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Exploitation of Latent Allostery Enables the Evolution of New Modes of MAP Kinase Regulation

Scott M. Coyle,[1,2] Jonathan Flores,[1] and Wendell A. Lim[1,3,*]
[1]Howard Hughes Medical Institute, Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94158, USA
[2]Program in Biological Sciences, University of California, San Francisco, San Francisco, CA 94143, USA
[3]UCSF Center for Systems and Synthetic Biology, San Francisco, CA 94143, USA
*Correspondence: lim@cmp.ucsf.edu
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SUMMARY

Allosteric interactions provide precise spatiotemporal control over signaling proteins, but how allosteric activators and their targets coevolve is poorly understood. Here, we trace the evolution of two allosteric activator motifs within the yeast scaffold protein Ste5 that specifically target the mating MAP kinase Fus3. One activator (Ste5-VWA) provides pathway insulation and dates to the divergence of Fus3 from its paralog, Kss1; a second activator (Ste5-FBD) that tunes mating behavior is, in contrast, not conserved in most lineages. Surprisingly, both Ste5 activator motifs could regulate MAP kinases that diverged from Fus3 prior to the emergence of Ste5, suggesting that Ste5 activators arose by exploiting latent regulatory features already present in the MAPK ancestor. The magnitude of this latent allosteric potential drifts widely among pre-Ste5 MAP kinases, providing a pool of hidden phenotypic diversity that, when revealed by new activators, could lead to functional divergence and to the evolution of distinct signaling behaviors.

INTRODUCTION

Eukaryotic signaling proteins display highly diverse and divergent allosteric regulation. Although any one genome might contain many evolutionarily related signaling molecules, such as protein kinases, individual family members usually display divergent substrate specificity and unique allosteric regulation by various partner proteins. By controlling when and where signaling proteins are activated, these allosteric regulatory interactions play a central role in determining the specific wiring of the molecular networks that control cellular behavior (Figure 1A).

Despite their importance, little is known about how these complex allosteric regulatory partnerships in signaling networks evolve. The molecular complexity of these systems represents a challenge for evolution: allosteric activators and the target proteins that they act on must seemingly acquire their complementary regulatory properties simultaneously for these systems to be functional and provide a selective advantage. These allosteric activators must also be specific enough to ensure that they do not inadvertently target homologous signaling components in the cell. The viable paths by which such multicomponent regulatory systems can evolve are therefore unclear.

In other complex systems, many new features appear to evolve by taking advantage of pre-existing or latent behavior: an active site that catalyzes a particular reaction can, with increased promiscuity, perform similar reactions on other substrates; a binding pocket that favors binding of one nuclear hormone can be adapted to accommodate a yet-to-be evolved hormone with somewhat similar structural features (Aharoni et al., 2005; Baker et al., 2012; Bridgham et al., 2006; O'Brien and Herschlag, 1999; Khersonsky and Tawfik, 2010; Wise et al., 2005). Although such latent capacities provide clear toeholds for new enzymatic activities or ligand binding capacities, these changes represent a shift in an already well-established and constitutive molecular activity. It is thus unclear the extent to which these evolutionary models apply to allosteric systems in which new protein partnerships must develop that are unrelated to any existing form of regulation and that must produce complex structural reorganization. Computational and protein engineering studies suggest that certain features of protein structure and dynamics may endow proteins with some latent capacity for allosteric regulation (Lee et al., 2008; Reynolds et al., 2011). Whether natural systems have harnessed such latent features to produce new allosteric regulation during evolution, however, has not been established.

Comparative studies that track the appearance of specific molecular properties across related species were instrumental in uncovering how new enzymatic activities, receptor/ligand pairs, and transcriptional circuits evolve (Afriat et al., 2006; Booth et al., 2010; Gerlt and Babbitt, 2001; O'Brien and Herschlag, 2001; Roodveldt and Tawfik, 2005; Taylor Ringia et al., 2004). However, applying these approaches to multicomponent allosteric regulation of signaling proteins has been hindered by a lack of model systems that can be biochemically interrogated over species spanning a considerable window of evolutionary time.

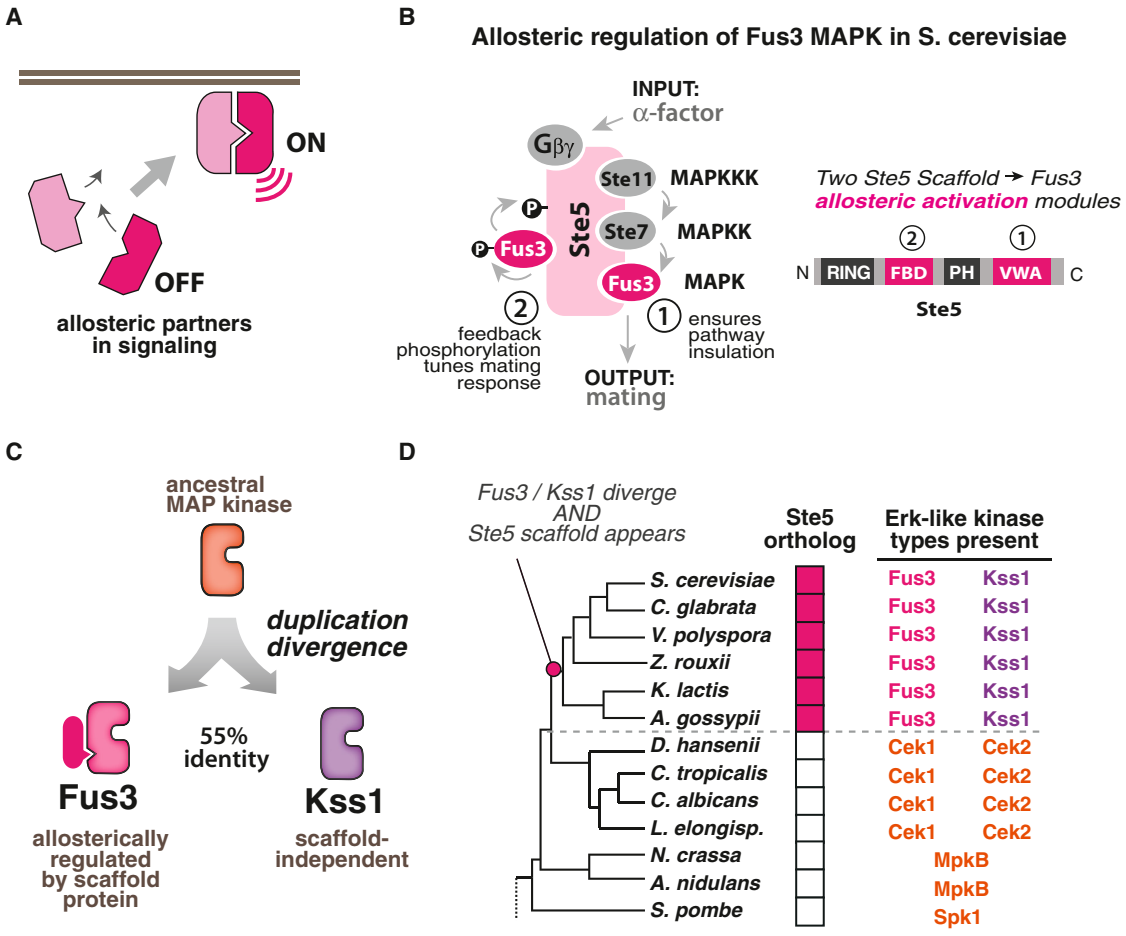

Figure 1. Fungal Erk Kinase Signaling Repertoires Provide a Model System for Biochemically Interrogating the Evolution of Novel and Divergent Allosteric Activation Mechanisms

(A) Allosteric interactions between signaling partners control when and where signaling molecules are activated in cells.

(B) In the *S. cer.*-mating MAP kinase pathway, two unique allosteric activities of the Ste5 scaffold regulate the MAP kinase Fus3, but not its paralog Kss1: (1) a VWA domain in Ste5 (Ste5-VWA) that is required to allosterically prime Fus3 for phosphorylation by the upstream MAPKK Ste7; and (2) a FBD that stimulates Fus3 autophosphorylation as part of a negative feedback loop that shapes the morphological response of cells to mating pheromone.

(C) Fus3 and Kss1 are Erk-like kinases that are 55% identical and arose from a duplication of an ancestral MAP kinase.

(D) Abbreviated phylogeny of fungal species from Ascomycota with the signaling repertoire (number and types of ERK-like kinases present; presence or absence of Ste5 scaffold) indicated for each species (see also Figures S1A–S1C).

The budding yeast MAP kinase network presents a unique model system with which to take a comparative approach to understand how complex multicomponent allosteric regulation might have evolved. Prior biochemical studies have shown that, in *S. cerevisiae* (*S. cer.*), the function of the mating-pathway-specific MAP kinase Fus3 requires its allosteric activation by the scaffold protein Ste5 (Figure 1B). This scaffold-mediated allosteric activation ensures that Fus3 is only activated in signaling complexes that are organized in response to pheromone stimulation, thus preventing inappropriate crosstalk in which distinct MAP kinase-mediated pathways might trigger mating (Zalatan et al., 2012). Interestingly, the closely related starvation-responsive MAP kinase, Kss1, functions independently of Ste5 scaffold regulation, despite the fact that Fus3 and Kss1 are 55% identical, both are targets of the MAPKK Ste7, and both likely arose from duplication of the same Erk-like MAP kinase ancestor (Figure 1C) (Madhani and Fink, 1998).

Given their common MAPK ancestor, how did Fus3 become dependent on allosteric regulation, whereas Kss1 did not? The availability of a large number of sequenced fungal genomes provides an opportunity to gain insights into this evolutionary question by exploring the regulatory properties of scaffold and MAP kinase orthologs throughout the fungal tree. Comparison of Erk-like MAP kinase sequences from across the Ascomycota fungi (to which *S. cer.* belongs) indicates that these kinases are highly divergent and fall into distinct classes (Figures S1A–S1C available online). Interestingly, only those species that have both a Fus3 and Kss1 ortholog also have a Ste5 scaffold ortholog (Figure 1D; detailed in Extended Experimental Procedures). It is unclear how both a potent allosteric activator (Ste5)

and its regulated target (Fus3) could simultaneously evolve as a two-part complementary system. However, because we have access to signaling repertoires from species that clearly diverged from the *S. cer.* lineage prior to the appearance of Fus3, Kss1, and Ste5, we have the potential to uncover the mechanism by which this allosteric partnership evolved.

Here, we expressed and purified Erk-like MAP kinases and Ste5 orthologs (if present) from 13 diverse fungal species that span from *S. pombe* to *S. cer.* (~1 billion years of divergence—comparable to the divergence between sea squirt and human). Using an in-vitro-reconstituted system, we determined the ability of these orthologs to cross-activate one another, even for species that do not contain a Ste5 protein. These quantitative data allowed us to determine when specific kinase and scaffold biochemical features arose during evolution and to formulate a model for the evolution of the allosteric regulatory schemes observed in *S. cer*.

First, we find that the Ste5 allosteric interaction required for Fus3 activation by the MAPKK Ste7 (Ste5-von Willebrand type A [VWA] domain) is a conserved scaffold feature of all Fus3/Kss1-containing species, whereas a second allosteric region in Ste5 (Ste5-Fus3-binding domain [FBD]) that tunes the ultrasensitivity of the mating response is, in general, not conserved outside of *S. cer*. This is consistent with a model in which a core function of the Ste5 scaffold protein has been to functionally insulate Fus3 and Kss1 since their divergence but also suggests that Ste5/Fus3 interactions might continue to evolve to meet specific organismal needs. Second, and surprisingly, we find that the Ste5 scaffold can allosterically activate orthologous MAP kinases from species that diverged prior to the evolution of Ste5, i.e., kinases that are likely to never have coexisted with the Ste5 scaffold. This result suggests that the Ste5 allosteric interactions evolved by tapping into latent, pre-existing dynamic properties of the ancestral MAP kinase. The magnitude of this latent allostery appears to drift significantly within the pre-Ste5 MAP kinases—some orthologs are primed for Fus3-like regulation (strong allosteric response), whereas others are primed for Kss1-like regulation (inability to respond). We propose that hidden diversity in these latent allosteric properties provides a toehold that new partner molecules can exploit to develop novel, component-specific allosteric regulatory relationships, simplifying the evolutionary paths to allosteric controls that shape pathway behavior and distinguish functional identity.

RESULTS

The *S. cer.* Ste5 Scaffold Protein Allosterically Activates the Fus3 MAP Kinase via Two Mechanisms

In prior work, we identified two modes by which the Ste5 scaffold protein allosterically activates the Fus3 MAP kinase in budding yeast *S. cer*. (Figure 1B). The first allosteric interaction involves a VWA domain in the Ste5 scaffold protein that is required to allosterically unlock Fus3 to allow for its dual phosphorylation and activation by the upstream MAP kinase kinase (MAPKK), Ste7 (Figure 2A). This VWA allosteric coactivation is essential for the transmission of the mating signal but has no influence on activation of the paralogous starvation-specific MAP kinase Kss1, which is interestingly also a substrate for the MAPKK Ste7 (Good et al., 2009). In the resting Ste5 molecule, Ste5-VWA activity is autoinhibited by other domains in Ste5. This autoinhibition prevents Fus3 from being activated until mating inputs relieve this inhibition, providing insulation from alternative inputs that activate the upstream MAPKK Ste7, such as starvation (Zalatan et al., 2012).

The second allosteric interaction involves a linear motif in Ste5 called the Ste5-FBD, which binds Fus3 and allosterically activates autophosphorylation of the MAP kinase on its activation loop tyrosine (Figure 2B) (Bhattacharyya et al., 2006). This partially activated form of Fus3 back phosphorylates Ste5 to downregulate mating pathway output and reshapes the morphological response of cells to α factor ("shmooing") to be switch like (ultrasensitive) instead of graded (Malleshaiah et al., 2010). The FBD allosteric activation is not essential for mating signaling but instead appears to fine-tune the quantitative aspects of the mating response.

The Ste5-VWA Allosteric Interaction Dates Back to Fus3/Kss1 Divergence, whereas the Ste5-FBD Allosteric Interaction Is a Recent Innovation that Tunes Mating Behavior in a Few Specific Lineages

We first examined when Ste5-VWA allosteric activity appeared relative to the emergence of Fus3 and Kss1 kinases families. We purified Ste5-VWA domain orthologs from diverse fungal species that contain the Ste5 scaffold and determined if they could allosterically coactivate *S. cer.* Fus3 phosphorylation by the *S. cer.* Ste7 MAPKK (henceforth, Ste7) (Figures 2A and S2A). As observed previously, phosphorylation of *S. cer.* Fus3 by Ste7 is very slow in the absence of *S. cer.* Ste5-VWA (k_{cat} = 6.0 ± 0.4 × 10^{-7} s^{-1}), and the addition of saturating *S. cer.* Ste5-VWA stimulates this rate by greater than three orders of magnitude (6,250 ± 610-fold). When saturating amounts of other Ste5-VWA orthologs were provided instead, rate enhancements were nearly identical to that of *S. cer.* Ste5-VWA. The most parsimonious interpretation of these data is that the Ste5-VWA domain possessed potent allosteric activity toward Fus3 in the last common ancestor of these species (Figure 2C). Consistent with this, chimeric *S. cer.* Ste5 molecules in which the native VWA domain was replaced with the VWA domain from other Ste5 orthologs were able to support robust mating in vivo (Figure S2E).

Is the Ste5-VWA domain of other orthologs subject to autoinhibition, as in *S. cer.*? A simple diagnostic for Ste5 autoinhibition is that full-length Ste5 provides a smaller rate enhancement for Fus3 phosphorylation than the isolated VWA domain (Zalatan et al., 2012). Thus, we compared the rate enhancement provided by the longest Ste5 construct we could express for each ortholog to that of the corresponding isolated VWA domain (Figures S2A and S2C). As in *S. cer.*, every ortholog we examined was less effective than the isolated VWA domain in enhancing the Ste7 → Fus3 reaction. The extent of this autoinhibition ranged from values comparable to the *S. cer.* inhibition (~10-fold) to values that were as much as 80-fold inhibited. Additional experiments indicate that the molecular mechanism of this inhibition is likely the same as in *S. cer.* Ste5 (Figure S2D), and thus, the simplest explanation for these data is that this mechanism to control Ste5 VWA allosteric activation was a conserved Ste5 feature present in the last common ancestor of these species.

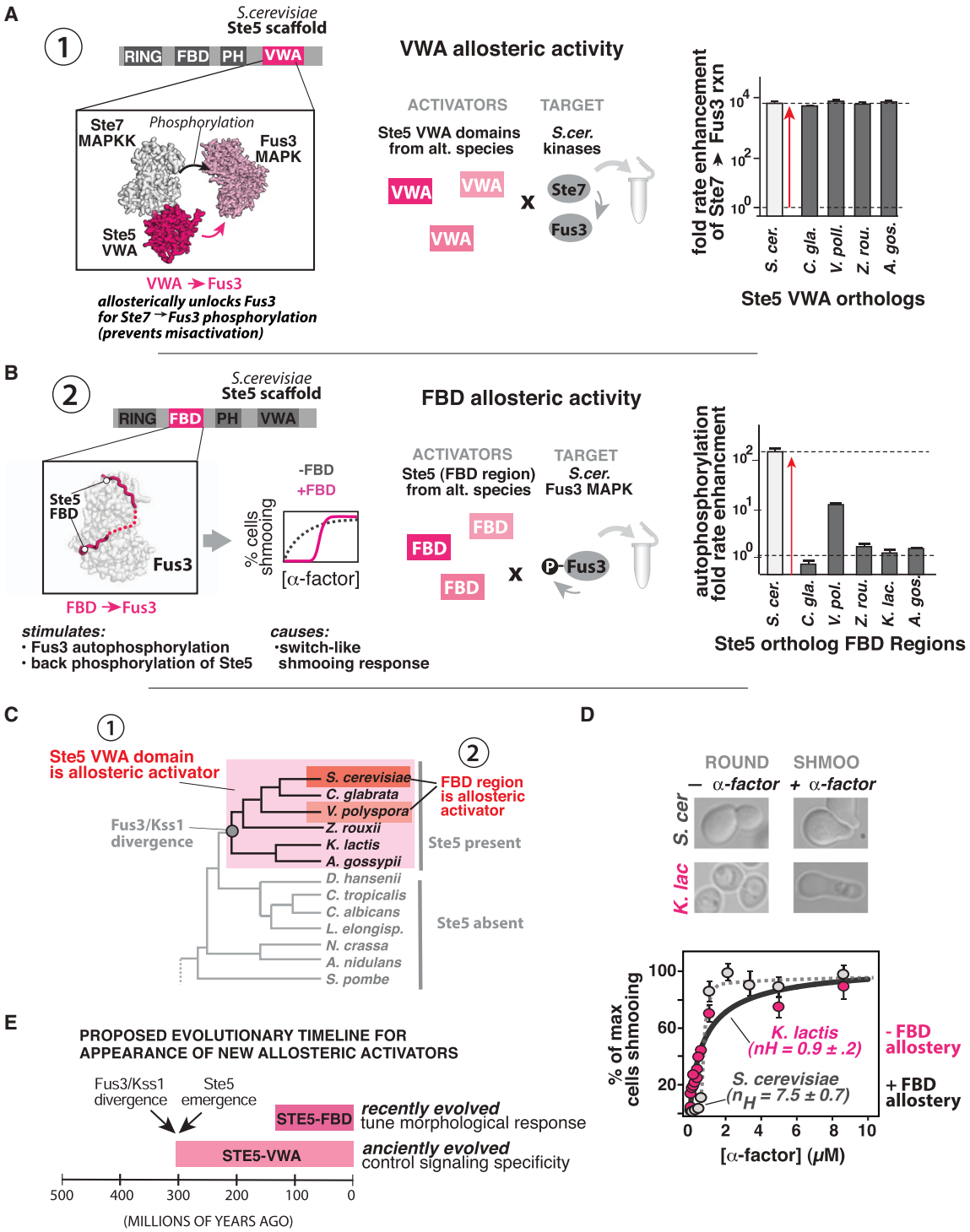

Figure 2. Tracking the Emergence of MAPK Allosteric-Activating Domains within the Ste5 Scaffold Protein

(A) In *S. cer.*, Ste5-VWA is required to allosterically unlock Fus3 for phosphorylation by Ste7; Ste7 cannot effectively phosphorylate Fus3 in the absence of this domain. Fold rate enhancements (mean ± SEM) for Ste7-catalyzed phosphorylation of *S. cer.* Fus3 in the presence of saturating amounts of the indicated Ste5-VWA ortholog are shown (see also titration curves in Figure S2A).

(B) In *S. cer.*, the Ste5-FBD is a linear motif between the Ste5 RING and PH domains that binds Fus3 and stimulates its autophosphorylation activity as part of a mechanism that results in a switch-like morphological dose-response profile to α factor. Rate enhancements for *S. cer.* Fus3 autophosphorylation (mean ± SEM) provided by addition of 25 μM of the indicated Ste5-FBD are shown (see also titration curves in Figure S3B).

(C) Phylogeny of Ascomycota indicating the appearance of Ste5-VWA and Ste5-FBD scaffold activities as inferred from (A) and (B).

(legend continued on next page)

We next examined the evolutionary history of the *S. cer.* Ste5-FBD allosteric regulatory interaction. Orthologous Ste5-FBD sequences (detailed in Extended Experimental Procedures) were purified and assayed for the ability to stimulate *S. cer.* Fus3 autophosphorylation (Figures 2B, S3A, and S3B). As observed previously, *S. cer.* Ste5-FBD potently stimulated the rate of *S. cer.* Fus3 autophosphorylation (149.7 ± 13.5-fold rate enhancement). In contrast, the FBD region from all but one Ste5 ortholog failed to provide a detectable rate enhancement for *S. cer.* Fus3 autophosphorylation. The one exception was the FBD sequence from *Vanderwaltozyma polyspora* (*V. pol.*), which provided an intermediate effect (12.8 ± 0.3-fold rate enhancement). One possible explanation for the lack of allosteric activity we observed for most Ste5-FBD sequences is that perhaps each FBD motif is optimized for its corresponding Fus3 ortholog. However, no differences were observed when the Fus3 ortholog from the same source species was used as a target instead of *S. cer.* Fus3 (Figure S3D). Thus, unlike Ste5-VWA regulation, the Ste5-FBD regulation found in *S. cer.* is not conserved in every organism that contains Fus3, Kss1, and Ste5 (Figure 2C).

Most likely, the FBD interaction evolved as a recent lineage-specific feature to tune the mating behavior of *S. cer.* It has previously been shown that mutating the Ste5-FBD in *S. cer*. converts a switch-like (ultrasensitive) shmooing response to α factor into a graded (linear) response (Malleshaiah et al., 2010). This model would predict that species lacking an active FBD motif would show a linear shmooing response. To test this model, we quantitatively examined the morphological responses of *K. lactis*—a species that lacks an active FBD motif but retains an active VWA domain (Figure 2D). As predicted, the morphological dose response of *K. lactis* to α factor was graded (n_H = 0.9 ± 0.2) in comparison to the switch-like response observed in *S. cer.* (n_H = 7.7 ± 0.7). The fact that *K. lactis* cultures must undergo prolonged phosphate starvation to be mating competent (Booth et al., 2010; Tuch et al., 2008a) may complicate a direct comparison of these two profiles. Nonetheless, together with our biochemical analyses, these data suggest that the Ste5-FBD interaction arose well after the divergence of Fus3 and Kss1 as a mechanism to fine-tune quantitative mating responses. We note, however, that we cannot definitively rule out repeated loss of the Ste5-FBD from multiple lineages as an alternative explanation of these data.

Together, the simplest evolutionary model for these data is that a potent but tightly regulated Ste5-VWA activity was present in the last common ancestor of the species that contain both Fus3 and Kss1 MAP kinase types, whereas the Ste5-FBD activity was likely layered on top of the core-conserved Ste5 activities to reshape the morphological response to mating pheromone in only certain species (Figure 2E). This suggests that a core function of the Ste5 scaffold protein has been to functionally insulate Fus3 and Kss1 since their divergence but also suggests that Ste5 scaffold interactions with the Fus3 kinase might continue to evolve to meet specific organismal signaling needs.

Latent Allostery: Ste5 Allosteric Activator Domains Can Stimulate MAP Kinases that Diverged prior to the Evolution of Ste5

We then turned to the converse question of understanding how the Fus3 MAPK acquired the necessary features to serve as a target of these two Ste5 allosteric interactions. Here, we reversed our in vivo cross-reaction components and tested the extent to which Fus3 and Kss1 orthologs from other species could be regulated by the *S. cer.* Ste5 scaffold activities. Starting with the *S. cer.* Ste5-VWA domain (Figure 3A), we found that all Fus3 orthologs were strongly allosterically regulated by the VWA domain: they were poor substrates for Ste7 in the absence of *S. cer.* Ste5-VWA (k_{cat} <5 × 10^{-6} s^{-1}), but the addition of *S. cer.* Ste5-VWA enhanced phosphorylation of each MAPK by greater than 2,000-fold. This strong allosteric activation was identical when other Ste5-VWA orthologs were used in place of *S. cer.* Ste5-VWA (Figure S2B).

In contrast, all of the Kss1 orthologs we tested were not targets for VWA activation—these MAPKs were ideal substrates for Ste7 (k_{cat} >1 × $10^{-3}s^{-1}$) in the absence of any other molecules and were unaffected by the addition of *S. cer.* Ste5-VWA (rate enhancement <1.5-fold). From these data, we infer that Fus3 and Kss1 likely possessed their divergent responses to Ste5-VWA regulation in the last common ancestor of the species that contain these kinases. The ability of Fus3 orthologs to be activated by the VWA domain, thus, appears to be tightly conserved after the functional divergence of the Fus3 and Kss1 MAPKs.

We then tested whether Erk-like kinases from species that diverged from *S. cer.* prior to the evolution of Ste5—henceforth referred to as "pre-Ste5" Erk-like kinases—had the capacity to be regulated by the modern *S. cer* Ste5 VWA domain (Figure 3A). Unlike *S. cer* Fus3, these pre-Ste5 kinases were intrinsically good substrates for Ste7-catalyzed phosphorylation in vitro (k_{cat} >7 × 10^{-5} s^{-1}). However, addition of *S. cer.* Ste5-VWA surprisingly stimulated phosphorylation of many of these kinases by as much as a 42-fold rate enhancement. Thus, these pre-Ste5 MAP kinases are similar to Fus3 in that they have a modest capability to serve as a target for allosterically activation by the Ste5 VWA domain, despite the fact that the species from which they come lack Ste5.

We then analogously examined when the ability to serve as a target for the Ste5-FBD interaction arose within the MAP kinase family (Figure 3B). Although only the *S. cer* Ste5 ortholog possessed potent Ste5-FBD activity, we surprisingly found that the Fus3 orthologs from nearly every species that we examined were targets for FBD activation—like *S. cer* Fus3, they all displayed a FBD-enhanced rate autophosphorylation of greater than 100-fold. In contrast, *S. cer.* Ste5-FBD did not significantly enhance the rate of autophosphorylation of the Kss1 orthologs we tested. We conclude that Fus3 was primed for regulation by a *S. cer.* Ste5-FBD mechanism in the common ancestor of these species, even before the FBD activity had evolved in

(D) Morphological response to α factor (mean ± SEM for percentage [%] of maximum cells shmooing; n ≥ 500 cells) of *S. cer*. (indicated by gray dots and dashed line) and *K. lactis* (shown in pink dots and solid line). Data were fit to a Hill equation to extract the parameter n_H.

(E) Timeline indicating the proposed appearance of Ste5 allosteric activators relative to the appearance of the Ste5 and the Fus3/Kss1 divergence (dating estimates from Dujon, 2006; Taylor and Berbee, 2006). See also Figures S2 and S3.

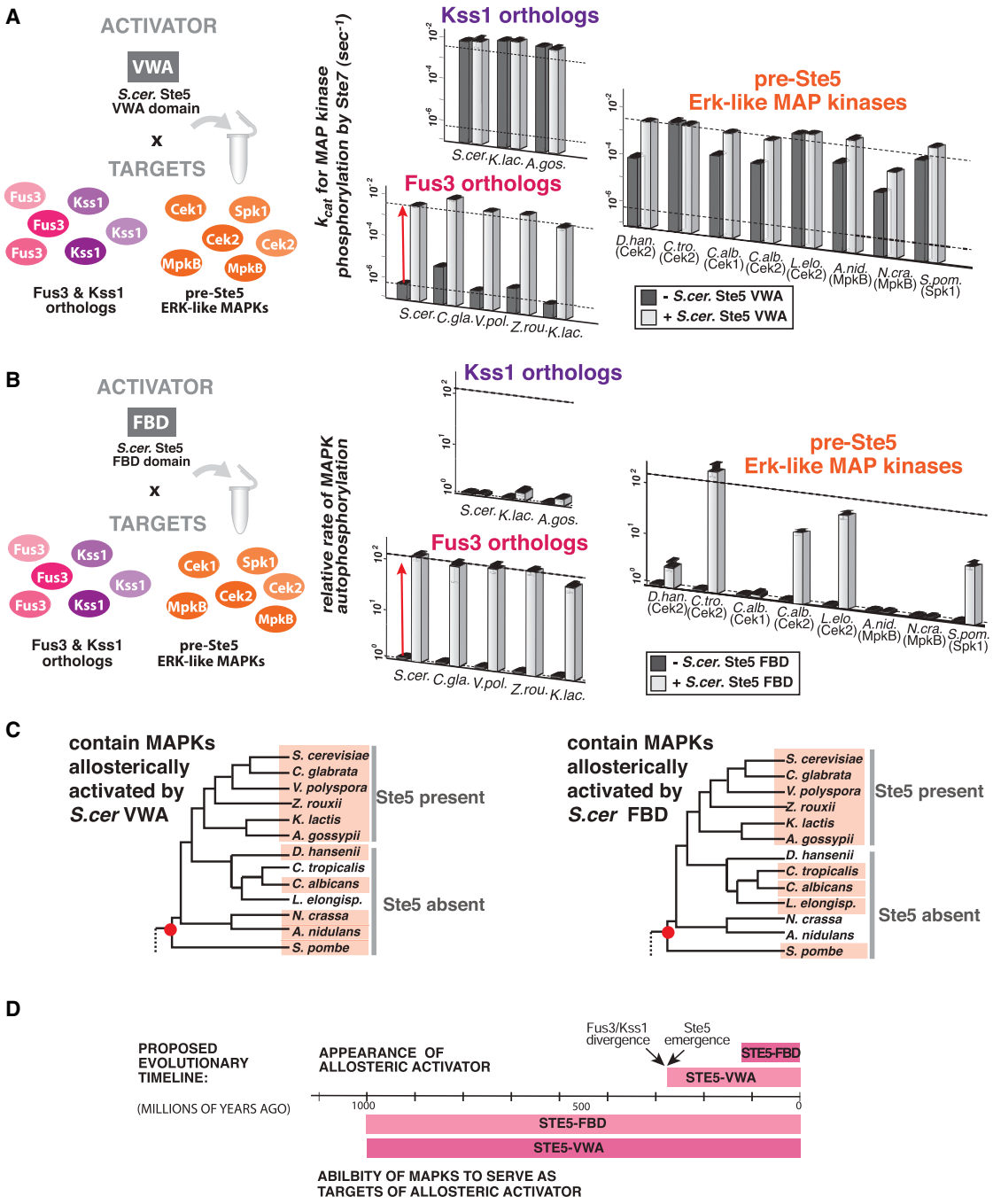

Figure 3. Ste5 Scaffold Domains Can Allosterically Activate Erk-like MAP Kinases that Diverged prior to the Evolution of Ste5

(A) Rate constants (mean ± SEM) for Ste7-catalyzed phosphorylation of the indicated MAP kinase substrate in the presence (white) or absence (gray) of saturating amounts of *S. cer*. Ste5-VWA for the indicated Fus3 orthologs, Kss1 orthologs, or pre-Ste5 Erk-like MAP kinases.

(B) Relative rates (mean ± SEM) for MAP kinase autophosphorylation (normalized to the no Ste5-FBD rate) in the presence (white) or absence (gray) of saturating amounts of *S. cer*. Ste5-FBD for the indicated Fus3 orthologs, Kss1 orthologs, or pre-Ste5 Erk-like MAP kinases.

(C) Phylogeny of Ascomycota fungi with species that contains an Erk-like kinase that can be regulated by the indicated Ste5 activator domain marked in pink. The red dot represents the last common ancestor that likely contained an Erk-like kinase that could be regulated by the indicated Ste5 activator.

(D) Proposed timeline (date estimates from Dujon, 2006; Taylor and Berbee, 2006) indicating when the capacity for regulation by a Ste5 activator appeared in the fungal Erk-like MAP kinase family relative to the appearance of Ste5 scaffold activator domains that target that capacity for regulation.

the Ste5 scaffold; little change to the kinase was necessary for the *S. cer.* Ste5-FBD to be able to influence the rate of autophosphorylation.

We then tested whether *S. cer.* Ste5 FBD could enhance the rate of autophosphorylation of the pre-Ste5 Erk-like kinases (Figure 3B). We observed a broad range of capacities for regulation by *S. cer.* Ste5-FBD. Several kinases were not allosterically affected by the *S. cer.* Ste5-FBD (*D. han.* Cek2, *C. alb.* Cek1, *A. nid.* MpkB, *N. cra.* MpkB) even though these kinases readily bound to *S. cer.* Ste5-FBD (Figure S4A). Some kinases, however, showed intermediate effects (*S. pom.* Spk1, *C. alb. Cek2*); and still others showed allosteric responses that approached or even exceeded the enhancement in autophosphorylation that is seen for *S. cer.* Fus3 (*C. tro.* Cek2, *L. elo.* Cek2). Thus, many of the pre-Ste5 MAPKs display the ability to serve as a target for both VWA and FBD-mediated allosteric activation.

These findings suggest that both the VWA and FBD allosteric interactions evolved by tapping into latent allosteric features that pre-existed within this family of kinases. Because the pre-Ste5 Erk-like kinases—including the Spk1 kinase from *S. pombe*, which is the most distantly related to *S. cer.* Fus3—broadly show modest regulation by both Ste5-VWA and Ste5-FBD, the most parsimonious explanation of these data is that some capacity for both of these forms of allosteric regulation was likely present in the ancestral kinase of all the orthologs we inspected (Figure 3C). Although it is formally possible that there exist other alternative allosteric regulators that capitalize on these modest features in the pre-Ste5 lineages, several lines of reasoning argue against this. First, we tested several likely candidate proteins present in these organisms for such activity and found no evidence in support of this (Figures S4B, S4D, and S4E). Second, the extensive variation in the latent allosteric features of the pre-Ste5 MAPKs, including the absence of these features in particular orthologs, suggests that these features are not under selective pressure. That is, these particular allosteric regulations of the MAPK substrate have not been fixed in all of the pre-Ste5 branches of the Ascomycota to the extent that they have been fixed in the post-Ste5 species, casting doubt on the existence of other critical allosteric regulators that are using the latent allosteric features. Third, given that the Ste5-VWA regulation is functionally required for pathway specificity, i.e., discriminating between the Fus3 and Kss1 kinases, it is unclear why such allosteric effectors would exist in lineages that contain only a single Erk-like kinase. Finally, the observed allosteric effects on the pre-Ste5 kinases are in most cases relatively small: all of the kinases were adequate MAPKK substrates in the absence of any additional Ste5 regulation. Thus, it is unlikely that these species functionally require such allosteric effectors. As such, we favor a model in which the capacity for the allosteric regulation we observed was already present in the ancestral kinases, providing a toehold for the emergence of new forms of allosteric regulation.

Drift in Latent Allostery Produces Evolutionary Related Kinases that Are Primed for Divergent Responses to New Allosteric Activators

A model of latent allostery within the MAP kinase family provides a simple framework for how new allosteric regulators such as the Ste5-FBD and Ste5-VWA domain may have evolved. However, it also raises an important question in terms of divergent regulation: how then is it that Fus3 orthologs are targets for this allosteric regulation, whereas Kss1 orthologs are not?

To gain insights into this question, we examined the diversity in the distribution of properties observed for the pre-Ste5 Erk-like kinases (Figure 4A). When only considered as substrates for the MAPKK Ste7, pre-Ste5 Erk-like kinases generally cluster together and appear to be similar quality substrates in the absence of any scaffold coactivator. However, the latent capacity for allosteric regulation in each of these substrates results in additional dimensions of MAPK phenotypic diversity beyond their basic properties as substrates for phosphorylation by the MAPKK Ste7. This diversity is easily visualized by plotting each of the kinases on phenotypic morphospace plots in which one dimension is the rate of Ste7→Fus3 phosphorylation—the apparent kinase diversity in the absence of any allosteric activators—and a second dimension is the allosteric enhancement of either of the two Ste5 allosteric interactions—the hidden phenotypic diversity that is only revealed upon interaction with scaffold effectors (Figure 4A; see also Figure S4C). For both Ste5-VWA and Ste5-FBD activities, the highly divergent regulation of Fus3 and Kss1 orthologs places them in opposite regions of this space, whereas most of the pre-Ste5 Erk-like kinases are "hybrids" that, as a set, occupy a region of space in between Fus3 and Kss1. Importantly, these plots reveal that kinases that may appear close together in the one-dimensional perspective as substrates for MAPKK phosphorylation can be far apart along these hidden allosteric dimensions. Thus, drift in these hidden phenotypic properties (latent allostery) results in a distribution of family members, with some much closer to Fus3 in behavior and others much closer to Kss1.

These findings suggest a simple and general mechanism for the evolution of novel and divergent allosteric regulation of paralogous signaling components such as the Kss1 and Fus3 MAP kinases (Figures 4B and 4C). Neutral drift in a latent capacity for allosteric regulation produces paralogous variants that are primed for divergent responses to regulation. Appearance of a new interaction partner with weak activity against this latent allosteric feature "reveals" the pre-existing diversity and provides a toehold for Darwinian processes to exploit these differences and drive these kinases into divergent regulatory modes by selection, as was observed for the divergent responses of Fus3 and Kss1 to the Ste5-VWA domain. Such selection events have the potential to fix other latent allosteric properties within the newly selected lineage owing to founder effects or hitchhiking, which could explain why all of the Fus3 and Kss1 orthologs we tested also display divergent responses to Ste5-FBD regulation even before this activity evolved.

Dissection of the *V. pol.* Ste5-FBD with Intermediate Allosteric Activity Reveals Alternative Paths for Co-Opting the Same Latent Regulatory Features

Our data demonstrate that a significant capacity for allosteric regulation is present in kinases prior to the evolution of the effectors that provide that regulation in *S. cer*. How, at a molecular level, does evolution discover activators that can tap into these hidden allosteric features and co-opt this pre-existing capacity

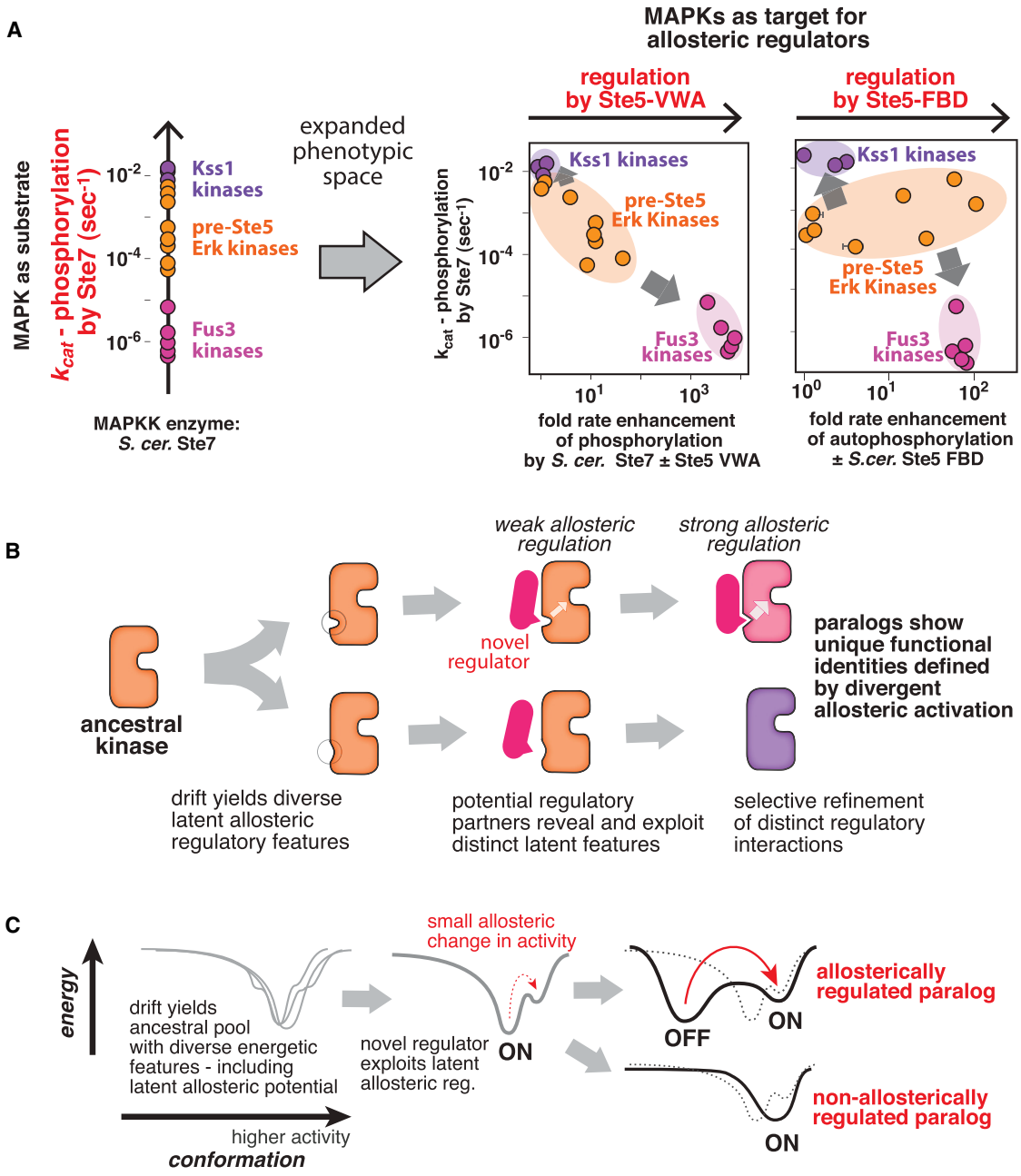

Figure 4. Drift in Latent Protein Allostery Provides a Path for Evolution of Divergent Regulatory Phenotypes within Seemingly Equivalent Kinase Paralogs

(A) Morphospace visualizations of MAP kinase biochemical diversity found in this study, either only as substrates for phosphorylation by the upstream MAPKK or taking into account the additional hidden dimensions of diversity in substrates upon interaction with the Ste5 scaffold. Circles in the plots correspond to individual MAP kinases (Fus3-type kinases are indicated in pink; Kss1-type kinases are shown in purple; pre-Ste5 Erk-like kinases are represented in orange) that we examined and indicate their associated properties. See also Figure S4C.

(B) Evolutionary model for novel and divergent regulation by exploitation of latent allosteric diversity. An ancestral kinase (orange) with some capacity for allosteric regulation is duplicated. In the absence of an effector, drift yields paralogous kinases with distinct latent regulatory features. Potential regulatory partners can reveal and exploit differences in these distinct latent features, providing a foothold for selection to refine and optimize the targets and effectors by coevolution to produce paralogous kinases with divergent allosteric responses to an effector molecule.

(C) The model in (B) is illustrated in terms of the conformational energy landscape of the proteins. See also Figure S4.

for new regulation? Our biochemical screen of Ste5-FBD motifs identified a sequence with intermediate allosteric activity from *V. pol.* Ste5-FBD that gives us an opportunity to biochemically dissect how this FBD-mediated allosteric activity may have arisen (Figure 5A). (Addressing this question for Ste5-VWA domain regulation is difficult because of the lack of any forms of Ste5 that show intermediate VWA activities.)

We wanted to determine whether the *V. pol.* Ste5-FBD functions through a related mechanism to that used by the *S. cer.* Ste5-FBD. We previously showed that the *S. cer.* Ste5-FBD sequence binds to Fus3 in a bipartite manner to allosterically activate the Fus3 kinase: an "A site" motif binds to the N lobe of the kinase, whereas a second "B site" motif binds to a canonical-docking groove on the C lobe of the MAP kinase (albeit in a noncanonical reverse C-toN-terminal orientation); linking these two binding sites is thought to constrain the two kinase domains into a more active conformation that promotes autophosphorylation (Figure 5C) (Bhattacharyya et al., 2006). Inspection of the *V. pol.* Ste5-FBD sequence reveals a sequence that resembles the A site motif of *S. cer.* Ste5-FBD, but there is no obvious sequence that resembles the B site motif.

To better understand the mechanism of the *V. pol.* Ste5-FBD interaction, we used deletion analysis to map the regions of this sequence that were required for its allosteric activity (Figures 5B and S5A). Like *S. cer.* Ste5-FBD, we found that two distinct regions were required for activity. One of these regions contained the motif that resembles the A site of the *S. cer.* Ste5-FBD, suggesting that both *V. pol.* Ste5-FBD and *S. cer.* Ste5-FBD use this A site sequence to engage the N lobe of Fus3. Unlike in the *S. cer.* Ste5-FBD, however, the second region of *V. pol.* Ste5-FBD required for allosteric activity was on the opposite side of the A site (N terminal to it, i.e., the opposite orientation relative to the *S. cer.* Ste5-FBD). This second required region in the *V. pol.* Ste5-FBD motif resembles the consensus MAPK-docking motif ($[R/K]_{1\text{-}2}$-$X_{2\text{-}6}$-Φ−x-Φ−x-Φ) that is used by many signaling partners to interact with MAPKs (Reményi et al., 2005; Tanoue et al., 2000). Consistent with this, mutation of the residues within this motif that would disrupt a MAPK-docking interaction completely abolished the allosteric activity of *V. pol.* Ste5-FBD (Figure 5B).

From these data, we infer a model for how *V. pol.* Ste5-FBD interacts with Fus3 to exert allosteric influence (Figure 5C). At low resolution, both the *S. cer.* and *V. pol.* FBD mechanisms appear very similar: they both bind at the same two sites on the MAPK, potentially constraining the kinase N and C lobes relative to one another in a manner that increases autophosphorylation. Nonetheless, whereas the *S. cer.* Ste5-FBD binds Fus3 with an A site-B site bipartite polypeptide, the *V. pol.* Ste5-FBD appears to bind to Fus3 with a "docking motif"-A site bipartite polypeptide (where the docking motif functionally replaces the B site motif). In both cases, functionally analogous motifs that bind the C lobe docking groove cooperate with binding of the A site motif to the N lobe of the kinase to achieve allosteric activation. We postulate that this distinct but analogous bipartite binding represents a case of convergent evolution—both bipartite peptides can constrain the kinase lobes required to stimulate autophosphorylation, albeit to different degrees (Figure S5B).

Is the *V. pol.* Ste5-FBD motif, despite its detailed differences, tapping into the same latent allosteric features present in the fungal MAPK family as those exploited by the *S. cer.* Ste5-FBD? If so, then we predict that the effects of the *V. pol.* Ste5-FBD motif on diverse members of the Erk-like fungal kinase family should mirror those observed for the *S. cer* Ste5-FBD motif. Indeed, we observe a linear relationship across fungal species between the degree to which the *V. pol.* Ste5-FBD and the *S. cer* Ste5-FBD motifs can activate individual MAPK family members (Figure 5E; see also Figure S3D). Thus, even this weak activator appears to reveal the same latent potential for allosteric regulation that is present in many fungal MAP kinases, including the pre-Ste5 Erk-like kinases. During the course of evolution, once a weak effector like the *V. pol.* Ste5-FBD uncovers these latent kinase regulatory features, Darwinian processes can proceed to optimize this allosteric regulation. Because different yeast species occupy distinct environments and exhibit different mating preferences (Booth et al., 2010), the outcomes of these Darwinian processes will differ depending on local selective pressures and organismal niche: the activity can be optimized to increase potency (as in *S. cer.* Ste5-FBD), it can be maintained as a weak effector (as in *V. pol.* Ste5-FBD), or it can be turned over to a state in which Ste5-FBD regulation is lost (as observed in *C. gla.*; see Figure 2B).

DISCUSSION

Our analysis of kinase and scaffold properties from across Ascomycota fungi allowed us to determine when particular Ste5 scaffold allosteric activator functions most likely arose, as well as when the capacity of a MAP kinase to serve as a target for such allosteric regulation arose. From this analysis, we made the surprising observation that many kinases from species that diverged from *S. cer.* prior to the evolution of the Ste5 scaffold can still be regulated by the allosteric motifs within Ste5. These findings suggest that a latent capacity for allosteric regulation was present within this MAP kinase family long before the evolution of effectors that target this allostery for regulation.

Exploitation of an existing latent capacity to derive a new molecular regulatory relationship is similar to proposed models for the evolution of new catalytic activities by catalytic promiscuity (O'Brien and Herschlag, 1999; Khersonsky and Tawfik, 2010) and new hormone receptor signaling responses by molecular exploitation (Bridgham et al., 2006). In this case, however, the latent allosteric kinase features we have described in this study are not obviously similar to some pre-existing regulatory interaction but represent new regulatory connections that can redirect and reshape information flow in cell signaling pathways.

The Dynamic Protein Kinase Structure as a Source of Latent and Diverse Allosteric Behaviors

We postulate that the dynamic nature of the protein kinase structure itself may provide the latent allosteric potential and diversity observed in these fungal MAP kinases. Indeed, both Ste5-VWA domain and Ste5-FBD motif are thought to allosterically activate the Fus3 MAP kinase by altering the kinase flexibility (Bhattacharyya et al., 2006; Good et al., 2009), and such flexibility is an innate but variable feature of protein kinases (Huse and

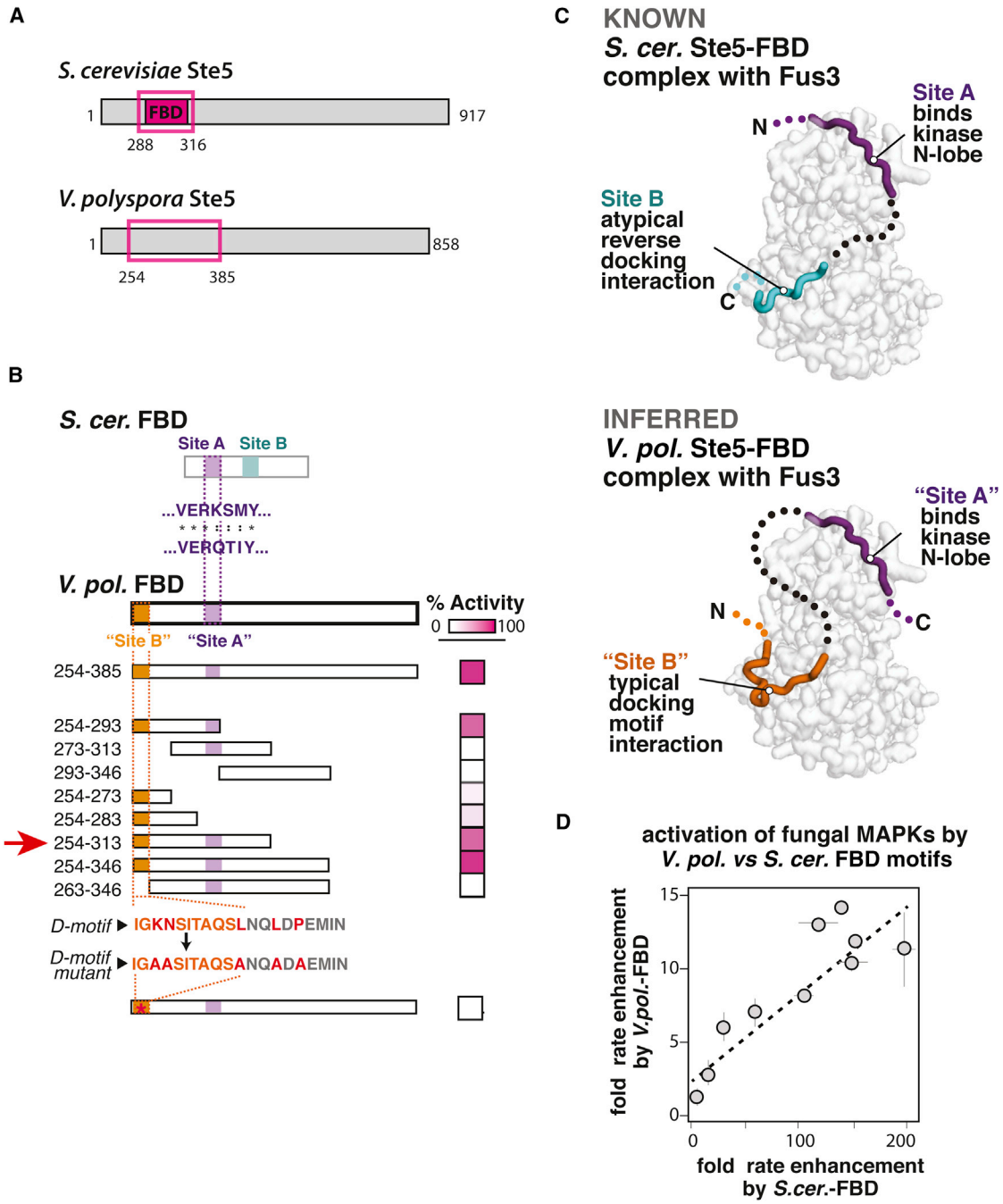

Figure 5. Dissection of the *V. pol*. Ste5-FBD Motif with Intermediate Allosteric Activity Reveals Alternative Paths for Co-Opting the Same Latent Regulatory Features

(A) Position of minimal *S. cer*. Ste5-FBD sequence mapped previously (Bhattacharyya et al., 2006) and initial region of *V. pol*. Ste5 that showed FBD activity that serves as the starting point for further analysis.

(B) Truncation mapping of the *V. pol*. Ste5-FBD fragment, showing the relative activity of an indicated truncation or fragment (see also Figure S5). This analysis identifies two sites that are required for activity. One site ("Site A") is similar to a sequence required for activity in the *S. cer*. Ste5-FBD fragment. A second site ("Site B") resembles a traditional MAPK-docking peptide sequence.

(C) Comparison of the known structure of the *S. cer*. Ste5-FBD•Fus3 complex to the inferred structure based on homology and the truncation mapping from (B). The two kinases appear to use similar Site A sequences to bind the N lobe of the kinase but use distinct mechanisms to engage the docking groove of the MAP kinase C lobe.

(D) A plot indicating the magnitude (mean ± SEM) of the *S. cer*. FBD effect and *V. pol*. FBD effect; each point corresponds to an individual kinase we inspected. The tight linear relationship between these effects suggests that both the *S. cer*. and *V. pol*. Ste5-FBD sequences target the same allosteric features and diversity present in the MAP kinases we inspected. See also Figure S5.

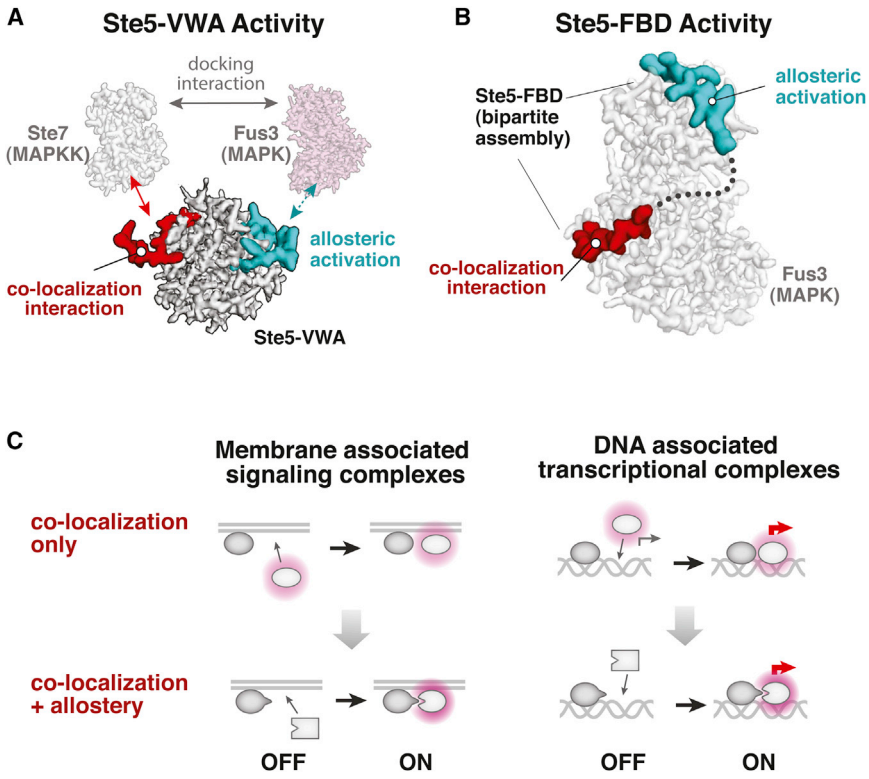

Figure 6. The Role of Colocalization in the Evolution of New Allosteric Regulation

(A) Colocalization interactions that are distinct from the essential allosteric surface of the Ste5-VWA assemble a Ste5•Fus3•Ste7 ternary complex that is essential for the Ste5-VWA to allosterically activate Fus3. These colocalization interactions are sufficient for tight complex assembly on their own.

(B) Binding of the Ste5-FBD A site motif to the Fus3 MAP kinase, which is essential for allosteric activation of autophosphorylation, requires a second-site interaction with the docking groove of MAP kinase. This docking interaction is, by itself, a nonallosteric colocalization interaction that can be sufficient for complex formation.

(C) Colocalization-based activation mechanisms—whether on scaffolds, membranes, or DNA—can facilitate the evolution of allosteric interactions between the colocalized components and yield the tighter, precise spatiotemporal control of activation that is observed in modern pathways.

Kuriyan, 2002). Thus, rather than requiring that evolution create unprecedented structural features to produce allosteric innovation, the ruggedness of the MAP kinase conformational landscape itself may provide toeholds for weak, but specific, activators that act by selecting and stabilizing particular kinase conformations (Ma et. al., 1999) (Figure 4C). These relationships can then be strengthened by evolutionary mechanisms that tune and modulate the stability of certain states of the molecule or widen the difference in activity between alternative states. These findings are consistent with patterns observed in many other members of the protein kinase family: all kinases appear to require the proper assembly of the same core catalytic and structural elements in order to adopt an active state; but different kinases adopt a wide array of distinct inactive conformations, each of which requires a different set of inputs to stabilize the conserved active conformation (Huse and Kuriyan, 2002; Kornev et al., 2006). More generally, modes of flexibility intrinsic to particular protein folds may provide the starting point for future regulatory evolution (Halabi et al., 2009).

The hidden regulatory diversity that we find in the fungal MAP kinases may be a more general feature of many protein kinases as well as other dynamically regulated macromolecules. In fact, many drugs may act as effectors that uncover this regulatory potential. Indeed, such hidden conformational toeholds serve as the basis of action of the Abl-specific tyrosine kinase inhibitor Gleevec, which stabilizes an inactive conformation that is uniquely accessible to that kinase (Schindler et al., 2000). Similarly, some small molecules have been found to allosterically activate regulatory proteins, despite the lack of a clear physiologic analog that normally targets that site (Hardy et al., 2004).

Colocalization May Facilitate the Evolution of New Allosteric Regulation

The latent allosteric properties in MAP kinases we have described must be "revealed" by an effector in order for selection to be possible. How do such primitive allosteric regulators evolve? The mechanism of the Ste5 VWA and FBD motifs suggests that colocalization may facilitate this process because both interactions involve the interplay between allosteric interactions and colocalization interactions. The VWA domain allosterically regulates Fus3 in the context of a higher-order molecular complex (a Ste5•Fus3•Ste7 ternary complex) that is assembled by nonallosteric colocalization interactions that are sufficient for tight complex formation (Figure 6A). Similarly, binding of the Ste5-FBD A site motif to the Fus3 kinase depends on a second-site interaction with the docking groove of MAP kinase, which is sufficient for complex formation on its own (Figure 6B). A simple model is that colocalization of the future allosteric target and regulator was an early step in the evolution of these allosteric relationships. Such colocalization establishes effective concentrations of the components in the millimolar range in which fleeting and weak interactions occur more readily (Kuriyan and Eisenberg, 2007; Reynolds et al., 2011), thus enhancing the likelihood of uncovering a weak interaction that reveals a latent allosteric feature in a target.

This evolutionary "colocalization first" strategy is similar to the novel "tethering" approach used for developing small molecule allosteric effectors, in which a library of disulfide-containing small molecules is localized to a particular cysteine residue on the drug target (Erlanson et al., 2004) allowing for the identification of weak effectors that bind to surprising new allosteric protein sites (Hardy and Wells, 2004). The synergy between evolutionary and engineering approaches suggests that genetically encoded libraries that "tether" a variable protein or RNA

library to a target might offer an effective in vivo screening approach for identifying new allosteric effectors.

Finally, these observations indicate that the colocalization of signaling components on scaffolds or at the membrane may play a more active role in the evolution of new signaling pathways and behaviors than previously appreciated, by producing local environments in which hidden allosteric diversity and the ruggedness of conformational landscapes are revealed by high effective concentrations and potential new effector interactions. Indeed, many primitive signaling pathways may have initially simply consisted of components that became colocalized upon stimulation with an input signal (Figure 6C). These assemblies, however, might then provide a context that would facilitate the evolution of allosteric regulation, as described here, that yielded the diverse forms of precision control that we observe in modern pathways. An analogous progression of regulatory evolution is suggested to take place among DNA binding factors that are tethered at a promoter (Baker et al., 2012; Tuch et al., 2008b).

EXPERIMENTAL PROCEDURES

Identification, Sequence Analysis, and Cloning of Kinase and Scaffold Orthologs

S. cer. Fus3, Kss1, and Ste5 sequences were used to query the fungal orthogroups database to identify orthologous sequences in the Ascomycota (additional details in Extended Experimental Procedures), which were subsequently cloned from gDNA or synthesized directly. Phylogenetic analysis of these sequences is detailed in the Extended Experimental Procedures. A complete list of all constructs used in this study is in Table S1.

Protein Purification

MAP kinases, Ste5 scaffold fragments, and the SR13 Fab antibody were expressed in BL21(T1R) *E. coli* cells. The *S. cer.* Kss1 ortholog and the constitutively active form of the MEK Ste7 (Ste7EE) were expressed from *S. frugiperda* (SF9) cells. Proteins were purified similarly as described previously by Good et al. (2009), Reményi et al. (2005), and Zalatan et al. (2012), with minor modification as detailed in the Extended Experimental Procedures.

In Vitro Kinase Activity Assays

Initial rates for Ste7-catalyzed phosphorylation of a MAPK as well as MAPK autophosphorylation were measured by quantitative western blotting as described and detailed in the Extended Experimental Procedures. Under saturating conditions, VWA reactions contained 50 nM of MBP-Ste7EE, 5 μM MAPK substrate, and (if present) 5 μM Ste5-VWA ortholog; saturating FBD reactions contained 10 μM MAPK and, if present, 25 μM of a Ste5-FBD sequence.

Morphological Dose Response to α Factor

Morphological responses to α factor were performed for *S. cer.* (strain W303) and *K. lactis* (strain yLB17a; Booth et al., 2010) as described previously by Malleshaiah et al. (2010). For *K. lactis*, the response was measured after 6 hr of growth in SCD media lacking phosphate to ensure that cells were mating competent (detailed in Extended Experimental Procedures). The percentage of cells shmooing at a given concentration of pheromone was determined by microscopy, and the resulting dose-response curves were fit to a Hill equation to extract the Hill coefficient parameter n_H.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.07.019.

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REFERENCES

Afriat, L., Roodveldt, C., Manco, G., and Tawfik, D.S. (2006). The latent promiscuity of newly identified microbial lactonases is linked to a recently diverged phosphotriesterase. Biochemistry *45*, 13677–13686.

Aharoni, A., Gaidukov, L., Khersonsky, O., McQ Gould, S., Roodveldt, C., and Tawfik, D.S. (2005). The 'evolvability' of promiscuous protein functions. Nat. Genet. *37*, 73–76.

Baker, C.R., Booth, L.N., Sorrells, T.R., and Johnson, A.D. (2012). Protein modularity, cooperative binding, and hybrid regulatory states underlie transcriptional network diversification. Cell *151*, 80–95.

Bhattacharyya, R.P., Reményi, A., Good, M.C., Bashor, C.J., Falick, A.M., and Lim, W.A. (2006). The Ste5 scaffold allosterically modulates signaling output of the yeast mating pathway. Science *311*, 822–826.

Booth, L.N., Tuch, B.B., and Johnson, A.D. (2010). Intercalation of a new tier of transcription regulation into an ancient circuit. Nature *468*, 959–963.

Bridgham, J.T., Carroll, S.M., and Thornton, J.W. (2006). Evolution of hormone-receptor complexity by molecular exploitation. Science *312*, 97–101.

Dujon, B. (2006). Yeasts illustrate the molecular mechanisms of eukaryotic genome evolution. Trends Genet. *22*, 375–387.

Erlanson, D.A., Wells, J.A., and Braisted, A.C. (2004). Tethering: fragment-based drug discovery. Annu. Rev. Biophys. Biomol. Struct. *33*, 199–223.

Gerlt, J.A., and Babbitt, P.C. (2001). Divergent evolution of enzymatic function: mechanistically diverse superfamilies and functionally distinct suprafamilies. Annu. Rev. Biochem. *70*, 209–246.

Good, M., Tang, G., Singleton, J., Reményi, A., and Lim, W.A. (2009). The Ste5 scaffold directs mating signaling by catalytically unlocking the Fus3 MAP kinase for activation. Cell *136*, 1085–1097.

Halabi, N., Rivoire, O., Leibler, S., and Ranganathan, R. (2009). Protein sectors: evolutionary units of three-dimensional structure. Cell *138*, 774–786.

Hardy, J.A., and Wells, J.A. (2004). Searching for new allosteric sites in enzymes. Curr. Opin. Struct. Biol. *14*, 706–715.

Hardy, J.A., Lam, J., Nguyen, J.T., O'Brien, T., and Wells, J.A. (2004). Discovery of an allosteric site in caspases. Proc. Natl. Acad. Sci. USA *101*, 12461–12466.

Huse, M., and Kuriyan, J. (2002). The conformational plasticity of protein kinases. Cell *109*, 275–282.

Khersonsky, O., and Tawfik, D.S. (2010). Enzyme promiscuity: a mechanistic and evolutionary perspective. Annu. Rev. Biochem. *79*, 471–505.

Kornev, A.P., Haste, N.M., Taylor, S.S., and Eyck, L.F.T. (2006). Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. Proc. Natl. Acad. Sci. USA *103*, 17783–17788.

Kuriyan, J., and Eisenberg, D. (2007). The origin of protein interactions and allostery in colocalization. Nature *450*, 983–990.

Lee, J., Natarajan, M., Nashine, V.C., Socolich, M., Vo, T., Russ, W.P., Benkovic, S.J., and Ranganathan, R. (2008). Surface sites for engineering allosteric control in proteins. Science *322*, 438–442.

Ma, B., Kumar, S., Tsai, C.J., and Nussinov, R. (1999). Folding funnels and binding mechanisms. Protein Eng. *12*, 713–720.

Madhani, H.D., and Fink, G.R. (1998). The riddle of MAP kinase signaling specificity. Trends Genet. *14*, 151–155.

Malleshaiah, M.K., Shahrezaei, V., Swain, P.S., and Michnick, S.W. (2010). The scaffold protein Ste5 directly controls a switch-like mating decision in yeast. Nature *465*, 101–105.

O'Brien, P.J., and Herschlag, D. (1999). Catalytic promiscuity and the evolution of new enzymatic activities. Chem. Biol. *6*, R91–R105.

O'Brien, P.J., and Herschlag, D. (2001). Functional interrelationships in the alkaline phosphatase superfamily: phosphodiesterase activity of *Escherichia coli* alkaline phosphatase. Biochemistry *40*, 5691–5699.

Reményi, A., Good, M.C., Bhattacharyya, R.P., and Lim, W.A. (2005). The role of docking interactions in mediating signaling input, output, and discrimination in the yeast MAPK network. Mol. Cell *20*, 951–962.

Reynolds, K.A., McLaughlin, R.N., and Ranganathan, R. (2011). Hot spots for allosteric regulation on protein surfaces. Cell *147*, 1564–1575.

Roodveldt, C., and Tawfik, D.S. (2005). Shared promiscuous activities and evolutionary features in various members of the amidohydrolase superfamily. Biochemistry *44*, 12728–12736.

Schindler, T., Bornmann, W., Pellicena, P., Miller, W.T., Clarkson, B., and Kuriyan, J. (2000). Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. Science *289*, 1938–1942.

Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000). A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat. Cell Biol. *2*, 110–116.

Taylor, J.W., and Berbee, M.L. (2006). Dating divergences in the Fungal Tree of Life: review and new analyses. Mycologia *98*, 838–849.

Taylor Ringia, E.A., Garrett, J.B., Thoden, J.B., Holden, H.M., Rayment, I., and Gerlt, J.A. (2004). Evolution of enzymatic activity in the enolase superfamily: functional studies of the promiscuous o-succinylbenzoate synthase from *Amycolatopsis*. Biochemistry *43*, 224–229.

Tuch, B.B., Galgoczy, D.J., Hernday, A.D., Li, H., and Johnson, A.D. (2008a). The evolution of combinatorial gene regulation in fungi. PLoS Biol. *6*, e38.

Tuch, B.B., Li, H., and Johnson, A.D. (2008b). Evolution of eukaryotic transcription circuits. Science *319*, 1797–1799.

Wise, E.L., Yew, W.S., Akana, J., Gerlt, J.A., and Rayment, I. (2005). Evolution of enzymatic activities in the orotidine 5′-monophosphate decarboxylase suprafamily: structural basis for catalytic promiscuity in wild-type and designed mutants of 3-keto-L-gulonate 6-phosphate decarboxylase. Biochemistry *44*, 1816–1823.

Zalatan, J.G., Coyle, S.M., Rajan, S., Sidhu, S.S., and Lim, W.A. (2012). Conformational control of the Ste5 scaffold protein insulates against MAP kinase misactivation. Science *337*, 1218–1222.