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Supplementary Materials

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Materials and Methods
Figs. S1 to S6
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Conformational Control of the Ste5 Scaffold Protein Insulates Against MAP Kinase Misactivation

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Cells reuse signaling proteins in multiple pathways, raising the potential for improper cross talk. Scaffold proteins are thought to insulate against such miscommunication by sequestering proteins into distinct physical complexes. We show that the scaffold protein Ste5, which organizes the yeast mating mitogen-activated protein kinase (MAPK) pathway, does not use sequestration to prevent misactivation of the mating response. Instead, Ste5 appears to use a conformation mechanism: Under basal conditions, an intramolecular interaction of the pleckstrin homology (PH) domain with the von Willebrand type A (VWA) domain blocks the ability to coactivate the mating-specific MAPK Fus3. Pheromone-induced membrane binding of Ste5 triggers release of this autoinhibition. Thus, in addition to serving as a conduit guiding kinase communication, Ste5 directly receives input information to decide if and when signal can be transmitted to mating output.

Cells use a complex network of signaling proteins to respond to diverse signals and stresses. Execution of proper decisions is complicated by the fact that individual cells contain many closely related signaling proteins (*1*). In fact, the same proteins are often reused in multiple signaling pathways (*2*, *3*). The resulting interlinked networks could lead to inappropriate cross talk between signaling pathways.

Scaffold proteins, which physically assemble components of a signaling pathway (*4*–*6*), provide a possible solution to this problem. By binding and organizing pathway components into complexes, scaffold proteins promote efficient signaling along a particular pathway. Scaffold proteins may also insulate against improper communication by physically sequestering signaling proteins into distinct pools (*7*–*15*). However, to prevent shared proteins from exchanging between pools, a scaffold must bind its partners with dissociation rates that are slow compared to the time scale for signaling. Direct evidence for this prevailing view of scaffold-based insulation is limited.

A prototypical scaffold protein is Ste5, which coordinates the yeast mating mitogen-activated protein kinase (MAPK) response by binding to all three components of the MAPK cascade and

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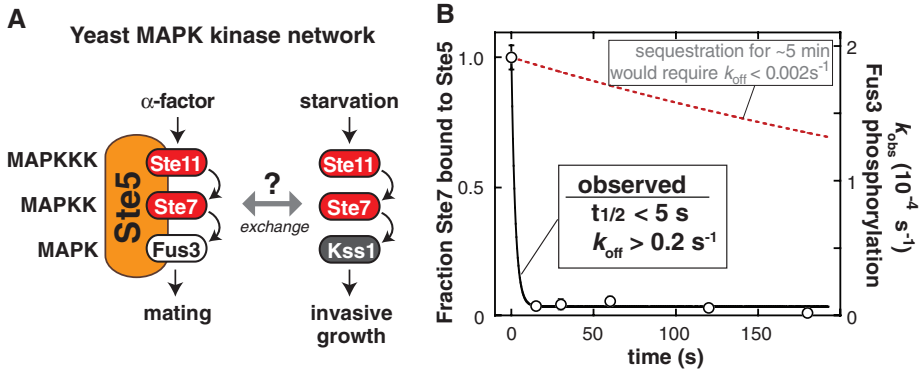
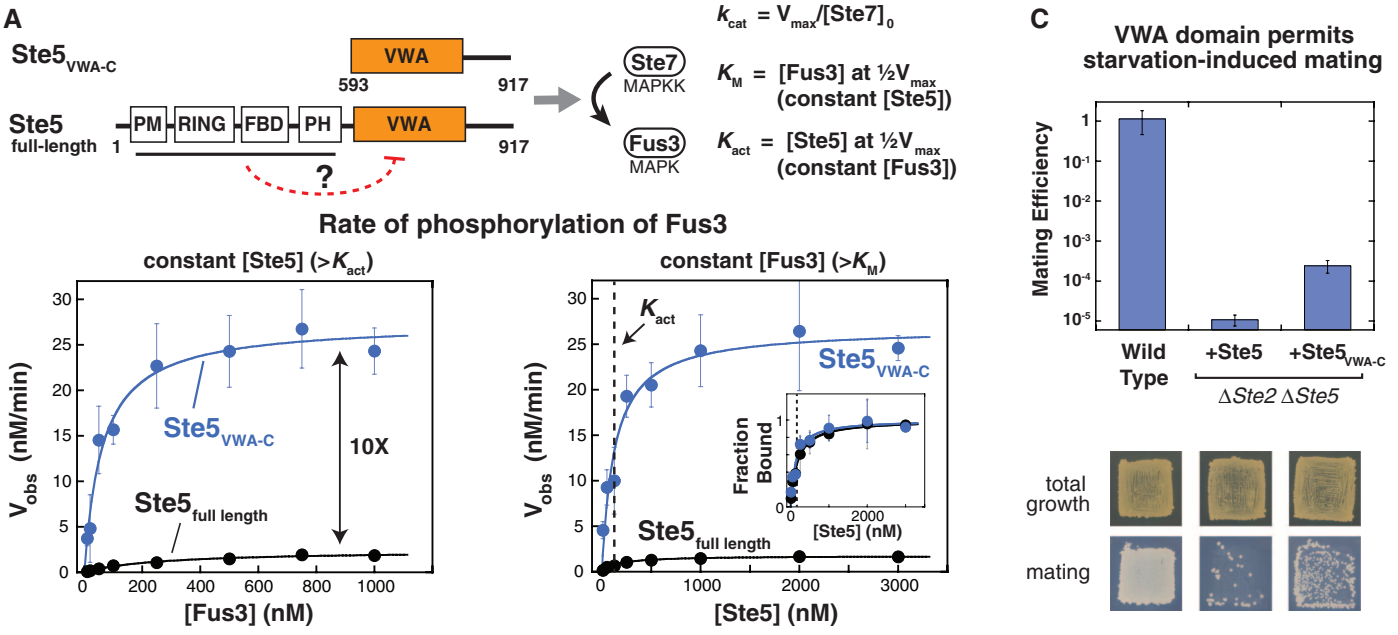


Fig. 1. Exchange of the Ste7 MAPKK from the Ste5 scaffold protein. (**A**) Shared components of the yeast mating and invasive growth pathways yield physiologically distinct input-output responses. (**B**) Dissociation rate of the MAPKK Ste7 from the Ste5 scaffold protein measured with purified recombinant Ste5, the MAPKKK Ste11, the MAPK Fus3, and a constitutively active form of the MAPKK Ste7 [Ste7EE, bearing phosphomimic mutations in the Ste7 activation loop (*16*)]. To a preassembled Ste5-Ste11-Ste7-Fus3 complex, an excess of a Ste7 binding domain [a minimal Ste7 binding domain from Ste5 (residues 759 to 810)] was added to capture Ste7 as it dissociated from Ste5 (fig. S1). At various times, adenosine 5′-triphosphate (ATP) was added, and the initial rate of Fus3 phosphorylation was measured (the amount of Ste5-Ste11-Ste7-Fus3 complex remaining at each timepoint). Error bars, mean ± SD. The observed k_{off} of 0.2 s^{-1} is a lower limit; dissociation occurred on a time scale faster than could be measured with mixing by hand.

serving as a required coactivator of the mating-specific MAPK, Fus3 (*16*, *17*). The Ste5 scaffold is thought to insulate the mating response from other MAPK pathways in yeast, such as the starvation response, which uses the identical MAPK kinase (MAPKK), Ste7, and MAPKK kinase (MAPKKK), Ste11, proteins, but activates a distinct starvation-specific MAPK Kss1 to produce an invasive growth response (Fig. 1A) (*2*, *17*). How the common MAPKK, Ste7, when activated by a specific input, is directed to the correct downstream MAPK is only partially understood. With mating input, both Fus3 and Kss1 are activated (binding to the Ste5 scaffold does not prevent the MAPKK Ste7 from activating Kss1) (*16*, *18*). However, activation of Kss1 by mating input does not lead to cross talk because activated Fus3 overrides the Kss1-induced starvation response by phosphorylating and down-regulating a starvation-specific transcription factor (*19*, *20*). Thus, proper starvation response hinges on preventing Fus3 misactivation by starvation inputs, which would both launch the mating program and directly inhibit the starvation response.

For Ste5 to act as a sequestration-based insulator would require exchange rates for the scaffold-bound shared kinases (Ste11 or Ste7) to be slow relative to the time scale of signaling. Otherwise, shared kinases activated by nonmating inputs would be able to exchange onto the Ste5 scaffold protein and activate the mating response [Fus3 can only be activated when the MAPKK Ste7 is bound to the Ste5 scaffold (*16*)]. We measured the dissociation of purified Ste7 from Ste5 to have a half time ($t_{1/2}$) of <5 s (Fig. 1B), faster by several orders of magnitude than the typical ~5 min time scale of MAPK signaling pathways, and far faster than the time scale of days on which the yeast starvation response operates. Thus, physical sequestration is unlikely to be the primary mechanism that prevents activation of the mating MAPK Fus3 by nonmating inputs.

An alternative model for insulation is that, in the absence of mating input, Ste5 adopts an inactive conformation that blocks its ability to co-catalyze Fus3 phosphorylation (*17*). To investigate this possibility, we measured rates of Fus3



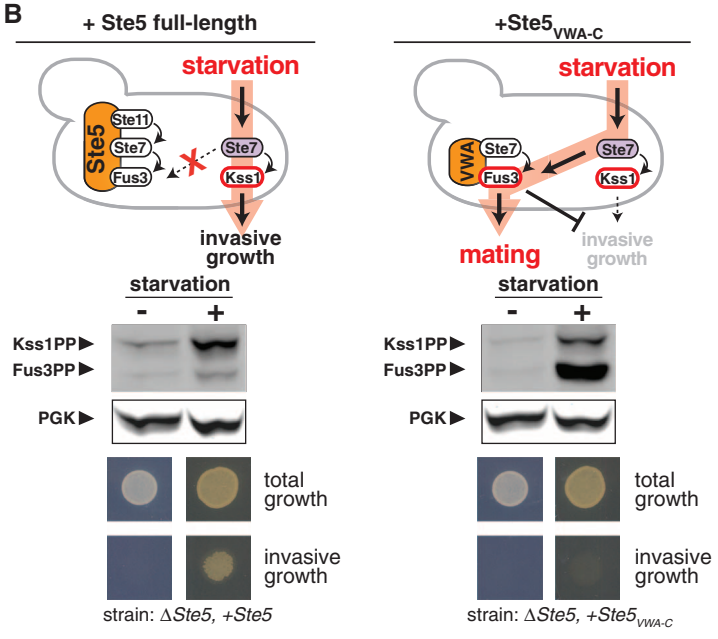


Fig. 2. Autoinhibition of the Ste5 scaffold protein insulates the MAPK Fus3 from activation by incorrect inputs. (**A**) Full-length Ste5 (residues 1 to 917) and $Ste5_{VWA-C}$ (residues 593 to 917) used for in vitro kinetic assays for phosphorylation of Fus3, Michaelis-Menten plot of V_{obs} versus [Fus3], and plot of V_{obs} versus [Ste5]. V_{obs} is the initial rate of Fus3 phosphorylation. K_{act} corresponds to the midpoint of the V_{obs} versus [Ste5] plot and represents the dissociation constant for Ste5-Ste7. Error bars, mean ± SD. It was unnecessary to measure binding affinity for Fus3 assembly into the ternary complex because the Ste5 VWA domain does not bind with any detectable affinity to Fus3 [Fus3 is recruited to this ternary catalytic complex via binding to Ste7; the interaction between the Ste5 VWA domain and Fus3 is a transient catalytic interaction (*16*)]. See figs. S2 to S4 and table S1 for values of fitted kinetic constants. (**B**) Fus3 misactivation in response to starvation in yeast cells expressing $Ste5_{VWA-C}$ (see supplementary materials for growth conditions). Fus3 and Kss1 phosphorylation was monitored with an antibody to phosphorylated MAPKs by protein immunoblotting, and phosphoglycerate kinase (PGK) is shown as a loading control for yeast lysates (see figs. S5 to S7 for quantitative analysis). The invasive growth response was assayed with yeast cells grown on solid agar plates (see supplementary materials). Similar results were obtained with constitutively active alleles of Ste11 and Ste7 (fig. S8). (**C**) $Ste5_{VWA-C}$ misdirects signaling to Fus3 and allows cells to mate in response to starvation. Mating efficiencies were determined using a quantitative mating assay, and patch assays were done as described (see supplementary materials). Error bars, mean ± SD.

phosphorylation by the MAPKK Ste7 with full-length Ste5 and with a minimal Ste5 fragment [$Ste5_{VWA-C}$, containing a von Willebrand type A (VWA) domain that is required for Fus3 coactivation, together with active MAPKK Ste7 (*16*)]. Under maximal rate (k_{cat}) conditions (saturating concentrations of all components), the rate of Fus3 phosphorylation with full-length Ste5 was one-tenth that in the presence of $Ste5_{VWA-C}$ (Fig. 2A). Assembly of the Ste5-Ste7-Fus3 complex was similar with either Ste5 construct (Fig. 2A), suggesting that the dominant contribution to the difference in activity is not a binding effect (disruption of kinase complex assembly) but rather a disruption of the catalytic coactivator function of the VWA domain.

To test the possibility that this activity difference contributes to insulation of the mating MAPK Fus3 in vivo, we introduced the fully active $Ste5_{VWA-C}$ fragment into yeast. In these cells, starvation led to substantial activation of Fus3, whereas cells with full-length Ste5 predominantly activated the starvation MAPK Kss1 (Fig. 2B). Thus, the minimal $Ste5_{VWA-C}$ fragment promotes Fus3 activation when the MAPKK Ste7 is activated, regardless of whether cells have received the mating signal or not. Because activated Fus3 cross-inhibits the starvation signaling pathway at the transcriptional level (*19*, *20*), this misactivation of Fus3 prevents the normal physiological

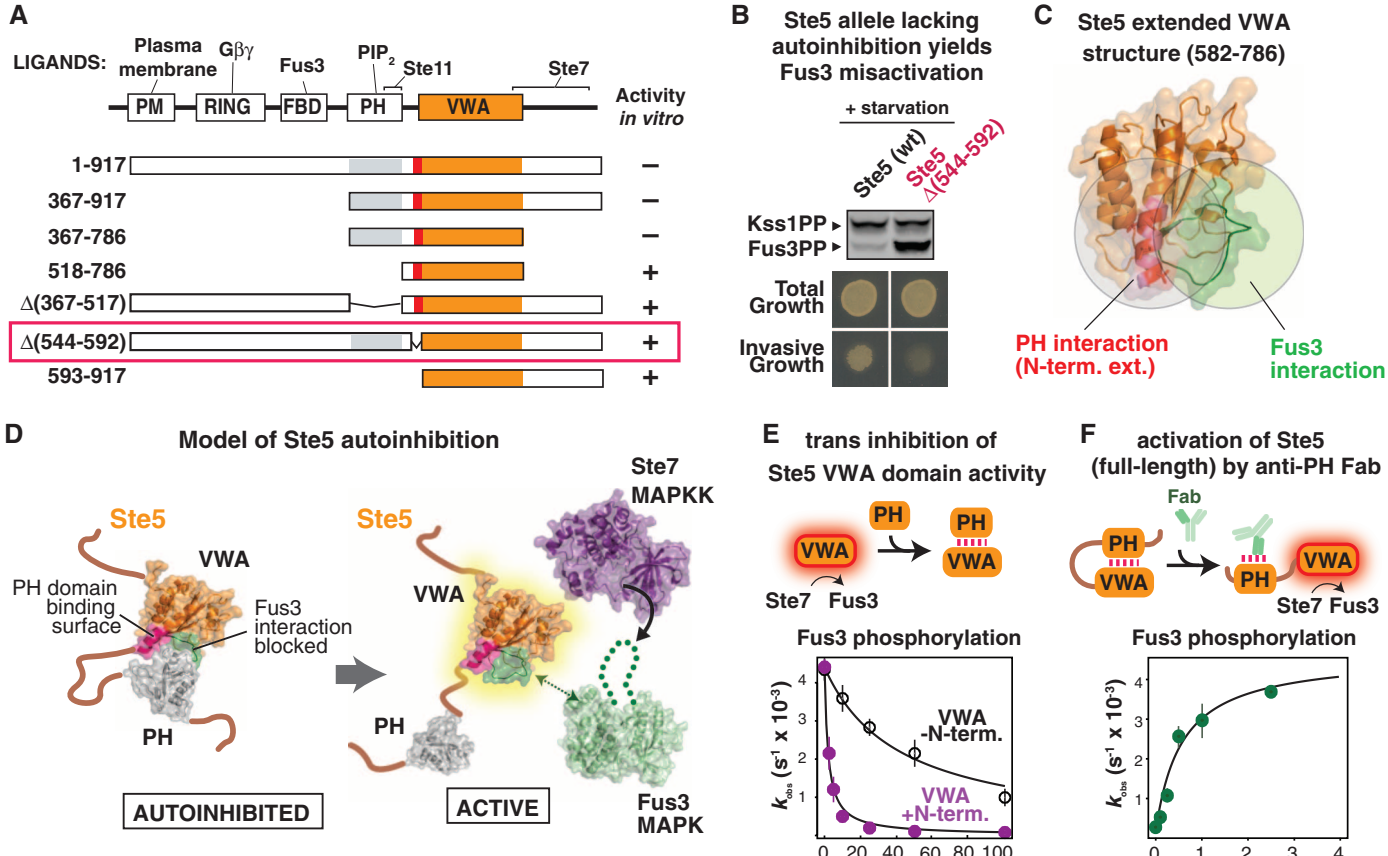


Fig. 3. Mechanism of Ste5 autoinhibition and activation by mating-specific input. (**A**) Diagram of truncation mapping of Ste5 (red is residues 544 to 592). The minimal autoinhibited fragment (367 to 786) contains all elements necessary to assemble the three-tiered MAPK cascade [Fus3 is recruited to the VWA domain by Ste7 (*16*)]. See fig. S9 for additional constructs. (**B**) Effect of deletion of the N-terminal extension of the VWA domain in Ste5 (residues 544 to 592) to disrupt pathway insulation in vivo. Activation of Fus3 measured by protein immunoblotting, and invasive growth assayed on solid agar plates (assays conducted as in Fig. 2B; also see fig. S6). (**C**) Crystal structure of $Ste5_{582-786}$ (see table S4 for crystallographic statistics). The N-terminal extension (582 to 592) is shown in pink, with surface residues necessary for trans inhibition by the PH domain (fig. S10) shown as sticks in red. The Fus3-coactivating loop of Ste5 (743 to 756) is shown in green. The spatial proximity of the PH-domain interface and the site of Fus3 coactivation is illustrated by overlapping circles. (**D**) Model for Ste5 autoinhibition inferred from truncation mapping data and $Ste5_{582-786}$ crystal structure, using structural models as described in the supplementary materials. (**E**) Titration of the VWA domain with the PH domain results in inhibition of the Fus3 coactivator activity (k_{obs}) of the VWA construct bearing the N-terminal extension (582 to 786, shown in pink), or lacking the N-terminal extension (593 to 786, shown in black). Error bars, mean ± SD. (**F**) Relief of autoinhibition in full-length Ste5 by a Fab antibody (SR13) that can bind the PH domain. Error bars, mean ± SD. (**G**) Recruitment of Ste5 to membranes with PIP_2 stimulates coactivation of Fus3. A minimal, autoinhibited Ste5 fragment bearing a hexahistidine tag can be recruited to small unilamellar vesicles of varying lipid compositions by the DGS-NTA(Ni) lipid (see fig. S13 for exact details of the Ste5 construct used here). Error bars, mean ± SD.

response to starvation (invasive growth) (Fig. 2B). Further, under starvation conditions, $Ste5_{VWA-C}$ restores a partial mating phenotype in cells that lack the mating receptor, Ste2 (Fig. 2C); full rescue of mating likely requires mating pathway components upstream of the kinase cascade that are not activated by starvation (*21*). Thus, cells expressing the unregulated $Ste5_{VWA-C}$ fragment misinterpret starvation as a signal to phosphorylate the mating MAPK Fus3, blurring the normally clear insulation between the starvation and mating pathways.

By deletion analysis, we identified two regions of Ste5 essential for autoinhibition of the VWA domain in vitro: the PH domain, which binds to phosphoinositol 4,5-bisphosphate (PIP_2) to facilitate membrane binding (*22*) and binds to the MAPKKK Ste11 (*23*), and an N-terminal extension to the VWA domain (residues 544 to 592), within the linker that connects the PH and VWA domains (Fig. 3A and fig. S9). When Ste5 was replaced by Ste5Δ(544-592) in vivo, activation of Fus3 in response to mating pheromone was normal (fig. S7), but Fus3 was misactivated in response to starvation (Fig. 3B). Because all kinase binding sites are intact in Ste5Δ(544-592), this result supports the idea that physical sequestration of kinases by Ste5 is not sufficient for pathway insulation.

We determined the crystal structure of an extended VWA fragment (residues 582 to 786; we were unable to obtain crystals for a PH-VWA complex) (Fig. 3C). This construct includes the minimal N-terminal extension that binds the PH domain (fig. S10). This extension forms an N-terminal α helix lying directly adjacent to the VWA domain coactivator loop that contains residues essential for Fus3 coactivation (*16*). The spatial proximity of the autoinhibitory PH domain binding site (the N-terminal extension) and the Fus3 coactivator loop indicates that PH domain binding and Fus3 activation might be mutually exclusive, providing a molecular mechanism for Ste5 autoinhibition (Fig. 3D). Indeed, the isolated PH domain of Ste5 inhibited the Fus3 coactivator function of the VWA domain in trans (Fig. 3E). Further, a Fab antibody fragment that binds the PH domain competitively relieved autoinhibition (Fig. 3F and fig. S11). Also, an allele of Ste5 (S770N) that was previously found to constitutively activate the mating pathway (*24*) is not autoinhibited in vitro (fig. S12).

An early step in mating pathway activation is pheromone-induced membrane recruitment of Ste5, which requires a cooperative set of membrane interactions that includes the PH domain binding to PIP_2 lipids (*22*). Thus, binding of Ste5 to PIP_2-containing membranes might disrupt the PH-VWA interaction and relieve autoinhibition. We designed a minimal, membrane-binding Ste5 construct that is autoinhibited, but PIP_2-containing lipid vesicles did not bind or activate this construct in vitro (fig. S13). Because pheromone-induced membrane recruitment of Ste5 is a cooperative process that requires several membrane-binding motifs (*21*), we induced association of the autoinhibited Ste5 construct to the lipid vesicles using other cooperative membrane interactions (Fig. 3G and fig. S13). Under these conditions, PIP_2 caused a 3-fold activation of Ste5 (Fig. 3G), suggesting that membrane recruitment of Ste5 and its interaction with PIP_2 contributes to relief of autoinhibition of Ste5. The inability of such membrane association to completely relieve autoinhibition of Ste5 (a 10-fold effect) (Fig. 2A) could result from incomplete binding to lipid vesicles in vitro (fig. S13) or because complete activation requires additional interactions present in vivo. Ste5 oligomerization has been suggested to contribute to pathway activation (*25*), but we find no evidence that oligomerization plays a direct role in relief of Ste5 autoinhibition (fig. S14).

We propose that, although the shared upstream kinases (MAPKKK Ste11 and MAPKK Ste7) can be activated by other inputs, only mating input activates both the kinase cascade and the Ste5 scaffold protein to permit Fus3 activation (Fig. 4A). Activation of the mating pathway recruits Ste5 to the membrane (*21*), thus activating the MAP kinase cascade by bringing the MAPKKK Ste11 in proximity to its upstream kinase Ste20. Membrane recruitment may also relieve autoinhibition in Ste5 when the PH domain interacts with PIP_2 at the membrane (Fig. 4B).

To further test this model, we decoupled the two functions of Ste5 by deleting the upstream

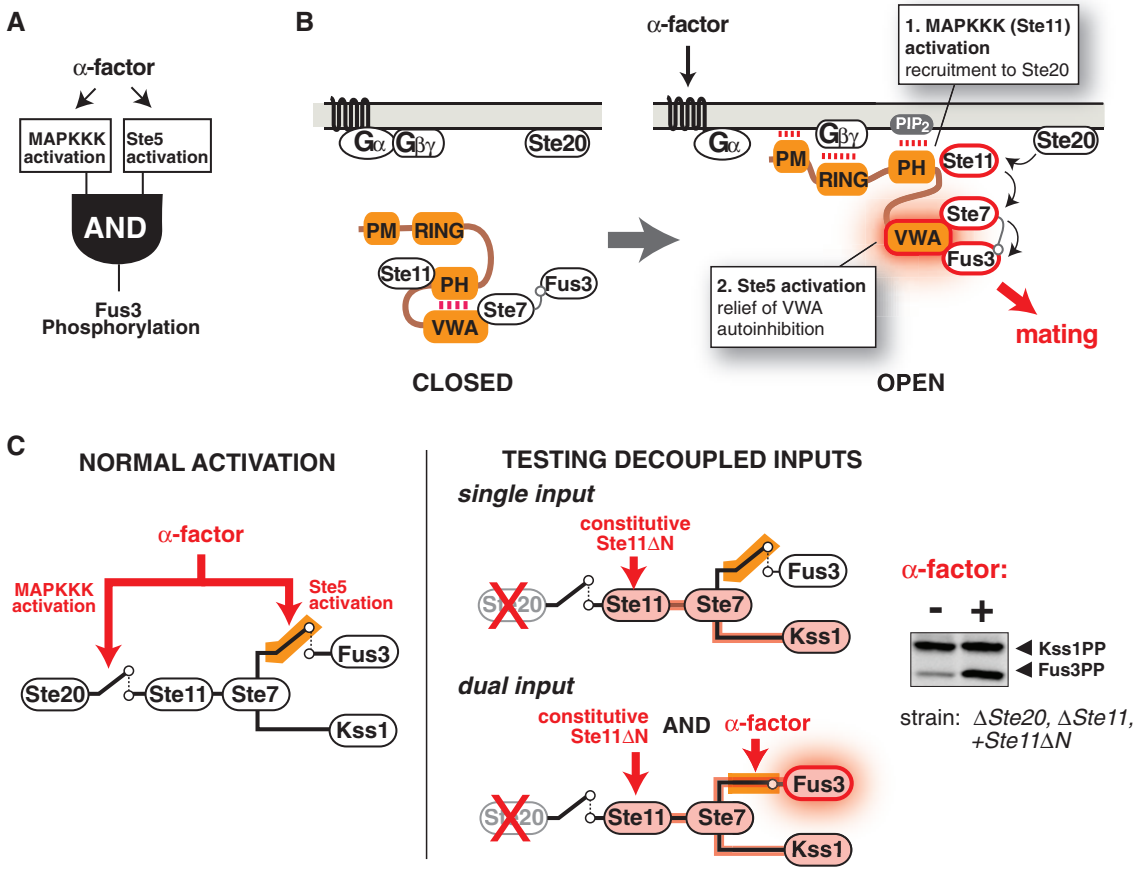


Fig. 4. Mating input-mediated conformational activation of Ste5. (**A**) Simple AND-gate model for specific mating pathway activation. Nonmating inputs that activate the shared MAPKKK do not activate Fus3. (**B**) Revised molecular model for mating pathway activation mediated by the Ste5 scaffold protein. Mating pheromone (α factor) activates a heterotrimeric G protein, leading to release of the Gβγ subunit from Gα and recruitment of Ste5 to free Gβγ at the membrane (*21*). Membrane recruitment triggers activation of the MAPKKK Ste11 and PH domain binding to PIP_2, leading to release of the VWA domain and relief of autoinhibition. (**C**) Fus3 activation in vivo when kinase cascade activation is decoupled from the mating signal (α factor), measured by protein immunoblotting. See fig. S15 for additional Ste11 alleles and controls.

kinase Ste20 (preventing normal activation of the MAPK cascade) and introducing a constitutively active allele of the MAPKKK Ste11, thus rendering activation of the kinase cascade independent of the mating signal. Previous experiments of this type demonstrated that full pathway activation still requires the mating input, suggesting that the input acts on a step downstream of kinase cascade activation (*24*, *26*). Here, we take this approach one step further by using a constitutively active allele, Ste11ΔN (*27*), which lacks the Ste5 binding site (*28*), so that any observed effects of Ste5 activation are likely to arise from promoting the Ste7→Fus3 reaction rather than the Ste11→Ste7 reaction. When wild-type Ste11 was replaced by Ste11ΔN in a yeast strain lacking Ste20, the MAPK Kss1 was preferentially phosphorylated, but when this strain was treated with α factor, activation of Fus3 was observed (Fig. 4C), supporting the idea that pheromone-induced membrane recruitment of Ste5 has two distinct and separable functions: to activate the MAPKKK Ste11 and to relieve autoinhibition in Ste5 to permit Fus3 activation.

Our data do not support the prevailing model that scaffold proteins primarily insulate signaling by sequestration of proteins. Instead, Ste5 appears to function as a conformational switch to gate the flow of information between two distinct signaling outcomes. This mechanism provides a potentially general means to control information flow in complex signaling networks with shared components.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S15
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References

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Rad51 Is an Accessory Factor for Dmc1-Mediated Joint Molecule Formation During Meiosis

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Meiotic recombination in budding yeast requires two RecA-related proteins, Rad51 and Dmc1, both of which form filaments on DNA capable of directing homology search and catalyzing formation of homologous joint molecules (JMs) and strand exchange. With use of a separation-of-function mutant form of Rad51 that retains filament-forming but not JM-forming activity, we show that the JM activity of Rad51 is fully dispensable for meiotic recombination. The corresponding mutation in Dmc1 causes a profound recombination defect, demonstrating Dmc1's JM activity alone is responsible for meiotic recombination. We further provide biochemical evidence that Rad51 acts with Mei5-Sae3 as a Dmc1 accessory factor. Thus, Rad51 is a multifunctional protein that catalyzes recombination directly in mitosis and indirectly, via Dmc1, during meiosis.

Meiosis reduces chromosome number as required for biparental reproduction. The meiotic program evolved by modification of the mitotic cell cycle, via duplication and specialization of key proteins, including the RecA family members Rad51 and Dmc1 (*1*, *2*). Rad51 and Dmc1 form nucleoprotein filaments on single-stranded DNA (ssDNA) tracts that flank double-strand break (DSB) sites. These filaments search for, and swap strands with, homologous double-stranded DNA (dsDNA) segments on unbroken chromatids to form homologous joint molecules (JMs). Rad51 is the only protein that acts directly in JM formation during mitotic recombination. Dmc1 is a meiosis-specific protein. Normal meiotic recombination depends on both Rad51 and Dmc1. Previous results reveal that both Rad51 and Dmc1 are capable of carrying out homology search and catalyzing the formation of JMs (*3*–*5*), but they do not reveal whether one, the other, or both proteins contribute these activities during wild-type (WT) meiosis.

The *Escherichia coli* RecA protein has two DNA binding sites, a high-affinity site (site I) sufficient for polymerization of proteins on ssDNA tracts and a low-affinity DNA binding site (site II) specifically required for interaction of the ssDNA-protein filament with a second DNA during homology search and JM formation (*6*) (fig. S1A). Site II in RecA includes positively charged residues Arg^{243} (R243) and Lys^{245} (K245) (*7*); a third residue, R227, completes a basic patch on the groove of the helical filament. This patch corresponds to a patch of three residues in Rad51 protein (R188, K361, and K371; fig. S1B). We mutated these three residues in Rad51 to alanine to form Rad51-II3A. This protein was then purified (fig. S2).

To test Rad51-II3A for site I binding activity, we used fluorescence polarization (FP) and electrophoretic mobility shift assays (EMSA). FP detected no difference in apparent binding affinity between the WT protein (Rad51-WT) and Rad51-II3A (Fig. 1A). EMSA analysis showed that both Rad51-WT and Rad51-II3A shift the mobility of both ss- and dsDNA under similar

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