

# Differential input by Ste5 scaffold and Msg5 phosphatase route a MAPK cascade to multiple outcomes

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**Pathway specificity is poorly understood for mitogen-activated protein kinase (MAPK) cascades that control different outputs in response to different stimuli. In yeast, it is not known how the same MAPK cascade activates Kss1 MAPK to promote invasive growth (IG) and proliferation, and both Fus3 and Kss1 MAPKs to promote mating. Previous work has suggested that the Kss1 MAPK cascade is activated independently of the mating G protein (Ste4)–scaffold (Ste5) system during IG. Here we demonstrate that Ste4 and Ste5 activate Kss1 during IG and in response to multiple stimuli including butanol. Ste5 activates Kss1 by generating a pool of active MAPKKK (Ste11), whereas additional scaffolding is needed to activate Fus3. Scaffold-independent activation of Kss1 can occur at multiple steps in the pathway, whereas Fus3 is strictly dependent on the scaffold. Pathway specificity is linked to Kss1 immunity to a MAPK phosphatase that constitutively inhibits basal activation of Fus3 and blocks activation of the mating pathway. These findings reveal the versatility of scaffolds and how a single MAPK cascade mediates different outputs.**

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## Introduction

Mitogen-activated protein kinase (MAPK) cascades form the cores of many receptor-linked signaling pathways in eukaryotes and respond to many external stimuli. The same MAPK cascade can be used by more than one signal transduction pathway to effect different responses, raising the question of how pathway specificity is achieved (Madhani and Fink,

1998; Tan and Kim, 1999). One level of pathway specificity arises from variations in the strength and duration of activation of a MAPK cascade by stimulus (Traverse *et al*, 1992; Marshall, 1995), which is influenced by the level of MAPK phosphatase (Bhalla *et al*, 2002).

Scaffold proteins also provide specificity (Yu *et al*, 1998; Burack and Shaw, 2000; Elion, 2001; Roy *et al*, 2002; Weston and Davis, 2002). In budding yeast, the Ste5 and Pbs2 scaffolds join different subsets of the same kinases to different plasma membrane anchors that sense different stimuli (Figure 1A; Choi *et al*, 1994; Whiteway *et al*, 1995; Maeda *et al*, 1995; Posas and Saito, 1997). Despite this overlap, each scaffold provides specificity based on the distinct phenotypes of *ste5* and *pbs2* null mutants. Ste5 binds the  $\beta$  subunit (Ste4) of a heterotrimeric G protein that is coupled to mating pheromone receptors, and joins MAPKKK Ste11 and MAPKK Ste7 with MAPK Fus3, which promotes mating in response to mating pheromone. Pbs2 binds Sho1, a plasma membrane sensor, and joins Ste11 with itself, a MAPKK, and MAPK Hog1, which promotes survival in high osmolarity. In each case, binding of scaffold to membrane-linked anchor is thought to facilitate activation of Ste11 by Ste20, a p21-activated kinase (PAK) that is anchored to Cdc42 GTPase in an active state. The scaffolds therefore specify two events, MAPKKK activation in response to stimulus and linkage of the activated MAPKKK to MAPKK and MAPK. This three-tier model of kinases with scaffold is also used in other pathways (Burack and Shaw, 2000; Morrison and Davis, 2003).

The Ste20, Ste11, and Ste7 kinases also activate the Kss1 MAPK to promote invasive growth (IG) in haploid cells, pseudohyphal development (PD) in diploid cells, and cell integrity during vegetative growth (sterile vegetative growth (SVG)) (Figure 1A; Madhani and Fink, 1998; Lee and Elion, 1999; Pan *et al*, 2000). They also activate Kss1 during mating (Gartner *et al*, 1992; Cherkasova *et al*, 2001; Sabbagh *et al*, 2001). IG and mating pathway specificity is linked to the ability of Kss1 and Fus3 to recognize distinct substrates such as different transcription factors (Elion *et al*, 1993; Madhani and Fink, 1997a; Bretkreutz and Tyers, 2002). This configuration of a MAPKKK and MAPKK activating multiple MAPKs is a common feature of MAPK cascades, and includes human extracellular regulated kinases (Erks) and c-Jun N-terminal kinases (Jnks) (Widmann *et al*, 1999; Pearson *et al*, 2001).

It is not understood how Kss1 is selectively activated during proliferative responses. Both IG and PD are induced by activated Ras2 and by nutritional changes, which has led to the hypothesis that these conditions activate the Kss1 MAPK cascade (Gimeno *et al*, 1992; Roberts and Fink, 1994; Mosch *et al*, 1996; Pan *et al*, 2000). Kss1 is activated by mutations in Och1, which is a mannosyltransferase required for synthesis of mannoproteins (Lee and Elion, 1999) and may be upregulated by Sho1 (Cullen *et al*, 2000). Since genetic evidence places Ras2 and Sho1 upstream of Ste11 and

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Och1 in parallel with Ste11 (Figure 1A), these regulators are unlikely to be part of a mechanism that distinguishes between Kss1 and Fus3 activation. The mating receptor, G protein, and Ste5 scaffold are not required for IG (Roberts and Fink, 1994). These observations led to the hypothesis that specificity is provided by an alternative scaffold that selectively activates a Ste11, Ste7, and Kss1 MAPK cascade in response to Ras2 input (Roberts and Fink, 1994).

The question of whether or not Ste5 regulates Kss1 during IG is unresolved. During mating, Kss1 is activated by the same receptor–G protein–Ste5 scaffold system that activates Fus3 (Ma *et al*, 1995; Breikreutz *et al*, 2001; Cherkasova and Elion, 2001; Sabbagh *et al*, 2001), but is subsequently attenuated by activated Fus3 (Sabbagh *et al*, 2001) (Figure 1A). Downregulation of Kss1 by Fus3 has been proposed to block erroneous crossactivation of the IG pathway during mating (Madhani *et al*, 1997b; Sabbagh *et al*, 2001). However, the fact that low levels of mating pheromone induce IG-like responses in a wild-type strain (Oehlen and Cross, 1998; Roberts *et al*, 2000; Erdman and Snyder, 2001) suggests that Ste5 could normally activate a Kss1-dedicated pathway as long as Fus3 activity is below a certain threshold. To date, a systematic analysis of positive regulators of basal Kss1 and Fus3 kinase activity has not been done, nor has it been established that conditions that induce IG lead to changes in Kss1 kinase activity.

In this study, we screened for upstream regulators of the Kss1 MAPK cascade and identified Ste4 and Ste5 as key positive regulators of Kss1 activation during vegetative and IG, with little contribution by Ras2 and Sho1. Ste4 and Ste5 also activated Fus3 during vegetative and IG. Pathway specificity was linked to constitutive inactivation of Fus3 by MAPK phosphatase Msg5. Our results support a model in which a single-core G protein–scaffold–kinase pathway responds to multiple stimuli and regulates two functionally distinct MAPKs, with a MAPK phosphatase determining the threshold for entry into two developmental choices. Interestingly, Ste5 provides different functions in activating the two MAPKs. Fus3 is dependent on multiple tethering functions of the scaffold and is likely to be activated on the scaffold. In contrast, Kss1 can be activated off of the scaffold by an active pool of Ste11 that is generated as a result of Ste5 recruitment to Ste4. This novel mechanism provides a means by which signal amplification might occur as a result of scaffold-induced activation.

## Results

### **Ste4 and Ste5 are essential for basal activation of Kss1, while Ras2, Sho1, Gpr1, and Gpa2 are not**

To identify positive regulators of the SVG pathway, we screened a library of yeast null mutants in the S288c strain background using the *FRE* (filamentation and invasion response element) (*Tec1*)-*lacZ* reporter gene (Madhani and Fink, 1997a). Control tests showed that the *FRE(Tec1)-lacZ* reporter was a sensitive monitor for both SVG and IG pathways under basal and induced conditions. *FRE(Tec1)-lacZ* expression was reduced by a *kss1Δ* mutation and increased by *fus3Δ* and *hog1Δ* mutations that abolish attenuating mechanisms (Figure 1B). Furthermore, *FRE(Tec1)-lacZ* expression was stimulated in an *och1Δ* strain with reduced cell integrity (Lee and Elion, 1999) and in low glucose

conditions (i.e. 2% glycerol/2% ethanol) that induce IG (Cullen and Sprague, 2000). Initial screening showed that *FRE(Tec1)-lacZ* expression is not regulated by a wide variety of plasma membrane-linked receptors and other proteins known to sense environmental conditions (Figure 1C; data not shown). However, a reproducible 50% reduction in  $\beta$ -galactosidase activity was found in strains that lacked Ras2, Sho1, Gpa2 or Gpr1, indicating that these proteins weakly stimulate but are not essential for signaling through the Kss1 MAPK cascade during VG in rich medium. This finding was consistent with the observation that a Ras2<sup>A19V</sup> mutant did not stimulate the activation of Kss1 to an obvious extent (Supplementary Figure 1).

In contrast, *FRE(Tec1)-lacZ* expression was abolished in S288c strains that lack either the G $\beta$  subunit Ste4 (*ste4Δ*) or the MAPK cascade scaffold Ste5 (*ste5Δ*), whereas mutation of the mating pheromone receptor Ste2 (*ste2Δ*) had no effect (Figure 1C). Similar results were found in W303 strains (data not shown). Ste4 and Ste5 were as critical as Ste20, Ste11, and Ste7, and more critical than Ste50, which positively regulates Ste11, and is essential for IG (Ramezani Rad *et al*, 1998). The requirement for Ste4 and Ste5 in basal activation of Kss1 was confirmed by immunoblot analysis of the level of active Kss1 protein in whole-cell extracts. Activated Kss1 was detected with an anti-MAPK antibody directed against dual phosphorylated forms of mammalian p42/p44 that crossreacts with activated Kss1 and Fus3, and compared to the total level of Kss1 protein with an anti-Kss1 antibody. The level of active Kss1 was nearly abolished in the *ste4Δ* and *ste5Δ* strains, but was slightly elevated in the *ste2Δ* mutant lacking the  $\alpha$  factor mating pheromone receptor (Figure 1D).

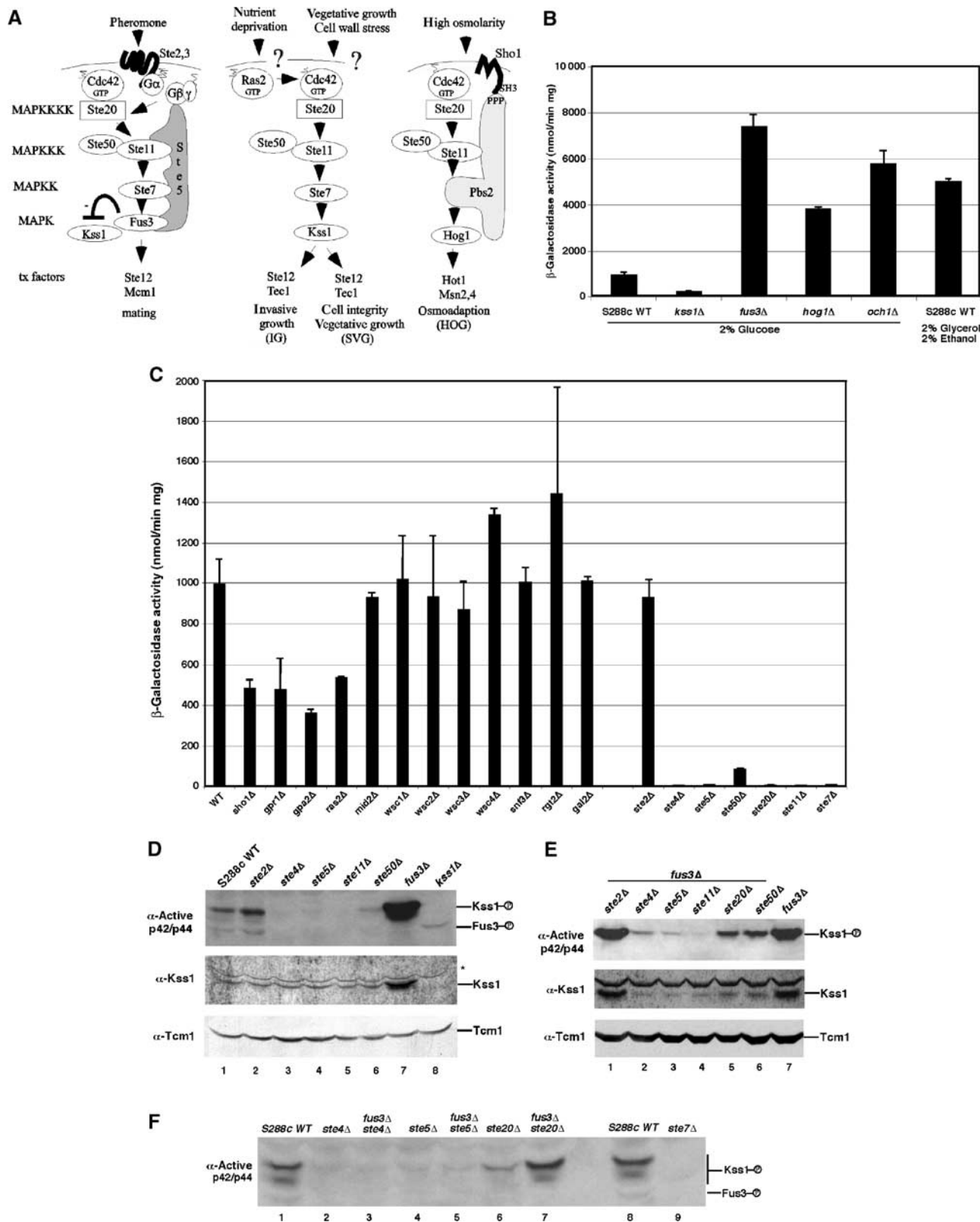
It was possible that the requirement for Ste4 and Ste5 was indirectly due to negative regulation of Kss1 levels by inactive Fus3, which blocks accumulation of active Kss1 and Kss1 protein (Cherkasova *et al*, 2003). However, Ste4 and Ste5 were required to activate Kss1 in a *fus3Δ* strain, which has greatly elevated levels of active Kss1 and Kss1 protein as a result of the loss of attenuation by Fus3 (Figure 1E). Active Kss1 was barely detected in *fus3Δste4Δ* and *fus3Δste5Δ* double mutants compared to a large amount of active Kss1 in the *fus3Δ* single mutant and residual active Kss1 in *ste20Δ fus3Δ* and *ste50Δ fus3Δ* double mutants (Figure 1E). The level of active Kss1 was as reduced in *ste5Δ fus3Δ* and *ste4Δ fus3Δ* double mutants as it was in *ste5Δ* and *ste4Δ* single mutants (Figure 1F). Therefore, signaling through the Kss1 MAPK cascade during proliferation is directly dependent on Ste4 and Ste5 and not due to a change in the status of Fus3.

### **Ste4 and Ste5 activate Kss1 under conditions that stimulate IG**

A prediction of our findings was that Ste4 and Ste5 should be required for Kss1 to be activated during conditions that induce IG. No direct evidence exists for stimulus-induced effects on Kss1 activation during proliferation. We tested three conditions that induce IG: growth on solid agar plates containing 2% glucose (Roberts and Fink, 1994), glucose depletion (Cullen and Sprague, 2000), and 1-butanol induction, which induces morphological changes resembling, but not identical to, pseudohyphal growth (Lorenz *et al*, 2000a; Zeitlinger *et al*, 2003). Assessment of IG in S288c strains in which the naturally occurring mutation in the *FLO8* gene was corrected with a *FLO8* plasmid (Liu *et al*, 1996) showed that

*ste4Δ* and *ste5Δ* strains were as deficient in invading the agar as *flo11Δ* and *ste20Δ* strains (Figure 2A and B). Kss1 activation was not detected in *ste4Δ* and *ste5Δ* mutants grown in liquid medium containing 2% glycerol/2% ethanol, although these conditions increased the level of active Kss1 in the

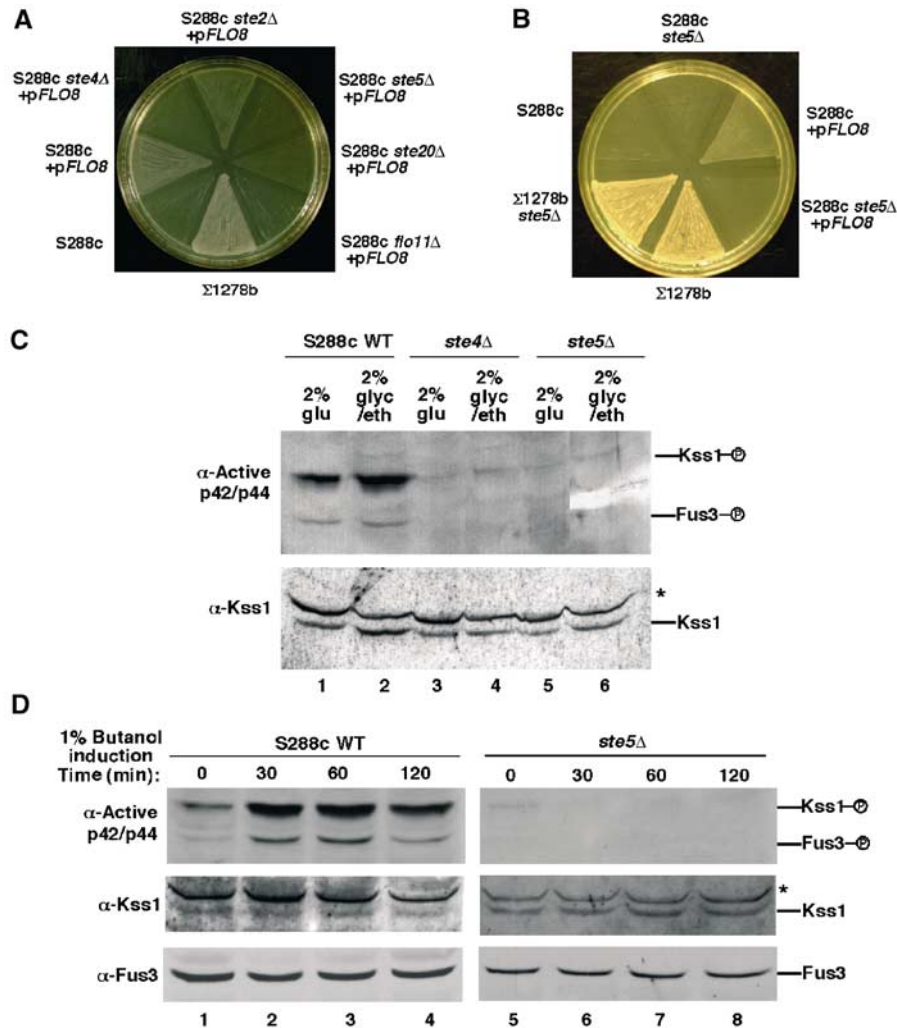
control strain (Figure 2C). Moreover, although 1-butanol induced the activation of both Kss1 and Fus3 within 30 min in the S288c wild-type strain (Figure 2D), no activation or morphological changes occurred in the *ste4Δ* and *ste5Δ* strains. Similar results were found in W303 (data not



shown). Thus, Ste4 and Ste5 are required for Kss1 to be activated by multiple stimuli that induce IG.

The requirement for Ste4 and Ste5 in activation of Kss1 during IG in S288c contrasted  $\Sigma$ 1278b, which does not require Ste4 or Ste5 for IG or SVG (Roberts and Fink, 1994; Lee and Elion, 1999).  $\Sigma$ 1278b shares only a small amount of genetic lineage with S288c (Winzeler *et al*, 2003) and undergoes significantly more robust IG compared to S288c/*FLO8*

strains (Figure 2A) (Liu *et al*, 1996). A comparison of Kss1 activation in  $\Sigma$ 1278b, S288c, and W303 showed that  $\Sigma$ 1278b does not require Ste5 to activate Kss1 and has significantly more active Kss1 and total Kss1 protein compared to S288c and W303, and a significantly greater ability to stimulate the *FRE(Tec1)-lacZ* reporter gene (Supplementary Figure 2A and B). The dependence on Ste5 for Kss1 activation in S288c was found to correlate with lower levels of basal activation of



**Figure 2** Activation of Kss1 during IG is dependent on Ste4 and Ste5. (A, B) IG of S288c strains harboring a *FLO8* plasmid requires *STE4* and *STE5*. (C) Enhancement of Kss1 activity as a result of growth in the absence of glucose requires *STE4* and *STE5*. Immunoblot analysis of extracts made from cells grown in liquid medium containing 2% glucose or 2% glycerol/2% ethanol. (D) 1-Butanol activates Kss1 and Fus3 in the presence of Ste4 and Ste5. S288c strains growing in liquid medium containing 2% glucose were induced with 1% 1-butanol for the indicated times.

**Figure 1** Activation of Kss1 during vegetative growth is dependent on the mating G protein  $\beta$  subunit (Ste4) and scaffold (Ste5), but not on upstream regulators of the high osmolarity growth (HOG), protein kinase A, protein kinase C, and sucrose nonfermenting pathways. (A) Cartoon of mating response, IG, SVG and HOG pathways in *Saccharomyces cerevisiae*. (B) *FRE(Tec1)-lacZ* reporter gene expression.  $\beta$ -Galactosidase assays were performed on mitotically dividing S288c strains harboring a *pFRE(TEC1)-lacZ* plasmid (Madhani and Fink, 1997a). Standard deviation is shown for triplicate samples. (C) *Fre(Tec1)-LacZ* activity in S288c strains with mutations in upstream regulators of a variety of protein kinase pathways. (D) Kss1 activity and protein in S288c strains with mating pathway mutations. (E) Kss1 activity and protein in S288c *fus3* $\Delta$  strains with mutations in the mating pathway. Immunoblot analysis was performed on whole-cell extracts prepared from vegetatively dividing cells. (F) Kss1 activity in *fus3* $\Delta$  and *ste* strains. The active form of Kss1 was detected with an anti-phospho p42/p44 peptide antibody and total Kss1 protein was detected with a polyclonal antibody. The asterisk indicates a protein that crossreacts nonspecifically with the Kss1 antibody. Tcm1 is a ribosomal protein that serves as a normalization control. Similar results were found in W303a. Depending on gel conditions, Kss1 migrates as a doublet when activated for unknown reasons.

Kss1 (Supplementary Figure 2C). The feral parent of S288c, EM93 (Mortimer and Johnston, 1986), had very low levels of active Kss1 (data not shown), suggesting that S288c and  $\Sigma$ 1278b reflect differences in basal signaling among naturally occurring yeast strains.

**Only the recruitment function of Ste5 that leads to Ste11 activation is required to activate Kss1**

The strict dependence of Kss1 activation on Ste4 and Ste5 in S288c and W303 and the absence of the requirement for Ste5 under conditions of higher basal activation in  $\Sigma$ 1278b raised the question of whether the G protein and Ste5 activate Kss1 and Fus3 by different mechanisms, since very little active Fus3 is detected in these strains. Ste5 activates Fus3 through multiple tethering functions, which can be divided into two key events: activation of Ste11 and activation of Fus3 by activated Ste11. Ste11 activation involves Ste5 binding to both Ste4 and Ste11 to facilitate phosphorylation of Ste11 by Ste20. Fus3 activation involves Ste5 binding to Ste11, Ste7, and Fus3, to facilitate activation of Ste7 and Fus3 (Choi *et al*, 1994; Inouye *et al*, 1997; Feng *et al*, 1998; Pryciak and Huntress, 1998; Bardwell *et al* 2000; Wang and Elion, 2003). To determine whether Ste5 activates Kss1 by a similar mechanism, we assessed whether Ste5 mutants with defects in binding to either Ste4 (Ste5<sup>C180A</sup>; Feng *et al*, 1998) or Ste11 (Ste5<sup>L482/485A</sup>; Wang and Elion, 2003) could activate Kss1. Neither Ste5<sup>C180A</sup> nor Ste5<sup>L482/485A</sup> supported the activation of Kss1 in either *ste5* $\Delta$  or *ste5* $\Delta$  *fus3* $\Delta$  strains, before or after induction with 1% butanol (Figure 3A). Therefore, Ste5 must be able to bind to both Ste4 and Ste11 in order to activate Kss1.

Two constitutively active derivatives of Ste11, Ste11-4 (Stevenson *et al*, 1992), and Ste11 $\Delta$ N (Cairns *et al*, 1992), were used to determine whether Kss1 must be bound to Ste5 to be activated by Ste11. Ste11-4 has a T596 $\rightarrow$ I mutation in subdomain VII (596TDFG599) of the catalytic domain and retains its ability to bind to Ste5. Ste11 $\Delta$ N lacks the auto-inhibitory N-terminal regulatory domain that binds Ste5. Ste11-4 activated Kss1 with equal efficiency in wild-type, *ste5* $\Delta$ , *ste5*<sup>C180A</sup>, and *ste5*<sup>L482/485A</sup> strains (Figure 3B, lanes 1–6). This behavior was similar to that of Hog1, which was also activated by Ste11-4 in the presence and absence of Ste5 (data not shown). In contrast, Ste11-4 did not activate Fus3 when Ste5 was either not present or unable to bind to Ste11-4 (i.e. *ste5* $\Delta$  and *ste5*<sup>L482/485A</sup>; Figure 3B, lanes 4, 6), but could activate Fus3 in pathway mutants that permitted binding of Ste11-4 to Ste5, including *ste5*<sup>C180A</sup> (Figure 3B, lane 5) and *ste4* $\Delta$ , *ste20* $\Delta$ , and *ste50* $\Delta$  (Figure 3C). The inability to detect active Fus3 in the absence of Ste5 was not a detection problem based on parallel analysis with Fus3-101 (Figure 3D), which autophosphorylates weakly in the absence of Ste5 (Brill *et al*, 1994). Furthermore, similar results were found with Ste11 $\Delta$ N, which activated Kss1 with equal efficiency in wild-type and *ste5* $\Delta$  strains, but did not activate Fus3 (Figure 3E).

These findings suggested that Kss1 does not need to bind to Ste5 to be activated by Ste11, whereas Fus3 must be bound. We tested this interpretation with a Ste5 $\Delta$ 241-336 mutant that lacks the binding site for Fus3 and Kss1 (Choi *et al*, 1994). Ste5 $\Delta$ 241-336 could support activation of Kss1 by Ste11-4; however, Fus3 was not activated (Figure 3F). Thus, Ste11 activates Fus3 while bound to Ste5, whereas Kss1 does not

have to be bound to Ste5 to be activated. This finding implies that Ste7 activates Fus3 within the Ste5 complex but activates Kss1 independently of Ste5.

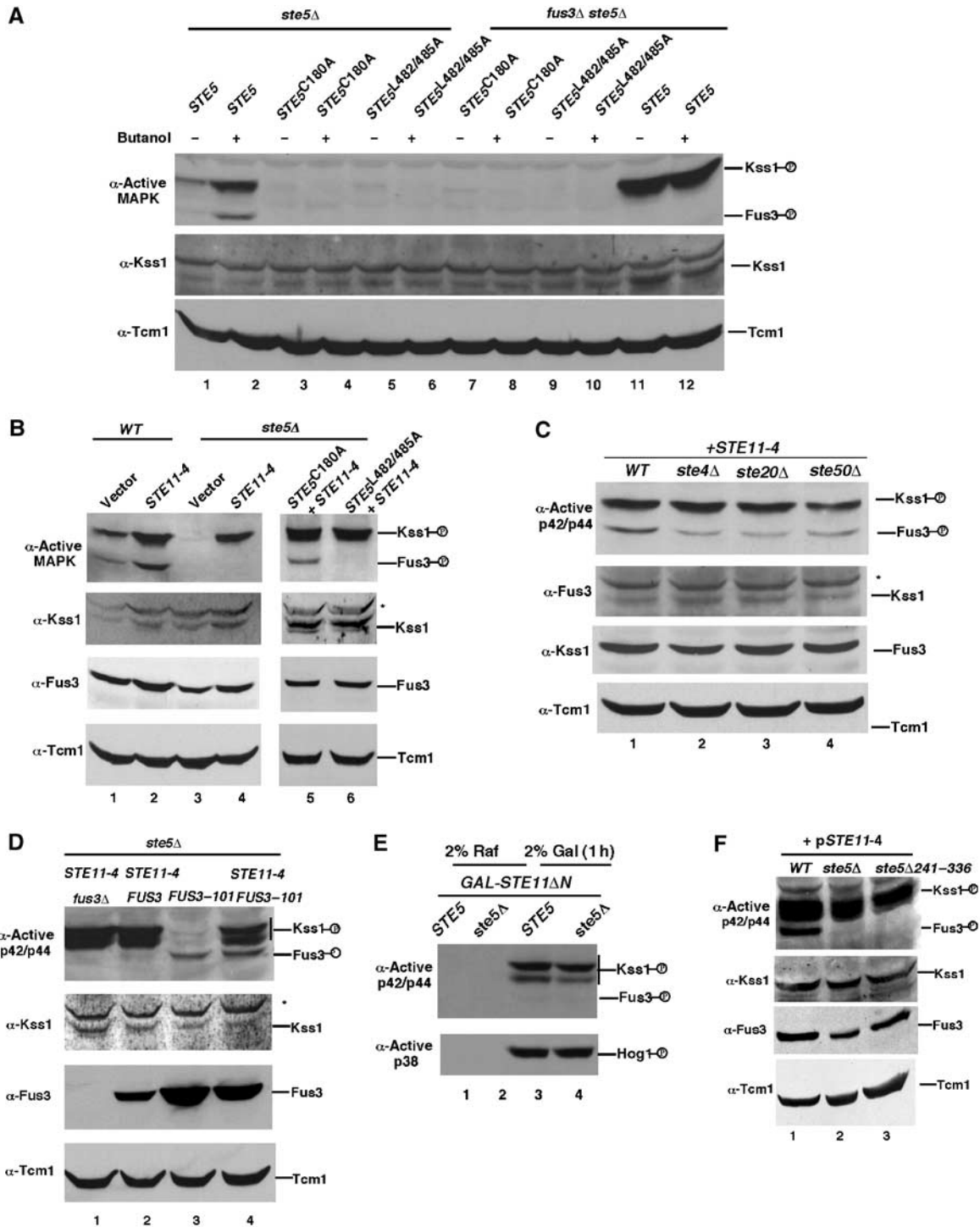
**Kss1 dependence on the Ste5 scaffold is bypassed by increasing the levels of Ste11 and Ste7**

Overexpression of Ste11 inhibits Fus3 activation unless Ste5 is also increased, which is consistent with Fus3 activation requiring a scaffold complex that contains Ste11 (Choi *et al*, 1994). We further tested the possibility that a scaffold is not required for Kss1 to be activated by Ste11, by determining whether increased levels of Ste11 or Ste7 would stimulate Kss1 activation in the absence of Ste5. Overexpression of either Ste11 or Ste7 increased the level of active Kss1 in a *ste5* $\Delta$  strain, with a nearly complete bypass when Ste11 and Ste7 were co-overexpressed (Figure 4A). Co-overexpression of Kss1 with Ste11 and Ste7 resulted in a further increase in active Kss1 (Figure 4B). In contrast, the level of active Fus3 did not increase under any of these conditions (Figure 4A and B). These results are consistent with active Ste11 and Ste7 being able to activate Kss1 independently of Ste5.

**Msg5 phosphatase inhibits constitutive activation of Fus3**

It was not clear how the same G protein-scaffold system selectively activates one MAPK during proliferation and two MAPKs during mating, since the only difference between these two states is pheromone activation of a receptor that is coupled to the G protein (Figure 1A). For example, the expression of Cdc42<sup>G12V</sup> increases the level of active Kss1 with negligible effect on Fus3, even though the stimulation of Kss1 requires Ste5 (Figure 4C). We tested whether the G protein-scaffold system activates Kss1 more efficiently than Fus3 by comparing the relative fold induction of Kss1 and Fus3 as a function of brief (15 min) exposure to physiological concentrations of mating pheromone ( $\alpha$  factor) and increasing the level of Ste5 in the absence of pheromone stimulation. The fold increase in Fus3 activation was greater than Kss1, with the preference for Fus3 greatest at highest  $\alpha$  factor concentration (Figure 5A and B). Increasing the level of Ste5 or expressing a hyperactive allele of Ste5, Ste5<sup>hyp1</sup> (Hasson *et al*, 1994) also caused a greater fold increase in the activity of Fus3 compared to Kss1 (Figure 5C and D). Therefore, the G protein-scaffold system is unlikely to activate Kss1 more efficiently than Fus3.

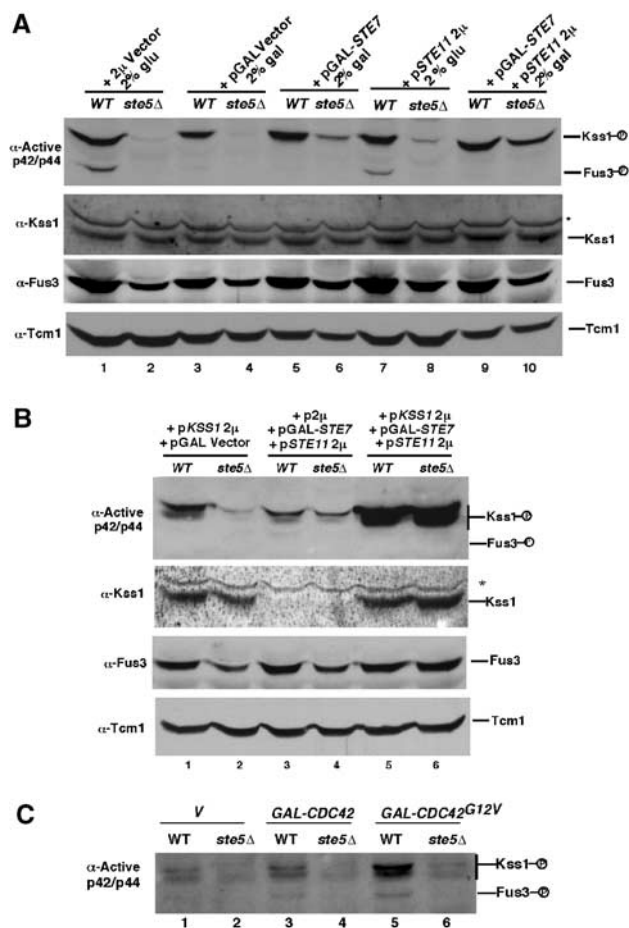
An alternative explanation for pathway specificity is that Fus3 and Kss1 are both basally activated by the G protein-scaffold system, but subsequent inactivation of Fus3 leaves Kss1 to effect responses. Fus3 is immediately inactivated upon mating pheromone withdrawal, consistent with it being subject to constitutive inactivation (Choi *et al*, 1999). To test this hypothesis, we compared the effect of mutations in the three major MAPK phosphatases (Msg5, Ptp2 and Ptp3) on the activity of Fus3 and Kss1 in addition to the two other MAPKs expressed in haploids, Hog1 and Mpk1. Msg5 is a dual-specificity MAPK phosphatase (Doi *et al*, 1994) that is thought to inactivate Fus3 during recovery from pheromone-induced G1 arrest (Doi *et al*, 1994), and also regulate Mpk1 (Martin *et al*, 2000) and Kss1 (Davenport *et al*, 2001). Ptp2 and Ptp3 are phospho-tyrosine phosphatases



**Figure 3** Kss1 only requires Ste5 to activate Ste11. (A) Kss1 activation is dependent on Ste5 binding to Ste4 and Ste11. Level of Kss1 activity in *ste5 $\Delta$*  and *ste5 $\Delta$  fus3 $\Delta$*  strains expressing Ste5, Ste5<sup>C180A</sup> or Ste5<sup>L482/485A</sup> from centromeric plasmids. The '+' indicates 1 h induction with 1% 1-butanol. (B) Ste11-4 activates Kss1 in the absence of Ste5. Levels of active Kss1 and Fus3 and total Kss1 and Fus3 proteins in wild-type and *ste5 $\Delta$*  cells expressing either a control vector or Ste11-4 from a centromeric vector. (C) Ste11-4 activates Kss1 and Fus3 in the absence of STE4, STE20, and STE50. (D) Ste5 is needed for Ste11-4 to activate FUS3-101. Active Kss1 and Fus3 in a *fus3 $\Delta$  ste5 $\Delta$*  strain expressing FUS3 or FUS3-101, with or without STE11-4. (E) Ste11 $\Delta$ N activates Kss1, but not Fus3, in the absence of Ste5. Ste11 $\Delta$ N was expressed from the GAL1 promoter. Cells were grown in 2% raffinose medium and then in 2% galactose medium for 1 h. The abundance of Kss1 and Fus3 is lower in 2% raffinose medium than in 2% galactose medium, resulting in very low basal activity. (F) Ste5 $\Delta$ 241-336 supports activation of Kss1, but not Fus3, by Ste11-4. Extracts were prepared from the *S288c ste5 $\Delta$*  pSTE11-4 strain with *CEN* vectors expressing Ste5 (lane 1), Ste5 $\Delta$ 241-336 (lane 3) or a control vector (lane 2).

(Watanabe *et al*, 1995) that have been linked to Fus3, Kss1 (Zhan *et al*, 1997) and Hog1 (Wurgler-Murphy *et al*, 1997; Mattison *et al*, 1999).

The *msg5 $\Delta$*  mutation caused a large increase in the levels of active Fus3 and Mpk1 during vegetative growth, with no obvious effect on the amount of active Kss1 or active



**Figure 4** Fus3, but not Kss1, must bind to Ste5 to be activated by Ste11. (A) Overexpression of either Ste11 or Ste7 bypasses the need for Ste5 in basal activation of Kss1. Wild-type and *ste5* $\Delta$  strains harbor a vector expressing *STE11* from its native promoter (pSTE11 2 $\mu$ ) on a multicopy plasmid or *STE7* from the *GAL1* promoter (pGAL-STE7) on a centromeric plasmid. (B) Co-overexpression of Ste7, Ste11, and Kss1 completely bypasses the need for Ste5 to activate Kss1. Relative levels of active Kss1 and total Kss1 protein in wild-type and *ste5* $\Delta$  cells overexpressing Kss1, Ste7, and/or Ste11. The strong intensity of the Kss1 band in strains expressing KSS1 2 $\mu$  obscures the weak band of endogenous Kss1. (C) Cdc42<sup>G12V</sup> does not stimulate Kss1 activity in a *ste5* $\Delta$  strain. GAL1p-CDC42<sup>G12V</sup> was expressed for 1 h in medium containing 2% galactose.

Hog1 (Figure 5E, lanes 1 and 2). In contrast, the *ptp2* $\Delta$  and *ptp3* $\Delta$  mutations, alone or in combination, had little effect on the level of active Fus3 or Mpk1, whereas the level of active Kss1 increased somewhat and that of Hog1 increased greatly (lanes 3–6). The increase in Fus3 activity in the *msg5* $\Delta$  strain is not the result of activation of Mpk1, because Mpk1 does not positively regulate mating pathway activation (Lee and Elion, 1999). Furthermore, the greater increase in Fus3 activity compared to Kss1 may be an underestimate, because the anti-active MAPK antibody is directed against a region in p42/p44 that is more conserved in Kss1 than in Fus3 (Materials and methods). Therefore, the G protein–scaffold system efficiently activates Fus3 in addition to Kss1 during vegetative growth, with the relatively greater pool of active Kss1 the result of selective inactivation of Fus3 by Msg5.

### Differential input by Ste5 and Msg5 is critical for pathway specificity

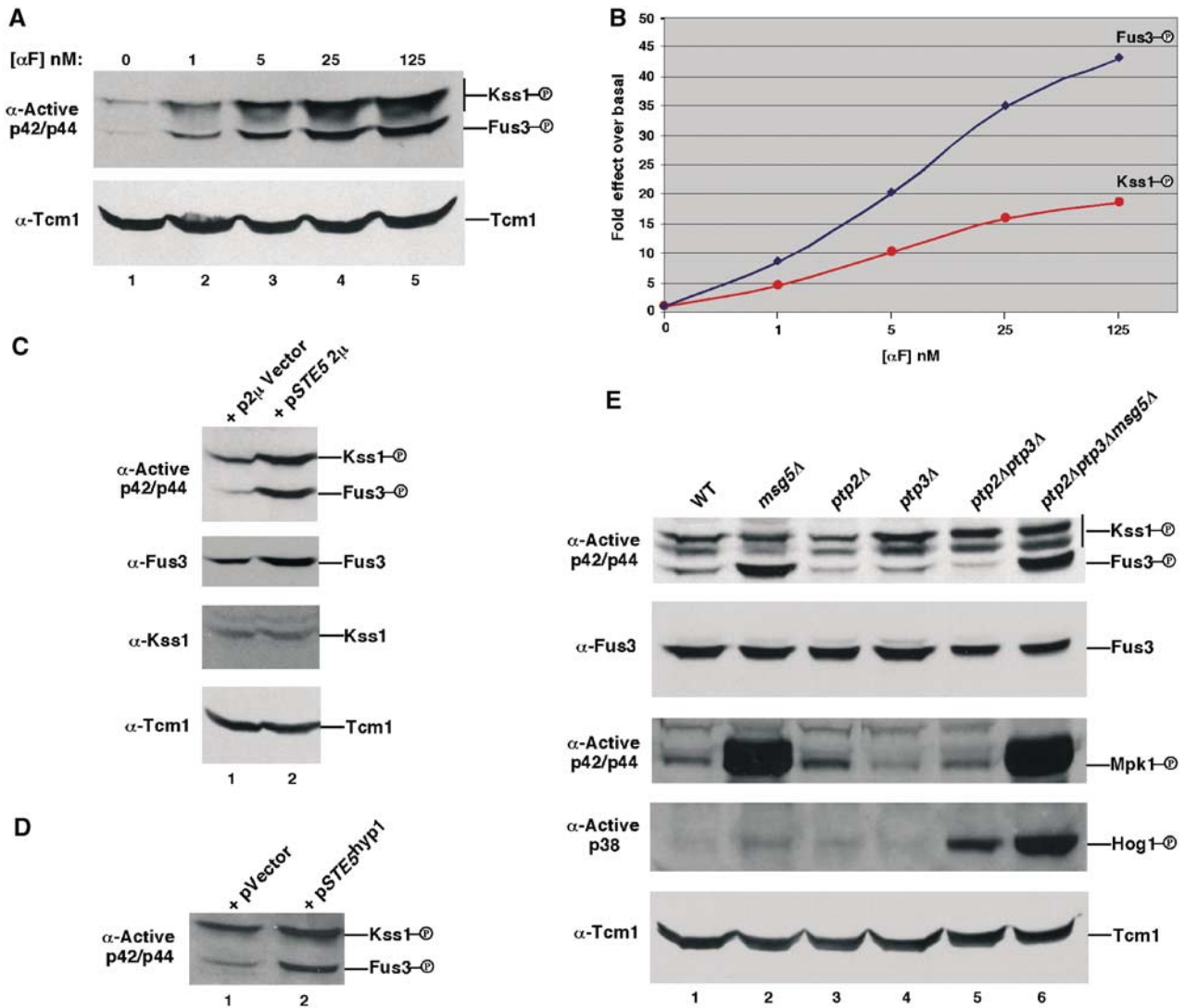
We determined whether Msg5 regulates pathway specificity by analyzing the phenotype of the *msg5* $\Delta$  mutant, using reporter genes and cell morphology as monitors. Loss of Msg5 constitutively activated the mating pathway, based on an increased level of active Fus3 (Figure 5E, lanes 1 and 2) and expression of *FUS1-lacZ* and *FUS1-GFP* reporter genes (Figure 6A), cell enlargement and morphological changes resembling shmoo formation in many cells (Figure 6A) and slower growth rate (data not shown). In contrast, IG was inhibited based on decreased expression of *FRE(Tec1)-lacZ* (Figure 6B), which correlated with a decreased level of active Kss1 (Figure 5E). This result is consistent with the inhibitory effect of Fus3 on IG and Kss1 activation as a result of its activation by pheromone (Sabbagh *et al*, 2001). Therefore, Msg5 prevents misactivation of the mating pathway and enforces the Kss1 fate.

The combined effect of Msg5 and Ste5 on pathway specificity during MAPK activation was further analyzed in *STE11-4* strains. The *msg5* $\Delta$  mutation, which increases the level of active Fus3 and Mpk1 compared to Kss1 (Figure 6C), also increased the degree of morphological changes resembling shmoos and caused a nearly complete cell cycle arrest compared to the *STE11-4* strain (Figure 6D). These phenotypes are consistent with the increased level of active Fus3 compared to Kss1, which likely results from coincident attenuation by Fus3. The introduction of a *ste5* $\Delta$  mutation into the *STE11-4* and *STE11-4 msg5* $\Delta$  strains abolished activation of Fus3 equivalently (Figure 6C) and restored efficient growth (Figure 6D) and a round budding morphology (Figure 6D). Although the *ste5* $\Delta$  mutation had no effect on Hog1 (data not shown), it did reduce the level of active Mpk1 (Figure 6C), which is activated as a consequence of morphological changes induced by mating pheromone (Zarzov *et al*, 1996; Buehrer and Errede, 1997). Thus, dual control of Fus3 and Kss1 by the G protein–Ste5 scaffold system, together with MAPK phosphatase selectivity, allows for proper differentiation and survival.

## Discussion

### Receptor-independent recruitment of a scaffold to G protein anchor stimulates a proliferative MAPK

Previous work defined the Ste20  $\rightarrow$  Ste11  $\rightarrow$  Ste7  $\rightarrow$  Fus3/Kss1 MAPK cascade as a mediator of mating, IG, and cell integrity, with specificity at the level of the MAPKs (Madhani and Fink, 1998; Breikreutz and Tyers, 2002). However, it has not been clear how different inputs into the same pathway selectively activate one MAPK (Kss1) to promote IG and both MAPKs (Fus3 and Kss1) to induce mating. Our genetic screen unexpectedly reveals that the Kss1 MAPK cascade utilizes the mating G protein and the Ste5 scaffold to signal during vegetative growth and IG, but not the receptor that is activated by mating pheromone. The dependence of Kss1 activation on the RING-H2 domain of Ste5 indicates that Kss1 is activated as a result of Ste5 recruitment to Ste4 in the absence of mating pheromone. These findings lead to a simplified view in which a single G protein-coupled scaffold system regulates one MAPK cascade under a variety of conditions and in the absence of an obvious external stimulus (Figure 6E). Inputs by other sensors of environment such as



**Figure 5** Fus3 is efficiently activated during vegetative growth, but is inhibited by Msg5 MAPK phosphatase. (A) Activation of Kss1 and Fus3 by mating pheromone. Vegetatively growing cells were treated with  $\alpha$  factor for 15 min. Parallel immunoblotting showed that the levels of Fus3 and Kss1 remained the same under these conditions (data not shown). (B) Fold effect of activation of Fus3 by  $\alpha$  factor is greater than that of Kss1 based on densitometric analysis. The level of active MAPK relative to total MAPK protein was determined by densitometry for each lane and the fold increase as a consequence of  $\alpha$  factor addition was plotted. Quantitation was done with the Scion image 1.62c densitometry program of the public domain software NIH image (available at <http://rsb.info.nih.gov/nih-image/>) using multiple exposures of immunoblots. (C) Effect of increasing Ste5 levels on Fus3 and Kss1 activity. (D) Effect of gain-of-function *STE5<sup>hyp1</sup>* mutant on Fus3 and Kss1. (E) Level of active Kss1, Fus3, Mpk1, and Hog1 in *mgs5 $\Delta$* , *ptp2 $\Delta$* , and *ptp3 $\Delta$*  MAPK phosphatase mutants. Kss1, Fus3, and Mpk1 were detected with anti-p42/p44 phosphopeptide antibody. Hog1 with anti-active p38 antibody. The positions of Mpk1 and Hog1 were deduced from *mpk1* and *hog1* mutants. The level of Kss1 protein did not change in the mutants compared to wild type (data not shown).

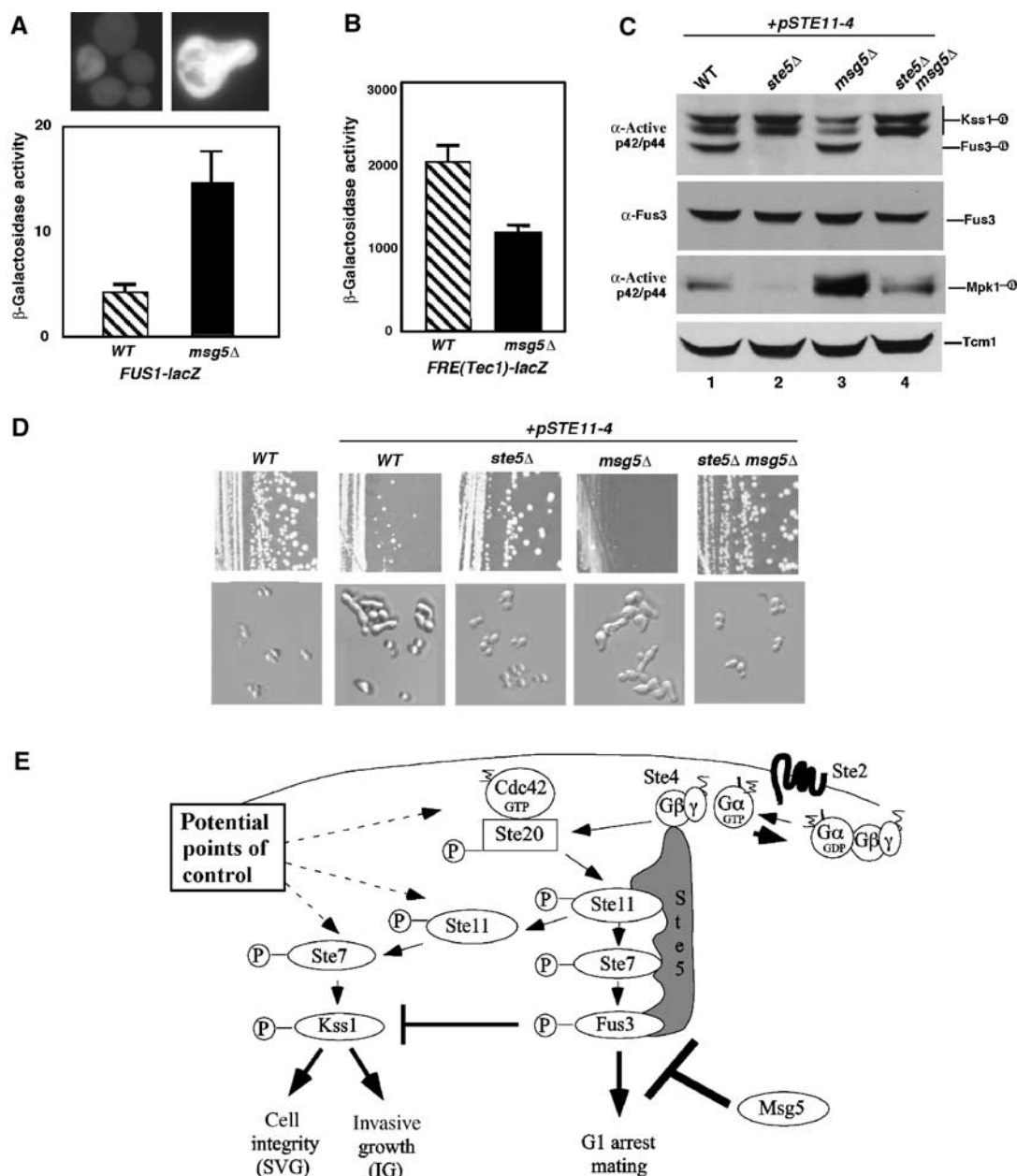
Ras2 and Sho1 may stimulate the Kss1 MAPK cascade, but to a much lesser degree than Ste4 and Ste5. Flexibility is achieved by a simpler role of Ste5 in Kss1 activation. While it is not possible to test the validity of release of active Ste11 from Ste5 in the physiological context of invasively growing cells, this interpretation is strongly supported by the behavior of specific Ste11 and Ste5 mutants, and the fact that activation by 1-butanol, activated Cdc42, and overexpressed Ste11 and Ste7 each cause preferential activation of Kss1 compared to Fus3.

#### Stimulation of the Kss1 MAPK cascade can occur at multiple points in the pathway

The results suggest that external and internal inputs could influence signaling through the Kss1/Fus3 MAPK cascade at

multiple steps; for example, by increasing the pool of active Ste20, the pool of free G $\beta$  $\gamma$  dimer, the level of Ste5 or the pool of active Ste11 or Ste7 (Figure 6E). Regulatory inputs that increase the pool of active Ste11 or Ste7 are predicted to be sufficient to stimulate Kss1 with no effect on Fus3, providing the potential for further flexibility in how the two MAPKs may be regulated. Furthermore, the absence of a requirement for Ste11 and Kss1 to bind to Ste5 for Kss1 to be activated suggests that the Ste5-independent signaling in  $\Sigma$ 1278b could occur without an alternative scaffold. The significantly higher level of basal activation of Kss1 in  $\Sigma$ 1278b raises the interesting possibility that the level of basal activation of the Kss1 pathway is a key determinant of whether a strain undergoes IG or PD. In contrast, the strict dependence of Fus3 for all of the tethering functions of Ste5 demonstrates that its activa-





**Figure 6** Differential regulation of Kss1 and Fus3 by Ste5 and Msg5 is required for pathway specificity. (A) Constitutive mating pathway activation in *msg5*Δ strain based on FUS1-lacZ, FUS1-GFP, and cell morphology. (B) Decreased *FRE(Tec1)-lacZ* expression in a *msg5*Δ strain. (C) Level of active Kss1, Fus3, and Msg5 in *STE11-4* strains with *ste5*Δ and/or *msg5*Δ mutations. (D) Growth and morphology of *STE11-4* strains with *ste5*Δ and/or *msg5*Δ mutations. Strains were streaked for single colonies on a SC-selective plate and incubated at room temperature. Nomarski images were taken of the cells. (E) Model for how a single G protein-scaffold and MAPK phosphatase system regulates IG and mating pathways.

tion mechanism is more dependent on direct contribution by the scaffold, less flexible, and more dependent on external pheromone stimulation.

#### MAPK phosphatase specificity limits pathway activation to Kss1 during growth

Previous work has shown that MAPK phosphatase can provide signaling flexibility by influencing the strength and duration of the effect of a given stimulus. Here, we show that constitutive inhibition of a MAPK by a specific MAPK phosphatase, Msg5, can determine the pathway fate. Although Msg5 was known to be important for pheromone-mediated inhibition of Fus3 (Doi *et al*, 1994), its involvement in basal

activation of Fus3 was not realized. Multiple lines of evidence support a role for Msg5 in pathway specificity, including a striking increase in the level of active Fus3 in the *msg5*Δ strain and phenotypes associated with mating differentiation. In addition, there is a decrease in active Kss1 and expression of the *FRE(Tec1)-LacZ* reporter gene, as predicted for an increased pool of active Fus3. These findings lead to the surprising conclusion that Ste5 normally activates Fus3 under a variety of growth conditions in the absence of pheromone stimulation, possibly to an even greater extent than Kss1. Msg5 constitutively inhibits Fus3, with no obvious effect on Kss1, allowing selective accumulation of active Kss1. The constitutive inhibition of Fus3 by Msg5 stabilizes signaling

through Kss1 under conditions meant to promote growth by preventing activation of mating pathway outputs and down-regulation of Kss1 by active Fus3. This interpretation is consistent with the observation that low levels of mating pheromone stimulate IG rather than mating differentiation.

Of the four MAPKs expressed in haploid cells, Kss1 is unique in that it is not dramatically affected by mutations in Msg5, Ptp2 or Ptp3 phosphatases, suggesting that it has evolved to be a default MAPK that is responsive to small changes in stimulus. Further work is needed to determine the molecular basis of this specificity. Our findings lead us to speculate that the level of Fus3 activation determines pathway specificity, with the level of stimulus required to achieve a pool of active Fus3 being set by the level of Msg5. When the level of Fus3 inhibition exceeds the level of pathway activation, such as under conditions of either no or weak stimulation, only Kss1 should accumulate. This appears to be the

case in glucose and glycerol/ethanol. Stronger stimuli such as 1-butanol and pheromone lead to the accumulation of active Fus3; however, at low levels of stimulation when the pool of active Fus3 is below a critical threshold (e.g. in 1-butanol and very low pheromone), cells are expected to divide and grow invasively rather than mate.

### Versatility of a MAPK scaffold in signaling

Previous work on Ste5 led to the hypothesis that it functions by joining together all the three kinases of a MAPK cascade (MAPKKK, MAPKK, MAPK) to allow MAPK activation on the scaffold. Similar models have been made for scaffolds in other pathways, including Pbs2, the JIPs, KSR and  $\beta$ -arrestin. Our analysis confirms that the MAPK can be activated on the scaffold. In addition, it shows that a MAPK cascade scaffold protein can be versatile by providing distinct functions that are differentially utilized by different MAPKs that share

**Table I** Yeast strains and plasmids used in this study

Strain name	Relevant genotype	Source or reference
<i>Isogenic to S288c</i>		
BY4741	<i>leu2 ura3 his3 met15</i>	Research Genetics
RG3042	<i>fus3Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG6981	<i>kss1Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG2724	<i>hog1Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG4406	<i>och1Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG6116	<i>sho1Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG3731	<i>gpr1Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG152	<i>gpa2Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG2978	<i>ras2Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG5241	<i>mid2Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG1784	<i>wsc1Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG1161	<i>wsc2Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG6255	<i>wsc3Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG935	<i>wsc4Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG7201	<i>snf3Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG3836	<i>rgt2Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG2692	<i>gal2Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG5645	<i>ste2Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG2468	<i>ste4Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG4038	<i>ste5Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG3439	<i>ste50Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG5271	<i>ste11Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG956	<i>ste20Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG3857	<i>ste7Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG1408	<i>sst1Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
RG5953	<i>flo11Δ::KAN<sup>r</sup> leu2 ura3 his3 met15</i>	Research Genetics
DSY28	<i>fus3Δ::KAN<sup>r</sup> ste2Δ::KAN<sup>r</sup></i>	This study
JAY458	<i>fus3Δ::KAN<sup>r</sup> ste4Δ::KAN<sup>r</sup></i>	This study
JAY408	<i>fus3Δ::KAN<sup>r</sup> ste5Δ::KAN<sup>r</sup></i>	This study
DSY19	<i>fus3Δ::KAN<sup>r</sup> ste11Δ::KAN<sup>r</sup></i>	This study
DSY34	<i>fus3Δ::KAN<sup>r</sup> ste20Δ::KAN<sup>r</sup></i>	This study
JAY493	<i>fus3Δ::KAN<sup>r</sup> ste50Δ::KAN<sup>r</sup></i>	This study
<i>Isogenic to W303a</i>		
EY957	<i>sst1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 GAL<sup>+</sup></i>	Elion <i>et al</i> (1993)
Y568	<i>trp1-1 his3-11,15 leu2-3,112 ura3-1</i>	Zhan <i>et al</i> (1997)
Y572	<i>ptp2Δ::LEU2 trp1-1 his3-11,15 leu2-3,112 ura3-1</i>	Zhan <i>et al</i> (1997)
Y567	<i>ptp3Δ::URA3 trp1-1 his3-11,15 leu2-3,112 ura3-1</i>	Zhan <i>et al</i> (1997)
Y578	<i>ptp2Δ::LEU2 ptp3Δ::URA3 trp1-1 his3-11,15 leu2-3,112 ura3-1</i>	Zhan <i>et al</i> (1997)
Y170	<i>msg5Δ::KAN<sup>r</sup> trp1-1 his3-11,15 leu2-3,112 ura3-1</i>	Zhan <i>et al</i> (1997)
Y173	<i>msg5Δ::KAN<sup>r</sup> ptp2Δ::LEU2 ptp3Δ::URA3 trp1-1 his3-11,15 leu2-3,112 ura3-1</i>	Zhan <i>et al</i> (1997)
DSY62	<i>ste5Δ::TRP1 trp1-1 his3-11,15 leu2-3,112 ura3-1</i>	This study
DSY63	<i>ste5Δ::TRP1 msg5Δ::KAN<sup>r</sup> trp1-1 his3-11,15 leu2-3,112 ura3-1</i>	This study
AFY49	<i>ste11Δ::ura3 ste5Δ::TRP1</i>	Flotho and Elion (submitted)
DSY116	<i>ste11Δ::ura3 ste5Δ::TRP1 fus3-8::ADE2</i>	This study
<i>Isogenic to Σ1278b</i>		
L5528	<i>his3::hisG ura3-52</i>	Roberts <i>et al</i> (1994)
L5554	<i>ste5Δ::HIS3<sup>+</sup></i>	Roberts <i>et al</i> (1994)

**Table 1** (Continued)

Plasmid name	Description	Source or reference
B1817	<i>HIS3 CEN</i>	Elion lab collection
B1819	<i>LEU2 CEN</i>	Elion lab collection
B1820	<i>URA3 CEN</i>	Elion lab collection
Yep13	<i>LEU2 2μ</i>	Elion lab collection
pDAD-2	<i>pGAL1,10 URA3 2μ</i>	Elion lab collection
pSKM49	<i>STE5-MYC9 CEN LEU2</i>	Elion lab collection
pYBS138	<i>STE5 URA3 CEN</i>	Kranz <i>et al</i> (1994)
pJB224	<i>STE5 LEU2 2μ</i>	Kranz <i>et al</i> (1994)
pCL-S5C180A-M9	<i>ste5<sup>C180A</sup>-MYC9 CEN LEU2</i>	A Flotho (unpublished)
pDJ174	<i>STE5<sup>typp1</sup> URA3 2μ</i>	Hasson <i>et al</i> (1994)
pDH27	<i>3'ste5(-509 to -13)::TRP1::5'ste5(+2395 to +2702)</i>	Leberer <i>et al</i> (1993)
pBN3	<i>STE11 URA3 2μ</i>	Elion lab collection
p11-4	<i>STE11-4 HIS3 CEN</i>	H Madhani
pYGL-11.1N	<i>pGAL1-STE11.1N LEU2 CEN</i>	Cairns <i>et al</i> (1992)
pJBK-008	<i>STE2 URA3 CEN</i>	Kanopka and Hartwell (1989)
Yep213-STE4	<i>STE4 LEU2 2μ</i>	Elion lab collection
pSTE20-5	<i>STE20 URA3 CEN</i>	Leberer <i>et al</i> (1992)
pMG5	<i>STE50 URA3 HIS3 CEN</i>	MR Rad
pXT1	<i>KSS1 LEU2 2μ</i>	Courchesne <i>et al</i> (1989)
pYEE81	<i>FUS3 URA3 CEN</i>	Elion <i>et al</i> (1990)
pJB237	<i>FUS3-101 LEU2 CEN</i>	Brill <i>et al</i> (1994)
pKCS5	<i>pGAL1-STE7-MYC HIS3 CEN</i>	Choi <i>et al</i> (1994)
YCpR2V	<i>RAS2<sup>A19V</sup> URA3</i>	Gimeno <i>et al</i> (1992)
pHL1	<i>FLO8 URA3 CEN</i>	Liu <i>et al</i> (1996)
B641	<i>pGAL1,10-HO URA3 LEU2 CEN</i>	Elion lab collection
BHM275	<i>FRE(TEC1)::LACZ URA3 2μ</i>	Madhani and Fink (1997)
FB80	<i>MATα</i>	Winston lab collection
pGAL1-42	<i>pGAL1-CDC42 LEU2 CEN</i>	D Johnson
pGAL1-VAL12	<i>pGAL1-CDC42<sup>G12V</sup> LEU2 CEN</i>	D Johnson
pYW-S5Δ241-336	<i>STE5(Δ241-336) URA3 CEN</i>	Elion lab collection

common upstream activators (Figure 6E). The recruitment function of Ste5 (defined here as binding of Ste5 to Ste4) serves both Kss1 and Fus3 by facilitating the activation of associated Ste11 by active Ste20 that is bound to Cdc42. However, the tethering function of Ste5 (defined here as the binding of multiple kinases in the MAPK cascade) is only essential for Fus3 activation. This conclusion agrees with the original biochemical evidence that Ste5 facilitates the activation of Fus3 as a multi-kinase complex and the observation that Fus3 of highest specific activity is associated with the scaffold (Kranz *et al*, 1994; Choi *et al*, 1994, 1999). The conclusion that the active form of Ste11 can dissociate from Ste5 and activate a pool of Ste7 and Kss1 that is not bound to scaffold is supported by the observation that a plasma membrane-localized form of Ste5 does not recruit Ste11 or Kss1 to the plasma membrane, although it is able to recruit Ste7 and Fus3 (van Drogen *et al*, 2001). Further work is needed to ascertain whether the pool of active Ste11 that is released from Ste5 is the same pool that phosphorylates Ste7 on the scaffold, and whether Ste11 is modified while on the scaffold.

It is noteworthy that the kinase that is not entrained to the scaffold is not sensitive to inhibition by Msg5, whereas the kinase that is strictly dependent on scaffold is subject to inhibition. The absence of a requirement for Ste11 to be bound to Ste5 during activation of Kss1, combined with a possible immunity to inhibition by phosphatase, may provide a means of amplifying the initial signal at multiple loci in the cell in response to small changes in stimulation. In contrast, the greater dependence of Fus3 on the scaffold may be tied to its functions that are not shared by Kss1 and are critical for mating differentiation, that is, cell cycle arrest, polarized growth and cell fusion, which may involve a localization

that is with or near Ste5 and potentially less subject to phosphatase inhibition. These possibilities are interesting to consider in light of the propensity of Msg5 to have access to Fus3 (Kholodenko *et al*, 2000). The fact that increasing the level of activating kinases in a cell can bypass the need for the recruitment function of a pathway scaffold supports the view that a key role of scaffolds is to enhance kinase-kinase interactions that are limited by diffusion. In addition, it reveals a new level of difficulty in assigning the appropriate pathway links for scaffold proteins *in vivo*.

## Materials and methods

### Strains, plasmids, media, microbiological techniques

Yeast strains and plasmids are listed in Table 1. Yeast strains are from three backgrounds, S288c (Research Genetics), Σ1278b (Roberts and Fink, 1994) or W303a (Elion lab). S288c deletion strains (Winzeler *et al*, 1999) were obtained from Research Genetics, Inc. Strains used for α factor induction were *bar1Δ* (*sst1Δ*) and lacked a protease that degrades α factor (Ciejek and Thorner, 1979). Standard microbiological and molecular biological techniques were used to transform *Escherichia coli*, prepare plasmid DNA, and transform yeast.

### Immunoblotting

Primary antibodies used in this study were anti-phospho p44/42 MAPK (Cell Signaling Technology, Inc, #9101), anti-Kss1 polyclonal anti-serum (Santa Cruz), anti-Fus3 polyclonal antiserum (Elion *et al*, 1993), and anti-Tcm1 monoclonal antibody (gift from J Warner, Albert Einstein College of Medicine). Secondary antibodies used were rabbit anti-goat Ig-HRP, goat anti-mouse Ig-HRP and goat-anti-rabbit Ig-HRP. The p42/p44 anti-active MAPK antibody is directed against a region in ERK1 that is more conserved in Kss1 than Fus3 (C Salvatore, personal communication, Cell Signaling Technologies).

Additional details can be found in Supplementary data.

**Supplementary data**

Supplementary data are available at *The EMBO Journal* Online.

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