**Negative Feedback Phosphorylation of Gγ Subunit Ste18 and the Ste5 Scaffold Synergistically Regulates MAPK Activation in Yeast**

**Graphical Abstract**

**Highlights**

- Yeast Gγ/Ste18 phosphorylation in response to GPCR activation requires Fus3
- Ste18/Ste5 phosphorylation represses the rate and amplitude of Fus3 activation
- Ste18/Ste5 phosphorylation controls the bulk rate of Ste5/plasma membrane association
- Ste18 and Ste5 phosphorylation, together, control switch-like mating in yeast

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**In Brief**

Choudhury et al. show that Gγ subunits, besides acting as anchors for their obligate Gβ subunits, have more complex roles in regulating G protein signaling. Furthermore, they show that this tuning of G protein signaling by the phosphorylated Gγ N-terminal tail is achieved by altering the interaction between Gβγ and downstream effectors in a PTM-dependent manner.
Negative Feedback Phosphorylation of Gγ Subunit Ste18 and the Ste5 Scaffold Synergistically Regulates MAPK Activation in Yeast

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https://doi.org/10.1016/j.celrep.2018.03.135

SUMMARY

Heterotrimeric G proteins (Gαβγ) are essential transducers in G protein signaling systems in all eukaryotes. In yeast, G protein signaling differentially activates mitogen-activated protein kinases (MAPKs)—Fus3 and Kss1—a phenomenon controlled by plasma membrane (PM) association of the scaffold protein Ste5. Here, we show that phosphorylation of the yeast Gγ subunit (Ste18), together with Fus3 docking on Ste5, controls the rate and stability of Ste5/PM association. Disruption of either element alone by point mutation has mild but reciprocal effects on MAPK activation. Disabling both elements results in ultra-fast and stable bulk Ste5/PM localization and Fus3 activation that is 6 times faster and 4 times more amplified compared to wild-type cells. These results further resolve the mechanism by which MAPK negative feedback phosphorylation controls pathway activation and provides compelling evidence that Gγ subunits can serve as intrinsic regulators of G protein signaling.

INTRODUCTION

Canonical G protein signaling systems—including 7-transmembrane G-protein-coupled receptors (GPCRs), heterotrimeric G-proteins (Gαβγ), and diverse effector proteins—constitute a highly conserved system enabling the transduction of extracellular signaling molecules such as hormones, neurotransmitters, and chemokines (Cabrera-Vera et al., 2003; Wetschereck and Offermanns, 2005). As one of the primary signal transduction mechanisms in eukaryotes, G protein signaling pathways control a wide range of processes in both single-cell and multi-cellular organisms such as yeast and humans (Bargmann, 2006; Fan et al., 1997; Kaul et al., 2001; Rockman et al., 2002; Rosenbaum et al., 2009). Owing to their high degree of structural conservation, fundamental mechanisms underlying the regulation of G protein signaling have emerged from empirical studies conducted across widely diverse organisms from yeast to human. Such attributes have also helped to solidify their prominent role as pharmaceutical targets for the treatment of human disease (Hauser et al., 2017).

The yeast model system for G protein signaling remains one of the most well-characterized signaling pathways in history and has been instrumental in the discovery of G protein regulatory mechanisms, including regulators of G protein signaling (RGS) proteins and post-translational-modification (PTM)-based regulators (Cappell et al., 2010; Clement et al., 2013; Deflorio et al., 2013; Dohlman et al., 1991, 1996; Dohlman and Thorner, 2001; Isom et al., 2013; Li et al., 1998; Stone et al., 1991; Wang et al., 2005). In budding yeast Saccharomyces cerevisiae, a single canonical G protein signaling pathway controls the process of mating, wherein two yeasts of opposite and complementary mating types, MATa and MATα, fuse to form an a/z diploid cell (Dohlman and Thorner, 2001). As in the case of multicellular organisms, including mammals, G protein signaling in yeast is initiated by agonist-dependent activation of a GPCR at the cell surface (Dohlman and Thorner, 2001). In MATa cells, a single peptide mating pheromone (α factor) serves as the agonist of the pheromone GPCR, Ste2, and upon binding promotes the exchange of GDP for GTP on the Gα subunit (Gpa1). GTP binding stabilizes a conformational change in Gα and dissociation of the heterotrimer into Gα and Gβγ (Ste4/Ste18) components (Dohlman and Thorner, 2001; Sprang, 1997). In yeast, Gβγ serves as the primary activator of the mating pathway, whereas Gβ serves primarily to sequester Gγ (Dohlman and Thorner, 2001). When not sequestered, Gγ nucleates the formation of a protein complex at the plasma membrane that includes a p21-activated protein kinase (PAK; Ste20) and a protein scaffold (Ste5) in complex with a mitogen-activated protein kinase (MAPKKK) (Ste11), MAPKK (Ste7), and mitogen-activated protein kinase (MAPK) (Fus3), as well as additional proteins important for cell polarization (Arkowitz, 2009; Nern and Arkowitz, 1999). Activation of the pheromone response pathway through this process drives a phosphorylation cascade resulting in double phosphorylation (referred to here as di-phosphorylation or activation) of Kss1T183,Y185 and Fus3T180,Y182—highly conserved orthologs of the human MAPKs Erk1 and Erk2—which phosphorylate several targets necessary for a complete mating response. Once activated, Fus3 translocates to the nucleus, wherein it phosphorylates the transcription factor Ste12 and other proteins necessary to drive a gene transcription program resulting in morphological change, cell-cycle arrest, and eventual mating (van Drogen et al., 2001; Elion et al., 1993). Signaling is terminated by hydrolysis of GTP to GDP on the Gα subunit and re-association of the heterotrimeric complex, a process that is accelerated by the RGS protein (Sst2) (Dohlman et al., 1996).
phosphorylated in high-throughput proteomics. Here, we show that the N-terminal tail of the yeast Gγ subunit, Ste18, together with the Fus3/Ste5 docking interaction, constitute a negative regulatory system that synergistically controls the activation of Fus3 and Kss1 through a dynamic phospho-inhibitory mechanism that prevents rapid and stable association of bulk Ste5 at the plasma membrane. Disruption of either side alone (by point mutation of Ste18 or Ste5) results in minor but significant and reciprocal changes to the rate and amplitude of Fus3 activation and Ste5/PM recruitment, whereas combined disruption of both elements results in ultra-rapid and robust Fus3 activation and Ste5/PM recruitment. We show that negative regulation is facilitated by weakened binding between Gβγ and Ste5 when both proteins are phosphorylated—a mechanism that may have emerged through co-evolution of the two distinct phospho-regulatory elements. Taken together, these data reveal a way in which Gβγ effector binding can be modulated to control signaling output through post-translational modifications.

**RESULTS**

**The N-Terminal Tail of Ste18 Is Rapidly Phosphorylated in Response to GPCR Activation**

Like other Gγ subunits throughout Eukarya, the terminal ends of Ste18, representing 20% of its residue content, are intrinsically disordered—most of which correspond to the N-terminal tail (Ste18Nt). This region in Ste18 (residues 1–13) harbors phosphorylation sites at Thr-2, Ser-3, and Ser-7, specifically (Dewhurst et al., 2015; Soufi et al., 2009). Phosphorylation of Ste18Nt produces a distinctive electrophoretic mobility shift in response to pheromone stimulation, which can be detected by SDS-PAGE.
and immunoblotting (Dewhurst et al., 2015). Treatment with alkaline phosphatase eliminates the mobility shift in a manner that depends on the presence or absence of phosphatase inhibitors (Figure S1A). Moreover, phospho-null mutations (Ste18T2A, S3A, S7A; referred to here as Ste18 3A) and phospho-mimic mutations (Ste18T2E, S3E, S7E; referred to here as Ste183E) eliminate or restore the mobility shift, respectively (Dewhurst et al., 2015). To determine a precise estimate of phosphorylation kinetics early and late after receptor activation, we surveyed the phosphorylation-dependent mobility shift of Ste18 in both long and short time course experiments (Figures 2A and S1B). We found that phosphorylation occurs almost instantaneously after receptor activation and is readily detectable within 30 s (Figures 2B and S1B). Maximum phosphorylation is achieved at ~80% of total Ste18 levels within 3 min, with time at half maximum (t½) of 1.25 min (Figure 2B, inset). Phosphorylation remains stable for the duration of pheromone exposure up to 90 min. Activation profiles for Fus3 and Kss1 MAPKs were consistent with their expected patterns of activation at 3-μM pheromone concentrations (Figure 2C) (Hao et al., 2008).

Surmising that Ste18 phosphorylation may depend on a kinase within the pheromone pathway, we monitored Ste18 phosphorylation in cells lacking single components of the pathway, including: Ste20, Ste11, Ste7, Fus3, and Kss1, in addition to the MAPK scaffold Ste5. We found that all kinases upstream of and including the scaffolded MAPK complex were necessary for robust phosphorylation of Ste18Nt in response to pheromone (Figures S1C and S1D). Kss1, which is rapidly phosphorylated after receptor activation but is not scaffolded by Ste5, is not required for pheromone-dependent Ste18 phosphorylation. Since Ste18 phosphorylation should not be lost in fus3Δ cells if MAPKs upstream of Fus3 are responsible for phosphorylation, these data suggest that Fus3, but not upstream MAPKs, are necessary. Other genes involved in the pheromone pathway that either regulate pheromone dependent kinase activation or are themselves pheromone-activated kinases had little to no effect when deleted from the genome (Figure S2). Taken together, these data demonstrate that Ste18 is rapidly phosphorylated in response to GPCR activation in a manner that requires Fus3.

**Phosphorylation of Ste18Nt and Ste5 Function Synergistically to Delay Fus3 Peak Activation in Response to Pheromone**

Considering that Ste18 is rapidly phosphorylated within seconds of receptor stimulation, we hypothesized that it must be a prerequisite for proper pathway activation. Consistent with this hypothesis, we found that preventing phosphorylation of Ste18 (Ste183A) resulted in a significant shift in peak activation of Fus3, which occurred twice as fast (15 min earlier) than in
wild-type cells (Figures S3A and S3C). In contrast, activation of Fus3 and Kss1 in cells harboring the phosphomimic form, Ste18\(^{3E}\), was no different from that in wild-type cells, as would be expected if phosphorylation is a prerequisite feature required for normal pathway activation (Figures S3A and S3B). Thus, Ste18 phosphorylation is required for delayed peak activation of the scaffolded MAPK Fus3 but has no effect on the activation of the un-scaffolded MAPK, Kss1.

Negative feedback phosphorylation of Ste5, like phosphorylation of Ste18, occurs early/before the mating response and also requires Fus3. Therefore, we hypothesized that both elements may function in concert to delay Fus3 peak activation. This hypothesis was inspired, in part, by two points of indirect evidence that converge on Fus3 as a negative regulator of the pheromone response. First, rapid receptor-activated phosphorylation of Ste18\(^{3E}\) requires Fus3 (Figure S1), and activated Fus3 has been previously shown to counteract the stability of Ste5/PM association during the pheromone response (Yu et al., 2008b). Second, Fus3-mediated negative feedback phosphorylation on Ste5 dampens the intensity of Fus3 activation in response to pheromone (Dowell et al., 1998; Hao et al., 2008; Inouye et al., 1997). Additionally, we considered as further evidence that stable Ste5/PM association is facilitated in part by the interaction of the Ste5\(^{\text{RING}}\) domain (Ste5\(^{138-214}\)) with residues in Ste4 (Ste4\(^{49-65}\)) that are located in the coiled-coil structure formed by both Gβ (Ste4) and Gγ (Ste18) subunits and in very close proximity to where phosphorylation occurs in Ste18\(^{3E}\) (Dowell et al., 1998).

To test the hypothesis, we monitored phosphorylation of Ste18, Kss1, and Fus3 in cells that endogenously express different combinations of Ste18 phosphosite and Ste5\(^{3E}\) docking-site mutants. Precise mutation of multiple sites within two essential binding surfaces (A and B) in the Ste5\(^{3E}\) (a.k.a. Ste5\(^{\text{ND}}\)) prevents Fus3 docking, allosteric activation, and negative feedback phosphorylation of Ste5 (Bhattacharyya et al., 2006). Interestingly, phosphorylation of Ste18\(^{3E}\) was unaffected by Ste5\(^{\text{ND}}\), suggesting that Fus3 docking to Ste5 is not necessary for Ste18 phosphorylation (Figures S3A and S3B). As observed in previous experiments, peak activation of Fus3 occurred significantly early in Ste18\(^{3E}\) compared to Ste18\(^{3E}\) or wild-type cells (Figures 3C and 3E), whereas Kss1 activation was unaffected (Figures 3C and 3D). In contrast, we found that peak activation of Fus3 (at 30 min) and Kss1 (between 5 and 30 min) was significantly elevated, but not early, in Ste5\(^{\text{ND}}\) cells harboring the wild-type form of Ste18, an observation that is nearly identical with previously reported evidence (Figures 3C–3E) (Hao et al., 2008).

We made several observations with yeast harboring both Ste18 and Ste5 mutations in combination. First, peak activation of Fus3 occurs rapidly in Ste18\(^{3E}/\text{Ste5}^{\text{ND}}\) cells, wherein neither protein can be phosphorylated (Figure 3E; Table S2). Significantly, this response was 25 min earlier and nearly 1.5-fold greater in amplitude than observed for Ste5\(^{\text{ND}}\) alone and 3.6-fold greater than the response in wild-type cells (Figure 3E; Table S2). Neither this nor any other mutant showed abrupt differences in the pattern of pheromone-dependent Fus3 expression level compared to wild-type cells, suggesting that differences in the intensity of activated Fus3 at early time points is due to Fus3 phosphorylation and independent of Fus3 expression (Figure S4A). Interestingly, Ste18\(^{3E}/\text{Ste5}^{\text{ND}}\), but not other cell types, also exhibited morphological defects in the absence of pheromone, showing pronounced elongation in multiple cases (Figure S4B). Second, in cells harboring the combination of Ste18\(^{3E}\) and Ste5\(^{\text{ND}}\), Fus3 peak activation occurs early (15 min) and with no change in amplitude—nearly identical to the response in Ste18\(^{3E}/\text{Ste5}^{\text{WT}}\) cells and indicative that phosphorylation restricted to either Ste18 or Ste5 is sufficient to elicit an equivalent outcome in Fus3 peak activation (Figure 3E; Table S2). Complementary phos-tag analysis of Fus3 phosphorylation further suggests that either Ste18\(^{3E}\) phosphorylation or Ste5 phosphorylation (controlled by Fus3 docking on Ste5) can effectively inhibit the aberrant di-phosphorylation of Fus3 in the absence of pheromone (Figure S5). Moreover, preventing such control on both proteins together results in aberrant Fus3 di-phosphorylation in the absence of pheromone. Unexpectedly, activation of Kss1 was also affected by combined disruption of Ste18/Ste5. However, in contrast to Fus3, Kss1 becomes hyper-activated in Ste18\(^{3E}/\text{Ste5}^{\text{ND}}\) cells (Figure 3D). Further confirmation of these results was achieved by endpoint immunoblot assays conducted within the first 15 min of pheromone stimulation (Figures S4C–S4E).

Evidence from MAPK activation analysis suggested that Ste18 and Ste5 phosphorylation behave synergistically so that the combined effect of both produces an effect that is greater than the sum of their separate effects. Indeed, a quantitative test for synergy revealed that phosphorylated Ste18 and Ste5 are synergistic in their control of Fus3 peak activation time but additive in their control of Fus3 peak amplitude, with an overall synergistic impact that is ~4x greater than the sum of effects contributed by Ste18 or Ste5 alone (Table S2). Taken together, these data suggest that the phosphorylation of Ste18 and MAPK docking/phosphorylation of Ste5 (referred to here as the Ste18/Ste5 phospho-inhibitory system) synergize to delay the activation and repress the amplitude of Fus3 activation in response to pheromone.

**Together, Ste18/Ste5 Phosphorylation Delays Bulk Recruitment Rate and Reduces the Duration of Ste5/PM Association**

Activation of Fus3 requires recruitment and stable association of Ste5 with the plasma membrane, and consequently, mutations that disrupt or enhance Ste5/PM association should be reflected by the kinetics and amplitude of Fus3 activation. To test this hypothesis, we monitored Ste5/PM association by fluorescence microscopy before, during, and after pheromone stimulation of yeast cells. We were unable to detect Ste5-GFP at the PM in any cell that was not treated with pheromone (Figure 4A, top). However, in as few as 23–26 min after pheromone stimulation, we observed robust PM accumulation of Ste5-GFP in all cell types, most noticeably in Ste18\(^{3E}/\text{Ste5}^{\text{ND}}\) cells (Figure 4A, bottom). Consistent with our hypothesis, preventing phosphorylation on both Ste18 and Ste5 (Ste18\(^{3E}/\text{Ste5}^{\text{ND}}\)) resulted in the robust accumulation of Ste5-GFP at the PM after 25 min of pheromone stimulation, reaching a level nearly 7-fold greater than that of wild-type cells measured at the same time (Figure 4B). However, no differences were observed in the protein levels of Ste5-GFP (Figure S6). Thus, the Ste5-GFP signal at the membrane reflects altered Ste5/PM accumulation rather than protein stability. Furthermore, these data suggest that the bulk
recruitment rate of Ste5 to the PM—reflected in the number of Ste5-GFP molecules that accumulate at the PM per unit time (i.e., total fluorescence intensity/time)—is significantly faster when phosphorylation on both proteins is prevented.

To compare the relative rates and stability of Ste5-GFP/PM accumulation at the population level, we quantified the percentage of the cell population with Ste5-GFP localized at the PM as a function of time. Measured in this way, Ste5/PM accumulation data are

**Figure 3. Phosphorylation on Ste18 and Ste5 Cooperate to Prevent Early and Maximal Fus3 Activation**
Cells harboring the indicated combination of wild-type or mutant versions of Ste18 and Ste5 were stimulated with 3 μM α-F followed by quantitative immunoblot analysis of HA-Ste18 or activated Fus3 and Kss1.

(A) HA-Ste18 immunoblot in cells harboring wild-type (WT/WT) or Ste5ND (WT/non-docking [ND]).

(B) Quantitative comparison of pHA-Ste18 from (A) (n = 4).

(C) Representative immunoblot for activated Kss1 and Fus3.

(D) Quantitative comparison of activated Kss1 relative to wild-type peak activation at 5 min from immunoblots shown in (C).

(E) Quantitative comparison of activated Fus3 relative to wild-type peak activation at 30 min from immunoblots shown in (C). Data represent mean ± SD; n = 12.

SE, short exposure; LE, long exposure; LC, loading control.

See also Figures S3, S4, and S5 and Table S2.
more comparable to MAPK activation data, which is a population-based analysis. Preventing phosphorylation on both Ste18 and Ste5 (Ste18<sup>3A</sup>/Ste5<sup>ND</sup>) significantly enhanced the rate at which the population of cells exhibited Ste5-GFP/PM accumulation, where 100% of the cells were responsive by 14 min (Figures 4C and 4D). This effect was reverted to a wild-type state in Ste18<sup>3E</sup>/Ste5<sup>ND</sup> cells, mimicking the effects of reciprocal mutations on MAPK activation seen earlier. In contrast, Ste5<sup>ND</sup> cells exhibited an intermediate phenotype. As expected, mutations to Ste18 alone had mild average effects on Ste5-GFP/PM accumulation.

Lastly, we also observed that Ste5/PM association was noticeably more stable in Ste18<sup>3A</sup>/Ste5<sup>ND</sup> cells compared to all other
Ste5 has been shown previously to be an important control point for the switch-like morphological response, which, in wild-type cells, is controlled by competitive Ste5FBD docking by Fus3 and the phosphatase Ptc1 (Malleshaiah et al., 2010). Consequently, non-docking Ste5ND mutants, which cannot bind to either Fus3 or Ptc1, exhibit a graded morphological dose-response. We hypothesized that Ste18 phosphorylation might also be involved in controlling the switch-like mating decision. To test this hypothesis, we performed microscopy-based dose-response experiments to quantify shmoo formation as a function of pheromone dose. Modeling the data with a sigmoidal dose-response curve with variable slope, we confirmed that wild-type cells exhibit switch-like dose-responsiveness with a hill slope well above 1 (hill coefficient $n_\text{H}^{\text{WT}} = 6.2$) (Figure 5A; Table S3). Similarly, Ste183A and Ste183E cells were also switch-like ($n_\text{H}^{3A} = 6.3; n_\text{H}^{3E} = 3.7$). We observed that Ste5ND cells are graded rather than switch-like ($n_\text{H}^{\text{ND}} = 0.97$), consistent with previous reports (Coyle et al., 2013; Malleshaiah et al., 2010) (Figure 5B; Table S3). Disabling both regulatory elements (Ste183A/Ste5ND) produced a slightly more graded response ($n_\text{H}^{3A/ND} = 0.85$), exhibiting a hill slope lower than that for Ste5ND. Not surprisingly, Ste183A/Ste5ND cells also exhibited an elevated basal response that was 3-fold greater than that for wild-type cells, consistent with earlier results showing elevated Fus3 activation in these cells (Figures 3, S4B, S4C, and S5). Expression of the phosphomimic form of Ste18 in the Ste5ND background (Ste183E/Ste5ND) resulted in a reversion of the analog response in Ste5ND cells back to a switch-like state ($n_\text{H}^{3E/ND} = 2.7$) (Figure 5B; Table S3). Taken together, these data suggest that phosphorylation on Ste18 regulates the morphological mating switch in cells when Ste5 is incapable of docking with Fus3. Furthermore, these data suggest that Ste18/Ste5 phosphorylation regulates the switch-like mating decision in yeast.

Phosphorylation of the $G_\gamma$ Subunit and MAPK Docking on Ste5 Appear Simultaneously in the Phylogeny of Yeasts

Recent phylogenetic and experimental evidence has revealed that the Ste5 FBD arose ~130 million years ago and is almost exclusively found in the clade to which Saccharomyces cerevisiae belongs but is also partially extant in V. polyspora species, which contain a partial FBD that is moderately active (Coyle et al., 2013). Having determined that the phosphorylation of the N-terminal tail of Ste18 and the Ste5FBD function synergistically to control signaling, we asked whether these two structural elements may have co-evolved. Comparison of the phylogeny of Ste18 and Ste5 fungal orthologs revealed compelling evidence in support of this hypothesis. Indeed, the appearance of the phosphorylation sites in the Ste18 intrinsically disordered region (phospho-IDR) coincides with the appearance of Ste5FBD (Figures 6A and S7A). Furthermore, all extant members of the clade retain nearly 100% identity for sites of phosphorylation and MAPK binding that are essential to either element (Figure 6B). A somewhat similar putative phospho-IDR was also found in the Ste18 ortholog from S. castellii and the distantly related Y. lipolytica, both species of which harbor 2 of the 3 phosphosites observed in Ste18NT and Ste5, together, control the bulk rate and stability of Ste5/PM association and MAPK activation.

strains, whereby the population of Ste183A/Ste5ND cells had maximal association for the longest time (141 min) compared to that of wild-type or Ste183E/Ste5ND cells (Figure 4E). In support of the hypothesis that this is promoted by more stable association between Ste4/18 and Ste5, co-immunoprecipitation (coIP) experiments revealed significantly more (2 × 5 x) Ste18 immunoprecipitated with Ste5-GFP in Ste183A/Ste5ND cells compared to all other cell types (Figures 4F and 4G). We conclude that rapid bulk recruitment of Ste5 to the PM and subsequent Fus3 activation in Ste183A/Ste5ND cells can be attributed in part to greater physical association between the two proteins in their non-phosphorylated states and support evidence that phosphorylation of Ste18NT and Ste5, together, control the bulk rate and stability of Ste5/PM association and MAPK activation.

Figure 5. The Switch-like Morphological Response to Pheromone Is Regulated by Ste18-NT Phosphorylation When Fus3 Cannot Bind to Ste5
The morphological dose-response to mating pheromone represented by the cellular formation of a mating projection (i.e., shmoo) was quantified as a percentage of total cells in a population by DIC microscopy. Data were fit to a sigmoidal dose-response curve with variable slope. (A) Effect of individual Ste18NT phosphorylation mutations on the mating response. The percentage of total cells in a population by DIC microscopy. Data were fit to a sigmoidal dose-response curve with variable slope. (B) Effect of Ste18NT phosphorylation mutations in strains exclusively expressing the Fus3 non-docking mutant Ste5ND. Error bars represent SEM across ≥200 cells per experiment.

See also Table S3.
S7P Site B

A. gossypii of Ste5-containing yeast species (excluding lengths (Figure S7B). When looking strictly within the phylogeny but exhibit dramatically different N-terminal IDR S. cerevisiae

Ste18 orthologs, culminating in a dramatic increase of 8–10 amino progressive lengthening over time of the N-terminal IDRs of harbors an extraordinarily long N-terminal IDR), we observed a membrane anchors for G

DISCUSSION

appear only once in the evolution of budding yeast. Ste5 phospho-regulatory elements arose at similar times and signaling systems (Cook et al., 1998; Kisselev et al., 1995; Muntz

function is generally perceived as benign. We previously re-

subunits are recognized as having limited function as mem-

brane proteins that are GTPase-activating proteins (GAPs) for heterotrimeric G protein signaling systems (Cook et al., 1998; Kisselev et al., 1995; Muntz et al., 1992). As such, their role in signal transduction beyond this function is generally perceived as benign. We previously re-

translate stimulus dose to MAPK response signals—a function that is facilitated by scaffolding of the MAPK cascade module (MAPKKK Ste11, MAPK Ste7, and MAPK Fus3). In resting cells, Ste5/MAPK and Ste5 inter-domain interactions, as well as phosphorylation, prevent signaling from occurring spontaneously. Under such conditions, Ste5 is distributed between the cytoplasm and the nucleus—a phenotype that is cell-cycle regulated in part (Maeder et al., 2007; Strickfaden et al., 2007). Cytoplasmic Ste5 can still interact with all three components of the MAPK cascade module (Takahashi and Pryciak, 2008). However, Ste5 is incapable of facilitating MAPK activation in this state due to inter-domain binding between Ste5PH and Ste5VWA domains (Zalatan et al., 2012) as well as inhibitory phosphorylation mediated by Fus3 and Ste5PH interactions (Bhattacharyya et al., 2006). Relief of inter-domain binding inhibition is mediated by pheromone-dependent accumulation of PIP2 in the PM, which, when stimulated, promotes Ste5PH/PM association, and de-inhibition of Ste5VWA allows for a 3-fold

Figure 6. Coordinated Phospho-regulation of Ste18 and Ste5 Evolved at the Same Time

(A) Bootstrapped phylogenetic tree of Ste18 orthologs from the Ascomycota phylum showing the evolutionary co-occurrence of regulatory phosphorylation sites on the N-terminal tail of the G_Y subunit and the FBD of orthologous Ste5 scaffolds. The presence of Ste5 orthologous proteins, functional Ste5-like FBDs, and N-terminal G_Y phospho-regulatory intrinsically disordered regions (IDRs) are shown next to yeast species harboring each element.

(B) Speciees that contain the synergistic regulatory element (Ste18Nt and Ste5FBD) are nearly 100% identical at phosphorylation site alignment positions in Ste18 and MAPK binding sites reported previously in Ste5 (Coyle et al., 2013).

See also Figure S7.

New Insights into the Regulation of Ste5/PM Association and MAPK Activation

For good reason, understanding the mechanism by which Ste5 coordinates mating pathway output has been a long-standing endeavor with many revisions over the past 10–15 years. The primary function of Ste5 is to properly

translate stimulus dose to MAPK response signals—a function that is facilitated by scaffolding of the MAPK cascade module (MAPKKK Ste11, MAPK Ste7, and MAPK Fus3). In resting cells, Ste5/MAPK and Ste5 inter-domain interactions, as well as phosphorylation, prevent signaling from occurring spontaneously. Under such conditions, Ste5 is distributed between the cytoplasm and the nucleus—a phenotype that is cell-cycle regulated in part (Maeder et al., 2007; Strickfaden et al., 2007). Cytoplasmic Ste5 can still interact with all three components of the MAPK cascade module (Takahashi and Pryciak, 2008). However, Ste5 is incapable of facilitating MAPK activation in this state due to inter-domain binding between Ste5PH and Ste5VWA domains (Zalatan et al., 2012) as well as inhibitory phosphorylation mediated by Fus3 and Ste5PH interactions (Bhattacharyya et al., 2006). Relief of inter-domain binding inhibition is mediated by pheromone-dependent accumulation of PIP2 in the PM, which, when stimulated, promotes Ste5PH/PM association, and de-inhibition of Ste5VWA allows for a 3-fold

S. cerevisiae but exhibit dramatically different N-terminal IDR lengths (Figure S7B). When looking strictly within the phylogeny of Ste5-containing yeast species (excluding A. gossypii, which harbors an extraordinarily long N-terminal IDR), we observed a progressive lengthening over time of the N-terminal IDRs of Ste18 orthologs, culminating in a dramatic increase of 8–10 amino acid residues that appeared coincidently with the Ste5FBD (Figure S7C). Taken together, these data suggest that Ste18 and Ste5 phospho-regulatory elements arose at similar times and appear only once in the evolution of budding yeast.

DISCUSSION

GY subunits are recognized as having limited function as mem-

brane anchors for G_Y subunits in heterotrimeric G protein signaling systems (Cook et al., 1998; Kisselev et al., 1995; Muntz et al., 1992). As such, their role in signal transduction beyond this function is generally perceived as benign. We previously re-

translate stimulus dose to MAPK response signals—a function that is facilitated by scaffolding of the MAPK cascade module (MAPKKK Ste11, MAPK Ste7, and MAPK Fus3). In resting cells, Ste5/MAPK and Ste5 inter-domain interactions, as well as phosphorylation, prevent signaling from occurring spontaneously. Under such conditions, Ste5 is distributed between the cytoplasm and the nucleus—a phenotype that is cell-cycle regulated in part (Maeder et al., 2007; Strickfaden et al., 2007). Cytoplasmic Ste5 can still interact with all three components of the MAPK cascade module (Takahashi and Pryciak, 2008). However, Ste5 is incapable of facilitating MAPK activation in this state due to inter-domain binding between Ste5PH and Ste5VWA domains (Zalatan et al., 2012) as well as inhibitory phosphorylation mediated by Fus3 and Ste5PH interactions (Bhattacharyya et al., 2006). Relief of inter-domain binding inhibition is mediated by pheromone-dependent accumulation of PIP2 in the PM, which, when stimulated, promotes Ste5PH/PM association, and de-inhibition of Ste5VWA allows for a 3-fold
increase in Fus3 phosphorylation over the inhibited state in vitro (Zalatan et al., 2012).

Our evidence suggests that Ste5/PM association is also modulated by phosphorylation on Ste18 in addition to Ste5, both of which function synergistically to control Fus3 activation output. In a wild-type cell, phosphorylation of both proteins slows the rate and restricts the amplitude of Fus3 activation, resulting in 30-min peak activation (Ste18 WT/Ste5WT). Engaging either only one element (Ste18 3E/Ste5ND) or the other (Ste18 3A/Ste5WT) produces an equivalent enhancement in peak activation and amplitude that is 2 times faster and 1.5 to 2 times more intense than observed in wild-type cells—a response that demonstrates synergy between the two phospho-regulatory elements (phosphorylated Ste18 and Ste5) in the system.

Figure 7. Phosphorylated Ste18Nt and Ste5FBD Constitute a Dynamic Phosphoregulatory System for Pheromone Signaling
(A) In response to pheromone, Ste18 is rapidly phosphorylated at its N-terminal tail (P-lollipop). Ste5 is simultaneously phosphorylated via negative feedback controlled by Fus3/Ste5FBD docking (P-lollipops). Together, this constitutes a phoso-inhibitory system that prevents otherwise rapid Ste5/PM association. While not shown outright here, previous work implicates pheromone-stimulated expression of Ptc1 phosphatase and removal of inhibitory phosphorylation on Ste5 as the inhibition release (Malleshaiah et al., 2010) (dashed orange box). Consequently, the mating pathway is activated with a kinetic delay, as evident by the slower rate of Ste5 association at the membrane and delayed peak activation of Fus3.

(B) Cells engineered to prevent activation of the Ste18/Ste5 system (Ste18 3A/Ste5ND) respond ~6 times faster with ~4 times greater intensity than observed in wild-type cells—a response that demonstrates synergy between the two phospho-regulatory elements (phosphorylated Ste18 and Ste5) in the system.

Consider the body of evidence in light of previous work by others, we propose a model in which the activation/deactivation of the Ste18/Ste5 phospho-inhibitory system is regulated differentially (Figure 7A). In a pre-stimulated state, Fus3 is monophosphorylated and, in this state, is capable of phosphorylating Ste5 at up to 4 positions near Ste5FBD (Figure S5; Malleshaiah et al., 2010), while Ste18 is also basally phosphorylated (Figures 2 and S1). This suggests that inhibition is partially engaged even before a pheromone stimulus. Upon the addition of pheromone, the Ste18/Ste5 inhibitory elements become fully activated, as both Ste18 and Ste5 become hyper-phosphorylated, demonstrated herein directly for Ste18 (Figure 2) and presumed by an in vitro Fus3 kinase assay with Ste5 (see Figure S9 in Malleshaiah et al., 2010). We propose that this constitutes the fully activated inhibitory element that is responsible for bulk inhibition of Ste5/PM association early in the pheromone response, as determined from the relative amounts of Ste5-GFP/PM accumulation observed in Ste18 3A/Ste5ND compared to other cells (Figure 4). Simultaneously, pheromone stimulates recruitment of Ptc1 phosphatase to Ste5FBD, which outcompetes Fus3 binding within 2 min post-stimulus and dephosphorylates the inhibitory feedback phosphosites on Ste5 (see Figure S18 in Malleshaiah et al., 2010). Since as few as one occupied Ste5 phosphosite leads to the inhibition of Fus3 di-phosphorylation (Malleshaiah et al., 2010), it is presumed that all four sites must be completely
dephosphorylated. We propose that this constitutes a release by partial de-inhibition of the Ste18/Ste5 inhibitory element, which leaves the Ste18 element intact (phosphorylated), as can be seen by the fact that Ste18 phosphorylation remains unchanged during this time (Figure 3B). We propose that, as a consequence of having one phospho-inhibitory element still intact (phospho-Ste18), the bulk recruitment of Ste5 to the membrane is permitted but relatively slow, and the activation of Fus3 is delayed, not reaching peak amplitude until ~30 min post-stimulus (Figures 2G, 3E, and 4). This half-activated state of the Ste18/Ste5 system also controls the degree to which Ste18 co-immunoprecipitates with Ste5 and the degree to which Ste5/PM association occurs (Figure 4G)—observations that correlate very well with Fus3 activation.

In preventing phosphorylation on Ste18 and Ste5 (Ste18Δ/Ste5ΔΔ), we show that this system is necessary to delay what is otherwise ultra-fast and intense Fus3 activation (Figure 7B). Consequently, Fus3 activation in this state appears to match the profile of Kss1 activation, which is not scaffolded (Figures 3D and 3E). Again, the rapid bulk recruitment of Ste5 under these conditions (Figure 4) can explain this effect, since rapid Ste5/PM association is known to drive rapid de-inhibition of the Ste5VWA domain by promoting the association of Ste5PH with the plasma membrane—a mechanism that has been demonstrated previously by the Wendell Lim lab as an essential step to drive phosphorylation of Fus3 by Ste7 (Zalatan et al., 2012). Switch-like behavior of the pathway is also modulated, in part, by the Ste18/Ste5 phospho-inhibitory system, which controls Ste5/PM association, an observation that is consistent with previous reports demonstrating that SteFBD and Ste5/PM association regulatehill slope (Coyle et al., 2013; Takahashi and Pryciak, 2008).

Several pieces of evidence, both from our work and others, suggest that Fus3 is the major (if not primary) kinase for activating the phosopho-inhibitory system. Fus3 phosphorylates proline +1 sites on Ste5 (Bhattacharyya et al., 2006) and on Ste18ΔΔ (Figures S3C and S3D)—of which, both sites emerged at the same time in the evolution of budding yeast (Figure 6). Second, Ste5/PM association is enhanced in fus3Δ compared to Fus3 cells treated with p44/42 MAPK antibody (Cell Signaling Technologies catalog #9101) exhibits reduced detection sensitivity for activated Fus3 compared to horseradish peroxidase (HRP)-conjugated secondary antibodies (goat-anti-rabbit, goat-anti-mouse, or rabbit-anti-goat) were used for the detection of reactant bands using chemiluminescence with ECL reagent (PerkinElmer catalog #NEL 10401EA). Immunoblots were quantified by high-resolution scanning and pixel densitometry using ImageJ software (Schindelin et al., 2015).
Morphological Response Assay
The morphological response of STE18/STE5 mutants to α-factor was measured as described previously (Coyle et al., 2013; Malleshaiah et al., 2010) and detailed in the Supplemental Information. The morphology of the cells was determined, 3 hr post-pheromone stimulation, by differential interference contrast (DIC) confocal microscopy using a PerkinElmer UltraVIEW spinning disk confocal microscope. The number of cells with mating projections was counted as a percentage of total.

STE5-GFP Localization Assay
Live cells endogenously expressing either Ste5-GFP or Ste5ND-GFP were visualized by microscopy. To ensure visibility across all strains, we exposed cells to 10 μM α-factor followed immediately by deposition onto agar pads saturated with 30 μM α-factor. Once deposited, cells were monitored using a PerkinElmer UltraVIEW VoX spinning disk confocal microscope. A detailed procedure for image acquisition and quantification is provided as Supplemental Information.

CoIP
Cells endogenously expressing different combinations of wild-type or mutant hemagglutinin epitope (HA)-STE18 and STE5-GFP were treated with 10 μM pheromone for 20 min followed by cell lysis and coIP using anti-HA monoclonal antibody (mAb)-agarose (MBL International, #D153-8). Eluted proteins were separated by SDS-PAGE and immunoblotting with anti-GFP (Invitrogen, A11122) and anti-HA (Supplemental Information).

Phylogenetic Analysis
Ascomycota protein sequences were retrieved from the Broad Institute Fungal Orthroups Repository (https://portals.broadinstitute.org/regev/orthogroups/). Multiple sequence alignments were achieved using MUSCLE with default parameters (Edgar, 2004). Phylogenetic and graphical analyses of Ste18 and Ste5 sequence alignments were achieved using Unipro UGENE software (Okonechkov et al., 2017). Bootstrap consensus trees were prepared using 100 (shown) and 500 (not shown) iterations. Presence or absence of Ste5 or the Ste5 FBD was determined from a combination of sequence alignment homology and previous phylogenetic analyses of Ste5 (Coyle et al., 2013).

Statistical Analysis
Statistical analysis for quantifying immunoblots and microscopy data was achieved using Prism software, v6/7 (GraphPad Software). Statistical significance was determined by ANOVAs.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.135.

ACKNOWLEDGMENTS
We would like to give special thanks to Aaron Lifland and the GT EBB1 microscopy core facility for support with confocal microscopy experiments; Prof. Kirill Lobachev and Zhiwei Sheng for graciously providing yeast deletion strains; Joseph Lachance for guidance in phylogenetic analysis; Dr. Tatiana Chernova for the CUP1 yeast expression plasmid; and Dr. Lucy Robinson for the CUP1 yeast expression plasmid. This work was supported by NIH grants R01GM117400 and R00GM094533 to M.P.T. and by startup funds to M.P.T. provided by the Georgia Institute of Technology.

AUTHOR CONTRIBUTIONS
S.C. conducted experiments; S.C. and P.B.-M. constructed plasmids and yeast strains; and M.P.T. and S.C. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: May 10, 2017
Revised: December 15, 2017
Accepted: March 29, 2018
Published: May 1, 2018

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Supplemental Information

Negative Feedback Phosphorylation
of $G_Y$ Subunit Ste18 and the Ste5 Scaffold
Synergistically Regulates MAPK Activation in Yeast

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**Supplemental Experimental Procedures**

**Yeast strains and plasmids** – Standard methods for cell growth, maintenance, and transformation of yeast, and for the manipulation of DNA were used throughout. Strain BY4741 (MATa leu2Δ met15Δ his3Δ ura3Δ) and BY4741-derived mutants were used. Details of strains used are listed in Table S1. The fus3Δ kss1Δ double deletion mutant was created by replacing the KSS1 gene with LEU2 in the fus3Δ background strain obtained from Open Biosystems. The non-docking mutants of Ste5 (Ste5ND, STE5 Q292A, I294A, Y295A, L307A, P310A, N315A) were created in HA-STE18wt, HA-STE18Δ, and HA-STE18ΔΔ background strains (Dewhurst et al., 2015), using the two-step delitto perfetto mutagenesis method (Storici and Resnick, 2006). Briefly, a CORE cassette comprising of counter selectable marker and reporter gene was inserted via homologous recombination, in the 829-990 region of STE5 coding sequence containing the Fus3 docking region on the protein. The CORE cassette was later replaced by oligonucleotides containing the desired mutations and regions of homology to STE5. Similarly, GFP-tagged STE5 strains were constructed by integrating a CORE cassette at the 3-prime end of STE5 and replacing it with the coding sequence for Green Fluorescent Protein (GFP). Strains were verified at each step by PCR amplification and dideoxy sequencing (Eurofins MWG Operon). LRB341 and LRB345 strains harboring yck1Δ and temperature-sensitive yck2ts alleles were graciously provided by Dr. Lucy Robinson (Robinson et al., 1993). Plasmids used in this study for kinase deletion screening (pRS316-CUP1-HA-STE18), were graciously provided (gift from T. Chernova).

**Yeast cell culture and treatments** – Yeast Strains were grown in YPD growth medium (Yeast Extract, Peptone, 2% Dextrose media) unless otherwise noted. Cells were grown at 30°C with shaking at 250 rpm and cell culture density was determined by absorbance at 600nm (OD600). All experiments were conducted with log phase cells between OD600 0.75-0.85. Log-phase cells were then treated with α-factor peptide hormone (Genscript) at 3μM final concentration. 10μl aliquots of treated cells were harvested with 0.5ml trichloroacetic acid (TCA) on ice and centrifuged at 3724 x g in Allegra X-14R Beckman Coulter Centrifuge. Cells were then washed with 10ml MiliQ water, followed by transfer to microcentrifuge tubes that were immediately frozen at -80°C.

For kinase screening, deletion strains carrying the pRS316-CUP1-HA-STE18 plasmid were grown in synthetic media lacking uracil and other appropriate amino acids as necessary. An overnight saturated culture was then diluted to OD600 0.05 and allowed to grow until OD600 0.2. Expression of HA-STE18 was then induced by addition of 100μM copper sulfate. Cells were grown to log-phase (OD 0.75-0.85), followed by treatment with 3μM α-factor for an hour. The cells were harvested with TCA as described earlier. For yckΔ strains, HA-STE18 expression was induced as above. At OD600 0.8, each culture was split into two parts: One subjected to pheromone treatment and the other incubated at 37°C for 30 minutes prior to stimulation with pheromone.

**Cell extracts and Immunoblotting** - Protein extracts were resolved by 7.5% or 12.5% SDS-PAGE and immunoblotted with epitope-specific antibodies specific to: the hemaglutinin antigen epitope (HA) (Cell Signaling Technologies, Cat #3724) at 1:5000; activating phosphorylated sites in Fus3 and Kss1 (Phospho-p44/42 MAPK) (Cell Signaling Technologies, Cat #9101) at 1:500; Fus3 protein (Santa Cruz, anti-Fus3 yC-19, Cat # sc-67737) at 1:350; Kss1 protein (Santa Cruz, anti-Kss1 yC-19, Cat # sc-6775R) at 1:1500; GFP (Invitrogen, Cat# GF28R MA5-1526-HRP) at 1:1000; and glucose-6-phosphate dehydrogenase (loading control; LC) (Sigma-Aldrich, Cat #A9521) at 1:50,000.

**Phosphatase Assay** – Pheromone-treated cells were harvested as described earlier. The frozen pellets were resuspended in 1x phosphatase buffer mix (New England Biolabs), comprising of 1x PMP buffer (50M HEPES (pH 7.5), 100mM NaCl, 2 mM DTT, 0.01% Brij 35), 1mM MnCl2, and 1x EDTAfree protease inhibitors (Roche). The resuspended pellet was subjected to glass bead lysis in the absence (Control and AP sample) or presence (AP/I sample) of phosphatase inhibitors (50mM NaF and 1.3mM sodium orthovanadate). The lysates were centrifuged at 21.1 x g in Thermo Pico21 centrifuge and the supernatant was collected in a fresh tube. The AP and AP/I samples were treated with phosphatase enzyme (final concentration of 2.25U/μl) for 30 mins at 30°C; whereas the control sample was left untreated. The reaction was stopped by addition of 6x SDS loading dye, and the samples were immediately run on SDS-PAGE gel and subjected to immunoblotting.

**Phos-tag gel analysis** – Protein extracts were resolved by 100μM Mn2+-Phos-tag in 7.5% acrylamide SDS-PAGE as per manufacturer’s instructions (Wako Chemicals, Richmond, VA). The gel was run at a constant current of 30 mA per gel till dye ran off. Next, the gel was soaked in transfer buffer (0.125M tris, 0.96M glycine, 20% methanol v/v) containing 1mM EDTA, and later in transfer buffer without EDTA for 30 min each with gentle shaking. This process chelates Mn2+ and increases transfer efficiency of both phosphorylated and non-phosphorylated proteins. This process...
transfer in buffer containing 0.1% SDS was used to effectively transfer proteins onto nitrocellulose membrane. The proteins were then subjected to immunoblotting.

**Morphological response assay** – The morphological response of STE18/STE5 mutants to α-factor was measured as described previously (Coyle et al., 2013; Malleshaiah et al., 2010). Briefly, an overnight culture was washed twice with MilliQ water, and then diluted to an OD600 0.05 in synthetic complete (SC) media. After 4h of growth at 30C with shaking at 250 rpm, the cultures were distributed into separate tubes, and serially diluted pheromone was added to each. The morphology of the cells was determined, 3h post-stimulation, by differential interface contrast (DIC) confocal microscopy using a PerkinElmer UltraVIEW spinning disk confocal microscope. Number of cells with mating projections were counted as a percentage of total.

**Ste5-GFP localization assay** – Live cells endogenously expressing either Ste5-GFP or Ste5ND-GFP were visualized by microscopy. Log phase cultures (OD 600 0.7-0.8) grown in SC media were briefly sonicated, centrifuged, and resuspended in SC media containing 10μM α-factor. Cells were immediately mounted on a SC agar (2.5%) pad with 30μM α-factor and the GFP fluorescence was monitored for 200 minutes. Cells were visualized by fluorescence microscopy using a PerkinElmer Ultraview VoX spinning disk confocal scanner with a Hamamatsu ORCA FLASH 4 camera on a Nikon Ti-e microscope stand. Photomicrographs were obtained using a 60x NA 1.49 apochromatic objective. The 488nm laser line was set at 10%, image exposure set to 500ms, and the Z-stacks were obtained for 4μm with 0.2μm step-size. A 525nm center, 50nm bandwidth emission filter was used for all fluorescence images. Images were analyzed using Volocity quantitation software (PerkinElmer). Find object module was used to identify objects with the threshold set to 18.4% for fluorescence intensity. Images were linearly contrast enhanced for visualization. Quantification was performed on raw data from which integrated pixel intensity was used for all further analyses.

**Co-immunoprecipitation** – 50 ml cultures of cells expressing different combinations of wild type or mutant HA-STE18 and STE5-GFP were grown to an OD of 0.8 and treated with 10μM pheromone for 20 mins. Cells were then harvested, washed with water, snap-chilled in liquid nitrogen and stored in -80C. Cell pellets were subjected to glass bead lysis in buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM MgCl2, 0.1% Triton X-100 (Sigma Aldrich), 5% glycerol, 1 mM TCEP, 0.5mM PMSF, and protease and phosphatase inhibitor tablets (Pierce #A32959). The soluble protein extract was then collected in a fresh tube after centrifugation at 21,000xg for 10 min. For immunoprecipitation of Ste5-GFP, anti-GFP mAb-agarose (MBL, #D153-8) pre-equilibrated with the lysis buffer was added to each lysate of fixed concentration and total mass followed by incubation with gentle agitation at 4C for 3 hours. Beads were washed with lysis buffer to reduce non-specifically bound proteins. Washed beads were resuspended in 1x SDS-PAGE buffer and boiled for 3 min to elute bound protein. Eluted proteins were separated by SDS-PAGE and immunoblotting with anti-GFP (Invitrogen, A11122) and anti-HA.

**Supplemental References**

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## Supplemental Data Items

Table S1. List of yeast strains used in this study. Related to experimental procedures.

| Strain    | Genotype                               | Ref                           |
|-----------|----------------------------------------|--------------------------------|
| BY4741    | MATα leu2Δ met15Δ his3Δ ura3Δ          |                                |
| YMT235    | BY4741 HA-STE18 WT                     | PMID 26070665                  |
| YMT236    | BY4741 HA-STE18 3A                     | PMID 26070665                  |
| YMT237    | BY4741 HA-STE18 3E                     | PMID 26070665                  |
| ste20Δ    | BY4741 ste20::KanMX                   | YKO collections from Open Biosystems. |
| ste11Δ    | BY4741 ste11::KanMX                   | YKO collections from Open Biosystems. |
| ste7Δ     | BY4741 ste7::KanMX                    | YKO collections from Open Biosystems. |
| fus3Δ     | BY4741 fus3::KanMX                    | YKO collections from Open Biosystems. |
| kss1Δ     | BY4741 kss1::KanMX                    | YKO collections from Open Biosystems. |
| ste5Δ     | BY4741 ste5::KanMX                    | YKO collections from Open Biosystems. |
| sst2Δ     | BY4741 sst2::KanMX                    | YKO collections from Open Biosystems. |
| akr1Δ     | BY4741 akr1::KanMX                    | YKO collections from Open Biosystems. |
| YMT286    | BY4741 fus3::KanMX kss1::LEU2         | this study                     |
| YMT 491   | BY4741 HA-STE18 WT STE5 ND (Q292A, I294A, Y295A, L307A, P310A, N315A) aka WT / ND | this study                     |
| YMT 492   | BY4741 HA-STE18 3E STE5 ND (Q292A, I294A, Y295A, L307A, P310A, N315A) aka 3A / ND | this study                     |
| YMT 501   | BY4741 HA-STE18 3A STE5 ND (Q292A, I294A, Y295A, L307A, P310A, N315A) aka 3E / ND | this study                     |
| YMT 581   | BY4741 HA STE18 WT STE5-GFP           | this study                     |
| YMT 582   | BY4741 HA STE18 3E STE5-GFP           | this study                     |
| YMT 583   | BY4741 HA STE18 3A STE5-GFP           | this study                     |
| LRB 341   | MATα his3 leu2 ura3-52                 | PMID 8474447                   |
| LRB 345   | MATα his3 leu2 ura3-52 yck1-Δ yck2-ts  | PMID 8474447                   |

Supplementary Table S2. List of yeast strains used in this study. Related to experimental procedures.
Table S2. Synergy tests for independent versus combined mutation of Ste18 phosphosites and Ste5FBD. Related to Figure 3. The independent or combined effects of Ste18/Ste5 phosphorylation on Fus3 peak activation time (F3PAT) and Fus3 mean peak amplitude (F3MPA) was compared as a test for synergistic or additive effects. (A) Summary table of F3PAT and F3MPA raw, relative and integrated data. Red text indicates mutants representing a Ste18 element off state (Ste183A/Ste5WT) or Ste5 element off state (Ste18WT/Ste5ND). Blue text indicates the mutant representing both elements off (Ste183A/Ste5ND). (B) Sub-tables showing synergy test results for F3PAT, F3MPA, and Integrated Outcomes. The total integrated rate and amplitude enhancement observed for Fus3 activation in Ste183A/Ste5WT and Ste18WT/Ste5ND was ~4x lower than that of Ste183A/Ste5ND cells (compare 5.6 to 21.7) (bottom table). The greatest degree of synergy between Ste18/Ste5 elements were observed for Fus3 peak activation time (top table). In contrast, peak activation amplitude results from additive rather than synergistic effects of the two elements (since the sum of effects for removal of either Ste18 or Ste5 elements alone were nearly equal to the elimination of both elements together) (middle table).

A

| Ste18/Ste5 | Fus3 Peak Activation Time (F3PAT) | Shift in F3PAT Relative to WT/WT (A) | Fold Rate Enhancement over WT/WT (B) | Fus3 Mean Peak Amplitude | Fold Amplitude Enhancement over WT/WT (C) | Integrated (B) * (C) |
|------------|----------------------------------|-------------------------------------|-----------------------------------|----------------------------|-----------------------------------------|---------------------|
| WT / WT    | 30                               | 0                                   | 1                                 | 100.00                     | 1.0                                     | 1.0                 |
| 3A / WT    | 15                               | -15                                 | 2                                 | 192.67                     | 1.6                                     | 3.3                 |
| 3E / WT    | 30                               | 0                                   | 1                                 | 114.80                     | 1.1                                     | 1.1                 |
| WT / ND    | 30                               | 0                                   | 1                                 | 235.01                     | 2.4                                     | 2.4                 |
| 3A / ND    | 5                                | -25                                 | 6                                 | 386.35                     | 3.6                                     | 21.7                |
| 3E / ND    | 15                               | -15                                 | 2                                 | 210.71                     | 2.1                                     | 4.2                 |

B

Synergy Test for Peak Activation Time (Column B)

- Sum of Fold Rate Enhancement for 3A/WT, WT/ND: 2
- Fold Rate Enhancement for 3A/ND: 6

Synergy Test for Peak Amplitude (Column C)

- Sum of Fold Amplitude Enhancement for 3A/WT, WT/ND: 4.0
- Fold Amplitude Enhancement for 3A/ND: 3.6

Synergy Test for Integrated Outcomes [Peak Activation Time * Peak Amplitude]

- Sum of Fold Integrated Enhancement [(B) * (C)] for 3A/WT & WT/ND: 5.6
- Fold Integrated Enhancement [(B) * (C)] for 3A/ND: 21.7
Table S3. Best-fit values for pheromone dose-response of independent or combined mutation of Ste18 phosphosites and Ste5FBD. Related to Figure 5. Sigmoidal dose-response curves with variable slope were fit to data shown in Figure 4 to estimate switch or graded morphological responses for each indicated yeast strain. A constraint value of 100 was applied to the top of the curve.

| Category      | WT   | 3A   | 3E   | WT / ND | 3A / ND | 3E / ND |
|---------------|------|------|------|---------|---------|---------|
| **Best-fit values** |      |      |      |         |         |         |
| Bottom        | 4.674| 5.923| 4.646| 7.79    | 12.61   | 2.151   |
| Top           | = 100| = 100| = 100| = 100   | = 100   | = 100   |
| HillSlope     | 6.201| 6.253| 3.681| 0.9708  | 0.8519  | 2.703   |
| EC50          | 0.4468| 0.6442| 0.4677| 0.5597  | 0.2966  | 0.9898  |
| **95% CI (asymptotic)** |      |      |      |         |         |         |
| Bottom        | 3.379 to 5.966| 4.381 to 7.464| 3.04 to 6.251| 5.08 to 10.5| 7.49 to 17.72| 0.6774 to 3.624|
| HillSlope     | 5.473 to 6.93| 4.908 to 7.597| 3.223 to 4.138| 0.9652 to 1.076| 0.6876 to 1.016| 2.292 to 3.114|
| EC50          | 0.4379 to 0.4559| 0.6031 to 0.6882| 0.4532 to 0.4827| 0.5009 to 0.6254| 0.2394 to 0.3674| 0.9331 to 1.05 |
| **Goodness of Fit** |      |      |      |         |         |         |
| Degrees of Freedom | 45   | 42   | 45   | 45      | 45      | 44      |
| R square      | 0.9933| 0.9875| 0.9904| 0.9779  | 0.9362  | 0.9879  |
| **Number of points** |      |      |      |         |         |         |
| # of X values | 48   | 48   | 48   | 48      | 48      | 48      |
| # Y values analyzed | 48   | 45   | 48   | 48      | 48      | 47      |
Figure S1. Ste18\(^{Nt}\) phosphorylation in response to pheromone and dependence on scaffolded MAPKs. Related to Figure 2. The abundance of phosphorylated HA-Ste18 in cells treated with pheromone for the indicated duration was determined by immunoblotting with anti-HA antibody. (A) Protein extracts from pheromone induced wild type cells were subjected to phosphatase treatment in the absence or presence of phosphatase inhibitor, and HA-Ste18 was detected by immunoblotting. (B) Immunoblots showing Ste18\(^{Nt}\) phosphorylation as well as Kss1 and Fus3 activation measured in 30 second intervals within the first 15 minutes of the pheromone response in wild type cells. Immunoblot of activated Kss1 and Fus3 has been contrast enhanced to facilitate visualization, and equivalent loading of lanes in immunoblot is demonstrated by comparable levels of G6PDH (LC). (C-D) Yeast single gene deletion mutants transformed with the pCUP1-HA-STE18 expression plasmid were stimulated with 3µM pheromone for 1 hour followed by immunoblot analysis of HA-Ste18, activated Kss1 and Fus3, and loading control proteins. The phosphorylation percentage of Ste18 (% of total) was compared between time 0 and 60 minutes with or without pheromone stimulation. (C) Representative immunoblots showing the phosphorylation of Ste18 in cells lacking the indicated kinase, or the MAPK scaffold (ste5\(^{Δ}\)). Activation of Kss1 and Fus3 were also measured to observe the effect of each mutant on pathway activation. (D) Average percent Ste18 phosphorylation and standard deviation for three independent colonies. Statistical significance determined by 2-way ANOVA: (#) significant difference from the same pheromone treatment in wild type, and (*) significant difference between 60-60+ pheromone treatment within the same strain. AP: alkaline phosphatase, AP/I: alkaline phosphatase + phosphatase inhibitor.
Figure S2. Effect of SST2 and YCK1/2 on the phosphorylation of Ste18Nt. Related to Figure 2. Yeast single gene deletion mutants transformed with the pCUP1-HA-STE18 expression plasmid were stimulated with 3µM pheromone for 1 hour followed by immunoblot analysis of HA-Ste18, activated Kss1 and Fus3, and loading control proteins (as in Figure S1). Representative immunoblots and quantification of Ste18 phosphorylation percentage are shown for three independent colonies with error bars representing standard deviation. Results are shown for yeast lacking: (A) the RGS protein (sst2Δ), (B) the yeast casein kinases (yck1Δ/yck2ts), and (C) the Yck1/2 palmitoyltransferase (akr1Δ).
Figure S3. N-terminal phosphorylation of Ste18 is required for delayed peak activation of Fus3 in response to pheromone. Related to Figure 3. (A) Yeast strains harboring wild type, phospho-null (3A) or phospho-mimic (3E) versions of HA-Ste18 expressed from the endogenous genomic locus and stimulated with 3µM pheromone for the indicated times followed by immunoblot analysis of pKss1 and pFus3. Both long and short film exposures (LE and SE, respectively) are shown. (B) Quantitative comparison of activated Kss1. (C) Quantitative comparison of activated Fus3. Average and standard error bars are shown for 8 independent colonies in 2 independent experiments.
Figure S4. Effect of phosphorylation synergy between Ste5 and Ste18 on cell polarization and MAPK activation level in response to GPCR activation. Related to Figure 3. Morphology and MAPK activation profile of cells harboring the indicated combinations of HA-Ste18 and Ste5 were analyzed in the absence or presence of pheromone stimulation respectively. (A) Immunoblots for Fus3 and Kss1 protein from experiments shown in Figure 3C. (B) The different Ste18/Ste5 mutants were visualized by differential interference contrast (DIC) microscopy in the absence of pheromone. Multiple fields are shown to provide additional views of the cell population. White arrows highlight polarized elongated growth that was exclusively observed in Ste18^3A/Ste5^ND cells. No other signs of similarly extreme morphology could be found in any other cell type. (C-E) Pheromone- dependent activation of Fus3 and Kss1 was measured for the desired amount of time. (C) Representative immunoblots showing the result of endpoint assays for HA-Ste18, activated MAPKs Kss1 and Fus3, and the loading control (LC). (D and E) Graphical representation of the mean ± SD of the activated MAPK levels (Kss1 in D, and Fus3 in E) in wild type and mutant cells. Statistical significance was determined by two-way ANOVA tests between each strain within a time point. Each test result is color matched to each compared strain for which statistically significant differences were observed. Black asterisks indicate significant difference from all other strains within the same time point.
Figure S5. Ste18/Ste5 phosphorylation cooperatively impact the mono- and di-phosphorylation of Fus3.

Related to Figure 3. Protein extracts from cells treated with 3µM α-factor for the desired duration (same as in Figure S4) were resolved on a phos-tag gel and immunoblotted with anti-Fus3 antibody. (A) Representative immunoblot showing the mono-phosphorylated (pFus3), di-phosphorylated (ppFus3) and non-phosphorylated Fus3 (Fus3). The relative abundance of Fus3 di-phosphorylation (ppFus3) confirms results from di-phospho-specific antibody blots shown in Figures 2 and 3. This same trend was also observed for mono-phosphorylated Fus3 (pFus3). (B) Quantification of the band intensity of ppFus3, pFus3 and Fus3 is denoted as percentage of total Fus3 in each lane. (C) Graphical representation of the percentage of mono- and di-phosphorylated Fus3 from B. Before pheromone stimulation (time 0), neither pFus3 nor ppFus3 are detectable in Ste18WT/Ste5ND cells since the Ste5FBD is needed for allosteric activation and mono-phosphorylation of Fus3. Both pFus3 and ppFus3 are significantly elevated in Ste183A/Ste5ND cells and moderately elevated in Ste183A/Ste5WT cells (relative to wild type cells) before the addition of pheromone, suggesting that phosphorylation of both proteins contributes to the inhibition of MAPK activation in the absence of pheromone. Consistent with this conclusion, we found that pFus3 was diminished or completely abolished in wild type or Ste183E/Ste5ND cells, which mimics partial inhibition. Overall, these data suggest that either Ste18Nt phosphorylation or Ste5 phosphorylation (controlled by Fus3 docking on Ste5), can effectively inhibit the aberrant di-phosphorylation of Fus3 in the absence of pheromone. Furthermore, simultaneously preventing phosphorylation on both proteins results in aberrant di-phosphorylation of Fus3 in the absence of pheromone. Data represent the mean ± SD for 3 independent colonies.
Figure S6. The steady state level of Ste5-GFP in cells used for PM translocation experiments. Related to Figure 4. Cells expressing either Ste5-GFP or Ste5\textsuperscript{ND}-GFP along with different phospho-mutants of Ste18 (same cells as used in Figure 3) were treated with 10\(\mu\)M \(\alpha\)-factor for the desired time. Protein extracts were separated by 7.5\% SDS-PAGE until higher molecular weight markers (110-160kDa markers of the Novex Sharp pre-stained protein standard [LC5800]) were well resolved, which was \(\sim\)20 minutes after the loading dye ran off of the gel. Ste5-GFP ran at its expected size of approximately 130kDa (Ste5: 102 kDa and GFP: 27kDa). The abundance of Ste5 was probed using anti-GFP antibody. LC: loading control.
Figure S7. Phylogenetic analysis of the Ste18/Ste5 phospho-regulatory system. Related to Figure 6. (A) Bootstrapped phylogenetic tree of Ste18 orthologs with bootstrap replicates conforming to the illustrated phylogenetic tree indicated (out of 100). (B) Multiple sequence alignment of Ste18 yeast orthologs correlated with the predicted secondary structure of Ste18, including the N-terminal intrinsically disordered region (N-term IDR) and alpha helical residues. (C) Alignment from B with all alignment gaps removed to illustrate the lengthening of the N-term IDR in S. mikatae, S. bayanus, S. paradoxus, and S. cerevisiae – yeast strains that also harbor Fus3 binding domains (FBD) in their Ste5-orthologous scaffolds. Sequences in the alignment shown are in order of appearance (from top to bottom) in A.