Analysis of the thresholds for transcriptional activation by the yeast MAP kinases Fus3 and Kss1

Matthew J. Winters and Peter M. Pryciak*
Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605

ABSTRACT
Signaling in the pheromone response pathway of budding yeast activates two distinct MAP kinases (MAPKs), Fus3 and Kss1. Either MAPK alone can mediate pheromone-induced transcription, but it has been unclear to what degree each one contributes to transcriptional output in wild-type cells. Here, we report that transcription reflects the ratio of active to inactive MAPK, and not simply the level of active MAPK. For Kss1 the majority of MAPK molecules must be converted to the active form, whereas for Fus3 only a small minority must be activated. These different activation thresholds reflect two opposing effects of each MAPK, in which the inactive forms inhibit transcription, whereas the active forms promote transcription. Moreover, negative feedback from Fus3 limits activation of Kss1 so that it does not meet its required threshold in wild-type cells but does so only when hyperactivated in cells lacking Fus3. The results suggest that the normal transcriptional response involves asymmetric contributions from the two MAPKs, in which pheromone signaling reduces the negative effect of Kss1 while increasing the positive effect of Fus3. These findings reveal new functional distinctions between these MAPKs, and help illuminate how inhibitory functions shape positive pathway outputs in both pheromone and filamentation pathways.

INTRODUCTION
Mitogen-activated protein (MAP) kinase cascades are ubiquitous signaling modules that mediate responses to a wide variety of signals in eukaryotic cells. In the budding yeast Saccharomyces cerevisiae, there exist several different MAP kinase cascade pathways (Chen and Thorner, 2007). One such pathway in yeast controls the process of mating, which is stimulated by extracellular mating pheromones (Dohlmans and Thorner, 2001; Bardwell, 2005; Alvaro and Thorner, 2016). These pheromones trigger responses via a G protein–coupled receptor and a downstream MAP kinase cascade that culminates in the activation of two distinct MAP kinases (MAPKs), Fus3 and Kss1 (Figure 1A). Of these, Fus3 is dedicated solely to mating, whereas Kss1 also functions in a separate pathway that regulates filamentous growth in response to nutrient limitation (Chen and Thorner, 2007; Cullen and Sprague, 2012). In the past, there has been debate over whether Kss1 participates in mating responses of wild-type cells or whether it only does so when Fus3 is defective or absent (Madhani et al., 1997). Currently, it is accepted that both Fus3 and Kss1 become activated in response to mating pheromones (Breitkreutz et al., 2001; Sabbagh et al., 2001; Andersson et al., 2004; Schwartz and Madhani, 2006), which occurs via Ste7, the upstream MAPK kinase (MAPKK). Moreover, during pheromone response, Fus3 has two effects that limit the potential ability of Kss1 to induce the filamentous growth pathway (Figure 1B): 1) it stimulates proteolysis of a key Kss1 target, the filamentation-specific transcription factor Tec1 (Bao et al., 2004; Bruckner et al., 2004; Chou et al., 2004); and 2) it triggers a negative feedback loop that reduces the activation of both MAPKs (Gartner et al., 1992; Sabbagh et al., 2001; Yu et al., 2008; Hao et al., 2012).

Despite considerable progress on this topic, one issue that has remained unresolved is the degree to which both Fus3 and Kss1 contribute to the transcription of pheromone-induced genes. It is...
RESULTS

Transcriptional induction by Kss1 requires its hyperphosphorylation

The studies described here arose from our efforts to understand puzzling prior observations involving a mutant form of the protein Ste5, which serves as a scaffold protein for the pheromone-responsive MAP kinase cascade (Dohlmans and Thormer, 2000; Bardwell, 2005; Alvaro and Thormer, 2016). Ste5 is required for the initiation and propagation of signal through the mating pathway, as it binds each kinase of the three-tier cascade as well as the upstream Gβγ dimer of the heterotrimeric G protein. A double point mutation in Ste5, V763A S861P (hereafter termed “VASP” [Flatauer et al., 2005]), disrupts binding to the MAPKK Ste7 (Figure 1C; Inouye et al., 1997a). This Ste5-VASP mutant is defective at activating Fus3, but appears capable of activating Kas1 (Flatauer et al., 2005; Schwartz and Madhani, 2006). Despite this ability to activate Kas1, ste5-VASP mutant cells cannot mate (Inouye et al., 1997a,b), which is surprising because fus3Δ cells are able to mate, albeit with reduced efficiency (Elien et al., 1990, 1991; Madhani et al., 1997; Breitkreutz et al., 2001). This behavior presented a paradox: although Kas1 can mediate mating responses in fus3Δ cells, it cannot do so in ste5-VASP cells.

To investigate this enigma, we compared the levels of Kas1 activation in the presence and absence of Fus3, and in cells harboring either Ste5 wt or Ste5-VASP (Figure 2A). In the presence of Fus3, Kas1 was activated to a similar extent in both STE5 and ste5-VASP cells, and yet the ste5-VASP cells did not induce transcription of a mating pathway reporter (FUS1-lacZ). When Fus3 was absent (fus3Δ), Kas1 could induce transcription in STE5 but not in ste5-VASP cells. Notably, in the STE5 background, deleting FUS3 was accompanied by an unusually high degree of Kas1 activation, whereas in the ste5-VASP background this did not occur; instead, Kas1 was activated to levels comparable to those seen in FUS3 cells, and it failed to induce transcription. We observed similar phenotypes in cells harboring a preactivated form of the MAPKKK Ste11 called Ste11-Asp3 (Drogen et al., 2000), thus verifying that the Ste5-VASP phenotypes are due to disruption of steps downstream from Ste11 activation (Figure 2B). Together, these findings reveal two notable points. First, the Ste5-VASP mutant shows a defect in Kas1 activation in fus3Δ cells that is not evident in FUS3 cells. This suggests that the defect is ordinarily masked by negative feedback from active Fus3 (see Discussion). Second, the results suggest that the capacity for Kas1 to induce transcription of mating genes is observed only under conditions that lead to abnormally high levels of Kas1 activation (fus3Δ STE5), whereas activation of Kas1 to levels similar to those seen in normal cells (FUS3 STE5) does not induce transcription.

Similar findings were obtained with a series of related transcriptional reporters in which the promoters of various genes are placed upstream of lacZ (Roberts et al., 2000). These include four genes induced by the mating pathway and four genes that are preferentially induced by Kas1 and the filamentation pathway (Figure 2C). All four mating pathway reporters behaved as described for the

FIGURE 1: Signaling pathways involving the MAPKs Fus3 and Kss1. (A) Diagram of Fus3 and Kss1 signaling pathways. Either MAPK can induce transcription of mating genes in response to mating pheromones, which requires the scaffold protein Ste5 and the downstream transcription factor Ste12. Separately, Kss1 can induce transcription of filamentation genes in response to nutrient limitation, which requires a heterodimer of Ste12 with Tec1. In each pathway, the MAPK promotes transcription by phosphorylating and displacing the repressor proteins Dig1 and Dig2 from the transcription factor. PRE, pheromone response element; FRE, filamentation response element. (B) Fus3 has two negative effects that reduce the ability of Kss1 to activate filamentation pathway signaling: it triggers negative feedback that reduces activation of both MAPKs, and it phosphorylates Tec1, which is thus marked for degradation. (C) The Ste5-VASP mutation disrupts binding to Ste7, which reduces propagation of signal through the kinase cascade (as indicated by gray dashed arrows).
preceding experiments; namely, they were induced in both FUS3 and ste5Δ cells, but could not be induced in ste5-VASP cells, regardless of Fus3 status. The filamentation pathway reporters were only induced by pheromone in fus3Δ cells and not in FUS3 cells, as described previously (Roberts et al., 2000). Interestingly, the ste5-VASP mutation blocked their strong pheromone induction in fus3Δ cells, indicating that transcription of these reporters depends not only on the removal of Fus3 but also on the strong Kss1 hyper-phosphorylation seen in fus3Δ cells with wild-type Ste5. (For three reporters—PGU1pr, KSS1pr, and YLR042Cpr—the elevated basal expression in fus3Δ cells was also reduced by the ste5-VASP mutation.) We also observed similar results in another yeast strain background, Σ1278b (Figure 2D), which is commonly used to monitor filamentous growth (Roberts and Fink, 1994; Cullen and Sprague, 2012). Again, transcriptional induction in fus3Δ cells was associated with hyperactivation of Kss1 to levels not seen in FUS3 cells, and when Kss1 activation was restricted to the lower level by the ste5-VASP mutation, induction of both mating (FUS1pr) and filamentation (PGU1pr) reporters was blocked. It was possible to detect residual transcriptional activation in fus3Δ ste5-VASP cells when using another common reporter, TEC1(FRE)-lacZ (which contains a short filamentation response element [FRE] from the TEC1 gene placed upstream of the CYC1 promoter; Madhani and Fink, 1997; Sabbagh et al., 2001), perhaps because it is harbored on a
high copy number plasmid. Altogether, the collective findings indicate that the ability of Kss1 to induce strong transcription is associated with its hyperactivation to levels markedly higher than those seen in wild-type cells.

Analysis of MAPK phosphorylation state

MAPK activation requires phosphorylation at both threonine and tyrosine residues (of a TXY motif) in the kinase activation loop (Canagarajah et al., 1997). Although in the preceding experiments Kss1 appeared to be phosphorylated to similar levels in STE5-wt and ste5-VASP cells, we considered the possibility that the ste5-VASP mutant might yield predominantly inactive, monophosphorylated (1P) forms of Kss1 rather than active, dual-phosphorylated (2P) forms, and that this might be overlooked if the antibodies used for detection did not adequately distinguish these forms. We addressed this issue in two ways: 1) we used mutant forms of Fus3 and Kss1 to test if phosphorylation of both Thr and Tyr residues is required for recognition by phospho-specific antibodies; and 2) we used Phos-tag gels (Kinoshita et al., 2006), which can resolve different phospho-isoforms of MAPKs (Maeder et al., 2007; Aoki et al., 2011; Nagiec et al., 2015).

First, mutations in the TXY motifs showed that the antibodies are not fully specific for the 2P forms (Figure 3A, top panels). For Fus3, neither of two antibodies was specific for the 2P form, because the signal was blocked only by the Thr to Ala mutation (TA) and not by the Tyr to Phe mutation (YF). For Kss1, recognition by one antibody (#9101) was impaired only mildly by the TA mutation and more strongly by the YF mutation, whereas the other antibody (#4370) showed the reverse pattern; thus, each antibody recognized the 2P form of Kss1 preferentially but not exclusively. Second, we analyzed the same samples using Phos-tag gels, and probed for total Fus3 or Kss1 (Figure 3A, bottom panels). For each MAPK the predominant forms seen with the wild-type proteins are likely to be 0P (unphosphorylated) and 2P. Detection of 1P forms (pY and pT) was clearly evident with the TA and YF mutants, although their mobility is somewhat altered compared with the 1P forms of wild-type proteins.

FIGURE 3: Analysis of MAPK phosphorylation state. (A) Activation loop mutations in Fus3 and Kss1 were tested for their impact on recognition by phospho-specific antibodies (top) and migration in Phos-tag gels (bottom). A wild-type strain (wt) and a fus3∆ kss1∆ strain harboring a FUS3 or KSS1 plasmid (wt or mutant) was incubated ± αfactor (5 µM, 15 min), as indicated. Note that the FUS3 alleles were expressed from the TPI1 promoter, which yields expression that is elevated but also is independent of pathway activity, unlike the native FUS3 promoter. Top panels, blots of standard gels were probed with two different phospho-MAPK antibodies; the results indicate that neither is specific for dual-phosphorylated MAPKs. Bottom panels, the same samples were separated on Phos-tag gels and analyzed with anti-Fus3 and anti-Kss1 antibodies. At left, black font and arrows indicate positions of different forms of each wt protein, whereas red font and bullets indicate the positions of mutant proteins (pY for TA mutants, pT for YF mutants); note that for the Fus3 panels, due to limited space, arrows and bullets were labeled on alternate panels. Strains: PPY640, PPY1173. Plasmids: pPP679, pPP4007, pPP4008, pPP4009, pPP4010, pPP4014, pPP4016, pPP4017, pPP4018. (B) The indicated strains were transformed with STE5-wt and ste5-VASP plasmids, and then treated ± αfactor. Protein samples were separated on Phos-tag gels and probed with two different phospho-MAPK antibodies as well as with antibodies against total Fus3 or Kss1. Strains: PPY858, PPY1667, PPY1669. Plasmids: pPP1969, pPP2861.
(Note that, as observed for mammalian ERK2 [Aoki et al., 2011], the pY forms of both MAPKs ran more slowly than the 2P forms, although the reverse has been seen for Fus3 [Nagiec et al., 2015], presumably due to different gel formulations.) It is noteworthy that the total amount of phosphorylated Fus3 was much higher for each single mutant (TA and YF) than for the wild-type form, which fits the expectation that only the active (2P) form of Fus3 can trigger negative feedback to dampen its own phosphorylation. By contrast, the total amount of phosphorylated Kss1 was largely similar between wild-type, TA, and YF forms, confirming that active Kss1 does not trigger comparable negative feedback.

When we used Phos-tag gels to analyze MAPK activation in STE5-wt and ste5-VASP cells (Figure 3B), the results validated the findings described earlier. Namely, deletion of FUS3 resulted in unusually high levels of the Kss1_2P form, but only in STE5-wt cells and not in ste5-VASP cells. These features were evident regardless of whether the Phos-tag gels were probed with phospho-specific or anti-Kss1 antibodies. Moreover, when using antibodies against total Fus3 or Kss1, it was possible to observe the fraction of each MAPK that became converted to the 2P form. Notably, stimulation of STE5 fun3 cells caused the majority of Kss1 molecules to convert to the 2P form, whereas this was not seen in FUS3 or ste5-VASP cells. This raised the possibility that surpassing a minimum threshold level of the 2P form is important for Kss1 function and that this threshold is not achieved in ste5-VASP cells.

Dose-response assays suggest a high activation threshold for Kss1

The preceding results suggested to us that Kss1 might become capable of potent transcriptional activation only when phosphorylated above a minimum threshold amount or proportion of total Kss1. To explore this issue further, we activated Kss1 to varying degrees by treating fun3 cells with increasing pheromone doses, and then monitored both the extent of Kss1 activation and the strength of transcriptional induction (Figure 4). This treatment yielded a gradual increase in both Kss1_2P (by Phos-tag) and phospho-Kss1 (by phospho-MAPK blots). Because the secreted protease Bar1 degrades pheromone over time (Ciejek and Thorner, 1979; Sprague and Herskowitz, 1981), we analyzed both Bar1 and bar1Δ strains; each yielded a similar overall pattern, although BAR1 strains showed a larger time-dependent change in Kss1 phosphorylation (comparing 15 vs. 60 min treatment), especially at lower pheromone doses, which is consistent with pheromone degradation. In either case, the transcriptional response reached a plateau at pheromone concentrations that converted the majority of Kss1 molecules to the active form (2P fraction = 0.55–0.6) for a sustained (60-min) period; at doses giving half-maximal transcription (~650 nM for BAR1, and ~7.5 nM for bar1Δ), the Kss1_2P fraction was still roughly half (0.45–0.55). By contrast, in FUS3 cells the maximum pheromone dose activated Kss1 to maximally lower extents (2P fraction = 0.2–0.3) that in fun3 cells could only barely induce transcription; for example, corresponding to roughly 50 nM in BAR1 fun3 cells or 1.5 nM in bar1Δ fun3 cells, which yielded transcriptional induction of roughly 10–15% of the maximum inducible by Kss1 (and only 4–5% the maximum inducible by Fus3). Thus, strong transcriptional induction by Kss1 was associated with activation levels that surpassed those obtainable in FUS3 cells.

We performed similar experiments to monitor both Fus3 and Kss1 simultaneously, in FUS3 KSS1 cells (Figure 5A), and then we compared the dose-response results with the behavior of Kss1 in fun3 cells (Figure 5B). Several points were notable. First, we never observed the majority of Fus3 molecules converted to the 2P form; instead, the 2P fraction for Fus3 plateaued at ~0.25–0.3. Second, in FUS3 KSS1 cells, negative feedback from Fus3 limited Kss1 activation so that the 2P fraction plateaued at ~0.35–0.4 (at 60 min) and was ~0.2 at half-maximal transcription; by comparison, in fus3Δ cells these levels were associated with minimal transcriptional output. Collectively, these results support the idea that positive transcriptional induction by Kss1 involves a level of activation at which a majority of molecules are converted to the active form, and that this level is ordinarily not reached in otherwise wild-type (FUS3) cells.

These experiments uncovered an additional noteworthy feature. In FUS3 KSS1 cells, the pheromone dose yielding a half-maximal response (EC50) for transcription was closely aligned to the EC50 for activation of Fus3 and Kss1, whereas in fus3Δ cells the activation of Kss1 was markedly left-shifted so that its EC50 was below 1 nM (Figure 5B). This shift in Kss1 activation is consistent with previous findings that negative feedback from Fus3 promotes “dose-response alignment” (Yu et al., 2008) of intracellular responses with receptor occupancy. Remarkably, however, the EC50 for transcription was not left-shifted in parallel. To ensure that the transcriptional reporter in these experiments was not unusual, we tested reporters driven by four different promoters—from two mating genes and two filamentation genes (Figure 5C)—and found that their EC50s in fus3Δ cells were all similar to each other (albeit slightly lower for the filamentation reporters). The finding that transcriptional responses do not shift concordantly with kinase activation implies the existence of additional mechanisms, separate from Fus3 negative feedback, that align transcriptional outputs to the dose of input stimulus. Moreover, the results show that the largest change in Kss1-dependent transcription does not coincide with the largest change in levels of activated Kss1, implying an unusual relationship between activity and output of Kss1.

Kss1 functional output depends on the ratio of active to inactive kinase

We sought to distinguish whether transcriptional induction by Kss1 requires a threshold number versus a threshold fraction of molecules to be in the active state. Although the former model is simpler, it is known that the inactive form of Kss1 represses transcription (Cook et al., 1997; Madhani et al., 1997; Bardwell et al., 1998); therefore, antagonism between inhibitory and activating forms of Kss1 could make the ratio of the two forms a critical factor. To explore this possibility, we varied the total amount of each MAPK (from roughly 0.1x to 10x native levels) by expressing them from foreign promoters of different strengths (Figure 6A). (Note that in these experiments the gene for the other MAPK was deleted, so that the results reflect only the status of a given MAPK and not competition between Fus3 and Kss1.) Using various doses of pheromone, we assayed both the amount and the fraction of MAPK that was phosphorylated, and compared this to transcriptional induction. The results revealed that Kss1 tolerates only a narrow range of expression levels. In particular, overexpression of Kss1 from the GPD1 promoter resulted in the near complete loss of transcriptional activation despite the fact that total phosho-Kss1 levels were equal or higher than normal (Figure 6A, left). Analysis in Phos-tag gels showed that the active (2P) pool of Kss1 was overwhelmed by an excess of inactive Kss1. Thus, transcriptional induction by Kss1 is not dependent solely upon reaching a minimal amount of active Kss1, and instead a threshold fraction is required. Kss1 was also unable to drive transcription when underexpressed from the CYC1 promoter, even though the majority of molecules were converted to the 2P form, presumably because total phosho-Kss1 levels were too low (e.g., lower than those seen without pheromone when Kss1 was expressed from its native promoter).
Thus, the 2P fraction is not the only important parameter, and (unsurprisingly) some minimal level of active kinase is required. Hence, Kss1 function is constrained to a narrow expression range.

Compared to Kss1, Fus3 was more tolerant of altered protein levels, as it could induce transcription even when its expression was varied over nearly two orders of magnitude (Figure 6A, right). Nevertheless, it is noteworthy that Fus3 was maximally functional when expressed from its native promoter, and some observations indicated that inactive Fus3 inhibits transcription mildly: 1) when expressed from the weak CYC1 promoter, Fus3 could induce transcription despite quite low levels of phospho-Fus3 that were not sufficient when expressed from other promoters (e.g., compare \( P_{CYC1} \)-FUS3 at 50 nM \( \alpha \) factor with \( P_{FUS3} \)-FUS3 at 0.5 nM \( \alpha \) factor); and 2) transcription was gradually dampened when Fus3 was expressed from the stronger ADH1 and GPD1 promoters, which yielded similar or slightly higher levels of active (phosphorylated) Fus3 but also increased inactive (unphosphorylated) Fus3. There-}

FIGURE 4: Transcription is induced when the majority of Kss1 molecules are activated. Dose-response analyses compare the profiles of Kss1 phosphorylation and transcriptional output in fus3Δ cells. Assays were performed in both BAR1 (left) and bar1Δ (right) backgrounds; in each case, FUS3 strains were tested in parallel, ± the maximum dose. Strains (PPY2335, PPY861, PPY2367, PPY2365) harbored STE5 plasmid pPP1969 and FUS1-lacZ plasmid pPP1044. Cells were treated with the pheromone concentrations shown, and then harvested at 15 or 60 min for protein analysis or at 90 min to assay transcriptional induction. Protein samples were resolved in standard gels and probed with phospho-MAPK antibodies, or in Phos-tag gels and probed with anti-Kss1 antibodies. Immunoblot signals were quantified by densitometry; representative examples are shown, and charts combine results from repeated trials (mean ± SD; \( n = 3 \)). (i) Top, phosphorylated Kss1 (P-Kss1) was plotted relative to the 60-min signal at the maximum dose. (ii) Middle, dual-phosphorylated Kss1 (Kss1_2P) was plotted as the fraction of total (0P + 2P). (iii) Bottom, FUS1-lacZ activity was plotted as \( \beta \)-galactosidase units.
Distinct transcription activity of MAPKs

Effect of Tec1 stabilization on Kss1 activation and filamentation gene transcription

Finally, although this study mainly concerned the role of Kss1 in transcription of mating genes, the results also bear on its role in the filamentation pathway. During pheromone response, the potential antagonism, possibly because of weaker induction by the active enzyme–substrate saturation (Ferrell and Ha, 2014); for Fus3, this condition might be either absent or counteracted by other factors such as negative feedback (see Discussion).

FIGURE 5: Fus3 controls both the extent and EC50 of Kss1 phosphorylation. (A) Dose-response analysis of kinase phosphorylation and transcriptional induction was performed as in Figure 4, but using bar1Δ FUS3 KSS1 cells in which activation of both Fus3 and Kss1 was monitored. Top two panels show phospho-MAPK blots, and bottom four panels show blots from Phos-tag gels probed with either anti-Kss1 or anti-Fus3 antibodies. Bottom, FUS1-lacZ induction (mean ± range; n = 2). Strain PPY2365 harbored STE5 plasmid pPP1969 and FUS1-lacZ plasmid pPP1044. (B) Quantification of results from experiments as in panel A. The top and middle sets of charts show quantification of Fus3 and Kss1 phosphorylation in bar1Δ FUS3 KSS1 cells, plotted as in Figure 4 (mean ± range; n = 2); all four charts show the same FUS1-lacZ results (mean ± range; n = 2) to facilitate comparison. The bottom set of charts show results in bar1Δ fus3Δ cells from Figure 4, replotted here to allow comparison to the FUS3 results above. Vertical axes plot P-MAPK levels as the fraction of maximum response (left) or levels of 2P species as the fraction of total MAPK (right); FUS1-lacZ levels are plotted relative to the maximum. (C) Dose-response assays measuring transcriptional induction in bar1Δ fus3Δ cells of lacZ reporters driven by promoters of four different genes: two targets of the mating pathway (FUS1 and FUS3) and two targets of the filamentation pathway (PGU1 and KSS1). Strain PPY2367 harbored a STE5 plasmid (pPP1969) plus a reporter plasmid (pPP847, pPP849, pPP852, or pPP854). Cells were treated with α factor for 90 min. Results (mean ± SD, n = 4) were expressed relative to the difference in mean β-galactosidase units measured at the lowest and highest dose, which were as follows: FUS1 (28, 400); FUS3 (31, 189); PGU1 (48, 208); KSS1 (105, 224).

et al., 2001), although Kss1 is considerably more sensitive to this antagonism, possibly because of weaker induction by the active form, stronger inhibition by the inactive form, or both.

A further noteworthy point was revealed when we plotted the 2P fraction for each kinase as a function of pheromone dose (Figure 6B). The results were strongly dependent on total expression level for Kss1, but not for Fus3. This behavior of Kss1 is unexpected for simple phosphorylation reactions in which the rate is directly proportional to substrate concentration, and hence it implies that the phosphorylation rate is limited in vivo by a condition such as enzyme–substrate saturation (Ferrell and Ha, 2014); for Fus3, this condition might be either absent or counteracted by other factors such as negative feedback (see Discussion).
Fus3 phosphorylates the filamentation pathway transcription factor, Tec1, which is then targeted for degradation (Bao et al., 2004; Bruckner et al., 2004; Chou et al., 2004). Mutant forms of Tec1 lacking the Fus3 phosphorylation site, such as Tec1-T273M, are resistant to this effect and hence allow pheromone to induce filamentation genes even in FUS3 cells (Bao et al., 2004; Bruckner et al., 2004; Chou et al., 2004). These findings suggest that, if Tec1 is not degraded, Kss1 is activated sufficiently strongly in Fus3 cells to induce filamentation genes, and it might not need to be hyperactivated to the degree observed in fus3Δ cells. To explore this further, we first tested the effect of the stabilized Tec1-T273M mutant on Kss1 protein and phosphorylation levels, in both FUS3 and fus3Δ backgrounds.

In FUS3 cells, Tec1-T273M caused an increase in phosphorylated Kss1 (P-Kss1; Figure 7A); this appeared to be due to a mild elevation in total Kss1 levels, which is not surprising as KSS1 transcription is induced by Tec1 and the filamentation pathway (Roberts et al., 2000; Chou et al., 2006). On Phos-tag gels it was evident that both OP and 2P forms were elevated in these cells (Figure 7A), and hence the 2P fraction was relatively unchanged and clearly did not reach the very high fraction seen in fus3Δ cells. Yet, we confirmed that Tec1-T273M could activate a filamentation reporter in FUS3 cells (Figure 7B). Therefore, unlike in fus3Δ cells, this transcriptional response did not depend on achieving a strong majority fraction of active Kss1. This contrast led us to consider that Kss1 might not mediate the response in FUS3 cells. Indeed, Tec1-T273M activated a filamentation reporter to the same extent in kss1Δ cells as in KSS1 cells (Figure 7C), indicating that the response can be activated by Fus3 and does not require Kss1. This finding agrees with results in a previous study (Chou et al., 2004), using a different reporter and stabilized Tec1 mutant (T273V). A potent response in fus3Δ cells confirmed that Kss1 is capable of inducing the reporter (Figure 7C) but, as emphasized throughout this study, Kss1 is hyperactivated in these cells. Results presented earlier, involving the STE5-VASP allele, indicate that when Kss1 is not hyperactivated it does not substantially activate filamentation genes even when Fus3 is absent and hence Tec1 is stabilized. We conclude that the genes induced by Tec1-T273M in FUS3 cells are likely activated by Fus3 rather than by Kss1. In other words, the critical role for Fus3-induced degradation of Tec1 is not to prevent activation of filamentation genes by Kss1, but to prevent their activation by Fus3.

**DISCUSSION**

The findings reported here reveal surprising differences in threshold activation properties of Fus3 and Kss1, the two pheromone-activated MAPKs in budding yeast. Our observations suggest that, during the normal pheromone response of wild-type cells, Kss1 is not activated to an extent needed for it to induce robust transcription. To do so, Kss1 needs to be activated to a high level that is reached only in fus3Δ cells and not in FUS3 cells, because Fus3 activates a negative feedback loop that limits activation of both MAPKs. Furthermore, our findings indicate that the activation threshold for Kss1 corresponds to a point where the majority of Kss1 molecules are converted to the active (phosphorylated) form, and that this requirement reflects a
need to overcome transcriptional repression by the inactive form of Kss1. In contrast to Kss1, the active form of Fus3 never accounts for the majority of Kss1 molecules to the active form. In pheromone-stimulated cells, Fus3 prevents Kss1 from being activated to this required level, and hence this can largely suffice to keep Kss1 from inducing filamentation. Fus3 also stimulates degradation of Tec1 (Bao et al., 2004; Bruckner et al., 2004; Chou et al., 2004), but this appears to be needed to prevent Tec1-dependent transcription from being stimulated by Fus3, rather than by the low level of activated Kss1.

Fus3 has two effects on Kss1 activation (summarized in Figure 8C): it reduces the maximum activation and increases the EC50. The EC50 effect is consistent with previous studies on the role of negative feedback in dose-response alignment (Yu et al., 2008). Curiously, however, there was not a parallel shift in the EC50 for transcriptional induction (Figure 8B, bottom). As a consequence, in fus3Δ cells the largest change in transcriptional output occurs over a range of pheromone doses in which the level of active Kss1 changes by only approximately twofold (Figure 8C, yellow highlighted region). It seems likely that the misalignment between Kss1 activation and downstream transcriptional responses involves the need for active Kss1 molecules to overcome the repressive effects of inactive Kss1. Other relevant factors might include the following: 1) the MAPKs are in substantial excess over the transcription factor Ste12 and its repressors Dig1/Dig2 (Thomson et al., 2001; Yu et al., 2008). Curiously, however, there was not a parallel shift in the EC50 for transcriptional induction (Figure 8B, bottom). As a consequence, in fus3Δ cells the largest change in transcriptional output occurs over a range of pheromone doses in which the level of active Kss1 changes by only approximately twofold (Figure 8C, yellow highlighted region). It seems likely that the misalignment between Kss1 activation and downstream transcriptional responses involves the need for active Kss1 molecules to overcome the repressive effects of inactive Kss1. Other relevant factors might include the following: 1) the MAPKs are in substantial excess over the transcription factor Ste12 and its repressors Dig1/Dig2 (Thomson et al., 2001; Yu et al., 2008), but this appears to be needed to prevent Tec1-dependent transcription from being converted to the derepressed state. Similar considerations might also pertain to other regulatory circuits, such as the G1-S transition of the cell cycle, in which transcriptional induction involves an analogous conversion from repressed to activated promoters (Bertoli et al., 2013).

Our finding that the active fraction of Kss1 molecules dictates its signaling output is reminiscent of recent findings on the pheromone receptor (Bush et al., 2016). That study showed that antagonistic effects of bound versus unbound receptors cause downstream responses to reflect the bound fraction of receptors, not the number (Bush et al., 2016). Such regulatory topologies in which pathway outputs are controlled by opposing effects of active and inactive forms of a single component are said to constitute a “push-pull” mechanism (Andrews et al., 2016). Theory suggests that, as with negative feedback, push-pull mechanisms can help align downstream outputs with upstream inputs (Andrews et al., 2016). In the case studied here, the two MAPKs can be considered to establish a joint push-pull circuit in which the activating ("push") and inhibiting ("pull") roles are performed separately by Fus3 and Kss1.
respectively. In this view, the misaligned EC50s for transcription and Kss1 activation seen in fus3Δ cells, discussed above, could be a consequence of unbalanced potency of push and pull effects; that is, strong inhibition by Kss1 is no longer balanced by strong activation from Fus3. It will be of interest in future studies to probe these concepts further via both theory and experiments.

The dependence of MAPK activation on concentration (summarized in Figure 8D) provides new insights into the in vivo reaction constraints. In particular, the active fraction of Kss1 depends on its expression level. This behavior is not expected for kinase reactions driven by simple, first-order kinetics (Ferrell and Ha, 2014), for which the phosphorylated fraction should depend solely on the level of input stimulus. Instead, it suggests the possibility that the upstream kinase, the MAPKK Ste7, is saturated with substrate, such that the phosphorylation rate cannot be increased by higher substrate concentrations. This condition limits the maximum active fraction, so that the highest fractions occur at the lowest expression level (e.g., when Kss1 is controlled by the CYC1 promoter [Figure 6] or in fus3Δ cells lacking Tec1 [Figure 7A]). Saturation conditions might be driven by high-affinity docking interactions between Ste7 and the MAPKs (Bardwell et al., 2001; Remenyi et al., 2005), and/or by a large excess of each MAPK over Ste7 (Thomson et al., 2011), and measurements of complex formation in vivo are consistent with the majority of Ste7 molecules being bound to MAPKs at steady state (Maeder et al., 2007). In contrast to Kss1, the active fraction of Fus3 did not show a strong dependence on expression level (Figure 8D, bottom). We postulate that this different behavior is not due to a difference in saturation of Ste7, but rather because negative feedback from Fus3 provides an additional self-limiting condition or because the required participation of a third component, Ste5, makes the reaction more complex (Good et al., 2009). Collectively, our findings will inform future efforts to develop accurate computational models for these pathways.

Finally, our findings also indicate that negative feedback can mask signaling defects. In particular, the Ste5-VASP mutant shows a defect in Kss1 activation that is evident only in fus3Δ cells and not in Fus3 cells. This suggests that the defect is concealed by negative feedback from active Fus3. That is, Fus3 dampens signaling, but this dampening does not occur in Ste5-VASP cells (because Fus3 is not activated), and so the reduced dampening compensates for the reduced MAPK activation. Because the Ste5-VASP mutation disrupts binding to the MAPKK Ste7 (Inouye et al., 1997a), the signaling defects presumably reflect reduced activation of Ste7, which then leads to reduced activation of both Fus3 and Kss1. Other mutations that disrupt Fus3 activation more specifically might also have effects masked by reduced negative feedback; for example, this might include mutations in the “coactivator” region of the Ste5 VWA domain (Good et al., 2009). In future studies of this and other signaling pathways, performing assays in cells with inactivated negative feedback circuits might allow hidden or subtle defects to be unveiled.

![Figure 8: MAPK activation behavior and control of transcription.](image-url)
TABLE 1: Yeast strains used in this study.

| Strain bkgd. | Name          | Relevant genotype | Source                        |
|--------------|---------------|-------------------|-------------------------------|
| a            | PPY640        | MATα FUS1::FUS1-lacZ::LEU2 | Pryciak and Huntress (1998)   |
| a            | PPY858        | MATα FUS1::FUS1-lacZ::LEU2 ste5::ADE2 | Pryciak and Huntress (1998)   |
| a            | PPy861        | MATα ste5::ADE2   | This study                    |
| a            | PPy1173       | MATα FUS1::FUS1-lacZ::LEU2 fus3::LEU2 kss1::ura3COA | Winters et al. (2005)          |
| a            | PPy1667       | MATα FUS1::FUS1-lacZ::LEU2 ste5::ADE2 fus3::LEU2 | This study                    |
| a            | PPy1669       | MATα FUS1::FUS1-lacZ::LEU2 ste5::ADE2 kss1::ura3COA | This study                    |
| a            | PPy2335       | MATα ste5::ADE2 fus3Δ::natMX6 | This study                    |
| a            | PPy2365       | MATα ste5::ADE2 bar1Δ::hphMX6 | This study                    |
| a            | PPy2367       | MATα ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6 | This study                    |
| a            | PPy2387       | MATα ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6 | This study                    |
| a            | PPy2389       | MATα ste5::ADE2 bar1Δ::hphMX6 kss1Δ::kanMX6 | This study                    |
| a            | PPy2408       | MATα ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6 kss1::natMX6::P CYC1-KSS1 | This study                    |
| a            | PPy2409       | MATα ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6 kss1::natMX6::P ADH1-KSS1 | This study                    |
| a            | PPy2411       | MATα ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6 kss1::natMX6::P GPD1-KSS1 | This study                    |
| a            | PPy2412       | MATα ste5::ADE2 bar1Δ::hphMX6 kss1::kanMX6 fus3::natMX6::P CYC1-FUS3 | This study                    |
| a            | PPy2413       | MATα ste5::ADE2 bar1Δ::hphMX6 kss1::kanMX6 fus3::natMX6::P ADH1-FUS3 | This study                    |
| a            | PPy2415       | MATα ste5::ADE2 bar1Δ::hphMX6 kss1::kanMX6 fus3::natMX6::P GPD1-FUS3 | This study                    |
| b            | PPy966        | MATα              | Lamson et al. (2002)          |
| b            | PPy968        | MATα tec1::HIS3   | This study                    |
| b            | PPy1276       | MATα fus3::LEU2   | This study                    |
| b            | PPy1284       | MATα ste5::HIS3   | This study                    |
| b            | PPy1285       | MATα ste5::HIS3 fus3::LEU2 | This study                    |
| b            | PPy1608       | MATα kss1::ura3COA | This study                    |
| b            | PPy1612       | MATα fus3::LEU2 kss1::ura3COA | This study                    |
| b            | PPy2484       | MATα tec1::HIS3 fus3Δ::kanMX6 | This study                    |

It remains an open question whether functional asymmetries similar to those shown by Fus3 and Kss1 are also exhibited by MAPKs in other systems. Their homologues in vertebrates also exist as a closely related pair, ERK1 and ERK2. As was originally thought to be true for Fus3 and Kss1, current evidence suggests that ERK1 and ERK2 are functionally redundant, with any functional differences being largely attributable to differences in expression patterns or levels (Busca et al., 2016; Saba-El-Leil et al., 2016). It is conceivable, however, that more subtle distinctions such as effects on dose-response alignment could have gone undetected, and/or that relevant biochemical distinctions are obscured by other circuit properties such as negative feedback.

**MATERIALS AND METHODS**

**Yeast methods**

Standard procedures were used for growth and genetic manipulation of yeast (Rothstein, 1991; Sherman, 2002). Cells were grown at 30°C in yeast extract/peptone medium with 2% glucose (YPD) or in synthetic (SC) medium with 2% glucose. Strains and plasmids are listed in Tables 1 and 2, respectively. Promoter replacement at endogenous FUS3 and KSS1 loci was performed by homologous recombination using PCR-generated fragments, using methods described previously (Longtine et al., 1998; Janke et al., 2004). The PCR templates (pPP4036, pPP4037, pPP4038) were created by transferring CYC1, ADH1, and GPD1 promoters as SacI-XbaI fragments from pRS413-based plasmids (Mumberg et al., 1995) in place of the TEF1 promoter in plasmid pYM-N19 (Janke et al., 2004); the products are near-identical reconstructions of previously described plasmids (pYM-N7, pYM-N11, and pYM-N15; Janke et al., 2004).

**Pheromone signaling assays**

Asynchronous cultures were treated with α-factor using concentrations and durations indicated in each figure. For dose-response assays in BAR1 strains, in order to minimize Bar1-mediated degradation of α-factor (Ciejek and Thorner, 1979; Sprague and Herskowitz, 1981; Manney, 1983), and thus increase reproducibility, cells were first pelleted and washed once with fresh media before adding α-factor. For all dose-response assays, α-factor was diluted in YPD medium.

To measure transcriptional responses, cells harboring integrated or plasmid-borne lacZ reporters were treated with α-factor as indicated, and then were collected and assayed for β-galactosidase activity. Specifically, the OD₆₆₀ of each culture was recorded, and then
TABLE 2: Plasmids used in this study.

| Name               | Alias             | Description                  | Source          |
|--------------------|------------------|------------------------------|-----------------|
| pPP679             | pRS314           | CEN ARS TRP1 vector          | Sikorski and Hieter (1989) |
| pPP681             | pRS316           | CEN ARS URA3 vector          | Sikorski and Hieter (1989) |
| pPP847             | p2985            | CEN ARS LEU2 PGU1-pr-lacZ    | Roberts et al. (2000) |
| pPP849             | p2987            | CEN ARS LEU2 KSS1-pr-lacZ    | Roberts et al. (2000) |
| pPP850             | p2988            | CEN ARS LEU2 YLR042Cpr-lacZ  | Roberts et al. (2000) |
| pPP851             | p3017            | CEN ARS LEU2 SVS1-pr-lacZ    | Roberts et al. (2000) |
| pPP852             | p3018            | CEN ARS LEU2 FUS3-pr-lacZ    | Roberts et al. (2000) |
| pPP854             | p3058            | CEN ARS LEU2 FUS1pr-lacZ     | Roberts et al. (2000) |
| pPP855             | p3079            | CEN ARS LEU2 PRM3-pr-lacZ    | Roberts et al. (2000) |
| pPP856             | p3081            | CEN ARS LEU2 FIG1pr-lacZ     | Roberts et al. (2000) |
| pPP1013            | p2972            | CEN ARS TRP1 PGU1pr-lacZ     | Roberts et al. (2000) |
| pPP1014            | p2982            | CEN ARS TRP1 KSS1-pr-lacZ    | Roberts et al. (2000) |
| pPP1038            | p3058-T          | CEN ARS TRP1 FUS1-pr-lacZ    | Roberts et al. (2000) |
| pPP1044            | pH-CFL           | CEN ARS HIS3 FUS1-lacZ       | Lamson et al. (2006) |
| pPP1926            | pH-FD11-Asp3     | CEN ARS HIS3 STE11-Asp3     | Lamson et al. (2006) |
| pPP1969            | p55kmyc          | CEN ARS URA3 STE5-myc<sub>13</sub> T<sub>CYC1</sub> | Winters et al. (2005) |
| pPP2861            | p55kmyc-VASP     | CEN ARS URA3 ste5(V63A S861P)-myc<sub>13</sub> T<sub>CYC1</sub> | This study |
| pPP4007            | pGA1840          | CEN ARS TRP1 P<sub>TP11</sub>-FUS3-wt | Gartner et al. (1992) |
| pPP4008            | pGA1881          | CEN ARS TRP1 P<sub>TP11</sub>-fus3(Y182F) | Gartner et al. (1992) |
| pPP4009            | pGA1894          | CEN ARS TRP1 P<sub>TP11</sub>-fus3(T180A) | Gartner et al. (1992) |
| pPP4010            | pGA1895          | CEN ARS TRP1 P<sub>TP11</sub>-fus3(T180A Y182F) | Gartner et al. (1992) |
| pPP4014            | YCplu-KSS1       | CEN ARS URA3 KSS1-wt         | Bardwell et al. (1998) |
| pPP4016            | YCplu-KSS1-Y185F | CEN ARS URA3 kss1(Y185F)     | Bardwell et al. (1998) |
| pPP4017            | YCplu-KSS1-AEF   | CEN ARS URA3 kss1(T183A Y185F) | Bardwell et al. (1998) |
| pPP4018            | YCplu-KSS1-T183A | CEN ARS URA3 kss1(T183A)     | Bardwell et al. (1998) |
| pPP4019            | YEplu-FT1Z       | 2 µm URA3 FRE[TEC1]-lacZ     | Sabbagh et al. (2001) |
| pPP4036            | pFA6a-nat-Pyc1   | CYC1 promoter natMX6 (PCR template) | This study |
| pPP4037            | pFA6a-nat-Padh1  | ADH1 promoter natMX6 (PCR template) | This study |
| pPP4038            | pFA6a-nat-Pgpd1  | GPD1 promoter natMX6 (PCR template) | This study |
| pPP4042            | YCplac33-TEC1    | CEN ARS URA3 TEC1            | Bao et al. (2004) |
| pPP4043            | YCplac33-tec1-T273M | CEN ARS URA3 tec1-T273M     | Bao et al. (2004) |

cells (usually 1 ml) were harvested by centrifugation, resuspended in 0.5 ml of Z buffer (82 mM sodium phosphate [pH 7.0], 10 mM KCl, 1 mM MgSO<sub>4</sub>, 40 mM β-mercaptoethanol), and permeabilized by vortexing in the presence of 0.01 ml of 0.4% SDS and 0.05 ml of chloroform. Reactions were started by adding 0.3 ml of o-nitrophenyl-β-d-galactopyranoside (2.4 mg/ml in Z buffer), incubated at 30° for 5–300 min, stopped by adding 0.5 ml of 1 M Na<sub>2</sub>CO<sub>3</sub>, and stored on ice until all reactions were finished. Reaction mixtures were clarified by spinning in a microcentrifuge (full speed, 5 min), and then 1 ml supernatant was collected for measurement of OD<sub>420</sub>. β-Galactosidase activity was calculated as (1000 × OD<sub>420</sub>) / (OD<sub>660</sub> × culture volume [ml] × reaction time [min]).

To measure MAPK phosphorylation, cells were treated ± α factor as indicated and then harvested by centrifugation; cell pellets were frozen in liquid nitrogen and stored at −80°C before cell extracts were prepared, as described below.

Cell extracts and immunoblotting

Whole cell extracts were prepared by lysis in trichloroacetic acid as described previously (Pope et al., 2014), using frozen cell pellets from 2 ml cultures; protein concentrations were measured by bicinchoninic acid (BCA) assay (Pierce #23225), and equal amounts (usually 20 µg) were loaded per lane. Proteins were resolved by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) in a submerged tank. Standard gels used 12% acrylamide. Analysis in Phos-tag gels followed methods described previously (Kinoshita et al., 2004), using 10% acrylamide (29:1 acrylamide: bisacrylamide), 50 µM MnCl<sub>2</sub>, and 25 µM Phos-tag AAL-107; after running, gels were washed (2 × 15 min in transfer buffer with 1 mM EDTA, then 1 × 10 min in transfer buffer) before the transfer procedure. Primary antibodies were rabbit anti-phospho-p44/42 (1:1000 or 1:2000; Cell Signaling Technology #9101 [lots 23, 26, 27] or #4370), rabbit anti-G6PDH (1:100,000; Sigma #A9521), rabbit anti-Kss1 (1:1000; Santa
Cruz Biotechnologies #sc-6775-R), and goat anti-Fus3 (1:5000; Santa Cruz Biotechnologies #sc-6773). Horseradish peroxidase–conjugated secondary antibodies were goat anti-rabbit (1:3000; Jackson ImmunoResearch #111-035-144), or donkey anti-goat (1:3000; Santa Cruz #sc-2020). Enhanced chemiluminescence detection used a Thermo Scientific SuperSignal West Pico substrate (#34080) or a BioRad Clarity substrate (#170-5060). Exposures were captured on x-ray film, and densitometry was performed using ImageJ (https://image.nih.gov/ij/).

ACKNOWLEDGMENTS
This work was supported by a grant from the National Institutes of Health (R01 GM057769). We thank Gustav Ammerer, Lee Bardwell, and Hiten Madhani for gifts of plasmids, as well as Jim Ferrell for helpful feedback. We also thank Lee Bardwell for informative discussions at early stages of this project. We are particularly grateful to Alejandro Colman-Lerner for valuable discussions and for suggesting that Fus3 and Kes1 can be regarded as a joint push-pull device.

REFERENCES
Alvaro CG, Thorner J (2016). Heterotrimeric G protein-coupled receptor signaling in yeast mating pheromone response. J Biol Chem 291, 7788–7795.
Andersson J, Simpson DM, Qi M, Wang Y, Elion EA (2004). Differential input by Ste5 scaffold and Msg5 phosphatase route a MAPK cascade to multiple outcomes. EMBO J 23, 2564–2576.
Andrews SS, Peria WJ, Yu RC, Colman-Lerner A, Brent R (2016). Push-pull and feedback mechanisms can align signaling system outputs with inputs. Cell Syst 3, 444–455.e2.
Aoki K, Yamada M, Kunita K, Yasuda S, Matsuda M (2011). Processive phosphorylation of ERK MAP kinase in mammalian cells. Proc Natl Acad Sci USA 108, 12675–12680.
Bao MZ, Schwartz MA, Cantin GT, Yates JR 3rd, Madhani HD (2004). Phospho-molecule-dependent detection of the Tec1 transcription factor is required for MAP kinase signaling specificity in yeast. Cell 119, 991–1000.
Bardwell L (2005). A walk-through of the yeast mating pheromone response pathway. Peptides 26, 339–350.
Bardwell L, Cook JG, Voora D, Baggott DM, Martinez AR, Thorner J (1998). Inhibitory and activating functions for MAPK Kes1 in the S. cerevisiae filamentous-growth signaling pathway. Nature 390, 85–88.
Cullen PJ, Sprague GF Jr (2012). The regulation of filamentous growth in yeast. Genetics 190, 23–49.
Dohlman HG, Thorner JW (2001). Regulation of G protein-initiated signal transduction in yeast: paradigms and principles. Annu Rev Biochem 70, 703–754.
Drogen F, O’Rourke SM, Stucke V, Jaquenoud M, Neiman AM, Peter M (2000). Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo.Curr Biol 10, 630–639.
Elion EA, Brill JA, Fink GR (1991). Fus3 represses CLN1 and CLN2 and in concert with KSS1 promotes signal transduction. Proc Natl Acad Sci USA 88, 9392–9396.
Elion EA, Grisafi PL, Fink GR (1990). Fus3 encodes a cdc2/cDC28-related kinase required for the transition from mitosis into conjugation. Cell 60, 649–664.
Ferrell JE Jr, Ha SH (2014). Ultrasensitivity part i: Michaelian responses and zero-order ultrasensitivity. Trends Biochem Sci 39, 496–503.
Flatauer LJ, Zadel SF, Bardwell L (2005). Mitogen-activated protein kinases with distinct requirements for Ste5 scaffolding influence signaling specificity in Saccharomyces cerevisiae. Mol Cell Biol 25, 1793–1803.
Gartner A, Nasmyth K, Ammerer G (1992). Signal transduction in Saccharomyces cerevisiae requires tyrosine and threonine phosphorylation of Fus3 and Kss1. Genes Dev 6, 1280–1292.
Good M, Tang G, Singleton J, Remyen A, Lim WA (2009). The Ste5 scaffold directs mating signaling by catalytically unlocking the Fus3 MAP kinase for activation. Cell 136, 1085–1097.
Hagen DC, McCaffrey G, Sprague GF Jr (1991). Pheromone response elements are necessary and sufficient for basal and pheromone-induced transcription of the FUS1 gene of Saccharomyces cerevisiae. Cell 69, 2925–2961.
Hao N, Nayak S, Behar M, Shanks RH, Nagiec MJ, Errede B, Hasty J, Elston TC, Dohlman HG (2008). Regulation of cell signaling dynamics by the protein kinase-scaffold Ste5. Mol Cell 30, 649–656.
Hao N, Yildirim N, Nagiec MJ, Parnell SC, Errede B, Dohlman HG, Elston TC (2012). Combined computational and experimental analysis reveals mitogen-activated protein kinase-mediated feedback phosphorylation as a mechanism for signaling specificity. Mol Biol Cell 23, 3899–3910.
Inouye C, Dhillon N, Durfee T, Zambrinsky PC, Thorner J (1997a). Mutualational analysis of STE5 in the yeast Saccharomyces cerevisiae: application of a differential interaction trap assay for examining protein-protein interactions. Genetics 147, 479–492.
Inouye C, Dhillon N, Thorner J (1997b). Ste5-RING-H2 domain: role in Ste5-promoted oligomerization for yeast pheromone signaling. Science 278, 103–106.
Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, Inouye C, Dhillon N, Durfee T, Zambryski PC, Thorner J (1997a). Mutational analysis of STE5 in the yeast Saccharomyces cerevisiae: application of a differential interaction trap assay for examining protein-protein interactions. Genetics 147, 479–492.
Inouye C, Dhillon N, Thorner J (1997b). Ste5-RING-H2 domain: role in Ste5-promoted oligomerization for yeast pheromone signaling. Science 278, 103–106.
Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, Moreno-Borchart A, Doenges G, Schwob E, Schiebel E, et al. (2004). A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21, 947–962.
Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T (2006). Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5, 749–757.
Lamson RE, Takahashi S, Winters MJ, Pryciak PM (2006). Dual role for membrane localization in yeast MAP kinase cascade activation and its contribution to signaling fidelity. Curr Biol 16, 618–623.
Lamson RE, Winters MJ, Pryciak PM (2002). Cdc42 regulation of kinase activity and signaling by the yeast p21-activated kinase Ste20. Mol Cell 10, 2939–2951.
Longtime MS, McKenzie A 3rd, Demarinii DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR (1998). Additional modules for versatile and
Roberts RL, Fink GR (1994). Elements of a single MAP kinase cascade in Saccharomyces cerevisiae mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev 8, 2974–2985.

Rothstein R (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol 194, 281–301.

Saba-El-Leil MK, Fremin C, Meloche S (2016). Redundancy in the world of MAP kinases: all for one. Front Cell Dev Biol 4, 67.

Sabbagh W Jr, Flatauer LJ, Bardwell AJ, Bardwell L (2001). Specificity of MAP kinase signaling in yeast differentiation involves transient versus sustained MAPK activation. Mol Cell 8, 683–691.

Schwartz MA, Madhani HD (2006). Control of MAPK signaling specificity by a conserved residue in the MEK-binding domain of the yeast scaffold protein Ste5. Curr Genet 49, 351–363.

Sherman F (2002). Getting started with yeast. Methods Enzymol 350, 3–41.

Sikorski RS, Hieter P (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27.

Sprague GF Jr, Herskowitz I (1981). Control of yeast cell type by the mating type locus. I. Identification and control of expression of the a-specific gene BAR1. J Mol Biol 153, 305–321.

Takahashi S, Pryciak PM (2008). Membrane localization of scaffold proteins promotes graded signaling in the yeast MAP kinase cascade. Curr Biol 18, 1184–1191.

Thomson TM, Benjamin KR, Bush A, Love T, Pincus D, Resnekov O, Yu RC, Gordon A, Colman-Lerner A, Endy D, et al. (2011). Scaffold number in yeast signaling system sets tradeoff between system output and dynamic range. Proc Natl Acad Sci USA 108, 20265–20270.

Winters MJ, Lamson RE, Nakanishi H, Neiman AM, Pryciak PM (2005). A membrane binding domain in the Ste5 scaffold synergizes with Gbeta-gamma binding to control localization and signaling in pheromone response. Mol Cell 20, 21–32.

Yu RC, Pesce CG, Colman-Lerner A, Lok L, Pincus D, Serra E, Holl M, Benjamin K, Gordon A, Brent R (2008). Negative feedback that improves information transmission in yeast signalling. Nature 456, 755–761.