Epigenetic control of pheromone MAPK signaling determines sexual fecundity in *Candida albicans*

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Several pathogenic *Candida* species are capable of heritable and reversible switching between two epigenetic states, "white" and "opaque." In *Candida albicans*, white cells are essentially sterile, whereas opaque cells are mating-proficient. Here, we interrogate the mechanism by which the white-opaque switch regulates sexual fecundity and identify four genes in the pheromone MAPK pathway that are expressed at significantly higher levels in opaque cells than in white cells. These genes encode the β subunit of the G-protein complex (*STE4*), the pheromone MAPK scaffold (*CST5*), and the two terminal MAP kinases (*CEK1/CEK2*). To define the contribution of each factor to mating, *C. albicans* white cells were reverse-engineered to express elevated, opaque-like levels of these factors, either singly or in combination. We show that white cells co-overexpressing *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. Moreover, engineered white cells recapitulate the transcriptional and morphological responses of opaque cells to pheromone. These results therefore reveal multiple bottlenecks in pheromone MAPK signaling in white cells and that alleviation of these bottlenecks enables efficient mating by these "sterile" cell types. Taken together, our findings establish that differential expression of several MAPK factors underlies the epigenetic control of mating in *C. albicans*. We also discuss how fitness advantages could have driven the evolution of a toggle switch to regulate sexual reproduction in pathogenic *Candida* species.

mating | sexual reproduction | signaling bottlenecks | transcriptional regulation | phenotypic switching

Mitogen-activated protein kinase (MAPK) cascades are used by eukaryotes to respond to a variety of extracellular signals, and there has been considerable interest in defining how these pathways regulate diverse processes such as apoptosis, stress adaptation, and tumorigenesis. Many insights have been gleaned from investigation of MAPK signaling in the model yeast *Saccharomyces cerevisiae*. Here, studies have examined pathway dynamics (1, 2), signaling crosstalk (3, 4), scaffold proteins (5–7), switch-like versus graded responses (6, 8–10), and pathway re-engineering (11, 12). Analysis of MAPK signaling in fungal species has also provided evidence as to how these pathways diverged during evolution (13, 14).

The pheromone MAPK pathway in *S. cerevisiae* has been extensively used as a paradigm for signal transduction (for reviews, see refs. 15 and 16). Here, extracellular pheromone activates a G-protein–coupled receptor, driving dissociation of Gβ and Gγ subunits from an inhibitory Gα subunit. The activated Gβγ complex recruits a scaffold protein, Ste5, to the membrane, tethered to which are the kinases Ste11, Ste7, and Fus3. 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 repressors Dig1 and Dig2, thereby activating Ste12 and initiating a transcriptional response to pheromone. In addition, pheromone signaling induces formation of mating projections via Gβγ interactions with Far1 and the cell polarization machinery, and activated Far1 also mediates cell-cycle arrest by interfering with Cdk activity.

Candida albicans is a hemiascomycete yeast related to *S. cerevisiae*, although these species diverged ∼235 Mya (17). *C. albicans* is a commensal of humans that is also capable of causing both superficial and invasive opportunistic infections (18). Many factors contribute to the ability of *C. albicans* to grow and invade host niches, including its ability to adopt distinct phenotypic states. A prominent example is provided by the white-opaque switch, in which *C. albicans* cells undergo a heritable, epigenetic transition between two alternative cell states termed "white" and "opaque" (19). Stochastic switching between the two states occurs, and white cells are essentially sterile whereas opaque cells mate up to a million times more efficiently (20). Mating of *C. albicans* opaque cells shows parallels to mating in *S. cerevisiae*, as pheromone signaling induces cell-cycle arrest, formation of mating projections, and expression of mating genes (21–24). In contrast, white cells respond to pheromone but do not undergo mating and instead can form "sexual biofilms" (25). Many of the same MAPK components transduce the pheromone signal in both white and opaque cells (26, 27), yet the mechanism by which the two cell states exhibit distinct mating capacities is unknown.

Regulation of the white-opaque switch involves a highly interconnected network of transcription factors (28) with additional

Significance

A central theme in biology is to understand how different signaling outputs can be accomplished by changes to signal transduction pathways. Here, we examined epigenetic differences between two cell states in the human fungal pathogen *Candida albicans*. We show that cells in the "white" state are sterile due to multiple bottlenecks in MAPK signaling relative to mating-competent "opaque" cells. Alleviation of these bottlenecks by reverse engineering effectively converts sterile white cells into sexually competent cells. These results have broad implications for understanding how epigenetic changes can impact MAPK expression and signaling output, including events associated with tumorigenesis. We also propose a model for how the white-opaque switch gained control of sexual reproduction in *Candida* during evolution.

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control mediated through chromatin modifications and the Mediator complex (29–31). The master regulator of the switch is Wor1, the expression of which is both necessary and sufficient to promote formation of the opaque state (32–34). The white-opaque switch is also regulated by the *MTL* (mating-type-like) locus. In *MTL***a**/α cells, the presence of the **a**1/α2 complex represses *WOR1* expression so that only *MTL* homozygous cells are fully competent for switching to the opaque state (20, 35). This switch has a profound effect on gene expression as more than 1,000 transcripts are differentially regulated between white and opaque states, representing one-sixth of the *C. albicans* transcriptome (36).

In this work, we define the mechanism by which the *C. albicans* white-opaque switch determines mating competence. We focus on differences in pheromone MAPK signaling between the two cell types and reveal that opaque cells express several key components at higher levels than in white cells. Ectopically increasing the expression levels of three MAPK components resulted in white-locked cells mating several orders of magnitude more efficiently, close to the mating efficiency of opaque cells. Analysis of protein levels established that ectopic expression of MAPK components in white cells produced expression levels comparable to those in opaque cells. These experiments therefore reveal how relatively small differences in the expression levels of key MAPK components can produce large differences in signaling output. Finally, given the conservation of MAPK-signaling pathways from yeast to humans, our results have implications for how the perturbation of signaling pathways can drive events such as carcinogenesis.

Results

Comparison of *C. albicans* White and Opaque 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 and show no obvious morphological response to pheromone (20). To address the mechanism underlying these differences, the published transcriptomes of white and opaque cells were compared (36). Approximately 1,300 genes are differentially expressed between white and opaque cells, indicative of massive transcriptional differences between cell types. We focused on the pheromone MAPK cascade as this pathway plays a conserved role in regulating sexual reproduction in *C. albicans* and other fungal species (23, 37) and identified four genes that are more than threefold up-regulated in opaque cells relative to white cells. These genes are *STE4*, encoding the β subunit of the heterotrimeric G-protein complex (8-fold up-regulated); *CST5* (ortholog of *ScSTE5*), encoding the MAPK scaffold protein (3.8-fold up-regulated); and *CEK1*/*CEK2* (orthologs of *ScKSS1*/*ScFUS3*), encoding the terminal MAP kinases (7.7- and 10.4-fold up-regulated, respectively) (Fig. 1).

Overexpression of MAPK Components Allows White-Locked Cells to Secrete and Respond to Pheromones. We hypothesized that limited expression of one or more MAPK components restricts pheromone signaling in *C. albicans* white cells. To test this, both copies of *WOR1* were deleted to lock **a**- and α-cell types in the white state (32–34), and strains were engineered to ectopically express each of the four MAPK components listed above, either singly or in combination. *STE4*, *CST5*, and *CEK1* were placed under the *ACT1* promoter and *CEK2* under the *ADH1* promoter, as these are constitutive promoters that confer high levels of gene expression. The resulting overexpression strains were analyzed for their ability to form mating projections and to undergo cell–cell conjugation in comparison with control white and opaque cells.

Analysis of mating projection formation was performed by coincubation of **a** and α cells under conditions conducive to pheromone signaling and mating (38). In control experiments, coincubation of opaque **a** and α cells resulted in ∼70% of cells forming polarized mating projections, whereas <0.3% of white-locked (*wor1*Δ/*wor1*Δ) **a** and α mixtures showed a morphological response (Fig. 2 *A* and *B*). When tested individually, only ectopic expression of *STE4* in mixtures of white-locked **a** and α cells resulted in detectable formation of mating projections (5% of cells

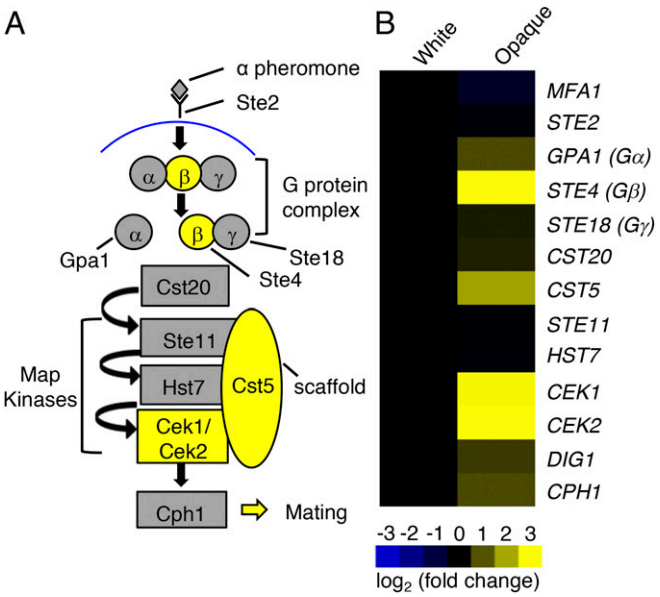

Fig. 1. Comparison of the pheromone MAPK pathway in *C. albicans* white and opaque cells. (*A*) Schematic of the *C. albicans* pheromone MAPK pathway in *MTL***a** cells. Components highlighted in yellow are those that are transcriptionally up-regulated in opaque cells relative to white cells. (*B*) Analysis of RNA-sequencing data (36) indicates that four pheromone MAPK components—*STE4*, *CST5*, *CEK1*, and *CEK2*—are expressed at more than threefold higher levels in *C. albicans* opaque cells relative to white cells.

formed projections) (Fig. 2*B*). In marked contrast, however, ectopic expression of both *STE4* and *CEK2* resulted in 20% of white-locked **a** and α cells forming mating projections, while coexpression of three MAPK components (*STE4*, *CST5*, and *CEK2*) resulted in 35% of white-locked cells forming mating projections, a frequency that is only twofold less than that of control opaque cells (Fig. 2 *A* and *B*). (We note that *CEK1* was not included in combinatorial assays because overexpression of this factor did not enhance mating responses in any of the experiments performed in this study.) We infer from these data that ectopic expression of MAPK factors enables mixtures of white-locked **a** and α cells to secrete and respond to pheromone, and combinatorial expression of multiple MAPK components can produce additive or even synergistic increases in pheromone responses.

To directly determine the role of pheromone in mediating these morphological responses, we also exposed white-locked **a** cells to synthetic α pheromone. Similar quantitative responses were observed to those described above, such that white-locked **a** cells engineered to overexpress MAPK components underwent an increased morphological response to α pheromone (*SI Appendix*, Fig. S1). This finding supports the idea that white cells can be engineered to form mating projections in response to exogenous pheromone through the overexpression of certain MAPK components.

White-Locked Cells Overexpressing MAPK Components Undergo Increased Mating. Engineered overexpression strains were analyzed for their abilities to undergo cell–cell conjugation in comparison with control white-locked and opaque cells. Mating assays were performed by coincubation of auxotrophic **a** and α cells on Spider medium for 5–7 d, and the fraction of prototrophic mating products was quantified. Control white-locked strains underwent mating at very low efficiency, with an average mating frequency of 4.7×10^{-6}, whereas control opaque cells mated ∼75,000 times more efficiently, with an average mating frequency of 0.35 (Fig. 2*C*). These results are consistent with previous comparisons of mating between white and opaque cells (20, 38). As was seen for mating projection formation, mating frequencies increased with the forced overexpression of specific

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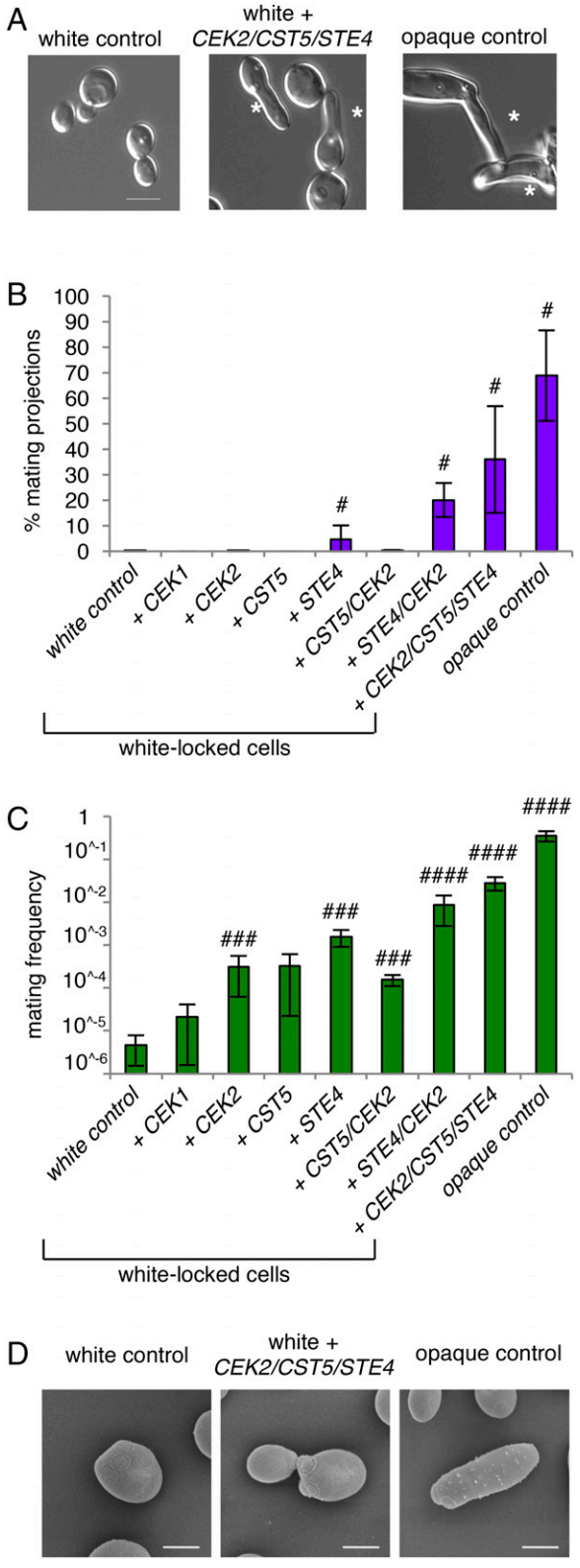

Fig. 2. Quantification of mating in *C. albicans* white cells ectopically expressing pheromone MAPK components. (*A*) Representative images of mating projection formation following coincubation of **a** and α cells that were white-locked (white control) and white-locked and overexpressing *CEK2/CST5/STE4* (white + *CEK2/CST5/STE4*) or wild-type opaque (opaque control). Cells were coincubated on Spider medium for 24 h and imaged. Asterisks indicate cells with mating projections. (Scale bar, 5 μm.) (*B*) Quantification of mating projections in mixtures of white-locked **a** and α cells overexpressing MAPK genes, either singly or in combination, compared with white-locked parental cells (white control) or opaque cells (opaque control). Significant differences are relative to the white control. $^{\#}P < 0.05$, Kruskal–Wallis (Mann–Whitney pairwise). $n = 3$ biological replicates. Error bars = SD. (*C*) Conjugation frequencies between white-locked **a** and α cells overexpressing MAPK genes, either singly or in combination, compared with white-locked cells (white control) or opaque cells (opaque control). Significant differences are relative to the white-locked control. $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, Kruskal–Wallis (Mann–Whitney pairwise). n = minimum of six biological replicates. Error bars = SEM. (*D*) Scanning electron micrographs of white-locked **a** cells (white control), white-locked **a** cells overexpressing *CEK2*, *CST5*, and *STE4* (white + *CEK2/CST5/STE4*), and opaque **a** cells. Cells were grown under nonmating, vegetative conditions. (Scale bar, 2 μm.)

pheromone MAPK components. Thus, white-locked strains overexpressing *CEK2* or *CST5* showed a 66- or 67-fold increase in mating efficiencies, respectively, whereas overexpression of *CEK1* had little effect on mating (Fig. 2*C*). 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 over parental strains (Fig. 2*C*). Overexpression of some combinations of MAPK components resulted in higher mating efficiencies than overexpression of individual components. For example, overexpression of *CEK2*/*STE4* resulted in an 1,800-fold increase in mating, whereas overexpression of all three MAPK components (*CEK2*, *CST5*, and *STE4*) produced a mating frequency of 2.9×10^{-2} (Fig. 2*C*). This frequency is within an order of magnitude of that of wild-type opaque cells and represents a 6,000-fold increase over the mating frequency of parental white-locked *wor1Δ/wor1Δ* strains.

Importantly, even upon combinatorial overexpression of the three MAPK genes and with mating behaviors traditionally attributed only to opaque cells, the white-locked cells did not exhibit the classic morphologies associated with opaque cells. That is, vegetative white-locked cells overexpressing *CEK2*, *CST5*, and *STE4* maintained their round shapes and failed to develop the bean-shaped morphology or pimpled cell surface characteristic of opaque cells (Fig. 2*D*). Taken together, these experiments establish that elevating the levels of pheromone MAPK components can have dramatic effects on the mating efficiency of *C. albicans* white cells and that combinatorial overexpression of only three components is sufficient to drive white-cell mating to levels approaching those of opaque cells. We also note that results from quantitative mating assays (Fig. 2*C*) parallel those of mating projection formation (Fig. 2*B*), indicating that enhancement of pheromone MAPK signaling had a similar impact on both of these processes in white cells.

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 (*i*) white-locked cells; (*ii*) white-locked cells ectopically overexpressing *STE4*, *CST5*, and *CEK2* (designated "white-OE" cells); and (*iii*) control opaque cells. Experiments were performed by coincubation of the corresponding **a** and α strains on Spider medium, and transcriptomes were compared at 8 h.

We found that mating mixes of opaque **a** and α cells differentially expressed 1,615 genes relative to equivalent mixes of white-locked cells (false discovery rate < 0.05, Benjamini–Hochberg corrected *P* value) (Dataset S1). Gene ontology (GO) term analysis revealed several gene categories that were significantly enriched in the opaque cell transcriptome including pheromone response, sexual reproduction, and G-protein response (*SI Appendix*, Fig. S2 and Dataset S2). We also noted differences in expression in many metabolic processes between white and opaque cells consistent with their distinct metabolic capacities (39–41). In contrast, mixtures of white-OE cells showed

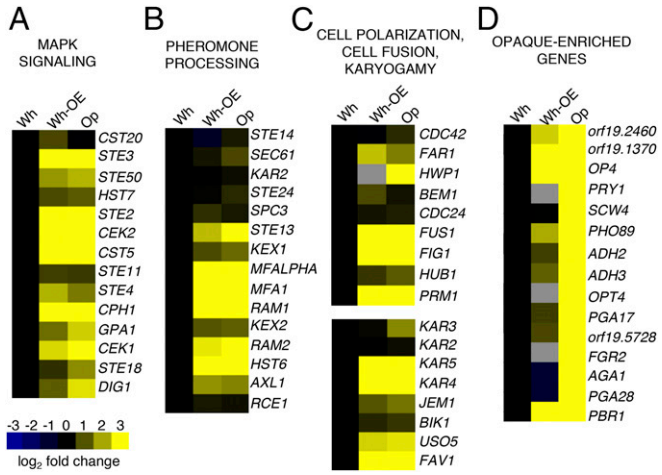

Fig. 3. Comparison of expression profiles between mating mixes of white cells (Wh), white cells engineered to overexpress MAPK genes (Wh-OE), and opaque cells (Op). Expression levels of genes involved in (*A*) the core pheromone MAPK-signaling cascade; (*B*) pheromone production and processing; (*C*) cell polarization, cell fusion, and karyogamy; and (*D*) opaque-enriched genes. In all cases, mating mixes of a and α cells were incubated on Spider medium for 8 h, and RNA expression was determined. Data are normalized to expression levels in mating mixes of control white cells. White cells are white-locked cells (*wor1Δ/wor1Δ*), and engineered white-locked cells are constitutively expressing *STE4*, *CST5*, and *CEK2* (white-OE cells). Opaque-enriched genes represent those most highly up-regulated in opaque cells relative to white cells (36). Average of two biological replicates.

significant expression differences in only 73 genes relative to control white-locked cells (Dataset S3). Despite these limited differences, white-OE cells showed significant enrichment in GO terms for pheromone response, sexual reproduction, and G-protein response relative to white cells (*SI Appendix*, Fig. S3 and Dataset S4). However, the large-scale differential gene expression of metabolic genes evident between white and opaque cells was not observed between white cells and white-OE cells (*SI Appendix*, Fig. S3).

We next compared the expression of individual mating-related genes between cell populations, including genes involved in pheromone processing, MAPK signaling, and karyogamy. Strikingly, a number of mating genes were expressed at a similarly high level in mixes of both white-OE cells and opaque cells relative to control white cells (Fig. 3 *A*–*C*), consistent with the mating efficiencies of these cell types. We also compared the expression of a set of "opaque-enriched genes" between strain backgrounds. These genes were highly expressed in opaque cells relative to white cells, whereas engineered white-OE cells did not express elevated levels of most of these genes (Fig. 3*D*). This establishes that white-OE cells largely retained the expression profile of the white phenotype.

Finally, we examined expression differences in cell-wall genes, given that white and opaque cells display notable differences in cell-wall structure (42–44). Using GO term analysis, we identified 32 cell-wall genes that showed significant differences in expression between white and opaque cells and found that the majority of these genes did not show differential regulation between white cells and white-OE cells (*SI Appendix*, Fig. S4). This result is also consistent with the observation that white-OE cells are superficially similar to control white cells in scanning electron micrographs (Fig. 2*D*).

Together, these results establish that manipulation of the expression of three MAPK components is sufficient for white cells to express mating-related genes at levels similar to those in opaque cells. Furthermore, engineered white cells are mating-competent while still maintaining an expression profile similar to that of natural white cells.

Relative Levels of MAPK Components in White, Opaque, and Engineered Cell Types. The previous experiments highlight that altered expression of MAPK genes converts "sterile" white cells into mating-competent cells. To compare the protein levels of key signaling components, MAPK genes were epitope-tagged, and Western blotting was performed. Epitope-tagged **a** cells were coincubated with non–epitope-tagged α cells on Spider medium for 24 h, and whole-cell lysates were analyzed by SDS/PAGE and Western blotting.

In general, relative protein levels reflected RNA expression differences for each of the three key MAPK components studied here. Thus, Cek2 protein was present at 181-fold higher levels in opaque cells than in white cells, whereas Cst5 and Ste4 proteins were expressed at 13- and 1.9-fold higher levels in opaque cells than in white cells, respectively (Fig. 4 and *SI Appendix*, Fig. S5). White-OE strains engineered to ectopically express *CEK2* produced 58-fold more Cek2 protein than control white cells, a level threefold lower than that in opaque cells (Fig. 4). For white-OE strains ectopically expressing *CST5*, these strains produced a similar level of Cst5 protein to opaque cells (a 19-fold increase over control white cells). Curiously, levels of Ste4 protein were only modestly elevated in white-OE cells ectopically expressing *STE4*, with a 2.5-fold increase relative to control white cells, although this is similar to the 1.9-fold difference between natural white and opaque cells (Fig. 4 and *SI Appendix*, Fig. S5).

Overall, these results establish that the protein levels of key MAPK components are present at higher levels in opaque cells than in white cells. Furthermore, the protein levels in white cells engineered to ectopically express MAPK factors generally reflect, at least for the most part, the levels of these proteins in opaque cells. Thus, engineered white-OE cells express physiologically relevant levels of MAPK components relative to opaque cells. Furthermore, *C. albicans* mating appears particularly sensitive to Ste4 levels, as even small changes in these levels have a profound effect on mating frequency.

Remodeling of MAPK Signaling Is Associated with a Fitness Cost. Studies in *S. cerevisiae* have revealed that elevated levels of basal signaling through the pheromone MAPK pathway increase mating efficiency but come with an associated fitness cost (45). This is because basal pheromone signaling drives Ste12-mediated expression of a number of mating genes, and the cost of expressing these genes is a decrease in growth rate (45). By analogy, we hypothesized that *C. albicans* white cells engineered to become mating-competent may also exhibit a concomitant defect in fitness due to the cost of increased signaling through the pheromone MAPK pathway. To test this, we compared the growth rates of white-locked OE cells that overexpress *CEK2*/*CST5*/*STE4* with control white-locked cells. Notably, we found that white-OE cells showed a significant decrease in growth rates relative to control white-locked cells when grown in YPD medium (doubling rates of

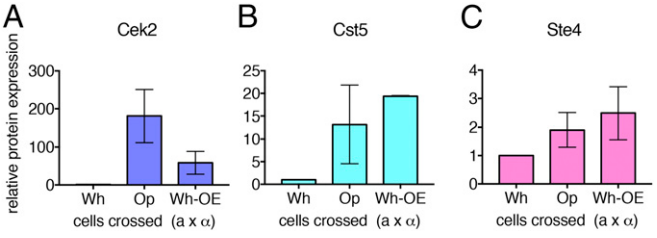

Fig. 4. Comparison of protein levels for pheromone MAPK components in white cells, engineered white cells, and opaque cells. Western blots were performed for strains expressing epitope-tagged versions of Cek2 (*A*), Cst5 (*B*), or Ste4 (*C*). Op: mating crosses between wild-type opaque **a** and α cells; Wh: mating crosses between *wor1Δ/wor1Δ* **a** and α cells. White-OE: mating crosses between white-locked **a** and α cells that ectopically express *CEK2* (*A*), *CST5* (*B*), or *STE4* (*C*). MAPK protein levels are relative to control white cells (normalized to tubulin signal). Cek2 and Ste4: $n = 4$ biological replicates. Cst5: $n = 2$ biological replicates. Graphs show mean ± SEM. See *SI Appendix*, Fig. S5, for Western blot images.

154 and 131 min, respectively, at 30 °C; *SI Appendix*, Fig. S6*A*). To further establish a fitness difference between these strains, a direct competition assay was performed between white-locked and white-OE cells. After 7 d in culture, white-locked strains composed 83% of the total population compared with 45% on day 1 (*SI Appendix*, Fig. S6*B*). This suggests that white-OE cells exhibit a significant fitness defect relative to control white cells due to ectopic overexpression of MAPK components.

Discussion

The regulation of sexual reproduction has undergone extensive remodeling between eukaryotic species. A striking example of remodeling occurred within the *Candida* clade, where sexual reproduction came under the control of an epigenetic switch so that cells in the opaque state are mating-competent, whereas genetically identical white cells are sterile. We demonstrate that quantitative differences in pheromone MAPK signaling are responsible for differences in mating competence between the two cell states and identify the key steps at which signaling through the MAPK pathway is regulated.

A critical observation is that white cells express several MAPK components at decreased levels relative to opaque cells and that correction of these differences enables white cells to become mating-competent. In particular, individual overexpression of the G-protein β subunit *STE4*, the MAPK scaffold *CST5*, or the terminal MAP kinase *CEK2* all enhanced mating ability. Moreover, when these factors were overexpressed in combination, engineered white cells mated four orders of magnitude more efficiently than control white cells. Engineered white cells recapitulated both the transcriptional and the morphological responses of opaque cells to pheromone. Furthermore, these cells expressed physiologically relevant levels of MAPK genes, as both RNA and protein levels of ectopically expressed factors were comparable to those in opaque cells. Together, these results establish that *C. albicans* white cells can be reverse-engineered to be mating-competent by restitution of the levels of the key signaling components to opaque cell-like levels.

Ectopic expression of any one of the *STE4*, *CST5*, or *CEK2* genes enhanced mating of white cells, yet expression of *STE4* had by far the strongest influence on mating. Elevating expression of this one gene enhanced mating 336-fold, and ectopic *STE4* expression also enhanced mating in cells overexpressing *CST5*/*CEK2*, further augmenting mating of these cells by 183-fold. Surprisingly, forced expression of *STE4* in white cells increased protein levels by only 2.5-fold, a similar value to *STE4* differences between natural white and opaque cells. *C. albicans* mating therefore appears exquisitely sensitive to Ste4 levels. A similar conclusion was reached for *S. cerevisiae*, as a twofold increase in Ste4 levels enhanced the pheromone response, whereas a comparable change in other G-protein subunits did not (46). However, it is worth noting that differences in G-protein signaling exist between *C. albicans* and *S. cerevisiae*; the G-protein α subunit promotes *C. albicans* mating whereas it is repressive for *S. cerevisiae* mating (47). Further dissection of the role of Ste4 is therefore necessary to determine how pheromone signaling is hypersensitive to changes in this component. One possibility is that while population levels show limited expression differences between white/opaque states, individual cells may exhibit much greater differences than the population average.

The white-opaque switch is currently thought to have evolved in the ancestor to *C. albicans* and *Candida tropicalis* and regulates sexual competency in both extant species (48, 49). Given our identification of MAPK signaling differences between *C. albicans* white and opaque cells, we examined *C. tropicalis* transcriptome data (50) and found that *STE4* was expressed at a twofold higher level in opaque cells than in white cells (*SI Appendix*, Fig. S7). Elevated levels of *STE4* are therefore likely to contribute to the enhanced sexual competency of opaque cells over white cells in both *Candida* species. We propose a model whereby *STE4* became white-opaque–regulated in the ancestor to *C. albicans* and *C. tropicalis*, whereas *CST5* and *CEK1*/*2* subsequently came under the control of this circuit specifically in the *C. albicans* lineage (*SI Appendix*, Fig. S8). The fact that *CST5*/*CEK1*/*CEK2* are not white-opaque–regulated in *C. tropicalis* may account for the more limited mating differences (only ∼100-fold) between white and opaque cells of this species (48) compared with that between *C. albicans* white and opaque cells. Furthermore, *STE4* is specific to pheromone signaling, whereas most other components in this pathway are shared by the filamentation MAPK pathway (51). Thus, manipulation of *STE4* expression may allow for modulation of mating signaling in *Candida* species without impacting other MAPK-signaling pathways. Targeting of pathway-specific components can thereby provide the means for evolution to remodel one signaling pathway while leaving related pathways that share overlapping components untouched.

What advantage(s) does the white-opaque switch provide to pathogenic *Candida* species? The switch has been shown to control expression of many metabolic genes, and these metabolic differences enable the two cell types to colonize different niches in the mammalian host (39, 41). In this study, we demonstrate that the white-opaque switch also limits basal signaling through the pheromone MAPK pathway in white cells. This is of significance because basal pheromone signaling is associated with a fitness cost in *S. cerevisiae* due to low-level expression of mating genes (45). Indeed, we show that restoring pheromone MAPK signaling to *C. albicans* white cells is associated with a significant fitness cost. We therefore propose that the white-opaque switch may provide an evolutionary advantage by restricting signaling through the pheromone MAPK pathway. This mechanism could allow *C. albicans* cells to be mating-competent (cells can switch to the sexually competent opaque state) without white cells having to pay the cost of constitutive expression of mating pathway genes. In this way, *C. albicans* may have the best of both worlds: it remains capable of sexual reproduction without paying the price of having cells that are always primed to undergo mating.

At a broader level, our work reveals how contrasting phenotypes are achieved between two epigenetic cell states due to relatively small differences in the levels of key signaling components. As such, our study has implications for understanding MAPK-signaling changes that occur in cancer cells. For example, it is now apparent that mutations in the MEK-upstream kinase BRAF are present in over 50% of melanomas (52, 53) and that ∼20% of all cancers involve mutations in G-protein–coupled receptors (54, 55). As such, there is considerable interest in determining how signaling mechanisms are altered in cancer cells, as well as how strategic targeting of specific MAPK components could be used for therapeutic intervention. There is also an increasing interest in the cancer epigenome with the realization that more than half of cancers involve mutations in chromatin organization factors (56, 57). Our analysis of a model MAPK-signaling pathway reinforces the importance of epigenetic events that are independent of the primary DNA sequence. Thus, further understanding of signaling bottlenecks and the mechanisms that impact these signaling points will be important for understanding signal transduction pathways across all eukaryotic species.

Materials and Methods

A complete description of all materials and methods can be found in *SI Appendix*.

Media, Strains, and Mating Assays. Media were prepared as described (58–60), and strains were constructed as described in *SI Appendix*. Mating assays were performed as described (61). Briefly, auxotrophic strains were grown overnight in SCD (Synthetic Complete Dextrose) medium, and ∼2.5×10^7 cells of each mating type were mixed and deposited onto 0.8-μm nitrocellulose filters on Spider medium. Plates were incubated for 7 d at 22 °C, and CFUs (colony forming units) were determined on SCD–leu, SCD–arg, and SCD–leu–arg media. Mating frequency was defined by the number of CFUs on SCD–leu–arg medium divided by the lowest number of CFUs on SCD–leu or SCD–arg medium. At least four independent strains were used with at least six biological replicates performed for each experiment.

RNA Sequencing. Individual cell populations or mating mixes of **a** and α cells were grown on Spider medium at 22 °C for 8 h. Cells were collected and RNA was purified using the RiboPure RNA purification kit for yeast. RNA sequencing was performed using the Illumina HiSeq 2500 platform generating 50-bp single-end reads. Reads were aligned to the *C. albicans* SC5314 genome (Assembly 21) using TopHat2 (62) as described in *SI Appendix*.

Western Blotting. Cell lysates were prepared from mating mixes of cells grown on Spider medium for 24 h at 22 °C. Briefly, Western blotting was performed on lysates using anti-myc or anti-tubulin antibodies, followed by a peroxidase-conjugated secondary antibody. Antibody binding was detected by chemiluminescence using Lumigen ECL Ultra.

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