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Evolutionary Reshaping of Fungal Mating Pathway Scaffold Proteins

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ABSTRACT **Scaffold proteins play central roles in the function of many signaling pathways. Among the best-studied examples are the Ste5 and Far1 proteins of the yeast *Saccharomyces cerevisiae*. These proteins contain three conserved modules, the RING and PH domains, characteristic of some ubiquitin-ligating enzymes, and a vWA domain implicated in protein-protein interactions. In yeast, Ste5p regulates the mating pathway kinases while Far1p coordinates the cellular polarity machinery. Within the fungal lineage, the *Basidiomycetes* and the *Pezizomycetes* contain a single Far1-like protein, while several *Saccharomycotina* species, belonging to the CTG (*Candida*) clade, contain both a classic Far1-like protein and a Ste5-like protein that lacks the vWA domain. We analyzed the function of *C. albicans* Ste5p (Cst5p), a member of this class of structurally distinct Ste5 proteins. *CST5* is essential for mating and still coordinates the mitogen-activated protein (MAP) kinase (MAPK) cascade elements in the absence of the vWA domain; Cst5p interacts with the MEK kinase (MEKK) *C. albicans* Ste11p (CaSte11p) and the MAPK Cek1 as well as with the MEK Hst7 in a vWA domain-independent manner. Cst5p can homodimerize, similar to Ste5p, but can also heterodimerize with Far1p, potentially forming heteromeric signaling scaffolds. We found direct binding between the MEKK CaSte11p and the MEK Hst7p that depends on a mobile acidic loop absent from *S. cerevisiae* Ste11p but related to the Ste7-binding region within the vWA domain of Ste5p. Thus, the fungal lineage has restructured specific scaffolding modules to coordinate the proteins required to direct the gene expression, polarity, and cell cycle regulation essential for mating.**

IMPORTANCE **The mitogen-activated protein (MAP) kinase cascade is an extensively used signaling module in eukaryotic cells, and the ability to regulate these modules is critical for ensuring proper responses to a wide variety of stimuli. One way that cells regulate this signaling module is through scaffold proteins that insulate related pathways against cross talk, improve signaling efficiency, and ensure that signals are connected to the correct response. The Ste5 scaffold of the *S. cerevisiae* mating response is a well-studied representative of this class of proteins. Using bioinformatics, structural modeling, and molecular genetic approaches, we have investigated the equivalent scaffold in the pathogenic yeast *Candida albicans*. We show that the *C. albicans* protein is structurally distinct from that of *Saccharomyces cerevisiae* but still provides similar functions. Increases in pathway complexity have been associated with changes in scaffold connectivity, and overall, the tethering capacity of the scaffolds has been more conserved than their structural organization.**

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Typical eukaryotic signaling pathways often involve components that are coordinated through protein-protein interactions, and a large number of protein motifs, including such extensively distributed elements as the SH2, SH3, PH, and SAM domains that serve to facilitate these interactions, have been identified (1). As well, signaling pathways can involve scaffold proteins, originally identified because they served a primarily noncatalytic function, but were involved in direct binding of separate catalytic elements of a signaling pathway (2, 3). One of the best-studied examples of a scaffolding protein is Ste5p of the yeast *Saccharomyces cerevisiae* (ScSte5p); this protein coordinates the elements of the mitogen-activated protein (MAP) kinase (MAPK) cascade involved in the pheromone response pathway and is critical for mating in yeast (4).

The function of the Ste5 scaffold is complex. Initial observations identified binding sites for all members of the MAP kinase cascade (5–7), while later evidence established physical association with the β subunit of the heterotrimeric G protein linked to the pheromone receptor protein (8). This association directs the MAP kinase module to the membrane as part of the pheromone-mediated activation of signaling (9). The Ste5 protein also appeared to provide specificity to the kinases Ste20p, Ste11p, and Ste7p, all of which functioned in other pathways separate from the mating pathway (10). More-recent evidence has established that Ste5p is more than just a scaffold but in fact plays a critical role in the activation of Fus3p, the MAP kinase specific for the pheromone response (11).

The Ste5 protein contains several structural motifs. The amino-terminal half of the protein contains a membrane-anchoring motif (12), a RING-H2 Zn finger domain (here termed RING) (13), and a cryptic PH (plextrin homology) domain (14),

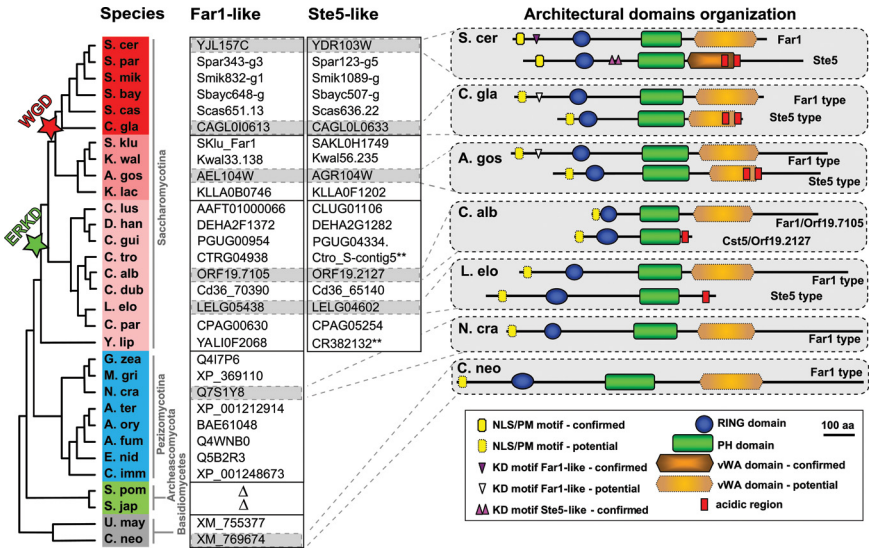

FIG 1 Phylogenetic evolution of mating scaffold proteins in the fungi. (A) Scaffold proteins with sequence similarity to *S. cerevisiae* Ste5p and/or Far1p could be identified throughout the entire fungal kingdom, with the noticeable exception of the *Archiascomycota* (represented by *S. pombe* and *S. japonicus*). Schematic representations of the protein structural organization of selected Far1/Ste5-like proteins featuring newly detected cryptic vWA domains of Far1-like proteins are displayed on the right. WGD, whole-genome duplication event; ERKD, ERK duplication; **, protein sequence manually assembled from multiple tBLASTn reads.

while the carboxy-terminal half of the protein consists primarily of a vWA (von Willebrand type A) module. The vWA module is involved in association with the Ste7p MEK protein (15), while the N-terminal RING/PH region is implicated in Ste5p homodimerization (16), Ste11p association (7), interaction with the MAPK Fus3 (17), and also Ste4p G protein β subunit binding (8). In addition, the RING/PH domain structure found in Ste5p is characteristic of the RING class of ubiquitin-ligating enzymes (18), suggesting that Ste5p could play a role in the proteolytic stability of the mating response pathway components.

In addition to Ste5p, the yeast *S. cerevisiae* contains a second scaffold protein that functions in ensuring a proper mating response. This protein, Far1p, has an overall structure similar to that of Ste5p, with similarly placed RING and PH domains (19–21), even though the overall level of sequence identity between the proteins is low (22). However, with the exception of the G protein β subunit Ste4p (23) and the MAPK Fus3p (24–26), which bind within the RING/PH region of both proteins, the proteins associating with the Far1p scaffold are distinct from those interacting with Ste5p. In addition to the G protein β subunit, Far1p associates with regulators of cell polarity, in particular Cdc24p, the GEF for Cdc42, and the SH3 domain protein Bem1p (23).

Far1p plays two distinct functions in the pheromone response process (27). It serves as a cyclin-dependent kinase inhibitor (CKI) (28) that triggers cell cycle arrest in response to pheromone signaling (29), and it regulates pheromone-directed mating projection formation through its coordination of the cellular polarity machinery regulated by Bem1p and Cdc42 (30, 31). However, Far1p plays no role in the regulation of pheromone-mediated gene expression (32). Epistatic analysis shows therefore that Ste5p functions near the beginning of the mating pathway and that Far1p acts later.

Recently, the investigation of this family of RING/PH domain scaffold proteins has been extended to other fungi. The fungal pathogen *Candida albicans* contains a clear Far1p ortholog, Orf19.7105 (21, 33–37), which has been analyzed for its roles in pheromone-mediated arrest and in mating. Intriguingly, loss-of-function mutants of the *C. albicans FAR1* gene result in loss of all aspects of pheromone response, including gene expression induction (33), in clear contrast to the situation for *S. cerevisiae*. Because a second RING/PH protein, similar to Ste5p, was not evident in the *C. albicans* genome, it was possible that in the pathogen Orf19.7105 played the roles of both the Ste5 and the Far1 proteins and that duplication of the gene after the whole-genome duplication (WGD) allowed subfunctionalization into the Ste5 and Far1 proteins. Here, we show that this is not the case; while the *Basidiomycetes* and the *Pezizomycetes* contain a single RING/PH ortholog and the *Archiascomycetes* lack such proteins entirely, the *Saccharomycotina* species of the CTG clade, such as *C. albicans*, contain Ste5p orthologs that lack the vWA domain. Analysis of the *C. albicans STE5* ortholog *CST5* shows that this gene is required for all aspects of the pheromone response but that in the absence of the vWA domain, the protein coordinates the MAP kinase elements in a distinct manner.

RESULTS

Ancestral evolution of RING/PH domain proteins in fungi. We explored the Far1p/Ste5p domain organization in a set comprising 29 Far1-like proteins and 19 Ste5-like proteins. We carried out 3D-Jury consensus structure predictions based on recent fold recognition and advanced sequence profile algorithms (38, 39) and confirmed the presence of the RING and PH domains in all analyzed representatives of Far1-like and Ste5-like proteins. Structurally similar proteins are found throughout the fungal kingdom (Fig. 1), although, intriguingly, none are identified in *Archiascomycetes* such as *Schizosaccharomyces pombe* (Fig. 1, green species). In many species, including those of the *Pezizomycetes* (Fig. 1, blue species) and the *Basidiomycetes* (Fig. 1, grey species), only one member of this class of proteins can be identified; these family members are most similar to *S. cerevisiae* Far1p. In contrast, members of the *Saccharomycotina* contain both a Far1p ortholog and a Ste5p ortholog (Fig. 1, pink and red species); the appearance of a pair of RING/PH scaffold proteins did not correlate with the yeast whole-genome duplication (WGD) (40) but preceded it at a phylogenetic point similar to that of the extracellular signal-regulated kinase (ERK) duplication that generated the *S. cerevisiae* mitogen-activated protein kinase (MAPK) duo comprising Fus3p and Kss1p. While we failed to identify RING/PH domain proteins in *S. pombe* and *Schizosaccharomyces japonicus* (*Archiascomycota* representatives), a fission yeast MAPK Fus3p ortholog, Spk1p, is easily identified (41, 42).

The vWA domain is ubiquitous in the Far1p family but not in Ste5 orthologs. In a subset of the *Saccharomycotina* that includes the CTG (*Candida*) clade, a classic Ste5 ortholog was not identified. Instead, a shorter class of RING/PH proteins (43) that lacked

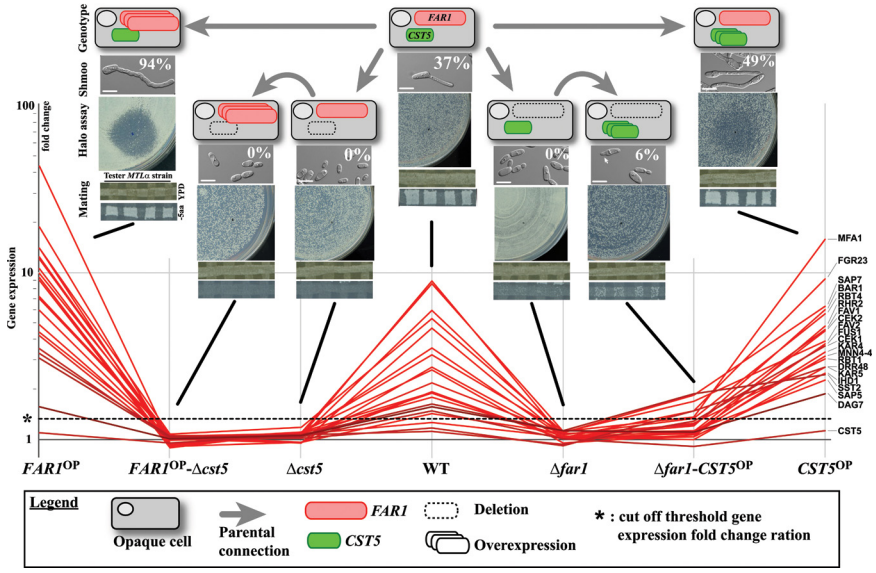

FIG 2 *C. albicans* possesses a functional *CST5* gene that can partially suppress loss of *FAR1*. We phenotypically characterized the genetic connection between *C. albicans FAR1* and *C. albicans STE5* homolog *CST5* (formally *ORF19.2127*) during pheromone response and mating assays. Morphological responsiveness was measured by shmoo formation. Pheromone-triggered cell cycle arrest was assessed by a halo assay. Transcriptional gene induction was investigated using DNA microarrays, and a set of modulated genes (representing the *Candida* pheromone-induced core set of genes) is displayed here. Finally, the mating capacity of each strain was illustrated by the patch-mating assay (YPD medium, no selection; −5 aa, progeny selection plate).

the vWA domain recently identified in *S. cerevisiae* Ste5p was observed (15). The vWA fold (44) is detected with high reliability in Ste5p from *Candida glabrata*, *Kluyveromyces lactis*, and other species within their respective clusters, having close homology to ScSte5p (Fig. 1). However, the vWA domain is predicted to be absent from Ste5p for a cluster of 9 related fungal species represented by Cst5p, whose sequences either end shortly after the PH domain (e.g., 39 amino acid [aa] residues in *C. albicans*) or are unrelated to the vWA fold (e.g., *Candida lusitaniae* and *Debaryomyces hansenii*). This structural variety may suggest rapid evolution of the Ste5 scaffold within the CTG clade.

In addition, we obtained reliable predictions, as judged by the statistically significant 3D-Jury scores, for the presence of a vWA fold in the sequence following the predicted PH domain for all Far1-like proteins, including those from *C. albicans* and *S. cerevisiae* (see Fig. S1 in the supplemental material). Consistent with the fold recognition data, this vWA domain follows the consensus secondary structure of the vWA-like fold in the Far1-like proteins (Fig. S1). Interestingly, the known structure of the ScSte5p vWA domain was not among the template hits from fold detection of the Far1p vWA domains, but other vWA fold structures, like those of integrins, were identified. Our structural predictions thus indicate that the vWA domains of Far1p and Ste5p are distinct variations of the vWA fold.

Genetic analyses of scaffold proteins in *C. albicans*. Recently, crystallographic analysis of the Ste5p C terminus identified that the MAPK kinase (MAPKK) Ste7 interaction domain on Ste5p, called Ste5-MS, involved the vWA structure of Ste5p (15), and mutant forms of the scaffold that lack this region or contain point mutations show major defects in pheromone-mediated responses and mating (15, 45). As this domain is completely missing in the *CST5* gene, we first determined if Cst5p actually functioned in the *C. albicans* mating pathway. We created both the Δ*cst5* deletion mutant (PCa63) and a strain overexpressing *CST5* (PCa68) in the *C. albicans MTL***a** reference background 3294 (complete genotypes are available in Table S1 in the supplemental material) and then tested for pheromone- and mating-associated defects (Fig. 2). In pheromone response assays, the Δ*cst5* strain fails to make mating projections (shmoos), fails to undergo cell cycle arrest and transcriptional induction, and fails to mate (Fig. 2); all of these phenotypes were reverted when *CST5* was reintegrated into the Δ*cst5* strain (see Fig. S2 in the supplemental material). In contrast, a mutant strain ectopically expressing higher levels of *CST5* (the *CST5*^{OP} strain) showed slightly enhanced mating-associated responses compared to those of its parental strain, 3294 (Fig. 2), with a slight increase in the percentage of shmooing cells (from 37% to 49%) and a more pronounced cell cycle arrest.

Therefore, both *FAR1* (33) and *CST5* loss-of-function mutants of *C. albicans* lead to the abolition of pheromone-induced gene expression (Fig. 2), while in budding yeast this phenotype is associated only with the *STE5* deletion (32). Because in budding yeast *STE5* overexpression could partially rescue the shmoo formation defect observed in a Δ*far1* strain, whereas *FAR1* overexpression failed to restore Δ*ste5*-associated phenotypes (22), we tested whether a similar relationship exists between the two scaffold proteins in *C. albicans*. We analyzed overexpression of both *FAR1* and *CST5* in backgrounds deleted for the other gene (Fig. 2); from these two strains, the Δ*far1-CST5*^{OP} (PCa67) and *FAR1*^{OP}-Δ*cst5* (PCa66) strains, we saw functional suppression only from the overexpression of *CST5* in the *far1*-deleted background. Shmoo formation was detectable at ~6%, and there was partial restoration of pheromone-dependent gene expression and mating, so the response of the Δ*far1-CST5*^{OP} strain is significant compared to that of either the *FAR1*^{OP}-Δ*cst5* strain or the Δ*far1* loss-of-function strain (Fig. 2). Together, these results suggest that, similar to *S. cerevisiae*, *C. albicans* possesses two distinct scaffold-like proteins that are functionally implicated in the mating pathway.

Interaction between CaSte11p and Cst5p in *C. albicans*. Although Cst5p is required for the *C. albicans* mating process, it could be that *C. albicans* Far1p provides all the kinase docking functions in pheromone signal transduction in the pathogen. We therefore looked for protein-protein interactions, based on the *S. cerevisiae* model, that are known to be specific to only one of the two scaffold proteins. One such specific interaction is between the MAP kinase kinase kinase Ste11p and the PH domain of Ste5p (19); the PH domain of Far1p has not been detected to interact with Ste11p. To investigate the possibility that this interaction occurs between the *C. albicans* proteins, we have used a yeast two-hybrid (Y2H) system (46) (C. Wu, G. Jansen, D. Y. Thomas, and M. Whiteway, unpublished data) to express protein domains of interest and screen for their potential interactions in

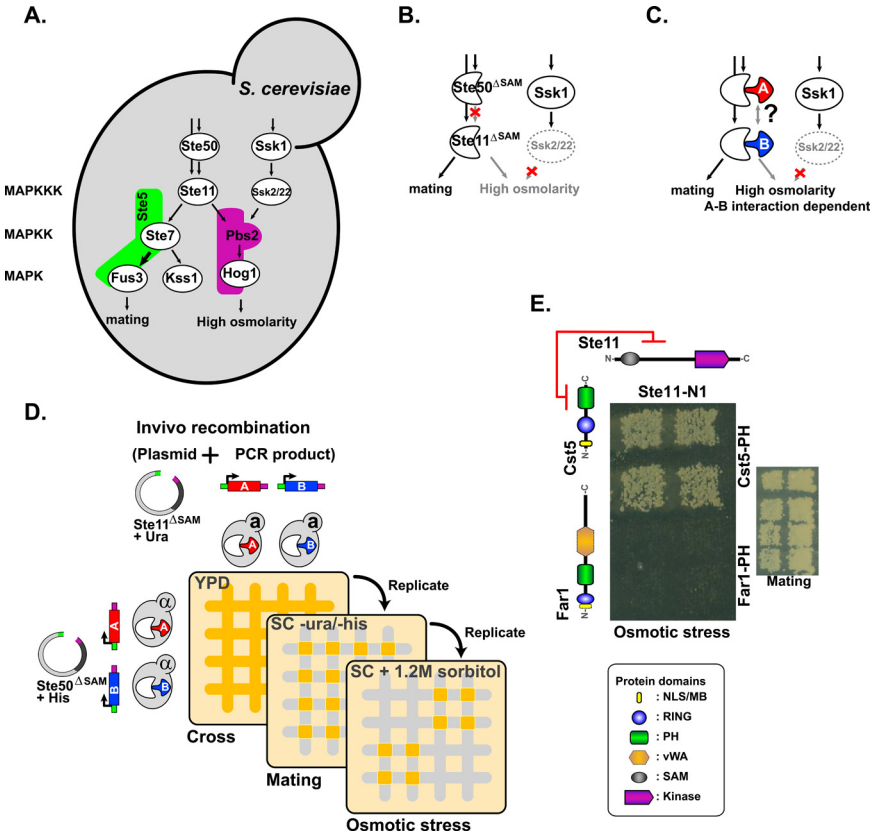

FIG 3 *C. albicans* Cst5p but not Far1p interacts with CaSte11p, as monitored by a yeast two-hybrid assay. (A) Shared components between the mating and high-osmolarity MAPK pathways in *S. cerevisiae*. Panels B and C are schematic representations of the engineered *S. cerevisiae* genetic background that is used to test the interaction between two protein fragments of interest. (D) Representation of the technical approach used to test the interaction *in vivo*. (E) Interaction between CaSte11p aa 136 to 378 and the Far1p or Cst5p PH domain was assessed using this yeast two-hybrid assay. Petri plates were scanned after 1 day of growth (mating plate) and 5 days (osmotic stress plate).

the cytoplasm (Fig. 3). We created a collection of protein fusion fragments covering the entire lengths of both Far1p and Cst5p and screened for their interactions with *C. albicans* Ste11p (CaSte11p). We found that Cst5p but not Far1p was able to interact with CaSte11p (Fig. 3E). We refined the Cst5-CaSte11 interaction with the PH domain of Cst5p and a region of CaSte11p localized to amino acids 136 to 378; similar assays for detecting the association of this region of CaSte11p with the Far1p PH region showed no interaction.

In addition, evidence in *S. cerevisiae* supports an interaction between the RING/PH regions of Far1p and Ste5p and the MAPK Fus3p. Although some of these interactions are phospho dependent (7, 24, 45, 47), a phospho-independent interaction exists between Ste5p and Fus3p (11). Therefore, we explored whether a similar interaction exists between MAPKs and scaffold proteins in *C. albicans*. Here, we found that Cek1p, the sequence homolog in *C. albicans* closest to *S. cerevisiae* Kss1p, binds to Cst5p, whereas neither Cek1p nor Cek2p was found to interact with Far1p (Fig. 4). Similar results, involving the interaction of Cst5p with Cek1p but not Cek2p, were observed in an independent two-hybrid system (48; B. Stynen and P. Van Dijck, personal communication); intriguingly, the results of co-immunoprecipitations (co-IPs) described in the paper accompanying the present one (65) show a Cst5p-Cek2p association. Thus, it appears that *C. albicans* Cst5p provides functions in the *Candida* pheromone response that are equivalent to those of *S. cerevisiae* Ste5p.

Kinase interaction network in *C. albicans*. We expanded our analysis to look at the interactions among all the kinases and scaffold proteins in the system. Figure 4 summarizes all the positive interactions identified, and Fig. S3 in the supplemental material shows the locations of the most-specific associating regions identified for each interaction. In addition to the previously mentioned interactions with CaSte11p and Cek1p, we found Far1p, Cst5p itself, and Hst7p all as associating partners with Cst5p. The Far1p-Cst5p interaction has been linked to a Far1p region of 229 residues covering the entire PH domain and part of the RING domain (amino acids 71 to 300) and to a mutated Cst5p region that includes its RING and PH domains as well (amino acids 67 to 360, including the C135R mutation). The same extensive region of Cst5p is required for its oligomerization and for Cek1p and Hst7p binding as well. Along with the identification of Cst5p-interacting partners, we found that both Hst7p and Cek1p were interacting with CaSte11p. We mapped the Cek1p interaction with CaSte11p adjacent to the Cst5p-interacting region (Fig. S3). Cek2p, the *C. albicans* ortholog of Fus3p, was not found to interact with any elements that we have tested. While the interaction between Cst5p and CaSte11p in *C. albicans* is similar to the one reported for *S. cerevisiae*, occurring between the Ste5p PH domain and an evolutionarily conserved region of Ste11p, the Cst5p-Hst7p interaction is different. Here, we found that the C-terminal part of Hst7p (residues 457 to 588), which includes the last part of the kinase domain and its adjacent region, is the region interacting with Cst5p (residues 61 to 360). PCR-based mutagenesis relying on leaky *Taq* polymerase was used to obtain a double mutation (T128A-Q216R) that disrupted the Cst5p-Hst7p interaction (Fig. 4). This double mutation specifically blocked the Cst5p association with Hst7p but not its homodimerization interaction or its CaSte11p binding (Fig. 4). This interaction surface is in sharp contrast to the situation in *S. cerevisiae*, where the entire Ste7p kinase domain interacts with Ste5p through the Ste7-binding loop located C terminal to the vWA domain of the scaffold (7, 15); it remains to be determined which region of the Ste7 C terminus is directly involved in the Ste5-Ste7 interaction.

Scaffold protein Cst5 homo- and heterodimerization with Far1. As mentioned above, PCR-based mutagenesis was used to generate mutants that disrupted protein-protein interactions. We were able to identify a point mutation (C135R) on Cst5p that prevents its homodimerization. Also, we found that Cst5p and Far1p are able to interact, but intriguingly, this interaction occurred only when homo-oligomerization of Cst5p was blocked (e.g., C135R) (Fig. 4). To gain further insights into the structural topology of this interaction, we screened for additional Cst5p point mutations to identify residues

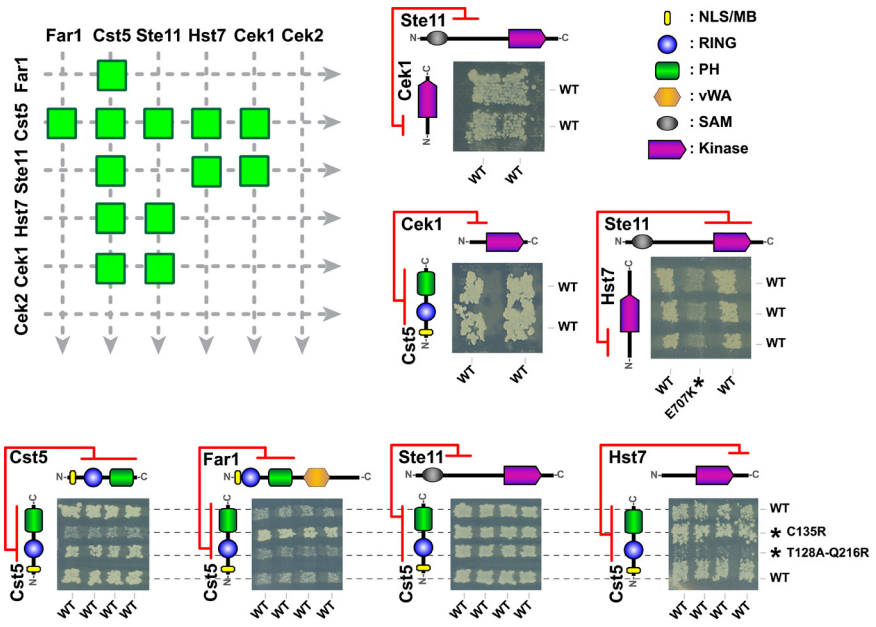

FIG 4 Cst5p is the central docking platform for Far1, Cst5, CaSte11, Hst7, and Cek1. A Y2H general map of all identified interaction between *C. albicans* proteins is illustrated in the top left corner. Green squares represent Y2H interactions. A closeup view indicates where within each of the protein-protein interactions the binding regions could be located. The associated colony patches represent individual clones that were sequenced and from which mutants could be identified (see text for further details). For clarity, square patches with a minimum of two wild-type clones (plus mutants) are displayed.

that were required for the Cst5-Far1 interaction. In total, we identified 9 mutations targeting 6 different residues located within the Cst5 RING domain (Fig. 5). Some mutations modify highly conserved residues (e.g., D104, C135, and C140) while other residues are less conserved (L98 and K99) (see Fig. S4A in the supplemental material). Although these mutations span over 40 residues within the RING domain primary peptide sequence, we observed that, when mapped on the folded structure of the RING domain, they are all within or in close proximity to the second zinc finger (Fig. 5). This suggests that the modification of the second zinc finger is an important contributor to the Cst5-Far1 interaction. Interestingly, we found that the G103V mutation within the zinc finger allows both Cst5-Cst5 and Cst5-Far1 complexes to be formed. Using the same approach, we identified four single point mutations and a double mutation preventing Far1p association with Cst5p (Fig. S4B). Five out of these six mutations could be mapped within or just before the Far1p PH domain. These mutation sites, coupled with the minimal region allowing the Cst5 interaction identified by Y2H, suggest that Far1p interacts with the Cst5p RING domain through its own PH domain.

Acidic region in the activation loop of CaSte11p. Not all the protein-protein interactions in the pheromone response MAP kinase module involve the scaffold protein directly. There is an interaction between the MEKK kinase domain and the MEK N terminus that is known to be transient and phospho dependent in budding yeast (49). Here, we found that the same elements in *C. albicans* show a strong interaction in the Y2H assay. Comparative sequence analysis shows that *C. albicans* and the group of closely related CTG clade species, which have Ste5p orthologs lacking the vWA domains, also have a common insert of acidic residues in the middle of the activation loop of the catalytic domain of their Ste11p-like kinase (Fig. 6A). We identified a point mutation on CaSte11 (E707K) that specifically prevented the CaSte11-Hst7 interaction. A homology model of the activated CaSte11p kinase domain phosphorylated at Ser719 predicts that this acidic region is highly solvent exposed (Fig. 6B; see also Fig. S5A in the supplemental material). Changes in this region, like the E707K mutation, would be unlikely to affect the overall fold of the kinase domain, making it suitable for protein-protein docking interactions. We constructed opaque strain CA217, which contained the E707K version of CaSte11 as the only functional allele. This strain mated 100-fold less efficiently than the wild-type (WT) control or the CaSte11 heterozygote (Fig. S6), suggesting that this identified interaction plays a significant role in mating pathway signaling.

DISCUSSION

Distinctive vWA domains in fungal scaffold proteins. Using comparative sequence analysis and consensus fold recognition, we were able to identify a vWA domain (44) within the Far1 family of fungal scaffold proteins. This domain differs structurally from the vWA domain recently characterized for the Ste5 scaffolding proteins from *S. cerevisiae* and its relatives, but this domain is a member of the same superfamily. Thus, the basic structures of the majority of both Far1-like and Ste5-like proteins contain sequential RING, PH, and vWA domains, making the Ste5 proteins of the *Candida* clade and its close relatives, which totally lack the C-terminal vWA domain, an interesting anomaly. Neither of the two regions of the ScSte5p vWA domain that function in promoting Ste7p-to-Fus3p activation is present in the Far1p vWA domain of any species, including those that lack Ste5p vWA domains.

Mutational studies of ScFar1p have implicated negative autoregulation involving both the RING domain and the C-terminal region corresponding to the newly identified vWA domain, either intramolecularly by their direct mutual interaction or intermolecularly by dimerization (21). As yet, a specific function has not been identified for vWA domains in intracellular proteins (44, 50), although the combination of these domains with a RING domain and/or another Zn finger-like domain is not unprecedented for intracellular yeast proteins; one example is provided by the structurally homologous multidomain proteins Sec23 and Sec24, involved in endoplasmic reticulum (ER)-to-Golgi protein trafficking. Their vWA domains not only participate directly in binding to the Sar1 small GTPase but also form a Sec23/Sec24 heterodimeric trunk-like interface (51), suggesting a possible geometry for a Far1p dimeric assembly. Another example is Ssl1, the *S. cerevisiae* TFIIH-p44 homolog (52), whose RING and vWA domains establish a connection between transcription and the ubiquitination system: the RING domain is responsible, at least in part, for the recently discovered E3 ubiquitin ligase activity of Ssl1 (53), while the vWA domain shares significant homology with the Rpn10 vWA domain, which facilitates the degradation of a number of proteasome substrates (54–57). It will be of interest to learn

whether fungal Far1 proteins also possess ubiquitin ligase activity and whether their newly predicted vWA domain may serve a facilitator role in proteasomal degradation.

Ste5-like scaffolding is more conserved than its architectural organization. Ample evidence suggests that point mutations preventing Ste5p scaffolding in *S. cerevisiae* are sufficient to inactivate proper signal transduction (15, 45). However, our understanding of the function of such scaffold proteins is currently limited to ScSte5p itself. Intriguingly, the *C. albicans* Ste5p homolog Cst5p, which lacks the vWA carboxy terminus characteristic of the Ste5 protein of yeast, and is essentially half the size of the yeast scaffold, is still capable of interacting with the MEKK CaSte11p, the MEK Hst7p, and the MAPK Cek1p. While the MEKK and MAPK interactions were anticipated because they occur at the amino-terminal ends of the scaffolds, which are structurally similar in both Ste5p and Cst5p, the MEK association was more unexpected since Cst5p lacks the entire vWA domain. Interestingly, the Cst5-Hst7 association shows distinct characteristics when compared to the *S. cerevisiae* Ste5-Ste7 interaction. In *S. cerevisiae*, the Ste7-binding loop at the end of the Ste5 vWA domain serves to interact with the amino-terminal end of Ste7, adjacent to its kinase domain (7). In *C. albicans*, Cst5p possesses a vWA-independent interface that interacts with the C-terminal end of Hst7p. Based on these observations, we suggest that the capacity of the Ste5 protein to interact with its MEK can be accommodated in different ways and can be accomplished in the absence of a vWA domain.

(Re)Modeling the *C. albicans* mating signaling cascade. The absence of a vWA domain in Cst5p has required a restructuring of protein interactions in the system of the mating and MAP kinase pathway in *C. albicans*. The absence of the vWA domain is correlated with the presence of a MEK-binding loop in CaSte11p that allows strong Hst7p docking. The similarity between this CaSte11 acidic loop and the Ste7-binding loop present on ScSte5p is striking (Fig. 6; see also Fig. S5B in the supplemental material). The presence of a MEK-binding loop directly on the MEKK could permit a strong, scaffold-independent interaction between the MEKK CaSte11p and the MEK Hst7p. This CaSte11 acidic loop is inserted in the middle of the kinase domain activation loop not far from its phosphorylation sites. In the active conformation (e.g., phosphorylated at Ser719), electrostatic repulsions would keep the negatively charged insert exposed and away from the phosphorylated site and the substrate-binding groove (Fig. 6B; see also Fig. S5 in the supplemental material). In this conformation, the acidic insert is highly accessible as a docking site for Hst7p. In support of this model, the E707K mutation, occurring within the CaSte11 acidic loop, is sufficient to disrupt the strong interaction between CaSte11p and Hst7p, and this disruption reduces mating efficiency by close to 2 orders of magnitude. Given a plethora of positively charged side chains in the activation loop, it is plausible that the acidic insert may also fold back onto the activation loop in the unphosphorylated state and compete with phosphorylation (Fig. S5). Hence, the acidic insert may have an additional role in modulating the enzymatic activity of CaSte11p itself. The mechanisms of how the structure and dynamics of the acidic insert in the activation loop of CaSte11p can modulate interactions with Hst7p, as well as how CaSte11p is altered upon the E707K charge reversal mutation, remain to be elucidated.

Homo- and heterodimerization of scaffolds. The observed two-hybrid interaction between Far1p and Cst5p suggests that the scaffolds can heterodimerize; however, this interaction is observed only when the second zinc finger of the Cst5p RING domain is modified (Fig. 5). Indeed, we found in 5 out of 6 cases that the Cst5-Far1 interaction was possible only when Cst5p oligomerization itself was compromised, suggesting a possible regulated switch between binding partners. *CST5* alone is an inefficient transmitter of the mating signal, as deletion of *FAR1* dramatically perturbs mating (Fig. 2). *CST5* expression is pheromone induced, and *CST5* is more of a "switch" for the pheromone signaling, as overexpressing it does not significantly activate pheromone-triggered responses (Fig. 2); on the other hand, *FAR1* expression is not regulated by pheromone stimulation, but when ectopically increased, *FAR1* enhanced all mating-associated responses (33). A shift in scaffold protein affinity has recently been described to occur between Ste5p and Fus3p (15); here, the Cst5p switch would occur between the two scaffold proteins Cst5p and Far1p to create an efficient signal transmitter complex. This speculative model is supported by one of our Cst5 mutations, G103V; in this case, as opposed to the 5 other RING domain mutations, we observed that the Cst5 RING domain containing the G103V modification is able to bind both scaffold proteins. The precise impact of a valine instead of a glycine on the RING structure of either Ste5 or Cst5 is unknown; however, based on various RING domain primary sequences (18), it is possible that the mutated zinc finger would result in a slightly modified form rather than a completely misfolded structure. Transposed onto the *S. cerevisiae* model, this could suggest that the Cst5p-Far1p complex in *C. albicans* might functionally correspond to *S. cerevisiae* Ste5p, which has been predicted to function as a homodimer.

Cst5-Cst5 and Cst5-Far1 complex formation also reveals new details about the white/opaque cell pheromone signaling cascades

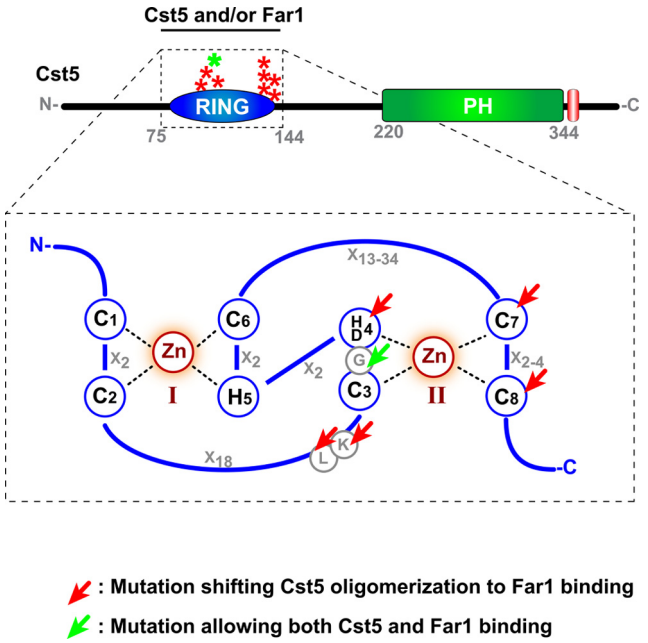

FIG 5 The second zinc finger of the Cst5 RING domain is crucial for Cst5p homo- or heterodimerization with Far1p. The mapping of Cst5p mutations on the primary sequence organization of the Ste5p family RING domain coordinating two zinc atoms is shown. Secondary sequence organization of the RING finger domain shows that all identified Cst5 mutations specifically affect the coordination of the second zinc atom (the schematic representation is based on data from reference 18).

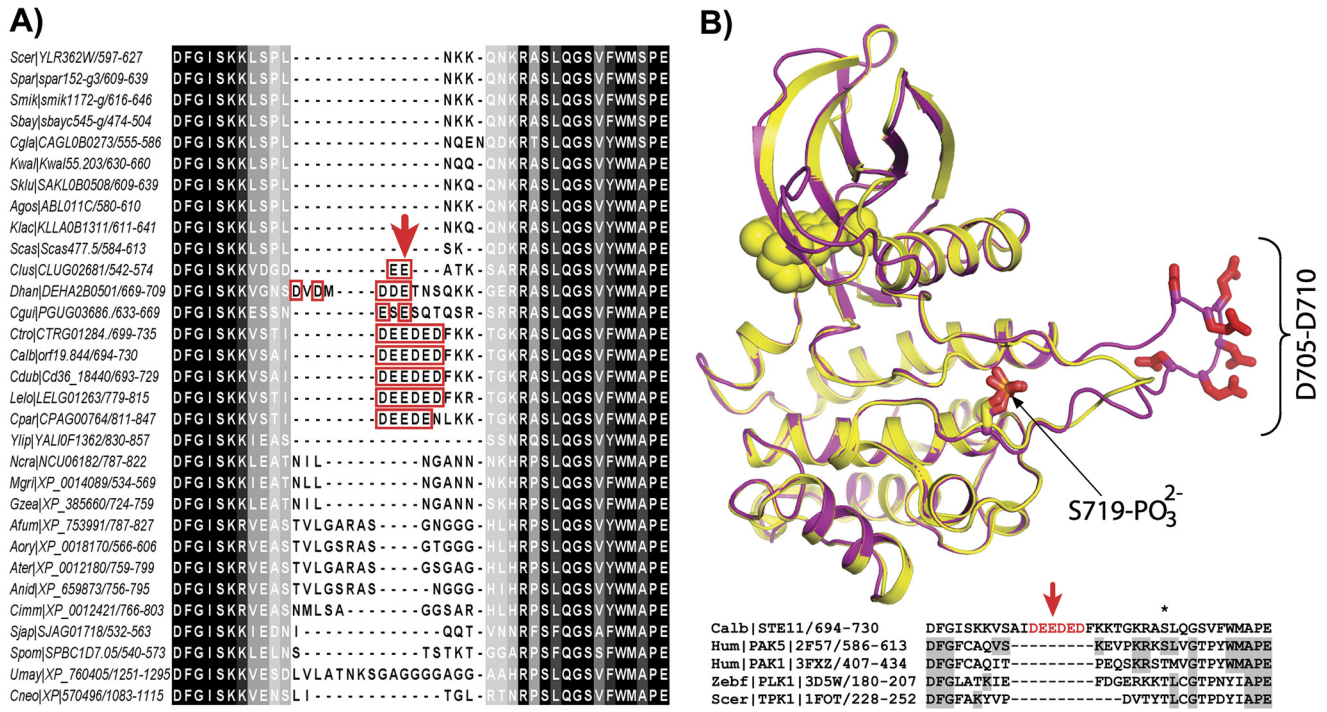

FIG 6 CaSte11-Hst7 mutant and interaction. The location of the acidic insert in the activation loop of CaSte11p is shown. (A) Multiple sequence alignment of the Ste11p kinase activation loop sequences from 31 fungal species. The shading represents the level of conservation, from high (black) to low (white). The acidic residues composing the insert loop are boxed in red. The position of the glutamic acid corresponding to CaSte11p mutation E707K is indicated with a red arrow. (B) Homology model of the CaSte11p kinase domain (magenta rendering) overlaid onto the human PAK5 crystal structure used as a template for model building (yellow rendering). The acidic insert in the activation loop of the CaSte11p kinase domain is shown, with side chains colored in red (D705 to D710). The E707K mutation and the phosphorylation sites are indicated by an arrow and an asterisk, respectively.

found in *C. albicans*. While the *S. cerevisiae* pheromone pathway is comparable in its inputs/outputs to the *C. albicans* opaque cell pheromone pathway described in this report, a new pheromone signaling response pathway has recently been discovered in *C. albicans*. Referred to as the "white pheromone signaling pathway" (58), this signaling cascade uses all the components of the conventional "opaque" pheromone pathway, with the exception of the transcription factor (36), where the "white" pheromone pathway uses the transcription factor Tec1 instead of Cph1 and activates genes associated with biofilm formation (59). Although the molecular mechanisms leading to the differential activation of Cph1p in the *C. albicans* opaque form versus Tec1p in the white form are still unknown, it is interesting to observe that Far1p is implicated only in the opaque cell signaling cascade: Δ*far1* strains are capable of responding at WT levels to pheromone stimulation in white-form cells (36) but not in opaque-form cells (33, 36). In this regard, the scaffold-scaffold interactions between Cst5p and Far1p lead us to propose a model in which Far1p, through its interaction with Cst5p, might be a contributor to the opaque cell pheromone pathway (see Fig. S7 in the supplemental material). On the other hand, Cst5p, through oligomerization and tethering of the MEKK, MEK, and MAPK enzymes, might be the central scaffold shared by the *C. albicans* white and opaque cell pheromone signaling cascades. In support of this model, Yi et al. confirmed in the report accompanying the present one (65) that Cst5 is essential to the pheromone signaling cascades of both *C. albicans* forms.

Phylogenetic reconstruction of the mating signaling pathway. Forces such as gene duplication and functional rewiring can generate new molecular mechanisms of gene regulation and coordination during evolution. For instance, during fungal genome evolution there have been multiple individual gene duplications (e.g., the MAPK duplication) (15) and a major whole-genome duplication (WGD) event (40, 60). Although comparing two distantly related species can offer insights into the ancestral form of the gene or protein of interest, the evolutionary path of regulatory circuits is more difficult to map. Here, we have combined protein structure analyses, domain prediction, and functional characterization, with the goal of gaining insight into the structural evolution of fungal mating scaffold proteins (Fig. 7).

The *Basidiomycetes* contain a single RING-PH-vWA scaffold protein of the Far1p type; this scaffold lacks candidate Ste7-binding and Fus3-coactivating regions. The *Basidiomycetes* also contain a single representative for each MEKK, MEK, and MAPK (Kss1p-like) with similarity to the kinases implicated in the yeast mating pathway. During the separation between *Pezizomycetes* and *Saccharomycotina* (prior to the *Yarrowia lipolytica* branching point), a new RING and PH domain protein can be identified, perhaps arising through duplication of the N terminus of Far1p; this protein does not contain the vWA domain. After the *Y. lipolytica* branching point, there are multiple changes in the structural organization of the MAPK pathway. MAPK duplication is observed; this event provided more regulatory potential to the system but also demanded a mechanism for discrimination between the newly acquired pair of kinases, which could be achieved by connecting Cst5p and one of the MAPKs. However, the observations that two-hybrid assays suggest a unique Cst5p-Cek1p asso-

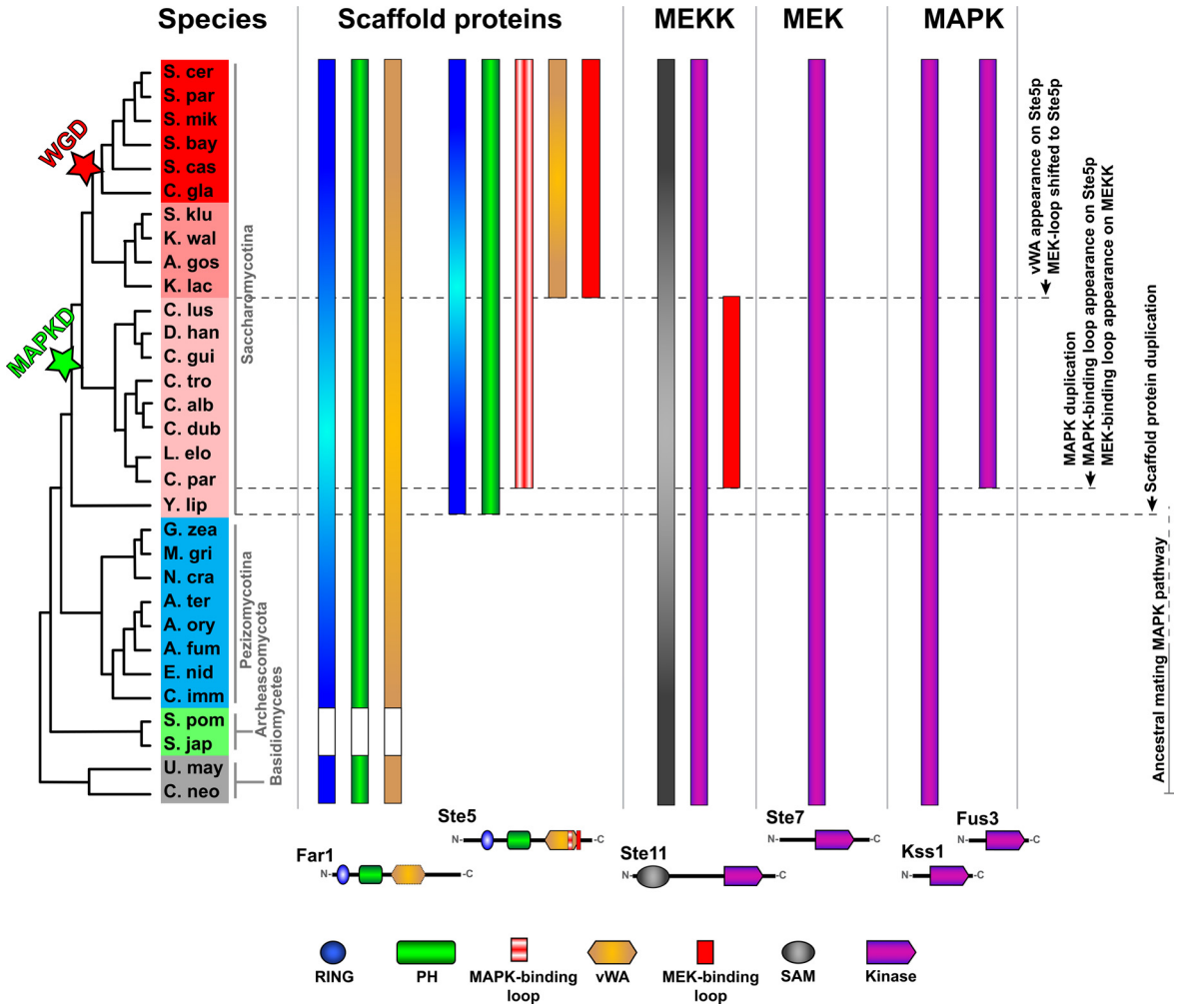

FIG 7 Architectural evolution of the mating MAPK signaling cascade during fungal evolution. Proteins are represented by their structural domains/motifs, which, if detected within a species, are marked in the table according to the color code indicated at the bottom. Major evolutionary steps are described on the right.

ciation and that co-IP experiments (65) are consistent with a Cst5-Cek2p interaction suggest that this potential discrimination process would be complex. This kinase-scaffold connection can use the MAP kinase coactivator loop described by Good et al. (15), which appears coincident with the kinase duplication and is subsequently maintained within the entire Ste5-like scaffold family (Fig. 7; see also Fig. S8 in the supplemental material). Independently, a strong direct interaction between the MEKK and the MEK that relies on an acidic loop found within the kinase domain of MEKK is identified. In *C. albicans*, this CaSte11 acidic loop is critical for Hst7p interaction, as insertion of a positively charged residue into it (E707K) compromised MEK binding and mating. Interestingly, this MEK-binding insert is absent from the MEKKs at the same phylogenetic point (prior to the *K. lactis* branching point) where the Ste5-like scaffold establishes both a vWA domain and a structurally and functionally equivalent MEK-binding acidic loop. In *S. cerevisiae*, mutations in this MEK-binding acidic loop of Ste5p specifically block the Ste5-Ste7 interaction, without compromising the other tethering interactions of Ste5p and Ste7p (15). This picture is consistent with a very modular structure for the protein-protein interaction networks established by the MAP kinase cascade members and the scaffold proteins (61) and indicates that evolution has used various combinations of these modules to achieve signaling specificity and efficacy.

MATERIALS AND METHODS

Strain creation, media, and growth conditions. All *C. albicans* strains and primers used in this study are described in Table S1 in the supplemental material. For gene deletion, we used a sequential cassette blaster strategy to remove the complete coding sequence of *ORF19.2127/CST5* from the 3294 strain background (62). Restoration and overexpression of *CST5* used a strategy similar to that previously applied to *FAR1* (33). Briefly, overexpression was done using the actin promoter (from pACT1) (63) fused to the coding sequence of *CST5* to generate the plasmid pCST5-act1, which was then targeted into the *RPS1* locus to create the 3294 derivative *CST5*^{OP} strain. The Δ*far1-CST5*^{OP} and Δ*cst5-FAR1*^{OP} epistatic strains were created from a combination of strains and plasmids described above (for Δ*cst5* and pCST5-act1) or previously described (for Δ*far1* and pFAR1-act1) (33). The Ste11^{E707K} strain was constructed in two steps. A strain heterozygous for *C. albicans STE11* (*CaSTE11*) was first created by disrupting one allele with the *HIS1* selectable marker, and then the wild-type allele was replaced with the Ste11^{E707K} mutant version; insertion of the Ste11^{E707K} allele was then confirmed by sequencing. For further comparison, a true null mutant was created

by replacing the WT allele present in Caste11/CaSTE11 with the *URA3* selectable marker. Two independent clones for each background were retained for subsequent experiments. Finally, growth, culturing, and selection of opaque cells were done as previously described (33).

Pheromone response and mating assays. Much of the work involves mating competent opaque-phase cells, and the conditions for the selection and maintenance of these cells were in accordance with standard procedures previously described (33, 64). Pheromone-triggered gene expression was assessed by DNA microarrays involving 3 different biological replicates per strain (microarray data are directly available at http://candida2.bri.nrc.ca/whitewaylab/index.html). The pheromone-induced set of genes was assembled from the most significantly induced genes (fold change, >1.8; $P < 0.05$). For the quantitative mating assay, opaque cells were grown overnight in supplemented minimal medium, mixed at a 10:1 tester/experiment ratio, filtered through a nitrocellulose filter, and incubated at room temperature on a yeast extract-peptone-dextrose (YPD) plate. On the following day, cells were washed off, diluted, and plated for the input strain and for tetraploid selection.

Comparative sequence analysis, domain annotation, and structural homology modeling. A complete description of the methods indicated this section is provided in Text S1 in the supplemental material. Briefly, homologous protein sequences were retrieved from the fungal genome database (http://www.yeastgenome.org/) and by a protein domain architecture search in the SMART database (http://smart.embl.de/). Structural domain detection and multiple-query/multiple-template sequence alignments were assembled using (i) MAFFT, (ii) 3D-Coffee, (iii) 3D-Jury, (iv) manual minor local improvements, and (v) three-dimensional (3D) structural alignment using the domain alternate fit in Swiss-PdbViewer version 4.0.1

Mapping of protein-protein interactions among Cst5 and other components of the mating pheromone pathway. To map protein-protein interactions among domains of the pheromone signaling pathway components, we used an alternative yeast two-hybrid (Y2H) system that we developed recently for the detection of protein-protein interactions in the cytoplasm (detailed information of this Y2H system is provided in Text S1 in the supplemental material). Briefly, this yeast two-hybrid system is based on the interaction of Ste11p (MEKK) and Ste50p that is required for HOG pathway activation and osmoadaptation, which is critical for the survival of yeast cells under hyperosmotic stress in the absence of the two-component osmosensing branch (Fig. 3A). The interaction of Ste11p and Ste50p through their respective SAM domains that is required for activation of the HOG pathway can be replaced by association of other protein-interacting modules (46). This property offers a unique potential to analyze bait-prey interactions by substituting them for the respective SAM domains and using the activation of the HOG pathway as a reporter (Fig. 3B and C). To query the bait-prey interaction, *in vivo* recombination (IVR)-positive clones of the baits in one of the two yeast strains were crossed to the IVR-positive clones of the preys in the other yeast strain, mating products were selected, and their ability to activate the HOG pathway was measured by the ability to grow on hyperosmolarity medium (Fig. 3D and E).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00230-10/-/DCSupplemental.

Text S1, DOC file, 0.083 MB.
Table S1, XLS file, 0.041 MB.
Figure S1, TIF file, 1.415 MB.
Figure S2, TIF file, 2.860 MB.
Figure S3, TIF file, 2.906 MB.
Figure S4, TIF file, 2.501 MB.
Figure S5, TIF file, 2.947 MB.
Figure S6, TIF file, 0.980 MB.
Figure S7, TIF file, 2.045 MB.
Figure S8, EPS file, 1.946 MB.

REFERENCES

1. **Bhattacharyya, R. P., A. Remenyi, B. J. Yeh, and W. A. Lim.** 2006. Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. Annu. Rev. Biochem. **75**:655–680.
2. **Dard, N., and M. Peter.** 2006. Scaffold proteins in MAP kinase signaling: more than simple passive activating platforms. Bioessays **28**:146–156.
3. **Cortese, M. S., V. N. Uversky, and A. K. Dunker.** 2008. Intrinsic disorder in scaffold proteins: getting more from less. Prog. Biophys. Mol. Biol. **98**:85–106.
4. **Elion, E. A.** 2001. The Ste5p scaffold. J. Cell Sci. **114**:3967–3978.
5. **Printen, J. A., and G. F. Sprague, Jr.** 1994. Protein-protein interactions in the yeast pheromone response pathway: Ste5p interacts with all members of the MAP kinase cascade. Genetics **138**:609–619.
6. **Marcus, S., A. Polverino, M. Barr, and M. Wigler.** 1994. Complexes between STE5 and components of the pheromone-responsive mitogen-activated protein kinase module. Proc. Natl. Acad. Sci. U. S. A. **91**: 7762–7766.
7. **Choi, K. Y., B. Satterberg, D. M. Lyons, and E. A. Elion.** 1994. Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. Cell **78**:499–512.
8. **Whiteway, M. S., C. Wu, T. Leeuw, K. Clark, A. Fourest-Lieuvin, D. Y. Thomas, and E. Leberer.** 1995. Association of the yeast pheromone response G protein beta gamma subunits with the MAP kinase scaffold Ste5p. Science **269**:1572–1575.
9. **Pryciak, P. M., and F. A. Huntress.** 1998. Membrane recruitment of the kinase cascade scaffold protein Ste5 by the Gbetagamma complex underlies activation of the yeast pheromone response pathway. Genes Dev. **12**: 2684–2697.
10. **Chen, R. E., and J. Thorner.** 2007. Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **1773**:1311–1340.
11. **Bhattacharyya, R. P., A. Remenyi, M. C. Good, C. J. Bashor, A. M. Falick, and W. A. Lim.** 2006. The Ste5 scaffold allosterically modulates signaling output of the yeast mating pathway. Science **311**:822–826.
12. **Winters, M. J., R. E. Lamson, H. Nakanishi, A. M. Neiman, and P. M. Pryciak.** 2005. A membrane binding domain in the ste5 scaffold synergizes with gbetagamma binding to control localization and signaling in pheromone response. Mol. Cell **20**:21–32.
13. **Borden, K. L.** 2000. RING domains: master builders of molecular scaffolds? J. Mol. Biol. **295**:1103–1112.
14. **Shaw, G.** 1996. The pleckstrin homology domain: an intriguing multifunctional protein module. Bioessays **18**:35–46.
15. **Good, M., G. Tang, J. Singleton, A. Remenyi, and W. A. Lim.** 2009. The Ste5 scaffold directs mating signaling by catalytically unlocking the Fus3 MAP kinase for activation. Cell **136**:1085–1097.
16. **Yablonski, D., I. Marbach, and A. Levitzki.** 1996. Dimerization of Ste5, a mitogen-activated protein kinase cascade scaffold protein, is required for signal transduction. Proc. Natl. Acad. Sci. U. S. A. **93**:13864–13869.
17. **Kranz, J. E., B. Satterberg, and E. A. Elion.** 1994. The MAP kinase Fus3 associates with and phosphorylates the upstream signaling component Ste5. Genes Dev. **8**:313–327.
18. **Deshaies, R. J., and C. A. Joazeiro.** 2009. RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. **78**:399–434.
19. **Garrenton, L. S., S. L. Young, and J. Thorner.** 2006. Function of the MAPK scaffold protein, Ste5, requires a cryptic PH domain. Genes Dev. **20**:1946–1958.
20. **Inouye, C., N. Dhillon, and J. Thorner.** 1997. Ste5 RING-H2 domain: role in Ste4-promoted oligomerization for yeast pheromone signaling. Science **278**:103–106.
21. **Wiget, P., Y. Shimada, A. C. Butty, E. Bi, and M. Peter.** 2004. Site-specific regulation of the GEF Cdc24p by the scaffold protein Far1p during yeast mating. EMBO J. **23**:1063–1074.

22. **Leberer, E., D. Dignard, D. Harcus, L. Hougan, M. Whiteway, and D. Y. Thomas.** 1993. Cloning of *Saccharomyces cerevisiae* STE5 as a suppressor of a Ste20 protein kinase mutant: structural and functional similarity of Ste5 to Far1. Mol. Gen. Genet. **241**:241–254.
23. **Butty, A. C., P. M. Pryciak, L. S. Huang, I. Herskowitz, and M. Peter.** 1998. The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. Science **282**:1511–1516.
24. **Elion, E. A., B. Satterberg, and J. E. Kranz.** 1993. FUS3 phosphorylates multiple components of the mating signal transduction cascade: evidence for STE12 and FAR1. Mol. Biol. Cell **4**:495–510.
25. **Peter, M., A. Gartner, J. Horecka, G. Ammerer, and I. Herskowitz.** 1993. FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. Cell **73**:747–760.
26. **Tyers, M., and B. Futcher.** 1993. Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase Cdc28 kinase complexes. Mol. Cell. Biol. **13**:5659–5669.
27. **Elion, E. A.** 2000. Pheromone response, mating and cell biology. Curr. Opin. Microbiol. **3**:573–581.
28. **Mendenhall, M. D.** 1998. Cyclin-dependent kinase inhibitors of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Curr. Top. Microbiol. Immunol. **227**:1–24.
29. **Chang, F., and I. Herskowitz.** 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. Cell **63**:999–1011.
30. **Lyons, D. M., S. K. Mahanty, K. Y. Choi, M. Manandhar, and E. A. Elion.** 1996. The SH3-domain protein Bem1 coordinates mitogen-activated protein kinase cascade activation with cell cycle control in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16**:4095–4106.
31. **Shimada, Y., M. P. Gulli, and M. Peter.** 2000. Nuclear sequestration of the exchange factor Cdc24 by Far1 regulates cell polarity during yeast mating. Nat. Cell Biol. **2**:117–124.
32. **Roberts, C. J., B. Nelson, M. J. Marton, R. Stoughton, M. R. Meyer, H. A. Bennett, Y. D. He, H. Dai, W. L. Walker, T. R. Hughes, M. Tyers, C. Boone, and S. H. Friend.** 2000. Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. Science **287**:873–880.
33. **Côte, P., and M. Whiteway.** 2008. The role of *Candida albicans FAR1* in regulation of pheromone-mediated mating, gene expression and cell cycle arrest. Mol. Microbiol. **68**:392–404.
34. **Atir-Lande, A., T. Gildor, and D. Kornitzer.** 2005. Role for the SCFCDC4 ubiquitin ligase in *Candida albicans* morphogenesis. Mol. Biol. Cell **16**: 2772–2785.
35. **Tsong, A. E., M. G. Miller, R. M. Raisner, and A. D. Johnson.** 2003. Evolution of a combinatorial transcriptional circuit: a case study in yeasts. Cell **115**:389–399.
36. **Yi, S., N. Sahni, K. J. Daniels, C. Pujol, T. Srikantha, and D. R. Soll.** 2008. The same receptor, G protein, and mitogen-activated protein kinase pathway activate different downstream regulators in the alternative white and opaque pheromone responses of *Candida albicans*. Mol. Biol. Cell **19**:957–970.
37. **Zhao, R., K. J. Daniels, S. R. Lockhart, K. M. Yeater, L. L. Hoyer, and D. R. Soll.** 2005. Unique aspects of gene expression during *Candida albicans* mating and possible G(1) dependency. Eukaryot. Cell **4**:1175–1190.
38. **Fischer, D.** 2006. Servers for protein structure prediction. Curr. Opin. Struct. Biol. **16**:178–182.
39. **Ginalski, K., A. Elofsson, D. Fischer, and L. Rychlewski.** 2003. 3D-Jury: a simple approach to improve protein structure predictions. Bioinformatics **19**:1015–1018.
40. **Wapinski, I., A. Pfeffer, N. Friedman, and A. Regev.** 2007. Natural history and evolutionary principles of gene duplication in fungi. Nature **449**:54–61.
41. **Toda, T., M. Shimanuki, and M. Yanagida.** 1991. Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. Genes Dev. **5**:60–73.
42. **Neiman, A. M., B. J. Stevenson, H. P. Xu, G. F. Sprague, Jr., I. Herskowitz, M. Wigler, and S. Marcus.** 1993. Functional homology of protein kinases required for sexual differentiation in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* suggests a conserved signal transduction module in eukaryotic organisms. Mol. Biol. Cell **4**:107–120.
43. **Butler, G., et al.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature **459**:657–662.
44. **Whittaker, C. A., and R. O. Hynes.** 2002. Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol. Biol. Cell **13**:3369–3387.
45. **Inouye, C., N. Dhillon, T. Durfee, P. C. Zambryski, and J. Thorner.** 1997. Mutational analysis of STE5 in the yeast *Saccharomyces cerevisiae*: application of a differential interaction trap assay for examining protein-protein interactions. Genetics **147**:479–492.
46. **Wu, C., G. Jansen, J. Zhang, D. Y. Thomas, and M. Whiteway.** 2006. Adaptor protein Ste50p links the Ste11p MEKK to the HOG pathway through plasma membrane association. Genes Dev. **20**:734–746.
47. **Remenyi, A., M. C. Good, R. P. Bhattacharyya, and W. A. Lim.** 2005. The role of docking interactions in mediating signaling input, output, and discrimination in the yeast MAPK network. Mol. Cell **20**:951–962.
48. **Stynen, B., P. Van Dijck, and H. Tournu.** 2010. A CUG codon adapted two-hybrid system for the pathogenic fungus *Candida albicans*. Nucleic Acids Res. **38**:e184.
49. **Schwartz, M. A., and H. D. Madhani.** 2004. Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*. Annu. Rev. Genet. **38**: 725–748.
50. **Ponting, C. P., L. Aravind, J. Schultz, P. Bork, and E. V. Koonin.** 1999. Eukaryotic signalling domain homologues in archaea and bacteria. Ancient ancestry and horizontal gene transfer. J. Mol. Biol. **289**:729–745.
51. **Bi, X., R. A. Corpina, and J. Goldberg.** 2002. Structure of the Sec23/24-Sar1 pre-budding complex of the COPII vesicle coat. Nature **419**: 271–277.
52. **Humbert, S., H. van Vuuren, Y. Lutz, J. H. Hoeijmakers, J. M. Egly, and V. Moncollin.** 1994. p44 and p34 subunits of the BTF2/TFIIH transcription factor have homologies with SSL1, a yeast protein involved in DNA repair. EMBO J. **13**:2393–2398.
53. **Takagi, Y., C. A. Masuda, W. H. Chang, H. Komori, D. Wang, T. Hunter, C. A. Joazeiro, and R. D. Kornberg.** 2005. Ubiquitin ligase activity of TFIIH and the transcriptional response to DNA damage. Mol. Cell **18**:237–243.
54. **Verma, R., R. Oania, J. Graumann, and R. J. Deshaies.** 2004. Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. Cell **118**:99–110.
55. **Fu, H., N. Reis, Y. Lee, M. H. Glickman, and R. D. Vierstra.** 2001. Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. EMBO J. **20**:7096–7107.
56. **Bounpheng, M. A., J. J. Dimas, S. G. Dodds, and B. A. Christy.** 1999. Degradation of Id proteins by the ubiquitin-proteasome pathway. FASEB J. **13**:2257–2264.
57. **Voges, D., P. Zwickl, and W. Baumeister.** 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. **68**:1015–1068.
58. **Daniels, K. J., T. Srikantha, S. R. Lockhart, C. Pujol, and D. R. Soll.** 2006. Opaque cells signal white cells to form biofilms in *Candida albicans*. EMBO J. **25**:2240–2252.
59. **Sahni, N., S. Yi, K. J. Daniels, G. Huang, T. Srikantha, and D. R. Soll.** 2010. Tec1 mediates the pheromone response of the white phenotype of *Candida albicans*: insights into the evolution of new signal transduction pathways. PLoS Biol. **8**:e1000363.
60. **Wolfe, K. H., and D. C. Shields.** 1997. Molecular evidence for an ancient duplication of the entire yeast genome. Nature **387**:708–713.
61. **Peisajovich, S. G., J. E. Garbarino, P. Wei, and W. A. Lim.** 2010. Rapid diversification of cell signaling phenotypes by modular domain recombination. Science **328**:368–372.
62. **Magee, B. B., M. Legrand, A. M. Alarco, M. Raymond, and P. T. Magee.** 2002. Many of the genes required for mating in *Saccharomyces cerevisiae* are also required for mating in *Candida albicans*. Mol. Microbiol. **46**:1345–1351.
63. **Nicholls, S., M. Straffon, B. Enjalbert, A. Nantel, S. Macaskill, M. Whiteway, and A. J. Brown.** 2004. Msn2- and Msn4-like transcription factors play no obvious roles in the stress responses of the fungal pathogen *Candida albicans*. Eukaryot. Cell **3**:1111–1123.
64. **Dignard, D., and M. Whiteway.** 2006. SST2, a regulator of G-protein signaling for the *Candida albicans* mating response pathway. Eukaryot. Cell **5**:192–202.
65. **Yi, S., N. Sahni, K. J. Daniels, K. L. Liu, G. Huang, A. M. Garnaas, C. Pujol, T. Srikantha, D. R. Soll.** 2011. Utilization of the mating scaffold protein in the evolution of a new signal transduction pathway for biofilm development. mBio **2**(1):e00237-10. doi:10.1128/mBio.00237-10.