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Evolution of Synthetic Signaling Scaffolds by Recombination of Modular Protein Domains

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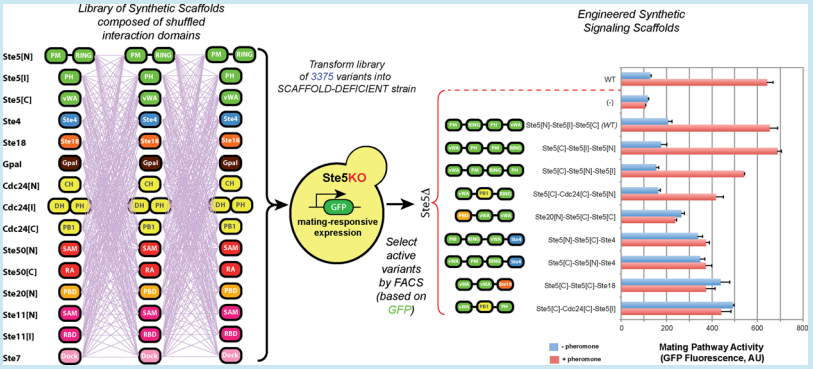
Supporting Information



ABSTRACT: Signaling scaffolds are proteins that interact via modular domains with multiple partners, regulating signaling networks in space and time and providing an ideal platform from which to alter signaling functions. However, to better exploit scaffolds for signaling engineering, it is necessary to understand the full extent of their modularity. We used a directed evolution approach to identify, from a large library of randomly shuffled protein interaction domains, variants capable of rescuing the signaling defect of a yeast strain in which Ste5, the scaffold in the mating pathway, had been deleted. After a single round of selection, we identified multiple synthetic scaffold variants with diverse domain architectures, able to mediate mating pathway activation in a pheromone-dependent manner. The facility with which this signaling network accommodates changes in scaffold architecture suggests that the mating signaling complex does not possess a single, precisely defined geometry into which the scaffold has to fit. These relaxed geometric constraints may facilitate the evolution of signaling networks, as well as their engineering for applications in synthetic biology.

KEYWORDS: *signaling networks, scaffold, domain shuffling, synthetic biology, evolution*

Signaling scaffolds direct the assembly of multiple signaling proteins into high-order complexes, required for the proper propagation of signaling information. Ste5, a scaffold in the MAPK-mediated yeast mating pathway, is one of the best characterized scaffolds thus far.[1] Ste5 is composed of a short N-t amphipathic helix, followed by three domains: a RING domain, a PH domain and a C-t von Willebrand type A (vWA) domain (Figure 1A). Before pathway activation, an intramolecular interaction between the PH and vWA domains maintains Ste5 in an inactive state. Pathway activation by the mating pheromone triggers the recruitment of Ste5 to the plasma membrane, an event mediated by multiple interactions: the N-t amphipathic helix binds to the membrane surface, the RING domain binds to the G protein β-subunit Ste4, and the PH domain binds plasma membrane-localized phosphatidylinositol 4,5-biphosphate.[1] In addition, the PH domain binds the MAP3K Ste11, while the vWA domain binds to the MAP2K Ste7 and allosterically activates the MAPK Fus3.[2,3] Subsequently, a cascade of phosphorylations (MAP3K > MAP2K > MAPK) enables yeast mating. MAPK-mediated signaling pathways in higher organisms, including humans, are also often organized around scaffolds. For example: ERK-mediated cellular proliferation requires the scaffolds KSR[4] or IQGAP1,[4,5] depending on the particular cell type, while JNK-mediated differentiation programs depend on the scaffold JLP.[6] Because scaffolds nucleate key signaling components, they have been preferred targets in synthetic biology efforts aimed at engineering signaling networks.[7−10]

Most protein domains are autonomous folding units with modular functions.[11] Thus, mutational events that rearrange domains among different genes can generate new domain combinations with novel functions.[12−14] The multidomain nature of many signaling scaffolds suggests that scaffolds could evolve (or be engineered) by domain accruement and shuffling. However, if scaffold function depends on spatially precise interactions with multiple signaling components, then it is hard to envision how random shuffling events could result in functional scaffolds. Here, we use a directed evolution approach to investigate how putative synthetic signaling scaffolds capable

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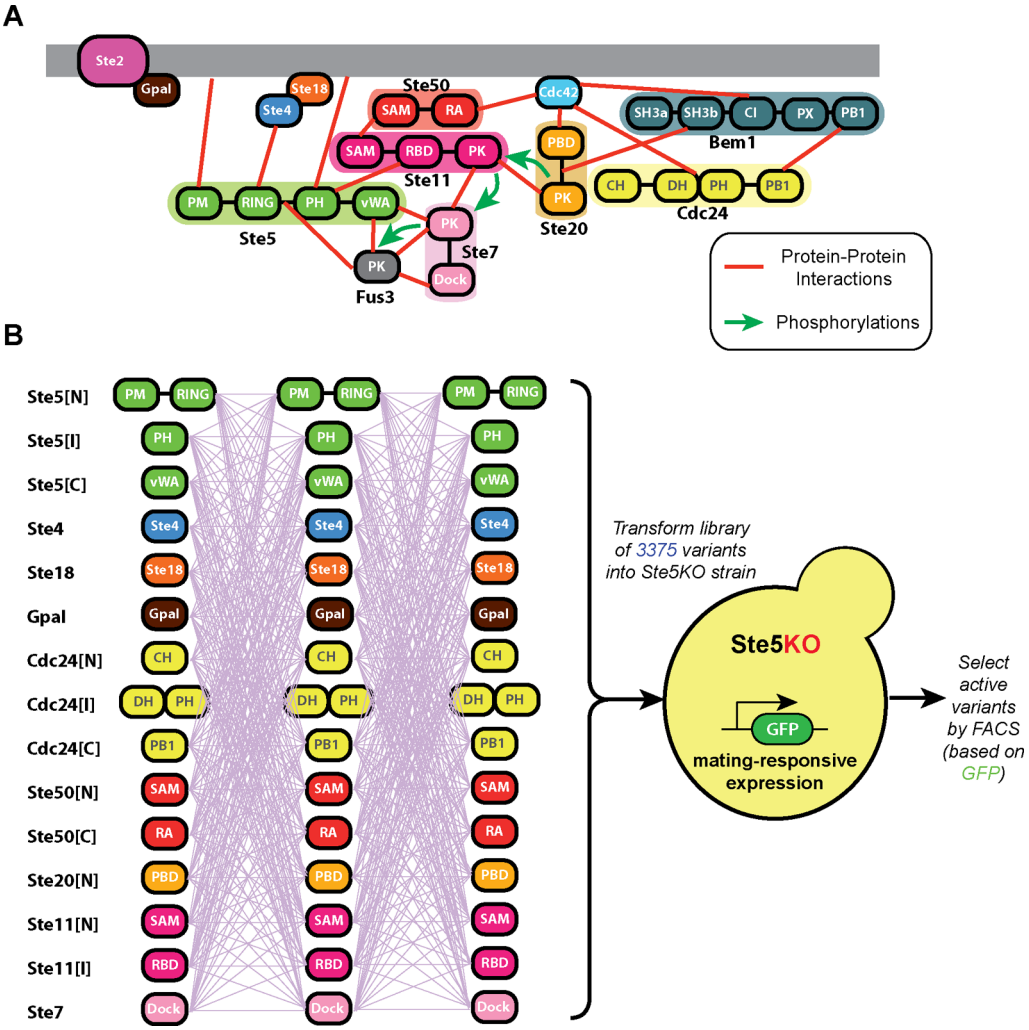

Figure 1. Design of a recombination-derived scaffold library to recover yeast mating pathway activity in a strain deficient in the wild-type scaffold Ste5. (A) The mating pathway is activated upon binding of pheromone to the G protein coupled receptor, Ste2, in a-type cells. Upon pheromone binding, the Gβ (Ste4) and Gγ (Ste18) dissociate from the Gα subunit (Gpa1), allowing the binding of the scaffold (Ste5) to Ste4. Membrane-localized Ste5 then recruits the MAPKKK (Ste11), MAPKK (Ste7), and MAPK (Fus3). Additionally, Ste11 is tethered by an adaptor (Ste50) to the GTPase Cdc42, where it is phosphorylated by the upstream p21-activated kinase Ste20. This triggers the phosphorylation cascade, where Ste11 phosphorylates Ste7, which in turn phosphorylates Fus3. The activated MAPK phosphorylates downstream effectors leading to cellular processes that culminate in mating of a-type and α-type cells. Note that the stoichiometry (and degree of heterogeneity) of the mating signaling complex is not well-defined; thus, for simplicity, we represented a single copy of each protein. (B) Domain recombination library of regulatory domains in the yeast mating pathway. The library of 3375 putative synthetic scaffolds was expressed under an ADH1 promoter and terminator in an a-type Ste5Δ strain. Fluorescence-activated cell sorting was used to isolate synthetic scaffolds able to restore mating pathway activity measured by a GFP reporter under the control of a mating responsive promoter from the FUS1 gene.

of mediating activation of the yeast mating pathway could be assembled by random shuffling of modular protein domains.

RESULTS AND DISCUSSION

Selection of Alternative Domain-Shuffled Scaffolds Capable of Rescuing Mating Pathway Activity in a Ste5Δ Strain. Using a high-throughput combinatorial cloning method that we have developed,[12] we designed a plasmid-borne library of potential scaffolds, in which interaction domains from nine proteins in the yeast mating pathway were combined in groups of three (Figure 1B and Supporting Information Figure 1, note that for simplicity we limited the diversity of our library to shuffling domains that belong to mating pathway proteins). The library of 3375 variants (all possible combinations = 15^3) was then transformed into a yeast strain in which the WT mating pathway scaffold Ste5 had been deleted and in which a mating responsive promoter (pFUS1) controls the expression of a fluorescent reporter (GFP).[8] We then used fluorescence activated cell sorting (FACS) to isolate yeast cells carrying library variants capable of rescuing mating pathway activity in the Ste5KO strain (Figure 1B and Supporting Information Figure 2). After sorting, plasmids were extracted from individual colonies, retransformed into fresh Ste5KO cells, and tested once more by flow cytometry to confirm that the selected variants were capable of mediating pathway activation. Active variants were then sequenced, and their domain composition was determined. The fact that each active variant was recovered multiple times independently suggests that our FACS-based selection method efficiently identifies most active variants present in the domain shuffling libraries.

Strikingly, as shown in Figure 2A, the loss of the WT Ste5 scaffold can be rescued by multiple proteins with diverse

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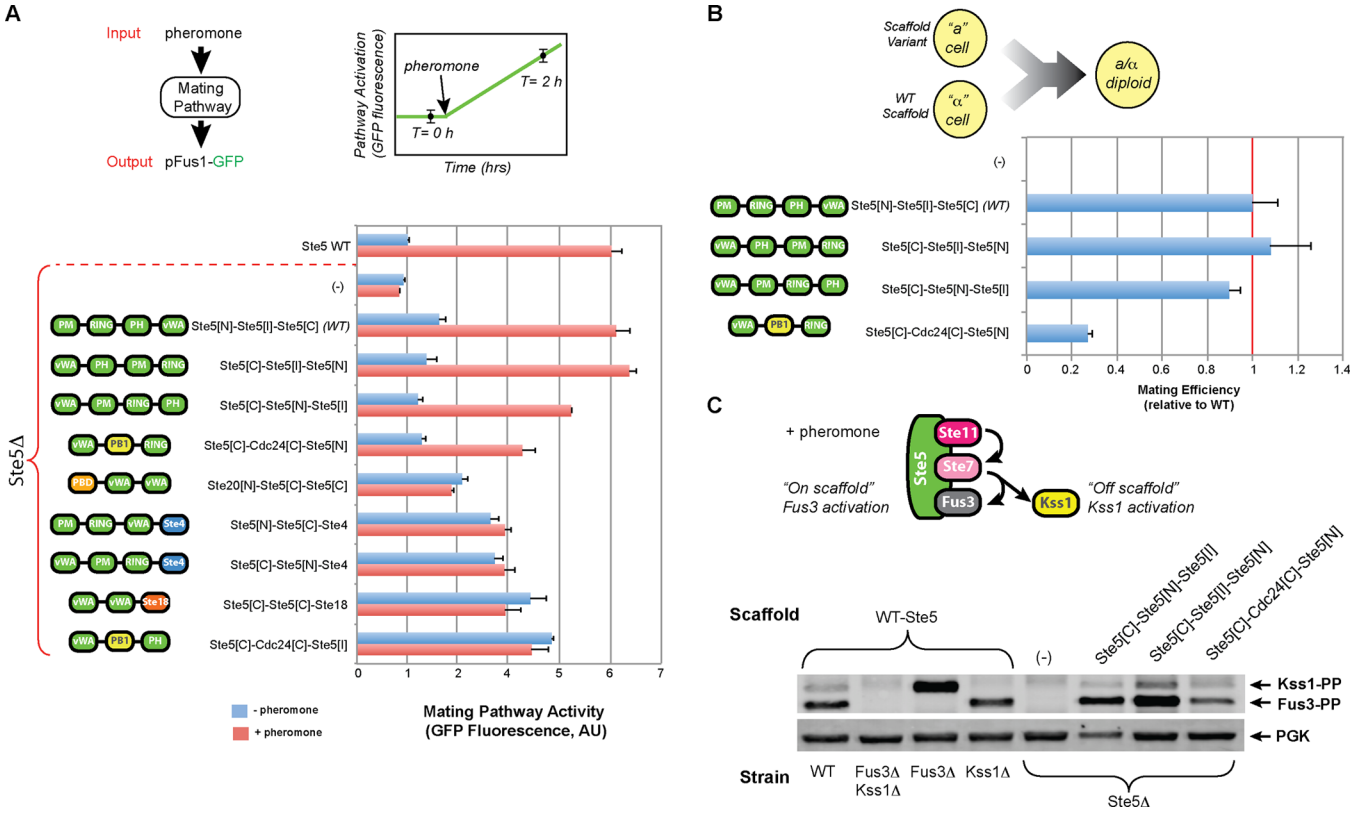

Figure 2. Synthetic scaffolds mediate a range of inducible and constitutive mating pathway responses. (A) Mating pathway activity mediated by the synthetic scaffolds was assessed by flow cytometry, measuring pFUS1-GFP in a Ste5Δ, Far1Δ, Bar1Δ strain, before and after pheromone induction (data shown are the mean and standard deviation of three independent measurements). (B) Quantitative mating efficiency was determined for the synthetic scaffolds capable of mediating a pheromone-dependent response. Specifically, synthetic scaffolds in an a-type Ste5Δ strain were incubated with α-type cells, and mating efficiency was calculated (data shown are the mean and standard deviation of three independent measurements). Our results show that all tested synthetic scaffolds are able to mediate a full mating response; interestingly, we observed that pFUS-GFP expression and mating efficiency correlate. (C) Synthetic scaffolds enable pheromone-dependent activation of Fus3 by Ste7 phosphorylation. Specifically, pheromone-dependent mating pathway activation mediated by WT Ste5 results in the phosphorylation of Fus3 and Kss1 (lanes 1−4). In contrast, deletion of Ste5 abolishes Fus3 and Kss1 phosphorylation even after pheromone activation (lane 5). Expression of the synthetic scaffolds rescues pheromone-dependent Fus3 and Kss1 phosphorylation (lanes 6−8). The ability of the synthetic scaffolds to enable pheromone-dependent Fus3 phosphorylation is particularly important, as it indicates that their catalytic vWA domain (required for Ste7-mediated Fus3 phosphorylation) is fully functional. Phosphoglycerate kinase (PGK) is shown as loading control. Experiments were repeated twice and found to be in good agreement.

domain compositions. The top three variants have similar, Ste5-like domain compositions, though arranged in three different orders: PM-RING-PH-vWA (as in WT Ste5), but also vWA−PH-PM-RING or vWA-PM-RING-PH. These results suggest that, though Ste5′s domain composition may be needed for high-efficiency pathway activation, the order in which the different domains are arranged in the polypeptide can be altered. In fact, variant vWA−PH-PM-RING has activity levels that are indistinguishable from those of WT Ste5. Still, it should be noted that, while the pathway tolerates several possible Ste5 domain rearrangements, there are many rearrangements that were not recovered in our selection scheme, suggesting that not all possible rearrangements lead to active Ste5 variants. A second group of active variants contains domain compositions in which some of the Ste5′s domains are replaced by domains from other mating pathway proteins. This suggests that some interactions that are normally mediated by Ste5′s domains, can actually be replaced by interactions mediated by alternative domains. In particular, we observed that Ste5′s localization to the membrane-bound signaling complex, which is mediated by the N-t PM helix, the RING and the PH domains, can be replaced in a number of ways (Figure 2A and Supl. Figure 3): (i) by Cdc24's PB1 domain, which localizes to the vicinity of Cdc42 via an interaction with Bem1,[15] (ii) by Ste20s PBD domain, which directly interacts with Cdc42,[16] or by the G protein β-subunit Ste4 or γ-subunit Ste18, both of which are membrane-localized.[17]

Identity of Membrane Recruitment Interactions Differentiates Inducible from Constitutive Scaffolds. In the WT pathway, pheromone binding to the Ste2 receptor triggers the dissociation of the Gα subunit (Gpa1) from the G$\beta\gamma$ heterodimer (Ste4/Ste18), enabling the recruitment of the Ste5 scaffold to the membrane and subsequently activating the phosphorylation cascade.[17] The stimulus-dependent change in Ste5 subcellular localization is a key event controlling pathway activation. As shown in Figure 2A, our selection procedure identified two different classes of scaffolds: one that signals in a pheromone-dependent manner and one that signals constitutively. A simple analysis of the domain composition of the selected scaffolds suggests that pheromone-dependent signaling results from scaffolds for which membrane recruitment is pheromone-dependent (e.g., membrane recruitment depends mainly on Ste5′s RING domain), while constitutive signaling results from scaffolds that constitutively localize to the plasma membrane (e.g., membrane recruitment is mediated by Ste20s PBD domain, or directly by the Gβ or Gγ subunits—even in

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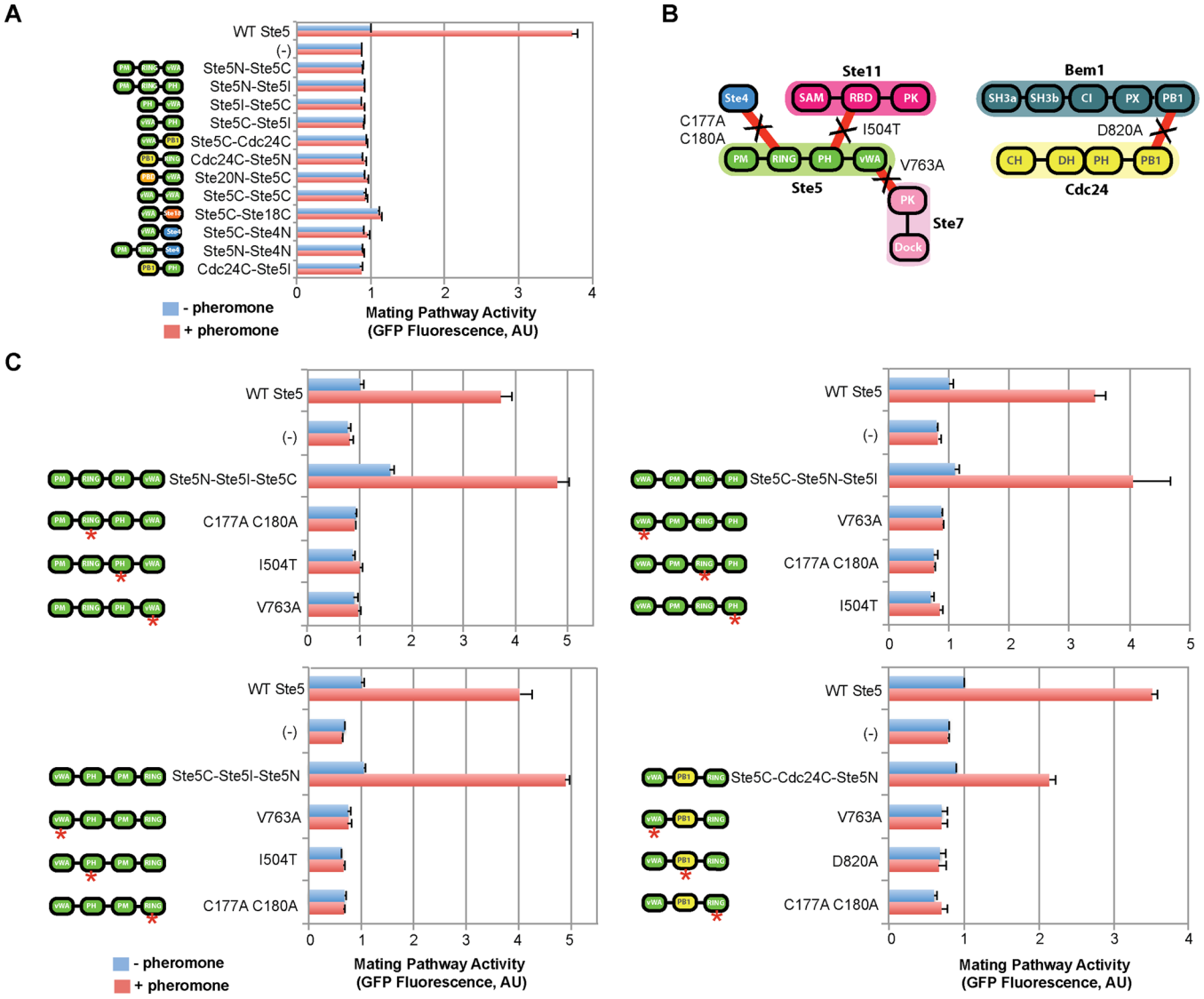

Figure 3. All protein−protein interactions are required for proper function of the synthetic scaffolds. (A) Removal of individual protein interaction domains from the synthetic scaffolds abolishes mating pathway responses, indicating that all domains are needed for scaffold function. (B) We introduced the following mutations, known to abolish specific protein−protein interactions: C177A/C180A in Ste5's RING domain, which disrupts binding to membrane-localized Ste4; I504T in Ste5's PH domain, which disrupts binding to the RBD domain of the MAPKKK Ste11; V763A in Ste5's vWA domain, which abolishes the interaction with the MAPKK Ste7; and D820A in the PB1 domain of Cdc24, which disrupts the interaction with the PB1 domain in Bem1. (C) Each individual point mutation known to disrupt key recruitment interactions, eliminates mating pathway activation mediated by either WT Ste5 or the synthetic scaffolds, as determined by pFUS1-GFP expression and flow cytometry (data shown are the mean and standard deviation of triplicate experiments).

scaffolds that also contain a RING domain). An interesting comparison is that of the selected scaffolds Ste5 vWA-Cdc24 PB1-Ste5 RING and Ste5 vWA-Ste5 PB1-Ste5 PH, as the former mediates inducible pathway activation, while the latter is constitutively active. While the RING domain is recruited to the plasma membrane only after the pheromone-induced dissociation of the Gβ Ste4 from the Gα Gpa1,[18,19] the PH domain binds phosphoinositide 4,5-diphosphate constitutively.[20] In addition, it is interesting to note that Cdc24 PB1 domain is known to bind Bem1's PBI domain, localizing Cdc24 to the proximity of membrane-bound Cdc42's polarity complex. We believe that Ste5 variants containing Cdc24 PB1 are likely to be localized to the proximity of the polarity complex as well. Still, the observation that Ste5[C]-Cdc24[C]-Ste5[N] activates pathway response in a pheromone-dependent manner, suggests that Cdc24's PB1-Bem1's PB1 interaction is not sufficient to recruit Ste5[C]-Cdc24[C]-Ste5[N] to the membrane in a signaling-productive way. Instead, it may be the integration of two independent interactions, Cdc24's PB1-Bem1's PB1 and Ste5's RING-Ste4/Ste18, that is required for the stable recruitment of Ste5[C]-Cdc24[C]-Ste5[N] to the membrane and subsequent pathway activation. Because Ste5's RING-Ste4/Ste18 interaction depends on the pheromone-induced release of Ste4/Ste18 from the heterotrimeric G protein complex, Ste5[C]-Cdc24[C]-Ste5[N]-mediated pathway activation may be pheromone-dependent. Furthermore, the lack of Ste5's PH domain in variant Ste5[C]-Cdc24[C]-Ste5[N] suggests that Ste11 can be activated in the absence of Ste5's PH domain, though the exact mechanism of activation remains unknown. We would like to speculate that the lack of Ste5's PH domain, at the same time, facilitates Fus3 activation by Ste5's vWA domain (as this domain would normally be

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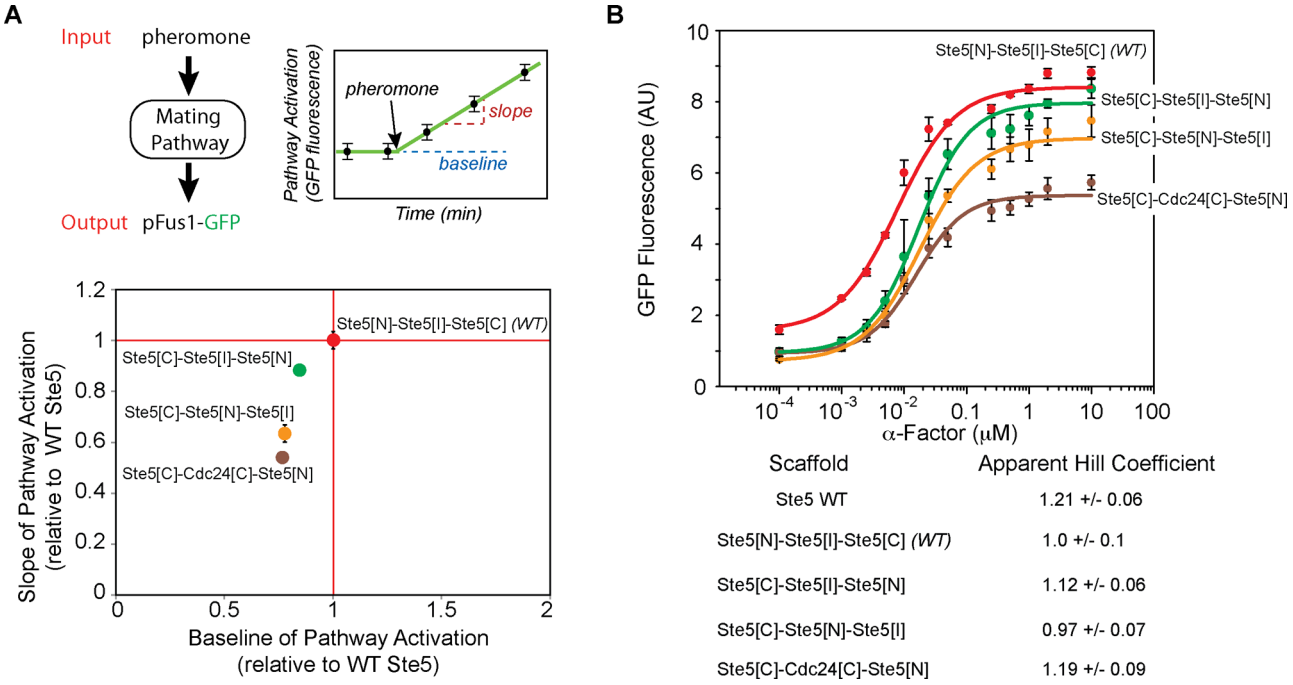

Scaffold	Apparent Hill Coefficient
Ste5 WT	1.21 +/- 0.06
Ste5[N]-Ste5[I]-Ste5[C] *(WT)*	1.0 +/- 0.1
Ste5[C]-Ste5[I]-Ste5[N]	1.12 +/- 0.06
Ste5[C]-Ste5[N]-Ste5[I]	0.97 +/- 0.07
Ste5[C]-Cdc24[C]-Ste5[N]	1.19 +/- 0.09

Figure 4. Effect of the synthetic scaffolds on mating pathway response kinetics and dose response. (A) Time-course measurements of pFUS1-GFP fluorescence were done to calculate the baseline and slope of mating pathway response, upon stimulation with 1 μM α-factor. The resulting baselines and slopes were normalized to wild-type values and plotted. While all selected scaffolds can mediate pathway response, scaffold domain architecture affect response kinetics, with the WT Ste5 mediating the fastest response. (B) Dose response curves for the WT Ste5 and the selected synthetic scaffolds. pFUS1-GFP fluorescence was measured 2 h after stimulation with the corresponding concentration of α-factor. Data were fitted to a Hill equation and apparent Hill coefficients were calculated.

inhibited by the PH domain), enabling efficient signaling propagation even with low levels of Ste11 activation. Because stimulus-dependence is a key aspect of the mating pathway (and in fact of most signaling pathways), for the remainder of this work, we focus only on synthetic scaffolds capable of mediating pheromone-dependent responses.

Synthetic Scaffolds Can Mediate Cell–Cell Fusion. The mating pathway response includes, in addition to changes in gene expression (represented here by the activation of a GFP reporter), complex changes in cell morphology that ultimately result in the fusion of the two cells.[17] To determine if the pheromone-dependent synthetic scaffolds were capable of mediating a full mating response, we quantitatively measured their mating efficiency,[21] as compared to cells expressing the WT Ste5 scaffold. As shown in Figure 2B (and Supporting Information Figure 4), all of the alternative scaffolds are able to mediate full mating responses. In particular, the scaffolds with domain compositions similar to those of the WT Ste5, though with domains arranged in different orders, have mating efficiencies that are indistinguishable from that of the WT scaffold. Thus, we conclude that the complex changes in cell morphology required for mating can be mediated by signaling scaffolds with alternative domain architectures. In addition, we also observed that for pheromone-dependent variants, mating efficiencies correlate well with pFUS1-GFP fluorescence (Supporting Information Figure 5), confirming that pFUS1-GFP is a good proxy for pheromone-dependent mating pathway activation.

Synthetic Scaffolds Can Catalytically Unlock the MAPK Fus3. WT Ste5 has two key roles in mating pathway activation: (i) it assembles a membrane-bound multiprotein signaling complex, and (ii) through the specific activity of its vWA domain, it catalytically unlocks Fus3, facilitating Ste7-mediated Fus3 activation.[2] In principle, the selected alternative scaffolds could mediate pathway activation via Fus3 (as WT Ste5), or via Kss1, a Fus3 paralog that can be activated by Ste7 in a Ste5-independent manner (Figure 2C). Interestingly, while the synthetic scaffolds (either inducible or constitutive, see Figure 2A) can differ in the identity of the domains responsible for membrane-recruitment, they all include at least one vWA domain. This observation suggests that the synthetic scaffolds mediate mating pathway activation through Fus3, similarly to WT Ste5. To confirm this hypothesis, we determined Fus3 phosphorylation state after addition of pheromone. As shown in Figure 2C, all pheromone-dependent scaffolds enable Fus3 phosphorylation, independently of whether the vWA domain is present at the N-t, C-t, or in the interior of the protein. This observation suggests that, while Fus3 unlocking requires a functional vWA domain, the exact location of the vWA domain within the signaling complex is not important.

Specific Domain-Mediated Interactions Are Required for the Proper Function of the Synthetic Scaffolds. Scaffolds' central role in the assembly of functional signaling complexes depends on their ability to interact with multiple signaling proteins (probably with some of them simultaneously). In the case of Ste5, individual domains mediate each of these multiple interactions (e.g., RING-Ste4, PH-Ste11, vWA-Ste7, etc.[1]). Thus, to increase the probability of selecting functional scaffolds in our directed evolution experiment, we designed our library by shuffling multiple combinations of at least three interaction domains (as opposed to, for instance, combinations of only two domains). Still, to investigate if all domains present in the synthetic scaffolds were actually needed for signaling function, we created variants lacking each

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individual domain and determined their ability to mediate mating pathway activation by flow cytometry. As shown in Figure 3A, all tested variants lacking one domain are inactive, confirming that the full domain composition of each synthetic scaffold is required for proper function. Interestingly, even though two of the constitutively active scaffolds selected in our experiments have only two domain types (i.e., two vWA domains connected to Ste20s PBD or to the Gγ Ste18, Figure 2A), in both cases, deletion of one of the repeated vWA domains resulted in inactive proteins. While further work will be needed to fully explain these observations, we may speculate that at least two vWA domains within a signaling complex could be needed for proper signal propagation. In the case of WT Ste5, oligomerization (mediated by the RING domain[18,22]) may ensure that two vWA domains are present within the complex.

To further confirm that the synthetic scaffolds require fully functional domains, we introduced mutations known to eliminate specific domain−domain interactions and determined the ability of the resulting scaffolds to mediate mating pathway activation by flow cytometry. In particular, we introduced the following mutations in Ste5-derived domains (numbering according to WT Ste5): C177A and C180A in the RING domain, known to disrupt Ste4 binding;[18] I504T in the PH domain, known to disrupt Ste11 binding;[23] and V763A in the vWA domain, known to disrupt Ste7 binding.[24] In addition, we introduced the mutation D820A in Cdc24's PB1 domain (numbering according to WT Cdc24), known to disrupt Bem1 binding[25] (Figure 3B). As shown in Figure 3C, disruption of each individual interaction abolishes mating pathway activation, confirming that all domain-mediated interactions are needed for scaffold (and pathway) function and further suggesting that the function of the synthetic scaffolds (as that of WT Ste5) depends on the assembly of a multiprotein signaling complex.

Kinetics of the Mating Pathway Response Depends on the Domain Architecture of the Signaling Scaffold. Mating pathway activation is a complex process that requires changes in multiple protein−protein interactions, as well as several catalyzed steps (e.g., GDP-GTP exchange reactions or phosphorylations). Pathway activation kinetics is therefore the result of this complex multistep process. The signaling scaffold could affect activation kinetics by facilitating certain interactions and, presumably, by positioning interacting partners in specific orientations. Thus, we reasoned that, even though signal flow can proceed with scaffolds with altered domain architectures, these changes in domain architecture could impact pathway kinetics. To explore this hypothesis, we determined the time course of mating pathway activation for the evolved alternative scaffolds. As shown in Figure 4A, the WT Ste5 scaffold (Ste5 PM-RING-PH-vWA) mediates the fastest pathway activation, followed by the two synthetic scaffolds with Ste5 domain compositions but altered order (vWA-PH-PM-RING and vWA-PM-RING-PH), and last by the synthetic scaffold Ste5 [vWA]-Cdc24 [PB1]-Ste5 [PM-RING]. Thus, while the selected synthetic scaffolds are capable of mediating mating pathway activation, the kinetics of the response is affected by the scaffold's domain architecture. Interestingly, from all the synthetic scaffolds selected from our library, the WT scaffold mediates the fastest response.

To confirm that the differences in kinetics were the result of the scaffolds different domain architectures, rather than of different protein levels, we determined the steady state protein levels for the different scaffolds by measuring the GFP fluorescence of strains expressing GFP-labeled scaffolds. As shown in Supporting Information Figure 6, all tested strains have similar levels of GFP fluorescence. While it is not possible to derive precise quantitative information from GFP fusions, the similar levels of fluorescence among the different variants suggest that their concentrations are somehow similar. Thus, it is unlikely that the differences in response kinetics are due to large differences in scaffold concentration.

Sensitivity of the Mating Pathway Response Does Not Depend on the Domain Architecture of the Signaling Scaffold. In wild type cells, the dependence of mating pathway response on pheromone concentration, as measured by the transcriptional expression of pathway-dependent genes, is graded, with an apparent Hill coefficient of 1.21 ± 0.06.[8] Interestingly, Pryciak and co-workers have shown that, while the kinase module of the mating pathway is intrinsically ultrasensitive (apparent Hill coefficient ≫1), membrane recruitment of Ste5 converts this ultrasensitive response into a graded response,[26] likely by allowing the faithful propagation of weak signals (at low pheromone concentration) that would otherwise be filtered out. Thus, we then asked whether this ability to transform a switch-like response into a graded response was exclusive to the WT Ste5 scaffold, or could also be achieved by the synthetic scaffolds. For that, we determined the dose response curves for pathways containing synthetic scaffolds capable of mediating inducible activation. As shown in Figure 4B, while the maximum levels of pathway activation vary for the different scaffolds, the cooperativity of the response is similar for all of them (with apparent Hill coefficients ranging from 0.97 ± 0.07 to 1.19 ± 0.09). Thus, we conclude that, similarly to the WT scaffold Ste5, the synthetic scaffolds can convert the intrinsically ultrasensitive mating response into a graded response. These results indicate that the sensitivity of the pathway response is robust to changes in scaffold domain architecture.

Synthetic Scaffolds Do Not Significantly Alter Growth Rate. Cell cycle progression is controlled by the Fus3-dependent phosphorylation of Far1. Thus, changes in mating pathway components may consequently alter growth rate. To determine whether the effects of the synthetic scaffolds on growth rate are different from that of WT Ste5, we measured growth rates in a strain *MATa, Ste5:: Trp1, his3, trp1, leu2, ura3, ADE2 can1* carrying selected synthetic scaffolds. As shown in Supplementary Figure 7, the effects of the synthetic scaffolds on growth rate are indistinguishable from that of WT Ste5.

Taken together, our results indicate that the mating signaling scaffold is remarkably modular, tolerating changes in domain architecture, both in terms of domain order and composition. The facility with which the mating signaling network accommodates changes in scaffold domain architecture may imply that the mating signaling complex does not possess a single, precisely defined geometry. This hypothesis supports recent computational and experimental analyses of MAPK-mediated signaling pathways, which indicated that rather than precisely assembled multiprotein machines, signaling complexes are heterogeneous ensembles of transient complexes.[27−30] While the mutational mechanisms that rearrange domains in natural proteins are obviously different from the 3-part shuffling method used to construct our library, our results suggest that scaffolds could evolve in nature by mutational events that alter proteins' domain compositions (e.g., duplications and fusions, recombinations, or transpositions, to name a few[31−36]). Finally, our results support the choice of protein scaffolds as platforms

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for signaling engineering, a central goal of synthetic biology.[7−10]

METHODS

Library Construction and Strains. The library of scaffolds was cloned using a multipart combinatorial cloning approach adapted from ref 12, which uses the type IIs restriction enzyme, Aar1. Protein interaction domains (Supporting Information Table 1) were amplified by PCR from a *Saccharomyces cerevisiae* genomic library (Invitrogen) with customized AarI overhangs GGAG ('A'), CCCT ('B'), GCGA ('C'), and TGCG ('D') (Supporting Information Figure S1). The library was produced in a single step by ligating all AarI-digested gel-purified PCR products into a pRS315 CEN-ARS acceptor plasmid in a 2:2:2:1 (domains with A−B overhangs/B−C overhangs/C−D overhangs/plasmid) molar ratio. To ensure that all possible clones in the library were represented, we harvested ∼35 000 colonies after transformation into *Escherichia coli* (the library's theoretical diversity is 15 × 15 × 15 = 3375). To confirm that the library was assembled properly, we sequenced 20 independent clones from the unselected library and observed that (i) every clone in the unselected library was identified only once and (ii) domains from all proteins present in the library were observed at least once. In all cases, clones were expressed from an ADH1 promoter and an ADH1 transcription terminator. Specific plasmids used in this study are described in Supporting Information Table 2. Yeast strains used in this study are described in Supporting Information Table 3.

Fluorescence-Activated Cell Sorting. The recombination-derived scaffold library was sorted to isolate variants capable of recovering mating pathway response in a scaffold deficient strain (Ste5Δ) by fluorescence activated cell sorting (FACS). In particular, the library was first transformed into a *S. cerevisiae* strain derived from W303, with the following genotype: *MATa, bar1::NatR, far1Δ, mfa2::pFUS1-GFP, Ste5:: Trp1, his3, trp1, leu2, ura3*. To achieve an adequate representation of the library's diversity, ∼35 000 transformants were harvested in complete synthetic dropout medium and vortexed for 5 min. A 20 μL aliquot of the library mixture was diluted in 50 mL selective medium and grown overnight at 30 °C. Overnight cultures were diluted and grown to early log phase (OD600 = 0.05−0.1). The mating pathway was activated by addition of 1 μM α-factor for 3 h at 30 °C. Following pathway induction, 100 000 cells were sorted using a BD FACSAria I cell sorter, according to GFP fluorescence (Supporting Information Figure 2). Sorted cells in which putative synthetic scaffolds were capable of recovering pathway activity (i.e., GFP expression) were plated and cultured. Scaffold variants were identified by extracting plasmids from cultured yeast lysates and sequenced with external primers.

Flow Cytometry. Analysis of pathway activity by flow cytometry was described in ref 12 with the following modifications: For all experiments, triplicate cultures were grown to early log phase. For time-course experiments, the pathway was initiated by 1 μM α-factor and aliquots were taken at 20 min intervals. For dose−response experiments, the pathway was initiated by indicated α-factor concentrations and measured after 2 h of induction. For all experiments, the cells were treated with cycloheximide immediately after indicated induction times and the fluorophore was allowed to mature for 30 min in the darkness. Cultures were then analyzed with a MACSQuant VYB (Miltenyi Biotech). The GFP signal of 10 000 cells was measured in each case. Data shown are the mean and standard deviation of triplicates. Dose−response curves were fit to a Hill equation with OriginPro (OriginLab) as follows:

Hill equation

$$F(a) = F_{\min} + (F_{\max} - F_{\min})\,(a^{nH}/(C_m{}^{nH} + a^{nH})$$

where F = mean GFP fluorescence, a = concentration of α-factor, $F_{\min}$ = mean basal fluorescence (no α-factor), $F_{\max}$ = mean fluorescence with maximal pathway output (saturating α-factor), C_m = α-factor concentration at which fluorescence is half-maximal, and nH = Hill coefficient. Hill coefficient errors are standard deviations were calculated with OriginLab and reflect errors among triplicate samples.

Quantitative Mating Assays. Quantitative mating assays were done as described in ref 21 with minor modifications. Plasmids containing specified constructs were transformed into a *S. cerevisiae* (S0992) strain with the following genotype: *MATa, Ste5:: Trp1, his3, trp1, leu2, ura3, ADE2 can1*. Triplicate cultures were then grown to mid log phase (OD600 = 0.9). Equal amounts of each a-type strain were mixed with α-type cells (*lys*) and deposited on a 0.45 μM cellulose nitrate membrane filter (Whatman). Filters were placed face up on YPD agar plates and incubated for 6 h at 30 °C. Cells were then redissolved in SD-minimal medium, serially diluted, and equal aliquots plated on SD-minimal and SD-lysine. Mating efficiency was calculated as the number of colonies on SD-minimal divided by the number of colonies on SD-lysine. Data shown are the mean and standard deviation of triplicates, normalized with respect to the wild type strain.

Site Directed Mutagenesis. Site-directed mutagenesis was done by Quick Change, following the manufacturer's protocol (Quick Change II Site-Directed Mutagenesis Kit; Agilent). Mutations were verified by DNA sequencing of the corresponding plasmids.

Western Blots. Western blots were done as described previously,[3] with minor modifications. Yeast cultures were induced with pheromone for 1 h, lysed, and run in 10% Tris-Gly, SDS polyacrylamide gels at 120−140 V for ∼1.5 h. Proteins were then transferred to low fluorescence BioRad PVDF membranes, using a Trans-Blot Turbo transfer system. Membranes were then blocked overnight using Odissey blocking buffer and incubated with the primary antibodies (for detection of doubly phosphorylated Fus3 and Kss1, we used a rabbit monoclonal phospho-p44/42 MAPK antibody, Cell Signaling Technology, #4370, at a dilution of 1:2000; for detection of PGK we used Invitrogen's mouse monoclonal antibody 459250). After washes, membranes were incubated with the secondary antibodies (for detection of doubly phosphorylated Fus3 and Kss1, we used a goat antirabbit Licor IRDYE 800 antibody, at a dilution of 1:10000; for detection of PGK, we used goat antimouse Licor IRDYE 680LT antibody, 929-68020, at a dilution of 1:20000. After washes, membranes were visualized on a Licor infrared scanner.

Growth Rates Measurements. Strains *MATa, Ste5:: Trp1, his3, trp1, leu2, ura3, ADE2 can1* carrying selected synthetic scaffolds were grown in triplicates in liquid culture and optical densities (OD) were measured at 600 nm every hour for 6 h. Growth rates (λ) were calculated by fitting the data to an exponential of the form: $OD = OD_o\, e^{\lambda t}$, where OD_o is the initial OD value and t is time. Growth rates were averaged and standard deviations calculated. The statistical significance of the

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observed differences was assessed by 2-tailed t tests (differences were considered significant if $p < 0.05$).

■ ASSOCIATED CONTENT

Supporting Information

Supporting methods and supplementary figures 1−7. This material is available free of charge via the Internet at http://pubs.acs.org

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†A.L. and P.M.S. contributed equally to this work. A.L., P.M.S., and S.G.P. designed experiments, analyzed results, and wrote the manuscript. A.L. and P.M.S. performed experiments.

Notes

The authors declare no competing financial interest.

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