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# Conformational Control of the Ste5 Scaffold Protein Insulates Against MAP Kinase Misactivation[‡]

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## Abstract

Cells re-use signaling proteins in multiple pathways, raising the potential for improper crosstalk. 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 MAP kinase 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 PH domain with the VWA domain blocks its the ability to co-activate 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.

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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 crosstalk 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.

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Supplementary Materials:
Materials and Methods
Figures S1–S15
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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 timescale 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 serving as a required co-activator 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 crosstalk because activated Fus3 overrides the Kss1-induced starvation response by phosphorylating and downregulating a starvation-specific transcription factor (19, 20). Thus, proper starvation response hinges upon 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 timescale of signaling. Otherwise, shared kinases activated by non-mating inputs would be able to exchange onto the Ste5 scaffold protein and activate the mating response. We measured the dissociation of purified Ste7 from Ste5 to have a $t_{1/2}$ of <5 seconds (Fig. 1B), faster by several orders of magnitude than the typical ~5 minute timescale of MAPK signaling pathways, and far faster than the timescale 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 non-mating 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 test this possibility, we measured rates of Fus3 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 co-activation 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 co-activator function of the VWA domain.

To test the possibility that this activity difference contributes to insulation of the mating pathway *in vivo*, we introduced the fully active Ste5$_{VWA-C}$ fragment into yeast. In these cells, starvation led to substantial activation of the mating MAPK Fus3, whereas cells with full-length Ste5 predominantly activated the starvation MAPK Kss1 (Fig. 2B). Thus the minimal Ste5$_{VWA-C}$ fragment appears to promote Fus3 activation when the MAPKK Ste7 is activated, regardless of whether cells have received the mating signal or not. Because Fus3 activation inhibits the invasive growth response at the transcriptional level (19, 20), misactivation of Fus3 by cells expressing Ste5$_{VWA-C}$ overrides the invasive growth phenotype (Fig. 2C). Further, under starvation conditions, Ste5$_{VWA-C}$ restores a partial

mating phenotype in cells that lack the mating receptor, Ste2 (Fig. 2D); full rescue of mating likely requires mating pathway components upstream of the kinase cascade that are not activated by starvation (21). Thus when Fus3 activation is promiscuous, cells misinterpret the starvation condition as a signal to initiate the mating program.

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 544–592), 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 582–786; 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 co-activator function of the VWA domain *in trans* (Fig. 3E). Further, a Fab antibody fragment that binds the PH domain completely 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 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. 4), 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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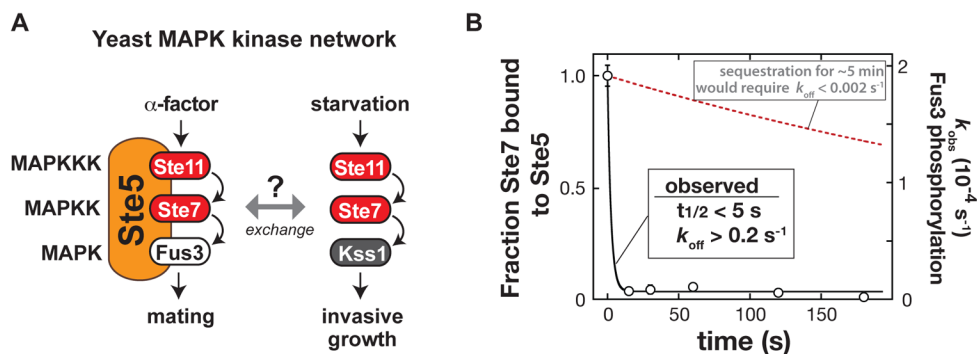
**Fig. 1. Exchange of the Ste7 MAPKK from the Ste5 scaffold protein**

(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 759–810]) was added to capture Ste7 as it dissociated from Ste5 (Fig. S1). At various times, ATP was added, and the initial rate of Fus3 phosphorylation was measured (the amount of Ste5-Ste7-Fus3 complex remaining at each timepoint). Error bars are standard deviations. The observed $k_{\mathrm{off}}$ of 0.2 s$^{-1}$ is a lower limit – dissociation occurred on a timescale faster than could be measured with mixing by hand.
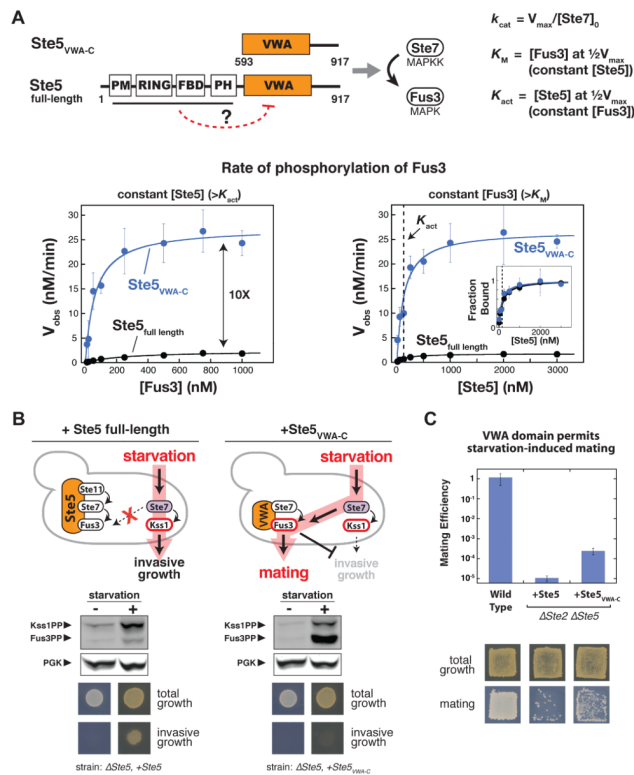
**Fig. 2. Autoinhibition of the Ste5 scaffold protein insulates the MAPK Fus3 from activation by incorrect inputs**

(A) Full-length Ste5 (residues 1–917) and Ste5$_{VWA-C}$ (residues 593–917) used for *in vitro* kinetic assays for phosphorylation of Fus3 by Ste7EE, Michaelis-Menten plot of V$_{obs}$ vs. [Fus3], and plot of V$_{obs}$ vs. [Ste5]. $K_{act}$ corresponds to the midpoint of the V$_{obs}$ vs. [Ste5] plot and represents the dissociation constant for Ste5-Ste7. Error bars are standard deviations. 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 fig. S2-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 Methods 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 fig. S5-S7 for quantitative analysis). The invasive growth response was assayed with yeast cells grown on solid agar plates (see Methods). 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 Methods). Error bars are standard deviations.
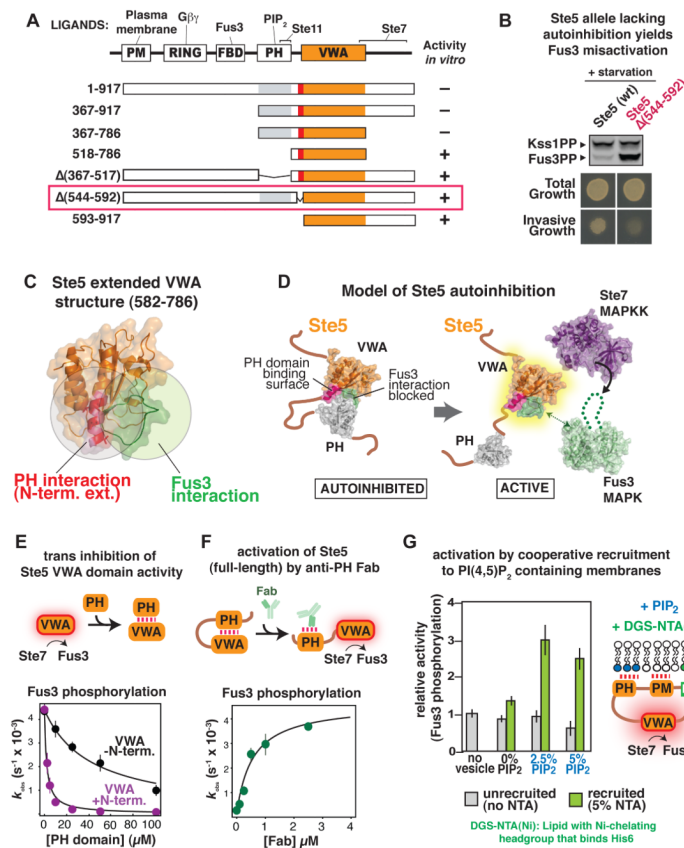
**Fig. 3. Mechanism of Ste5 autoinhibition and activation by mating-specific input**

(A) Diagram of truncation mapping of Ste5 (red is residues 544–592). The minimal autoinhibited fragment (367–786) 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 544–592) 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. 2C, see fig. S6).

(C) Crystal structure of $Ste5_{582-786}$ (see Table S4 for crystallographic statistics). The N-terminal extension (582–592) 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 (743–756) 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.

(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 (582–786, shown in pink), or lacking the N-terminal extension (593–786, shown in black). Error bars are standard deviations.

(F) Relief of autoinhibition in full-length Ste5 by a Fab antibody (SR13) that can bind the PH domain. Error bars are standard deviations.

(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 are standard deviations.
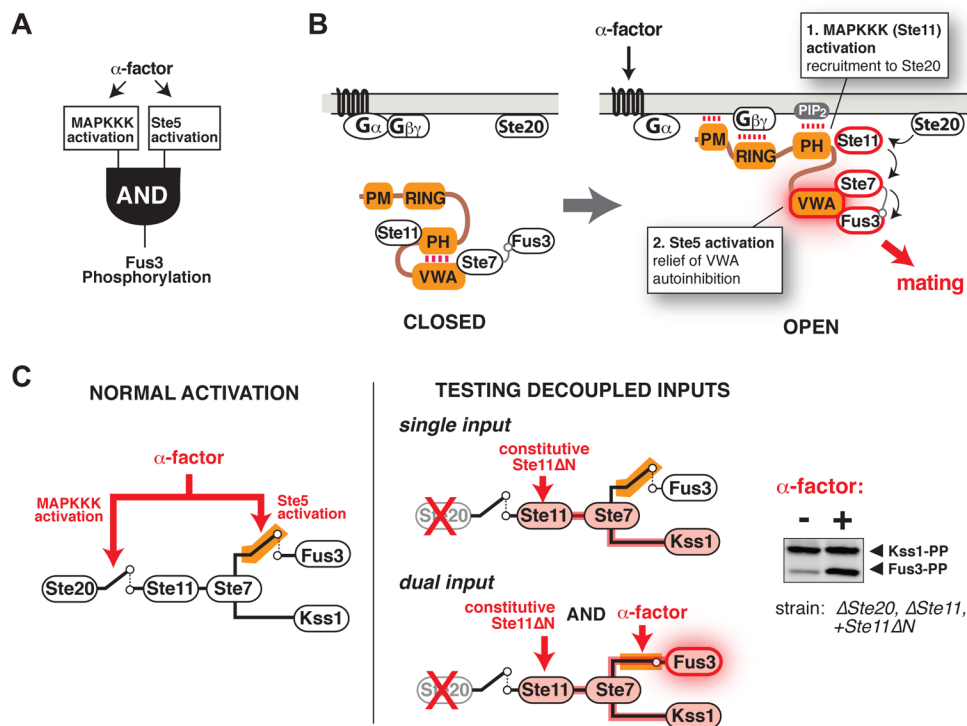
**Fig. 4. Mating input-mediated conformational activation of Ste5**

(A) Simple AND-gate model for specific mating pathway activation. Non-mating 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.