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

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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.**

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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 (Dohlman 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

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Abbreviations used: EC50, effective concentration for half-maximal response; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase.

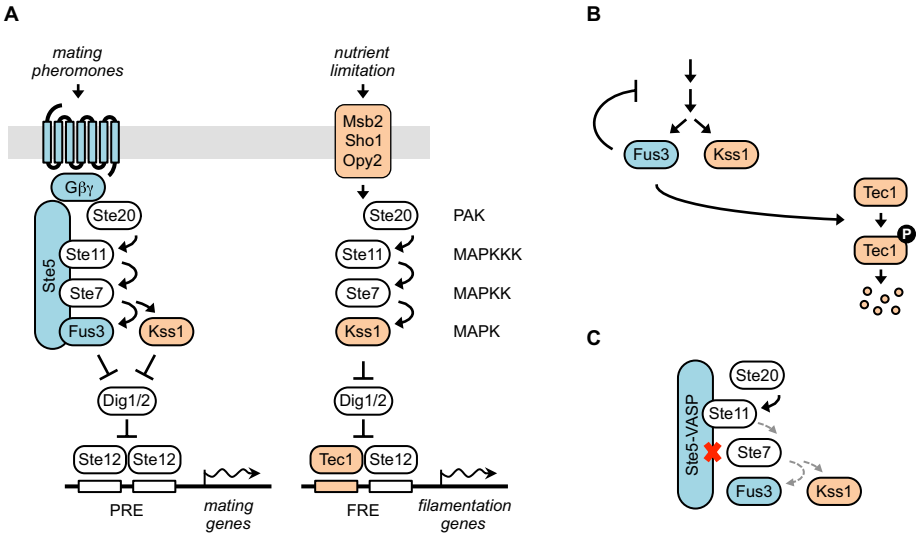

FIGURE 1: Signaling pathways involving the MAPKs Fus3 and Kss1. (A) Diagram of Fus3 and Kss1 signaling pathways. Either MAPK can induce transcription of mating genes in response to mating pheromones, which requires the scaffold protein Ste5 and the downstream transcription factor Ste12. Separately, Kss1 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).

clear that each MAPK is potentially capable of doing so, because pheromone can stimulate transcription in either *fus3*Δ or *kss1*Δ single mutant cells (but not in *fus3*Δ *kss1*Δ double mutants). Yet it is not clear whether each MAPK contributes to pheromone-induced transcription in wild-type cells that contain both Fus3 and Kss1 (or, if both do contribute, whether they do so to a comparable degree). In addition, there is little information for either MAPK about whether there is a threshold level of activation (such as a minimum number of molecules or fraction of the total) that must be activated in order to elicit downstream responses, or if instead the responses follow a continuum in direct proportion to the degree of MAPK activation. This issue is further complicated by findings that varying doses of pheromone might not simply affect the level of MAPK activity but also the duration of time spent in a highly active state (Behar *et al.*, 2008; Hao *et al.*, 2008).

In this study, we compare the threshold activation properties of Fus3 and Kss1. Surprisingly, our findings suggest that, in wild-type cells, Kss1 is not sufficiently activated by pheromone to induce appreciable transcription of either mating or filamentation genes. Instead, to induce transcription, Kss1 must be activated to a degree that is seen only in *fus3*Δ cells. This threshold level is not seen in *FUS3* cells because it is prevented by negative feedback from Fus3. The activation threshold for Kss1 correlates with a transition point at which a majority of Kss1 molecules are converted to the active form, reflecting a need to overcome transcriptional repression by the inactive form of Kss1. By contrast, Fus3 strongly limits its own activation so that the active form never accounts for the majority of molecules, and yet it is a potent transcriptional activator. Our findings reveal interesting functional contrasts between Fus3 and Kss1, and suggest a revised view of the contribution of each MAPK to gene induction in this model system.

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 (Dohlman and Thorner, 2001; Bardwell, 2005; Alvaro and Thorner, 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 Kss1 (Flatauer *et al.*, 2005; Schwartz and Madhani, 2006). Despite this ability to activate Kss1, *ste5-VASP* mutant cells cannot mate (Inouye *et al.*, 1997a,b), which is surprising because *fus3*Δ cells are able to mate, albeit with reduced efficiency (Elion *et al.*, 1990, 1991; Madhani *et al.*, 1997; Breitkreutz *et al.*, 2001). This behavior presented a paradox: although Kss1 can mediate mating responses in *fus3*Δ cells, it cannot do so in *ste5-VASP* cells.

To investigate this enigma, we compared the levels of Kss1 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, Kss1 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 (*fus3*Δ), Kss1 could induce transcription in *STE5* cells but not in *ste5-VASP* cells. Notably, in the *STE5* background, deleting *FUS3* was accompanied by an unusually high degree of Kss1 activation, whereas in the *ste5-VASP* background this did not occur; instead, Kss1 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 Kss1 activation in *fus3*Δ 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 Kss1 to induce transcription of mating genes is observed only under conditions that lead to abnormally high levels of Kss1 activation (*fus3*Δ *STE5*), whereas activation of Kss1 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 Kss1 and the filamentation pathway (Figure 2C). All four mating pathway reporters behaved as described for the

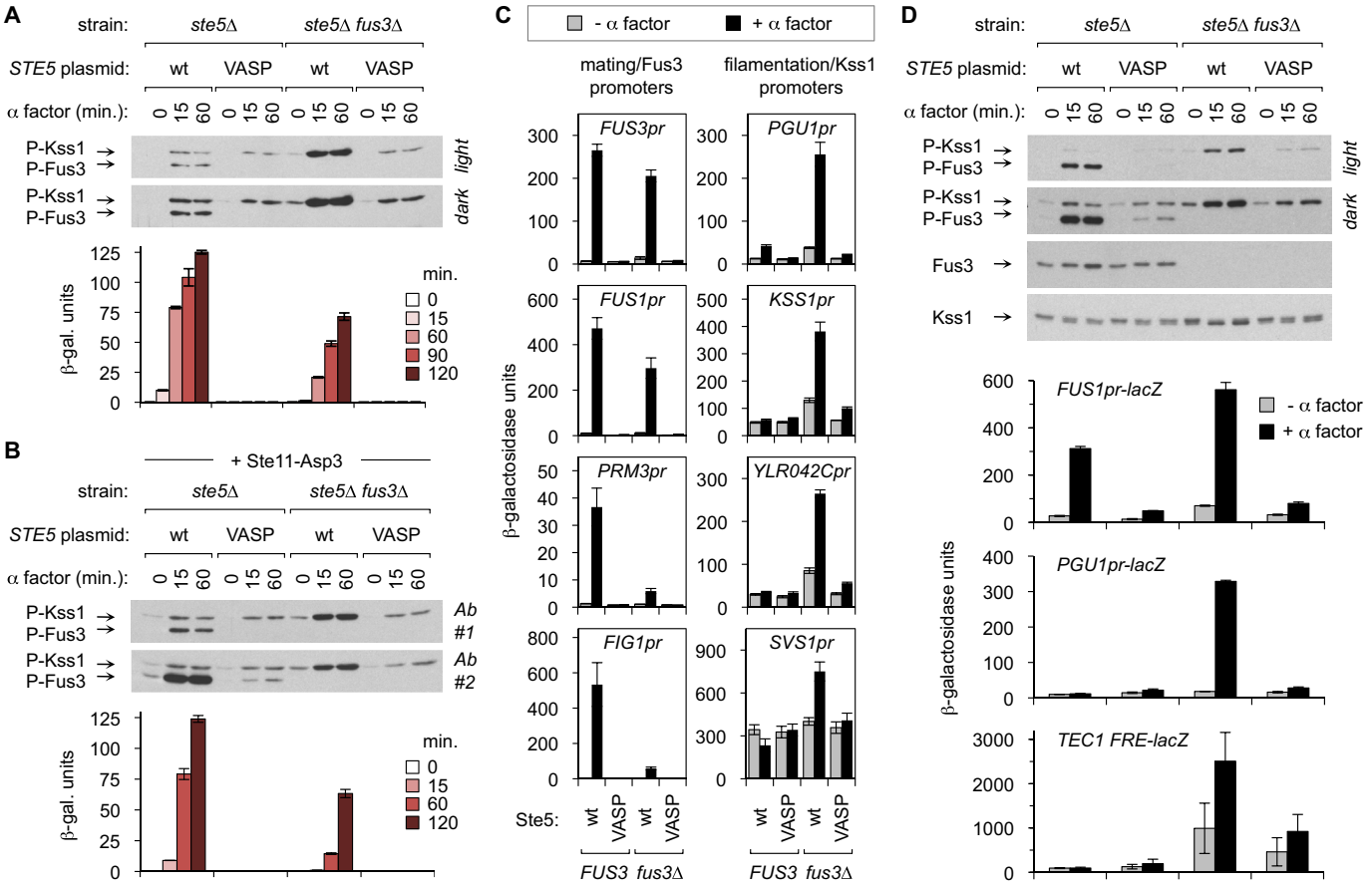

FIGURE 2: Transcriptional induction by Kss1 is correlated with its hyperphosphorylation. (A) Plasmid-borne *STE5-wt* and *ste5-VASP* alleles were expressed in *FUS3* and *fus3*Δ backgrounds and tested for pheromone response. Cells were treated with α factor (5 μM) for the times indicated. Top, representative assay of MAPK phosphorylation. Bottom, *FUS1-lacZ* assay of transcriptional induction (mean ± range; $n = 2$). Strains: PPY858, PPY1667. Plasmids: pPP1969, pPP2861. (B) Experiments were similar to those in panel A except that cells also harbored a plasmid expressing Ste11-Asp3, a constitutively active mutant. To ensure results were not antibody specific, phosphorylated MAPK was analyzed with two antibodies (CST cat. no. 9101, lot 23 [antibody #1], or lot 26 [antibody #2]); despite significant variation in reactivity toward P-Kss1 vs. P-Fus3, the effects of *STE5* and *FUS3* alleles were consistent between the two antibodies. *FUS1-lacZ* assays (bottom) show mean ± range ($n = 2$). Strains: PPY858, PPY1667. Plasmids: pPP1926 plus pPP1969 or pPP2861. (C) Transcriptional responses (mean ± SD; $n = 4$) were measured using reporters driven by eight different promoters that are activated preferentially by the mating or filamentation pathway. Cells harboring the indicated *STE5* and *FUS3* alleles were treated ± α factor (5 μM, 2 h). Strains: PPY861, PPY2335. *STE5* plasmids: pPP1969, pPP2861. Reporter plasmids: pPP847, pPP849, pPP850, pPP851, pPP852, pPP854, pPP855, pPP856. (D) Experiments were performed as in panel A, using cells of the Σ1278b strain background. Blots show both phosphorylated and total levels of Fus3 and Kss1. For transcription assays (bottom), cells were treated ± 5 μM α factor for 2 h; top two graphs show mean ± range ($n = 2$), and bottom graph shows mean ± SD ($n = 6$). Strains: PPY1284, PPY1285. *STE5* plasmids: pPP2857, pPP2863. Reporter plasmids: pPP1013, pPP1038, pPP4019.

preceding experiments; namely, they were induced in both *FUS3* and *fus3*Δ cells, but could not be induced in *ste5-VASP* cells, regardless of Fus3 status. The filamentation pathway reporters were only induced by pheromone in *fus3*Δ cells and not in *FUS3* cells, as described previously (Roberts *et al.*, 2000). Interestingly, the *ste5-VASP* mutation blocked their strong pheromone induction in *fus3*Δ cells, indicating that transcription of these reporters depends not only on the removal of Fus3 but also on the strong Kss1 hyperphosphorylation seen in *fus3*Δ cells with wild-type Ste5. (For three reporters—*PGU1pr*, *KSS1pr*, and *YLR042Cpr*—the elevated basal expression in *fus3*Δ 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 *fus3*Δ 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 *fus3*Δ *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.

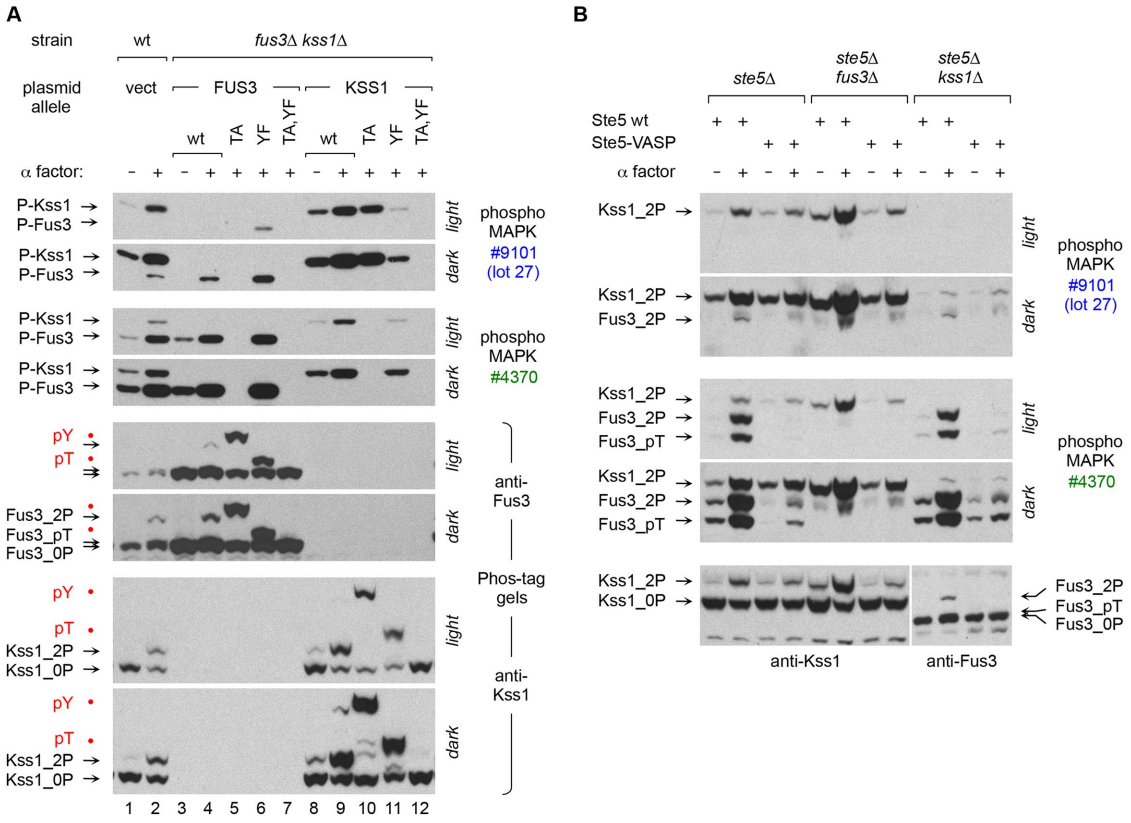

FIGURE 3: Analysis of MAPK phosphorylation state. (A) Activation loop mutations in Fus3 and Kss1 were tested for their impact on recognition by phospho-specific antibodies (top) and migration in Phos-tag gels (bottom). A wild-type strain (wt) and a *fus3*Δ *kss1*Δ strain harboring a *FUS3* or *KSS1* plasmid (wt or mutant) was incubated ± α factor (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 ± α factor. 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 fus3*Δ 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 *fus3*Δ 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 *bar1*Δ 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 *bar1*Δ), the Kss1_2P fraction was still roughly half (0.45–0.55). By contrast, in *FUS3* cells the maximum pheromone dose activated Kss1 to markedly lower extents (2P fraction = 0.2–0.3) that in *fus3*Δ cells could only barely induce transcription; for example, corresponding to roughly 50 nM in *BAR1 fus3*Δ cells or 1.5 nM in *bar1*Δ *fus3*Δ cells, which yielded transcriptional induction of roughly 10–13% 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 *fus3*Δ *KSS1* 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 *fus3*Δ 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 *fus3*Δ 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 *fus3*Δ 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 0.1× to 10× 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 phospho-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 phospho-Kss1 levels were too low (e.g., lower than those seen without pheromone when Kss1 was expressed from its native promoter).

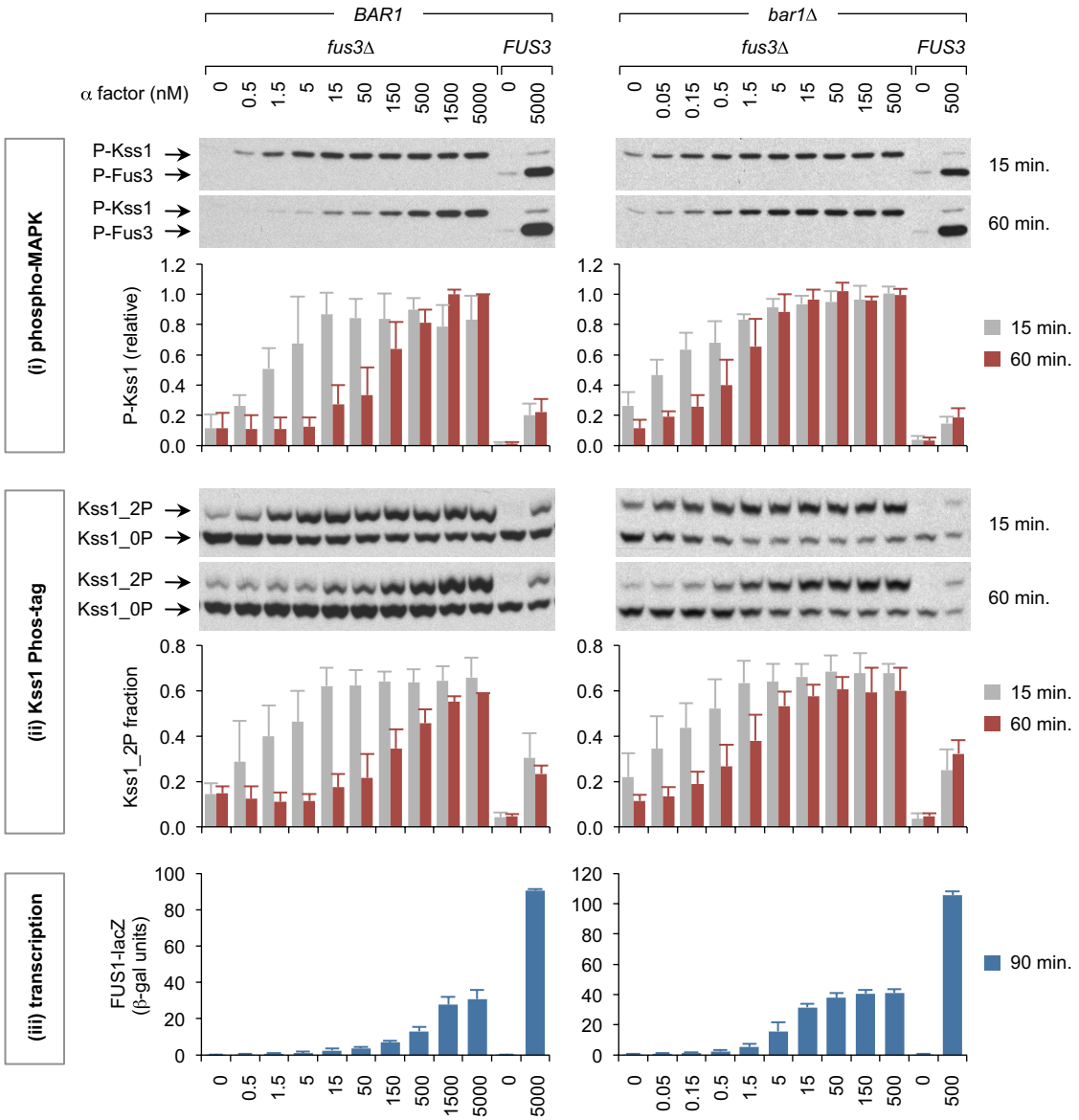

FIGURE 4: Transcription is induced when the majority of Kss1 molecules are activated. Dose-response analyses compare the profiles of Kss1 phosphorylation and transcriptional output in *fus3*Δ cells. Assays were performed in both *BAR1* (left) and *bar1*Δ (right) backgrounds; in each case, *FUS3* strains 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 β-galactosidase units.

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 α factor with P_{FUS3}-*FUS3* at 0.5 nM α 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. Therefore, as with Kss1, transcriptional induction by Fus3 also appears subject to antagonism by the inactive form (consistent with dominant-negative effects of kinase-inactive Fus3 mutants [Breitkreutz

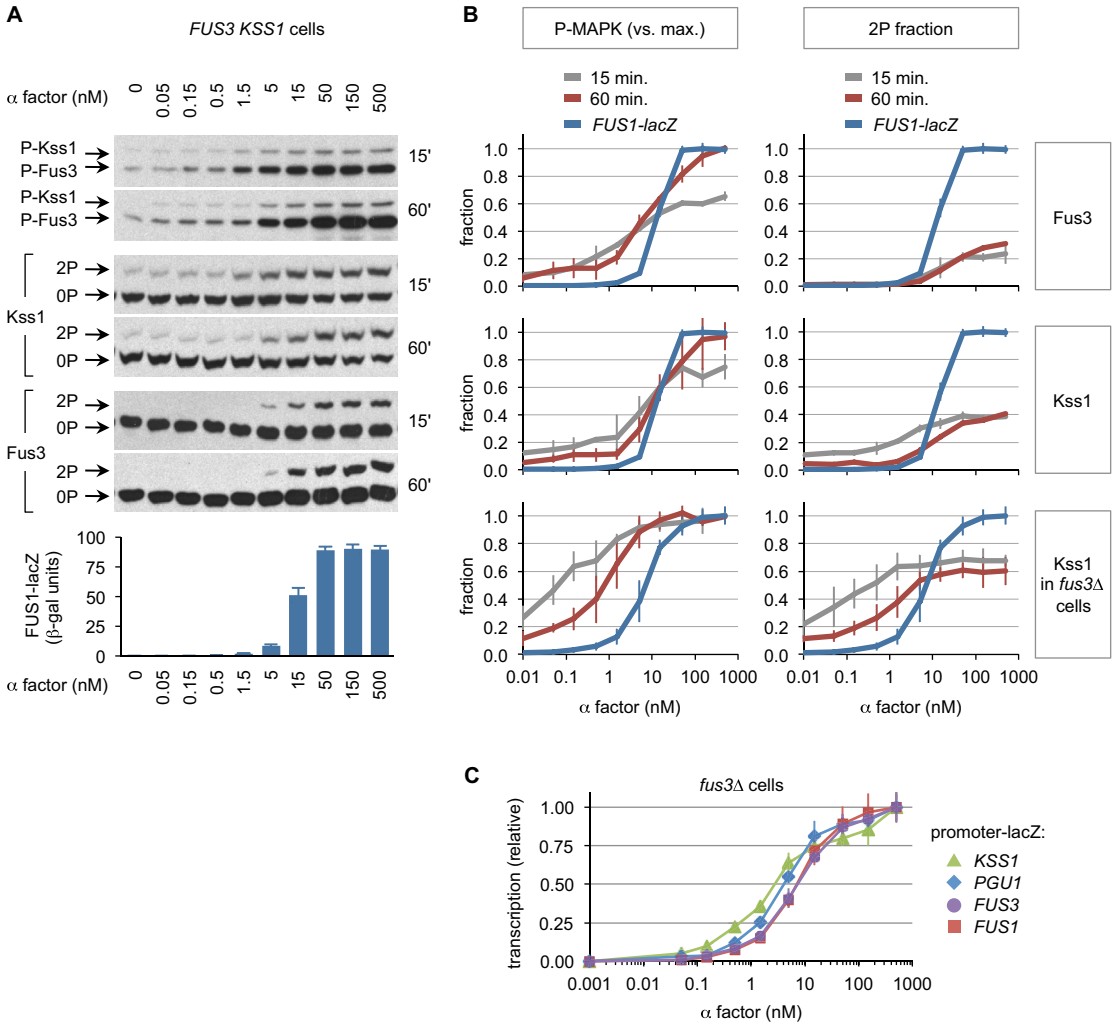

FIGURE 5: Fus3 controls both the extent and EC50 of Kss1 phosphorylation. (A) Dose-response analysis of kinase phosphorylation and transcriptional induction was performed as in Figure 4, but using *bar1*Δ *FUS3 KSS1* cells in which activation of both Fus3 and Kss1 was monitored. Top two panels show phospho-MAPK 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 *bar1*Δ *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 *bar1*Δ *fus3*Δ 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 *bar1*Δ *fus3*Δ 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*).

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 ability of Kss1 to activate filamentation genes is blocked because

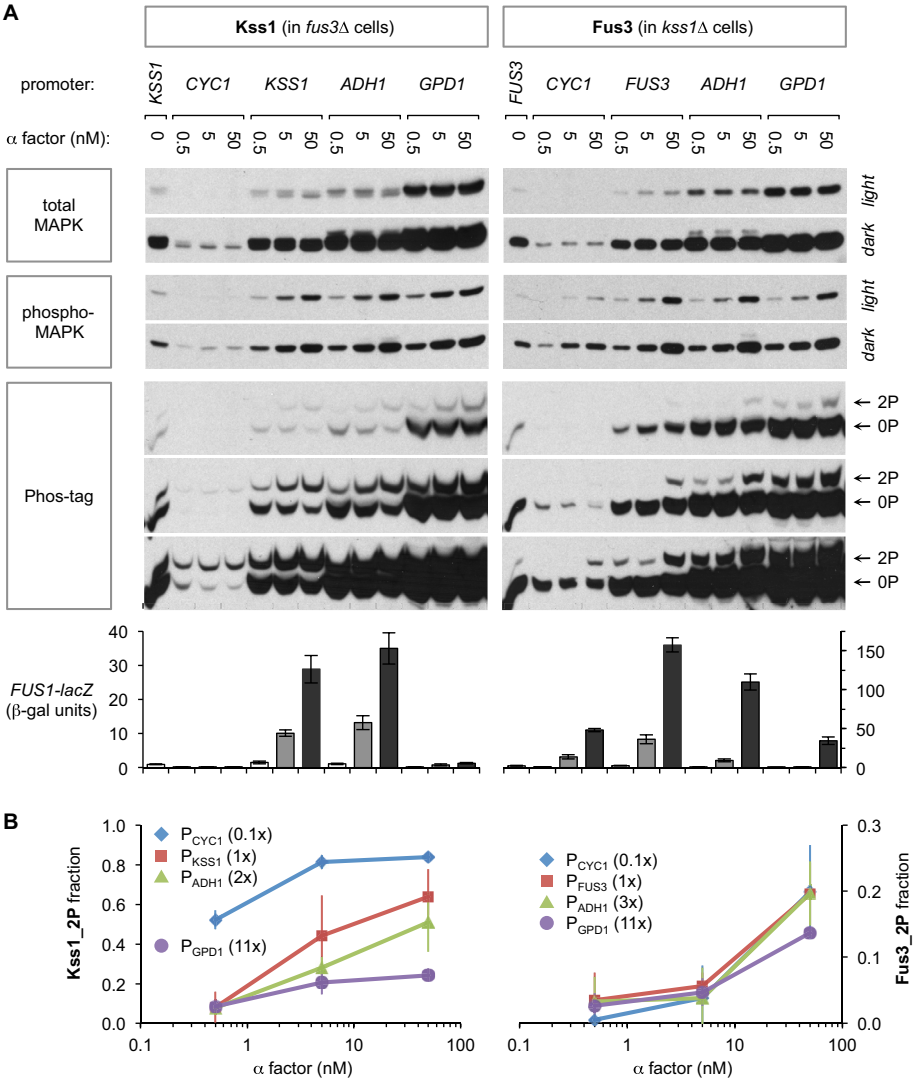

FIGURE 6: Varying Kss1 and Fus3 levels reveals distinct dependencies on active fraction. (A) *CYC1*, *ADH1*, and *GPD1* promoters were used to replace the native promoters of *KSS1* (in a *fus3*Δ *KSS1* strain) or *FUS3* (in a *FUS3 kss1*Δ strain). Cells were treated with the indicated concentration of α factor for 60 min (for protein analysis) or 90 min (for transcription analysis). Protein samples were run on standard gels to analyze total MAPK (top) or phospho-MAPK (middle), and on Phos-tag gels to resolve 0P and 2P forms (bottom). Transcriptional induction was assayed using a *FUS1-lacZ* reporter (mean ± SD; $n = 4$). Strains (PPY2387, PPY2408, PPY2409, PPY2411, PPY2389, PPY2412, PPY2413, PPY2415) harbored *STE5* plasmid pPP1969 and *FUS1-lacZ* plasmid pPP1044. (B) Quantification of the 2P form of each MAPK, expressed as a fraction of total MAPK and plotted as a function of α factor concentration (mean ± range, $n = 2$). Values in parentheses indicate the relative protein levels of MAPKs when expressed from each promoter, obtained by densitometry of total MAPK blots using both undiluted samples (as in panel A) and serial-diluted samples; results from each method were averaged to obtain the values shown.

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 *fus3*Δ 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 *fus3*Δ 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 0P 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 *fus3*Δ cells. Yet, we confirmed that Tec1-T273M could activate a filamentation reporter in *FUS3* cells (Figure 7B). Therefore, unlike in *fus3*Δ 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 *kss1*Δ 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 *fus3*Δ 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 *fus3*Δ 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

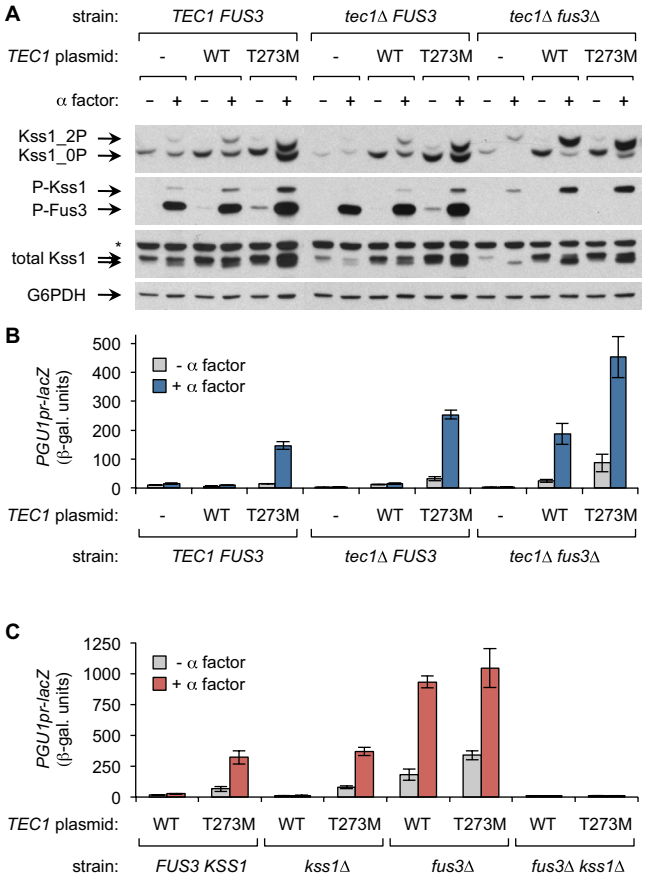

FIGURE 7: Effect of *TEC1* alleles on Kss1 and transcription in *FUS3* and *fus3*Δ strains. (A) The indicated strains harboring *TEC1* plasmids (empty vector, wt, or T273M) were treated ± α factor (5 μM, 60 min). Protein samples were analyzed on Phos-tag gels (probed with anti-Kss1) or on standard gels to detect phospho-MAPKs, total Kss1, and G6PDH (as a loading control). In the total Kss1 blot, the two arrows indicate partially resolved species corresponding to unphosphorylated (top) and phosphorylated (bottom) forms; the asterisk indicates a nonspecific band. Strains: PPY966, PPY968, PPY2484. Plasmids: pPP1013 plus pPP681, pPP4042, or pPP4043. (B) Strains harboring *TEC1* plasmids, as in panel A, were tested for induction of a filamentation reporter (*PGU1pr-lacZ*). Cells were treated ± α factor (5 μM, 2 h). Bars, mean ± SD ($n = 4$). Strains and plasmids were as in panel A. (C) The transcriptional effect of Tec1-T273M was compared in strains lacking *FUS3* and/or *KSS1*. Cells were treated ± α factor (5 μM, 2 h). Bars, mean ± SD ($n = 4$). Strains PPY966, PPY1276, PPY1608, and PPY1612 harbored plasmid pPP1013.

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 molecules, and yet it is a potent activator of transcription. Thus, Fus3 and Kss1 may further differ in the relative potency of their repressive versus activating effects on transcription (Figure 8A). Altogether, these findings reveal significant functional contrasts between Fus3 and Kss1, and they suggest a revised view of how each MAPK contributes to transcriptional output responses.

Previous studies established that Kss1 has both positive and negative effects on transcription; that is, transcription is not only promoted by active Kss1 but is also inhibited by inactive Kss1 (Cook *et al.*, 1997; Madhani *et al.*, 1997; Bardwell *et al.*, 1998). Our findings suggest that this negative effect outweighs the positive effect until the majority of Kss1 molecules are converted to the active form. Because Fus3 keeps Kss1 from being activated to this degree, in wild-type cells Kss1 can make little positive contribution to transcription, but its repressive effect can be gradually lifted as the stimulus increases. This view fits with other evidence that the presence of inactive Kss1 dampens *FUS1* activation at low pheromone doses (Sabbagh *et al.*, 2001; Paliwal *et al.*, 2007). It also fits with earlier experiments using adjustable expression of active Ste11, in which transcription that was mediated solely by Kss1 (in *ste5*Δ cells) required unusually high levels of Ste11 and Kss1 activation (Takahashi and Pryciak, 2008). We suggest that the transcriptional response in wild-type cells does not involve symmetric contributions from the two MAPKs but instead involves a division of labor in which inhibition mediated primarily by Kss1 is gradually replaced by activation that is mediated primarily by Fus3 (Figure 8B).

Our findings also pertain to the function of Kss1 in filamentation responses. As with mating-specific transcription, induction of filamentation-specific transcription was correlated with conversion of 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 *fus3*Δ 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.*, 2011); and 2) many mating and filamentation genes are controlled by multiple response elements (Hagen *et al.*, 1991; Baur *et al.*, 1997; Chou *et al.*, 2006), and it is unknown whether gene activation requires all or only some Ste12 complexes to be 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,

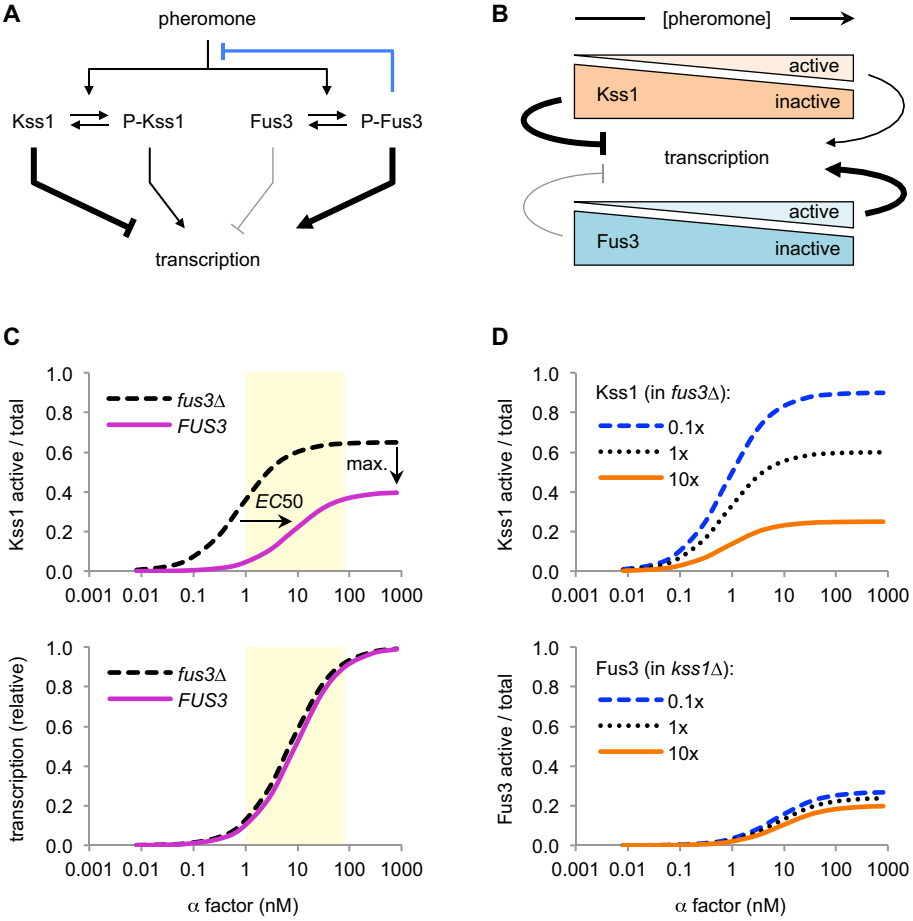

FIGURE 8: MAPK activation behavior and control of transcription. (A) General circuit by which pheromone controls phosphorylation of MAPKs and downstream transcriptional outputs. Fus3 and Kss1 may differ in the relative potency of repressive vs. activating effects on transcription. For simplicity, negative feedback is shown acting upstream of Fus3, but it could also promote Fus3 dephosphorylation (or both). (B) Schematic diagram illustrating that, in wild-type cells, the two MAPKs contribute asymmetrically to transcriptional induction: as pheromone concentrations increase, the strong inhibition from Kss1 is gradually replaced by strong activation from Fus3. For Kss1 to promote transcription, it must be hyperactivated beyond the degree seen in wild-type cells. (C) Idealized summary of observations regarding Kss1 activation behavior and transcriptional output as a function of pheromone dose. (The observed behaviors were assumed to follow a simple Hill function, $y = [x]/[x + \text{EC50}]$.) Comparing *FUS3* with *fus3*Δ cells suggests that negative feedback from Fus3 has two effects on Kss1 activation: increased EC50 and reduced maximum. In contrast, the EC50 for transcription is not strongly altered. The yellow region highlights the dose range over which the transcription response changes from 10% to 90% of maximum. In *fus3*Δ cells, levels of active Kss1 change by only approximately twofold over this range, and transcription is minimal when active Kss1 fraction is comparable to the peak levels seen in *FUS3* cells. (D) Idealized summary of observations regarding the effect of changing MAPK concentration on the active fraction. The active fraction of Kss1 is highly sensitive to concentration; this might suggest that the upstream kinase (Ste7) is saturated with substrate. By comparison, the active fraction of Fus3 is relatively insensitive to concentration. See the text for discussion.

respectively. In this view, the misaligned EC50s for transcription and Kss1 activation seen in *fus3*Δ 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 *fus3*Δ 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 *fus3*Δ 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.

Strain bkgd.[a]	Name	Relevant genotype	Source
a	PPY640	*MAT**a** FUS1::FUS1-lacZ::LEU2*	Pryciak and Huntress (1998)
a	PPY858	*MAT**a** FUS1::FUS1-lacZ::LEU2 ste5::ADE2*	Pryciak and Huntress (1998)
a	PPY861	*MAT**a** ste5::ADE2*	This study
a	PPY1173	*MAT**a** FUS1::FUS1-lacZ::LEU2 fus3::LEU2 kss1::ura3^{FOA}*	Winters *et al.* (2005)
a	PPY1667	*MAT**a** FUS1::FUS1-lacZ::LEU2 ste5::ADE2 fus3::LEU2*	This study
a	PPY1669	*MAT**a** FUS1::FUS1-lacZ::LEU2 ste5::ADE2 kss1::ura3^{FOA}*	This study
a	PPY2335	*MAT**a** ste5::ADE2 fus3Δ::natMX6*	This study
a	PPY2365	*MAT**a** ste5::ADE2 bar1Δ::hphMX6*	This study
a	PPY2367	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 fus3Δ::natMX6*	This study
a	PPY2387	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6*	This study
a	PPY2389	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 kss1Δ::kanMX6*	This study
a	PPY2408	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6 kss1::natMX6::P_{CYC1}-KSS1*	This study
a	PPY2409	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6 kss1::natMX6::P_{ADH1}-KSS1*	This study
a	PPY2411	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 fus3Δ::kanMX6 kss1::natMX6::P_{GPD1}-KSS1*	This study
a	PPY2412	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 kss1Δ::kanMX6 fus3::natMX6::P_{CYC1}-FUS3*	This study
a	PPY2413	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 kss1Δ::kanMX6 fus3::natMX6::P_{ADH1}-FUS3*	This study
a	PPY2415	*MAT**a** ste5::ADE2 bar1Δ::hphMX6 kss1Δ::kanMX6 fus3::natMX6::P_{GPD1}-FUS3*	This study
b	PPY966	*MAT**a***	Lamson *et al.* (2002)
b	PPY968	*MAT**a** tec1::HIS3*	This study
b	PPY1276	*MAT**a** fus3::LEU2*	This study
b	PPY1284	*MAT**a** ste5::HIS3*	This study
b	PPY1285	*MAT**a** ste5::HIS3 fus3::LEU2*	This study
b	PPY1608	*MAT**a** kss1::ura3^{FOA}*	This study
b	PPY1612	*MAT**a** fus3::LEU2 kss1::ura3^{FOA}*	This study
b	PPY2484	*MAT**a** tec1::HIS3 fus3Δ::kanMX6*	This study

[a]Strain background: (a) W303 [ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1]; (b) Σ1278b [his3::hisG leu2::hisG trp1::hisG ura3-52].

TABLE 1: Yeast strains used in 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 *Sac*I-*Xba*I 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 OD_{660} of each culture was recorded, and then

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 PGU1pr-lacZ	Roberts *et al.* (2000)
pPP849	p2987	CEN ARS LEU2 KSS1pr-lacZ	Roberts *et al.* (2000)
pPP850	p2988	CEN ARS LEU2 YLR042Cpr-lacZ	Roberts *et al.* (2000)
pPP851	p3017	CEN ARS LEU2 SVS1pr-lacZ	Roberts *et al.* (2000)
pPP852	p3018	CEN ARS LEU2 FUS3pr-lacZ	Roberts *et al.* (2000)
pPP854	p3058	CEN ARS LEU2 FUS1pr-lacZ	Roberts *et al.* (2000)
pPP855	p3079	CEN ARS LEU2 PRM3pr-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 KSS1pr-lacZ	Roberts *et al.* (2000)
pPP1038	p3058-T	CEN ARS TRP1 FUS1pr-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	pS5kmyc	CEN ARS URA3 STE5-myc$_{13}$ T$_{CYC1}$	Winters *et al.* (2005)
pPP2861	pS5kmyc-VASP	CEN ARS URA3 ste5(V763A S861P)-myc$_{13}$ T$_{CYC1}$	This study
pPP4007	pGA1840	CEN ARS TRP1 P$_{TPI1}$-FUS3-wt	Gartner *et al.* (1992)
pPP4008	pGA1881	CEN ARS TRP1 P$_{TPI1}$-fus3(Y182F)	Gartner *et al.* (1992)
pPP4009	pGA1894	CEN ARS TRP1 P$_{TPI1}$-fus3(T180A)	Gartner *et al.* (1992)
pPP4010	pGA1895	CEN ARS TRP1 P$_{TPI1}$-fus3(T180A Y182F)	Gartner *et al.* (1992)
pPP4014	YCpU-KSS1	CEN ARS URA3 KSS1-wt	Bardwell *et al.* (1998)
pPP4016	YCpU-KSS1-Y185F	CEN ARS URA3 kss1(Y185F)	Bardwell *et al.* (1998)
pPP4017	YCpU-KSS1-AEF	CEN ARS URA3 kss1(T183A Y185F)	Bardwell *et al.* (1998)
pPP4018	YCpU-KSS1-T183A	CEN ARS URA3 kss1(T183A)	Bardwell *et al.* (1998)
pPP4019	YEpU-FT1Z	2 μm URA3 FRE[TEC1]-lacZ	Sabbagh *et al.* (2001)
pPP4036	pFA6a-nat-Pcyc1	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)

TABLE 2: Plasmids used in this study.

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 $MgSO_4$, 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_2CO_3, 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_{420}. β-Galactosidase activity was calculated as $(1000 \times OD_{420}) \div (OD_{660} \times \text{culture volume [ml]} \times \text{reaction time [min]})$.

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.*, 2006), using 10% acrylamide (29:1 acrylamide:bisacrylamide), 50 μM $MnCl_2$, 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 chemilluminescence 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://imagej.nih.gov/ij/).

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