

Mechanisms of Sexual Reproduction in Human Fungal Pathogens

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CHAPTER 1: INTRODUCTION

Sections of this chapter (including Figures 1.2, 1.3, and Table 1.2) were published as:

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The diversity of fungal species

Fungi comprise a diverse group of eukaryotic organisms, which last shared a common ancestor with animals an estimated 1.5 billion years ago¹. Fungi are eukaryotes containing a cell wall, and can exist as unicellular organisms in budding yeast form or can form multicellular hyphae. Fungal species are responsible for a wide range of both benign and life threatening biological processes. These processes include the fermentation of food products^{2,3}, decimation of salamander populations⁴, the causal agent of athlete's foot⁵, and various additional biotechnological applications. Fungi have a long history of causing plant infections that greatly diminish crop production and impact food availability; perhaps the most infamous example of this is *Phytophthora infestans*, responsible for causing the Irish potato famine from 1845 to 1851⁶. More recently, an infectious strain of *Puccinia graminis*, termed Ug99, has emerged causing stem rust in wheat. If this strain were to reach the United States, the financial toll due to crop loss is estimated to be \$10 billion annually⁷. Fungal species are also responsible for fatal human infections, and are estimated to kill as many people as tuberculosis or malaria⁸.

Included amongst these disease-causing pathogens lie two non-threatening model yeasts: *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. These species have been instrumental in expanding our understanding of eukaryotic biological processes. However, *S. cerevisiae* has been described as “an excellent model for the basic features of eukaryotes and for experimentation, but a poor model for other fungi.”⁹ Additionally, pathogenicity does not seem to strictly

correlate with phylogeny, because the ability to cause disease has been lost and gained throughout fungal evolution¹⁰. Thus, the information required to combat the financial and life threatening burdens of fungal pathogens warrants a detailed examination of fungal biology and lifestyle.

Sexual reproduction in human fungal pathogens

Although once thought to be primarily clonal and asexual, the majority of human fungal pathogens are now recognized to undergo sexual or parasexual reproduction. In fact, the three most common human fungal pathogens, *Candida albicans*, *Cryptococcus neoformans/gattii* and *Aspergillus fumigatus*, all display extant sexual cycles¹¹. Recently, it has been demonstrated that a mating event led to the development of a particularly virulent *C. gattii* strain that has led to an outbreak in the Pacific Northwest of the United States¹². This work provides evidence that sexual recombination can lead to the emergence of strains with elevated virulence.

This thesis will examine sexual reproduction in two species of the *Candida* clade. The *Candida* clade is also known as the “CTG” clade because species within this clade translate the codon “CTG” as serine instead of leucine¹³. This clade lies within the large ascomycete phylum, of which *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are also members. Ascomycetes are named for the formation of an “ascus,” which is a sac-like structure used to hold the sexual

spores¹⁴. The *Candida* clade contains both non-pathogenic species, such as the saltwater fungus *Debaromyces hansenii*, as well as important opportunistic pathogens. Several *Candida* species reside in humans' gastrointestinal tracts as commensal organisms, but can transition to dangerous fungal pathogens in immunocompromised individuals. The most prevalent pathogen within this clade is *Candida albicans*, which is the fourth-most common source of nosocomial bloodstream infections in humans¹⁵. Other potential pathogens within this clade include *Candida tropicalis* and *Candida lusitanae*. Recently, there has been an outbreak of a multidrug resistant *Candida* species known as *Candida auris*. Sequencing has revealed that this species is closely related to *C. lusitanae*¹⁶.

Sexual reproduction can be sub-divided into two main steps: mating, or fusion of haploid gametes to form a diploid zygote, and meiosis, which is the regeneration of the haploid form. Much of what we know about this process in fungi has been gleaned from studies in *S. cerevisiae*. In this budding yeast, conventional sex occurs between two cells of opposite mating type, termed **a** and α (alpha). These cells express genes encoding cell type specific peptide pheromones (*MFa* or *MF α*), which are received by complementary receptors (Ste3 or Ste2, respectfully), thereby initiating the mating program. Mating proceeds by polarized growth towards a partner, cytoplasmic fusion, and karyogamy, generating an **a**/ α diploid cell. This process is facilitated by precise control over cell identity.

Regulation of cell identity in *S. cerevisiae*

Regulation of cell type identity is essential to ensure that mating can occur. In *S. cerevisiae*, mating type is governed by the presence of either the *MATa* or *MAT α* allele at the *MAT* (mating-type) locus, which encode transcription factors to activate or repress cell-type and haploid specific genes. The *MATa* allele encodes **a1**, a homeobox transcription factor, and the *MAT α* allele encodes two transcription factors: the homeobox protein $\alpha2$, and α domain transcription factor $\alpha1$. In **a** cells, **a** specific genes (asgs) are constitutively expressed via transcriptional activation by Mcm1 and Ste12, which bind directly to specific sites at asg promoters and haploid specific genes^{17,18}. Thus, asg expression does not require the binding of proteins encoded at the *MAT* locus. In α cells, asgs are downregulated 1000 fold¹⁸ by cooperative binding of the $\alpha2$ and Mcm1 proteins at their promoters¹⁹. A Tup1/Ssn6 complex is recruited by $\alpha2$ at asgs to ensure repression²⁰. Unlike **a** specific genes, α specific genes are not constitutively expressed and require the cooperative binding of $\alpha1$ and Mcm1 at their promoters and the recruitment of Ste12 by $\alpha1$ in order to initiate transcription^{21,22}. Diploid **a**/ α cells express both **a1** and $\alpha2$ proteins, which bind in a complex to repress haploid specific genes (hsgs)^{23,24}. In this way, initiation of the mating program is prevented in diploid **a**/ α cells.

Rewiring of cell identity in the *Candida* clade

Within the ascomycete lineage, many species contain homologs of *MAT* genes that allow mating to occur through a similar cell identification mechanism. However,

the molecular mechanism underlying cell identity in these species has striking differences from that in *S. cerevisiae*. For example, species within the *Candida* clade contain the “*MTL*” (mating type-like) locus, which harbors similar *MATa* and/or *MAT α* alleles²⁵. However, in addition to encoding **a1**, $\alpha1$, and $\alpha2$ proteins, the *C. albicans* *MTL* encodes an additional determinant of mating type: the HMG (high mobility group) transcription factor **a2**²⁶. Thus, in *C. albicans*, the **a**-specific genes are not constitutively expressed unlike the situation in *S. cerevisiae*, and instead are under positive regulation by expression of **a2** in **a** cells. In addition, **a**-specific genes are not negatively regulated by $\alpha2$ as in *S. cerevisiae* **a** cells, and instead are not expressed in *C. albicans* **a** cells due to the absence of $\alpha1$. Further analysis has demonstrated that positive activation of asgs by **a2** is likely the ancestral configuration of cell type regulation, and that the ability of **a** cells to constitutively express asgs evolved with the ability of $\alpha2$ to suppress asgs in the *Saccharomyces* lineage²⁷. It should be emphasized that although the molecular mechanism by which cell type is determined is different between *S. cerevisiae* and *C. albicans*, the overall logic of cell identity is the same, in that the *MTL* confers cell type specificity ensuring that **a** cells mate with α cells, and that heterozygous **a**/ α cells cannot undergo mating.

A similar mode of regulation exists in *C. lusitaniae*, in that $\alpha1$ and **a2** are required for mating, and *C. lusitaniae* is competent to undergo meiosis²⁸. However, $\alpha2$ has been lost in the *C. lusitaniae* lineage. Interestingly, *Candida guilliermondii*, another member of the *Candida* clade, retains the ability to undergo a full sexual

cycle but has lost both $\alpha 1$ and $\alpha 2$ ²⁵. Thus, how meiosis is regulated in these species and how the cells identify themselves as haploid or diploid remain open questions. These questions are addressed in a supplementary discussion in Appendix 1 of this thesis.

MAPK signaling drives mating in yeast

The ability to mate in yeast is governed by a (mitogen activated protein kinase) MAPK cascade, which transmits an environmental signal (in this case, pheromone binding) into a transcriptional response in the nucleus. The *S. cerevisiae* MAPK pheromone cascade is perhaps the most well understood signaling pathway in biology²⁹. From a wealth of studies, information concerning the components of the pathway and the order in which they function, the localization of each component, post-translational modifications of most proteins, and the effects of various perturbations on signaling output have been described³⁰⁻³². Additionally, with homologs of signaling components in diverse metazoans including *Drosophila melanogaster* (insects), *Caenorhabditis elegans* (worms), *Arabidopsis thaliana* (plants), *Mus musculus* (mice), and *Homo sapiens* (humans)³³, our understanding of this cascade has been essential in uncovering signaling mechanisms in development and disease.

Activation of the mating program begins with the binding of a peptide pheromone, either *MFa* (α factor) or *MF α* (alpha factor), to their cognate G-protein

coupled receptors (Ste2 or Ste3, respectively). The binding causes the exchange of GTP for GDP on the α subunit of the G protein ($G\alpha$, or Gpa1)³⁴, which changes its conformation, weakening its affinity for the $G\beta$ (Ste4)/ $G\gamma$ (Ste18) subunits and promoting $G\beta\gamma$ dissociation³⁰. Free $G\beta\gamma$ is considered the “activated” form of the G protein in *S. cerevisiae*, although active roles for $G\alpha$ have also been described^{35,36}. Interestingly, in cells which contain Ste4 fused to Gpa1 (Ste4-Gpa1 fusion protein), pheromone signaling can still proceed at a similar frequency to wild type³⁴. Thus, it appears that a conformational change in $G\alpha$ (due to GDP $\rightarrow$ GTP exchange) is sufficient to weaken its interactions with $G\beta\gamma$ and allow $G\beta\gamma$ to perform its effector functions.

The Ste4/Ste18 complex performs at least three effector functions that amplify pheromone signaling and serve to spatially localize enzymes and their substrates (Figure 1.1). The $G\beta\gamma$ unit is tethered to the membrane due to modifications on the C terminal tail of Ste18³⁷, and thus its binding of various effectors which are normally shuttled in and out of the nucleus recruits them near the membrane³⁰. First, $G\beta\gamma$ binds the scaffold protein Far1, which initiates cell cycle arrest and binds Cdc24. Cdc24 is a GEF (guanine nucleotide exchange factor) for Cdc42, a GTPase tethered to the plasma membrane. The resulting complex (Cdc24-Far1- $G\beta\gamma$) helps to orient the cell towards the pheromone gradient and directs polarized growth formation³⁸. Second, Ste4/Ste18 recruits Ste20 to the membrane, which is a p21-activated kinase and a substrate for Cdc42. Third, Ste4/Ste18 recruits the scaffold protein Ste5³⁹. Ste5 binds the major kinases in the cascade,

including Ste11 (MAPKKK), Ste7 (MAPKK) and Fus3 (MAPK). Ste20 (activated by Cdc42) phosphorylates Ste11 to initiate the signaling cascade, resulting in the phosphorylation of Fus3, which enters the nucleus and activates the master transcriptional regulator of mating, Ste12. Activation of Ste12 is achieved by phosphorylation of Ste12 by Fus3, as well as by phosphorylation of the repressors Dig1 and Dig2. An additional role for Fus3 is phosphorylation of Far1, which allows Far1 to inhibit Cdc28 and initiate cell cycle arrest⁴⁰. Fus3 also phosphorylates Bni1, which regulates actin polymerization and has a role in cell fusion⁴¹. (Figure 1.1).

The binding of Ste5 to Ste11, Ste7, and Fus3 represents a key paradigm of MAPK signaling: the presence of a scaffold protein with the ability to bring the necessary signaling molecules into close proximity, ensuring signaling efficiency and specificity. Ste5 exerts this ability via its modular domains.⁴² Ste5 contains a PM domain to tether it to the plasma membrane^{43,44} and a PH (pleckstrin-homology) domain which further anchors it to the lipid bilayer and overlaps with the binding site for Ste11⁴⁵⁻⁴⁷. The Ste5 vWA (von Willebrand type A) domain binds Ste7, facilitating phosphorylation by Ste11⁴⁸. Furthermore, the binding of Ste7 by Ste5 increases the k_{cat} of Fus3 phosphorylation by Ste7 5000-fold⁴². Without Ste5, Fus3 is a poor substrate for Ste7, and thus it is not activated in the filamentous growth pathway, which also signals through the Ste7 MAPKK but does not involve Ste5. Additionally, in the absence of pheromone signal, the Ste5 protein forms a homodimer in the cytosol, preventing membrane binding and misactivation^{29,49}. Thus, the scaffold protein plays a vital role in maintaining signaling fidelity. Not surprisingly, scaffold proteins play roles in directing signaling in in other eukaryotic

organisms, such as the KSR scaffold in the mammalian Ras-Raf-MEK-MAPK pathway, and the PSD95 scaffold present at neuronal synapses⁴⁸. However, a scaffold appears to be absent from the distantly related ascomycete *Schizosaccharomyces pombe*⁵⁰.

The enzymatic reactions described above initiate three outcomes for the cell: transcriptional activation of the mating program, cell cycle arrest, and polarized growth/cell fusion. *C. albicans* contains homologs for MAPK pathway proteins, as outlined in Table 1.1. Many of these MAPK pathway homologs have been characterized⁵¹⁻⁵⁶, and it is likely that they perform similar functions in *C. albicans* as in *S. cerevisiae*. However, studies that reveal significant mechanistic differences are described below. It is likely that additional differences in MAPK signaling between *C. albicans* and *S. cerevisiae* will be revealed as the pathway is further studied.

C. albicans contains a Ste5 homolog (Cst5) that retains the RING and PH domains of *S. cerevisiae* Ste5, but appears to be a truncated version that lacks the vWA domain. Yeast two hybrid assays have revealed that *C. albicans* Cst5 physically interacts with Ste11 via its PH domain, and interacts with Hst7 (homolog of *S. cerevisiae* Ste7) and Cek1 (homolog of *S. cerevisiae* Kss1) despite lacking the vWA domain⁵⁷. Co-immunoprecipitation assays have also revealed an interaction between Cst5 and Cek2 (homolog of *S. cerevisiae* Fus3)⁵². Deletion of Cst5 abrogates mating in *C. albicans* and Cst5 is localized to the polarized growth tip in pheromone treated cells^{52,57}. Thus, Cst5 coordinates the *C. albicans* mating cascade in a similar manner to Ste5, yet it binds Hst7 and Cek1 by a vWA-independent mechanism.

An additional difference for the role of the Far1 scaffold was revealed in a genetic study conducted in *C. albicans*⁵⁷. As described above, *S. cerevisiae* Far1 coordinates the machinery responsible for polarized growth and cell cycle arrest. However, deletion of Far1 in *S. cerevisiae* does not affect mating gene induction⁵⁸. Deletion of *FAR1* in *C. albicans* results in failure to undergo cell cycle arrest, failure to morphologically respond to pheromone, and decreased mating efficiency, as might be expected. However, *C. albicans* *FAR1* deletion cells also show decreased transcriptional gene induction in response to pheromone⁵⁹. Thus, *C. albicans* Far1 appears to have a positive role in the pheromone transcriptional response. Evidence for physical interaction of Cst5 and Far1 *in vitro* has led to the proposal that Far1 may act together with Cst5 to promote the transcriptional response to pheromone⁵⁷.

In addition to differences in the structure and potential interactions of scaffold proteins, the G α subunit appears to play contrasting roles in *S. cerevisiae* and *C. albicans*. In *S. cerevisiae*, genetic deletion of *GPA1* (G α subunit) is lethal in haploid cells⁶⁰. Creation of conditional mutants revealed that mutant cells arrest in the G1 phase of the cell cycle⁶¹. Additionally, overexpression of *GPA1* decreases mating⁶². These data are consistent with a model whereby the G α subunit serves as the negative regulator in the G protein complex. In contrast, a recent study in *C. albicans* demonstrated that deletion of *GPA1* results in lack of a morphological response to pheromone, lack of a transcriptional induction of mating genes, and sterility⁶³. These results establish *GPA1* as an activating factor in mating in *C. albicans*, and make the role of G α as a negative regulator of G $\beta\gamma$ seem unlikely.

Although G α as an activator is reminiscent of the mating program in *S. pombe*⁶⁴, a role for the G $\beta\gamma$ subunit has not been described in fission yeast⁵⁰, while the G $\beta\gamma$ unit is required for mating in *C. albicans*^{51,63}.

Perhaps the most striking difference between the regulation of mating in *S. cerevisiae* and *C. albicans* is an additional layer of regulation on mating in *C. albicans* known as the white-opaque switch. The description of this switch, its regulation, and its consequences for *Candida* biology are described below. In addition to mating, pheromone can direct *C. albicans* cells to form adherent cellular communities known as biofilms. The transcriptional regulation specifying mating versus biofilm formation is not entirely understood, as is discussed later in this introduction.

The white-opaque switch governs multiple aspects of C. albicans biology

C. albicans exhibits several forms of phenotypic switching in which cells undergo a reversible and heritable transition between alternative cell states. The best characterized of these epigenetic transitions is the white-opaque switch, named for the appearance of colonies on agar media. White cells are round and form brighter, dome-shaped colonies, whereas opaque cells are elliptical and form flatter, duller colonies⁶⁵. Miller and Johnson demonstrated that the switch to the opaque state is necessary for efficient sexual mating⁶⁶, stimulating interest into how phenotypic diversity impacts important physiological traits in *C. albicans*. Although

refractory to mating, white cells were subsequently shown to respond to mating pheromones resulting in 'sexual' biofilms⁶⁷, as further discussed below. White and opaque states exhibit many other distinct properties as outlined in Table 1.2. For example, white cells are predicted to have a fermentative metabolism, whereas opaque cells express genes associated with an oxidative metabolism⁶⁸. Opaque cells are better colonizers of the skin, whereas white cells are more virulent in a bloodstream model of infection^{69,70}. Furthermore, white cells are more susceptible to phagocytosis by host macrophages⁷¹ and secrete a leukocyte chemoattractant⁷², whereas opaque cells do not. These findings raise the possibility that the switch to the opaque state could promote immune evasion, although opaque cells are also more sensitive to reactive oxygen species, a common defense mechanism of host phagocytes⁷³.

Regulation of phenotypic switching

C. albicans strains are typically isolated in the white phenotypic state, in which the regulatory circuit is dependent on the transcription factor Efg1⁷⁴⁻⁷⁶. Conversely, expression of the Wor1 transcription factor is necessary and sufficient for stable transitioning to the opaque state⁷⁷⁻⁷⁹. Wor1 acts in concert with at least five other transcription factors as part of a network of positive and negative feedback loops to regulate bi-stability in *C. albicans*^{76,80}. Chromatin-level changes control the switching frequency, including a role for acetylation of histone H3K56 in promoting opaque cell formation⁸¹⁻⁸⁴ and deposition of H2A.Z in promoting the

white state⁸⁵. Mediator complex also regulates white-opaque switching, as deletion of certain mediator components can destabilize the white or opaque states⁸⁶. Recent experiments also demonstrated that the long 5' untranslated region (5' UTR) of *WOR1* regulates switching by reducing translational efficiency⁸⁷. These experiments reveal complex transcriptional, post-transcriptional and chromatin-mediated regulation of the switch, with parallels to other heritable circuits including embryonic stem cell differentiation⁸⁸.

The white-opaque switch is also controlled by transcription factors encoded at the *MTL*. Switching from white to opaque typically requires that diploid strains are homozygous at this locus (i.e., either “*MTLa/a*” or “*MTL α / α* ”)^{66,89}, as expression of *Wor1* is repressed by a complex of **a**1 and α 2 transcription factors that is present in *MTLa/ α* cells⁹⁰. However, recent work has shown that certain **a**/ α isolates can also switch to the opaque state under specific environmental conditions⁹¹. A similar white-opaque switch has been discovered in the related species *Candida tropicalis*^{92,93} and *Candida dubliniensis*⁹⁴. In the case of *C. tropicalis*, white-opaque switching appears to be independent of *MTL* regulation^{93,95}. The fact that the switch exists exclusively in three commensal fungi that co-evolved with the mammalian host suggests that this transition provides an advantage for survival *in vivo*.

Multiple environmental factors influence *C. albicans* switching between white and opaque states. Conditions that affect switching in laboratory cultures include temperature⁹⁶, carbon dioxide⁹⁷, anaerobiosis⁹⁸, N-acetylglucosamine⁹⁹ and genotoxic and oxidative stress¹⁰⁰. In the human host, opaque-to-white switching

occurs *en masse* during systemic infection¹⁰¹, whereas increased switching in the opposite direction, from white to opaque, was observed in one strain background during gastrointestinal colonization¹⁰². The sensitivity of the switch to a diverse array of environmental factors is reflective of multiple pathways impinging on white-opaque signaling. Some of these pathways have been defined and include the pheromone MAPK cascade¹⁰³, the cAMP/PKA pathway⁹⁹, and the Hog1 stress-activated protein kinase pathway¹⁰⁴.

Recently, *C. albicans* was shown to stably exist in two additional phenotypic states, termed the “gray” and “GUT” states, that are related to, but distinct from, white and opaque forms (Figure 1.2). Gray cells displayed a distinct metabolic profile from white and opaque cells, and were more successful than either of these states in an *ex vivo* mouse tongue infection model¹⁰⁵. Cells in the GUT state were observed following gastrointestinal colonization using a strain that overexpressed the *WOR1* gene¹⁰⁶. GUT cells were hypercompetitive when re-tested in this niche, indicating a potential role for this phenotypic state in promoting commensalism¹⁰⁶. The metabolic reprogramming of cells can therefore generate specialized forms that enable colonization and infection of different host niches.

***C. albicans* mating necessitates switching to the opaque state**

The most striking difference between white and opaque cells is their sexual fecundity. Opaque cells are the mating competent form of *C. albicans*, mating

approximately a million times more efficiently than white cells⁶⁶. Conventional heterothallic mating occurs between diploid **a** and α opaque cells and is driven by the secretion and response to sex-specific pheromones (Figure 1.3A). A conserved MAPK cascade transduces the pheromone signal resulting in activation of the transcription factor Cph1 (ortholog of *S. cerevisiae* Ste12). This Cph1 activation triggers polarized growth, cell fusion and karyogamy, resulting in the formation of tetraploid mating products^{53,55,56,107}. Meiosis has not been observed in *C. albicans* and instead tetraploid cells return to the diploid state by a series of reductional mitotic divisions¹⁰⁸⁻¹¹⁰. It was recently revealed that *C. albicans* diploid cells can reduce their ploidy to that of true haploids by a similar parasexual process, further extending the range of ploidy states that can be adopted by this species¹¹¹. Haploids contain only one copy of the *MTL* allele (either the *MTL α* or *MTL α*) and can readily switch to the opaque state and mate to reform diploids, or can undergo spontaneous diploidization¹¹². The transient formation of haploid **a** and α cells could potentially support mating events even in individuals that appear to be carrying a single **a**/ α diploid strain.

The population structure of *C. albicans* is predominantly clonal^{113,114}, although there is evidence of a mixed evolutionary history consistent with a sexually or parasexually reproducing species^{115,116}. In addition to heterothallic mating, experiments have shown that *C. albicans* is capable of unisexual, homothallic mating¹¹⁷. In particular, *C. albicans* opaque **a** cells lacking the α pheromone-degrading protease Bar1 experience autocrine pheromone signaling, resulting in

same-sex **a-a** mating. Same-sex mating was also detected in ménage-à-trois matings, where conjugation between cells of the same sex was induced by pheromone secreted by cells of the opposite sex (see Figure 1.3B)¹¹⁷. Subsequent experiments established that activation of the pheromone response was sufficient to cause opaque cells to undergo same-sex conjugation¹¹⁸.

Despite being unable mate, *C. albicans* white **a** and α cells can respond to pheromones produced by opposite-sex opaque cells. Synergistic signaling between the two cell types can enhance mating between rare opaque cells due to the amplification of pheromone levels¹¹⁹. White cells can thereby facilitate the mating of opaque cells, despite being essentially sterile themselves (Figure 1.3B). Interestingly, the role of such “helper” white cells can promote both opposite-sex mating and same-sex mating between minority opaque cells¹¹⁹. Thus, cooperation between white and opaque cells can support mating even in populations where only one cell type is in the “mating-competent” opaque state.

Pheromone signaling drives biofilm formation

Biofilms are communities of cells that adhere to inert or biological surfaces. They are often associated with an extracellular matrix and communal cells exhibit distinct phenotypic properties from free-floating planktonic cells¹²⁰. *C. albicans* causes a variety of device-associated infections that involve the formation of biofilms on catheters, heart valves, pacemakers, dentures, and prosthetic joints¹²¹.

Biofilm formation is initiated when a basal layer of unicellular yeast adheres to the substrate, upon which a layer of pseudohyphal and hyphal cells grows during maturation¹²². These microbial communities are often drug resistant due to the occlusion of antifungal drugs, the upregulation of drug efflux pumps, and the existence of persister cells within the biofilm^{123,124}. Conventional studies of biofilm formation in *C. albicans* have utilized *MTLa*/ α cell types; these make up the majority of clinical isolates whereas less than 10% of strains are naturally homozygous for *MTLa* or *MTL α* ^{115,125}.

More recently, it has been demonstrated that pheromone signaling in *C. albicans* can promote the formation of ‘sexual’ biofilms. These sexual biofilms were first observed in white **a** and α cells responding to pheromones secreted by opaque cells of the opposite sex⁶⁷. Responding white cells were induced to undergo substrate adhesion, filamentation, and development of a complex biofilm structure. Subsequently, it was shown that white cells also respond to pheromones secreted by opaque cells of the same sex, using a self-activation mechanism similar to the mechanism that drives same-sex mating¹²⁶. Sexual biofilms contain a basal layer of unicellular yeast cells upon which a layer of filamentous cells is formed, but appear to lack the drug resistance and small-molecule impermeability characteristic of ‘conventional’ biofilms generated by *MTLa*/ α cells¹²⁷. Interestingly, the permeability of sexual biofilms supports the build-up and maintenance of pheromone gradients within the biofilm. Thus, pheromones secreted by minority opaque cells form stable gradients within this structure, facilitating chemotropism

between opaque **a** and α partners⁶⁷. Presumably as a result of this mechanism, mating between minority opaque cells is 5- to 100-fold higher when these cells are seeded in a sexual biofilm compared to a conventional biofilm¹²⁸. These findings suggest that *C. albicans* has evolved two types of biofilms that are influenced by their *MTL* configuration; conventional **a**/ α biofilms that are 'pathogenic' based on their general impermeability and resistance to antifungals, and sexual biofilms that may provide an appropriate setting for mating to occur *in vivo*^{127,129,130}. It is also hypothesized that the white-opaque switch originally evolved to generate sexual biofilms that facilitate mating of *Candida* strains in the host¹³⁰.

The transcriptional regulation of both conventional and sexual biofilms has been examined in *C. albicans*. In conventional **a**/ α biofilms, a network of six major transcription factors regulates expression of ~2,200 genes¹³¹. A comparison between the transcriptional control of sexual biofilms and conventional biofilms revealed that four of the six transcriptional regulators involved in conventional biofilms are also required for sexual biofilms, indicating partial overlap of the two regulatory circuits¹³². Upstream signaling in conventional biofilms involves the Ras1/cAMP pathway, which activates the master transcriptional regulator Efg1^{127,133}. However, there have been conflicting reports concerning how the pheromone signal is transduced via the MAPK pathway to activate sexual biofilm formation. Experiments from our laboratory and others have shown that pheromone signaling in white cells requires the Cph1 transcription factor^{103,119,132}, and that this factor is essential for the transcriptional response and adhesive properties of pheromone-treated white cells¹³². In contrast, a separate set of studies

indicated that pheromone signaling acts through the Tec1 transcription factor, and that Cph1 is not required for sexual biofilm formation¹³⁴. Differences in experimental conditions may explain some of these differences¹³⁰, although all groups agree that Tec1 makes a significant contribution to both conventional and sexual biofilm formation^{103,132,134,135}, which reflects, at least in part, a key role for Tec1 in promoting hyphal growth^{136,137}. Overall, it is clear that questions remain concerning the precise signaling mechanisms contributing to the establishment of sexual biofilms, as well as the role of these biofilms during infection of the host.

Recent studies in *C. tropicalis* have revealed striking differences with *C. albicans* with respect to the properties of white and opaque cells. For example, while switching between states is influenced by multiple environmental conditions in *C. albicans*, only N-acetylglucosamine has been shown to also affect the transition in *C. tropicalis*^{92,93}. Furthermore, while pheromone signaling induces the formation of sexual biofilms in *C. tropicalis* as in *C. albicans*, it is responding opaque cells, not white cells, that contribute to biofilm development in the former¹³⁸. Thus, opaque cells are *both* the mating competent form *and* the biofilm forming cell type in *C. tropicalis*. These observations raise intriguing questions as to the evolution of the white-opaque switch in *Candida* species. At face value, they suggest that the switch did not simply evolve so that biofilms generated by white cells could facilitate mating between opaque cells, because white cells do not form sexual biofilms in *C. tropicalis*. It is possible that this mechanism represents a further refinement of the mating system in *C. albicans* that occurred after it diverged from *C. tropicalis*. There is also an incomplete understanding of the *in vivo* niches supporting the growth of

white and opaque cells in the mammalian host. Given recent advances in our understanding of the spatial and temporal heterogeneity of the human mycobiome¹³⁹, it is likely that we will soon learn more about the niches supporting white and opaque forms in the natural host. Distinguishing cells in different phenotypic states will likely be driven by emerging techniques such as RNA FISH (fluorescence *in situ* hybridization) to quantify the *in vivo* expression of state-specific gene transcripts.

Meiosis in yeast

As discussed above, *C. albicans* lacks a true meiosis and instead undergoes a program of concerted chromosome loss, in which chromosomes are lost mitotically until a diploid (or haploid) state is reached. However, other species within the *Candida* clade undergo a more conventional meiosis.

Meiosis is typically a reductional DNA division which causes diploid cells to generate gametes with a haploid set of chromosomes¹⁴⁰. This halving of genomic content occurs because two nuclear divisions immediately follow one round of DNA replication. Meiosis is thought to facilitate rapid evolution, owing to the mixing of two different genomes and the potential to generate recombinant alleles¹⁴¹. In *S. cerevisiae*, entry into meiosis has both a genetic requirement and a reliance on environmental cues. Cells must contain both *MATa* and *MATα* alleles, the gene product of which forms an **a1/α2** transcriptional complex to repress haploid-

specific genes as well as help to initiate the meiotic program^{23,24,142}. Additionally, meiosis is triggered during incubation in a low nitrogen, low glucose environment with no fermentable carbon source¹⁴¹. These cues, as well as others, converge on the master regulator of meiosis, Ime1 (inducer of meiosis 1)¹⁴³. Ime1 is a transcription factor that contains a promoter greater than 2 kilobases in length, and is one of the most highly regulated genes in *S. cerevisiae*¹⁴⁴. Overexpression of *IME1* is sufficient to bypass nutrient conditions and initiate the meiotic program in diploid cells¹⁴⁵. Additionally, overexpression of *IME1* in haploid cells causes meiosis and results in cell death¹⁴⁶.

Regulation of Ime1 is orchestrated by both transcriptional repressors and long non-coding RNA components. In haploid cells, the repressor Rme1 binds the *IME1* promoter and represses its expression¹⁴⁷. This repression is accomplished through the transcription of a long non-coding RNA on the Ime1 promoter, *IRT1*, which recruits the histone methyltransferase Set3 and histone deacetylase Set2 and establishes a repressive chromatin state¹⁴⁸. In diploid cells, the $\alpha 1/\alpha 2$ complex represses Rme1, relieving this regulation. In addition to Ime1, the RNA methyltransferase Ime4, which enhances Ime1 expression^{149,150}, is also repressed in haploid cells via a long non-coding RNA, known as *RME2*¹⁵¹. In diploid cells, *RME2* is repressed by direct binding of the $\alpha 1/\alpha 2$ complex to its promoter. Interference with *RME2* and *IRT1* expression is sufficient to induce meiosis in haploid cells¹⁴⁸, establishing them as key regulators of entry into the meiotic program.

The transcriptional cascade regulating meiosis is characterized by gene induction in three “waves”, termed “early,” “middle” and “late”¹⁴². Ime1 together with Ume6 binds to “early” meiosis genes, which are genes involved in entry into premeiotic S phase, crossing over, and transcription factors required for induction of “middle” genes^{152,153}. Among these genes are Spo11, an endonuclease responsible for making double-stranded DNA breaks during homologous recombination^{154,155}, Ime2, and Ndt80. Ime2 is a serine/threonine kinase with several important roles in meiosis. Ime2 promotes degradation of Sic1, a cyclin-dependent kinase inhibitor, which allows the cells to progress to meiotic S phase¹⁵⁶. Additionally, Ime2 promotes the transcription of Ndt80 by phosphorylating an Ndt80 repressor, Sum1, thereby antagonizing its interactions with the histone deacetylase Hst1¹⁵⁷. Lastly, Ime2 phosphorylates Ime1, targeting it for degradation and providing a negative feedback loop to ensure that Ime1 expression is appropriately regulated as meiosis progresses¹⁵⁸.

The induction of the transcription factor Ndt80 by Ime1 and Ime2 represents a “commitment” point for meiosis, because its expression triggers the onset of meiotic divisions (meiosis I and meiosis II)¹⁵². Ndt80 binds “middle” meiosis genes, which include regulators of chromosome segregation and spore formation. The master regulator of the “late” genes in meiosis is unknown, and the timing of expression of these genes is less understood¹⁵³. The best characterized late genes are *DIT1* and *DIT2*, which function in spore wall formation and spore maturation.

C. lusitaniae is one of the few species in the *Candida* clade that exhibits a true meiosis, characterized by spore formation and the halving of genomic content^{25,28}. Interestingly, *C. lusitaniae* undergoes meiosis despite lacking key genes important for recombination, including Dmc1, proteins that form the synaptosomal complex (Zip1-Zip4), and those that promote crossing over (Msh4/Msh5, Mer3).¹⁵⁹. Interestingly, about 1/3 of meiotic products in *C. lusitaniae* are aneuploid, a rate that is much higher than that observed in *S. cerevisiae* meiosis²⁸. Furthermore, meiosis in *C. lusitaniae* is not under control of the *MTLa1*/ α 2 complex, as the α 2 protein has been lost in this species. Perhaps most strikingly, meiosis proceeds in *C. lusitaniae* despite the fact that this species does not contain a homolog for *IME1*. Chapter 2 of this thesis addresses transcription factor regulation of meiosis in *C. lusitaniae* and establishes a regulatory network as a model for meiosis in the *Candida* clade.

Current Work

This thesis examines the regulation of sexual reproduction in two clinically relevant human fungal pathogens, *C. albicans* and *C. lusitaniae*. In Chapter 2 of this thesis, I examine how meiosis can proceed in *C. lusitaniae* despite lacking the major regulator of meiosis in *S. cerevisiae*, Ime1, as well as important recombination machinery. I will demonstrate that the entire *C. lusitaniae* sexual cycle has come under the control of Ste12, a transcription factor that regulates mating in many ascomycete species but that has not previously been associated with meiosis. I will propose that this Ste12 regulation is an example of convergent evolution, because *S.*

pombe exhibits a similar mechanism whereby an unrelated transcription factor regulates both mating and meiosis. In Chapter 3, I will investigate how epigenetic states regulate signaling output. More specifically, I will dissect the factors that distinguish the mating competency of *C. albicans* white and opaque cells. I will show that differential expression of several pheromone MAPK components between the two cell types is responsible for differences in mating efficiencies. I will further demonstrate that it is possible to recapitulate opaque-like levels of key MAPK factors in white cells, and to make normally sterile white cells into mating-competent cells. In the Discussion, I will examine why the white-opaque switch may have evolved to control mating in *C. albicans*, and the benefits of different ploidy states as they relate to *C. lusitaniae*. Overall, this work increases our knowledge of the regulation of sexual reproduction and has implications for understanding both transcriptional and epigenetic regulation in eukaryotic organisms.

S. cerevisiae

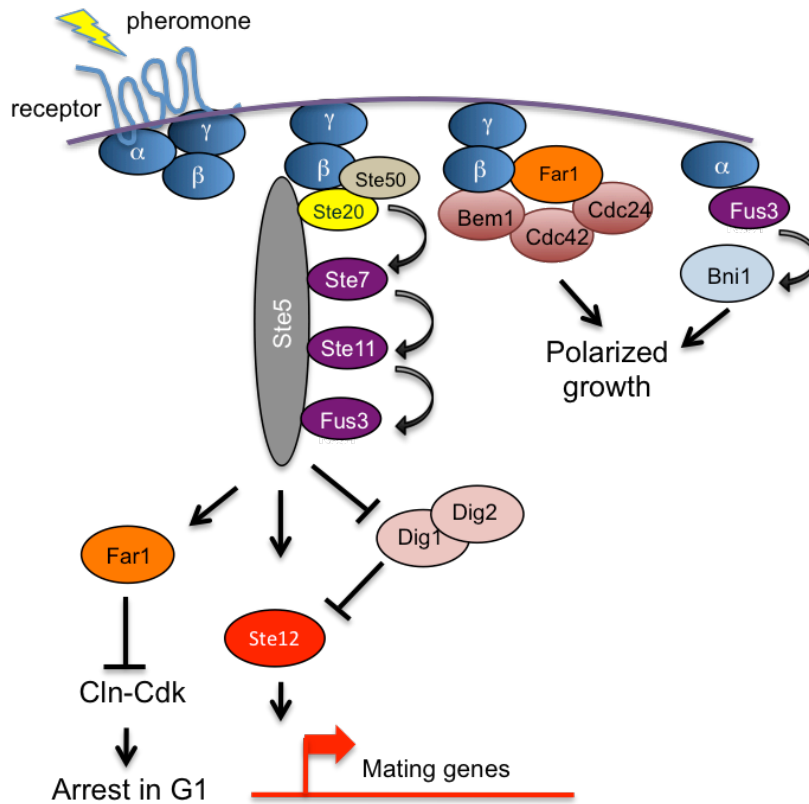


Figure 1.1: Major events of the MAPK signaling cascade.

Pheromone signaling is activated by pheromone binding to a G protein coupled receptor (lightening bolt) which causes dissociation of the G protein. Active Gβγ recruits the MAPK cascade and polarization machinery to the membrane by direct binding to scaffold proteins. Far1 is phosphorylated by Fus3 and initiates cell cycle arrest. Reproduced from *Bennett & Turgeon 2016*.¹⁴

Table 1.1. List of homologs of MAPK pathway genes between <i>S. cerevisiae</i> and <i>C. albicans</i>		
Gene Name (Sc)	Gene name (Ca)	Function
<i>MFALPHA1/MFALPHA2</i>	<i>MFALPHA</i>	α pheromone
<i>MFA1/MFA2</i>	<i>MFA</i>	a pheromone
<i>STE2</i>	<i>STE2</i>	α cell pheromone receptor
<i>STE3</i>	<i>STE3</i>	✓ cell pheromone receptor
<i>STE4</i>	<i>STE4</i>	β subunit G protein
<i>STE18</i>	<i>STE18</i>	γ subunit G protein
<i>GPA1</i>	<i>GPA1</i> or <i>CAG1</i>	α subunit g protein
<i>STE20</i>	<i>CST20</i>	Pak kinase
<i>STE5</i>	<i>CST5</i>	Scaffold (MAPKs)
<i>STE11</i>	<i>STE11</i>	MAPKKK
<i>STE7</i>	<i>HST7</i>	MAPKK
<i>FUS3</i>	<i>CEK2</i>	MAPK
<i>KSS1</i>	<i>CEK1</i>	MAPK (minor)
<i>STE12</i>	<i>CPH1</i>	Master transcriptional regulator
<i>DIG1/DIG2</i>	<i>DIG1</i>	Ste12 repressor
<i>FAR1</i>	<i>FAR1</i>	Scaffold (polarization)
<i>CDC42</i>	<i>CDC42</i>	Rho-type GTPase
<i>CDC24</i>	<i>CDC24</i>	GDP-GTP exchange factor for Cdc24
<i>CDC28</i>	<i>CDC28</i>	Cyclin dependent protein kinase

Table 1.1: List of homologs of MAPK pathway genes between *S. cerevisiae* and *C. albicans*.

Homologs with different names in *S. cerevisiae* (Sc) and *C. albicans* (Ca) are in blue.

Table 1.2. Properties of phenotypic states in <i>C. albicans</i>						
State	<i>MTL</i> Configuration	Major Regulator	Cues supporting formation of each state	Metabolism	Preferred niche	Pheromone response
White	Heterozygous or homozygous	Efg1 ^{74 75,80}	Stable at 25°C or 37° C ⁹⁶ , aerobic conditions ⁹⁸	Fermentative ⁶⁸	Commensal, Bloodstream ⁷⁰	Biofilm formation ⁶⁷
Opaque	Usually homozygous ^{66 89} but can be heterozygous ⁹¹	Wor1 ^{78,79,160}	25°C ⁹⁶ , CO ₂ ⁹⁷ , anaerobiosis ⁹⁸ , N-acetyl glucosamine ⁹⁹	Oxidative ⁶⁸	Skin ⁷⁰	Mating ⁶⁶
Gray	Heterozygous or homozygous ¹⁰⁵	N/A	Stable at 25°C or 37°C on rich media (YPD) ¹⁰⁵	Unique carbohydrate metabolism ¹¹⁹	Skin ¹⁰⁵	Mating at low efficiency ¹⁰⁵
GUT	Heterozygous ¹⁶¹	Requires Wor1 over-expression ¹⁶¹	Passage through the mammalian gastrointestinal tract ¹⁶¹	Adapted to the gastro-intestinal tract, high iron ¹⁶¹	Commensal in gastro-intestinal tract ¹⁶¹	No response ¹⁶¹

Table 1.2: Properties of phenotypic states in *C. albicans*.

Cells in each state have unique phenotypic properties. *MTL*, mating-type-locus. Predicted metabolic preferences based on gene expression studies.

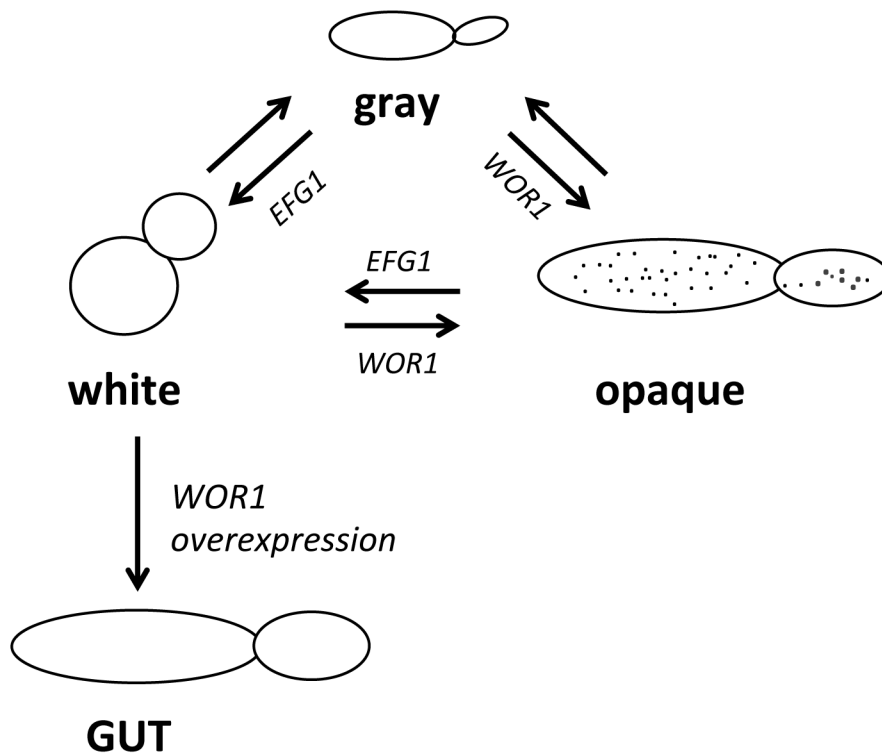


Figure 1.2: *Candida albicans* cells can exist in four related heritable states.

The white state is positively regulated by the transcription factor Efg1, while the opaque state requires expression of the transcription factor Wor1. Other transcription factors that co-regulate the white-opaque switch with Wor1 and Efg1 are not shown. Opaque cells often display characteristic ‘pimples’ on the cell surface when analyzed by scanning electron microscopy. Deletion of both of Wor1 and Efg1 can promote formation of the grey state. White cells overexpressing the Wor1 transcription factor can be induced to adopt the GUT phenotypic state after passage through the murine gastrointestinal tract. GUT cells were characterized in α/α cell types and appear similar in shape to opaque cells, but unlike opaque cells they show increased fitness in colonizing the gastrointestinal tract.

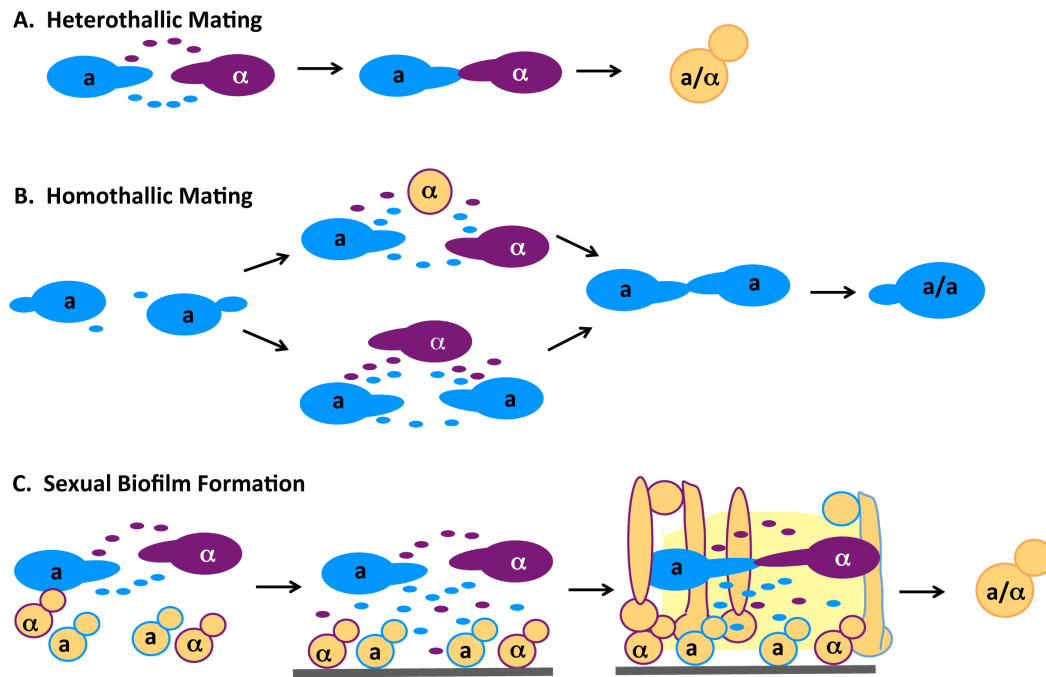


Figure 1.3: Interactions between white and opaque cells in a population.

A. Conventional heterothallic mating occurs between diploid opaque *C. albicans* cells of opposite mating types. Pheromone signaling stimulates mating projection formation and cell fusion to generate **a/α** tetraploid products.

B. Same-sex opaque cells can undergo mating in the presence of an opposite-sex white cell (top) or opaque cell (bottom). Pheromone signaling between these cell types leads to amplification of the pheromone response and conjugation between cells of the same sex.

C. Pheromone signaling from opaque cells can stimulate responding white **a** or **α** cells to adhere to a surface and undergo formation of a 'sexual biofilm' that includes filamentous cells. Within this immobilized structure, pheromone gradients between opaque cells are stabilized thereby leading to more efficient chemotropism and conjugation.

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CHAPTER 2: CONVERGENT EVOLUTION OF A FUSED SEXUAL CYCLE PROMOTES THE HAPLOID LIFESTYLE

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Author Contributions:

R.K.S. and S.M.T. made *C. lusitaniae* *IME2* and *STE12* deletion strains, conducted quantitative mating and meiosis assays on these strains, and made *IME2* and *STE12* deletion strains in *K. lactis*. R.K.S. performed microarray profiling for wild type cells undergoing mating and meiosis and *IME2* null cells undergoing mating and meiosis, analyzed mating genes induced in meiosis, and performed Ime2 qPCR. C.M.S. and R.K.S. analyzed wild type meiosis data. R.J.B performed the fluorescent-Ste2 experiment, quantified meiosis in Ste12 deletion and addback strains, and quantified mating and meiosis in *Pichia pastoris*. C.M.S. performed microscopy, *STE12* deletion microarray profiling, constructed *C. lusitaniae* *IME2* addback strains and quantified mating frequency, performed sporulation timecourse, constructed *S. cerevisiae* *STE12* mutants and quantified mating and meiosis, and quantitated mating and meiosis in *K. lactis* *STE12* deletion strains. R.J.B, R.K.S., and C.M.S. wrote the paper.

Abstract

Sexual reproduction is the prerogative of eukaryotic species and involves the fusion of haploid gametes to form a diploid cell that subsequently undergoes meiosis to generate recombinant haploid forms. This process has been extensively studied in the unicellular yeast *Saccharomyces cerevisiae*, which exhibits separate regulatory control over mating and meiosis. Here we address the mechanism of sexual reproduction in the related hemiascomycete species *Candida lusitanae*. We demonstrate that in contrast to *S. cerevisiae*, *C. lusitanae* exhibits a highly integrated sexual program in which the programs regulating mating and meiosis have fused. Profiling of the *C. lusitanae* sexual cycle revealed that gene expression patterns during mating and meiosis were overlapping, suggestive of co-regulation. This was particularly evident for genes involved in pheromone MAPK signaling, which were highly induced throughout the sexual cycle of *C. lusitanae*. Furthermore, genetic analysis showed that the ortholog of *IME2*, a 'diploid-specific' factor in *S. cerevisiae*^{3,4}, and *STE12*, the master regulator of *S. cerevisiae* mating^{5,6}, were each required for progression through both mating and meiosis in *C. lusitanae*. Together, our results establish that sexual reproduction has undergone significant rewiring between *S. cerevisiae* and *C. lusitanae*, and that a concerted sexual cycle operates in *C. lusitanae* that is more reminiscent of the distantly related ascomycete, *Schizosaccharomyces pombe*. We discuss these results in light of the evolution of sexual reproduction in yeast, and propose that regulatory coupling of mating and meiosis has evolved multiple times as an adaptation to promote the haploid lifestyle.

Introduction

Sexual reproduction is divided into two programs: gamete fusion, in which haploid cells merge to form a diploid cell, and gametogenesis, in which a specialized cell division, meiosis, generates recombinant haploid gametes. Sexual reproduction in *S. cerevisiae* involves mating between cells of opposite mating type, generating diploid products⁷ that subsequently undergo meiosis upon nutritional limitation^{1,8}. It is unclear, however, how representative this sexual cycle is of that in other yeast species. Here, we examine regulation of sexual reproduction in the related hemiascomycete *Candida lusitanae*, a member of the *Candida* clade of human pathogens⁹. *C. lusitanae* was recently shown to have a complete sexual cycle despite lacking orthologs of key meiosis genes such as *IME1*, encoding the master transcriptional regulator of meiosis in *S. cerevisiae*⁹⁻¹¹. In addition, whereas *S. cerevisiae* is predominantly diploid, *C. lusitanae* cells preferentially exist in the haploid form and therefore exhibit a transient diploid state^{9,10}.

Results and Discussion

Transcriptional profiling of *C. lusitanae* cells progressing through mating and meiotic programs was performed and compared to those of *S. cerevisiae*. In total, 406 genes were induced >4-fold during *C. lusitanae* mating, including highly conserved MAPK genes that regulate pheromone signaling in diverse fungal species^{2,12,13} (Figure 2.1a and Supplemental Figure 2.1). Interestingly, we also

observed elevated expression of several genes that are orthologs of ‘meiosis-specific’ genes in *S. cerevisiae*, including *SPO11*, *REC8*, and *IME2*^{3,14,15,16} (Fig. 2.1a). We similarly performed expression profiling on *C. lusitaniae* diploid **a**/ α cells induced to enter meiosis. Cells became enlarged by 8 h, exhibited a morphological change by 12 h, and by 18-36 h dyad (2-cell) spores were formed, as is typical of this species¹⁰ (Supplemental Figure 2.2a-c). Profiling revealed that a total of 618 genes were induced during *C. lusitaniae* meiosis, compared to 480 genes during *S. cerevisiae* meiosis¹⁷. In *S. cerevisiae*, meiotic gene expression includes early, middle, and late stages of expression¹⁷. We were similarly able to distinguish three temporal classes of meiotic gene expression in *C. lusitaniae* (Figure 2.1b and d, and Supplemental Figure 2.2d). In total, we observed increased expression (>3-fold) of 255 early genes, 307 middle genes, and 56 late genes during *C. lusitaniae* meiosis (Figure 2.1e). Thus, despite lacking an ortholog of *IME1*, many downstream components of meiosis are regulated in a similar stage-specific manner in *C. lusitaniae* as in *S. cerevisiae*.

The most striking aspect of *C. lusitaniae* meiosis was the expression of many genes whose orthologs are expressed specifically during mating in *S. cerevisiae*. In particular, multiple components of the pheromone MAPK signaling cascade were highly induced, including the terminal transcription factor *STE12* (Figure 2.1c and f). In addition to MAPK genes, pheromone, pheromone receptor, and pheromone processing genes were also induced in *C. lusitaniae* meiosis (Supplemental Figure 2.1). We therefore surmised that MAPK signaling might play a role in regulating *C.*

lusitaniae meiosis, in addition to its conserved function in directing cell-cell communication and conjugation.

Given that program-specific expression of many *S. cerevisiae* mating and meiosis genes does not occur in *C. lusitaniae* orthologs, genetic experiments were performed to determine whether this transcriptional rewiring has functional consequences for the sexual cycle. First, the role of *C. lusitaniae* Ime2 in mating and meiosis was analyzed. *S. cerevisiae* Ime2 (Inducer of meiosis 2) is a conserved serine/threonine kinase that acts in tandem with the cyclin-dependent kinase Cdk1 to promote meiosis^{3,4,18,19}. As predicted based on *IME2* function in *S. cerevisiae*, loss of *IME2* (*CLUG_00015*) in *C. lusitaniae* blocked meiosis; wildtype diploid cells formed haploid progeny with ~40% efficiency after 3 days, whereas <1% of haploids were formed by *ime2Δ/ime2Δ* cells (Figure 2.2a). In addition, whereas wildtype diploids generated dyad spores, *ime2Δ/ime2Δ* mutants failed to sporulate (Figure 2.2b). These results establish a conserved role for *IME2* in regulating meiosis in hemiascomycete yeast, even in species for which *IME1* is absent. Profiling of *C. lusitaniae ime2Δ/ime2Δ* mutants revealed that most early meiosis genes were still induced in this background, while induction of many middle and late meiosis genes was lost (Supplemental Figure 2.3). We also note that induction of *NDT80* (*CLUG_05634*) and genes encoding cell cycle regulators was compromised in the *ime2Δ/ime2Δ* mutant (Supplemental Figure 2.3b). *NDT80* is responsible for the induction of middle meiosis genes in *S. cerevisiae*, where its expression is dependent on Ime2²⁰. The diminished expression of *NDT80*, together with the loss of

expression of cell cycle genes, is likely responsible for the inability of *C. lusitaniae* *ime2Δ/ime2Δ* cells to proceed through meiosis.

We next addressed the role of *IME2* in *C. lusitaniae* mating. In contrast to *S. cerevisiae* where *IME2* has no role in mating, *C. lusitaniae* *IME2* was induced during mating (Figure 2.2c) and was required for the efficient formation of mating products. Thus, co-incubation of *C. lusitaniae* **a** and α cells resulted in approximately 0.5% of cells forming **a**/ α diploids, while bilateral crosses between *ime2Δ* **a** and *ime2Δ* α cells resulted in a mating frequency of less than 0.03% (a 16-fold decrease, Figure 2.2d). Unilateral crosses between wildtype and *ime2Δ* mutants showed mating frequencies similar to those between wildtype partners (Figure 2.2d). Complementation by reintegration of the *IME2* gene into the mutant background restored mating competency in the bilateral cross (Supplemental Figure 2.4). These results establish a novel role for *IME2* in *C. lusitaniae* mating, and indicate that the *ime2Δ* defect is limited to bilateral crosses. The latter observation is consistent with a late role for Ime2 during mating following the fusion of **a** and α partner cells. Microscopic analysis of *ime2Δ* crosses showed the formation of zygote structures further supporting a late role for Ime2 during mating (Figure 2.2e), and additional studies will be required address the precise role of Ime2 in this process.

Next, we investigated the role of pheromone MAPK signaling and the transcription factor Ste12 in the *C. lusitaniae* sexual cycle. First, we tested whether *C. lusitaniae* cells undergoing meiosis actively secrete pheromone. A pheromone reporter assay was developed in which the *C. lusitaniae* α pheromone receptor was

heterologously expressed in the related species *Candida albicans* (Figure 2.3a). Such heterologous expression of receptors has been shown to result in successful signaling in response to species-specific pheromones²¹. Co-incubation of the GFP-labeled reporter strain with *C. lusitaniae* cells undergoing meiosis showed induction of a morphological response in the reporter cells (Figure 2.3b), demonstrating that *C. lusitaniae* cells are actively secreting α pheromone during meiosis.

STE12 is the master regulator of mating and pheromone signaling in *S. cerevisiae* and many other hemiascomycetes, including *Candida* species^{2,12}. To test the role of *STE12* in the sexual cycle of *C. lusitaniae* we constructed haploid and diploid *ste12Δ* mutants. Loss of *STE12* (*CLUG_02576*) in haploid cells led to a complete block in zygote formation and mating between **a** and α cells (Figure 2.3c, d), as expected based on previous studies²². Surprisingly, however, loss of *STE12* in *C. lusitaniae* **a**/ α diploids also led to a block in meiosis and sporulation. Thus, formation of meiotic progeny was reduced ~100-fold in *ste12Δ/ste12Δ* mutants (Figure 2.3e) and spore formation was abolished (Figure 2.3f and Supplemental Figure 2c). Reintegration of the *STE12* gene into the *ste12Δ/ste12Δ* background restored the ability to undergo meiosis (Supplemental Figure 2.5). Expression profiling revealed that the meiotic transcriptional profile was essentially absent from *ste12Δ/ste12Δ* mutants (Figure 2.3g). Our results therefore establish that *STE12* plays a critical role in regulating *both* mating and meiosis in *C. lusitaniae*, in contrast to *S. cerevisiae* where *STE12* specifically regulates mating only.

Taken together, our studies demonstrate that a fundamental rewiring of the sexual cycle has occurred between the two hemiascomycetes, *S. cerevisiae* and *C. lusitaniae*. Mating and meiosis are distinct programs in *S. cerevisiae*, yet these programs are integrated in *C. lusitaniae* (Figure 2.4a and Supplemental Figure 2.7). This is evident both from transcriptional profiles that reveal overlap of gene expression patterns in mating and meiosis, as well as genetic analysis of key regulators of the sexual cycle. In particular, whereas *STE12* and *IME2* are necessary for *S. cerevisiae* mating and meiosis, respectively, the *C. lusitaniae* orthologs are required for efficient progress through both stages of the sexual cycle.

The regulation of sexual reproduction in *C. lusitaniae* exhibits striking parallels to that in the distantly related ascomycete *S. pombe*, even though these lineages diverged from one another more than 330 million years ago²³. In *S. pombe*, mating and meiosis are also tightly coupled and both are dependent on pheromone MAPK signaling and the downstream transcriptional regulator, Ste11^{24,25}. In *C. lusitaniae*, we show that the pheromone MAPK-associated transcription factor *STE12* is similarly essential for both mating and meiosis. Thus, in both *S. pombe* and *C. lusitaniae* the programs regulating mating and meiosis are fused (Figure 2.4a).

To address if Ste12 also regulates meiosis in other hemiascomycete species, we deleted the *STE12* ortholog from related sexual species including *S. cerevisiae*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Yarrowia lipolytica* (Supplemental Figure 2.6). In each species loss of *STE12* failed to block meiosis, suggesting that the meiotic function of *STE12* evolved relatively recently in the *C. lusitaniae* lineage

(Figure 2.4b). In support of this hypothesis we note that *C. lusitaniae*, but not the other hemiascomycete species, lost the transcriptional regulator $\alpha 2$ during evolution^{9,10}. The $\alpha 2$ gene acts to prevent expression of haploid-specific genes (including MAPK genes) in diploid $\mathbf{a}/\alpha$ cells in diverse yeast species^{26,27}. Thus, as previously hypothesized^{9,10}, the loss of $\alpha 2$ could have preceded the rewiring of meiotic control in *C. lusitaniae*. In particular, we propose that it facilitated MAPK signaling and enabled Ste12 to assume control of meiosis in this species.

Why have such distinct modes of sexual regulation evolved in diverse unicellular yeast? The most parsimonious explanation is that these differences reflect a species' preference for one ploidy state over another. Both *C. lusitaniae* and *S. pombe*, despite being highly divergent, exhibit haploid lifestyles that contrast sharply with the predominantly diploid lifestyle of *S. cerevisiae*. Co-regulation of the transcriptional programs controlling mating and meiosis therefore ensures that cells, once mated, immediately enter meiosis and return to the haploid state. This could represent an adaptive advantage for haploid cells in both *C. lusitaniae* and *S. pombe*. Alternatively, the selective pressure to keep these two sexual programs separate may have been lost during evolution, leading to the amalgamation of these programs in the two species. While the relative advantages of haploidy and diploidy continue to be debated^{28,29}, it is clear that distinct transcriptional circuits can evolve to promote either haploid or diploid lifestyles. That this mode of regulation evolved independently in *S. pombe* and *C. lusitaniae* further suggests that examples of fused sexual cycles will be found throughout the fungal tree of life.

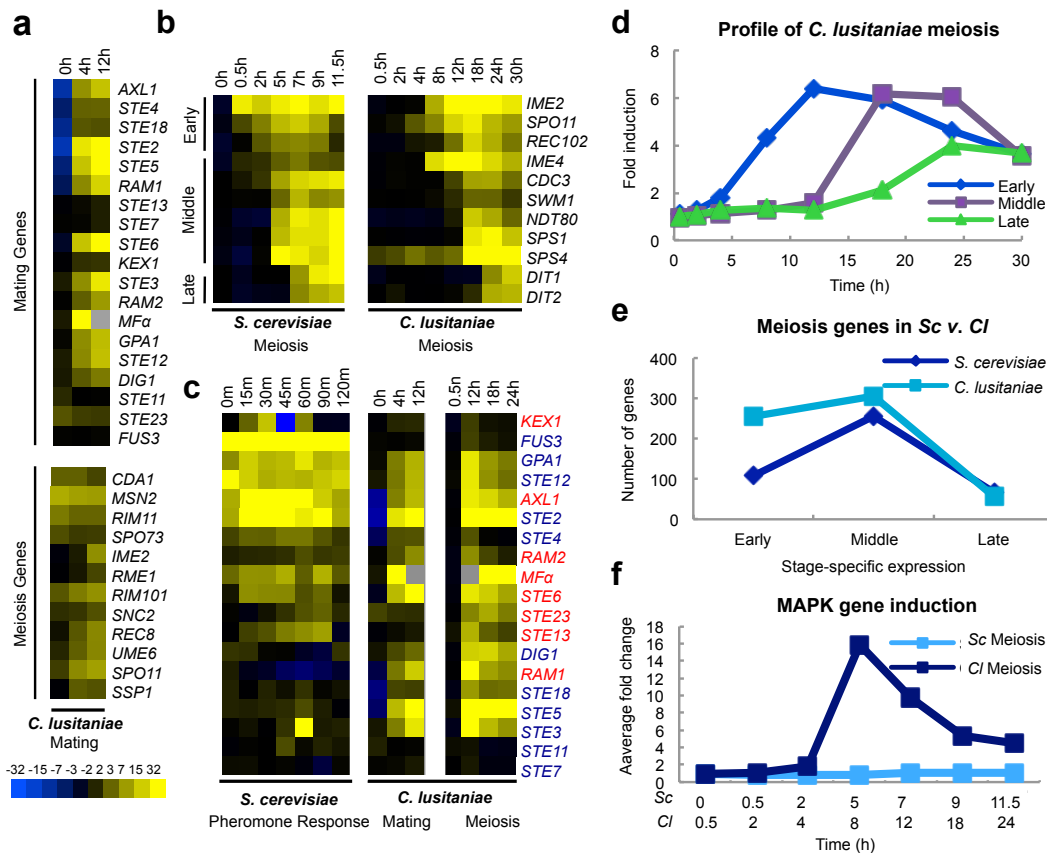


Figure 2.1: Transcriptional profiling of mating and meiosis in *C. lusitaniae*.

a, Profiling of *C. lusitaniae* mating. Top panel, induction of *C. lusitaniae* pheromone-signaling genes. Bottom panel, induction of genes characteristic of the meiotic program in *S. cerevisiae*. b, Stage-specific meiotic gene expression in *S. cerevisiae* and *C. lusitaniae*. Early, Middle, and Late meiosis genes in *C. lusitaniae* are defined as those >3 fold induced after 12, 18, and 24 h, respectively. c, Expression of MAPK pathway genes (blue text) and pheromone-processing genes (red text) during *C. lusitaniae* mating and meiosis. Expression changes for *S. cerevisiae* genes30 were enhanced 3-fold to be comparable to expression changes in *C. lusitaniae* genes. d, Average fold induction of early, middle, and late meiosis genes in *C. lusitaniae*. e, Comparison of meiosis gene expression changes in *C. lusitaniae* and *S. cerevisiae*. f, Fold induction of MAPK pathway genes during meiosis in *C. lusitaniae* and *S. cerevisiae*.

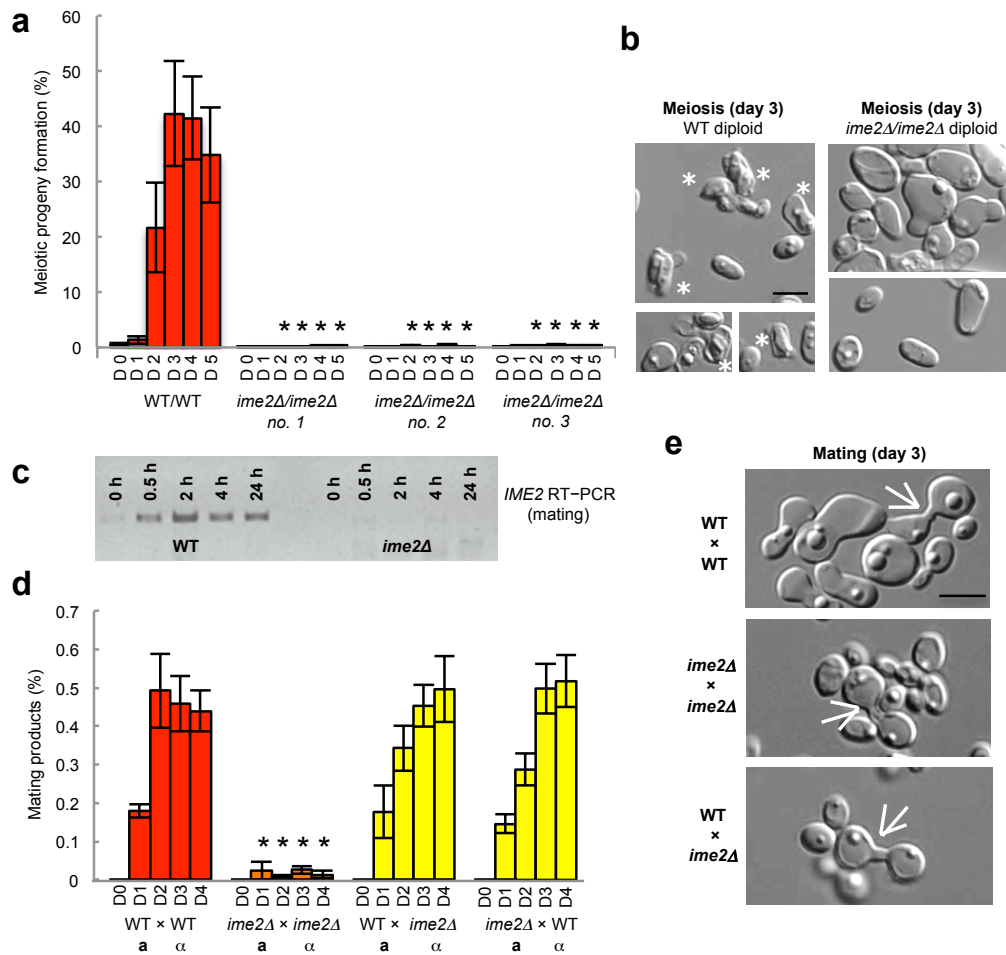


Figure 2.2: *IME2* is required for mating and meiosis in *Candida lusitaniae*.

a, Deletion of *IME2* blocks meiosis in *C. lusitaniae* (5-day time course, D1-D5). # represents $p < 0.01$, one way ANOVA, $n=3$. **b**, Wildtype diploid cells generate dyad spores (asterisks) after 3 days on PDA medium, whereas *ime2Δ/ime2Δ* diploids do not sporulate, $n=3$. **c**, RT-PCR data demonstrates induction of *IME2* during *C. lusitaniae* mating, $n=3$. **d**, Loss of *IME2* in both **a** and α cells leads to decreased mating (4 day time course, D1-D4). “#” represents $P < 0.05$, Kruskal Wallis, $n=4$. **e**, *ime2Δ* mutants generate mating zygotes, indicating the block to mating occurs post-cell fusion, $n=4$. Scale bars, 5 μm . Data represented as mean $\pm$ s.e.m.

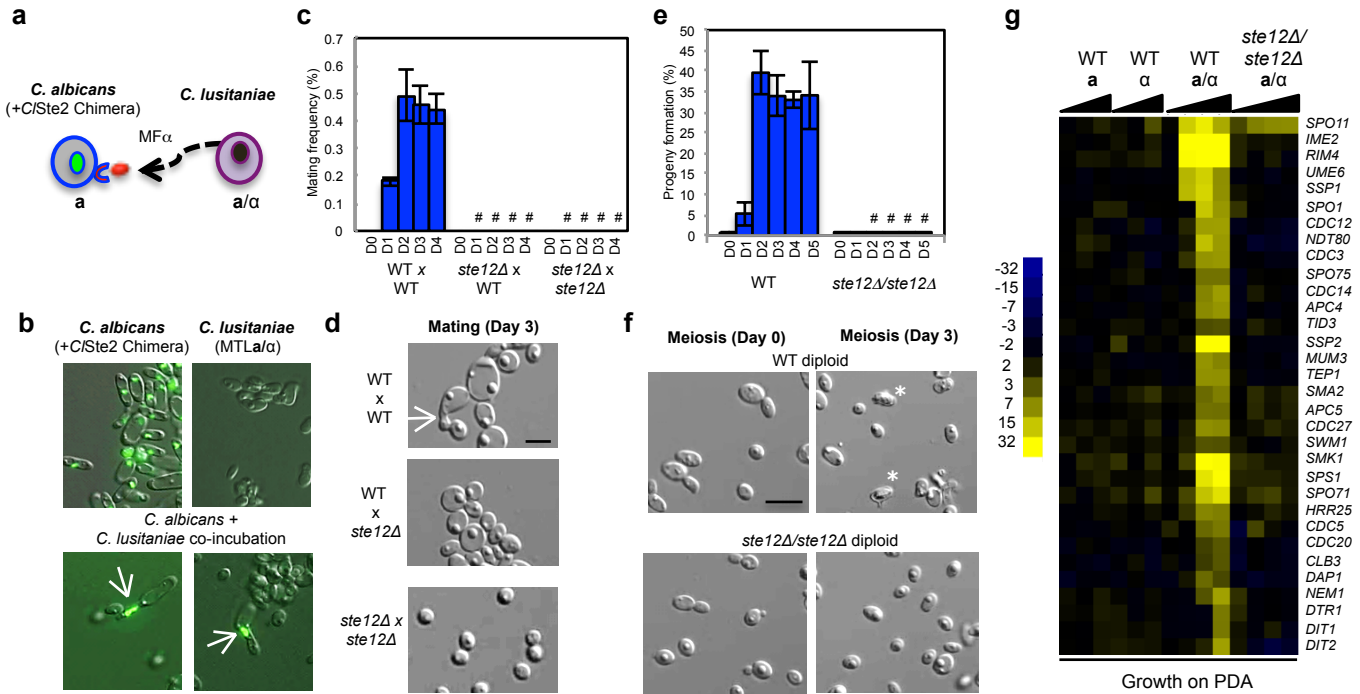


Figure 2.3: STE12 is essential for both mating and meiosis in *Candida lusitaniae*.

a, Schematic of assay to detect pheromone secretion from *C. lusitaniae* cells. *C. lusitaniae* a/α cells were co-incubated with GFP-labeled *C. albicans* a cells expressing the *C. lusitaniae* α pheromone receptor. **b**, *C. albicans* cells generate polarized mating projections (arrows) in response to *C. lusitaniae* pheromone, demonstrating that *C. lusitaniae* cells actively secrete pheromone during meiosis, $n=2$. **c**, Deletion of *STE12* abolishes mating in *C. lusitaniae* (4 day time course, D1-D4). “#” represents $P < 0.05$, Kruskal Wallis, $n=3$. **d**, Mating occurs between wildtype *C. lusitaniae* cells (arrow) but not between *ste12 Δ* mutant cells, $n=3$. **e**, Loss of *STE12* inhibited the formation of meiotic haploid progeny (4 day time course, D1-D4). “#” represents $P < 0.05$, unequal variance t test, $n=3$. **f**, Absence of meiotic spores in *C. lusitaniae* cells lacking *STE12*, $n=5$. **g**, Gene expression changes when *C. lusitaniae* haploid, diploid, or *ste12 Δ /ste12 Δ* strains are incubated on PDA medium. Deletion of *STE12* abolished most meiosis-specific gene expression changes. Where appropriate, scale bars, 5 μ m; data represented as mean $\pm$ s.e.m.

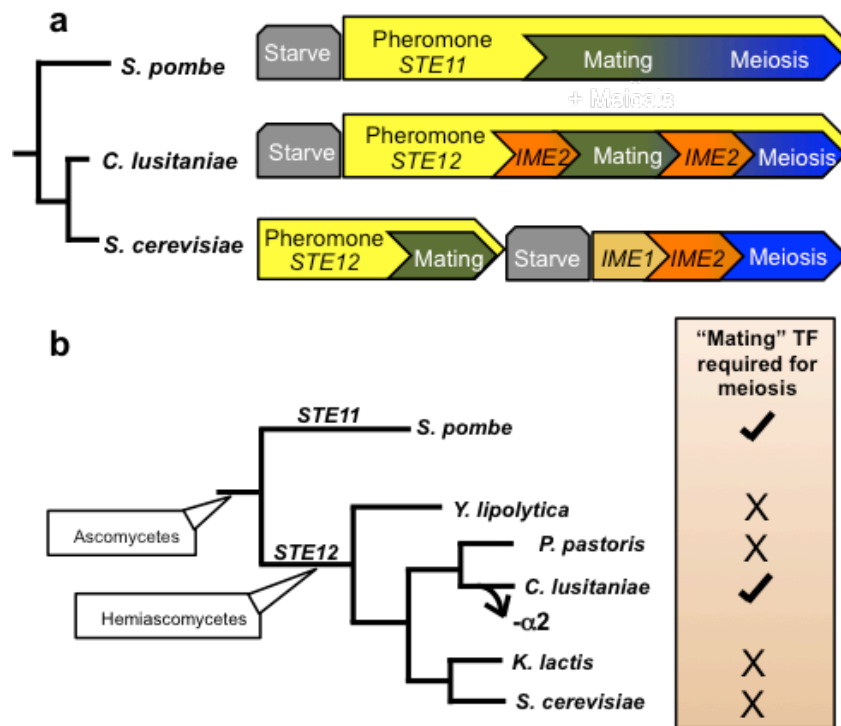
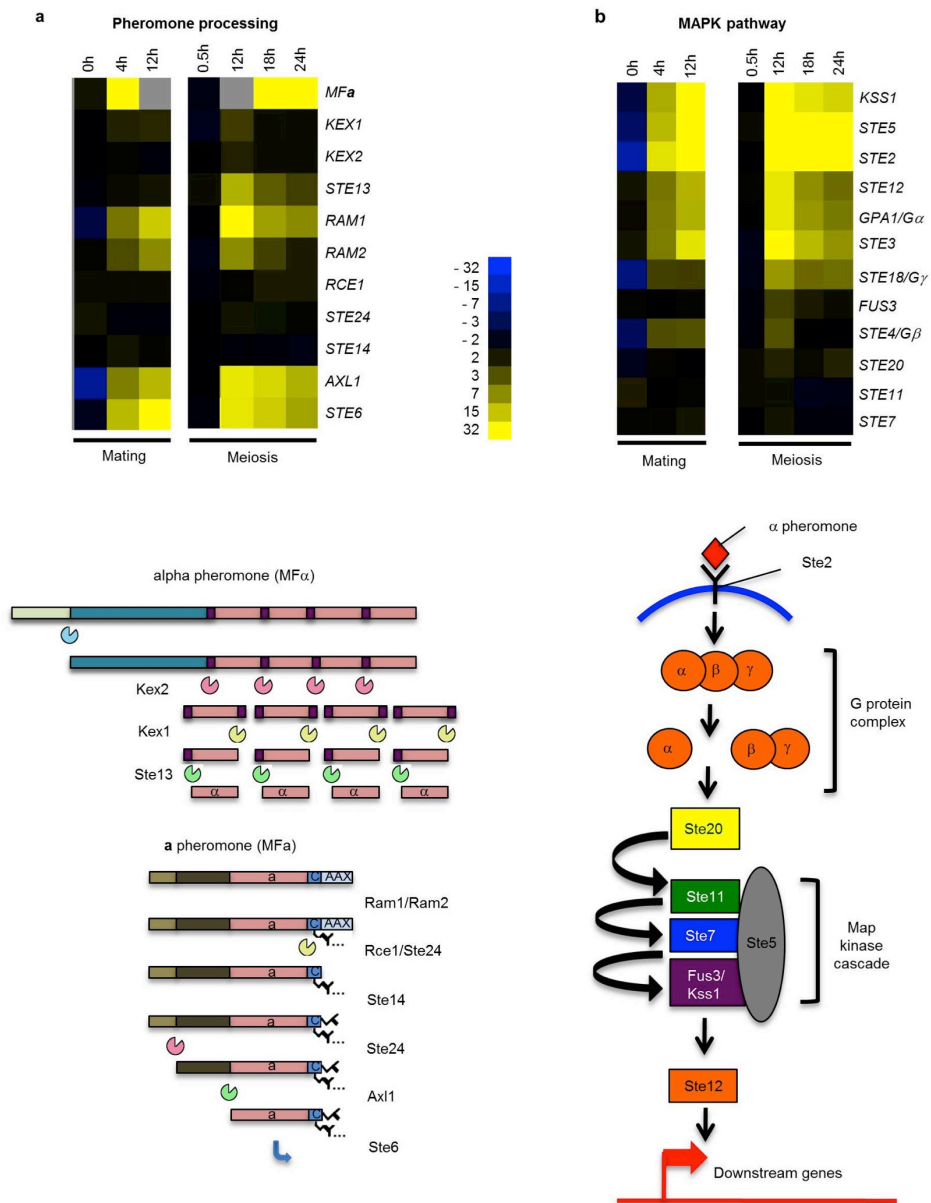


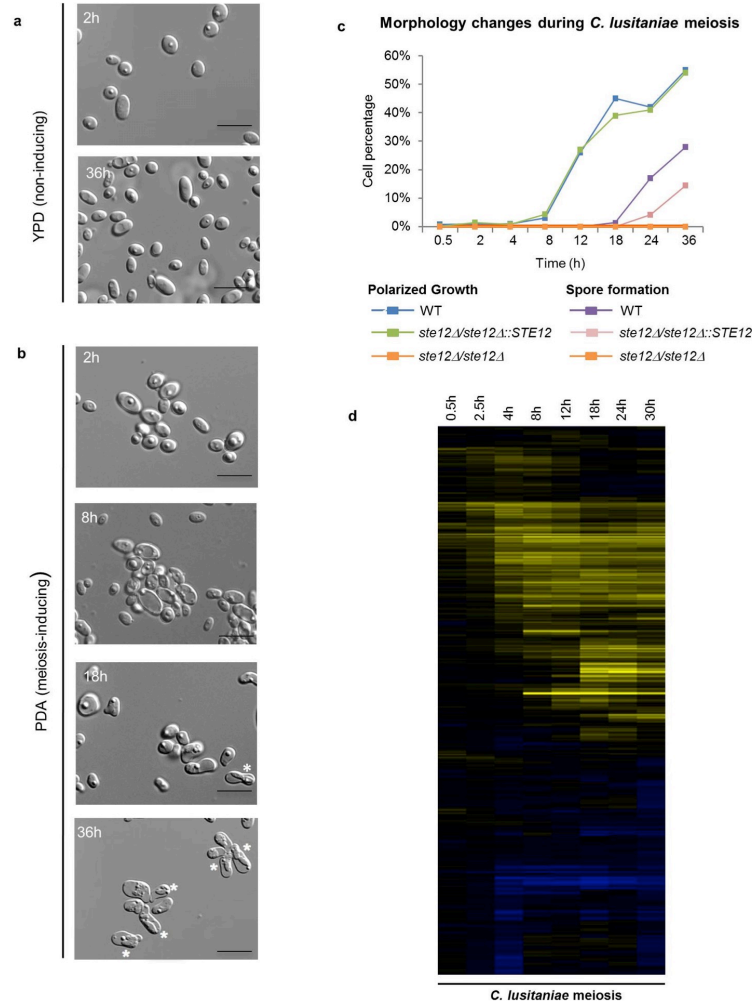
Figure 2.4: Sexual Reproduction in Yeast.

a, Model comparing the sexual lifecycles of *S. cerevisiae*, *C. lusitaniae*, and *S. pombe*. The diploid species *S. cerevisiae* exhibits distinct regulation of mating and meiosis; MAPK signaling via Ste12 regulates mating, while Ime1 regulates meiosis. In contrast, *S. pombe* exhibits integrated control of its sexual cycle; MAPK signaling and Ste11 regulate entry into both mating and meiosis. *C. lusitaniae* exhibits similar control over its sexual cycle to that in *S. pombe*. In particular, *C. lusitaniae* mating and meiosis are highly coupled, and both are regulated by the transcription factor Ste12. **b**, *C. lusitaniae* is the only hemiascomycete tested that requires *STE12* to undergo meiosis. Loss of the *MTLα2* transcription factor specifically in the *C. lusitaniae* lineage could have been a key step during evolution of this regulatory control, as it would permit expression of 'haploid-specific genes' (including MAPK genes) in diploid cells.



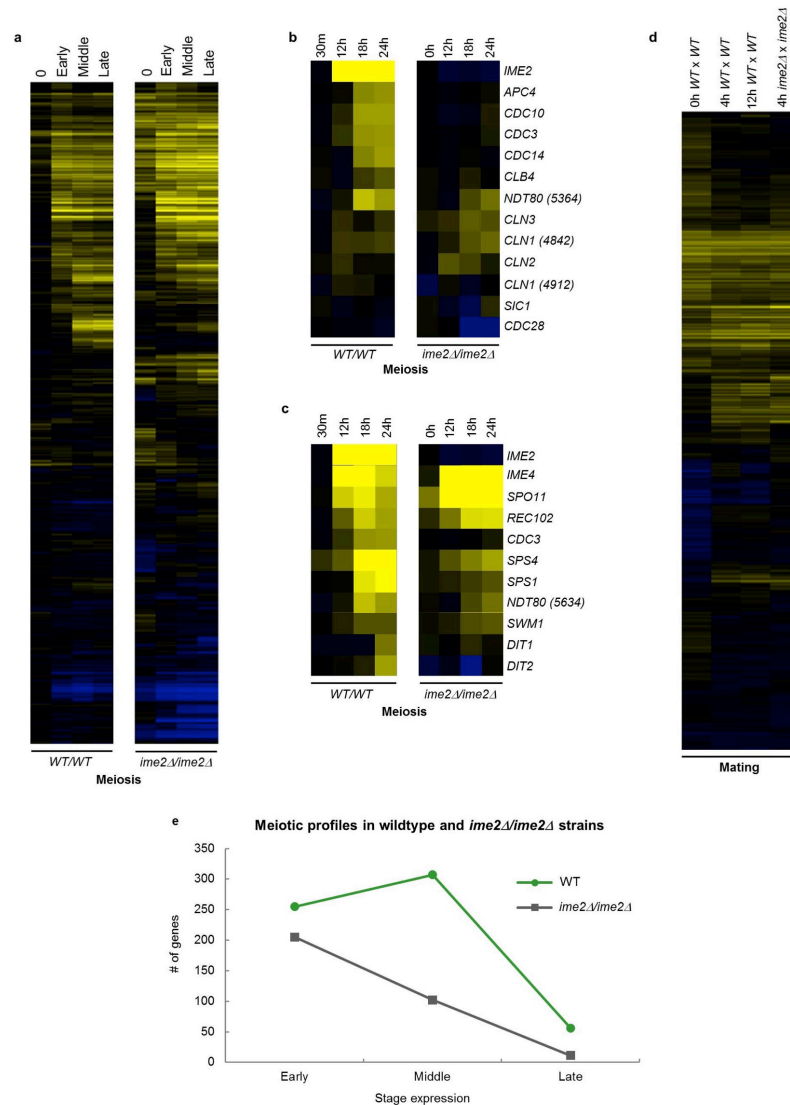
Supplemental Figure 2.1: Schematic showing induction of pheromone processing genes (a) and pheromone MAPK genes (b) during both mating and meiosis in *C. lusitaniae*.

Expression changes highlight genes induced during co-incubation of **a** and **✓** cells on PDA medium (mating), as well as during growth of diploid **a/✓** cells on PDA medium (induces meiosis and sporulation). In contrast, these genes are mating-specific in the related yeast *S. cerevisiae*.



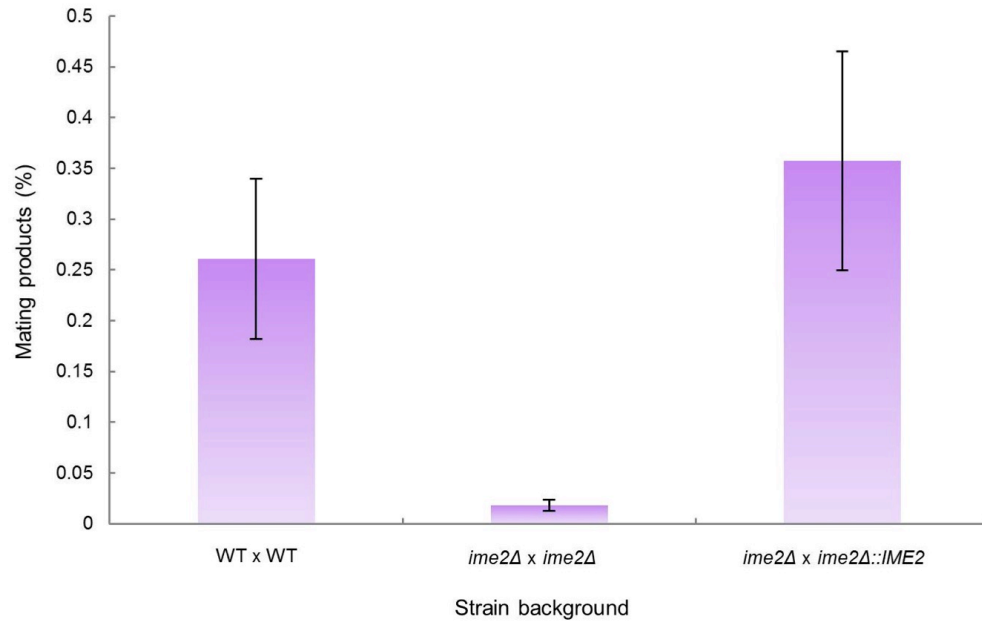
Supplemental Figure 2.2: Temporal analysis of meiosis in *C. lusitaniae*.

a, *C. lusitaniae* α/α cells divide as stable diploid cells on YPD medium. $n=3$. **b**, On PDA medium, α/α cells are induced to enter meiosis and dyad spores (asterisks) begin to appear at 18 h. $n=3$. **c**, Time course of meiosis in wildtype, *ste12Δ/ste12Δ*, and *STE12* complemented *C. lusitaniae* strains. A morphology change (polarized growth) is evident in wildtype diploid strains grown on PDA medium starting at 12 h, while spore formation is apparent at 18-36 h. In the *ste12Δ/ste12Δ* mutant, both sporulation and morphology change are absent. **d**, Global transcriptional profile of meiosis in *C. lusitaniae* showing induced genes (yellow) and repressed genes (blue). *C. lusitaniae* diploid α/α cells (RSY432) were grown on PDA (sporulating) medium and expression changes compared to those on YPD (non-sporulating) medium. Genes changing more than 4-fold in expression are shown.



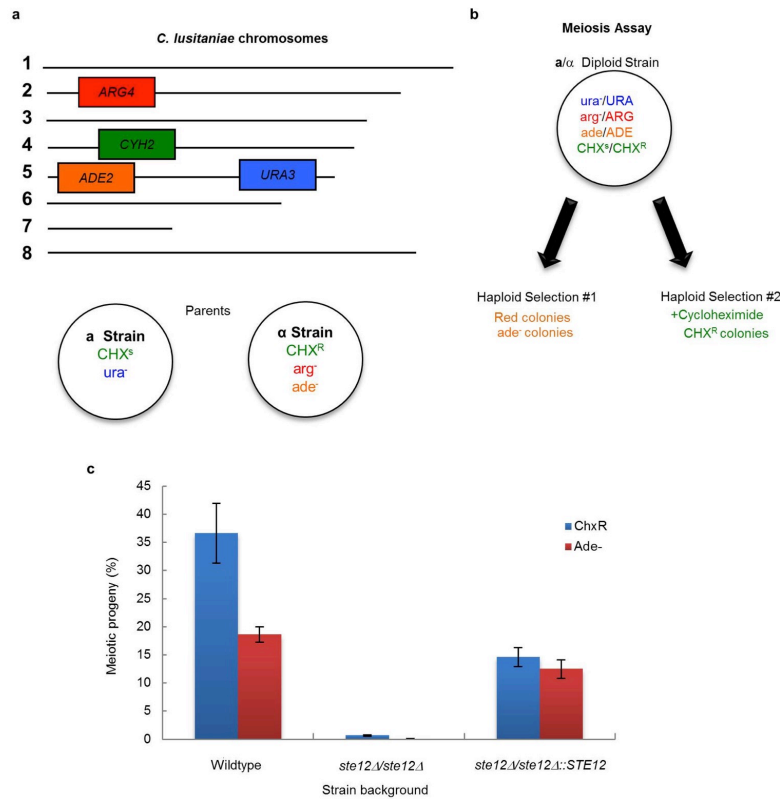
Supplemental Figure 2.3: Transcriptional profiling of *C. lusitaniae* wildtype and *ime2Δ* mutants during mating and meiosis.

a, Full profile of wildtype and *ime2Δ/ime2Δ* strains during meiosis indicates that many transcriptional changes occur in *ime2Δ/ime2Δ* mutants as in wildtype strains. **b**, Analysis of cell cycle regulating genes during *C. lusitaniae* meiosis. Several cell cycle genes are induced in wildtype cells undergoing meiosis but not in *ime2Δ/ime2Δ* mutants (e.g., *APC4*, *CDC3*, *CDC10*, and *CDC14*). **c**, Comparative expression of early (*IME2*, *IME4*, *SPO11*), middle (*REC102*, *CDC3*, *SPS4*, *SPS1*, *NDT80*, *SWM1*) and late (*DIT1*, *DIT2*) meiosis genes. **d**, In contrast to the meiotic profiles, the mating profiles of wildtype and *ime2Δ* strains were very similar. **e**, Comparison of meiosis genes induced in wildtype and *ime2Δ* mutants.



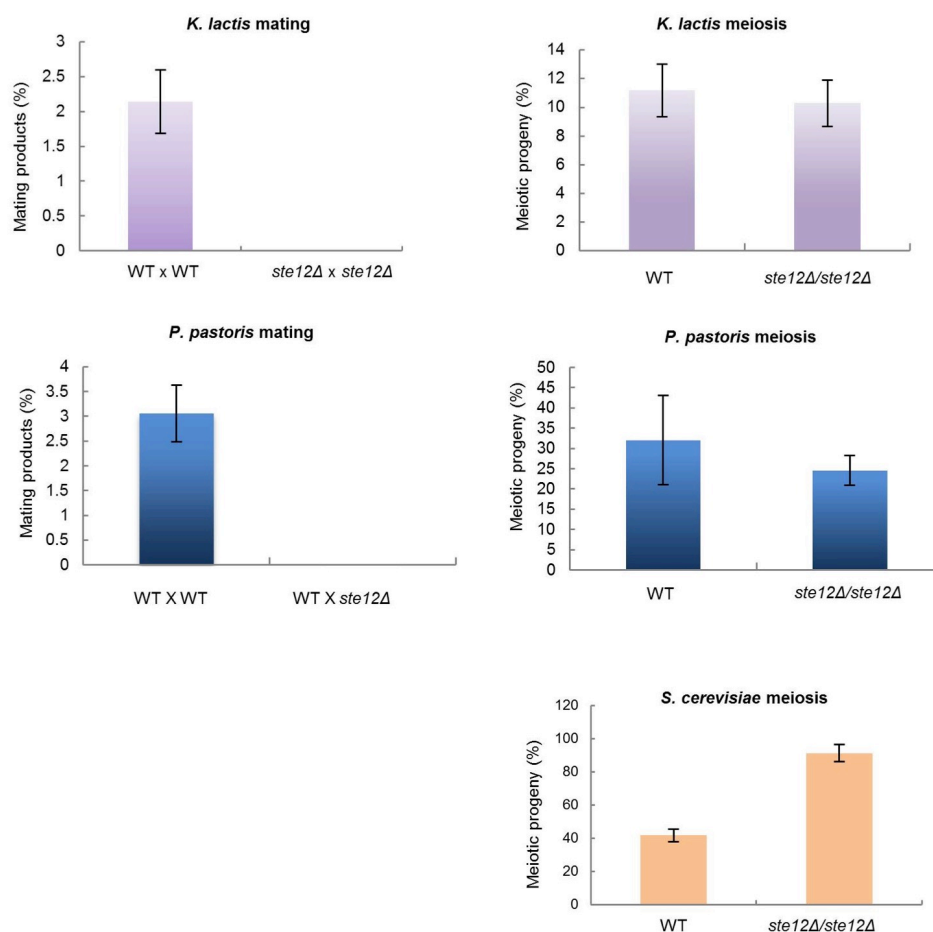
Supplemental Figure 2.4: Reintegration of *IME2* restores mating efficiency.

IME2 was reintegrated at the endogenous locus in an *ime2Δ* mutant (RSY437). Mating frequency was quantified by monitoring the formation of prototrophic products from auxotrophic parents. WT cross, RSY411 x RSY284, *ime2Δ* mutant cross, RSY406 x RSY437, *IME2* complemented cross, RSY406 x CAY5022. Differences between the WT cross and the *ime2Δ* mutant cross, and between the *IME2* complemented cross and the *ime2Δ* mutant cross were both significant. (n=7, p<0.001, Kruskal Wallis test). Data are representative of mean +/- s.e.m.



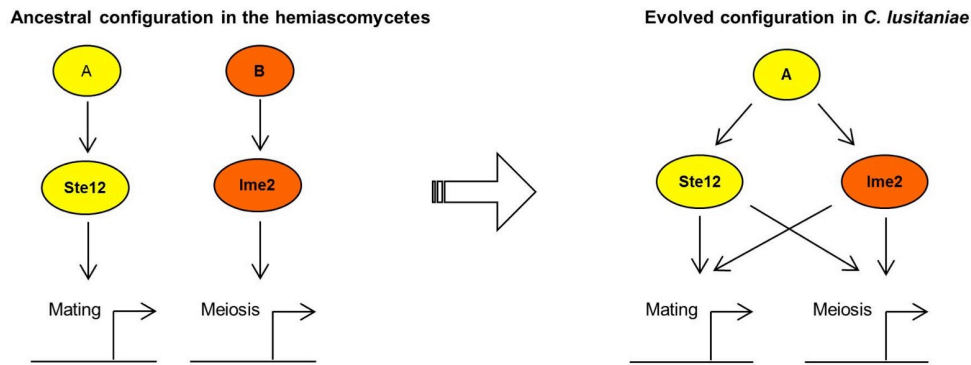
Supplemental Figure 2.5: Schematic of genetically marked *C. lusitaniae* strains and control of meiosis by STE12.

a, *C. lusitaniae* mating experiments were performed between RSY284 (**a** strain) and RSY411 (α strain) and mating products selected based on auxotrophic makers. **b**, The **a/α** diploid strain RSY432 was induced to undergo meiosis on PDA medium and meiotic progeny identified by their red color on YPD medium (ade⁻ colonies) or by growth on medium containing cycloheximide (CHX resistant colonies). **c**, Diploid **a/α** strains of *C. lusitaniae* were incubated on PDA medium for 3 days and analyzed for the frequency of formation of meiotic progeny. Two independent *ste12Δ* mutants were constructed in the **a/α** background and tested for meiotic progeny using both the *CHXR* and *ADE2* markers. Mutants lacking *STE12* were unable to undergo meiosis to produce haploid progeny, while reintegration of *STE12* into the mutant background restored meiosis. Differences between both wildtype strains and *ste12Δ* mutants, and between *ste12Δ* mutants and *STE12*-complemented strains were significant (n=3, p < 0.05, student's t test, two tailed). Data representative of mean +/- s.e.m.



Supplemental Figure 2.6: Analysis of *STE12* function in mating and meiosis in diverse hemiascomycete species.

The *STE12* gene was deleted from haploid and diploid strains of *K. lactis*, *P. pastoris*, and *S. cerevisiae*. The resultant strains were tested both for mating competency and the formation of meiotic progeny. Whereas deletion of *STE12* blocked mating in haploid strains of all three species, loss of *STE12* from diploid a/α strains did not have a significant effect on the formation of meiotic progeny. Thus, *C. lusitaniae* is unique among the hemiascomycete species tested in that *STE12* is essential for meiosis only in this species. *K. lactis* mating and meiosis, $n=2$, data combined for 3 independent mutants. *P. pastoris* mating, $n=3$. *P. pastoris* meiosis $n=4$. *S. cerevisiae* meiosis $n=3$. All data presented as mean $\pm$ s.e.m.



Supplemental Figure 2.7: Rewiring of the genetic programs that control sexual reproduction in hemiascomycetes

In many hemiascomycete species, including the model yeast *S. cerevisiae*, mating and meiosis are controlled by two distinct transcriptional programs with *STE12* as the master regulator of mating and *IME2* as a key regulator of meiosis. However, in the opportunistic pathogen *C. lusitaniae*, *STE12* has retained its role in regulating mating, but has also acquired control over meiosis. Similarly, *C. lusitaniae* *IME2* has a conserved role in regulating mating, but also has a role in promoting mating. The transcriptional programs controlling these two processes have therefore fused in *C. lusitaniae*, perhaps to facilitate a transient diploid state and the more efficient return to the predominant haploid state.

Methods

Strain Construction

All strains constructed for this study are listed in Table 2.1, and oligonucleotides used to construct these strains are listed in Table 2.2. Starting strain RSY148 was genetically marked by deleting both *ARG4* and *ADE2* genes. To accomplish this, the 5' flank of *ARG4* was amplified with oligonucleotides RS84/RS85. The 3' flank of *ARG4* was amplified with oligonucleotides RS86/RS87. The 5' flank was digested with *ApaI* and *XhoI* and ligated between *ApaI* and *XhoI* sites in pSFS2a³³. Next, the *ARG4* 3' flank was digested with *PstI* and *NotI* and ligated between the same sites within the plasmid containing the 5' flank of *ARG4*. This construct (pRB243) was linearized with *ApaI* and *NotI* and transformed into RSY148 to create RSY289. Integration was checked by PCR across the 5' and 3' gene boundaries using oligonucleotides RS90/BL70 and BL71/RS91, respectively. Loss of the *ARG4* open reading frame was confirmed by PCR using oligonucleotides RS88/RS89. RSY289 was cultured on SC-Maltose to induce expression of the *MAL2* promoter resulting in expression of *caFLP* and subsequent excision of the *SAT1* cassette at the FRT sites³³ creating RSY297. Loss of the *SAT1* marker from the deletion construct was confirmed by PCR analysis using RS90 and BL70.

The *ADE2* was deleted from RSY297 by first PCR amplifying the 5' flank of *ADE2* using oligonucleotides RS230/RS231 and the product ligated between *ApaI* and *XhoI* sites in pSFS2a. The 3' flank of *ADE2* was PCR amplified using oligonucleotides RS228/RS229 and ligated into the plasmid using *NotI* and *SacII* sites, generating pRB243. The *ADE2* deletion cassette was generated by digesting

with *ApaI* and *SacII* and transformed into RSY297 to create RSY379. Correct integration of the construct was verified by PCR analysis across the 5' and 3' junctions using oligonucleotides RS226/BL70 and BL71/RS227. Loss of the *ADE2* ORF was confirmed by PCR using oligonucleotides RS276/RS277. RSY379 was cultured on SC-Maltose to induce expression of the *MAL2* promoter resulting in excision of the *SAT1* cassette³³ to generate RSY411.

IME2 deletions were constructed in **a** and α strains RSY411 and RSY284, respectively. The 5' flank of *IME2* was amplified using oligonucleotides RS224/RS265. pSFS2a and the 5' *IME2* flank were digested with *KpnI* and *ApaI* and ligated together. Next, the 3' flank of *IME2* was PCR amplified using oligonucleotides RS263/RS225. The pSFS2a plasmid containing the 5' flank of *IME2* and the 3' *IME2* PCR product were digested with *NotI* and *SacII* and ligated to generate pRB245. pRB245 was linearized with *KpnI* and *SacII* and transformed into RSY284 to generate *ime2Δ* strain RSY410. The same construct was transformed into RSY411 creating *ime2Δ* strains RSY406, RSY407, and RSY408. Accurate integration of the construct was confirmed by PCR across 5' and 3' junction boundaries using oligonucleotides RS167/BL70 and BL71/RS148. Loss of the *IME2* ORF was verified by PCR analysis using oligonucleotides RS165/RS166. RSY410 was cultured on SC-Maltose to induce expression of *caFLP* and excision of the *SAT1* cassette³³ creating RSY437. To reintegrate *IME2* at the endogenous locus the *IME2* ORF, together with ~ 1 kb of promoter, was PCR amplified using primers RS281 and RS282. The PCR product was digested with *KpnI* and *XhoI* and cloned into pSFS2a to generate plasmid pRB278. pRB278 was linearized using *PshAI* and transformed into RSY437.

Integration at the endogenous *IME2* locus was checked by PCR using primers RS224/BL70. Diploid $\Delta ime2/\Delta ime2$ strains RSY427, RSY428 and RSY429 were generated by mating haploid RSY406 and RSY410 strains. Strains were verified by flow cytometry, PCR verification of an a/α *MTL* configuration, and prototrophy.

STE12 double deletion strains were constructed in RSY432. The 5' flank of *STE12* was amplified using oligonucleotides RS323/RS324. The 5' flank and pSFS2a were digested with *KpnI* and *ApaI* and ligated together. The 3' flank of *STE12* was PCR amplified using oligonucleotides RS325/RS326 and integrated into this vector using *NotI* and *SacII*. The *STE12* deletion construct was excised by digestion with *KpnI* and *SacII* and transformed into diploid strain RSY432 to generate the *STE12/ste12Δ* heterozygous strain RSY541. RSY541 was incubated on SC-Maltose to induce expression of the *MAL2* promoter resulting in excision of the *SAT1* cassette³³ to generate RSY542. RSY542 was transformed with the *STE12* deletion construct to generate RSY543, a *ste12Δ/ste12Δ* deletion mutant. RSY543 was incubated on SC-Maltose to excise the *SAT1* cassette to generate RSY564. In a separate transformation, RSY432 was transformed with the *STE12* deletion construct to generate an independent heterozygous *STE12/ste12Δ* strain, RSY575. The *SAT1* cassette was excised to generate CAY4160, and then transformed with the *STE12* deletion construct to generate RSY610, RSY611, and RSY612. The *SAT1* cassettes from these strains were excised following growth on SC-Maltose to generate CAY4240, CAY4241, and CAY4242, respectively.

Haploid *ste12Δ* strains RSY539, RSY535, RSY544, and RSY545 were generated by incubating RSY541 (*STE12/ste12Δ* heterozygote) on PDA medium for

3 days to generate haploid progeny. The *MTL* of these progeny was checked by PCR using oligonucleotides RS66/67 and RS68/69. PCR using primers annealing to the *STE12* ORF (RS399/RS400) were used to verify the absence of *STE12*.

The *C. lusitaniae STE12* gene was reintegrated into a *ste12Δ/ste12Δ* strain (RSY564). The *STE12* ORF, together with ~1 kb of the promoter, was PCR amplified using oligonucleotides RS401/RS402. pSFS2a and the *STE12* PCR product were digested with *KpnI* and *ApaI* and ligated together. The resulting construct was linearized using *BmgBI* and transformed into RSY564 to generate complemented strains RSY601, RSY602, and RSY603. Accurate integration of the construct was determined by PCR using oligonucleotides RS403 and RS345.

Construction of *K. lactis* strains

To obtain diploid *K. lactis* strains, CAY571 and CAY572 were mated on malt medium at 30°C and selected on medium lacking tryptophan and uracil to generate RSY467, RSY468, RSY469, RSY470, and RSY478. *K. lactis* mutants were generated using an electroporation protocol previously described³⁷. To make gene deletion cassettes, *STE12* 5' and 3' homologous flanks were generated by PCR of genomic DNA using oligonucleotides BL1581/BL1568 and BL1569/BL1582 respectively. Nourseothricin and Kanamycin resistant genes were amplified by PCR using plasmids pKANmx4 and pNATmx4⁵⁰ with primers BL1313 and BL1414. Resistance genes were fused to target gene-flanking regions by fusion PCR⁴⁹ using oligonucleotides BL1581 and BL1582. This created two linear DNA constructs for transformation, *STE12-NAT* and *STE12-KAN*. *STE12* double mutants were generated

by two sequential rounds of transformation. In the first round RSY478 was transformed with the *STE12-KAN* cassette to obtain RSY551 and RSY555. Accurate integration of this construct was verified by PCR across 5' and 3' junctions using oligonucleotides BL1500/BL1329 and BL1501/BL1330. RSY551 and RSY555 strains were transformed with the *STE12-NAT* construct to obtain *ste12Δ/ste12Δ* strains RSY571/RSY572 and RSY574, respectively. Loss of the *STE12* ORF was verified using PCR with primers specific to the ORF (BL1498/BL1499). Haploid *K. lactis STE12* mutants were generated by transforming CAY571 with the *STE12-NAT1* cassette to generate RSY567 and RSY568. CAY572 was transformed with *STE12-KAN* to generate RSY550 and with *STE12-NAT* to generate RSY570.

Construction of *P. pastoris* and *Y. lipolytica* strains

To delete the *STE12* gene in *Pichia pastoris*, the 5' and 3' flanks of the gene were amplified by PCR using oligonucleotides 2357/2358 and 2359/2360, respectively. The *HYG* or *NAT1* selection markers were amplified by PCR using oligonucleotides BL1309 and BL1310 from plasmids pNatMX4 and pHYGMX4. The *KAN* marker was amplified from plasmid pKANB⁵¹ using oligonucleotides 2365/2366. *STE12* flanks and selection markers were joined by fusion PCR using oligonucleotides 2357/2360. PCR constructs were then transformed into *P. pastoris* strains JC304 and JC306 using an established protocol³⁴.

To delete the *STE12* gene in *Yarrowia lipolytica*, the *STE12* ORF and flanking regions were first amplified by PCR using oligonucleotides BL2618/BL2619 and cloned into pCR-Blunt II-TOPO (Invitrogen) to generate pRB339. The *LEU2* gene

was also PCR amplified from *Y. lipolytica* using oligonucleotides BL2620/BL2621 and cloned into pCR-BluntII-TOPO. *LEU2* was then excised from this vector using *Bam*HI/*Kpn*I and used to replace the *STE12* ORF in pRB340. A cassette was released from this vector using *Hind*III/*Eco*RV and used to transform *Y. lipolytica* to delete the *STE12* gene. The *URA3* gene was also amplified from vector pINA156 (gift from Carmen Lisset-Flores Mauriz, Instituto de Investigaciones Biomédicas Alberto Sols CSIC-UAM, Madrid, Spain) using primers BL2622/BL2483 and cloned into pSC-B-amp/kan (Agilent). *URA3* was excised from this vector and used to replace *STE12* in vector pRB341. Subsequently, a cassette was released from this vector using *Hind*III/*Eco*RV and transformed into *Y. lipolytica* to delete *STE12*. Transformations in *Y. lipolytica* were achieved using the lithium acetate method of Barth and Gaillardin⁵².

Construction of *S. cerevisiae* strains

Diploid *S. cerevisiae* strains were generated by mating CAY4584 and CAY4585 on YPD medium for 24 h, then selecting for diploid products on SCD medium lacking tryptophan and adenine. The resulting mating product, CAY4595, was verified as being diploid by its ability to sporulate as well as PCR of the *MAT* locus using primers 2461/2562 to amplify *MAT* α 1 and 2565/2566 to amplify *MAT* α 1. Subsequent transformations were performed as previously described⁵⁴. To delete the *STE12* gene, oligonucleotides BL2468 and BL2469 were designed to PCR amplify selection markers from pNATMX4 and pKANMX4 and contained sequences that were complementary to the 5' and 3' regions of the *STE12* ORF. The first round

of transformation using a *NATMX4-STE12* PCR construct generated strain CAY4770, which was genotyped by PCR using primers BL1329/BL2470 and BL1330/BL2471. CAY4770 was transformed with a *KANMX4-STE12* PCR construct to generate CAY4808, a *ste12Δ/ste12Δ* deletion mutant. Loss of the ORF was confirmed by PCR using primers BL2472/BL2473.

Table 2.1: Strains Used in the *C. lusitaniae* Study

Strain	Organism	Genotype	Source
CAY3705	<i>C. albicans</i>	MTLa/a <i>ste2Δ::LEU2/ste2Δ::HIS1 C. lusitaniae</i> Chimeric <i>STE2</i> <i>HTB/HTB::YFP-SAT</i>	Lin et al. ⁹ , This Study
RSY147 (CL143)	<i>C. lusitaniae</i>	MTLa <i>chx^R</i>	Reedy et al. ¹⁰
RSY148 (CL145)	<i>C. lusitaniae</i>	MTLa <i>chx^R</i>	Gift of J. Heitman
RSY253 (JLR853)	<i>C. lusitaniae</i>	MTLa/α <i>LEU2/leu2Δ::SAT1 URA3/ura3 chx^S/chx^R</i>	Gift of J. Heitman
RSY284 (CL6936)	<i>C. lusitaniae</i>	MTLa <i>ura3Δ</i>	Gift of T. Noel
RSY289	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::SAT1 chx^R</i>	This Study
RSY297	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT chx^R</i>	This Study
RSY379	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::SAT chx^R</i>	This study
RSY539	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::FRT ste12Δ::SAT1 chx^R</i>	This Study
RSY406	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::FRT ime2Δ::SAT1 chx^R</i>	This Study
RSY407	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::FRT ime2Δ::SAT1 chx^R</i>	This Study
RSY408	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::FRT ime2Δ::SAT1 chx^R</i>	This Study
RSY410	<i>C. lusitaniae</i>	MTLa <i>ura3Δ ime2Δ::SAT1</i>	This Study
RSY411	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::FRT CHX^R</i>	This Study
RSY427	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ</i> <i>chx^S/chx^R ime2Δ::SAT1/ime2Δ::SAT1</i>	This Study
RSY428	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ</i> <i>chx^S/chx^R ime2Δ::SAT1/ime2Δ::SAT1</i>	This Study
RSY429	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ</i> <i>chx^S/chx^R ime2Δ::SAT1/ime2Δ::SAT1</i>	This Study
RSY432	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ</i> <i>chx^S/chx^R</i>	This Study
RSY535	<i>C. lusitaniae</i>	MTLa <i>ura3Δ arg4Δ::FRT ade2Δ::FRT ste12Δ::SAT1</i>	This Study
RSY538	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::FRT ste12Δ::SAT1 CHX^R</i>	This Study
RSY541	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ</i> <i>chx^S/chx^R STE12/ste12Δ::SAT1</i>	This Study
RSY542	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ</i> <i>chx^S/chx^R STE12/ste12Δ::FRT</i>	This Study
RSY543	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ</i> <i>chx^S/chx^R ste12Δ::FRT/ste12Δ::SAT1</i>	This Study
RSY544	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ura3Δ, ste12Δ::SAT1</i>	This Study
RSY545	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ura3Δ, ste12Δ::SAT1</i>	This Study
RSY547	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::FRT ste12Δ::SAT1 CHX^R</i>	This Study

RSY548	<i>C. lusitaniae</i>	MTLa <i>arg4Δ::FRT ade2Δ::FRT ste12Δ::SAT1</i>	This Study
RSY564	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::FRT</i>	This Study
RSY575	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R STE12/ste12Δ::SAT1</i>	This Study
RSY601	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::STE12-SAT1</i>	This Study
RSY602	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::STE12-SAT1</i>	This Study
RSY603	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::STE12-SAT1</i>	This Study
RSY610	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::SAT1</i>	This Study
RSY611	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::SAT1</i>	This Study
RSY612	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::SAT1</i>	This Study
CAY4160	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R STE12/ste12Δ::FRT</i>	This Study
CAY4240	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::FRT</i>	This Study
CAY4241	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::FRT</i>	This Study
CAY4242	<i>C. lusitaniae</i>	MTLa/α <i>ARG4/arg4Δ::FRT ADE2/ade2Δ::FRT URA3/ura3Δ chx^S/chx^R ste12Δ::FRT/ste12Δ::FRT</i>	This Study
RSY437	<i>C. lusitaniae</i>	MTLa <i>ura3Δ ime2Δ::FRT</i>	This study
CAY5022	<i>C. lusitaniae</i>	MTLa <i>ura3Δ ime2Δ::IME2-SAT</i>	This study
CAY571	<i>K. lactis</i>	MTLa <i>hmlalphaΔ::URA3 nej1Δ::LEU2 lysA1Δ trp1Δ leu2Δ metA1Δ uraA1Δ</i>	Gift of A. Johnson
CAY572	<i>K. lactis</i>	MTLa <i>hmraΔ::TRP1 nej1Δ::LEU2 ade1Δ trp1Δ leu2Δ metA1Δ uraA1Δ</i>	Gift of A. Johnson
RSY467	<i>K. lactis</i>	MTLa/α <i>hmlalphaΔ::URA3 hmraΔ::TRP1 nej1Δ::LEU2 ade1Δ/ade1Δ trp1Δ/trp1Δ leu2Δ/leu2Δ metA1Δ/metA1Δ uraA1Δ/uraA1Δ</i>	This Study
RSY468	<i>K. lactis</i>	MTLa/α <i>hmlalphaΔ::URA3 hmraΔ::TRP1 nej1Δ::LEU2 ade1Δ/ade1Δ trp1Δ/trp1Δ leu2Δ/leu2Δ metA1Δ/metA1Δ uraA1Δ/uraA1Δ</i>	This Study
RSY469	<i>K. lactis</i>	MTLa/α <i>hmlalphaΔ::URA3 hmraΔ::TRP1 nej1Δ::LEU2 ade1Δ/ade1Δ trp1Δ/trp1Δ leu2Δ/leu2Δ metA1Δ/metA1Δ uraA1Δ/uraA1Δ</i>	This Study
RSY470	<i>K. lactis</i>	MTLa/α <i>hmlalphaΔ::URA3 hmraΔ::TRP1 nej1Δ::LEU2 ade1Δ/ade1Δ trp1Δ/trp1Δ leu2Δ/leu2Δ metA1Δ/metA1Δ uraA1Δ/uraA1Δ</i>	This Study
RSY478	<i>K. lactis</i>	MTLa/α <i>hmlalphaΔ::URA3 hmraΔ::TRP1 nej1Δ::LEU2 ade1Δ/ade1Δ trp1Δ/trp1Δ leu2Δ/leu2Δ metA1Δ/metA1Δ uraA1Δ/uraA1Δ</i>	This Study

RSY551	<i>K. lactis</i>	MTLa/ α hmlalpha Δ ::URA3 hmra Δ ::TRP1 nej1 Δ ::LEU2 ade1 Δ /ade1 Δ trp1 Δ /trp1 Δ leu2 Δ /leu2 Δ metA1 Δ /metA1 Δ uraA1 Δ /uraA1 Δ ste12 Δ ::KanMX/STE12	This Study
RSY555	<i>K. lactis</i>	MTLa/ α hmlalpha Δ ::URA3 hmra Δ ::TRP1 nej1 Δ ::LEU2 ade1 Δ /ade1 Δ trp1 Δ /trp1 Δ leu2 Δ /leu2 Δ metA1 Δ /metA1 Δ uraA1 Δ /uraA1 Δ ste12 Δ ::KanMX/STE12	This Study
RSY571	<i>K. lactis</i>	MTLa/ α hmlalpha Δ ::URA3 hmra Δ ::TRP1 nej1 Δ ::LEU2 ade1 Δ /ade1 Δ trp1 Δ /trp1 Δ leu2 Δ /leu2 Δ metA1 Δ /metA1 Δ uraA1 Δ /uraA1 Δ ste12 Δ ::KanMX/ste12 Δ ::NatMX	This Study
RSY572	<i>K. lactis</i>	MTLa/ α hmlalpha Δ ::URA3 hmra Δ ::TRP1 nej1 Δ ::LEU2 ade1 Δ /ade1 Δ trp1 Δ /trp1 Δ leu2 Δ /leu2 Δ metA1 Δ /metA1 Δ uraA1 Δ /uraA1 Δ ste12 Δ ::KanMX/ste12 Δ ::NatMX	This Study
RSY574	<i>K. lactis</i>	MTLa/ α hmlalpha Δ ::URA3 hmra Δ ::TRP1 nej1 Δ ::LEU2 ade1 Δ /ade1 Δ trp1 Δ /trp1 Δ leu2 Δ /leu2 Δ metA1 Δ /metA1 Δ uraA1 Δ /uraA1 Δ ste12 Δ ::KanMX/ste12 Δ ::NatMX	This Study
RSY567	<i>K. lactis</i>	MTLa hmlalpha Δ ::URA3 nej1 Δ ::LEU2 lysA1 Δ trp1 Δ leu2 Δ metA1 Δ uraA1 Δ ste12 Δ ::NatMX	This Study
RSY568	<i>K. lactis</i>	MTLa hmlalpha Δ ::URA3 nej1 Δ ::LEU2 lysA1 Δ trp1 Δ leu2 Δ metA1 Δ uraA1 Δ ste12 Δ ::NatMX	This Study
RSY570	<i>K. lactis</i>	MTLa hmra Δ ::TRP1 nej1 Δ ::LEU2 ade1 Δ trp1 Δ leu2 Δ metA1 Δ uraA1 Δ ste12 Δ ::NatMX	This Study
JC304	<i>P. pastoris</i>	ade1 Δ his4 Δ	Lin Cereghino et al. ⁵⁴
JC306	<i>P. pastoris</i>	arg4 Δ ura3 Δ	Lin Cereghino et al. ⁵⁴
RBV4440	<i>P. pastoris</i>	ade1 Δ his4 Δ ste12 Δ ::HYG	This study
RBV4441	<i>P. pastoris</i>	ade1 Δ his4 Δ ste12 Δ ::HYG	This study
RBV4327	<i>P. pastoris</i>	ade1 Δ /ADE1 his4 Δ /HIS4 arg4 Δ /ARG4 ura3 Δ /URA3 (product of JC304 x JC306 mating)	This study
RBV4461	<i>P. pastoris</i>	ade1 Δ /ADE1 his4 Δ /HIS4 arg4 Δ /ARG4 ura3 Δ /URA3 ste12 Δ ::KAN/ste12 Δ ::NAT	This study
RBV4462	<i>P. pastoris</i>	ade1 Δ /ADE1 his4 Δ /HIS4 arg4 Δ /ARG4 ura3 Δ /URA3 ste12 Δ ::KAN/ste12 Δ ::HYG	This study
RBV586	<i>Y. lipolytica</i>	lys Δ /LYS his1 Δ /HIS1 ura3 Δ /ura3 Δ leu2 Δ /leu2 Δ	This study
UC8	<i>Y. lipolytica</i>	lys Δ /LYS his1 Δ /HIS1 ura3 Δ /URA3 leu2 Δ /leu2 Δ ste12 Δ ::LEU2/ste12 Δ ::URA3	This study
UC13	<i>Y. lipolytica</i>	lys Δ /LYS his1 Δ /HIS1 ura3 Δ /URA3 leu2 Δ /leu2 Δ ste12 Δ ::LEU2/ste12 Δ ::URA3	This study
CAY4584	<i>S. cerevisiae</i>	MATa, ho::hisG ura3(Δ Sma-Pst) ade2 Δ lys2 trp1 Δ cup1 Δ cyh-r GAL3+ cha1::ADE2 leu2-r CEN3::URA3 thr4-B chrIII(260097)::CUP1	Gift of N. Hunter
CAY4585	<i>S. cerevisiae</i>	MATa, ho::hisG ura3(Δ Sma-Pst) ade2 Δ lys2 trp1 Δ cup1 Δ can-r GAL3+ chrIII(37755)::TRP1 his4-B CEN3::LYS2 glt1::hphMX4	Gift of N. Hunter
CAY4595	<i>S. cerevisiae</i>	MATa/a, ho::hisG/ho::hisG ura3(Δ Sma-Pst)/ura3(Δ Sma-Pst) ade2 Δ /ade2 Δ lys2/lys2 trp1 Δ /trp1 Δ cup1 Δ /cup1 Δ cyh-r/CYH2 CAN1/can-r GAL3+ cha1::ADE2/CHA1 leu2-r/LEU2 CEN3::URA3/CEN3::LYS2 thr4-B/THR4 chrIII(260097)::CUP1 chrIII(260097)/chrIII(37755)/chrIII(37755)/chrIII(37755)::TRP1 HIS4/his4-B	This study

		<i>GIT1/git1::hphMX4</i>	
CAY4770	<i>S. cerevisiae</i>	<i>MATα/a</i> , <i>ho::hisG/ho::hisG</i> <i>ura3</i> (Δ Sma-Pst)/ <i>ura3</i> (Δ Sma-Pst) <i>ade2</i> Δ / <i>ade2</i> Δ <i>lys2/lys2</i> <i>trp1</i> Δ / <i>trp1</i> Δ <i>cup1</i> Δ / <i>cup1</i> Δ <i>cyh-r/CYH2</i> <i>CAN1/can-r</i> <i>GAL3+</i> <i>cha1::ADE2/CHA1</i> <i>leu2-r/LEU2</i> <i>CEN3::URA3/</i> <i>CEN3::LYS2 thr4-B/THR4 chrIII(260097)::CUP1 chrIII(260097)/</i> <i>chrIII(37755)/ chrIII(37755)/chrIII(37755)::TRP1 HIS4/his4-B</i> <i>GIT1/git1::hphMX4</i> <i>STE12/ste12::natmx4</i>	This study
CAY4808	<i>S. cerevisiae</i>	<i>MATα/a</i> , <i>ho::hisG/ho::hisG</i> <i>ura3</i> (Δ Sma-Pst)/ <i>ura3</i> (Δ Sma-Pst) <i>ade2</i> Δ / <i>ade2</i> Δ <i>lys2/lys2</i> <i>trp1</i> Δ / <i>trp1</i> Δ <i>cup1</i> Δ / <i>cup1</i> Δ <i>cyh-r/CYH2</i> <i>CAN1/can-r</i> <i>GAL3+</i> <i>cha1::ADE2/CHA1</i> <i>leu2-r/LEU2</i> <i>CEN3::URA3/</i> <i>CEN3::LYS2 thr4-B/THR4 chrIII(260097)::CUP1 chrIII(260097)/</i> <i>chrIII(37755)/ chrIII(37755)/chrIII(37755)::TRP1 HIS4/his4-B</i> <i>GIT1/git1::hphMX4</i> <i>ste12::kanmx4/ste12::natmx4</i>	This study

Table 2.2: *Oligonucleotides used in the C. lusitaniae study.*

Oligo	Name	Sequence
RS66	MTL alpha fwd	CATCAGGCTGACACTACGCTT
RS67	MTL alpha rev	CCCTACAGAAGATCCGTAAT
RS68	MTL a fwd	CATCAGGCTGACACTACTCTT
RS69	MTL a rev	CTCGTGCCGAAACCTACAGTT
RS84	5' Flank Fwd ARG (ApaI)	CCGGCGGGGCCCCGGACTGGTTAAACGAGCATGGC
RS85	5' Flank Rev ARG (XhoI)	CGGCCGCTCGAGCGCGGCTAGCACTCAATTGCCC
RS86	3' Flank Fwd ARG (PstI)	GGCCGGCTGCAGTTGGTCTATCGAAACGTGCGTG
RS87	3' Flank Rev ARG (NotI)	GCCCGCGCGGCCGCCAGATAGCTAATTTTATACCA
RS88	5' Arg4 ORF Check	CGATGCTGACTTGACAGGTAC
RS89	3' Arg4 ORF Check	AAGTCGGTGGCCAACATGTCC
RS90	5' Arg4 Flank Check Right	AGTCCCACAAATGCCATTCAA
RS91	3' Arg4 Flank Check Left	AGGGTCGTGAAGAGAGATTA
RS148	Ime2 3' flank check rev	TCATAATTTGTTTCAAGTGGCGG
RS165	5'cl Ime2 ORF 0015	CTATTTCTTCTTCACTCTCTTCC
RS166	3'cl Ime2 ORF 0015	ATTTGCCGCCAATGTCGTTTCGG
RS167	5 IME2 Fl Check fwd	CAGCCATAATAATAGCATGGCTC
RS184	Ime2 Fwd 00015	CGACTACAAAATGGTGAGCAAGT
RS185	Ime2 rev 00015	CGAACGACTCGTCGTAATGATCT
RS224	5' Flank IME2 Fwd KpnI	GCGGCGGGTACCCGGAACGTGAGAGAAAACCTCAA
RS225	3' Flank IME2 Reverse SacII	GCCCGCCCCGCGGGCTGAACGAGGTATCTTTGCGG A
RS226	5' Flank ADE2 Check Fwd	TCGTGCCCTCTGCTTTAACACGT
RS227	3' Flank ADE2 check rev	CAAGTGAAGACTTTGACCATCAA
RS228	3' Flank ADE2 Fwd NotI	GGCCGGGCGGCCGCATACTGAATGATTGTTTGACG AG
RS229	3' Flank ADE2 Rev SacII	GCCCGCCCCGCGGGTTTCTTGCTTAGAAGACTTC G
RS230	5' Flank ADE2 Fwd (ApaI)	GCGGCGGGGCCCCGGCTGTATGCAATAAATTTCTAA
RS231	5' Flank ADE2 Rev (XhoI)	GGGCCGCTCGAGGATGTGTGCAAGTATATCTTCCA
RS263	3' Flank IME2 Fwd NotI	GGCCGGGCGGCCGCCTGAGCTTAAAGACTGTTGTT TC
RS265	5' Flank IME2 Reverse ApaI	GCGGCGGGGCCCAAACCTTCTTTTTTGCAACTTTCC
RS276	cl Ade 2 ORF FWD 04693	CCCGAAACCATCCGTCTCATCCA
RS277	cl Ade 2 ORF REV 04693	CCCGTTATGTCTGTTGGTGCCAA
RS281	Ime2 Addback Fwd KpnI(-948)	CCGGGCGGTACCTCAAAGAGAGAGTATTCTACGC
RS282	Ime2 Addback Rev XhoI (2616)	CCGGGCCTCGAGCTAGCATGCGATTTTGTGACTAT
RS323	5'FL fwd clSte12 02576 KpnI	GGCGGGGTACCCAACGGAGCTGAAATTGGTTCAT

RS324	5'FL Rev clSte12 02576 ApaI	GGGCCGGGGCCCCGGTATTCCTTCTATACAGCGGA
RS325	3'FL fwd clSte12 02576 NotI	GCCCGCGCGGCCGCTGTTCTATCTCCACTCATTTCA
RS326	3'FL rev clSte12 02576 SacII	GGCCGGCCGCGGATGCGACCGTTTCAACACTAGTG
RS327	5' Flank fwd Check clSte12 02576	GAACATTTACGTGATCGAGTCCA
RS328	3'Flank rev Check clSte12 02576	GCAATCTTACTATCTCTGACCGA
RS347	3' Flank Ste12 Check reverse (+3574)	ACGGTACGCAATAATCGGCGATG
RS344	5' Flank Ste12 check (-2082)	GCAACAGCCACTATTACAAATCA
RS345	5' Flank Ste12 check (-2034)	GATCTAGTTTTCAGAGCCTAGTGA
RS399	5' STE12 ORF	TTCGGCCGTACAATCACAGACC
RS400	3' STE12 ORF	TTCTGTAGTTGCTTTCTCTTCTTC
RS401	clSTE12ab 02576 Fwd KpnI	GGGCCGGGTACCATCGAGTCCAAAGTATAGTGGCA
RS402	clSte12ab 02576 Reverse ApaI	GGGCCGGGTACCATCGAGTCCAAAGTATAGTGGCA
RS403	5' Ste12 ab Check 02576 REV (206)	CGGCCGAAATGCTGGAATTTGT
RS436	3' Flank Ste12 Check reverse (+3487)	GCGTCCCTTGTTTTTCAGAGGGCG
BL70	pSFS2a Left	CTCAACCATAGCAATCATGG
BL71	pSFS2 Right	CTCCGGGCGGCATTGACC
BL164	HTB 5' Fwd	AGGTGAATTGGCTAAACATGCTGTTTCTGAAGGTA CTAGAGCTGTTACCAAATACTCTTCTGCTTCTAAT GGTGGTGGTGATAACACTGAAGATGTTATT
BL165	HTB FP/SAT1	TATTAATATACAATACAAAATAAAACAACAATAA TTTTGGAGAAATAAACCATTTCATGACAAAACCTCT CTCTTGTAACGACGGCCAGTG
BL1306	Kl Ime2 Oligo 3 (0)	CACGGCGCGCCTAGCAGCGGGTGGTCAAAG TTAAAGCTGGC
BL1308	Kl Ime2 Oligo 4(+2150)	GTCAGCGGCCGCATCCCTGCCTGCTGGAGA GGGCAGTACA
BL1309	Oligo 2 – MX series	CCGCTGCTAGGCGCGCCGTGCCAGCTGAAG CTTCGTACGC
BL1310	Oligo 5 – MX series	GCAGGGATGCGGCCGCTGACGCATAGGCCA CTAGTGGATC
BL1313	Kl Ime2 Oligo 1 (-800)	TTGCACCAGAATTAGGCGCC
BL1314	Kl Ime2 Oligo 6 (+2900)	CCAGTATCCCATTGCTTCCAC
BL1329	P-TEF check	CAAGACTGTCAAGGAGGGTATTC
BL1330	T-TEF check	TGCCCAGATGCGAAGTTAAGTG
BL1498	Kl Ste12 5 orf (+250)	GCGCATATAGGATGCAGAAA
BL1499	Kl Ste12 3 orf (+1000)	AGGCCATGTGTAAGGAATA

BL1500	Kl Ste12 5 check (-950)	CTTAACACGGAATCGGATGG
BL1501	Kl Ste12 3 check (+2900)	GAAATCAGGAATCCTGCCGC
BL1568	Kl Ste12 oligo 3 (0)	CACGGCGCGCCTAGCAGCGGGTTAATGTTG AGATCCAGTCTC
BL1569	Kl Ste12 oligo 4 (+2000)	GTCAGCGGCCGCATCCCTGCCTCGACAAAG GAGGACTCTA
BL1581	Kl Ste12 oligo 1 (-850)	GACATGTTTCAGGTGTGGGAC
BL1582	Kl Ste12 oligo 6 (+2800)	GAAACGTTAGCTTGGAAGGG
BL2468	S.c. 5' forward -50MX	CTTTTATAGCGGAACCGCTTTCTTTATTTGAATTG TCTTGTTCCACCAAGGCCAGCTGAAGCTTCGTACGC
BL2469	S.c. 3' reverse +2000 MX	CTGGCCCGCATTTTTTAATTCTTGTATCATAAATT CAAAAATTATATTATAGCATAGGCCACTAGTGGAT C
BL2470	Sc. STE12 5' flank -600 F	GTCCAGTTTGCACAAGACAC
BL2471	Sc STE12 3' flank R +3000	ATCTCCGCATCGTGACTA
BL2572	Sc STE12 5' orf +89F	CGGGCGAAGTTGAAGAAT
BL2473	Sc STE12 3'ORF +1679 R	GGTGCTCTCGAACTATAAGG
BL2483	Yl URA3 KpnI (+1200)	GCCGGCGGTACCCTTCTGCCTCCAGGAAGTCC
BL2618	Yl STE12 HindIII (-900)	GGCGCCAAGCTTCGCACACACTGCACTGTAGC
BL2619	Yl STE12 EcoRV/NheI (+3000)	GCCGGCGATATCGCTAGCGAAGATGTCGACCAGGA CGGA
BL2561	Sc matalpha1 +204 3' jcn F	GATTCCTGTTCTTCCTCTC
BL2562	Sc matalpha1 +1191 3' jcn R	CAAGGGAGAGAAGACTTGTG
BL2565	Sc mata1 +283 3' jcn F	CAATATCACCCCAAGCAC
BL2620	Yl LEU2 PmlI	GGCGCCACGTGCATGGATGTAGATCACCAAGC
BL2621	Yl LEU2 KpnI	GCCGGCGGTACCGCACTGAGCTCGTCTAACGG
BL2622	Yl URA3 (PmlI) for	GGCGCCACGTGGTCGACGAGTATCTGTCTGA
BL2666	Sc mata1 +1282 3' jcn R	CAAGGGAGAGAAGACTTGTG

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CHAPTER 3: EPIGENETIC REWIRING OF THE PHEROMONE MAPK SIGNALING PATHWAY DETERMINES FUNGAL FECUNDITY IN *CANDIDA ALBICANS*

Author contributions: C.M.S. performed all experiments, except William Scheving performed pheromone biofilm experiments with experimental design and guidance by C.M.S. R.J.B. made the *act1-cst5-myc* plasmid. Shail Kabrawala made the *pACT1-STE4* plasmids and strains and the *pACT1-CST5* plasmid and strains.

Abstract

Several clinically relevant *Candida* species are capable of reversible switching between two heritable states, “white” and “opaque”, which determine the ability to undergo sexual reproduction. In *Candida albicans*, white cells are essentially sterile whereas opaque cells are highly proficient at mating. Here, we investigated the mechanism by which the white-opaque switch regulates fecundity, and focused on four pheromone MAPK genes that are transcriptionally upregulated in *C. albicans* opaque cells relative to white cells. These genes are *STE4*, encoding the β subunit of the G protein complex, *CST5*, encoding the pheromone MAPK scaffold, and *CEK1/2*, encoding the two terminal MAP kinases. To test the role of these factors in *C. albicans* mating, cells were genetically locked in the white state and engineered to express elevated levels of each factor, either singly or in combination. We demonstrate that white cells expressing elevated levels of three genes, *STE4*, *CST5*, and *CEK2*, undergo mating four orders of magnitude more efficiently than control white cells, and at a frequency approaching that of opaque cells. Engineered white cells recapitulate the transcriptional and morphological response of opaque cells to pheromone, and also ectopically express MAPK components at levels physiologically relevant to opaque cells. These results identify multiple MAPK signaling bottlenecks in *C. albicans* white cells, and show that correction of these bottlenecks enables efficient mating by these cell types. Our findings therefore establish that the differential expression of pivotal MAPK factors

underlies epigenetic control of mating in *C. albicans*, and we propose a model for the emergence of this mechanism in *Candida* species.

Introduction

Mitogen-activated protein kinase (MAPK) cascades are used by eukaryotes to respond to a variety of extracellular signals. There has been considerable interest in defining how these networks transduce environmental signals to regulate diverse cellular processes such as apoptosis, stress adaptation and tumorigenesis. Many mechanistic insights have been gleaned from investigation of MAPK signaling in the model yeast *Saccharomyces cerevisiae*. *S. cerevisiae*, studies have examined MAPK pathway dynamics¹⁻³, crosstalk between different MAPK pathways^{4,5}, the role of MAPK scaffold proteins⁶⁻⁸, how MAPK signaling can provide switch-like or graded behavior^{7,9-11}, and how these pathways can be mathematically modeled and manipulated^{12,13}. Analysis of MAPK signaling in fungal species has also provided evidence as to how these pathways have diversified during evolution¹⁴⁻¹⁶.

The pheromone MAPK pathway in *S. cerevisiae* has been particularly intensively studied as a paradigm for MAPK signal transduction^{17,18}. In *S. cerevisiae*, extracellular pheromone activates a G protein-coupled receptor, driving the dissociation of G β and G γ subunits from an inhibitory G α subunit. The activated G $\beta\gamma$ complex recruits the scaffold protein Ste5 to the membrane. The kinases Ste11, Ste7, and Fus3, that are at the center of MAPK signaling, are tethered to Ste5. Phosphorylation of Ste11 initiates a phosphorylation cascade from Ste11 to Ste7 to Fus3, and results in translocation of activated Fus3 to the nucleus. Fus3 in turn phosphorylates the transcription factor Ste12, as well as the repressors Dig1 and Dig2, thereby activating Ste12 and driving the transcriptional response to

pheromone. In addition, pheromone signaling induces formation of mating projections via G β γ interactions with Far1 and the cell polarization machinery, and activated Far1 also mediates cell cycle arrest by interfering with Cdk activity.

Candida albicans, like *S. cerevisiae*, is a hemiascomycete yeast, although these species diverged approximately 840 million years ago¹⁹. *C. albicans* is a natural commensal in ~70% of people²⁰, but is also a prevalent opportunistic pathogen, capable of causing both superficial and invasive infections²¹. Many factors contribute to the ability of *C. albicans* to grow and invade different host niches, including its ability to adopt multiple phenotypic states. A prominent example is provided by the white-opaque switch, in which *C. albicans* cells undergo a heritable, epigenetic switch between 'white' and 'opaque' forms²². Stochastic and reversible switching between the two states occurs once every ~10,000 generations²³ and regulates multiple *C. albicans* phenotypes. In particular, whereas white cells are essentially sterile, opaque **a** and α cells mate up to a million times more efficiently²⁴. The response of opaque cells to pheromone shows close parallels to that in *S. cerevisiae*; pheromone signaling induces cell cycle arrest, formation of mating projections, and expression of mating genes²⁵⁻²⁹. In contrast, white **a** and α cells can respond to pheromone but do not undergo mating and instead become adherent and generate 'sexual biofilms'³⁰. Many of the same MAPK components transduce the pheromone signal in both white and opaque cells^{31,32}, yet it is still unknown how the two cell states exhibit distinct mating capacities.

Regulation of the white-opaque switch involves a highly interconnected network of transcription factors. The master regulator of the switch is the transcription factor Wor1³³⁻³⁵, whose expression is necessary and sufficient to promote formation of the opaque state³⁶. Additional control occurs at the chromatin level via changes in histone modifications and Mediator complex³⁷⁻⁴⁰. The white-opaque switch is also regulated by the *MTL* (mating-type-like) locus; in **a**/ α cells, the **a**1/ α 2 complex represses *WOR1* expression, so that *MTL* heterozygous cells are less likely to undergo white-to-opaque switching than *MTL* homozygous cells^{24,41}. In total, 1306 transcripts are differentially expressed between white and opaque cells, representing approximately one-sixth of all *C. albicans* transcripts⁴², and revealing the dominant role of the switch on the transcriptome.

In this work, we sought to determine how *C. albicans* opaque cells are able to undergo efficient mating, whereas cells in the white state are essentially sterile despite being pheromone responsive. We focused on differences in pheromone MAPK signaling between white and opaque cells. Opaque cells express several MAPK components at higher levels than in white cells. By ectopically increasing the levels of three MAPK components, we created white cells that were able to mate several orders of magnitude more efficiently than control white cells. Analysis of protein levels established that elevated expression of these components in white cells produced comparable expression levels to those in opaque cells. These experiments therefore reveal how relatively small differences in the expression of key MAPK components between different cell states can drive large-scale differences in signal output. Given the conservation of MAPK signaling pathways

from yeast to man, our results have consequences for understanding signaling differences that underlie important fungal phenotypes and beyond to signaling differences that contribute to carcinogenesis.

Materials and Methods

Media and reagents

Media were prepared as previously described⁴³. Mating assays were performed on Spider medium consisting of 1.35% agar, 2% mannitol, 0.4% potassium phosphate, 1% nutrient broth, adjusted to pH 7.2. Nourseothricin-resistant transformants were selected on YPD (yeast peptone dextrose) supplemented with 200 $\mu\text{g ml}^{-1}$ nourseothricin, as previously described⁴⁴. SCD (synthetic complete dextrose) is synthetic complete medium supplemented with 2% glucose. Maltose medium is synthetic complete medium + 2% maltose. Lee's + N-acetyl glucosamine is Lee's medium⁴⁵ supplemented with 1.25% N-acetyl glucosamine.

Plasmid and Strain Construction

All strains, plasmids, and oligonucleotides used in this study are listed in Tables 3.1, 3.2, and 3.3, respectively. *C. albicans* white strains were engineered to constitutively express *C. albicans* *STE4*, *CST5*, *CEK1*, and *CEK2*, either singly or in combination. *STE4*, *CST5* and *CEK1* were placed under the control of the *C. albicans*

ACT1 promoter. The *ACT1* promoter was PCR amplified using oligonucleotides BL610 and BL611. The *STE4* ORF was PCR amplified using oligonucleotides BL1230/BL1232, the *CST5* ORF amplified using BL2227/BL2228, and the *CEK1* ORF amplified using BL2699/BL2605. *ACT1* and ORF sequences were then combined by fusion PCR using oligonucleotides BL610/BL1232 (*STE4*), BL610/BL2228 (*CST5*) or BL610/BL2605 (*CEK1*). PCR products were cloned into plasmid pSFS2a⁴⁴ using *ApaI* and *XhoI* to generate plasmids pRB241 (*pACT1-STE4*), pRB284 (*pACT1-CST5*) and pRB439 (*pACT1-CEK1*). These plasmids were linearized with *BglII* (partial digest), *BsrGI* (partial digest) or *BglII*, respectively. The pRB241 plasmid (*pACT1-STE4*) was linearized and transformed into strains CAY3337, CAY3339, and CAY3341 to generate CAY3581/3582, CAY3499, and CAY3500, respectively. Correct integration at the *ACT1* promoter was verified by PCR using oligonucleotides BL596/BL2234. The pRB284 plasmid (*pACT1-CST5*) was linearized and transformed into strains CAY3336/CAY3337/ CAY3339/CAY3341 to generate CAY3921, CAY3922/3923, CAY3924/3925/3926, and CAY3927, respectively. PCR was used to verify integration using oligonucleotides BL596/BL2238. Plasmid pRB439 (*pACT1-CEK1*) was linearized and transformed into CAY3337 and CAY3339 to generate strains CAY6220/6221/6222 and CAY6223/6224/6225, respectively.

To constitutively express *CEK2*, plasmid RB348 was generated containing a *pADH1-CEK2* cassette. The *ADH1* promoter was PCR amplified using oligonucleotides BL1246/BL2127 and the *CEK2* ORF amplified using BL2059/BL2063. Fusion PCR using oligonucleotides BL1246/BL2063 was used to

generate the *pADH1-CEK2* fusion construct. The construct was digested with *Apal* and *XhoI* and ligated into pSFS2a. This plasmid was linearized with *PmlI* and transformed into CAY3337 and CAY3339 to generate CAY4722-4724, and CAY4728-4730, respectively, and correct integration at the *ADH1* promoter was PCR verified using oligonucleotides BL1256/BL2061.

To generate strains constitutively expressing *STE4* and *CEK2*, the *pADH1-CEK2* construct was integrated into strains containing *pACT1-STE4*. Strains CAY3581/3499 were incubated on maltose medium to induce excision of *SAT1*, generating CAY5426/5428. Plasmid pRB348 was linearized and transformed into CAY5426/5428 to generate CAY5497-5500 and CAY5660/61, respectively.

Strains constitutively expressing *CST5* and *CEK2* were generated by transformation of the *pADH1-CEK2* construct into strains containing *pACT1-CST5*. Strains CAY3921 and CAY3927 were incubated on maltose medium to excise the *SAT* cassette, generating strains CAY5380 and CAY5379 respectively. Plasmid pRB348 was linearized and transformed into CAY5379/80 to generate strains CAY5505/5506, and CAY5657-59. Correct integration at the *ADH1* locus was verified by PCR using oligonucleotides BL1256/BL2061.

To generate strains constitutively expressing *STE4*, *CEK2*, and *CST5*, the *pACT1-STE4* construct was integrated into strains constitutively expressing *CEK2* and *CST5*. Strains CAY5506 and CAY5657 were incubated on maltose medium to induce *SAT1* excision to generate strains CAY5997/98 and CAY5999/6000, respectively. Plasmid pRB281 was linearized and transformed into strains

CAY5997/98 and CAY5999/6000 to generate strains CAY6066-6071 and CAY6068/6069/6072/6073, respectively. Strains were checked for integration of the *pACT1-STE4* construct at the *STE4* locus by PCR using oligonucleotides BL1672/BL2803.

Strains were constructed in which a 13x myc tag was added to the C terminal end of endogenously expressed Ste4, Cst5, or Cek2. The *STE4* ORF plus promoter were PCR amplified with oligonucleotides BL1649/BL1650. The PCR product was cut with *SmaI*/*Bam*HI and ligated into pMG2093⁴⁶ which contains the 13x myc tag to create pRB296. pRB296 was linearized with *KpnI* and transformed into RBY1132. Positive transformants were selected based on histidine prototrophy and integration examined by PCR using oligonucleotides BL3489/BL3434 to generate CAY6889 and CAY6890. CAY6889 and CAY6890 were incubated on Lee's + N-acetyl-glucosamine medium to generate the opaque form of these strains, CAY6917 and CAY6916 respectively. To generate strains with *CEK2* containing a myc tag at the endogenous locus, the *CEK2* ORF plus promoter were PCR amplified using oligonucleotides BL2946/2947. The resulting PCR product was cloned into pMG2093 using *Sall* and *SmaI* to generate pRB442. pRB442 was linearized with *SacI* and transformed into RBY1132, transformants selected on -his medium, and integration confirmed by PCR using oligonucleotides BL2948/BL3434 to generate strains CAY6891-6893. These strains were incubated on Lee's + N acetyl glucosamine to generate opaque forms of these strains, CAY9992, CAY6993, and CAY6915, respectively. To generate strains with *CST5* containing a myc tag at the endogenous locus, the *CST5* ORF and promoter were PCR amplified using

oligonucleotides BL3605/BL3606. The PCR product was cloned into pMG2093 using *XhoI* and *SmaI* to generate plasmid pRB325, which was linearized with *PmlI* and transformed into RBY1132. Transformants were selected on SCD medium lacking histidine and checked by PCR using oligonucleotides BL3488/BL3433 to generate strains CAY7230/31. CAY7230/31 were incubated on Lees+ N-acetylglucosamine to generate opaque strains CAY7237/36, respectively.

Myc tagged alleles were generated for constitutively expressed *STE4*, *CST5* and *CEK2* genes. The *STE4* ORF and 13x myc tag were PCR amplified from plasmid pRB296 using oligonucleotides BL2203/BL2244 and cloned into pRB281 using *PpuMI* and *XhoI* to generate pRB466 containing p*ACT1p-STE4-MYC*. The plasmid was linearized with *BglII* (partial digest) and transformed into CAY3339 to generate CAY6562/CAY6563. Integration at the *ACT1* promoter was verified by PCR using oligonucleotides BL596/BL2804. For *CST5*, a two step fusion PCR strategy was used in which *ACT1-CST5* (including 300 bp of *CST5* ORF) was PCR amplified from pRB284 using oligonucleotides BL610/BL2198 and the remainder of the *CST5* ORF and myc tag were PCR amplified from pRB325 using oligonucleotides BL2199/BL2204. PCR products were combined by fusion PCR (oligonucleotides BL610/BL2204) and the resulting product cloned with *ApaI/XhoI* into pSFS2a to generate pRB294. Plasmid pRB294 was linearized with *BsrGI* (partial digest) and transformed into CAY3339 to generate strains CAY6566/CAY6567. Integration at the *ACT1* promoter was verified by PCR using oligonucleotides BL596/BL2198. For *CEK2*, the ORF and myc tag was amplified from pRB442 using oligonucleotides BL2795/BL2244. The PCR product was cloned into pRB348 with *BglII* and *XhoI* to

generate pRB485. This plasmid was linearized with *Pml*I and transformed into CAY3339 to generate strains CAY7034-36. Integration at the *ADH1* locus was PCR verified using oligonucleotides BL1256 and BL2061.

Quantitative mating assays

Mating assays were performed essentially as described ⁴⁷. Auxotrophic strains were grown overnight in SCD medium at 22°C. Approximately 2.5×10^7 cells of each mating type (**a/a** or α/α) were mixed and deposited on 0.8 μ M nitrocellulose filters (Millipore Cat. No. AAWG01300) placed on Spider medium. Plates were incubated for 5-7 days at 22°C, and cells removed and plated for single colonies onto SCD – leu, SCD – arg and SCD –leu –arg selection media. Colony forming units (CFUs) were counted after 2 days. Mating frequency was defined by the number of CFUs on double selection media divided by the lowest number of CFUs on single selection media. For each experiment, at least 4 independent strains (two **a/a** and two α/α strains) were used, and at least 6 biological replicates were performed.

Pheromone-Stimulated Biofilm Assays

Biofilm assays were performed essentially as described ³¹. White **a** cells were grown overnight in liquid Spider medium at 30°C, and opaque **a** cells were grown 22°C in the same medium. Approximately 5×10^7 cells were added to 12 well

dishes (Costar, Corning Inc.) and mixed with 1 ml Lee's + glucose medium. Cells were treated with either 1 μ l 10 mg/ml *C. albicans* α pheromone or 0.01% DMSO and incubated at 22°C for 48 h without shaking. Supernatant was removed and wells were washed with 1 ml phosphate-buffered saline (PBS). The remaining adherent cells were resuspended in 1 ml sterile H₂O and OD₆₀₀ measured using a ThermoScientific Nanodrop 2000c spectrophotometer. Experiments were performed at least in biological triplicate.

Isolation of Cells for RNA sequencing analysis

Overnight cultures of CAY3337 (*wor1 Δ /wor1 Δ α/α*), CAY3339 (*wor1 Δ /wor1 Δ , a/a*) CAY6066 (*wor1 Δ /wor1 Δ , pACT1-STE4, pACT1-CST5, pADH1-CEK2, a/a*), CAY6068 (*wor1 Δ /wor1 Δ , pACT1-STE4, pACT1-CST5, pADH1-CEK2, α/α*), RBY1177 (WT opaque **a/a**), and RY1180 (WT opaque α/α) were grown at room temperature in 10 ml's SCD medium. Approximately 10⁸ cells of each mating type were mixed on Spider medium. Additionally, 10⁸ white **a** cells (CAY3339) and 10⁸ opaque **a** cells (RBY1177) were incubated alone on Spider medium to control for nutritional changes in each cell type. Cells were harvested by removal of cells off plates at 8 h and 24 h, flash frozen in liquid nitrogen, and stored at -80°C.

Library Preparation and RNA sequencing analysis

RNA was extracted from cells using the RiboPure™ RNA purification kit for yeast (ThermoFisher Scientific AM1926). RNA was treated with TURBO™ DNase (ThermoFisher Scientific AM2238) to remove contaminating DNA. Samples were run on an Agilent 2100 Bioanalyzer to verify RNA integrity. RNA-seq libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina RS-122-2103). High throughput sequencing was performed at the University of Missouri DNA Core Facility using the Illumina HiSeq 2500 platform generating 50 bp single end reads. Reads were aligned to the *C. albicans* SC5314 genome (Assembly 21) using TopHat2⁴⁸. For all libraries, a minimum of 86% of raw sequences aligned to the *C. albicans* genome. Differential expression analysis was conducted using Cuffdiff software⁴⁸ based on two biological replicates at each time point.

Cell collection for western blotting

50 ml cultures of cells were grown overnight at 22°C. 4×10^8 cells for each mating type were mixed and placed on Spider plates for 24 h at 22°C. Cells were removed from plates, flash frozen in liquid nitrogen, and stored at -80°C.

Protein extraction and western blotting

Cell pellets were washed in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 50 mM NaF) containing additional protease inhibitors (Pierce™ Protease and Phosphatase Inhibitor Mini Tablets, EDTA-free, #PI88669) and centrifuged at 16,000 rpm. Cells were resuspended in 250 µl lysis

buffer and lysis achieved by bead beating (1 min vortexing followed by 1 min on ice for a minimum of 10 cycles). Cell debris was removed by centrifugation at 16,000 rpm and protein concentration determined using Bradford assays. Approximately 10 µg of total protein for each sample was analyzed by SDS-PAGE. Protein was transferred to a nitrocellulose membrane using a Panther™ Semidry Electroblotter. Membranes were blocked for 1 h in PBS-T (PBS with 0.05% Tween) containing 5% milk, and incubated in α -tubulin antibody (MCA78G, Accurate Chemical, Westbury, New York) at a 1/500 dilution in PBST+5% milk overnight at 4°C with gentle shaking. The membrane was washed 3 times for 15 min in PBS-T and peroxidase-conjugated secondary antibody was added (α -rat, 1/1000 dilution, Jackson ImmunoResearch 712-036-150) and incubated for 2 h at 22°C with gentle shaking. Washes were repeated 3 x 15 min and antibody binding detected by chemiluminescence using Lumigen ECLA Ultra (TMA-100). To detect myc antibody, the membrane was washed 6 times for 5 min in PBS-T, then incubated in stripping buffer (1.4% β -mercaptoethanol, 2.5% SDS, 12.5% 1M Tris-HCl, pH 7) at 50°C for 30 min with shaking. The membrane was washed 6 times for 10 min in 25ml PBS-T and blocked for 1 h in PBS-T + 5% milk. Primary antibody (α -myc 1/10,000, clone 4A6 Millipore) in PBS-T+5% milk was added and incubated overnight at 4°C with gentle shaking. The membrane was washed 3 x 15 min in PBS-T and incubated in peroxidase-conjugated secondary antibody (α -mouse 1/20,000, Jackson ImmunoResearch 715-036-151) in PBS-T +5 % Milk for 2 h at 22°C with gentle shaking. Membranes were visualized as described above and experiments performed at least in biological duplicate.

Microscopy

Differential interference contrast (DIC) images were captured using a Zeiss inverted microscope (Axio Observer Z1) fitted with an AxioCam HR. Images were processed with AxioVision Rel 4.8 software (Zeiss, Germany). For quantification of mating projections, at least 300 cells were counted per biological replicate. At least four biological replicates were genotype were conducted.

Results

Comparison of white and opaque cell transcriptomes

Opaque cells are the mating competent form of *C. albicans*, forming mating projections in response to pheromone and efficiently undergoing cell-cell conjugation. In contrast, white cells are virtually sterile in mating crosses and show no morphological response to pheromone. To address the mechanism underlying this difference, we examined the transcriptomes of white and opaque cells grown in standard *in vitro* conditions⁴². Approximately 1,300 genes are differentially expressed between the two cell types, indicative of large-scale transcriptional differences. However, we focused on genes encoding components of the pheromone MAPK signaling cascade and noted that four MAPK genes are upregulated in opaque cells relative to white cells in non-pheromone treated cells. These genes are *STE4* encoding the β subunit of the heterotrimeric G protein complex (9 fold up), *CST5* encoding the MAPK scaffold protein (3.7 fold up), and *CEK1* and *CEK2* encoding the

terminal MAP kinases (8.8 and 11.5-fold up, respectively, Figure 3.1). We hypothesized that limited expression of one or more of these components prevents efficient pheromone signaling in *C. albicans* white cells. To test this hypothesis, we deleted both copies of *WOR1* in **a** and α cell types to lock them in the white state³³⁻³⁵. We then engineered white-locked strains to overexpress each of the four MAPK components listed above, either singly or in combination, using constitutive gene promoters. Engineered strains were analyzed for their ability to form mating projections and to undergo cell-cell conjugation in comparison to control white and opaque cells.

Overexpression of MAPK components allows white-locked cells to secrete and respond to mating pheromone

We first tested whether *C. albicans* white-locked strains engineered to overexpress MAPK components could secrete and/or respond to mating pheromones. We co-incubated **a** and α cells on Spider medium, conditions which are conducive to mating⁴⁹. Mixtures of white-locked (*wor1* Δ /*wor1* Δ) **a** and α cells rarely formed mating projections at 24 h (<0.3%) under these conditions (Figure 16). In contrast, co-incubation of opaque **a** and α cells resulted in ~70% of cells forming mating projections at 24 h. We next tested white-locked strains engineered to overexpress different MAPK components using constitutively active promoters. White-locked strains were engineered to express *STE4*, *CST5*, or *CEK1* under the *ACT1* promoter, or *CEK2* under the *ADH1* promoter. We found that white-locked

cells ectopically expressing *CEK1*, *CEK2*, or *CST5* rarely resulted in mating projection formation (<0.3%), whereas ectopic expression of *STE4* resulted in ~ 5% of cells forming mating projections (Figure 3.2). The efficiency of mating projection formation was enhanced by combinatorial overexpression of these MAPK components. Thus, ectopic expression of *STE4* and *CST5* increased mating projection formation to 10%, whereas overexpression of *STE4* and *CEK2* resulted in 20% of cells forming mating projections. Overexpression of all three components (*STE4*, *CST5*, and *CEK2*) resulted in 35% of cells forming mating projections, a frequency that is only 2-fold lower than that of natural opaque cells (Figure 3.2). Thus, increasing the expression of specific MAPK components enables white-locked cells to secrete and respond to pheromone, and expressing multiple components in combination has either an additive or synergistic effect on pheromone signaling.

A similar quantitative response was observed when performing experiments between **a** cells and synthetic α pheromone in liquid culture, supporting the contention that engineered white cells exhibit an increased morphological response to exogenous pheromone (Supplemental Figure 3.1). Furthermore, the characteristic round shape of white cells was maintained in the engineered strains and during mating projection formation. This indicates that *C. albicans* white cells can form mating projections even though they lack the intrinsic cell polarity exhibited by elongated opaque cells.

White-locked cells overexpressing MAPK components undergo elevated levels of mating

The mating efficiency of wildtype white and opaque cells was compared with white-locked strains engineered to ectopically express different MAPK components. Auxotrophic **a** and α cells were co-incubated on Spider medium for 5-7 days and mating frequencies determined by the fraction of prototrophic products (see *Methods*). White-locked strains underwent mating at very low efficiency, with an average mating frequency of 4.7×10^{-6} , whereas opaque cells mated approximately 75,000 times more efficiently, with an average mating frequency of 0.35 (Figure 3.3), consistent with previous observations^{24,49}. In white-locked **a** and α strains overexpressing single MAPK components, we found that overexpression of *CEK1* had little effect on mating frequency, whereas overexpression of *CEK2* or *CST5* increased mating 66 and 67-fold, respectively (Figure 3.3). Strains overexpressing *STE4* showed the largest increase in mating for any single MAPK component with an average mating frequency of 1.5×10^{-3} , representing a 336-fold enhancement of mating compared to the parental *wor1 Δ /wor1 Δ* strains (Figure 3.3).

The effect of overexpressing MAPK components in combination was also evaluated. Overexpression of *CEK1* gave only a minimal change in mating frequency so this gene was not examined in combinatorial experiments. Overexpression of both *CEK2* and *CST5* produced a 33-fold increase in mating, whereas overexpression of *CEK2* and *STE4* resulted in a 1,800-fold increase in mating (Figure 3.3). Overexpression of all three of these components, *STE4*, *CST5* and *CEK2*, produced a

mating frequency of 2.9×10^{-2} (Figure 3.3). This mating efficiency is within 12 fold that of wildtype opaque cells, and represents a 6,000 fold increase over parental *wor1Δ/wor1Δ* crosses. Together, these experiments demonstrate that elevating the levels of several pheromone MAPK components has a dramatic effect on the mating efficiency of *C. albicans* white cells, and that combinatorial overexpression further enhances mating so that engineered white cells undergo mating at levels approaching those of opaque cells. We note that the results from quantitative mating assays paralleled those of mating projection formation (Figure 3.2), indicating that restoration of the pheromone MAPK signaling had a similar relative impact on both of these phenotypes.

The effect of overexpression of MAPK components on mating efficiency was unequal when **a** and α cells were compared. Thus, overexpression of MAPK components in engineered white-locked **a** cells mated to opaque α cells mated 52 times more efficiently than the converse experiment, in which engineered white-locked α cells were mated to opaque **a** cells (Supplemental Figure 3.2). This result suggests that engineered **a** cells are more proficient at mating than the equivalently engineered α cells, and that additional components may be white-opaque regulated only in α cells. In line with this possibility, we note that Tsong *et al.*⁵⁰ previously demonstrated that the α -specific genes *MFLPHA* (encoding α pheromone) and *STE3* (receptor for **a** pheromone) are both highly upregulated in opaque cells relative to white cells. In contrast, the equivalent **a**-specific genes, *MFA* (encoding **a** pheromone) and *STE2* (receptor for α pheromone), are not differentially expressed

between white and opaque cells (Figure 3.1). We therefore propose that white **a** cells engineered to overexpress *STE4/CST5/CEK2* are more proficient at mating than the corresponding α cells due to the additional regulation of mating genes (*MFALPHA* and *STE3*) by the white-opaque switch only in α cells. However, we did not test this possibility in this study.

Influence of MAPK overexpression on pheromone-induced biofilms

C. albicans white cells, while mating incompetent, are able to respond to pheromone and form adherent biofilms on artificial substrates³⁰. The formation of these biofilms involves signaling via the same pheromone MAPK cascade as that operating in opaque cells³¹. We examined the effect of ectopic expression of different MAPK components on the ability of white cells to form pheromone-induced biofilms. To do this, we incubated engineered white-locked **a** cells, as well as control white and opaque cells, in 12 well polystyrene dishes in Lee's medium in the presence or absence of 10 μ M pheromone. After 24 h, wells were washed to remove non-adherent cells and adherent cells resuspended and absorbance at 600 nm recorded. We found that overexpression of several MAPK components increased the adherence of white-locked cells in these assays. Thus, ectopic expression of *STE4* or *CST5*, combinatorial overexpression of *STE4+CEK2*, as well as ectopic overexpression of *STE4+CST5+CEK2*, all increased white cell adherence (Supplemental Figure 3.4). These results show that in addition to an increased ability to undergo sexual mating, strains engineered to have increased MAPK

expression have an increased ability to undergo adherence and biofilm formation in response to pheromone.

Engineered white cells show enhanced expression of mating genes while retaining the white phenotypic state

To test how the manipulation of MAPK signaling components affects the transcriptome of engineered strains, RNA sequencing was performed on mating mixes of white-locked cells, white-locked cells overexpressing *STE4*, *CST5*, and *CEK2* genes, and control opaque cells. Experiments were performed by co-incubation of the appropriate **a** and α strains on Spider medium and transcriptomes compared at 8 and 24 hours (see *Methods*). We also compared expression profiles of mixes of **a** and α cells with those of cultures consisting of only a single mating type.

We observed that co-incubation of white **a** and α cells resulted in an expression profile very similar to that of white **a** cells alone, consistent with white cells showing an inability to efficiently secrete or respond to mating pheromones. In contrast, co-incubation of opaque **a** and α cells led to increased expression of 697 genes (FDR<0.05, Benjamin-Hochberg corrected p value) relative to cultures of opaque **a** cells alone. Many of the genes induced in mating mixtures of opaque cells included those implicated in conjugation, as outlined below. Next, we compared the transcriptome of white-locked cells constitutively expressing three MAPK genes (*STE4/CST5/CEK2*) with control white-locked cells. Mixed cultures of engineered **a**

and α cells (designated “OE strains”) showed differential expression of 62 genes relative to control mixtures of white **a** and α cells. In contrast, mating mixes of opaque cells expressed 1,624 genes differentially to those of mixes of white **a** and α cells. GO term analysis revealed that genes upregulated in mating mixes of both engineered white-locked strains and opaque cells relative to crosses between control white cells included numerous mating genes (Supplemental Figure 3.5). Genes implicated in pheromone processing, MAPK signaling, cell fusion, and karyogamy were expressed at similar levels in engineered white cells and in control opaque cells cultured under mating conditions (Figure 3.4). These transcription patterns show that manipulation of the expression of key MAPK components is sufficient for white cells to express mating genes at similar levels to those in opaque cells.

We emphasize that the targeted changes to MAPK signaling in white cells had little overall impact on the expression of genes regulated by the white-opaque switch. As noted above, ~1300 genes are differentially regulated between single sex cultures of white and opaque cells⁴², and we observed 1624 genes to be differentially expressed between mating mixtures of opaque **a** and α cells and equivalent mixtures of white **a** and α cells. In contrast, only 62 genes were significantly upregulated in the mating mixtures of engineered white cells relative to control white cells. These results establish that engineered white strains can recapitulate the upregulation of pheromone-responsive genes observed in opaque

cells, while still maintaining a global expression profile similar to that of wildtype white cells.

Analysis of protein levels of MAPK signaling components in white, opaque and engineered cell types

The previous experiments highlight that altered expression of MAPK genes enables mating competency in ‘sterile’ white cells. To examine how changes in MAPK protein levels modulate pheromone signaling, we compared Ste4, Cst5, and Cek2 proteins between white cells, opaque cells, and white cells engineered to ectopically express these components. MAPK proteins were therefore epitope tagged at the C-terminus in *MTLa* strain backgrounds. Tagged α and non-tagged α cells were then incubated on Spider medium for 24 hours, and whole cell lysates analyzed by SDS-PAGE and Western blotting (see *Methods*).

In general, protein expression levels between white and opaque cells closely paralleled RNA expression levels, and the three key MAPK proteins were consistently expressed at higher levels in opaque cells than in white cells. Thus, Cek2 was present at 186-fold higher levels in opaque cells than white cells, whereas Cst5 and Ste4 were expressed at 13-fold and 2-fold higher levels in opaque cells than white cells, respectively (Figure 3.5). White-locked strains engineered to overexpress *CEK2* produced 67-fold more Cek2 protein than control white cells, a level similar to that in opaque cells (Figure 3.5). For white-locked strains overexpressing *CST5*, these strains also produced a similar level of Cst5 protein to

control opaque cells under mating conditions (a 19-fold increase over control white cells). Levels of Ste4 protein were only slightly elevated in white cells ectopically expressing *STE4*, with a 3-fold increase relative to control white cells when both were cultured under mating conditions.

Overall, these results establish that the protein levels of several key MAPK components are present at higher levels in opaque cells than in white cells, and that the protein levels in re-engineered white cells accurately reflect, at least for the most part, the levels of these proteins in natural opaque cells. Furthermore, they reveal that mating in *C. albicans* appears especially sensitive to the levels of Ste4, as even small changes in the levels of this factor have a profound effect on mating frequency.

Discussion

The regulation of sexual reproduction has undergone extensive rewiring throughout the tree of life. A particularly striking example of rewiring occurred within the *Candida* clade, where sexual reproduction came under the control of a unique phenotypic switch. As a result, cells in the opaque state are mating competent whereas genetically identical cells in the alternative white state are virtually sterile. We establish that quantitative differences in pheromone MAPK signaling are responsible for differences in mating competence between the two cell states, and identify the critical steps at which signaling flux through the pheromone MAPK pathway is controlled.

We demonstrate that white cells express three key MAPK components at decreased levels relative to opaque cells, and that correction of these expression differences enables white cells to be mating competent. In particular, ectopic expression of the G protein β subunit encoded by *STE4*, the MAPK scaffold encoded by *CST5*, and the terminal MAP kinase encoded by *CEK2* enhanced mating ability. Each of these factors significantly increased mating of white cells when individually overexpressed, and when used in combination white cells mate 4 orders of magnitude more efficiently than control cells. These engineered white cells recapitulate both the transcriptional response of opaque cells to pheromone, as well as the morphological response to this stimulus. Furthermore, we emphasize that engineered white cells express physiologically relevant levels of ectopically expressed MAPK genes, as both RNA and protein levels of these factors are similar to opaque cell levels. Together, these results establish that *C. albicans* white cells can be engineered to be mating competent by restoration of the levels of key MAPK components, and are within 6-fold of opaque cells in terms of their sexual competence (Figure 3.6).

While ectopic expression of *STE4*, *CST5* or *CEK2* enhanced mating of *C. albicans* white cells, expression of *STE4* had by far the strongest influence on mating frequency, as elevating expression of this one gene enhanced white cell mating by 336 fold. Elevated expression of *STE4* also increased mating in white cells overexpressing *CST5* and *CEK2*, further augmenting mating in these cells by 183 fold (Fig 3.3). Surprisingly, however, overexpression of *STE4* protein levels by only ~3 fold in white cells, a similar value to the 2-fold difference in protein level between

natural white and opaque cells (Figure 3.5). This Ste4 pattern contrasts with Cek2 and Cst5, whose protein levels increased 13 fold and 180 fold, respectively, when ectopically expressed. Mating is therefore exquisitely sensitive to Ste4 levels, so that even small changes in expression can have a dramatic effect on cellular phenotypes. This result indicates that Ste4 levels are limiting in the cell, and we note that *C. albicans* α and γ subunits of the G protein complex are more highly expressed than *STE4* (by 9.6 and 38 fold, respectively, at the RNA level). A similar conclusion was reached for *S. cerevisiae*, as increasing Ste4 protein levels by two-fold caused a marked increase in the pheromone response, whereas a similar expression change in Ste2 or other G protein subunits did not⁵¹. However, it is worth noting that G protein signaling has been rewired between *C. albicans* and *S. cerevisiae* such that the α subunit promotes the mating response in *C. albicans* whereas it is repressive for *S. cerevisiae* mating⁵². Further dissection of the role of Ste4 will help shed light on how pheromone MAPK signaling is hypersensitive to this G protein subunit. One possible explanation is that while population levels show only limited expression differences between white and opaque states, individual cells may exhibit greater differences than the population average. Alternatively, there may be different Ste4 subcomplexes or differential localization of Ste4 subpopulations that are not captured by our analysis. Attempts to directly detect Ste4 protein in individual *C. albicans* cells, however, have so far proven unsuccessful.

We also considered how MAPK genes are differentially regulated between *C. albicans* white and opaque states. The white-opaque switch is regulated by an

interconnected network of transcription factors, of which Wor1 is considered the master regulator of the opaque state⁵³. Analysis of published chromatin immunoprecipitation data reveals that Wor1 is enriched at the *STE4* promoter in opaque cells⁴², making it likely that Wor1 directly promotes *STE4* expression in these cells. In contrast, none of the transcriptional regulators of the white-opaque switch show binding to the promoters of *CST5* or *CEK2*³⁶ (Supplemental Figure 3.6). We therefore propose that *STE4* is directly regulated by the white-opaque regulatory circuit, whereas *CST5* and *CEK2* are controlled indirectly by downstream components of the circuit.

At a broader level, our work reveals how contrasting phenotypes are achieved between epigenetic cell states simply by varying the expression of key MAPK components. Many mechanisms have been described to regulate MAPK signaling output and specificity in eukaryotic cells. These include the spatiotemporal regulation of signaling components, as well mechanisms for pathway feedback, and cross-pathway activation or inhibition^{1,3,4,54}. We propose that several signaling bottlenecks exist in the pheromone MAPK pathway in *C. albicans* white cells, and that alleviation of these bottlenecks enables white cells to undergo efficient pheromone signaling and mating. The white-opaque switch appears to have evolved in the ancestor of the lineage leading to *C. albicans* and *C. tropicalis*, and has been retained by these species as they diverged. Opaque cells are also the mating competent form in *C. tropicalis*, although the difference in mating efficiencies between white and opaque cells is only 100-fold, which is considerably less than the 5 orders of magnitude difference in mating between *C. albicans* white

and opaque forms. Inspection of transcriptional data from *C. tropicalis*⁵⁵ reveals that *STE4* is also expressed at a higher level (2-3 fold) in opaque cells than white cells from this species. Elevated *STE4* levels may therefore contribute to the enhanced sexual competency of opaque cells in both *C. tropicalis* and *C. albicans*. The fact that most other MAPK genes are not white-opaque regulated in *C. tropicalis* could account for the more limited mating differences between the two cell states than in *C. albicans*. Together, these results suggest a model in which *STE4* came under the control of the white-opaque regulatory circuit in the ancestor to *C. tropicalis* and *C. albicans*, and that additional MAPK genes (*CST5* and *CEK1/2*) subsequently became white-opaque regulated specifically in the *C. albicans* lineage. The white-opaque circuit therefore gained some level of mating control in the ancestor to both *Candida* species, before evolving additional degrees of control only in the *C. albicans* lineage (Figure 3.6).

Our study also has consequences for understanding the signaling changes that occur in cancer cells due to alterations in G proteins, G protein-coupled receptors (GPCRs) or MAPK transduction. For example, it is now apparent that mutations in the MEK-upstream kinase BRAF are present in over 50% of melanomas^{56,57}, and that approximately 20% of all cancers involve mutations in GPCRs^{58,59}. There is therefore considerable interest in determining how signaling mechanisms are rewired in cancer cells, and in the strategic targeting of specific MAPK components for therapeutic intervention. Our results emphasize that even small differences in the expression levels of critical signaling components can have large effects on signal output. Thus, understanding of signaling bottlenecks and the

mechanisms that impact these critical signaling points will be important for informing our understanding of signal transduction and cellular phenotypes across diverse eukaryotic species.

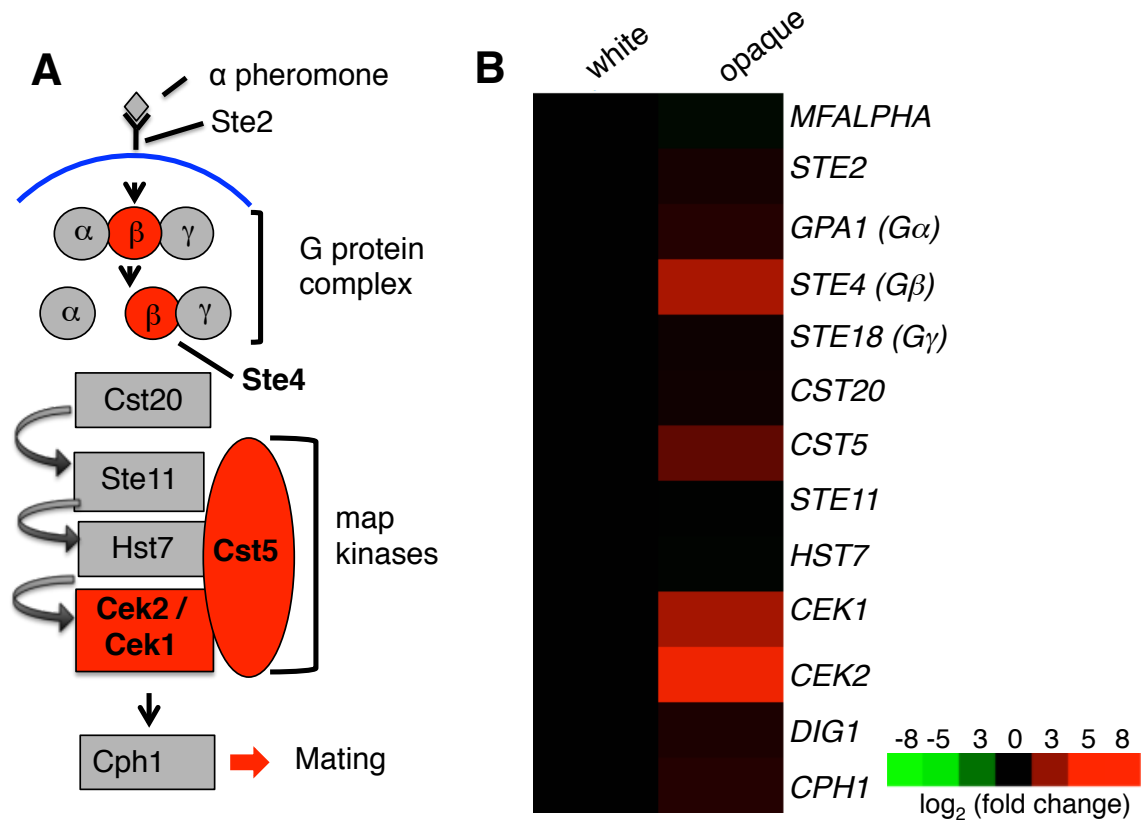


Figure 3.1: Gene expression of pheromone MAPK components in *C. albicans* white and opaque cells.

A. Major components of the *C. albicans* pheromone MAPK kinase pathway. Components highlighted in red are those that are transcriptionally upregulated in opaque cells relative to white cells.

B. Analysis of RNA sequencing data⁴² indicates that four pheromone MAPK components, *STE4*, *CST5*, *CEK1*, and *CEK2*, are induced 9 fold, 3.7 fold, 8.8 fold, and 11.5 fold higher in opaque cells than white cells.

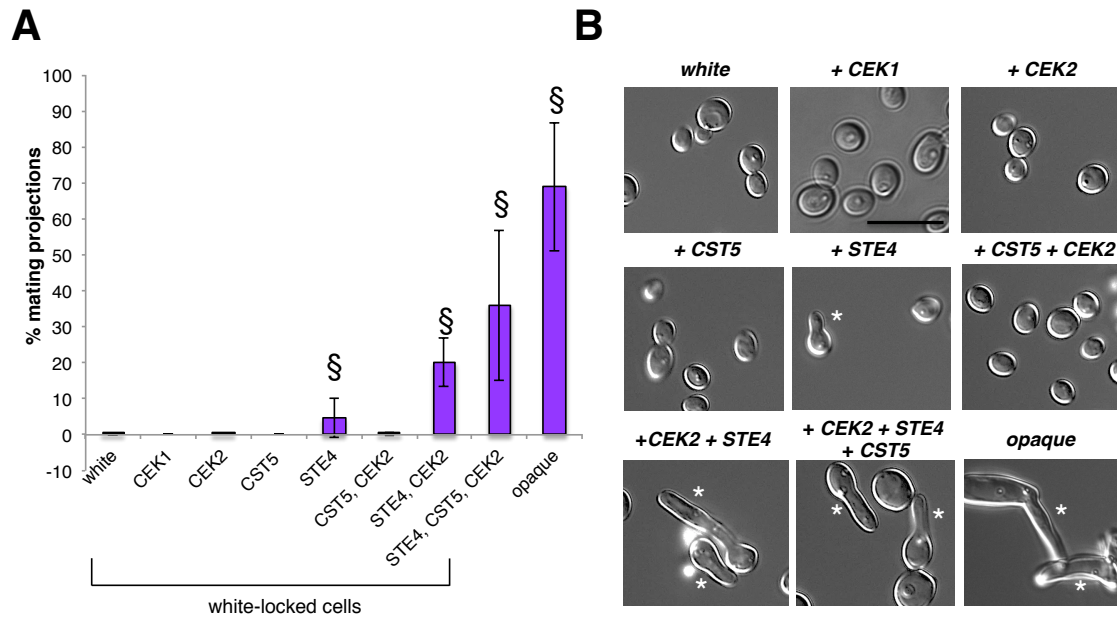


Figure 3.2: Overexpression of pheromone MAPK components enables *C. albicans* white cells to undergo an efficient morphological response to pheromone.

A. Quantification of mating projection formation for white-locked ($\Delta wor1/\Delta wor1$) **a** cells overexpressing different MAPK genes and comparison with control opaque **a** cells. § = $p < 0.05$ Kruskal Wallis (Mann-Whitney pairwise). Mean is plotted and error bars = s.d. n = minimum of 4 biological replicates.

B. Representative images of cell phenotypes after co-incubation of white-locked **a** and α cells overexpressing the indicated MAPK components. Cells were co-incubated on Spider medium for 24 h and imaged. Wildtype opaque cells are shown for comparison. * denotes mating projections.

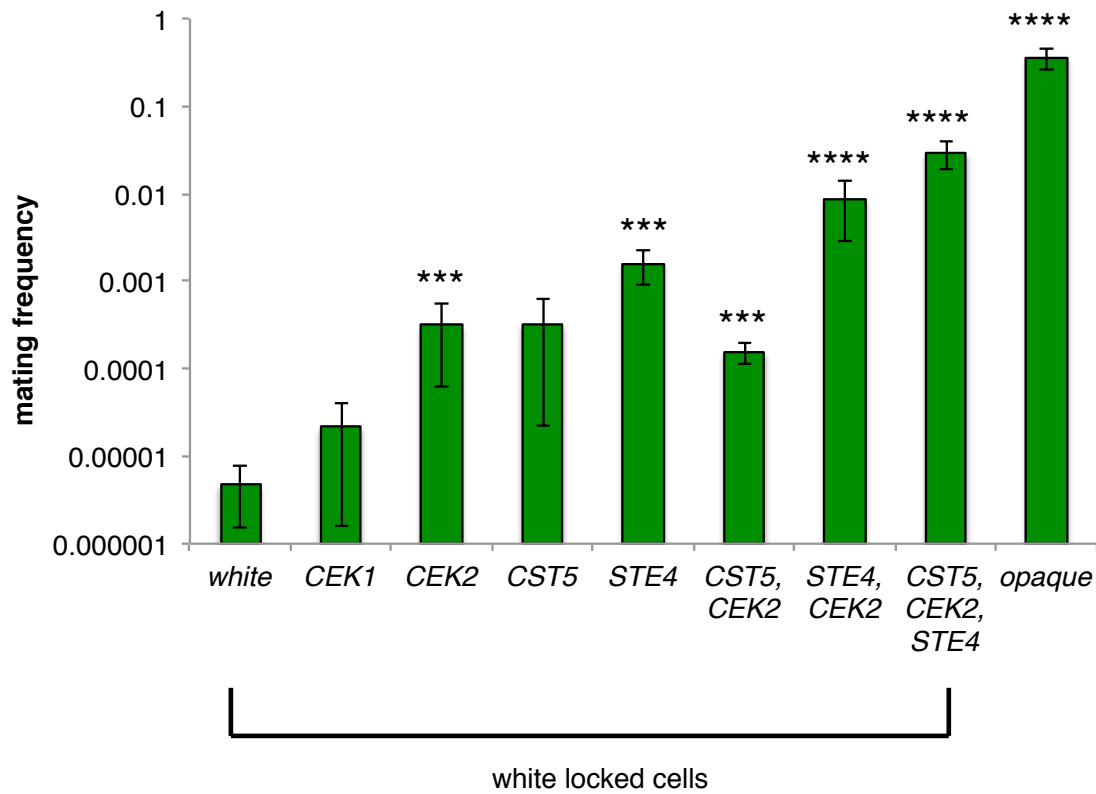


Figure 3.3: Quantification of mating in *C. albicans* white cells ectopically expressing pheromone MAPK components.

Mating of **a** and α cells was quantified in white-locked cells, opaque cells, and white-locked cells engineered to overexpress the MAPK components *STE4*, *CST5* and *CEK2* either singly or in combination. Significant differences compared to mating experiments with control white-locked cells are shown. *** = $P < 0.001$ **** = $p < 0.0001$ Kruskal Wallis (Mann-Whitney pairwise). Mean is plotted and error bars are s.e.m. n = minimum of 6 biological replicates per genotype.

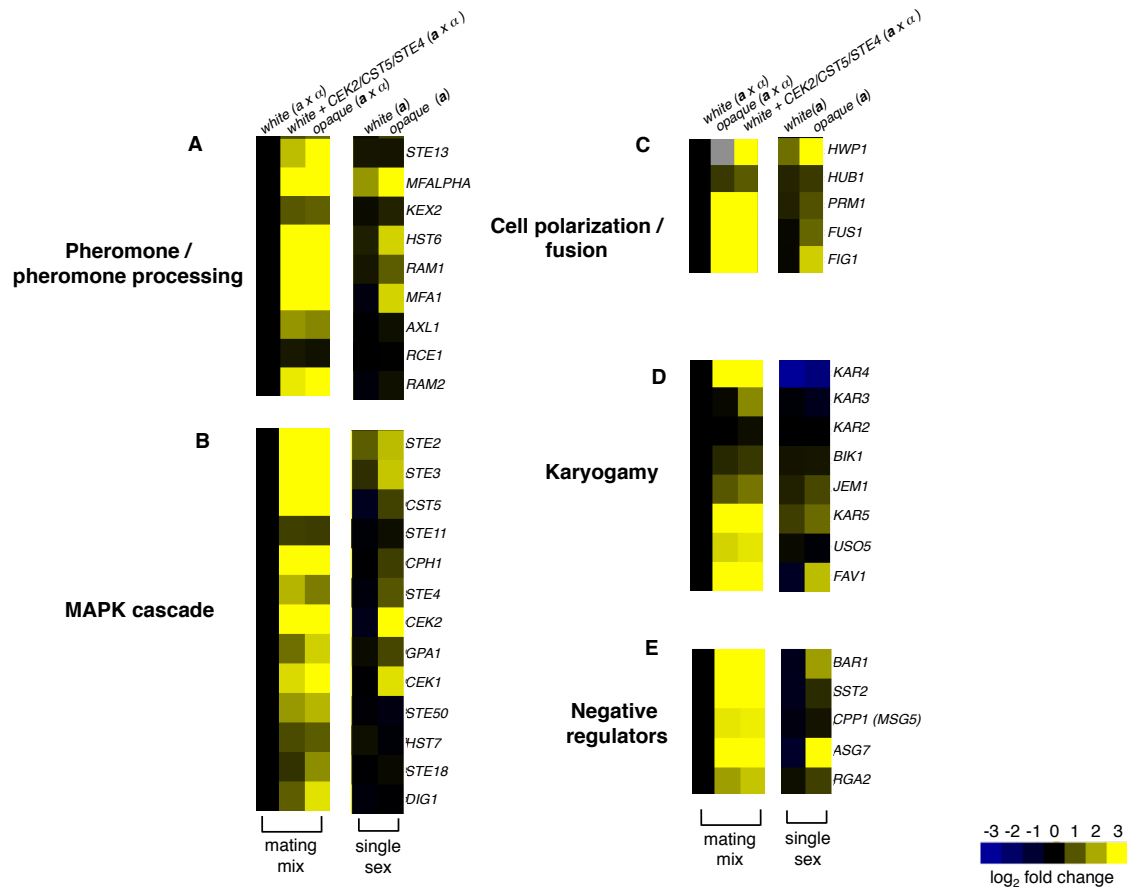


Figure 3.4: Comparison of expression profiles between mating mixes of *C. albicans* white cells, white cells engineered to overexpress MAPK genes, and opaque cells.

Expression levels of genes involved in pheromone production and processing (A), the core pheromone MAPK signaling cascade (B), cell polarization and fusion (C), karyogamy (D), and negative regulators of the MAPK pathway (E) are shown. Mating mixes of **a** and α cells were incubated on Spider medium for 8 h and RNA expression profiles determined.

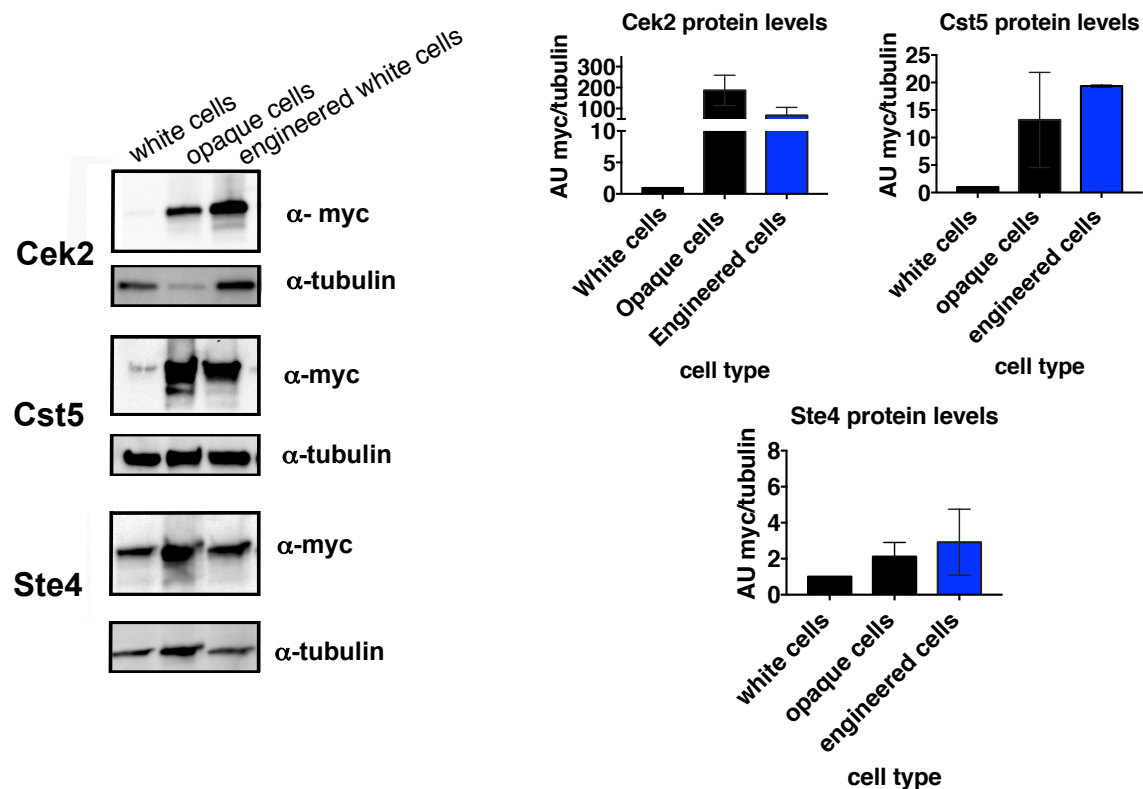


Figure 3.5: Comparative analysis of the protein levels of pheromone MAPK components in *C. albicans* white cells, opaque cells, and white cells ectopically expressing MAPK genes.

A. Representative images of Western blots conducted on strains containing 13x-myc epitope-tagged versions of *CEK2*, *CST5*, or *STE4*. White cells are mating crosses between *wor1* Δ /*wor1* Δ **a** and α cells. Opaque cells are mating crosses between opaque **a** and α cells. Engineered cells are crosses between white-locked cells (**a** x α) that ectopically express *STE4*, *CST5* or *CEK2*.

B. Quantitation of myc signal relative to tubulin (control) signal for the indicated protein in each cell type. For *Ste4* and *Cst5* $n = 4$ biological replicates. For *Cek2* $n = 2$ biological replicates. The mean is plotted and error bars are s.e.m.

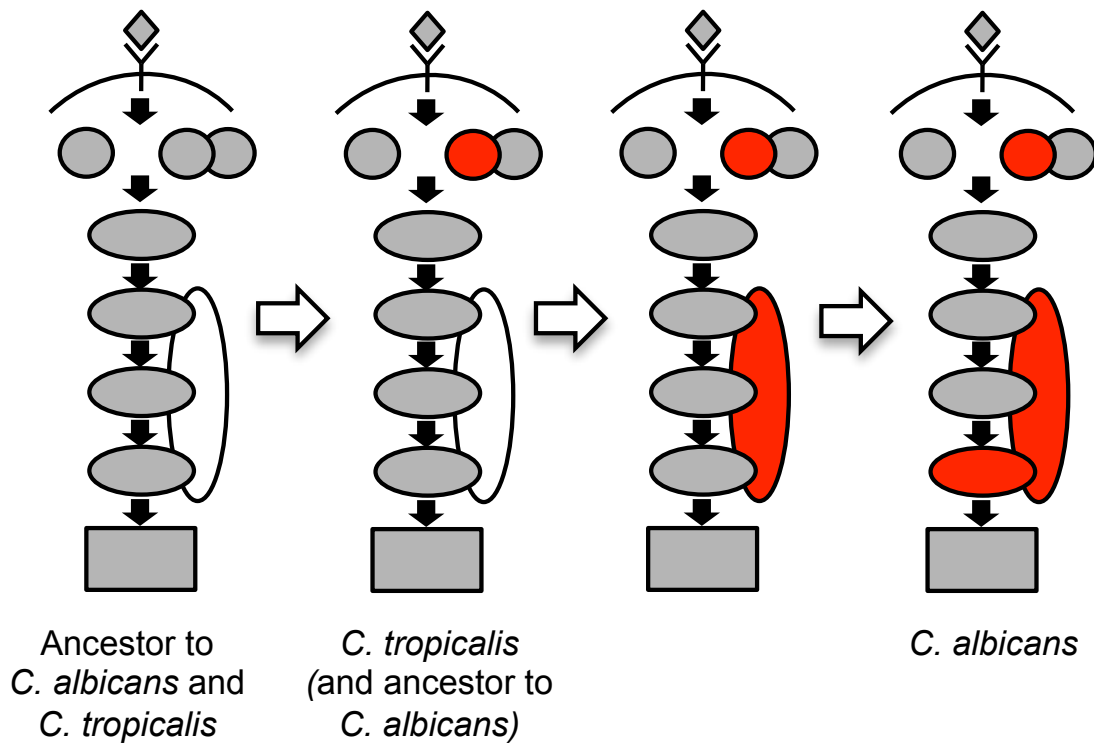
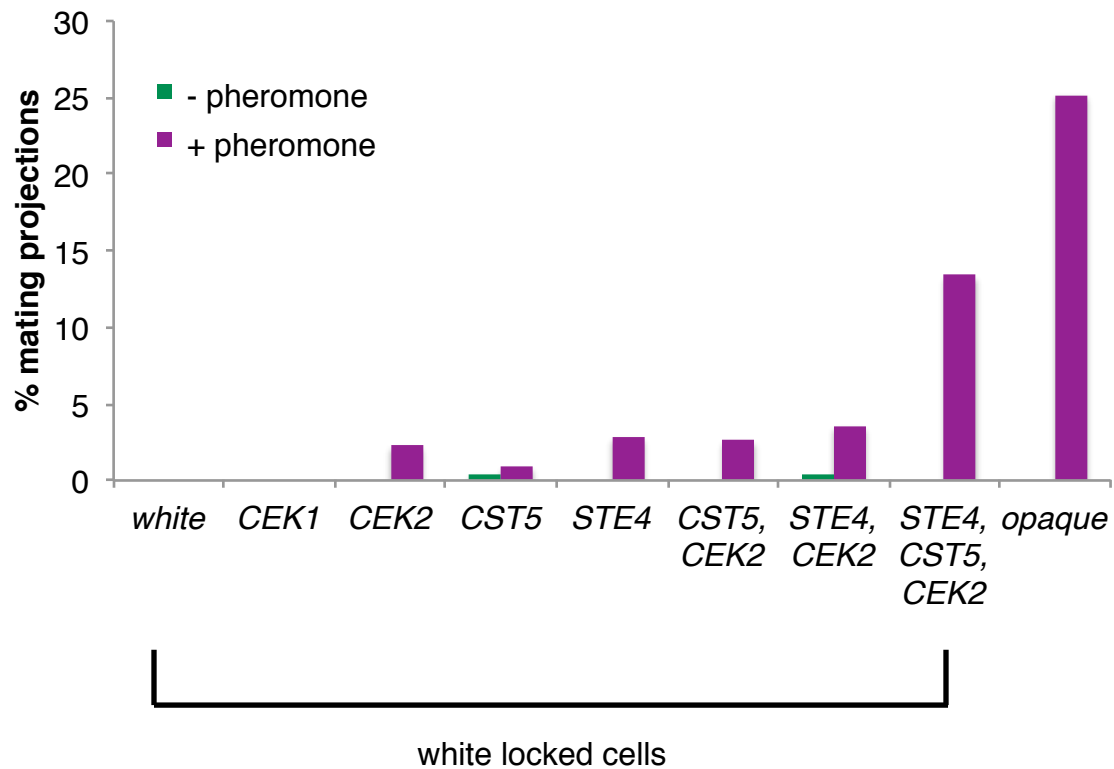


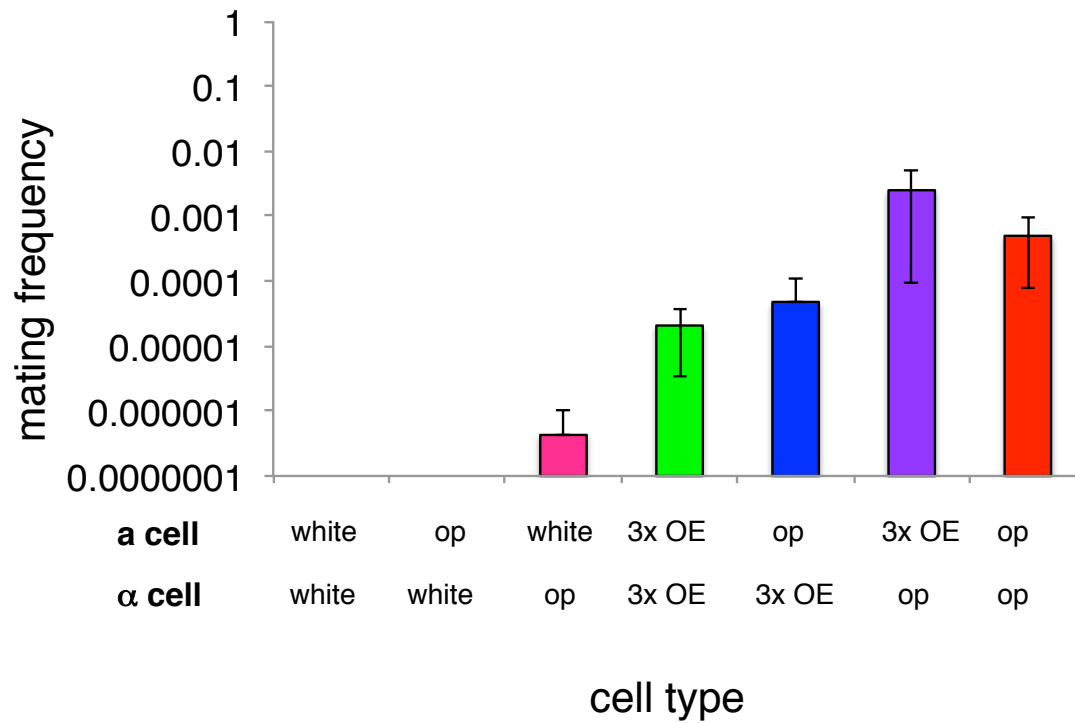
Figure 3.6: Model.

Model for how the pheromone MAPK signaling pathway came under the control of the white-opaque regulatory circuit in *Candida* species. Components in red are those that came under control of the white-opaque switch. Since only Ste4 is regulated in the white-opaque switch in *C. tropicalis*, it is hypothesized to have come under control of the switch first, followed by Cst5 and Cek2 in *C. albicans*.

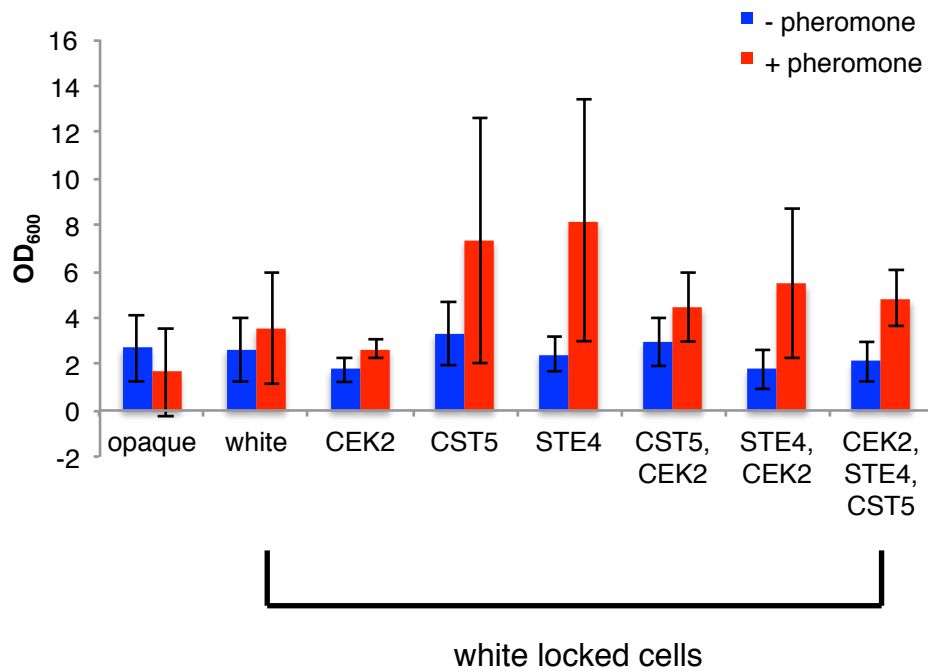


Supplemental Figure 3.1: Formation of mating projections in MTLa cells exposed to mating pheromone in Spider medium.

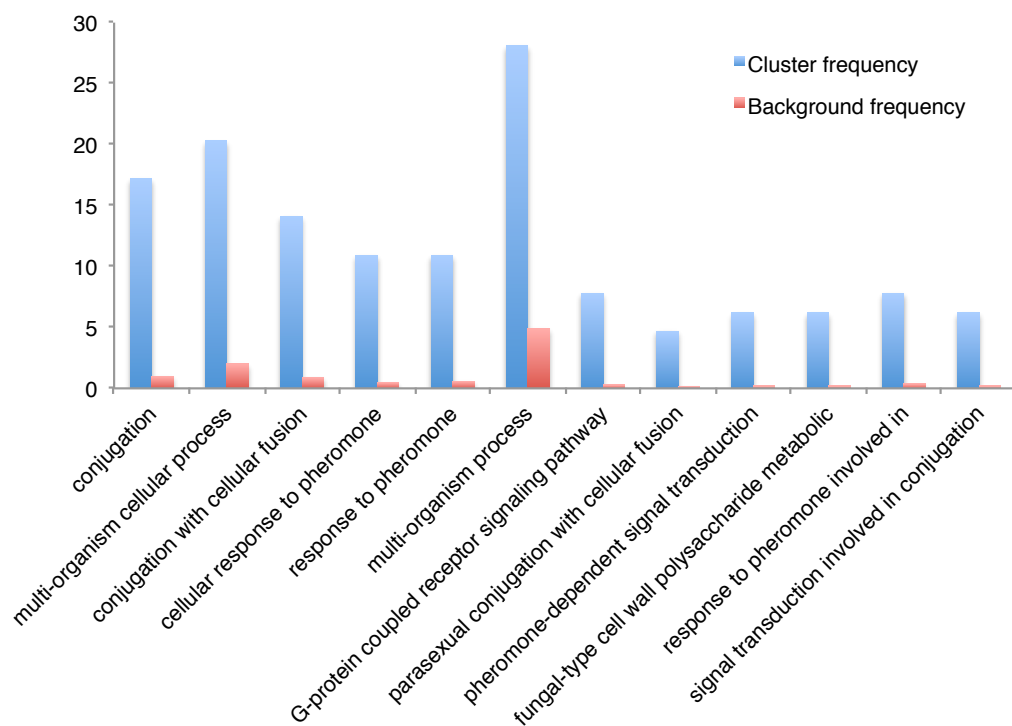
Control white-locked **a** cells (*wor1Δ/wor1Δ*) or white-locked **a** cells overexpressing the indicated MAPK components were incubated in Spider medium with or without the addition of 10 μ M synthetic α pheromone. The response of opaque **a** cells to pheromone is shown for comparison. Cells were analyzed after 6 h of pheromone treatment and at least 300 cells were counted for each genotype.



Supplemental Figure 3.2: Comparison of mating frequencies between *a* and α cells that are ectopically expressing STE4, CST5, and CEK2. *MTLa* and α cells were co-incubated on Spider medium for 5 days at 22°C. White = white locked cells (*wor1Δ/wor1Δ*). White-locked cells constitutively expressing STE4, CEK2, and CST5 are indicated as 3 x OE cells.

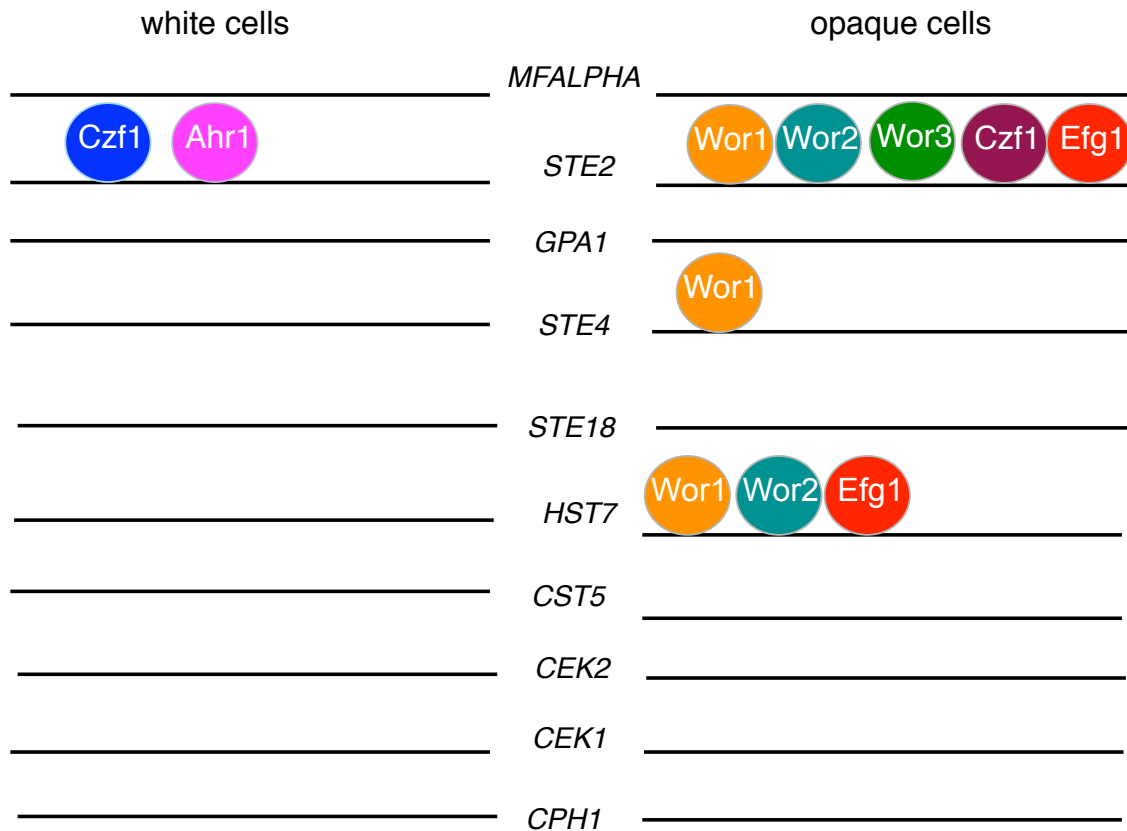


Supplemental Figure 3.4: Engineered cells form biofilms in response to pheromone. Control locked white **a** cells (*wor1Δ/wor1Δ*) or locked white **a** cells overexpressing the indicated MAPK component were incubated in 12 well polystyrene plates for 28 hours without shaking. Error bars are s.d. The results show the average of 3 biological replicates.



Supplemental Figure 3.5: GO term analysis of genes that exhibit significant expression differences in both engineered white cells and opaque cells under mating conditions.

Genes significantly altered in expression in crosses between opaque a and α cells and between white-locked a and α cells that are ectopically expressing *STE4/CST5/CEK2* in comparison to control crosses between white-locked a and α cells.



Supplemental Figure 3.6: Analysis of the binding of *C. albicans* white-opaque transcription factors to pheromone MAPK genes.

Schematic of the major-white opaque regulators that are present at the promoters of pheromone MAPK genes based on published ChIP-Chip data ³⁶. Black lines represent promoters in white cells (left) or opaque cells (right).

Table 3.1: Strains Used in *C. albicans* study

Strain	Genotype	White/opaque	Source
CAY1917	α/α , $ura3::imm^{434}::URA3/ura3::imm^{434}$ $iro1::IRO1/iro1::imm434$ $his1::hisG/his1::hisG$ $leu2/leu2$, $wor1\Delta::FRT/wor1\Delta::SAT1-FLP$	White	This study
CAY1919	α/α , $arg4::hisG/arg4::hisG$, $his1::hisG/HIS1$, $wor1\Delta::FRT/wor1\Delta::SAT1$	White	This study
CAY1925	a/a , $ura3::imm^{434}::URA3/ura3::imm^{434}$ $iro1::IRO1/iro1::imm434$ $his1::hisG/his1::hisG$ $leu2/leu2$, $wor1\Delta::FRT/wor1\Delta::SAT1-FLP$	White	This study
CAY1928	a/a , $arg4::hisG/arg4::hisG$, $his1::hisG/HIS1$ $wor1\Delta::FRT/wor1\Delta::SAT1-FLP$	White	This study
CAY3336	α/α , $ura3::imm^{434}::URA3/ura3::imm^{434}$ $iro1::IRO1/iro1::imm434$ $his1::hisG/his1::hisG$ $leu2/leu2$, $wor1\Delta::FRT/wor1\Delta::FRT$	White	This study
CAY3337	α/α , $arg4::hisG/arg4::hisG$, $his1::hisG/HIS1$ $wor1\Delta::FRT/wor1\Delta::FRT$	White	This study
CAY3339	a/a , $ura3::imm^{434}::URA3/ura3::imm^{434}$ $iro1::IRO1/iro1::imm434$ $his1::hisG/his1::hisG$ $leu2/leu2$, $wor1\Delta::FRT/wor1\Delta::FRT$	White	This study
CAY3341	a/a , $arg4::hisG/arg4::hisG$, $his1::hisG/HIS1$ $wor1\Delta::FRT/wor1\Delta::FRT$	White	This study
CAY3499	a/a , $ura3::imm^{434}::URA3/ura3::imm^{434}$ $iro1::IRO1/iro1::imm434$ $his1::hisG/his1::hisG$ $leu2/leu2$, $wor1\Delta::FRT/wor1\Delta::FRT$, $pACT1-$ $STE4::SAT1-FLP$	White	This study
CAY3500	a/a , $arg4::hisG/arg4::hisG$, $his1::hisG/HIS1$ $wor1\Delta::FRT/wor1\Delta::FRT$, $pACT1-$	White	This study

	<i>STE4::SAT-FLP</i>		
CAY3581	<i>α/α, arg4::hisG/arg4::hisG, his1::hisG/HIS1wor1Δ::FRT/wor1Δ::FRT, pACT1-STE4::SAT-FLP</i>	White	This study
CAY3582	<i>α/α, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pACT1-STE4::SAT1-FLP</i>	White	This study
CAY3921	<i>α/α, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::SAT1-FLP</i>	White	This study
CAY3922, CAY3923	<i>α/α, arg4::hisG/arg4::hisG, his1::hisG/HIS1wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::SAT1-FLP</i>	White	This study
CAY3924, CAY3925, CAY3926	<i>α/α, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::SAT1-FLP</i>	White	This study
CAY3927	<i>α/α, arg4::hisG/arg4::hisG, his1::hisG/HIS1 wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::SAT1-FLP</i>	White	This study
CAY4722, CAY4723, CAY4724	<i>α/α, arg4::hisG/arg4::hisG, his1::hisG/HIS1wor1Δ::FRT/wor1Δ::FRT, pADH1-CEK2::SAT1-FLP</i>	White	This study
CAY4728, CAY4729, CAY4730	<i>α/α, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pADH1-CEK2::SAT1-FLP</i>	White	This study
CAY5379	<i>α/α, arg4::hisG/arg4::hisG, his1::hisG/HIS1 wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::FRT</i>	White	This study
CAY5380	<i>α/α, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::FRT</i>	White	This study

CAY5426	<i>α/α, arg4::hisG/arg4::hisG, his1::hisG/HIS1wor1Δ::FRT/wor1Δ::FRT, pACT1-STE4::FRT</i>	White	This study
CAY5428	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pACT1-STE4::FRT</i>	White	This study
CAY5429	<i>a/a, arg4::hisG/arg4::hisG, his1::hisG/HIS1 wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::FRT</i>	White	This study
CAY5493, CAY5494, CAY5495, CAY5496	<i>a/a, arg4::hisG/arg4::hisG, his1::hisG/HIS1 wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::FRT, pADH1-CEK2::SAT1-FLP</i>	White	This study
CAY5498, CAY5499, CAY5500	<i>α/α, arg4::hisG/arg4::hisG, his1::hisG/HIS1wor1Δ::FRT/wor1Δ::FRT, pACT1-STE4::FRT, pADH1-CEK2::SAT1-FLP</i>	White	This study
CAY5505, CAY5506	<i>a/a, arg4::hisG/arg4::hisG, his1::hisG/HIS1 wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::FRT, pADH1-CEK2::SAT1-FLP</i>	White	This study
CAY5657, CAY5658, CAY5659	<i>α/α, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::FRT, pADH1-CEK2::SAT1-FLP</i>	White	This study
CAY5660, CAY5661	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pACT1-STE4::FRT, pADH1-CEK2::SAT1-FLP</i>	White	This study
CAY5663, CAY5664	<i>α/α, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pACT1-CST5::FRT, pADH1-CEK2::SAT1-FLP</i>	White	This study

CAY5997, CAY5998	a/a , <i>arg4::hisG/arg4::hisG</i> , <i>his1::hisG/HIS1</i> <i>wor1Δ::FRT/wor1Δ::FRT</i> , <i>pACT1-CST5::FRT</i> , <i>pADH1-CEK2::FRT</i>	White	This study
CAY5999, CAY6000	a/a , <i>ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴</i> <i>iro1::IRO1/iro1::imm434</i> <i>his1::hisG/his1::hisG leu2/leu2</i> , <i>wor1Δ::FRT/wor1Δ::FRT</i> , <i>pACT1-CST5::FRT</i> , <i>pADH1-CEK2::FRT</i>	White	This study
CAY6066, CAY6067, CAY6070, CAY6071	a/a , <i>arg4::hisG/arg4::hisG</i> , <i>his1::hisG/HIS1</i> <i>wor1Δ::FRT/wor1Δ::FRT</i> , <i>pACT1-CST5::FRT</i> , <i>pADH1-CEK2::FRT</i> , <i>STE4/ste4::pACT1-STE4::SAT1-FLP</i>	White	This study
CAY6068, CAY6069, CAY6072, CAY6073	a/a , <i>ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴</i> <i>iro1::IRO1/iro1::imm434</i> <i>his1::hisG/his1::hisG leu2/leu2</i> , <i>wor1Δ::FRT/wor1Δ::FRT</i> , <i>pACT1-CST5::FRT</i> , <i>pADH1-CEK2::FRT</i> , <i>STE4/ste4::pACT1-STE4::SAT1-FLP</i>	White	This study
CAY6220, CAY6221, CAY6222	a/a , <i>arg4::hisG/arg4::hisG</i> , <i>his1::hisG/HIS1 wor1Δ::FRT/wor1Δ::FRT</i> , <i>pACT1-CEK1::SAT1-FLP</i>	White	This study
CAY6223, CAY6224, CAY6225	a/a , <i>ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴</i> <i>iro1::IRO1/iro1::imm434</i> <i>his1::hisG/his1::hisG leu2/leu2</i> , <i>wor1Δ::FRT/wor1Δ::FRT</i> , <i>pACT1-CEK1::SAT1-FLP</i>	White	This study
CAY6562, CAY6563	a/a , <i>ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴</i> <i>iro1::IRO1/iro1::imm434</i> <i>his1::hisG/his1::hisG leu2/leu2</i> , <i>wor1Δ::FRT/wor1Δ::FRT</i> , <i>pACT1-STE4-MYC::SAT1-FLP</i>	White	This study
CAY6566, CAY6567	a/a , <i>ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴</i> <i>iro1::IRO1/iro1::imm434</i> <i>his1::hisG/his1::hisG leu2/leu2</i> , <i>wor1Δ::FRT/wor1Δ::FRT</i> , <i>pACT1-CST5-MYC::SAT1-FLP</i>	White	This study
CAY6889, CAY6890	a/a , <i>ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴</i> , <i>iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2 arg4/arg4</i> , <i>STE4/STE4-MYC-</i>	White	This study

	<i>HIS1</i>		
CAY6891, CAY6892, CAY6893	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴, iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2 arg4/arg4, CEK2/CEK2-MYC-HIS1</i>	White	This study
CAY6915, CAY6992, CAY6993	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴, iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2 arg4/arg4, CEK2/CEK2-MYC-HIS1</i>	Opaque	This study
CAY6916, CAY6917	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴, iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2 arg4/arg4, STE4/STE4-MYC-HIS1</i>	Opaque	This study
CAY7034, CAY7035, CAY7036	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2, wor1Δ::FRT/wor1Δ::FRT, pADH1-CEK2-MYC::SAT1-FLP</i>	White	This study
CAY7230, CAY7231	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴, iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2 arg4/arg4, CST5/CST5-MYC-HIS1</i>	White	This study
CAY7236, CAY7237	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴, iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2 arg4/arg4, CST5/CST5-MYC-HIS1</i>	Opaque	This study
RBY1118	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2</i>	Opaque	Schaefer <i>et al</i> 2007
RBY1119	<i>a/a, ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴ iro1::IRO1/iro1::imm434</i>	Opaque	Schaefer <i>et al</i>

	<i>his1::hisG/his1::hisG leu2/leu2</i>		2007
RBY1132	a/a , <i>ura3::imm⁴³⁴::URA3/ura3::imm⁴³⁴</i> , <i>iro1::IRO1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2 arg4/arg4</i>	White	Schaefer <i>et al</i> 2007
RBY1173	a/a , <i>leu2::hisG/leu2::hisG his1::hisG/HIS1</i>	White	Bennett <i>et al</i> 2006
RBY1174	α/α , <i>leu2::hisG/leu2::hisG his1::hisG/HIS1</i>	White	Bennett <i>et al</i> 2006
RBY1177	a/a , <i>leu2::hisG/leu2::hisG his1::hisG/HIS1</i>	Opaque	Bennett <i>et al</i> 2006
RBY1178	α/α , <i>leu2::hisG/leu2::hisG, his1::hisG/HIS1</i>	Opaque	Bennett <i>et al</i> 2006
RBY1179	a/a , <i>arg4::hisG/arg4::hisG, his1::hisG/HIS1</i>	Opaque	Bennett <i>et al</i> 2006
RBY1180	α/α , <i>arg4::hisG/arg4::hisG, his1::hisG/HIS1</i>	Opaque	Bennett <i>et al</i> 2006

Table 3.2: *plasmids used in the C. albicans study*

Plasmid Number	Description
RB34	Wor1 KO
RB281	Act1-Ste4 in psfs2a
RB284	Act1-Cst5 in psfs2a
RB294	Act1-Cst5-myc in psfs2a
RB296	Ste4-myc
RB325	Cst5-myc
RB348	Adh1-Cek2 in psfs2a
RB439	Act1-Cek1 in psfs2a
RB442	Cek2-myc
RB466	Act1-Ste4-myc in psfs2a
RB485	Adh1-Cek2-myc in psfs2a

Table 3.3: Oligonucleotides used in *C. albicans* study

Oligonucleotide number	Description	Sequence
BL596	ACT1 (-1100) FOR	CTTTGCCTTACTACCACGACTAAGG
BL610	Act1 (-950) Apal	GGAGCGGGGCCCCGGACCAAATAGAACTGTTCTCAGG
BL611	Act1 (0)	GAATGATTATATATTTTTTTAATATTAATATCGAGATAATG
BL1230	Ste4-Act1 Fusion	CATTATCTCGATATTAATATTAATAAAAAATATAATCATTCAAATGTCCGATTATCTTGCTCAATCG
BL1232	Ste4 (xho1) +1650	GCCGGCCTCGAGCACACTGGTGAAGCTTAAAG
BL1246	Adh1 (-800) Apal	ggagcgGGGCCCCAACGGTTGAGATGGAGCCG
BL1256	Adh1 (-800) check	GCCAGATGTTATCTGCAACTG
BL1649	Ste4 (Sall) for	ggcgccGTCGACATGTCCGATTATCTTGCTCAATCG
BL1650	Ste4 (BamHI) rev	gcgcggGGATCCCTCGAGTTCTCTACCCTCAGTCTCGC
BL1672	Alternate pSFS2a left	CGCCTCCCCCACTCCCCAATCTT
BL2059	Adh1-Cek2 fwd new	AACAACAACAATACAAAAACAATTATGAAGAAATCTACTGGCCCCACT
BL2061	Ca cek2 checkR 374	TACTGAACGTGTTGATCGCTC
BL2063	Adh1-Cek2 rev XhoI +1430 R	ggccggCTCGAGTGGTAAAAGAGCCAGGAACGTC
BL2127	Adh1 0 reverse new	AATTGTTTTTGTATTTGTTGTTGTT
BL2198	Cst5 (+300) rev	TCACTGTGGGTGGAGTCACC
BL2199	Cst5 (+300) for	GGTGACTCCACCCACAGTGA
BL2203	pMG2093 (BsrGI) rev	GGCGCCTGTACACCAAGATCACGCGTCTCGAG
BL2204	pMG2093 (BamHI) rev	GGCGCCGGATCCCCAAGATCACGCGTCTCGAG
BL2227	act1-cst5 fusion	CATTATCTCGATATTAATATTAATAAAAAATATAATCATT

		CAAATGCTACAATGTACG
BL2228	Cst5 (xhoI)	GGCGCCCTCGAGGTCGATACCCATTACAGTCCC
BL2234	Ste4 5' orf +150	ggaccaagatgccacaattc
BL2238	Cst5 (+200) Rev	GACTTGTTGAGAATATTGGC
BL2244	pMG2093 (+800) Xho1	GGCGCCCTCGAGACAGTTTTCTGGAGCACAGTG
BL2605	cek1 +1665 XhoIR	gcccgcctcgagCAACTCCAACACTCCTCCTT
BL2699	Cek1 (0) act1 fusion fwd	CATTATCTCGATATTAATATTAATAAAAAATATAATCATT CAAAtgAATATAGATCAACAT
BL2795	Ca cek2 q F1 +686	TGTTCCCTGGATCTGACTAC
BL2803	Ca ste4 q F1 +45	GGTACTGCAACAGGAACAAC
BL2804	Ca ste4 q R1 +248	CCTCGCAAGGTGTTGTATAG
BL2946	Cek2 myc Sall -846 F	ggcgccGTCGACGAGATCACGCCAGGTTATAG
BL2947	Cek2 myc SmaI +1096 R	ggcgccCCCGGGCGACATGACTATTTTGAAAA
BL2948	Cek2 -960 check forward	CAATGTCTGTGTTGCACGTT
BL3433	pmg2093 check 1000 rev	CTGGTGTCCAAGATCACGCG
BL3434	pmg2093 check +1140 rev	GAGAGGTAAACCCAGAAAGC
BL3488	Cst5 -985 check F	GGATATAAGAAGGAACTTCA
BL3489	Ste4 -980 check F	TTTATTTCCATGTAGCATTA
BL3605	Cst5 -675 XhoI F	GGCGCCCTCGAGGGAATCTACAATCAGGATCA
BL3606	Cst5 1152 SmaI R	GCGGCGCCCCGGGCGGGTCAGATGTAAGTAACG

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CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Mechanisms of Signaling Specificity: Cell states transcriptionally regulate signaling components in order to achieve distinct outputs

There are multiple ways that MAPK signaling cascades are regulated. Our work has identified that one way epigenetic programming can determine cellular output is through transcriptional regulation of signaling components. Rewiring of cells in one epigenetic state (the white state) can enable them to produce an output that is normally associated with a different epigenetic state (the opaque state). Through rewiring MAPK signaling, we believe that we have recapitulated the “natural” difference between *C. albicans* white and opaque cells, because opaque cells naturally have higher levels of three key MAPK proteins (Ste4/Cst5/Cek2) under steady-state conditions. Thus, we provide a molecular mechanism for a long-standing question as to the differences that make opaque cells the mating competent form of *C. albicans*. Furthermore, our work provides understanding into a novel mechanism for the regulation of sexual reproduction in fungi.

C. albicans phenotypic switching and restricting mating to the opaque state

Since the discovery of the white opaque switch in *C. albicans*, there has been much work to define the differences between the two states, and speculation as to why the switch may have evolved. The evolutionary advantage, if any, of the restriction of mating to the opaque state has remained unclear.

A thorough global analysis was recently completed in our laboratory that tested the fitness of each state in over 1,440 conditions¹. This analysis revealed that white cells are more fit than opaque cells under most *in vitro* conditions, consistent with *C. albicans* existing mostly in the white state in the host. At physiological temperature (37°C), opaque cells were stably propagated in <5% of conditions tested, some of which were alternative carbon sources, such as culture in the presence of sorbitol or N-acetylglucosamine. These studies support previous work indicating that white and opaque cells have distinct metabolic preferences², and that the opaque state represents a more specialized form of *C. albicans*, whereas white cells are seemingly the default state.

The discovery that white cells are able to form biofilms in response to pheromone, and that these biofilms stabilize pheromone gradients between opaque cells, led to the hypothesis that the white-opaque switch evolved to facilitate mating in the host, where opaque cells are assumed to be rare^{3,4}. However, the white-opaque switch also exists in the closely related species *C. tropicalis* and *C. dubliniensis*. In *C. tropicalis*, it has been demonstrated that opaque cells are both the biofilm-forming and mating competent form⁵. This information weakens the hypothesis that the white-opaque switch evolved solely so that white cells could form biofilms to facilitate mating events between opaque cells, although this is still a possibility for *C. albicans*.

In *S. cerevisiae*, researchers directly tested the effect of MAPK gene expression on cell fitness⁶. In this work, mutants that were defective in MAPK

signaling were identified by failure to undergo cell cycle arrest after treatment with synthetic α factor. These strains contained loss-of-function mutations in *STE4*, *STE5* and *STE7*. Microarray analysis revealed that these strains failed to induce downstream targets of the MAPK pathway at wild type levels. Interestingly, these signaling-defective strains had a significant growth advantage over wild type strains. Similarly, mutants carrying a deletion for *GPA1*, the α subunit of the G protein complex, had a 2% growth rate increase compared to wild type cells. The difference in growth rates between wild type and cells mutant for MAPK genes is thought to be because many genes are basally expressed at higher levels with increased MAPK signaling⁶. Tuning down the MAPK pathway results in a lower “cost” of gene expression, and a higher growth rate⁶. These results establish that signaling through the MAPK pathway causes a fitness defect in the model yeast.

Interestingly, when the authors sequenced natural strains of *S. cerevisiae*, they found that several strains contained a mutant allele of *GPA1* that actually *increased* signaling through the MAPK pathway and mating efficiency. Furthermore, it has been shown that cells with the capacity to sexually reproduce (mixes of **a** and α cells) have an evolutionary advantage when serially passaged as compared to **a** cells alone⁷. Thus, it appears that while expression of mating genes decreases fitness in the short term, there may be benefits to retaining this ability for natural selection during evolution.

By analogy, we reasoned that there could be a fitness cost associated with increased levels of basal MAPK signaling in *C. albicans*. To test this hypothesis, we

compared the growth rates of *C. albicans* white cells expressing natural or elevated levels of MAPK components. Preliminary results indicate a small fitness defect in strains overexpressing the three MAPK components *STE4*, *CST5*, and *CEK2* (Figure 4.1). These results are therefore consistent with the experiments in *S. cerevisiae* suggesting that increased levels of basal MAPK signaling may promote mating competence but have negative consequences for cell fitness in both fungal species.

These findings suggest an interesting hypothesis as to why the white-opaque switch may have evolved to regulate mating in *Candida* species. By essentially turning off basal MAPK signaling in the white state, cells would have gained a significant fitness advantage over cells that still exhibit basal signaling, but these cells were still capable of mating by undergoing the switch to the mating-competent opaque form. In this way, *Candida* cells would have the “best of both worlds” – they would limit the cost associated with MAPK signaling while still being able to undergo infrequent mating. When evolution found this novel solution to promote fitness, selection would ensure that this mechanism would spread through the population of *C. albicans* cells.

What are other features of opaque cells that contribute to mating competence?

Although white cells overexpressing key MAPK components can mate efficiently, their average mating frequency is 6-fold less than wild type opaque cells (Chapter 3, Figure 17). While this level of mating represents a dramatic increase

from parental locked white cells (~6000 fold), there is still a significant defect in engineered white cell mating efficiency as compared to opaque cells. This observation raises the question as to what else is contributing to mating efficiency in opaque cells, or what other factors make white cells refractory to mating. Addressing this question may have implications for what contributes to MAPK signaling efficiency in other systems.

One possibility is that the negative regulation, or inhibitory feedback, is different in the two cell types. On the surface, this does not seem likely, because the major negative regulators of the MAPK pathway are transcriptionally regulated at similar levels in engineered vs. opaque cells. (Chapter 3, Figure 3.4, Panel E). For example, the *C. albicans* homolog of *MSG5*, a pheromone-induced gene whose product dephosphorylates Fus3, is upregulated 7.3 fold in engineered cell crosses as compared to parental white cell crosses. *MSG5* is similarly induced 7.8 fold in opaque mating cells. Similar expression patterns can be seen for the pheromone α factor protease, *BAR1*, and the GTPase *SST2*, which returns G α to its inactive GDP-bound state.

However, one mode of negative regulation that has not been examined in *C. albicans* is the endocytosis of the pheromone receptor Ste2. In *S. cerevisiae* and humans, receptor endocytosis is a major form of signaling desensitization. In budding yeast, the binding of pheromone to Ste2 or Ste3 triggers rapid phosphorylation on the C terminal tail by kinases Yck1 and Yck2⁸⁻¹⁰. Ubiquitination then occurs near the site of phosphorylation, and the receptors are targeted for

degradation via clathrin-mediated endocytosis^{8,10}. *C. albicans* contains a Yck2 homolog, as well as homologs for proteins that bind ubiquitinated Ste2 and mediate its recruitment to clathrin coated pits (Ent2 and Ede1)¹¹. In the absence of pheromone, Ste2 is endocytosed at a slower rate^{10,12}. Recent studies in *S. cerevisiae* have demonstrated that a fluorescently-labeled synthetic α pheromone can be used to visualize receptor mediated endocytosis¹³. By adopting this technique for *C. albicans*, it may be possible to assess the rate at which Ste2 is internalized in opaque cells, and whether there are any differences in internalization rate between white and opaque cells either under mating or non-mating conditions.

Interestingly, the *C. albicans* Ste2 protein contains an extended cytoplasmic loop in its first intracellular tail (N-terminal), known as intracellular domain 1 (IC1) that is absent from *S. cerevisiae*¹⁴. Because this 55 amino acid tail is unique to *C. albicans*, it has been investigated for its role in mediating the white and opaque cell responses to pheromone. There are conflicting reports regarding its role in white cell biofilm formation^{14,15}. In mating projection assays, opaque cells show only a slight defect when lacking the IC1 domain¹⁵. Thus, it remains to be tested whether this *Candida*-specific domain has any role in regulating white and opaque phenotypes. This domain could recruit a repressor of the mating program in white cells. This hypothesis could be assayed by measuring mating projection formation or mating frequency in cells lacking the IC1 domain. Because mating frequency and mating projection formation are difficult to detect in natural white cells, this experiment could also be performed by deleting this domain in our engineered white strains overexpressing MAPK components.

Investigating G protein subunit levels and function

Our work in Chapter 3 shows that MAPK signaling is particularly sensitive to the amount of Ste4 present in the cell. Our strains expressing the *pACT1-STE4* construct have the highest mating efficiency of any strain overexpressing a single component, and integrating *pACT1-STE4* into a strain expressing *pADH1-CEK2* and *pACT1-CST5* further increases mating efficiency (Chapter 3, Figure 3.4, and Chapter 3, Methods). Curiously, protein levels of Ste4 vary by approximately two fold between white and opaque cells, and about 1.5 fold between white cells and strains ectopically expressing *STE4*. Furthermore, at the RNA level, Ste4 seems to be the “limiting component” to making active $\beta\gamma$ subunits, because it is expressed at much lower levels than the γ subunit (encoded by *STE18*). This expression pattern could, in part, explain why increasing Ste4 levels have such a dramatic effect on mating efficiency. Furthermore, the idea that Ste4 represents a key regulator subunit is consistent with studies in *S. cerevisiae* haploid cells, which demonstrate that overexpression of *STE4* alone, but not the γ subunit *STE18* alone, initiates a pheromone response¹⁶. However, the dynamics of G protein interactions and the specific role of Ste4 warrant further investigation, particularly given that the activating/inhibitory roles of the G complex subunits differ between *S. cerevisiae* and *C. albicans* (see Introduction).

One way to investigate G protein dynamics is to myc tag each complex component at its endogenous locus and measure protein levels in mating and non-

mating conditions in both white and opaque cells. Currently, it is unclear whether differences in mRNA expression hold true at the protein level, or whether translation of these components is tightly regulated to ensure 1:1 levels. It is also unclear whether protein levels of the α (*GPA1*) and γ (*STE18*) units are influenced by stimulation of signal transduction.

In addition to comparing protein levels among various G protein subunits, it will be important to directly address Ste4 binding partners after pheromone activation in white and opaque cells. As described in the Introduction of this thesis, Ste4 has at least three important effector functions in *S. cerevisiae*, which serve to amplify pheromone signaling. These functions are the recruitment of the scaffold Ste5 to the membrane, the recruitment of the p21-like kinase Ste20 to the membrane, and the recruitment of polarization machinery to the membrane (reviewed in¹⁷). Whether all these functions occur in opaque cells, and whether they occur differently in white versus opaque cells remain open questions. To test these questions, an immunoprecipitation (IP) of myc-tagged Ste4 could be performed, with immunoprecipitated proteins run on an SDS-PAGE gel. Silver staining of the gel could reveal proteins that are differentially bound to Ste4 in white and opaque cells. This difference would be assayed by the presence/absence of protein bands (or strength of protein bands) comparing lanes with Ste4-IP performed in white and opaque cells. These bands could be excised and identified by mass spectrometry. Immunoprecipitation of myc-tagged proteins and silver staining followed by mass spectrometry has recently been demonstrated to be an effective assay for identifying protein complexes in *C. albicans*¹⁸.

De-repression of the MAPK pathway in white cells

If overexpressing MAPK components enables mating in white cells, it should follow that de-repressing the MAPK pathway also promotes mating. In *S. cerevisiae*, the MAPK pathway can be de-repressed by genetic deletion of *DIG1* and *DIG2*, two paralogs that bind Ste12 in the nucleus, causing its repression. *C. albicans* contains one homolog of these proteins, orf19.1666 (now renamed *DIG1*). We created genetic deletions of this gene in white cells and measured the ability of the deletion mutants to undergo both the white and opaque cell pheromone responses (biofilm formation and mating, respectively). We also created strains in which the only copy of *DIG1* was under the control of the methionine promoter, allowing us to repress *DIG1* expression in medium containing methionine and cysteine. Using these repressable *DIG1* strains, we performed the same assays. Deletion of *DIG1* gave cells a phenotype that appeared intermediate between conventional white and opaque forms (Figure 4.2).

We found that deletion of *DIG1* significantly increased pheromone-stimulated biofilm formation. This result was observed for two independent isolates in which both copies of *DIG1* were deleted (Figure 4.4A, B), and also in *pMET3-DIG1* strains incubated in biofilm conditions plus the addition of methionine and cytosine (Figure 4.4C, D). In fact, biofilm formation was independent of pheromone addition in these strain backgrounds. This result likely indicates high induction of the pheromone MAPK pathway. However, *DIG1* also appears to have a role in negative

regulation of filamentation (Regan *et al.*, submitted), and the ability to filament is known to contribute to biofilm formation. Thus, this role also could have contributed to the strong biofilm forming phenotype.

We also performed quantitative mating assays on *dig1Δ/dig1Δ* cells by crossing them to either white or opaque *MTLα* cells. We found that cells deleted for *DIG1* mated almost as efficiently as opaque cells (Figure 4.5 compare right-most gray bar to right-most purple bar). These results support the hypothesis that de-repression of the MAPK pathway facilitates mating in white cells. To further investigate the role of *DIG1* in mating, *dig1Δ/dig1Δ* deletions could be made in a *wor1Δ/wor1Δ* background, which could rule out the possibility of cells switching to the opaque state.

A potential role for an Efh1-Efg1 interaction in mating

As discussed in Chapter 3, transcriptional levels of *CST5*, *STE4*, and *CEK2* are upregulated in opaque cells in non-mating, basal expression conditions (growth on SCD medium at 25°C). The promoter of *STE4* is bound by Wor1¹⁹, which makes it likely that Wor1 directly controls *STE4* expression. However, the mechanism by which *CST5* and *CEK2* are upregulated is less clear, particularly because their promoters are not predicted to be bound by any of the master white-opaque transcription factors ²⁰ (Chapter 3, Figure 25). In order to identify other transcription factors that regulate mating, we performed a screen using a library of

over 300 *C. albicans* transcription factors that are inducible by the addition of doxycycline to the medium¹. This library was constructed in white **a** cells auxotrophic for arginine. One to three isolates of each strain was mated to an opaque *MTL α* strain auxotrophic for adenine by incubation on Spider medium for 48 hours, followed by analysis on selection medium to test for formation of prototrophic products. This screen identified two factors, *EFH1* and *ASH1*, which increased mating in white cells above background levels. We deleted both copies of *EFH1* or *ASH1* in *wor1 Δ /wor1 Δ* deletion cells and repeated the quantitative mating assays in order to determine if they have a role in mating that is independent of white-opaque switching. While overexpression of *ASH1* in the *wor1 Δ /wor1 Δ* deletion background did not increase mating frequency, overexpression of *EFH1* increased mating frequency approximately 100-fold (Figure 4.6). Interestingly, this mating phenotype was more pronounced in **a** cells as compared to α cells (Figure 4.6). Furthermore, we found that *wor1 Δ /wor1 Δ* cells overexpressing *EFH1* exhibited a more rod-like phenotype, which was distinct from the conventional round phenotype of white cells (Figure 4.7).

In *C. albicans*, *EFH1* is a basic helix-loop-helix transcription factor that regulates an overlapping set of genes with the related transcription factor *EFG1*, a major regulator of the white-opaque switch, filamentation, and virulence²¹. In most cases, deletion of *EFH1* has no phenotype unless *EFG1* is also deleted, indicating that these two proteins are partially redundant. The change of cell shape in the *wor1 Δ /wor1 Δ* *pDOX-EFH1* strains leads us to hypothesize that Efh1 may occupy

promoter sites normally bound by Efg1, and that this binding pattern directly or indirectly inhibits mating. Thus, overexpression of *EFH1* could lead to displacement of Efg1 from critical promoters. If this is the mechanism responsible for increased mating in *wor1Δ/wor1Δ dox-EFH1* strains, then this increase should be abrogated in cells that are also lacking *EFG1*. We are currently working on constructing these strains and performing mating assays. We are also performing qPCR to test *EFH1* levels in *pDOX-EFH1 a* and α cells to investigate why this phenotype is more pronounced in **a** cells.

The benefits of existing in different ploidy states

In Chapter 2, we propose that *C. lusitaniae* exhibits a fused sexual cycle in order to promote propagation in the haploid state over the diploid state. This proposal raises the broader question as to why a cell would “want” (or be evolutionarily wired) to maintain the haploid state? Are there advantages to existing as a haploid over other ploidy states?

From one perspective, a diploid, heterozygous genome can be seen as more advantageous, because it protects from deleterious alleles. Indeed, although haploid cells exist in *C. albicans*, they are far less fit both *in vitro* and in mouse models of infection²². Diploid cells are more prone to certain types of mutations and genomic rearrangements, such as large deletions or aneuploidies²³, which may confer an advantage in some environments. Conversely, in asexually reproducing

populations, haploid cells are predicted to evolve faster, because any arising beneficial allele is automatically expressed. In diploid cells, the arising beneficial allele would have to have a dominant effect over the original allele that at that locus^{24,25}. This theory was tested across seven stressful environments in *S. cerevisiae* isogenic haploid and diploid cells. Haploids evolved significantly faster in three environments tested, and the trend held true for all seven stressors²⁴.

However, additional reports have refined or conflicted this study. For example, the concentration of a stressor can also have an effect on mutation rates in haploid and diploid populations. One study found that *S. cerevisiae* diploid cells evolved fluconazole resistance more rapidly when the fluconazole concentration was low, but haploid cells evolved faster when the fluconazole concentration was higher²⁶. A recent study compared the influence of ploidy on fitness across a wide range of environments in two yeast species, *S. cerevisiae* and *Saccharomyces paradoxus*. This study concluded that in both species, it is a fine interplay between ploidy and environment that determines fitness, rather than any one ploidy state having an advantage. This conclusion is reflected throughout the tree of life, because ploidy is inherently dynamic; varying in different organisms or within different times of one organism's life.

Closing remarks

In this work, I present two different mechanisms of regulation of sexual reproduction in related human fungal pathogens. The *Candida* species represent a

particularly interesting clade in which to study mating and meiosis, in order to understand the benefits and costs associated with each process and appreciate the diversity of sexual reproduction between closely related species. Although these species have clearly retained the capacity to sexually reproduce, they exert tight control over their reproductive cycles, which contrasts with the control seen in the model yeast *S. cerevisiae*. This regulation could be a consequence of adapting to living within the human host. For example, *C. albicans* may have developed a white-opaque switch to ensure that mating occurs infrequently and mating genes are only expressed when necessary. This pattern would help preserve beneficial combinations of alleles by ensuring clonal propagation, but still support sexual reproduction when stressful conditions are encountered and allelic recombination is beneficial. The fact that most human fungal pathogens maintain the ability to undergo sexual reproduction makes it likely that this process plays a role in ensuring fitness.. Continued investigation into understanding how and when sexual reproduction occurs in *Candida* species will further contribute to our understanding of the diverse mechanisms of sexual reproduction and may provide a link between sexual reproduction and virulence.

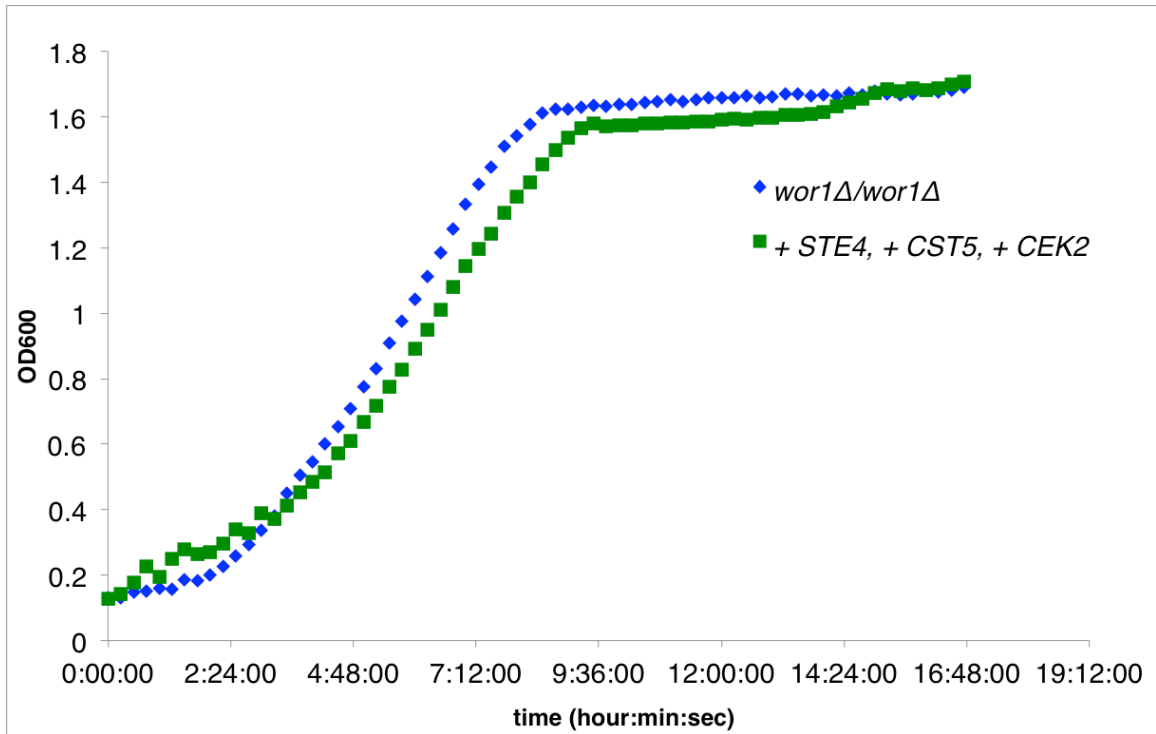


Figure 4.1: Growth rates of white cells and engineered cells grown at 30C in YPD.

OD₆₀₀ (absorbance at 600 nanometers) of triple overexpression strains (green) and white locked cells (blue) cultured in YPD liquid medium over sixteen hours. The genotype of the triple overexpression strains is: *wor1Δ/wor1Δ pACT1-STE4, pACT1-CST5, pADH1-CEK2*. Timepoints are measured every 15 minutes for 18 hours.

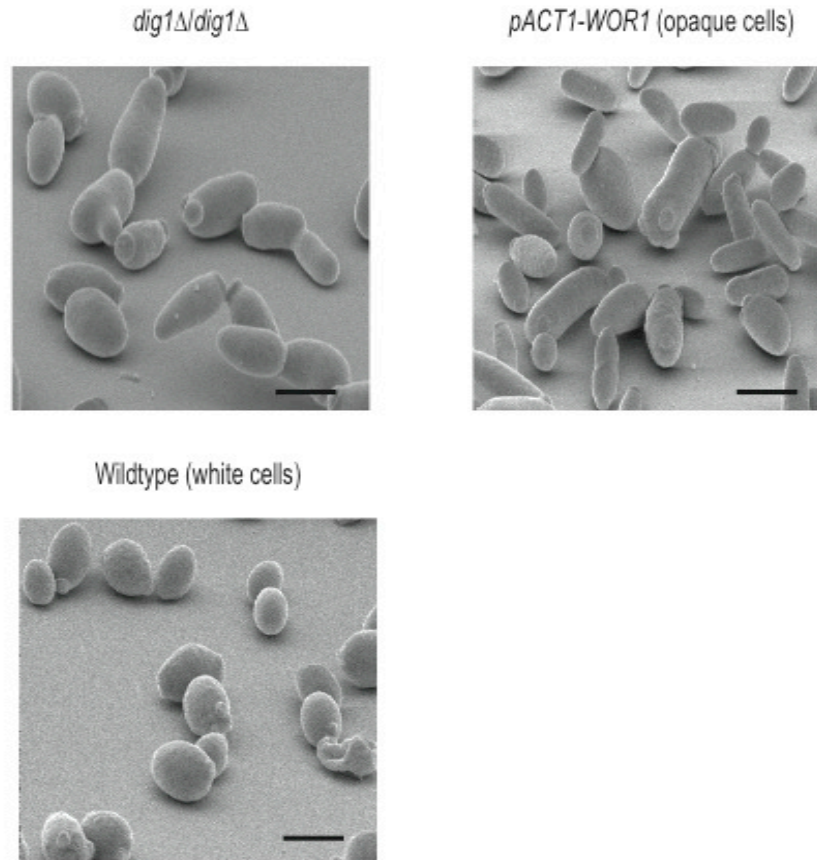


Figure 4.2: Scanning electron microscopy reveals intermediate morphology of *dig1Δ/dig1Δ* cells.

C. albicans were inoculated onto YPD agar and incubated at 30°C for 24 hours before processing for scanning electron microscopy. Comparison with locked opaque cells and wild type white cells is shown. Scanning electron microscopy images were taken by Matthew P. Hirakawa.

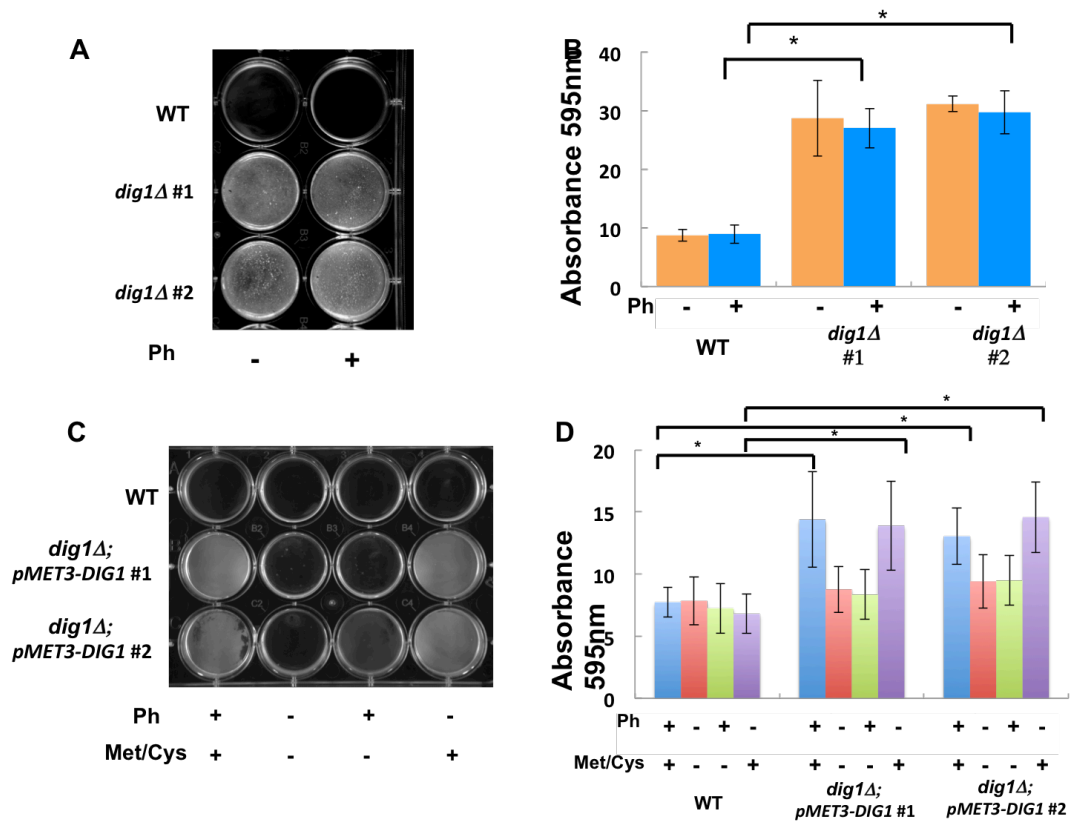


Figure 4.4: Enhanced biofilm formation in the *dig1Δ/dig1Δ* mutant.

A. Representative image of biofilm formation in two independent *dig1Δ/dig1Δ* mutants and the wild type control. Cells were grown as pheromone-stimulated biofilms in SCD media for 48 hours at room temperature without shaking. Ph, 10mM α pheromone.

B. Cells were washed with PBS and adherence quantified by crystal violet staining. *= p <.01, Student's t-test. error bars = standard deviation. n= 3 biological replicates.

C. *pMET3-DIG1* cells were grown as pheromone stimulated biofilms in Lee's + glucose media under repressive (+2.5mM methionine, +2.5mM cysteine) or non-repressive conditions for 48 hours at room temperature without shaking.

D. Quantitation of adherence by crystal violet staining as described above. * = p <0.05, one-way ANOVA. Comparisons made between the same condition. Error bars = standard deviation. n = 5 biological replicates

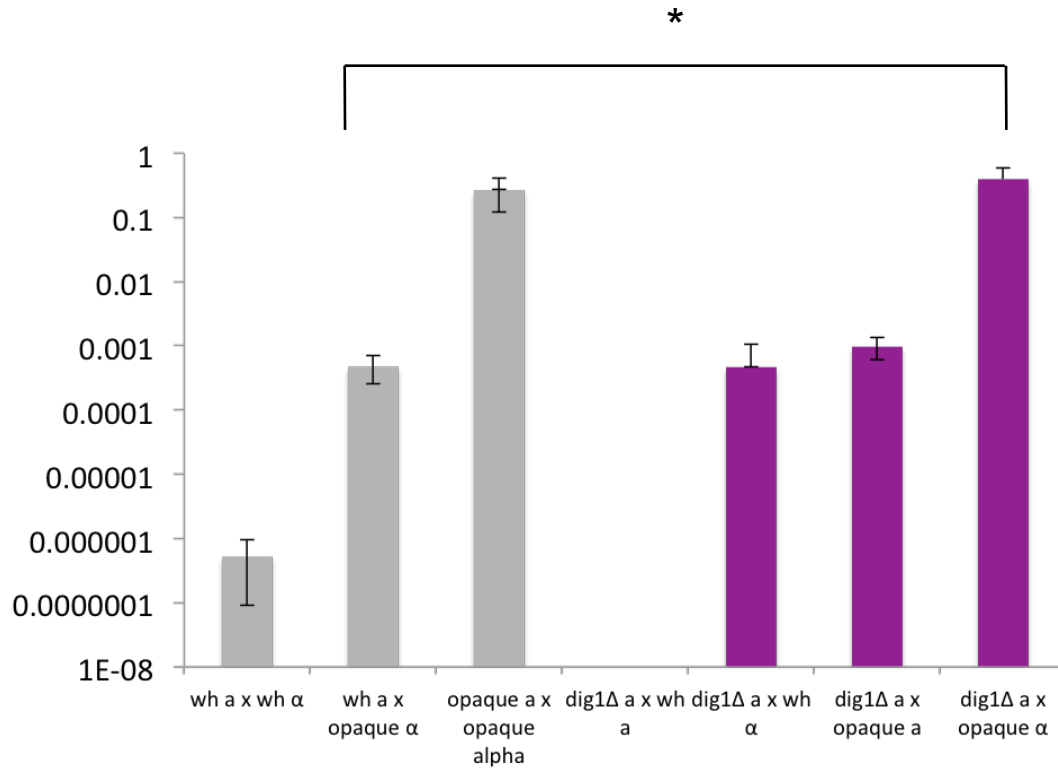


Figure 4.5: Mating in the *dig1Δ/dig1Δ* mutant.

Quantitative mating assays of crosses between the *dig1Δ/dig1Δ MTL α* strain and an opaque *MTL α* mating tester show an increase in mating frequency compared with the wild type white-opaque cross. The assay also identifies low frequency homothallic mating between the *dig1Δ/dig1Δ MTL α* and the *MTL α* mating tester. wh, white cells. op, op cells. * = $p < .05$, Kruskal- Wallis. error bars, s.e.m. $n = 3-6$ biological replicates per cross.

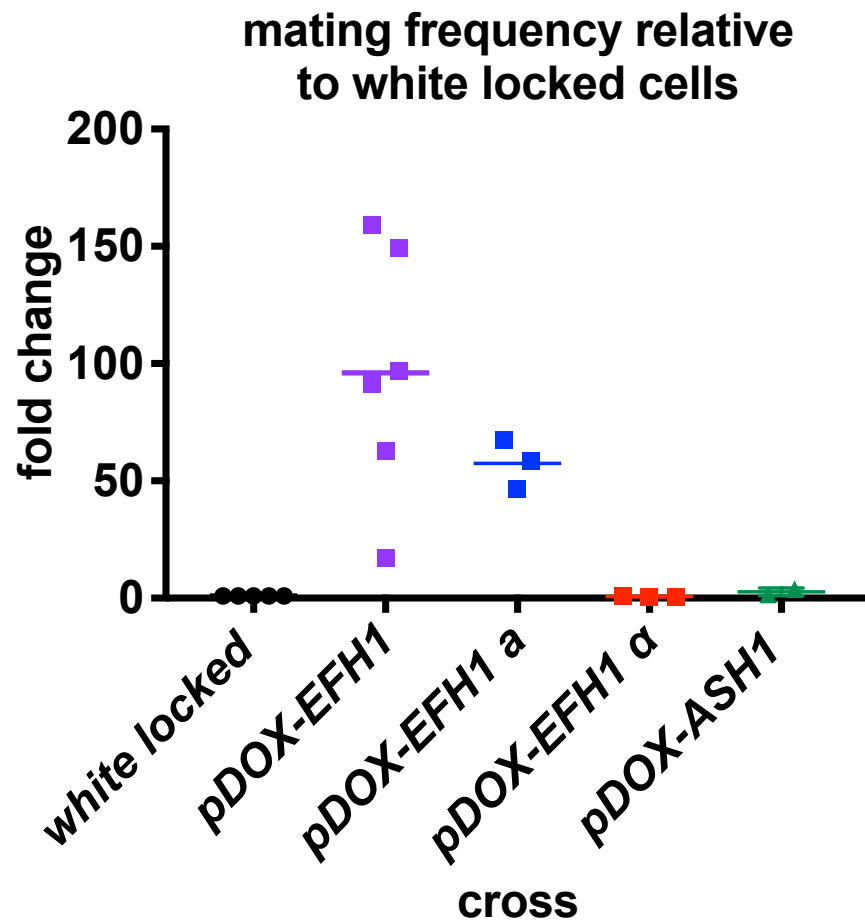


Figure 4.6: Mating frequency locked white cells with inducible expression of EFH1.

Locked white strains are *wor1Δ/wor1Δ*. Each symbol is a biological replicate. Horizontal bars represent the mean. Mating assays were performed as described in Methods, Chapter 3.

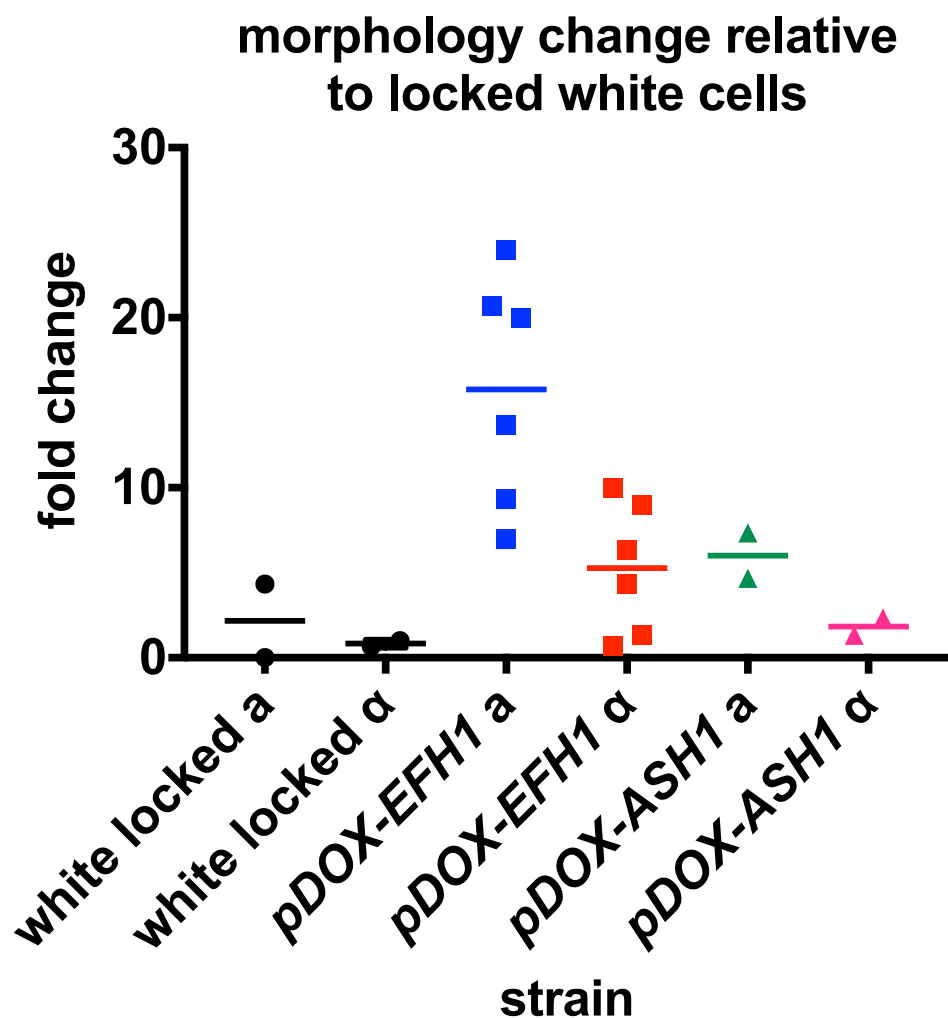


Figure 4.7: Morphology change in locked white cells with inducible expression of EFH1.

Morphology change is the number of rod-shaped cells, counted by microscopy. Each symbol is a biological replicate. 300 cells were counted per biological replicated. The horizontal bar represents the average. Locked white cells are *wor1Δ/wor1Δ*.

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Appendix 1: Supplemental Discussion for “Convergent Evolution of a Fused Sexual Cycle Promotes the Haploid Lifestyle”

Analysis of meiosis in C. lusitaniae

C. lusitaniae **a** and α cells undergo mating on dilute PDA (potato dextrose agar) medium to form diploid **a**/ α cells, that then typically proceed directly into meiosis^{10,42}. However, diploid **a**/ α cells can be isolated from PDA and stably propagated on rich YPD medium. Diploid cells can then be induced to re-enter the sexual cycle by growth on PDA medium (see Chapter 2, Supplemental Figure 2.2a, b). Microscopic analysis revealed that *C. lusitaniae* **a**/ α cells grown on PDA became enlarged by 8 hours, largely due to increased size of the vacuole, and exhibited a distinct change in shape after 12 hours. This morphological change was quantified (see Supplemental Figure 2.2c) in which cells demonstrated polarized growth, similar to that of haploid cells forming mating projections in response to pheromone. Dyad spores became visible at 18-36 hours (Supplemental Figure 2.2b, c). Deletion of *STE12* blocked all morphological changes (including both polarized growth and sporulation) in *C. lusitaniae* diploid strains, while reintegration of the *STE12* gene restored these phenotypes (Supplemental Figure 2.2c).

Genome-wide expression changes during *C. lusitaniae* meiosis are shown in Supplemental Figure 2.2d and are compared to those in *S. cerevisiae* in Figure 2.1e. The most striking aspect of this comparison was the upregulation of orthologs of conserved *S. cerevisiae* mating genes during *C. lusitaniae* meiosis (Figure 2.1c, f). On average, there was a 16-fold upregulation of *C. lusitaniae* MAPK and pheromone-

processing genes at the 8 hour time point during meiosis, whereas the equivalent genes are not induced during *S. cerevisiae* meiosis. Thus, there was increased expression of pheromone and pheromone processing genes during both mating and meiotic programs of *C. lusitaniae* (Supplemental Figure 2.1). Together, these observations led us to hypothesize that *STE12* is a key regulator of meiosis, as well as mating, in *C. lusitaniae*.

Transcriptional profiling of C. lusitaniae ime2Δ/ime2Δ mutants in meiosis

C. lusitaniae **a**/α diploids lacking the *IME2* gene failed to progress through meiosis and sporulate (Figure 2.2a, b), establishing the role of this gene as a conserved regulator of meiosis in hemiascomycete yeasts. To further investigate the role of *IME2* in *C. lusitaniae*, the transcriptional profile of diploid *ime2Δ/ime2Δ* **a**/α cells was compared to that of wildtype cells at early, middle, and late stages of meiosis (Supplemental Figure 2.3). Many of the genes induced during wildtype meiosis were similarly induced in the *ime2Δ/ime2Δ* mutant background, although notable differences were also observed and are discussed here.

Ime2 plays multiple roles in promoting progression through meiosis in *S. cerevisiae*³. It is involved in premeiotic DNA synthesis, as synthesis is delayed by 8-9 hours in *ime2Δ/ime2Δ* diploids⁴³. This role is mediated by Ime2 degradation of Sic1, which allows increased activity of a kinase/Clb5/Clb6 complex that promotes DNA synthesis⁴⁴. The Clb5, Clb6 cyclins of *S. cerevisiae* are missing in the *Candida* clade of yeast⁹, and the equivalent function is performed by Clb4 in *Candida albicans*⁴⁵. We

observed that several cyclin genes are induced during meiosis in *C. lusitaniae*, including *CLB4* and *CLN1* (Supplemental Figure 2.3b). Interestingly, expression of *CLB4* was compromised in the *C. lusitaniae ime2Δ/ime2Δ* mutant, suggesting that premeiotic DNA synthesis could be affected. Expression of several other cell cycle-dependent genes was decreased in the *ime2* mutant including *APC4*, *CDC3*, *CDC10*, and *CDC14*, while expression of *CLN1-3* genes was increased in the mutant (Supplemental Figure 2.3b). Together, these results indicate that regulation of cell cycle genes during *C. lusitaniae* meiosis is defective in the absence of *IME2*.

In addition to its role in regulating DNA synthesis, *S. cerevisiae* Ime2 directly promotes the expression of middle meiosis genes by phosphorylating Ndt80 and Sum1 transcription factors. Sum1 inhibits *NDT80* expression and Ime2 phosphorylation of this protein removes this repression, thereby increasing *NDT80* transcription⁴⁶. Phosphorylation of Ndt80 by Ime2 also directly increases Ndt80 transcriptional activity and expression of middle meiosis genes²⁰. We observed decreased expression of *NDT80* (*CLUG_05634*) in *C. lusitaniae ime2Δ/ime2Δ* mutants (Supplemental Figure 2.3b) as well as a reduction in expression of certain middle meiosis genes (e.g. *SPS1* and *SPS4*) and late meiosis genes (e.g. *DIT1* and *DIT2*, Supplemental Figure 2.3c). The defect in *NDT80* expression could contribute to the inability of *C. lusitaniae ime2Δ/ime2Δ* mutants to complete meiosis. Middle meiosis genes include the B type cyclins in *S. cerevisiae*, so it is possible that the defective expression of cyclin genes in *C. lusitaniae ime2Δ/ime2Δ* mutants is a direct result of reduced Ndt80 activity.

Transcriptional profiling of C. lusitaniae ime2Δ mutants during mating

The mating frequency of *C. lusitaniae ime2Δ a* and α mutants was significantly decreased relative to wildtype cells (Figure 2.2d). Wildtype or *ime2Δ a* and α cells were therefore mixed and plated onto PDA medium to profile gene expression changes during mating. Cells were harvested after 4 hours on PDA, and mating expression profiles compared to cells grown on YPD (non-mating) medium. As shown in Fig. 10d, most genes showed a very similar pattern of expression in both wildtype and *ime2Δ* cells during mating. This pattern is consistent with *ime2Δ* mutants being able to undergo effective formation of mating zygotes similar to wildtype cells (Figure 2.2e). Presumably the defect in *ime2Δ* mating occurs at a later step (post cell fusion) and is not revealed by transcriptional data. A post-cell fusion defect is also supported by the fact that unilateral matings (wildtype x *ime2Δ*) are not mating defective, while bilateral matings (*ime2Δ* x *ime2Δ*) are mating defective (Figure 2.2d).

STE12 is required for C. lusitaniae to undergo meiosis

Deletion of the *STE12* gene prevented *C. lusitaniae a/a* cells from undergoing meiosis and sporulation (Figure 2.3e, f). To determine whether the defect in meiosis was due to loss of *STE12*, independent deletions of *STE12* were constructed and tested for their ability to complete meiosis. In each mutant, deletion of both *STE12* alleles resulted in a block in meiosis, as measured by the frequency of cycloheximide resistant progeny formation and the absence of dyad meiotic spores (Figure 2.3e, f).

Supplemental Figure 2.5 shows the frequency of meiosis following either the cycloheximide resistance marker (ChxR) or the adenine marker (*ADE2*). For both markers, loss of the *STE12* gene essentially abolished the formation of haploid meiotic progeny. In addition, the *C. lusitaniae* *STE12* gene was reintegrated into a *ste12Δ/ste12Δ* diploid mutant and shown to restore the ability of these mutants to undergo meiosis and sporulation (Supplemental Figure 2.5). Together, our results establish that the *STE12* gene is essential for *C. lusitaniae* **a**/ α diploids to undergo meiosis and sporulation.

Rewiring of regulatory circuits controlling meiosis in yeast

Ste12 is a regulator of mating in several hemiascomycete yeasts, yet we demonstrate here that this transcription factor also controls meiosis in *C. lusitaniae*. To determine when Ste12 evolved control of meiosis in hemiascomycete species, we constructed *ste12Δ/ste12Δ* mutants in *S. cerevisiae*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Yarrowia lipolytica*. Analysis of Ste12 orthologs in multiple species allows us to infer if the meiotic function of Ste12 represents an ancestral role for this protein, or if it is more likely the result of a recent evolutionary event in the lineage leading to *C. lusitaniae*. The four species chosen represent a diverse collection of hemiascomycete yeast and all undergo full sexual cycles, including both mating and meiosis. Diploid **a**/ α *ste12Δ/ste12Δ* mutants were incubated on meiosis-inducing medium and analyzed for the ability to form meiotic progeny. As shown in Supplemental Figure 2.6, loss of Ste12 did not inhibit the formation of

meiotic products in *S. cerevisiae*, *K. lactis* or *P. pastoris*. For *Y. lipolytica*, non-quantitative analysis of meiosis revealed that *ste12Δ/ste12Δ* mutants in this species were also able to progress through meiosis and generate sexual spores (data not shown). In contrast, Ste12 is essential for sexual mating in *S. cerevisiae*^{5,47}, *K. lactis*, and *P. pastoris* (Supplemental Figure 2.6) in addition to *C. lusitaniae* (Figure 2.3). We therefore conclude that Ste12 is as a conserved regulator of mating in many hemiascomycete species, while also evolving specialized control over meiosis relatively recently in the *C. lusitaniae* lineage.

We further propose that Ste12 control of *C. lusitaniae* meiosis could have occurred as a direct result of the loss of the conserved regulator, *MTLα2*. In many hemiascomycete yeasts, $\alpha 2$ forms a heterodimer with **a1** to restrict the expression of haploid-specific genes (hsgs). The hsgs are therefore expressed specifically in **a** or α cell types, but not in **a**/ α cells²⁶. The most conserved hsgs encode components of the pheromone MAPK signaling pathway (e.g. *STE4*, *STE18*, and *GPA1*²⁷), and regulation by **a1**/ $\alpha 2$ therefore ensures that these genes are not expressed in **a**/ α cells. However, loss of $\alpha 2$ from the *C. lusitaniae* genome⁹ could have resulted in permissive expression of hsgs in *C. lusitaniae* **a**/ α cells. In this way, reconfiguration of the *MTL* locus was perhaps an important precursor to the rewiring of the meiotic program seen in the *C. lusitaniae* lineage. Indeed, it was previously proposed that the transient diploid state in *C. lusitaniae* could make repression of hsgs by $\alpha 2$ obsolete^{9, 10, 13, 48}. We also note that loss of $\alpha 2$ appears to have occurred at least three independent times in the *Candida* lineage⁵³. Whether loss of $\alpha 2$ correlates with meiotic expression of hsgs in these species and/or the existence of a fused

sexual cycle remains to be determined. Despite loss of $\alpha 2$, *MTL* control of meiosis still occurs in *C. lusitaniae*, as the **a1** gene is necessary for this process and both **a/a** and α/α diploids are unable to undergo meiosis¹⁰. It therefore remains to be seen how **a1** controls meiosis in *C. lusitaniae*, and if it has been reprogrammed to act in concert with the $\alpha 1$ gene as hypothesized¹⁰.

Finally, we note that examples of meiotic rewiring have recently been described in other hemiascomycete species. In particular, *K. lactis* exhibits a coupled mating and meiosis cycle similar to that in *C. lusitaniae*; mating of haploid **a** and α cells occurs in response to starvation, and diploid **a/ α** mating products immediately enter meiosis. In this case, however, coupling of mating and meiosis occurs by a very different mechanism to that in *C. lusitaniae*. Whereas in *S. cerevisiae* starvation turns on expression of *IME1* in diploids, starvation induces expression of *RME1* in haploid *K. lactis* cells, and this drives expression of hsgs and promotes mating²⁷. Once mated, *RME1* is repressed by **a1/ $\alpha 2$** in *K. lactis* and cells progress directly into meiosis²⁷. Thus, the integration of key starvation signals has diverged between these two species, and is thought to be a direct reflection of their contrasting lifestyles. While *S. cerevisiae* exists preferentially as a diploid species, *K. lactis* has a predominantly haplontic lifestyle where cells propagate preferentially as haploids. Together, these studies illustrate the plasticity of mechanisms regulating sexual reproduction, and demonstrate how alternative rewiring of circuits can directly promote haploid versus diploid lifestyles.

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