CGCCTCTA	<i>CCH1</i> 5' flank KpnI
JLR127	TCAACTTCTCGAGGGTATTGCTGTTATCC ATG	<i>CCH1</i> 5' flank XhoI
JLR125	TCAATCATTGCGGCCGCGAACTGTCACT TGGAAAAATGC	<i>CCH1</i> 3' flank NotI
JLR124	TACTTACAAGAGCTCCTGATCCAGAAGAT TTAGCT	<i>CCH1</i> 3' flank SacI
JLR280/JOHE16667	ATGTGCTATTCATTAACCACTAGCCTTCA TCATGATACCACCTTTTATTCTACTACTAT TTCTAATAATGAACTCATCATGGCCCCC CTCGAGGAAGTT	<i>MID1</i> 5' deletion primer
JLR281/JOHE16668	AGAACATAAACAATCAATCAATCGATTCT TCTTCTCTTTTCTATCTATTATATTCTATT TAAATGAACTATATGACAACGCTCTAGAA CTAGTGATCT	<i>MID1</i> 3' deletion primer
JLR163/JOHE14641	TCAACTTCTCGAGGAAGATTTGTCGAGTA CTAG	<i>MID1</i> complementation 3' XhoI

JLR133/JOHE14340	TCAATCATTGCGGCCGCCCTTTACTTTAA TGGTTGTCA	<i>MID1</i> 3' flank NotI
JLR132/JOHE14339	TACTTACAAGAGCTCGAGCTACATTAGTC ATAGCAT	<i>MID1</i> 3' flank SacI
JLR294/17110	ATTTCCCTAACAGCATCTTCCAAGTTC AAATATTTTCCCCTTTTATATCTAAATTT CATAAATCCAATCATGTCTGGCCCCCCC TCGAGGAAGTT	<i>CRZ1</i> 5' deletion primer
JLR295/17111	TAGAATAAAAAACAACCAACCAACCAAC CAACAGGAATAACTATCGTGAATGACAA CAACCTCAAAAAAACTAAGTGCTCTAG AACTAGTGGATCT	<i>CRZ1</i> 3' deletion primer
JLR470	TCAGGTAAGCTGATGCCAAGGAAAC CAACAAAT	<i>Ca RCN1</i> overexpression 5' PvuII
JLR473	ACGTACTAGATCTAGAGTATGTTGACTTG GATAGAAC	<i>Ca RCN1</i> overexpression 3' XbaI
JLR463	TCAGGTAACAGCTGATGGGTAATATTA TAACGGAT	<i>Sc RCN1</i> overexpression 5' PvuII
JLR466	ACGTACTAGATCTAGAAAACAATTAACGA GCCATGG	<i>Sc RCN1</i> overexpression 3' XbaI

5. Exploring the potential role of FKBP12 and homoserine dehydrogenase inhibitors in combinatorial drug therapy in *C. albicans* and *C. glabrata*

5.1 Introduction

Approximately 80% of the population carries *Candida spp.* as normal components of their microbiota, however under conditions of host immunosuppression these species have the ability to invade and cause disease (Odds 1988). The spectrum of disease caused by *Candida* varies from mucocutaneous infections of the skin, nails, mouth, esophagus, and vagina to life-threatening disseminated disease. Historically, *C. albicans* was the causative agent of most candidal disease; however, with the advent of azole antifungal therapy other, more drug resistant, species such as *C. glabrata* and *C. krusei* have increased in prevalence (Merz, Karp et al. 1986; Hazen 1995; Nguyen, Peacock et al. 1996). Despite the appearance of drug resistance and the fact that most therapeutics are fungistatic rather than fungicidal the antifungal armamentarium has changed relatively little over the past decade. Hindering the development of good antifungals with limited side-effects is the fact that fungal cells are eukaryotic and, unlike prokaryotic bacteria (that have many distinct cellular features distinguishing them from our own eukaryotic cells), thus share many similar features with our own cells. Two major difference between fungal cells and our own is the existence of a cell wall and the utilization of ergosterol rather than cholesterol in cell membranes, These fungal unique properties are the targets of most clinical antifungals.

Another striking difference between fungal cells and our own is the existence of a threonine and methionine biosynthetic pathway in fungi that is missing in humans. Interestingly, a genetic screen in *S. cerevisiae* revealed synthetic lethality between mutations in homoserine dehydrogenase (*HOM6*) and FKBP12 (*FPR1*) (Arevalo-

Rodriguez, Pan et al. 2004). Hom6 catalyzes the last step in converting aspartic acid into homoserine, which is a common precursor to both methionine and threonine (Black and Wright 1955). FKBP12 is a prolyl-isomerase that is also the target of two immunosuppressive drugs, FK506 and rapamycin. The FKBP12-FK506 complex binds to and inhibits the function of calcineurin, while FKBP12-rapamycin binds to and inhibits the Tor1 kinase (Blankenship, Steinbach et al. 2003). However, the synthetic lethality of *fpr1* and *hom6* mutation is independent of either calcineurin or Tor1 effects. Previous studies have shown that FKBP12 physically interacts with the enzyme aspartokinase (Hom3), the first step in the methionine-threonine biosynthetic pathway, regulating its feedback inhibition by products of the pathway (Alarcon and Heitman 1997; Arevalo-Rodriguez, Pan et al. 2004). Thus, in the absence of FKBP12 activity flux through the pathway is deregulated and in combination with a *hom6* deletion results in the accumulation of the toxic intermediate aspartate β -semialdehyde (ASA) (Arevalo-Rodriguez, Pan et al. 2004).

Inhibitors of both FKBP12 (FK506) (Blankenship, Steinbach et al. 2003) and Hom6 (HON; 5-hydroxy-4-oxonorvaline) (Jacques, Mirza et al. 2003; Ejim, Mirza et al. 2004) are available and could potentially be used as combination therapy. The clinical utility of FK506 has been limited by its immunosuppressant effects due to calcineurin inhibition (Cardenas, Muir et al. 1995; Cardenas, Zhu et al. 1995); however, other FKBP12 inhibiting compounds could be developed that would spare calcineurin function while inhibiting FKBP12. Thus far, all studies have focused on *S. cerevisiae*. Therefore, we studied this pathway in two pathogenic fungi, *C. albicans* and *C. glabrata* to determine whether this synthetic lethality exists in other clinically relevant fungal species and could be exploited as a new therapeutic strategy.

In this study, we identified the *C. albicans* and *C. glabrata* homologs of *HOM6* and generated deletion mutants in both species. Both deletion strains are auxotrophic for

both threonine and methionine, as expected. However, in *C. albicans* FK506 had no effect on the *hom6/hom6* mutant strains, suggesting that the synthetic lethality does not exist in this species or the intermediate is not toxic. However, results with *C. glabrata* suggest that FK506 does inhibit a *hom6* mutant and thus the function of the methionine and threonine biosynthetic pathway in this species is similar to *S. cerevisiae*, with which it is more closely related compared to *C. albicans*.

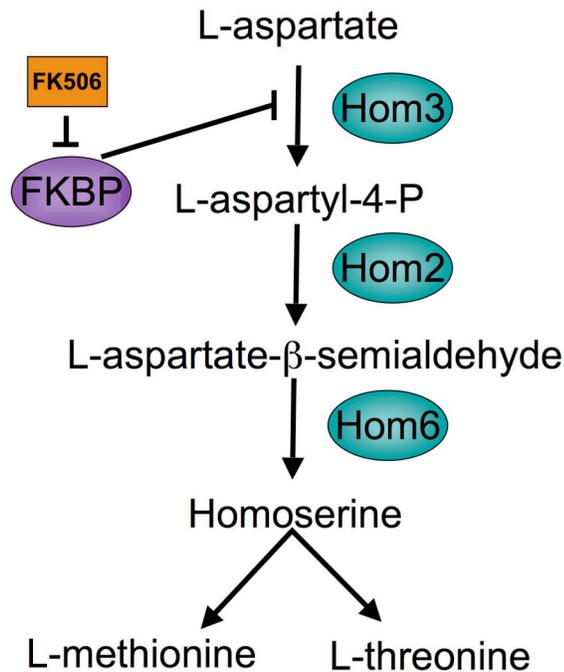


Figure 30: Methionine-threonine biosynthetic pathway

Enzymes of the methionine-threonine biosynthetic pathway of *S. cerevisiae* are shown in blue. FKBP12 (purple) interacts with Hom3 to feedback inhibit flux through the pathway. The immunosuppressive drug FK506 binds to and inhibits FKBP12 function, and causes deregulation of the pathway. Mutation or inhibition of Hom6 in a strain treated with FK506 results in the buildup of the toxic intermediate, L-aspartate- β -semialdehyde (ASA).

5.2 Materials and Methods

5.2.1 Strains and Media

Strains were routinely propagated on YPD (1% yeast extract, 2% BactoPeptone, 2% glucose, 2% Bactoagar; Difco). Auxotrophies were tested on Synthetic Dextrose Media (Difco) lacking either methionine, threonine, or both amino acids.

The *HOM6* disruption cassette was created by amplifying the *SAT1* flipper cassette using long primers with ~90 bp of homology to the region flanking *HOM6*. PCRs were carried out using the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 68°C for 4 minutes, and a final extension of 68°C for 15 min. All PCRs were performed using exTaq polymerase (Takara), and 5% DMSO. An ~4.2 kb PCR fragment was gel purified and wild-type strain SC5314 was transformed with 1 µg of DNA by electroporation as previously described (Reuss, Vik et al. 2004). For all deletion strains, at least 3 independent transformations were performed at each step of disruption and an independent transformant selected from each transformation for further analysis. Nourseothricin resistant isolates were selected on YPD + 200 µg/ml NAT (Werner). Correct integrants (*HOM6/hom6::SAT1*) were confirmed by colony PCR and Southern blot. At least 3 independent strains that had correctly integrated the disruption cassette were grown overnight in YPM (1% yeast extract, 2% bacto peptone, 2% maltose, and 2% bacto agar (Difco)) at 30°C and then plated onto YPD + 25 µg/ml NAT. Small colonies which represented those that had lost the *SAT1* cassette (*HOM6/hom6::frt*), via intergenic recombination at the *frt* site, were selected and confirmed by Southern blot. Three independent transformants were selected to undergo a second round of transformation to disrupt the remaining *HOM6* allele.

The *C. glabrata* *HOM6* disruption cassette was created by overlap PCR (Davidson, Blankenship et al. 2002). Three fragments were used for overlap PCR: 1) ~1 kb of homology to the promoter of *HOM6*, 2) the *C. glabrata* nourseothricin drug resistance cassette from plasmid pBM46 (from Brendan Cormack) 3) ~1 kb of homology to the terminator of *HOM6*. These three fragments were PCR amplified, gel purified and all three were mixed together in a final PCR reaction as template to generate the final overlap disruption construct. Strain BG2 was transformed in LiOAc as previously described (Cormack and Falkow 1999). Positive transformants were selected on medium containing 100 µg/ml NAT. Correct transformants were identified by colony PCR.

All primers and strains are listed in Table 8 and Table 9, respectively.

5.2.2 Disk diffusion assays

All yeast strains were grown overnight in YPD at 30°C. Cells were washed twice with PBS and counted with a hemocytometer. 5×10^6 cells were suspended in 4 ml of top agar (0.8% Bactoagar; Difco) and overlaid onto YPD plates. Diffusion disks impregnated with FK506 (Prograf; Astellas Pharma US, Inc) or control solvent (EtOH) were placed on top of the dried top agar. Cells were incubated at 30 or 37 °C for 24 to 48 hours and monitored for the appearance of inhibition halos surrounding the drug diffusion disks.

5.3 Results

5.3.1 Identification of the *C. albicans* and *C. glabrata* *HOM6* homolog

To identify the *C. albicans* and *C. glabrata* *HOM6* homologs, reciprocal BLAST searches were performed using the *S. cerevisiae* *HOM6* gene. The *C. albicans* *HOM6* gene

was identified as orf 19.2951 / orf 19.10468 on chromosome 1. The *C. glabrata* *HOM6* gene was identified as orf CAGL0M00330g. Three independent disruption mutants of the *C. albicans* *HOM6* gene were isolated using the *SAT1* flipper cassette as previously described (Reuss, Vik et al. 2004). The *C. glabrata* *HOM6* gene was deleted using overlap PCR to generate a cassette carrying the nourseothricin resistance cassette from plasmid pBM46 and ~1 kb of flanking homology to the promoter and terminator regions of *HOM6*. Three independent disruption mutants were isolated. The *hom6* deletion strains of both *Candida* species were auxotrophic for both methionine and threonine, consistent with the function of Hom6 as a key enzyme in this amino acid biosynthetic pathway.

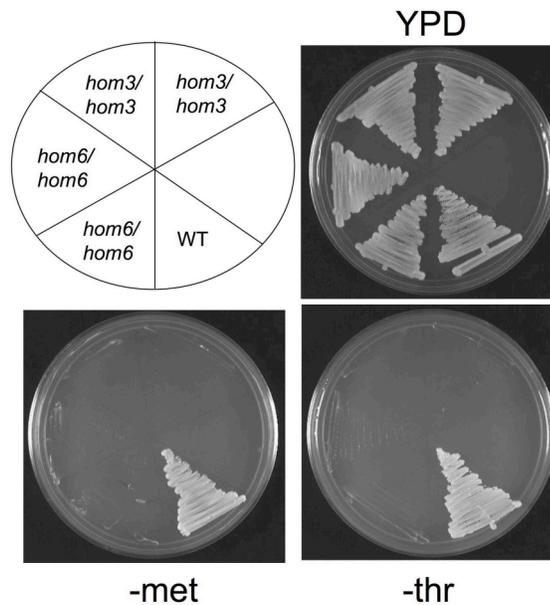


Figure 31: *C. albicans* *hom6/hom6* strains are auxotrophic for methionine and threonine

Two independent *C. albicans* *hom6/hom6* strains (JLR649 and JLR652), two independent *hom3/hom3* strains (CJK48 and CJK49) and wild-type (SC5314) were grown on YPD plates, or SD medium lacking with methionine or threonine.

5.3.2 *C. albicans hom6/hom6* is not sensitive to FK506

Three independent *hom6/hom6* *C. albicans* mutants were tested for their sensitivity to FK506 in disk diffusion assays. Drug diffusion disks were impregnated with varying concentration of FK506 (50 to 500 μg) and placed on the surface of a YPD plate that had been overlaid with top agar containing 5×10^6 yeast cells. The plates were incubated at 30 or 37 $^{\circ}\text{C}$ and were monitored for growth surrounding the disks. As a positive control the *S. cerevisiae hom6* mutant was analyzed in parallel. Although the *S. cerevisiae hom6* strain showed a clear halo of inhibition at 250 μg of FK506, there was no inhibition seen in *C. albicans hom6/hom6* strains, even at 500 μg FK506.

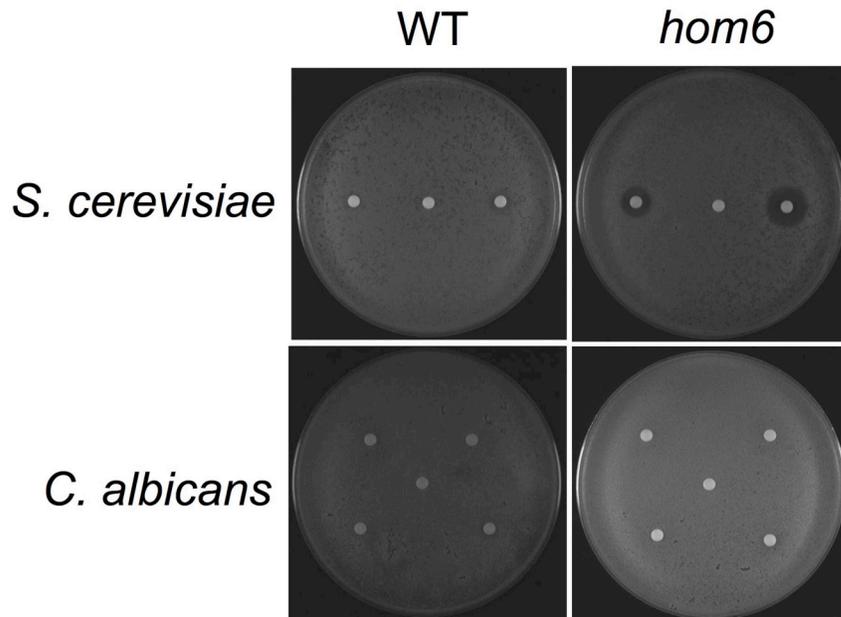


Figure 32: *C. albicans hom6/hom6* are not sensitive to FK506

The top panel shows *S. cerevisiae* wild-type or *hom6* strain in the presence of 250 μg FK506, solvent control, or 500 $\mu\text{g}/\text{ml}$ FK506 (disks from left to right respectively). The bottom panel shows wild-type (SC5314) and representative *hom6/hom6* *C. albicans* strain (JLR649) in the presence of solvent control (middle disk), or 50, 100, 250, and 500 $\mu\text{g}/\text{ml}$ FK506 (clockwise starting at lower left corner). Strains were overlaid in top agar onto YPD plates. Plates were incubated at 30 $^{\circ}\text{C}$ for 24 hours.

5.3.3 *C. glabrata hom6* is sensitive to FK506

Three independent *hom6* deletion strains were tested similarly to *C. albicans* for sensitivity to FK506 in disk diffusion assays and in liquid culture. Preliminary testing suggests that *C. glabrata hom6* strains are sensitive to 250 µg FK506; however repetition of this experiment is necessary.

5.4 Discussion

Due to the advent of drug resistance among pathogenic fungi, new antifungal targets are in demand. However, the discovery of new drug targets is hindered by the similarity of fungal cells to our own, limiting the number of unique targets that can be exploited as drug targets. The methionine –threonine biosynthetic pathway is one such target as it is unique to fungal cells. Previous studies in *S. cerevisiae* demonstrated that deletion of *hom6* in combination with inhibition of FKBP12 was lethal to yeast cells (Arevalo-Rodriguez, Pan et al. 2004). Here we wished to extrapolate that finding to the pathogenic yeasts, *C. albicans* and *C. glabrata*. However, our findings suggest that regulation of this pathway in *C. albicans* has been reconfigured such that *hom6/hom6* strains are not sensitive to FK506. Several hypothesis exist including a different mechanism of feedback control not dependent on FKBP12, the lack of toxic intermediate accumulation due to pathways that allow ASA to be converted into less toxic compounds, or potentially the inability of FK506 to sufficiently inhibit FKBP12 function in *C. albicans*. However, our preliminary results suggest that the synthetic lethality still exists in *C. glabrata*. The fact that *C. albicans* signaling pathway may be more diverged is not surprising as *C. glabrata* is actually more closely related to *S. cerevisiae* than it is to *C. albicans* (Diezmann, Cox et al. 2004).

The functional utility of this synergism could also be explored in other pathogenic fungi, such as *Aspergillus fumigatus* and *Cryptococcus neoformans*. Additionally *S.*

cerevisiae hom3 mutants were attenuated for virulence in murine models suggesting that the methionine-threonine pathway may be involved in virulence (Kingsbury, Goldstein et al. 2006). Thus, inhibition of the pathway by *HOM6* inhibitors alone could serve as potential antifungals for this species. Further investigation is needed to determine the universality of this synthetic lethality among pathogenic fungi, and whether the combination of Hom6 and FKBP12 inhibition could be utilized in the clinic for the treatment of specific fungal infections.

Table 8: Primers used in Chapter 5

Name	Sequence	Notes
JLR423	TTCACITTTCAATACTTACTATACTTTACTTAAAAA TCACTTGATAAAAATGTCAAAGGTCAGTTAATG TTGCAATTATTGGATCCGGTGTGGGCCCCCCC TCGAGGAAGTT	<i>C. albicans HOM6</i> deletion primer 5'
JLR424	CTACAGAACAAAATACAAAATAAACTACTGTATT GACAAAAACAATTGCTATATATATATAGTCTAA ATCAATTGGCAATTCTTTCAGCAAGCTCTAGAAC TAGTGGATCT	<i>C. albicans HOM6</i> deletion primer 3'
JAY11/JOHE18 841	AATTGCATACCAACTTAAGAAG	<i>Cg HOM6</i> 5' overlap
JAY13/JOHE18 842	CAATTCGCCTATAGTGAGTCTTAACACTCTTGT TAGACATTG	<i>Cg HOM6</i> 5' overlap
JAY12/JOHE18 843	CAATGTCTAACAAGAGTGTTAAGACTCACTATA GGGCGAATTG	<i>Cg HOM6 NAT</i>
JAY15/JOHE18 844	CAGTCTTTGAGCAGCCTTAATAACAGCTATGAC CATGATTACGC	<i>Cg HOM6 NAT</i>
JAY14/JOHE18 845	GCGTAATCATGGTCATAGCTGTTATTAAGGCTG CTCAAAGACTG	<i>Cg HOM6</i> 3' overlap
JAY16/JOHE18 846	AACTAATCGATAATATGCTTG	<i>Cg HOM6</i> 3' overlap

Table 9: Strains used in Chapter 5

Name	Genotype	Background	Source
SC5314			(Gillum, Tsay et al.1984)
JLR649	<i>hom6::frt/hom6::frt</i>	SC5314	this chapter

JLR652	<i>hom6::frt/hom6::frt</i>	SC5314	this chapter
CJK48	<i>hom3::frt/hom3::frt</i>	SC5314	From J. Kingsbury
CJK49	<i>hom3::frt/hom3::frt</i>	SC5314	From J. Kingsbury
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>		(Brachmann, Davies et al. 1998)
MAY308	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hom6Δ::kanMX2</i>	BY4742	(Arévalo-Rodríguez, Pan et al. 2004)

6. Conclusions and future directions

Despite the morbidity and mortality associated with fungal infections, our ability to effectively diagnose and treat fungal diseases is still limited. Thus, increasing our understanding of the life cycles of pathogenic fungi and how genetic diversity is created within these populations is of considerable importance. Additionally, expanding our knowledge of key signal transduction cascades that are required for cell survival and response to stress will aid in developing new antifungal therapies and strategies. This thesis has strived to further our understanding in both of these key areas.

6.1 Transcriptional rewiring of mating and meiosis in the pathogenic *Candida* spp. clade

In Chapter 2, we reported the characterization of the *MAT* locus of the sexual *Candida* spp., *C. lusitaniae* and *C. guilliermondii*. Interestingly, these species lack either one or both members of the canonical $\alpha 1 / \alpha 2$ heterodimer that is found in both *C. albicans* and *S. cerevisiae*. In *C. lusitaniae*, $\alpha 1$ has retained a function in promoting entry into meiosis and sporulation despite lacking its canonical binding partner. Previous studies have shown that the parasexual species *C. albicans* was missing key genes involved in mating and meiosis (Hull, Raisner et al. 2000; Jones, Federspiel et al. 2004). *A priori*, we expected that both sexual species would contain homologs for some or all of these key meiotic genes, which would provide a parsimonious explanation for the inability of *C. albicans* to undergo meiosis. However, to our surprise the sexual species were missing the same genes as *C. albicans* and many additional genes involved in meiosis. Despite lacking these key meiotic genes, meiotic mapping, *SPO11* mutant analysis, and CGH analysis demonstrate that *C. lusitaniae* is indeed a meiotic organism.

This project has spawned numerous interesting questions that can be pursued in further studies. Firstly, it is clear from diploid sporulation studies that a component from both the *MATa* and the *MAT α* locus is required for sporulation. Mutant analysis suggests that the *MATa* factor is **a1**, however further study is needed to determine the *MAT α* factor. The most likely candidate is $\alpha 1$; however, due to the inability of $\alpha 1$ strains to mate, to address the role of $\alpha 1$ in sporulation this gene will need to be disrupted in a diploid **a/ α** strain and the sporulation of this strain examined.

Secondly, this chapter has raised interesting questions regarding the possibility that *C. albicans* may undergo a previously unrecognized meiosis. Understanding the genes required for meiosis in *C. lusitaniae* may allow us to identify homologs in *C. albicans* and determine their functional significance. The meiosis that occurs in *C. lusitaniae* may be very different from canonical meiosis, and be mediated through a novel set of meiotic factors. The hypothesis that *C. albicans* is parasexual is based on the presence of aneuploidy and low recombination (Bennett and Johnson 2003; Forche, Alby et al. 2008). However, in *C. lusitaniae* meiosis appears to generate aneuploid progeny at a high frequency. The functional consequences of this aneuploidy remain to be determined.

Thirdly, our study focuses on *C. lusitaniae* as this species mates more robustly than *C. guilliermondii*. It would be of interest to understand how mating and meiosis are regulated in this other sexual species, particularly since *C. guilliermondii* is also lacking the **a1** transcription factor, and may therefore differ from *C. lusitaniae* in the regulation of meiotic entry.

Finally, one pervasive question is whether clonal or asexual reproduction is the ancestral state in the *Candida* complex, a question which comparative genomics between the sexual and asexual *Candida* species could help elucidate. Interestingly, there appears to be an inverse relationship between the ability to form ascospores and success as a

commensal/pathogen. Species with incomplete sexual cycles, such as *Candida albicans*, *Candida glabrata*, and *Candida parapsilosis*, account for the majority of candidal infections suggesting that meiosis may be disadvantageous. A similar phenomenon occurs among dermatophytic fungi which cause cutaneous infections, where those species with complete sexual cycles are less successful pathogens (personal communication, J. Kwon-Chung). That sexual *Candida* species exist argues that loss or restriction of sexual reproduction is the evolved state, possibly due to the energy expenditure required to undergo meiosis or to limit genetic exchange in a pathogen highly evolved to its host niche (Heitman 2006). Perhaps avoiding sporulation enables the pathogen to evade the host immune response to potentially antigenic spore proteins. Thus, continued study of the *Candida* species complex will provide insight on interesting evolutionary questions regarding the evolution of signal transduction pathways, sexual reproduction, and commensalism/pathogenesis.

6.2 Harnessing the calcineurin pathway as an antifungal drug target

In Chapter 3 we examined clinical *Candida* isolates from liver transplant recipients treated with FK506, and determined that none of the isolates demonstrated resistance to calcineurin inhibitors, suggesting that targeting of this pathway has potential utility in the clinical setting. In Chapter 4, we used a candidate gene approach to identify additional components of the calcineurin pathway: Rcn1, Mid1, and Cch1. Although we demonstrated by heterologous expression that *C. albicans* Rcn1 is likely able to inhibit calcineurin in a manner similar to other members of the RCAN family, further study is needed to specifically characterize the interaction between Rcn1 and calcineurin in *C. albicans*. Specifically, future studies should address whether Rcn1 can directly bind *C. albicans* calcineurin and inhibit its function. Additionally, the effect of deletion of

MID1 and *CCH1* on calcineurin activation should also be analyzed. One way to address this question would be to analyze the activation/shuttling of Crz1 in a *mid1/mid1* or *cch1/cch1* mutant strain, or to explore the regulation of calcineurin dependent transcription in these mutants.

Relatively few direct targets of calcineurin are known, and some, such as the *S. cerevisiae* Hph1 /Hph2 proteins (Heath, Shaw et al. 2004), do not have identifiable homologs in *C. albicans*. Thus, other approaches, such as large scale screening studies, may be required to identify additional pathway components. However, since *C. albicans* is a diploid organism libraries currently available for screening purposes are limited. In experiments not reported in this thesis, we conducted a screen for mutants with phenotypes similar to a calcineurin mutant (i.e . sensitivity to calcium, azoles, and serum) or potential *cnb1/cnb1* suppressor mutations (i.e. allow survival of cells in the presence of FK506 and azole, or FK506 and serum). Two libraries were screened, a heterozygous disruption library and a homozygous transcription factor deletion library (Uhl, Biery et al. 2003; Nobile and Mitchell 2005). Several potential candidates were identified from the transcription factor library screen, including *CRZ1* and *CAS5*. Although *cas5/cas5* strains have some similar sensitivities to calcineurin mutants they also have some additional phenotypes (Bruno, Kalachikov et al. 2006). Additionally, studies conducted in other labs supports the idea that Cas5 functions in promoting cell wall integrity. Based upon studies thus far, it is likely that Cas5 and calcineurin impact some similar cellular targets, but whether they act through the same or parallel pathways is not yet known. As the number of available homozygous mutant libraries expands, additional screens for calcineurin pathway components can be undertaken. Other screening methods, such as phosphorylation studies to define targets of calcineurin and yeast-two hybrid studies to identify binding partners, could also be conducted.

Additionally, the development of drugs that are fungal calcineurin specific, and thus would circumvent the immunosuppressive activities of the current calcineurin inhibitors, would be of great clinical utility. Such drugs could be used in combination therapy with current antifungals to enhance their efficacy. In the meantime, continued exploration of the use of combination therapy in topical applications, where the immunosuppressive effects are less profound (compared with systemic use), should be undertaken particularly for the treatment of oral candidiasis, skin and nail infections or candidal vaginitis.

6.3 Amino acid biosynthetic pathways as antifungal drug targets

In Chapter 5, we explored the interaction between the methionine and threonine biosynthetic pathway and inhibition of FKBP12 in both *C. albicans* and *C. glabrata*. Although it appears that the synthetic lethality observed in *S. cerevisiae* may not be universal among the fungi, the relationship may still hold true in a subset of species. Further studies are required to analyze the synthetic lethal interaction in *C. glabrata*, and further studies could be undertaken to characterize this pathway in both *C. neoformans* and *A. fumigatus*. Due to the high degree of similarity between fungal cells and our own, the continued elucidation of fungal specific pathways and regulators is key for designing new antifungal therapies.

The goal of this thesis was to further our understanding of sexual reproduction and signal transduction within the pathogenic *Candida* species complex. Our studies have raised numerous interesting questions, particularly regarding the evolution of mating and meiosis, with implications within and beyond the fungal kingdom.

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Biography

Jennifer Reedy was born April 15, 1978 in Syracuse, NY. After living in Syracuse for two months, she moved to Vestal, NY and graduated from Vestal Senior High School in 1996. She then moved on to Cornell University, College of Agriculture and Life Sciences where she majored in biology with a concentration in genetics and development. In 2000, she earned her B.S. from Cornell and began her M.D.-Ph.D. at Duke University. After two years of medical school, she began her Ph.D. studies in the Cell and Molecular Biology program, and the department of Molecular Genetics and Microbiology, eventually joining the laboratory of Joseph Heitman.

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Conference Poster Presentations:

FEBS, 2005

Candida and candidiasis, 2006

ISHAM, 2006

FEBS, 2007

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Candida and candidiasis, 2008

Oral Presentations:

MGM departmental meeting, 2007

FEBS, 2007

MGM retreat, 2007

MGM departmental meeting, 2008

Duke Genome Biology Meeting, 2008

Candida and candidiasis conference, 2008