

# Recruitment interactions can override catalytic interactions in determining the functional identity of a protein kinase

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The yeast *Saccharomyces cerevisiae* has four distinct mitogen-activated protein kinase kinases (MAPKKs), each of which has a distinct functional identity characterized by communication with specific upstream and downstream partners to form distinct functional pathways. These four kinases belong to one family, sharing closely related catalytic domains. How have these four related kinases diverged to take on four distinct functional roles? The specificity of an enzyme for a particular substrate is often thought to reside in differences in the catalytic domain. However, many kinases, including MAPKKs, have modular interaction domains and motifs that have been shown to play an important role in determining the specificity of kinases through recruitment to specific partners and complexes. Here we probe the relative importance of catalytic domain interactions versus recruitment interactions in defining the functional identity of MAPKKs by asking whether we can use recruitment interactions to force other MAPKK catalytic domains to play the functional role of the mating MAPKK, Ste7. We find that two alternative MAPKKs, Pbs2 and Mkk2, can be forced to functionally replace the mating MAPKK Ste7, but only if the proper set of recruitment interactions are grafted onto their catalytic domains. These results show that within a family of kinases, recruitment interactions can play a dominant role in defining functional identity, and is consistent with a model in which new kinase functions can arise through recombination of existing catalytic domains with new interaction modules.

protein interactions | signal transduction | evolution | synthetic biology

Eukaryotic cells have hundreds of protein kinases (human approximately 500, yeast approximately 100) that act in a coordinated fashion to regulate diverse cellular processes such as metabolism, growth, and differentiation (1). Proper signal transduction requires that a protein kinase be activated via the correct upstream mechanism and communicate with the correct subset of downstream cellular substrates.

The protein kinase superfamily shares a conserved catalytic domain consisting of 250–300 amino acid residues (1). Despite this conserved catalytic domain, distinct functional specificities are observed for individual kinases (2). Such specificities are thought to have arisen by divergent evolution, following expansion of the kinase family. A key question is how related kinases achieve these distinct functional specificities (3).

Historically, active sites within enzyme catalytic domains were thought to be the most important determinant of enzyme specificity because of the requirement for precise stereochemical complementarity with the substrate (e.g., a phospho-acceptor peptide, in the case of a protein kinase). Nonetheless, the study of eukaryotic signal transduction enzymes has made clear that these signaling proteins often also utilize modular protein interactions to form new substrate input and output relationships (4, 5). For example, some kinases have been found to use docking interactions to achieve selectivity (6–8). Docking sites—surface pockets outside of the enzyme’s catalytic surface—interact with cognate peptide docking motifs found on interacting proteins and

have been described in several Ser/Thr kinase families, including MAP kinases (6–9). Docking motifs have been found on the direct downstream substrate of the kinase, on upstream kinases, and on regulatory partners (phosphatases and scaffold proteins) (8–13). In addition to docking interactions, kinase specificity can also be in part determined by scaffold proteins. Scaffold proteins bind to multiple proteins in a pathway, often acting to physically wire them together in a single complex (4, 5).

Previous work has shown that modification of these modular recruitment interactions can be used to redirect signaling information flow in mitogen-activated protein kinase (MAPK) pathways. The core of a MAPK pathway is a three-tiered cascade of kinases that sequentially phosphorylate each other (MAPKKK → MAPKK → MAPK). The yeast MAPKKK, Ste11, is part of both the yeast mating response and the high osmolarity response. Signaling in the two pathways is kept functionally distinct because scaffolding interactions organize Ste11 into two different populations—one that is in complex with upstream and downstream mating partners, and another that is in complex with upstream and downstream osmolarity partners. Park et al., showed that the information flow through Ste11 could be rewired to accept mating input and transmit osmolarity output, by expressing a chimeric diverter scaffold that organized Ste11 into a complex with a novel combination of upstream and downstream partners (14). Moreover, Harris et al., showed that covalent fusion of Ste11 to one set of specific partners could restrict the protein to only function in one of the two possible pathways (15).

The work described above demonstrates that a single MAPK component can be redirected through protein interactions to functionally communicate with distinct combinations of its natural alternative partners. To what degree, however, can a kinase be forced to adopt a new function and to communicate with upstream and downstream partners that it normally does not communicate with? How functionally interchangeable are kinases? At one extreme, is much of kinase specificity intrinsic to the catalytic domain, such that novel communication cannot be forced? Might related kinases that function in the same cell be under strong selective pressure to evolve highly distinct catalytic preferences through negative selection, so as to avoid inadvertent crosstalk (16)? Alternatively, do catalytic domains encode limited specificity, such that accessory protein recruitment interactions are sufficient to force communication with novel, nonnative partners? To examine this question, we chose to test whether yeast MAPKK (MAP kinase kinase) proteins could be forced to adopt new

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functional identities—i.e., to mimic the function of their related kinases from the same organism. MAPKKs are the central node of MAPK pathways. MAPKKs are regulated by upstream MAPKKKs, which phosphorylate conserved threonine residues within the MAPKK active site in response to stimulus. In turn, MAPKKs activate specific cognate MAPKs and propagate signal through phosphorylation of conserved threonine and tyrosine residues within the activation loop of MAPKs.

There are four functionally distinct MAPKKs in *S. cerevisiae*, Ste7, Pbs2, Mkk1, and Mkk2 (Fig. 1A), that act to regulate mating, filamentous growth, osmolarity response, and cell wall integrity. These MAPKKs are highly similar to each other in sequence (Fig. 1B), but little is known about their intrinsic catalytic specificities. We chose to focus on the function of the mating MAPKK, Ste7: We asked whether the other functionally distinct yeast MAPKKs could be forced to take on the Ste7 identity. For an alternative MAPKK to adopt Ste7 functionality requires two basic criteria be satisfied. First, the alternative MAPKK must respond to activation of the pheromone pathway (be activated by Ste11 activated by the mating pathway). Second, the alternative MAPKK must successfully activate the downstream mating MAP kinase, Fus3, thereby triggering the transcriptional response and cellular reorganization cues that will ultimately lead to a proper mating response.

Ste7 has two recruitment interactions that are known to be important for function—two docking interactions and an interaction with the scaffold protein, Ste5. The two docking motifs within the Ste7 amino terminus (Fig. 1B) allow it to bind to the MAPK Fus3 (8). In the mating pathway, the scaffold protein Ste5 selectively binds the MAPKKK Ste11, the MAPKK Ste7, and the MAPK Fus3 (16–18).

Here we tested whether alternative MAPKKs could be forced to functionally replace the mating MAPKK, Ste7, by adding Fus3 docking motifs and/or artificially tethering them to the Ste5 scaffold protein. We find that two of the three alternative MAPKKs in yeast can functionally replace Ste7, but only if they have both of the native Ste7 recruitment interactions. In instances where only docking or only scaffolding interactions are present, the alternative MAPKK is unable to successfully maintain mating signal flow. These results suggest that recruitment interactions can, in some cases, play a dominant role in determining the func-

tional identity of a kinase, and that there is limited intrinsic discriminatory specificity encoded in the kinase domains of most yeast MAPKKs.

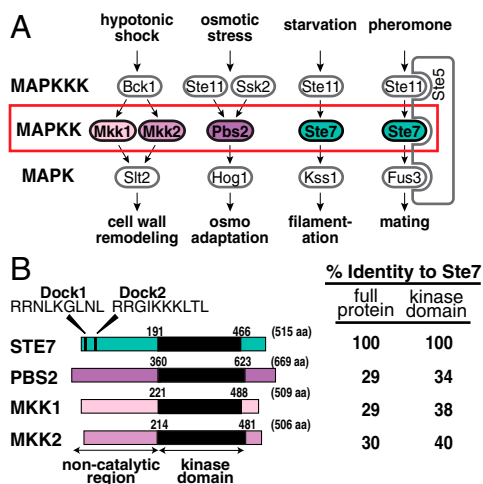
## Results

**Both Catalytic and Recruitment Interactions Are Necessary for Ste7 Function.** We first explored what recruitment interactions of the MAPKK Ste7 are required for its function. Mutations to specific Ste5 scaffold residues V763A/S861P (termed Ste5\*\*) selectively destroy recruitment of Ste7 to the scaffold, resulting in strong abrogation of the mating response (19). However, Park et al. showed that artificial recruitment of Ste7 to Ste5\*\* using an interacting PDZ domain pair fused to the proteins restores signal flux to the mating pathway, albeit at lower than wild-type mating levels in a quantitative mating assay (14). This restoration of signal argued that the passive tethering and increase in local concentration of Ste7 in relation to Ste5 was sufficient to propagate mating signal without the need for precise stereochemical orientation of the components. Here we show that if Ste7 is covalently fused to the Ste5\*\* protein, wild-type levels of mating were observed (Fig. 2A). Thus tethering of Ste7 to the Ste5 scaffold complex appears to be required for function, though artificial covalent tethering is functionally equivalent to the native recruitment interaction.

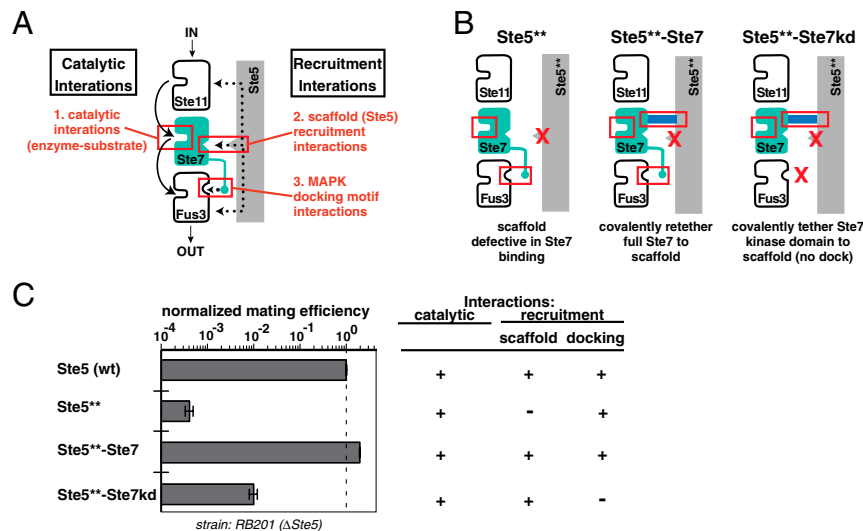
We then tested whether covalent tethering of the Ste7 kinase domain (Ste7kd) alone to Ste5\*\* was sufficient to yield signaling (Fig. 2B). We find that if the noncatalytic amino terminus of Ste7 is removed, even covalent fusion to the Ste5\*\* scaffold is not sufficient to restore full signaling (Fig. 2C). This observation is consistent with an important functional role for docking interactions between Ste7 and Fus3, even if the two kinases are already assembled into a single scaffolded complex. Two Fus3 MAPK docking motifs are found in this noncatalytic N-terminal region of Ste7. In summary, these results show that efficient Ste7 function requires at least two distinct types of recruitment interactions—tethering of the MAPKK to the Ste5 scaffold, and docking interactions between the MAPKK and the MAPK Fus3.

**Scaffold Tethering of Alternative Yeast MAPKKs Is Insufficient to Functionally Replace Ste7.** We then explored whether alternative yeast MAPKKs—Mkk1, Mkk2, and Pbs2—could be forced to assume the identity of Ste7 in the mating response. We first tested whether tethering of the alternative MAPKKs to the Ste5\*\* scaffold was sufficient to rescue mating pathway activation. We therefore fused either the full-length alternative MAPKK or its kinase domain alone to Ste5\*\* (Ste5\*\*-Mkk1, Ste5\*\*-Mkk2, Ste5\*\*-Pbs2) (Fig. 3A). These Ste5\*\*-alt kinase chimeras were expressed from the Ste5 promoter. None of the three alternative yeast MAPKKs were able to rescue mating to any significant level (unlike analogous fusions of Ste7), either when fused in full-length form or in kinase domain form only (Fig. 3B).

**Fusion of Ste7 Amino Terminus to the Alternative Yeast MAPKK Kinase Domains Is Insufficient for Functional Replacement of Ste7.** Given the importance of the Ste7-Fus3 docking interactions, we tested whether chimeric MAPKKs bearing the Ste7 N-terminal region could replace Ste7. We fused the full 191 amino acid amino-terminal portion of Ste7, which includes the two previously reported Ste7 docking peptides (Fig. 1B) to the kinase domains of the alternative MAPKKs (Ste7N-Mkk1KD, Ste7N-Mkk2KD, Ste7N-Pbs2KD) and tested their ability to replace Ste7 (Fig. 3A). To increase the likelihood of the chimeric MAP2Ks associating with the scaffold without a tethering interaction, we also overexpressed the chimeric MAP2Ks using an Adh1 promoter in relation to Ste5\*\* (expressed from the weaker Ste5 promoter). We tested the ability of these chimeric kinases to functionally replace Ste7 through coexpression of these chimeric MAPKKs with



**Fig. 1.** *S. cerevisiae* MAPKK proteins serve distinct functional roles despite a high degree of sequence similarity. (A) Schematic of the core of four MAPK signaling pathways in *S. cerevisiae*. A red box indicates the MAPKKs used in this study. Ste7 is colored green. The alternative yeast MAPKKs are shades of pink. (B) Comparison of the amino acid and structural conservation for the four MAPKKs in A. In this study, the kinase domain for each MAPKK is defined as the area between the numbered residues. The Fus3 docking motifs in the Ste7 N terminus are represented by black vertical bars.



**Fig. 2.** Both catalytic and recruitment interactions are necessary for MAPKK Ste7 function. (A) Schematic of the kinase cascade of the pheromone response pathway. Red boxes indicate the methods utilized by Ste7 for network connectivity: active site recognition, docking interactions, and interactions with the Ste5 scaffold. (B) Strategy for testing contribution of scaffolding and docking interactions to Ste7 function. Mating assays were performed with a Ste5 scaffold with selective mutations V763A/S861P that destroys native Ste7 binding to Ste5 (Ste5\*\*) (19) (depicted by red X). Ste7 was either untethered to Ste5, tethered to Ste5 by being expressed as a fusion to the Ste5 C terminus (indicated by blue bar), or tethered to Ste5 but with its Fus3 docking sites (green arm) deleted. (C) Quantitative mating assays with Ste7 recruitment mutants. Mating efficiencies were normalized to that of Ste5 (wild-type). Background mating efficiency is  $\leq 10^{-4}$ . Chimeric kinases were expressed from the Fus3 promoter on a pRS516 derived plasmid, in a  $\Delta Ste5$  strain (RB201). Data are average of three repeats.

Ste5\*\* (Fig. 3B). None of these three chimeric kinases was able to rescue mating.

**Alternative MAPKKs Can Functionally Replace Ste7 Only in the Presence of Proper Scaffold Tethering and Docking Interactions.** Our previous results indicated that two classes of recruitment interactions—scaffold tethering and docking—were critical for Ste7 function in the mating pathway. Thus, we then tested whether addition of both of these classes of recruitment interactions could overcome any intrinsic specificity encoded within the enzymes' catalytic domains. We therefore combined both scaffold tethering and the docking interactions to create tethered chimeric kinases (Ste5\*\*-Ste7N-Mkk1KD, Ste5\*\*-Ste7N-Mkk2KD, Ste5\*\*-Ste7N-Pbs2KD). These three-part chimeras contained the Ste5\*\* scaffold fused to the N terminus of Ste7 (containing docking motifs) fused to the alternative MAPKK kinase domain (Fig. 3A). We tested the ability of these chimeras to rescue mating when expressed from the native Ste5 promoter (Fig. 3B). Here, two of the three chimeric MAPKKs, Ste5\*\*-Ste7N-Pbs2KD and Ste5\*\*-Ste7N-Mkk2KD, mated efficiently (within an order of magnitude of wild-type levels) (Fig. 3B).

Western blot data (chimeras are tagged with HA-epitope) indicate that the alternate kinase domains when fused to Ste5\*\* were expressed at lower levels than the native Ste5 (Fig. S1). We therefore expressed each of the constructs using a stronger promoter, pAdh1. In this case the third alternative MAPKK chimera—Ste5\*\*-Ste7N-Mkk1KD—showed a slightly higher amount of mating when expressed on the Adh1 rather than the Ste5 promoter, but it was still about 100-fold below that of the Ste7, Mkk2, or Pbs2 constructs (Fig. S2).

To further determine the ability of the alternative chimeric kinases to replace Ste7, we asked whether they were able to induce mating pathway reporters (Figs. S3 and S4). Using antiphospho Fus3 (mating MAPK) antibody Western blots, we observed that the alternative MAPKK chimeras yielded alpha-factor-dependent Fus3 phosphorylation, albeit at a lower level than observed for WT Ste7 or a Ste5\*\*-Ste7 fusion (Fig. 4A). Interestingly, the Mkk1 chimera also seemed to yield some degree of Fus3 phosphorylation, suggesting that even this alternative MAPKK chimera has partial function. Western blots performed when

expressing the chimeras from the stronger Adh1 promoter showed slightly stronger levels of induced Fus3 phosphorylation (Fig. S4A). We also performed Western blots probing for phosphorylation of Hog1, the osmolarity MAP kinase, to determine if these MAPKK chimeras, when stimulated by alpha factor, activate MAP kinases besides the mating MAPK Fus3 (Fig. S5). No phosphorylation of Hog1 was detected, even for the Pbs2 chimera, indicating that this chimera no longer communicates with its native MAPK partner.

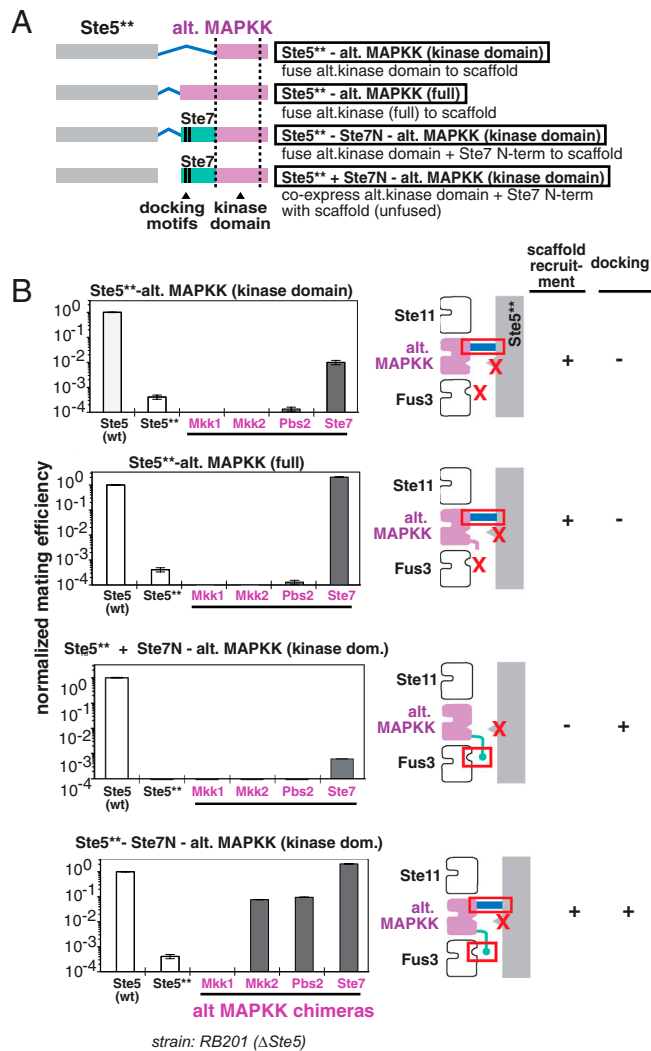
We also used flow cytometry to assay the ability of cells containing the alternative chimeric kinases to induce a mating reporter, Fus1-GFP, after pheromone stimulus. We see that both the Ste5\*\*-Ste7N-Mkk2KD and Ste5\*\*-Ste7N-Pbs2KD constructs are able to induce the mating transcription reporter above the levels that Ste5\*\* is capable of (Fig. S3).

Thus in summary, at least two out of the three alternative MAPKK catalytic domains could be forced to adopt a Ste7 function by several assays, but only when both scaffold tethering and docking interactions were grafted on to them.

**Functional MAPKK Chimeras Are Dependent on Proper Upstream and Downstream Signaling Partners.** Although a number of the alternative MAPKK chimeras were able to functionally replace the mating MAPKK Ste7, we tested whether the chimeras were properly interacting with other components in the mating pathway. To achieve Ste7 functionality, the tethered chimeric kinases must respond to pheromone stimulus and require passage of mating signal from the upstream MAPKKK Ste11 and to the downstream MAPKs, Fus3, or Kss1. Alternatively, the addition or removal of interactions could have potentially eliminated regulatory information, rendering the new kinase constitutively active in the absence of upstream input. As expected these chimeras did not function in strains deleted for either the mating MAPKKK, Ste11, or the mating MAPKs, Fus3 and Kss1 (Fig. 4C). Thus the chimeras replace rather than bypass the role of Ste7.

**Recruitment Interactions Alter Pbs2 Kinase Domain Functional Identity.** We have shown that the chimeric MAPKKs require upstream and downstream mating pathway components to replace Ste7 and that the addition of docking and scaffold association





**Fig. 3.** Alternative MAPKKs can functionally replace Ste7 if proper scaffold tethering and docking interactions are added. (A) Constructs used to test Ste7 functional replacement using alternative yeast MAPKKs. The green box represents a.a. 1–190 of Ste7, containing the docking motifs for Fus3. Although omitted for simplicity, each construct is HA-tagged (C terminus) and expressed from the Ste5 promoter. (B) Quantitative mating assays with alternative MAPKK constructs. Mating efficiencies were normalized to that of Ste5 (wild-type). Background mating efficiency is  $\leq 10^{-4}$ . The blue bar in the schematic represents the covalent fusion to Ste5. The green arm represents a.a. 1–190 of Ste7. The magenta arm represents the endogenous N terminus of each alternative MAPKK. Chimeric constructs were expressed from the Ste5 promoter on pRS316 derived plasmids, in a  $\Delta$ ste5 strain (RB201). In the case of the Ste5\*\* + Ste7N-altMAPKK (kinase domain) pairs of constructs, Ste5\*\* was expressed from the Ste5 promoter on pRS316 derived plasmids, while the Ste7N-altMAPKK kinase domain was expressed from the Adh1 promoter on pRS314 derived plasmids. Data are average of three repeats.

are sufficient to impart new functionality. However, have we successfully converted these kinases identities? We therefore asked whether the Pbs2 chimera, with its new Ste7 interaction motifs, would be able to still function in mediating response to high osmolarity (Fig. S5). The Pbs2 chimera that functionally replaces the Ste7 (Ste5\*\* - Ste7N - Pbs2KD) is unable to confer osmoresistance in a  $\Delta$ Pbs2 background. Thus this chimera is no longer able to function in the original osmoresponse MAPK pathway.

## Discussion

The problem of how related signaling proteins achieve functional specificity within the cell is a fundamental question in cell biology (21–23). In the case of a protein kinase family, such as the

MAPKKs, one might expect that there would be a high level of selective pressure for the individual MAPKKs that exist in the same cell to diverge in their catalytic specificity, in order to maximally avoid crosstalk. On the other hand, kinases like MAPKKs appear to utilize many accessory protein interactions, such as scaffolding or docking interactions, to direct their specificity. Here we wanted to evaluate the importance of accessory recruitment interactions versus intrinsic catalytic specificity in determining the functional identity of individual yeast MAPKKs.

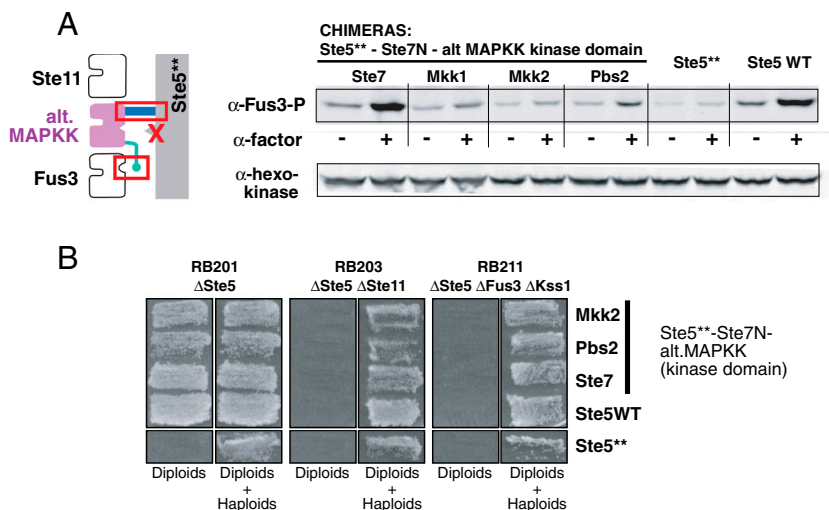
In this study, we have examined the use of docking motifs and scaffolding interactions to recruit alternative yeast MAPKKs to the same cellular complexes as the mating pathway MAPKK Ste7. Our analysis shows that we can change MAPKK identity through the simple addition of these recruitment interactions to the catalytic domains of several alternative yeast MAPKKs. Both docking and scaffold interactions are necessary. The simple addition of tethering to the scaffold was insufficient to force signal flux through an alternative yeast MAPKK, as evidenced by the absence or extremely low ability of Ste5\*\* - Mkk1, Ste5\*\* - Mkk2, Ste5\*\* - Pbs2, or any of the corresponding tethered kinase domain constructs to mate with appreciable frequency. Independently, the use of Ste7 docking interactions to recruit Fus3 to the chimeric kinases was also insufficient to permit mating flux. Thus, this work yields a picture in which there are relatively modest differences in the intrinsic catalytic specificity of the MAPKK catalytic domains (with the possible exception of Mkk1, discussed below), but that the functional specificity of the kinase is achieved through a layering of several distinct types of specific recruitment interactions.

Though our findings indicate that protein interactions may have a dominant role over catalytic specificity, the conversion of just two of the three alternative MAPKKs indicates that intrinsic kinase selectivity cannot always be overcome by forced recruitment and increased local concentration. Some intrinsic specificity is tied to kinase identity, either in the active site, or alternatively, in other pathway specific docking-type interactions that may reside within the kinase domain. Moreover, even the best chimeras do not perform as efficiently as the wild-type Ste7, suggesting that there are additional optimized properties of this particular kinase domain.

It is perhaps not so surprising that Pbs2 chimeras can substitute for Ste7: In the osmolarity pathway, the MAPKKK Ste11 act as a native input to Pbs2. This sharing of an upstream component between the mating and osmolarity pathway indicates that Ste11 may more easily recognize and activate a Pbs2 kinase domain, even in the context of mating pathway signal and interactions. Indeed, through the use of a diverter scaffold that fused mutant Ste5 and Pbs2 together, mating input was successfully converted to osmolarity output signal (14). Nonetheless, Pbs2 does not natively activate Fus3, the mating MAPK, and thus the grafted recruitment interactions are likely to play a role in generating this nonnative link.

The ability of the Ste5\*\* - Ste7N - Mkk2KD chimeric kinase to recapitulate mating cannot simply be explained by the sharing of an upstream MAPKKK with Ste7. Mkk2 normally receives upstream information from the MAPKKK Bck1 and transmits signals to the MAPK Slt2, as part of the hypotonic shock pathway. Why Mkk1 behaves differently from Mkk2 is challenging to explain, given that their kinase domains share over 80% in identity (24). Thus intrinsic specificity differences between the MAPKK catalytic domains does not appear to correlate in a simple way with overall sequence identity.

A growing body of work supports the idea that sophisticated cellular signaling networks in complex eukaryotes have arisen through the generation of new circuitry using a limited toolbox of parts rather than the evolution of novel proteins (21, 25, 26). Here, we examine the specificity of MAPKKs through attempts to use existing connections to convert MAPKK identity. We have



**Fig. 4.** Alternative MAPKK chimeras that able to functionally replace Ste7 signal through the proper upstream and downstream partners. (A) The alternative MAPKK chimeras that functionally replace Ste7 show alpha-factor induced phosphorylation of the mating MAPK Fus3. Western blots are shown for samples after 10 min of alpha-factor stimulation, probed with the anti-p42/44 antibody (cell signaling #4370). Here MAPKK chimeras are expressed from the Ste5 promoter. (B) Complementation of mating deficiency in *ste5* $\Delta$ , *ste5* $\Delta$ *ste11* $\Delta$ , or *ste5* $\Delta$ *fus3* $\Delta$ *kss1* $\Delta$  cells when Ste7 is replaced by alternative MAPKKs that contain both Ste5 scaffold recruitment and Ste7 docking interactions. Growth on diploid selective media indicates cells are capable of mating. Here, the absence of growth indicates that the alternative kinases require both Ste11 and Fus3/Kss1 for a mating phenotype. Chimeric kinases were expressed from Ste5 promoter on pRS316 derived plasmids.

successfully converted two alternative MAPKKs to Ste7 functionality, showing that we can use these simple protein interaction elements to redefine kinase behavior. However, we were unable to find an absolutely generic formula for converting kinases, as we find that some intrinsic contributions to specificity cannot be overcome by recruitment interactions.

## Materials and Methods

**Constructs and Strains.** Plasmids used in this study are shown in Table S1. All plasmids were constructed using the pRS316 CEN-ARS unless otherwise noted. Promoters were amplified from *S. cerevisiae* genomic DNA using 500 bp upstream (Ste5) or 1,400 bp upstream (Adh1) sequence. To determine expression of the constructs, three tandem copies of the HA epitope were fused to the C terminus of the kinases. Yeast strains used in this study are shown in Table S2. Standard gene disruption techniques were used to create the knockouts.

**Domain determination.** MAPKK sequences were obtained from the Saccharomyces Genome Database (<http://www.yeastgenome.org/>). The sequences were entered into the Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>) to determine the kinase domain boundaries. Using the SMART results, each MAPKK was split into two pieces, an N terminus (a.a. 1 to start of kinase domain) and a kinase domain (start of kinase domain to end of protein).

**Alternative kinase constructs.** Full-length alternative MAPKKs (Mkk1, Mkk2, Pbs2) or kinase domains only (Mkk1KD, Mkk2KD, Pbs2KD) were amplified from *S. cerevisiae* genomic. The chimeric kinases (Ste7N-Mkk1KD, Ste7N-Mkk2KD, Ste7N-Pbs2KD) were created using two-step PCR. The Ste7 N terminus (a.a. 1–191) and the alternative MAPKK kinase domains were amplified in separate reactions and fused using two-step PCR.

**Tethered alternative kinase constructs.** For Ste5\*\* -MAPKK recruitment, the alternative kinase constructs described above were fused C-terminal to the Ste5 V763A/S861P mutant (Ste5\*\*) using a Gly-Ser linker. Control plasmids of either Ste5 wild type (Ste5WT) or Ste5\*\* were also created without a kinase fusion. All plasmids and their chimeric junction sequences are listed in Table S1.

**Mating Assays. Quantitative mating assays.** Mating assays have previously been described (27). Briefly, RB201, RB203, or RB211 transformants were grown to midexponential phase strains in selective medium at 30 °C.  $2 \times 10^6$  cells were mixed with 1 ml ( $10^7$ ) of Maya12 cells ( $\alpha$  tester strain) and collected on a .45  $\mu$ m nitrocellulose membrane (Whatman). Filters were

recovered on YPD medium for 5 h. Cells were resuspended in YPD medium, serially diluted, and plated onto SD minimal or SD-lysine plates. Colonies were counted after 2–3 d to determine mating efficiency. Data shown are the average of three experiments.

**Qualitative mating assays.** Maya12 cells were grown to midexponential phase and spread onto YPD plates. Plates containing patches of the transformants were replica-plated onto the Maya12 lawns. Plates were incubated for 12–16 h to allow for mating, then replica-plated onto SD minimal plates to assess for diploid formation.

**Anti-phospho MAPK Western blot assays.** Cells (strain CB011) in midlog phase ( $OD_{600}$  between 0.7–0.8) were treated with  $\alpha$ -factor (1  $\mu$ M) for 10 min. 12–25 mL of treated or untreated culture was spun down and the pellet was snap-frozen in liquid nitrogen and stored at  $-80$  °C. Lysates were prepared using an adapted TCA precipitation method (28). Briefly, ice-cold TCA solution (10 mM Tris.HCl, pH 8.0, 10% Trichloroacetic acid (TCA), 25 mM  $NH_4OAc$ , 1 mM  $Na_2EDTA$ ) was added to each pellet along with glass beads (1/2 of total volume). Samples were vortexed  $4 \times 1$  min at 4 °C with 2 min rests on ice between bursts or broken open with a FastPrep at 6m/sec for 30 sec. Lysates were moved to a new tube and centrifuged. Proteins were recovered by boiling the pellets at 100 °C for 5 min in resuspension buffer (0.1 M Tris.HCl, pH 11.0, 3% SDS). 50–100  $\mu$ g of lysate was used per lane for immunoblotting. Samples were run on a 10% Tris-glycine gel until Fus3 was near the bottom and then blotted to nitrocellulose. Phospho Fus3 was detected using a p44/42 antibody (Cell Signaling Technology, #4370) at 1:2,000 dilution and a LiCor secondary IRDye800. HA was detected using a mouse HA antibody (Pierce). Phospho-Pbs2 was detected with P-p38 MAPK T180/Y182 (D3F9) mAb (Cell Signaling Technology, #4511) at 1:2,500 dilution. Blots were incubated with primary antibody at 2 h at room temperature in TTBS with 5% milk, then with Licor IRDye 800CW secondary antibody at 1:10,000 for 1 h.

**Osmotic Stress Plate Assays.** SH001 transformants were grown to midlog phase ( $OD_{600} = 0.5$ ). Tenfold dilutions starting at  $10^4$  cells were spotted onto selective plates containing 0, 0.5, or 1.0 M KCL. Cells were grown for 2–3 d at 30 °C and scored for growth or no growth.

**Flow Cytometry.** CB011 transformants were grown to early log phase and then diluted down to  $OD_{600} = 0.01$ . After 4 h of growth at 30 °C, cells were induced with 2  $\mu$ M  $\alpha$  factor. Aliquots were taken at each time point and mixed with cyclohexamide to stop protein synthesis. Cells were sonicated and run on a BD LSRII to determine GFP induction levels. Results were analyzed using FlowJo software. For these assays, chimeric kinases were expressed from the Adh1 promoter, and integrated using pNH605 derived plasmids.

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