Pheromone-induced morphogenesis and gradient tracking are dependent on the MAPK Fus3 binding to Ga

Beverly Errede, Lior Vered, Eintou Ford, Matthew I. Pena, and Timothy C. Elston

Department of Biochemistry and Biophysics, Department of Chemistry, and Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599

ABSTRACT Mitogen-activated protein kinase (MAPK) pathways control many cellular processes, including differentiation and proliferation. These pathways commonly activate MAPK isoforms that have redundant or overlapping function. However, recent studies have revealed circumstances in which MAPK isoforms have specialized, nonoverlapping roles in differentiation. The mechanisms that underlie this specialization are not well understood. To address this question, we sought to establish regulatory mechanisms that are unique to the MAPK Fus3 in pheromone-induced mating and chemotropic fate transitions of the budding yeast Saccharomyces cerevisiae. Our investigations reveal a previously unappreciated role for inactive Fus3 as a potent negative regulator of pheromone-induced chemotropism. We show that this inhibitory role is dependent on inactive Fus3 binding to the α-subunit of the heterotrimeric G-protein. Further analysis revealed that the binding of catalytically active Fus3 to the G-protein is required for gradient tracking and serves to suppress cell-to-cell variability between mating and chemotropic fates in a population of pheromone-responding cells.

INTRODUCTION

Cell fate decisions are mediated by growth factors that signal through mitogen-activated protein kinase (MAPK) pathways (Corson et al., 2003; Shilo, 2003, 2005). Depending on context, the same growth factor either induces or inhibits the ability of a cell to differentiate. In unicellular organisms such as budding yeast (Saccharomyces cerevisiae), mating pheromone specifies two alternative cell fates that are dependent on stimulus dosage (Figure 1). Haploid α and α cells in proximity to each other respond to the localized high dose of pheromone secreted by the opposite cell type (Schrick et al., 1997). Cells arrest in the G1 phase of the cell cycle and polarize their growth to form a specialized structure called a mating projection or “shmoo,” which is the site of contact and fusion between mating partners. When haploid cells are exposed to lower doses of pheromone, they transiently arrest in the G1 phase of the cell cycle and exhibit hyperpolarized growth that gives the cells an elongated, worm-like morphology. As cells reenter the cell cycle, they exhibit a polar rather than an axial budding pattern (Erdman and Snyder, 2001). We call this cell type “chemotropic” because growth is hyperpolarized in the direction of increasing pheromone concentration (Segall, 1993; Paliwal et al., 2007; Hao et al., 2008).

The developmental programs leading to either pheromone-induced mating differentiation or chemotropic growth depend on the identical signaling pathway (Figure 1; Dohlman and Thorner, 2001; Erdman and Snyder, 2001). This pathway is activated when peptide pheromone secreted by one cell type binds to its G-protein–coupled receptor in cells of the opposite cell type (α-factor to Ste2 in α cells or a-factor to Ste3 in α cells). The pheromone–receptor complex activates a heterotrimeric G-protein (Gα-Gαi1/Gβ-Ste4/Gγ-Ste18), which propagates the signal by activating a MAPK cascade. The scaffold-Ste5 organizes the core enzymes of the MAPK cascade by binding directly to the MAPKKK-Kss1, the MAPKK-Ste7, and the MAPK-Fus3. The MAPK-Kss1 is
part of the complex through its binding interaction with the MAPKKSte7. The scaffold-Ste5 also binds to G1-Ste4, and this interaction serves to colocalize the assembly with the p20-activated kinase (PAK) Ste20 (Whiteway et al., 1995; Leeuw et al., 1998). PAK-Ste20 functions in the MAPK module by phosphorylating and activating the MAPKKK-Ste11 (Wu et al., 1995; van Drogen et al., 2000).

In addition to mating projection formation and chemotropism, haploid yeast undergo a distinct transition from a vegetative to a pseudofilamentous polarized growth mode on rich medium in response to glucose depletion or high alcohol (for review, see Palecek et al., 2002; Truckses et al., 2004). Cells emerging from the periphery of colonies under these nutrient-limited conditions invade the substrate and form chains of highly elongated cells, resulting in an invasive growth phenotype. This transition requires the integration of MAPK and protein kinase A (PKA)-mediated responses. Without the exception of the receptors, G-protein, Fus3, and Ste5, the MAPK branch of the network has the same signaling components as the pheromone response pathway (Figure 1; Liu et al., 1993; Roberts and Fink, 1994).

Strains with different single or double deletions of FUS3 and KSS1 exhibit differences in their ability to differentiate, revealing specialized roles for the two MAPKs in the mating and filamentous growth programs (Breitkreutz and Tyers, 2002). Both Fus3 and Kss1 promote pheromone-induced transcription (Roberts et al., 2000; Breitkreutz et al., 2001). However, active Fus3 has a predominant role in mating differentiation, as most laboratory strains have more severe mating defects in fus3Δ than in kss1Δ mutants (Elion et al., 1991). This principal role is attributed to phosphoproteins essential for mating that are preferential substrates for Fus3 rather than Kss1 (Farley et al., 1999; Breitkreutz et al., 2001). For example, Fus3 more efficiently phosphorylates Far1 than does Kss1. This phosphorylation promotes Far1 function as a Cdc28-cyclin inhibitor and polarity complex scaffold during the mating response (Peter et al., 1993; Butty et al., 1998; Blondel et al., 1999; Shimada et al., 2000). In addition, Fus3 binding to Go-Gpa1 and phosphorylation of Bni1 facilitates pheromone-induced morphogenesis (Metodiev et al., 2002; Matheos et al., 2004). Active Kss1 is believed to be the principal MAPK required for promoting nutrient-regulated filamentous growth, whereas active Fus3 has an inhibitory role. This interpretation stems from the observation that haploid kss1Δ strains are not invasive, whereas fus3Δ strains invade more robustly than wild-type strains (Cook et al., 1997; Breitkreutz et al., 2003). Although the roles of Fus3 and Kss1 in the chemotrophic program are less well understood, both have a positive regulatory role. Strains with a single kss1Δ or fus3Δ mutation are completely defective (Erdman and Snyder, 2001). Again, Fus3 has additional roles in this developmental program that are not shared with Kss1. First, fus3Δ strains are defective in directing hyperpolarized growth toward increasing pheromone concentration (gradient tracking; Hao et al., 2008). Second, fus3Δ strains fail to restrict hyperelongation to conditions of low pheromone concentrations (Esh et al., 2006; Hao et al., 2008).

Although the three morphology transitions described in the foregoing are mediated in whole or in part by the signaling cascade dependent on the MAPKK-Ste7 and the transcription factor Ste12, the developmental outcome depends on whether Fus3, Kss1, or both are active. Specifically, Fus3 but not Kss1 is required for proper shmoo formation (Farley et al., 1999; Matheos et al., 2004) and gradient tracking (Hao et al., 2008), whereas Kss1 appears to be required for cells to respond properly to low pheromone concentration (Palival et al., 2007; Hao et al., 2008) and nutrient limitation (Cook et al., 1997; Breitkreutz et al., 2003). Similarly, mammalian Erk1 and Erk2 are MAPK isoforms that are coactivated by different stimuli, but it is becoming increasingly apparent that these isoforms have specialized roles in determining developmental fates (Sarbassov et al., 1997; Fischer et al., 2005; Shin et al., 2010; He et al., 2011; Chang et al., 2012). The mechanisms that underlie these differences among MAPK isoforms have not been fully established. To address this issue, we compared the pheromone-induced responses of transcription, G1 arrest, and morphogenesis under conditions in which Kss1 is active either in the absence of Fus3 (fus3Δ) or the presence of a Fus3 variant that is inactive because it cannot be phosphorylated (fus3ΔA180F182); Gartner et al., 1992). We found that the cells responded differently, depending on whether they lacked Fus3 completely or expressed the Fus3 variant. Further investigation of the phenotypic differences allowed us to distinguish Fus3-specific roles in the pheromone response and furthered our understanding of why chemotropism is repressed under conditions in which Kss1 but not Fus3 is active.

**RESULTS**

Pheromone responses are more defective in strains expressing nonactivatable Fus3 than in strains completely lacking Fus3

We previously observed that cells undergo invasive growth but are inhibited for pheromone-induced mating differentiation under
conditions in which Ste7 activates Kss1 but does not activate Fus3 (Maleri et al., 2004). Our interpretation is that under such conditions, inactive Fus3 is present and has a role in the inhibition of mating differentiation. This inhibitory function could serve to ensure against an inappropriate differentiation response, for example, by prevent mating differentiation under nutrient limiting conditions in which Kss1 is active but Fus3 is not. This inhibition could impinge not only on mating differentiation but also on chemotrophic growth through mechanisms that affect one or more processes, including transcription, G1 arrest, and polarization. To assess more directly what role(s) inactive Fus3 plays in protecting against inappropriate fate transitions, we generated isogenic strains with wild-type Fus3 (FUS3), no Fus3 (fus3Δ), or a nonactivatable Fus3 variant (fus3A<sup>180F<sub>182</sub>Δ) expressed from the endogenous locus.

To examine the effects of nonactivatable Fus3 on transcription of mating and filamentation gene expression, we measured expression of reporter genes representative of the two transcriptional programs. Consistent with previous observations, the absence of Fus3 (fus3Δ) has little effect on pheromone-induced expression of the prototypical mating-specific PRE<sub>3</sub>-LacZ reporter (Table 1; Roberts et al., 2001; Breitkreutz et al., 2001). In contrast, the strain expressing nonactivatable Fus3 (fus3A<sup>180F<sub>182</sub>Δ) shows reduced basal expression and remains at nearly baseline PRE<sub>3</sub>-LacZ expression after exposure to pheromone. Although Breitkreutz et al. (2001) reported that catalytically inert versions of Fus3 altered neither the pheromone-induced transcriptional profile nor the basal level of pheromone-induced genes, the data in the supplement they provided are qualitatively consistent with our findings. We suggest that the mechanism of repression of the mating-specific program by Fus3A<sup>180F<sub>182</sub></sup>Δ is analogous to the role inactive Kss1 plays in the inhibition of genes required for the filamentation program (Cook et al., 1997; Madhani et al., 1997; Tedford et al., 1997; Bardwell et al., 1998a; Chou et al., 2006). As previously reported, basal and pheromone-induced expression of the prototypical filamentation-specific TCS-PRE-lacZ reporter is significantly elevated (three to four times) in fus3Δ and fus3A<sup>180F<sub>182</sub></sup>Δ strains compared with the reference FUS3 strain (Table 1; Madhani et al., 1997; Sabbagh et al., 2001). Thus, in contrast to mating-specific gene expression, Kss1-promoted filamentation specific gene expression is the same in strains without Fus3 or with nonactivatable Fus3 (fus3Δ).

To compare morphological transitions characteristic of pheromone-induced fates in strains with different Fus3 alleles, we used microfluidic chambers to expose cells to a pheromone gradient (5–50 nM) over a 5-h time course. Morphological transitions that occur in these gradients depend on pheromone dosage (position in the gradient), time of exposure, and Fus3 genotype. Cells were placed into one of three morphological categories that are characteristic of different cell types: vegetative (no morphological response to pheromone), shmoo (mating competent), or hyperelongated (chemotrophic or filamentous; Figure 2D). The vegetative category includes cells with round or oval shape (whether budded or unbudded). The shmoo category includes unbudded cells with one or more tight projections. The hyperelongated category includes G1-arrested cells with an elongated or peanut shape and mitotic cells in which the buds are hyperelongated (whether the mother has an elongated or round shape).

Before establishing the pheromone gradient, all strains have a round or oval morphology characteristic of vegetative cells. After 5 h in the pheromone gradient,shmoo, hyperelongated, and vegetative morphologies are observed for the wild-type reference cells (FUS3), which are largely confined to zones of the microfluidic chamber corresponding to high, intermediate, and low pheromone concentrations, respectively (Hao et al., 2008; Figure 2A). Cells lacking Fus3 (fus3Δ) do not shmoo. Instead, they exhibit hyperelongated and vegetative morphologies in all zones of the chamber, a behavior consistent with a prior report (Hao et al., 2008; Figure 2B). Cells expressing nonactivatable Fus3 (fus3A<sup>180F<sub>182</sub>Δ) have significantly fewer elongated cells than those without Fus3 (fus3Δ) and remain largely in the vegetative growth phase in all zones of the chamber (Figure 2C). The same phenotype was observed with an independently constructed fus3A<sup>180F<sub>182</sub></sup>Δ strain (C699-192; unpublished data). The contrasting behaviors of cells without Fus3 and those that express the Fus3 variant show that nonactivatable Fus3 is an inhibitor of morphogenesis characteristic of chemotrophic cells.

In addition to activating pheromone-specific transcription, Fus3 promotes cell cycle arrest in G1 by phosphorylating Far1. Therefore, in the same microfluidic experiments, we compared efficiency of the G1 arrest response to pheromone based on the accumulation of unbudded cells in the chamber. Before loading cells into the chamber, all strains had an unbudded cell index (35–45%) characteristic of log-phase cultures in synthetic medium. The initial unbudded cell percentage reported for these microfluidic experiments is slightly higher (45–55%) because the geometry of the chamber causes a slight bias toward loading unbudded G1 cells. After 2 h in the pheromone gradient, >90% of the wild-type reference cells (FUS3) in the high- and intermediate–pheromone concentration zones of the gradient were unbudded and remained arrested throughout the remainder of the time course. In the low–pheromone concentration zone, only ~75% of the cells were unbudded, and most of these reentered the cell cycle during the remainder of the time course (Figure 3A). Cells lacking Fus3 (fus3Δ) show a less-homogeneous G1

| Reporter gene | β-Galactosidase activity (AU/min OD<sub>660</sub>) |
|---------------|-----------------------------------------------|
| PRE<sub>3</sub>-LacZ | FUS3 fus3Δ fus3A<sup>180F<sub>182</sub></sup>Δ |
| PHEROMONE | 1374 ± 78 1051 ± 125 175 ± 23 |
| No pheromone | 62 ± 5 50 ± 10 16 ± 1 |
| TCS-PRE-LacZ | fus3Δ fus3A<sup>180F<sub>182</sub></sup>Δ |
| PHEROMONE | 148 ± 12 1153 ± 28 1016 ± 45 |
| No pheromone | 30 ± 6 707 ± 30 544 ± 36 |
| PRE<sub>3</sub>-LacZ | P<sub>GAL1</sub>STE5CTM FUS3 P<sub>GAL1</sub>STE5CTM fus3Δ P<sub>GAL1</sub>STE5CTM fus3A<sup>180F<sub>182</sub></sup>Δ |
| GALACTOSE | 643 ± 17 43 ± 8 6 ± 1 |
| 2% | 1 ± 0.1 1 ± 0.2 1 ± 0.3 |
| No galactose | 1 ± 0.1 1 ± 0.2 1 ± 0.3 |

**TABLE 1: Comparison of reporter gene expression in strains with different FUS3 alleles.**
FIGURE 2: Developmental fate morphologies of strains with different \( FUS3 \) alleles in pheromone gradients. Cells from \( \text{bar}1\Delta \) strains with \( FUS3 \) (C699-5), \( \text{fus}3\Delta \) (C699-200), or \( \text{fus}3\Delta^{180F182} \) (C699-207) as indicated, were exposed to a linear gradient of pheromone (50–5 nM) in microfluidic chambers. (A–C) Left, micrographs showing representative fields of cells with the indicated \( FUS3 \) alleles after 5 h of growth in pheromone gradient. Right, bar graphs showing the percentage of cells in the upper (High), intermediate (Int), or lower (Low) one-third of the gradient with vegetative (\( \square \)), hyperelongated (\( \square \)), or shmoo (\( \square \)) morphology after 5 h of exposure to pheromone. Error bars show the 95% confidence limit using data from three or more gradient chamber experiments. The total number of cells scored in each portion of the gradient is given above the bars. (D) Micrographs of cells illustrating representative morphologies for the following scoring categories: vegetative, cells with a round or oval shape (whether budded or unbudded); shmoo, G1 (unbudded) cells with one or more pointed projections; hyperelongated, G1 (unbudded) cells with an elongated or peanut shape and mitotic cells (budded) with a hyperelongated shape and/or hyperelongated bud.

FIGURE 3: G1 arrest efficiency of strains with different \( FUS3 \) alleles in pheromone gradients. The percentage of unbudded cells in the microfluidic chambers used for data shown in Figure 2 was determined at hourly intervals after introduction of the pheromone gradient. (A–C) Unbudded cell percentage vs. time for the upper (black or red symbols; dotted line), intermediate (gray symbols; dashed line), and bottom (white symbols; solid line) one-third of the gradient for the strains with the indicated \( FUS3 \) alleles. The accumulation of unbudded cells is an indication of G1 arrest.
arrest response than do wild-type cells, with only 75–80% accumulating as unbudded cells. Of interest, the arrest response is the same regardless of position in the pheromone gradient (Figure 3B). The arrest response for cells expressing nonactivatable Fus3 (\(\text{fus3A}^{180F}{\text{F}^{182}}\)) is also independent of position in the pheromone gradient. However, consistent with a previous study, \(\text{fus3A}^{180F}{\text{F}^{182}}\)-expressing cells are more refractory to G1 arrest than are cells lacking Fus3 (\(\text{fus3A}\); Figure 3C; Breitkreutz et al., 2001). Thus nonactivatable Fus3 has the capability of more potently inhibiting pheromone-induced mating specific transcription, morphogenesis, and G1 arrest than absence of Fus3 in the cells.

Kss1 dual phosphorylation is elevated in strains without Fus3 activity

The contrasting behavior of cells without Fus3 and those expressing the Fus3 variant support the conclusion that nonactivatable Fus3 is an inhibitor of the mating and chemotropic transitions. One, albeit unlikely, explanation is that some strains compensate for loss of activity from having no or catalytically inert Fus3 by elevating the amount of active Kss1 (Sabbagh et al., 2001; Hao et al., 2012), but this compensation does not occur in the strain background used here. To eliminate this possibility, we compared the dual-phosphorylation status of Fus3 and Kss1 in the strains with different \(\text{FUS3}\) alleles before and at indicated times after pheromone induction. Extracts from samples of the different cultures were fractionated by SDS–PAGE and transferred to nitrocellulose for immune blot analysis to detect the amount of each MAPK that is dually phosphorylated (pT-E-pY) at the activation loop by using anti–phospho-p42/p44 antibodies (Sabbagh et al., 2001). Consistent with previous observations, the \(\text{fus3A}\) strain has significantly elevated basal and pheromone-induced amounts of dual-phosphorylated Kss1 compared with the \(\text{FUS3}\) reference strain (Figure 4; Sabbagh et al., 2001; Hao et al., 2012). A similar elevation is observed in the \(\text{fus3A}^{180F}{\text{F}^{182}}\) strain (Figure 4). This finding is pertinent because it shows that elevated Kss1 activity is insufficient to overcome the inhibitory effect of inactive Fus3 on mating and chemotropic transitions.

Activation of the pathway by Ste5-CTM suppresses the morphogenetic but not the transcriptional defect caused by nonactivatable Fus3

Fus3 activity and binding interactions have documented roles in pheromone-induced transcription and morphogenesis (Madhani et al., 1997; Tedford et al., 1997; Bardwell et al., 1998b; Roberts et al., 2000; Metodiev et al., 2002; Matheos et al., 2004; Figure 1). We considered the possibility that the binding of inactive Fus3 to relevant targets could obstruct compensating phosphorylation by Kss1 and therefore result in more severe defects than seen in the complete absence of Fus3. To test whether active receptor and/or G-protein might be the relevant target(s) for the effect that nonactivatable Fus3 has on morphogenesis, we exploited a variant of the scaffold Ste5 that is artificially targeted to the plasma membrane by its fusion to a carboxy-terminal transmembrane (CTM) sequence (Pryciak and Huntress, 1998). Overexpression of this fusion was shown to activate the mating pathway in the absence of pheromone or activated G-protein (Pryciak and Huntress, 1998). We generated isogenic \(\text{FUS3}\), \(\text{fus3A}\), and \(\text{fus3A}^{180F}{\text{F}^{182}}\) strains with Ste5-CTM under control of the galactose-regulated promoter (\(\text{P}_{\text{GAL1}}\)) expressed from the endogenous locus. We then used microfluidic chambers and time-lapse imaging to follow cells during galactose induction of Ste5-CTM.

Cells from the time-lapse images were scored as budded or unbudded (independently of their morphology) to monitor G1 arrest. At the start of the time course \((t = 0)\), the raffinose-grown cells for each of the strains in the microfluidic chambers showed a budding index (~35–45% unbudded) typical of mitotic cultures (Figure 5B). Nearly 100% of the reference cells (\(\text{FUS3}\)) were arrested in G1 as unbudded cells after 12 h of galactose induction (Figure 5B). At the same time point, cells lacking Fus3 (\(\text{fus3A}\)) showed a less homogeneous G1 arrest response than wild-type cells, with only ~65% accumulating as unbudded cells (Figure 5B). Cells expressing nonactivatable Fus3 (\(\text{fus3A}^{180F}{\text{F}^{182}}\)) failed to arrest, as they maintained the same unbudded percentage as the mitotic raffinose culture (Figure 5B). Thus the relative robustness of G1 arrest induced by expression of Ste5-CTM in the three strains (\(\text{FUS3} > \text{fus3A} > \text{fus3A}^{180F}{\text{F}^{182}}\)) is the same as seen under pheromone-inducing conditions.

Ste5-CTM expressing cells from the aforementioned images were also scored for cell morphology (vegetative, shmoo, or hyperelongated) as described for the pheromone induction experiments in Figure 2. The population of reference cells (\(\text{FUS3}\)) was heterogeneous with respect to vegetative, elongated, and shmoo morphologies after 12 h of galactose induction (Figure 5C). Cells lacking Fus3 (\(\text{fus3A}\)) failed to shmoo but were equally distributed between vegetative and elongated morphologies (Figure 5C). Cells expressing nonactivatable Fus3 (\(\text{fus3A}^{180F}{\text{F}^{182}}\)) showed a distribution of vegetative and elongated morphologies that was comparable to that of \(\text{fus3A}\) cells (Figure 5C). Thus activation of the pathway by Ste5-CTM
A. Raffinose Vegetative Galactose (12 hr) Elongated Shmoof

B. Bar graph comparing unbudded cell percentage

C. Bar graph comparing cell morphology

FIGURE 5: Developmental fate morphologies and G1 arrest promoted by overexpression of Ste5-CTM in the absence of pheromone. Cells from bar1Δ, P\textsubscript{GAL1}\textsubscript{5}-ST5-CTM strains with FUS3 (C699-218), fus3A (C699-214), or fus3A\textsuperscript{180F182} (C699-219) as indicated were grown in raffinose medium and switched to uniform 2% galactose medium in microfluidic chambers to induce overexpression of Ste5-CTM. (A) Micrographs of cells in the chambers before (t = 0) and after switching to 2% galactose medium (t = 12 h) show representative morphologies of the indicated categories used for scoring. (B) Bar graph comparing unbudded cell percentage (independent of morphology) in raffinose at the start of the time course (t = 0) and 12 h after switching to 2% galactose (t). Total number masks the inhibitory effect of nonactivatable Fus3 on morphogenesis. This outcome points to a role for Fus3 in morphogenesis at the level of the active receptor or G-protein.

We also compared expression of the prototypical mating-specific reporter gene (PRE\textsubscript{3}-lacZ) in these three strains after 8 h of growth in galactose medium (Table 1). In contrast to reporter expression induced by pheromone, PRE\textsubscript{3}-lacZ expression activated by Ste5-CTM was significantly less in cells without Fus3 (fus3Δ) than in the reference cells (FUS3; Table 1). We infer that Kss1 may be less potently activated by targeting Ste5 to the membrane than by pheromone induction. Nevertheless, the strain expressing nonactivatable Fus3 (fus3A\textsuperscript{180F182}) has much lower reporter gene expression (nearly baseline) than even the strain without Fus3 (fus3Δ) (Table 1).

Thus, in Ste5-CTM–expressing strains, nonactivatable Fus3 inhibits transcription but not the morphological transition. This outcome reveals that Fus3 regulates morphogenesis and transcription by independent mechanisms.

The binding of nonactivatable Fus3 to Gα inhibits pheromone-induced morphogenesis

It was previously shown that Fus3 binds directly to the Gα subunit of the activated G-protein (Metodiev et al., 2002). This interaction poises Fus3 for phosphorylating the formin homologue Bni1, which in turn promotes actin assembly and polarized growth (Matheos et al., 2004; Figure 1). To test whether binding to Gα underlies the effect that nonactivatable Fus3 has on morphogenesis, we exploited a MAPK-docking-site Gα variant (Gpa1E\textsuperscript{21}E\textsuperscript{22}) that abrogates binding (Metodiev et al., 2002) and generated isogenic FUS3, fus3Δ, and fus3A\textsuperscript{180F182} strains with the GPA1E\textsuperscript{21}E\textsuperscript{22} allele expressed from the endogenous locus. As before, we used microfluidic chambers to expose cells to a pheromone gradient (5–50 nM) over a 5-h time course. After 5 h in the pheromone gradient, the strain coexpressing the nonactivatable Fus3 and Gα nondocking variants (fus3A\textsuperscript{180F182} GPA1E\textsuperscript{21}E\textsuperscript{22}) showed the same distribution of elongated and vegetative cells as did the strains devoid of Fus3 either with or without the Gα nondocking variant (fus3Δ GPA1E\textsuperscript{21}E\textsuperscript{22}, fus3Δ Gpa1; Figures 6, B and C, and 2B). We conclude that binding of nonactivatable Fus3 to Gpa1 leads to its negative effect on morphogenesis. This effect is not observed in the absence of Fus3 because Kss1 has free access to the critical targets and is able to compensate.

Fus3 binding to Gα is required for gradient tracking and suppression of noise in the morphogenetic response to pheromone

The reference strain expressing the Gα nondocking variant (FUS3 GPA1E\textsuperscript{21}E\textsuperscript{22}) exhibited different pheromone gradient behavior from that of the wild-type strain (FUS3 Gpa1; compare Figures 6A and 2B). One striking difference for the FUS3 GPA1E\textsuperscript{21}E\textsuperscript{22} strain was the apparent inability of cells to track a gradient. To assess gradient tracking quantitatively, we focused on cells residing in the intermediate zone of chamber, where the pheromone gradient is most linear. Cell polarization was quantified by measuring the final angle of polarized growth and is represented as an angle distribution of cells scored at t = 0 and 12 h is given above the bars. (C) Bar graph comparing percentage of cells in galactose (t = 12 h) that have vegetative (●), hyperelongated (□), or shmoof (■) morphology (independent of budding status). Total number of cells scored is given above the bars for each FUS3 allele. Error bars in B and C show 95% confidence limit using data from three experiments.
FIGURE 6: Developmental fate morphologies of GPA1-E21E22 strains with different FUS3 alleles in pheromone gradients. Cells from bar1Δ GPA1-E21E22 strains with FUS3 (C699-223), fus3Δ (C699-331), or fus3A180F182 (C699-221) as indicated were exposed to a linear gradient of pheromone (50–5 nM) in microfluidic chambers. (A–C) Left, micrographs showing representative fields of cells with the indicated FUS3 alleles after 5 h of growth in pheromone gradient chambers. Right, bar graphs showing the percentage of cells in the upper (High), intermediate (Int), or lower (Low) one-third of the gradient with vegetative (●) or hyperelongated (▲) morphology after 5 h of exposure to pheromone. (A) <1% of the cells at High and Int. pheromone were scored as shmoos; (B and C) no cells scored as shmoos. Error bars show the 95% confidence limit for data from three or more gradient chamber experiments. The total number of cells scored in each portion of the gradient is given above the bars. Micrographs of cells in Figure 2D illustrate representative morphologies of the indicated categories used for scoring.

FIGURE 7: Comparison of gradient tracking in GPA1 and GPA1E21E22 strains with different FUS3 alleles. Histograms show the frequency distribution of the angle of cell polarization with respect to the pheromone gradient for the strains analyzed in Figures 2 and 6 after 4.5 h of growth in the gradient chambers. Zero degrees represents perfect alignment toward the gradient. Histograms report the percentage of cells with the angles (in degrees) as specified for each wedge on the circle periphery. The scale for cell percentage (0–15%) is indicated by rings emanating from the center of the circle. Data were collected from three or more independent experiments. Only cells in the intermediate zone of the gradient chamber were scored; the number scored (n) for each strain is given above the corresponding histogram.

(A) GPA1 FUS3 (n=169) (B) GPA1E21E22 FUS3 (n=85) (C) GPA1 fus3Δ (n=93) (D) GPA1E21E22 fus3Δ (n=99) (E) GPA1 fus3A180F182 (n=64) (F) GPA1E21E22 fus3A180F182 (n=103)

As expected, wild-type cells (FUS3 GPA1) oriented their growth in the direction of high pheromone, typically within ±45° of the gradient (Figure 7A). By contrast, cells expressing the Gα nondocking variant (FUS3 GPA1E21E22) oriented randomly (Figure 7B). The random growth orientation of the FUS3 GPA1E21E22 cells in the gradient is typical of cells without Fus3 activity (Hao et al., 2008; Figure 7, C–F). Taken together, these results show that the docking of Fus3 on Gα is essential for chemotropism.

Another difference for the strain expressing the Gα nondocking variant was the absence of a significant number of shmoos even in the high-concentration region of the gradient. Such a defect could be attributed to decreased sensitivity to pheromone. To assess this...
For comparison, the analysis was done using the reporter gene plasmid after 90 min of induction with indicated concentrations of pheromone. Data points are the mean of four (A) or three (B) independent experiments. Error bars show the 95% confidence limit. FUS3, circles; fus3Δ, triangles; fus3ΔA180F182, diamonds; GPA1 strains, solid symbols with dashed lines; GPA1E21E22 strains, open symbols with solid lines.

The possibility, we conducted dose-response comparisons for transcription using the reporter gene representative of the filamentation program (TCS-PRE-LacZ). Typical of Kss1-promoted responses, the amount of reporter expression is relatively insensitive to pheromone dosage. Again the profiles show the expected trends for the different FUS3 alleles and are essentially the same whether strains express wild-type Gα(GPA1) or the nondocking variant (GPA1E21E22; Figure 8B). Results with Ste5-CTM given earlier revealed that transcription and morphogenesis can be regulated independently. Therefore we also assessed dose-response behavior of the GPA1 and GPA1E21E22 strains with different FUS3 alleles for G1 arrest and morphogenesis. For these experiments, parallel cultures were treated with pheromone at concentrations ranging from 3.2 × 10⁻⁷ to 3.2 × 10⁻⁶ M (log scale −6.5 to −8.5). After 5 h, cells were fixed in formaldehyde and scored for cells in G1 (unbudded cells, independently of morphology) and polarized growth characteristic of pheromone response (either hyperelongated or shmoo, independently of budding). These comparisons show that there is no difference in the dosage dependence for the G1-arrest response based on whether the strains with different FUS3 alleles express wild-type Gα (GPA1) or the nondocking variant (GPA1E21E22; Figure 9A). There was no difference in the percentage of FUS3 or fus3Δ cells exhibiting polarized growth, which depended on whether cells had the GPA1 or GPA1E21E22 allele (Figure 9B). However, the percentage of fus3ΔA180F182Δfus3 cells exhibiting polarized growth was significantly less at all pheromone concentrations than for the fus3ΔA180F182GPA1E21E22 cells. Of importance, the pheromone dependence of polarized growth for the strain coexpressing the nonactivatable Fus3 and nondocking Gα variants (fus3ΔA180F182GPA1E21E22) was indistinguishable from that of the strains without Fus3 (fus3Δ; Figure 9B).

We did not distinguish between shmoo and hyperelongated morphologies for the purpose of assessing dose response of the different strains for polarized growth. However, we did observe a significant difference between the GPA1 FUS3 and GPA1E21E22FUS3 strains upon assessing the fraction of responding cells that have a shmoo compared with a hyperelongated morphology (Figure 9C). Specifically, the dose for transition of the population from hyperelongated to shmoo morphology is sharper for GPA1 cells than for GPA1E21E22. As a consequence, there is a more heterogeneous distribution of morphologies over a broader range of pheromone concentrations for the cells expressing the nondocking Gα variant compared with wild type. This behavior shows that the binding of Fus3 to Gα reduces heterogeneity and has a noise-suppressing effect on morphogenesis.

**DISCUSSION**

A Fus3–Gα complex regulates gradient tracking and morphogenesis

To further define regulatory roles for pheromone-activated MAPKs in chemotropism, we compared the pheromone-induced responses of transcription, G1 arrest, and morphogenesis under conditions in which Kss1 is active either in the absence of Fus3 (fus3Δ) or the presence of a Fus3 variant that is inactive because it cannot be phosphorylated (fus3ΔA180F182; Gartner et al., 1992). These comparisons revealed a previously unappreciated role for inactive Fus3 as a negative regulator of pheromone-induced chemotropism. The analyses further showed that Fus3 binding to the G-protein α-subunit is required for gradient tracking and acts to suppress cell-to-cell variability between mating and chemotropic fates in a population of pheromone-responding cells.

To explain these observations, we speculate that Gα provides a docking motif that serves to colocalize Fus3 with a substrate whose phosphorylation is critical for morphogenesis. We assume that the
MAPK docking site on Gα binds to Fus3 but poorly or not at all to Kss1. We further speculate that the target to be phosphorylated lacks a MAPK docking motif. This colocalization of the three components increases the efficiency of target phosphorylation (Figure 10A, top). In the case in which Fus3 is absent, a ternary complex does not form, so that Kss1 has access to the target. In this case, the phosphorylation reaction may not be as efficient as when colocalization is imposed by docking to Gα (Figure 10A, middle). A stable complex between inactive Fus3 and Gα would impose steric constraints for access of other kinases to that target. As such, the inactive ternary complex physically obstructs Kss1 from phosphorylating the target substrate. In the unphosphorylated state, the target is unable to promote morphogenesis (Figure 10A, bottom).

In the case in which there is active Fus3 but it is prevented from docking on Gα (by mutation of the docking site), both Fus3 and Kss1 have access to the target. However, without the ternary complex, the phosphorylation reaction may not be as efficient as when colocalization is imposed (Figure 10B, top). The reduced efficiency in target protein phosphorylation by Fus3 or the more significant competition with Kss1 could underlie a greater heterogeneity in shmoo versus hyperelongated morphology compared with when Gα has a functional docking site for Fus3. The morphogenesis phenotype of a strain expressing nonactivatable Fus3 reverts to that of the strain devoid of Fus3 simply by masking the Gα MAPK docking site. This masking can occur either by bypassing G-protein activation, as seen with Ste5-CTM, or by introducing amino acid substitutions in the Gα MAPK docking site that eliminate the Fus3–Gα binding interaction. In either case, the phenotype is reversed specifically because there is no ternary complex to obstruct Kss1 from phosphorylating the target (Figure 10B, middle and top). An obvious candidate for a critical substrate is Bni1 because its phosphorylation by Fus3 has a known role in pheromone-induced morphogenesis.

**FIGURE 9:** Comparison of G1 arrest and morphological dose responses in GPA1 and GPA1E21E22 strains with different FUS3 alleles. Budding and cell morphologies were scored for strains (as specified in the legend to Figure 8) with the indicated alleles using formaldehyde-fixed aliquots of cultures after 5 h of incubation at indicated concentrations of pheromone ($3 \times 10^{-9}$ to $3 \times 10^{-7}$ M α-factor). (A) Plots of the percentage of G1 (unbudded) cells vs. log pheromone concentration. (B) Plots of the percentage of cells with pheromone-induced polarized growth (either hyperelongated or shmoo) vs. log pheromone concentration. (C) Left, plots comparing the fraction of responding cells with hyperelongated (green lines and symbols) or shmoo morphology (black lines and symbols) vs. log pheromone concentration. Right, micrographs showing a representative field of GPA1 and GPA1E21E22 cells as indicated. Error bars show 95% confidence limit. FUS3, circles; fus3Δ, triangles; fus3A180F182, diamonds; GPA1 strains, solid symbols with dashed lines; GPA1E21E22 strains, open symbols with solid lines.
Normally, the pheromone dose at which cells transition from chemotropic to shmoo morphology is relatively sharp. Consequently the population distribution at a given pheromone concentration is fairly homogeneous for one type or the other. However, disruption of the Fus3–Gα binding interaction results in a more heterogeneous distribution of the two morphologies over a broader range of pheromone concentrations.

The importance of a MAPK–Gα docking interaction in developmental decisions is not unique to the yeast mating pathway. The soil amoeba Dictyostelium discoideum has 12 different Gα subunits and two MAPKs. Recently, roles for Erk1 or Erk2 binding to two of the Gα subunits have been found to be important during multicellular development of this organism. Gα4 binding to Erk2 was shown to be required for morphogenesis associated with culmination but not for its chemotaxis to folate (Nguyen and Hadwiger, 2009). Gα5 binding to Erk1 was similarly found to be important for Gα5 function in regulation of aggregate formation and tip development but not in the inhibition of folate chemotaxis (Raisley et al., 2010). Raisley et al. (2010) identified the presence of MAPK-docking sites in Gα subunits of not only yeast and Dictyostelium, but also mammalian cells. They highlighted the implication that MAPK interactions with Gα subunits might be widespread among eukaryotes and are likely to play important roles in G-protein–coupled receptor signaling.

Specialized functions for MAPK isoforms

It has been appreciated for some time that Fus3 and Kss1 have specialized regulatory roles in the mating differentiation and invasive growth transitions. The positive regulatory role of active Fus3 in the establishment of a stable G1 arrest and formation of mating projections explains why strains lacking Fus3 ( fus3Δ ) only transiently arrest in G1 and are unable to produce mating projections in response to saturating pheromone (Elion et al., 1990, 1991). Both Fus3 and Kss1 positively regulate Ste12 transcriptional activity at pheromone-inducible genes by phosphorylating and destabilizing the repressor complex formed with Dig1 and Dig2 at these promoters (Madhani et al., 1997; Tedford et al., 1997; Bardwell et al., 1998b; Roberts et al., 2000). Active Fus3 also specifies Ste12 promoter selectivity under pheromone-induced conditions through phosphorylation of Tec1 and its subsequent SCF-catalyzed ubiquitination and degradation (Bao et al., 2004; Bruckner et al., 2004; Chou et al., 2004; Wang and Dohlman, 2006). This additional regulatory role for active Fus3 promotes the transcriptional program specifying mating differentiation while inhibiting the program specifying invasive growth. Impairment of Tec1 turnover also accounts for the hyperinvasive growth phenotype of fus3Δ strains (Cook et al., 1997). Kss1 has both negative and positive regulatory roles in the invasive growth transition. Inactive Kss1 binds directly to Ste12 and stabilizes the Dig1–Ste12–Tec1 repressor complex at filamentation-specific genes (Madhani et al., 1997; Tedford et al., 1997; Bardwell et al., 1998a). Ste7 phosphorylation of Kss1 both promotes Kss1 catalytic activity and weakens Kss1–Ste12 binding. Kss1 phosphorylation of Dig1 (and Dig2) is believed to have a positive role in regulating transcription of filamentation-specific genes analogous to its role in regulating transcription of mating-specific genes (Cook et al., 1996; Tedford et al., 1997).

The roles of Fus3 and Kss1 in the chemotropic transition are less well studied. Strains lacking Fus3 ( fus3Δ ) respond to (high and low) pheromone by transiently arresting in G1 and adopting an elongated morphology even though they are unable to track pheromone gradients. The elongated morphology of fus3Δ strains is attributed to an overlapping function provided by Kss1, which becomes hyperactivated in the absence of Fus3 activity. In contrast to...
Fus3Δ strains, strains expressing nonactivatable Fus3 (Fus3•A180p•182) proliferate and retain largely vegetative morphology in the presence of pheromone. The contrasting phenotype of strains with nonactivatable Fus3 compared with strains lacking Fus3 reveals an inhibitory role for the inactive kinase in the pheromone-induced chemotactic program.

Chemotropism in low pheromone gradients is believed to increase the probability of an encounter with a distant mating partner (Erdman and Snyder, 2001). The extensive growth entailed in this search undoubtedly requires significant energy expenditure. Because Kss1 supports elongated growth but not gradient tracking (Hao et al., 2008), the inhibition by inactive Fus3 provides a beneficial “checkpoint” to guard against futile elongation and adds a layer of insulation between the invasive growth and mating pathways.

Unraveling the regulatory roles for the MAPKs Fus3 and Kss1 in different fate transitions will reveal aspects of signaling circuitry that are likely to be more broadly relevant to MAPK-mediated mammalian development. Erk1 and Erk2 are generally believed to possess redundant or overlapping functions because they are coactivated in mammalian cells in response to multiple stimuli. However, several reports revealed scenarios in which there are specialized roles for the different isoforms. For example, Erk1 appears to have a more important role in osteoclast development and bone resorptive activity (He et al., 2011) and thymocyte maturation (Fischer et al., 2005), whereas Erk2 has essential roles in mesoderm development, including myogenesis (Sarbassov et al., 1997) and the epithelial to mesenchymal transformation (Shin et al., 2010). Recent studies using genetic ablation of Erk1 or Erk2 revealed differences in T-cell proliferation, expansion, and differentiation, depending on which Erk is inactivated (Chang et al., 2012). Studies on the mechanisms through which Erk1 and Erk2 regulate transcription in pancreatic β-cells revealed that the kinases themselves are components of chromatin-bound transcription factor complexes on both the insulin and c-fos genes. In this example, the kinases appear on promoters when they are active and are no longer captured on chromatin if their activity is blocked (Lawrence et al., 2008). Evidence is also accumulating that Erk1 and Erk2 can play functionally important roles in chromatin remodeling, DNA transcription, and cell cycle regulation independently of their regular catalytic activities (for review, see Rodriguez and Crespo, 2011). The finding of context-specific MAPK functions in mammals indicates that the mechanisms underlying those for fate decisions in yeast are likely conserved.

MATERIALS AND METHODS
Plasmids
The plasmids used in these studies have been described previously. Table 2 provides their source and/or reference.

Yeasts strains and genetic procedures
Table 3 lists yeast strains used in these studies. Table 4 lists the sequence of oligonucleotides used for PCR fragment amplification, mutagenesis, and DNA sequence confirmation involved in the construction of these strains. Media preparation and standard yeast genetic methods for transformation, gene replacement, crosses, and tetrad dissection were as described in Amberg et al. (2005).

The following two strains were constructed using the one-step gene replacement method (Rotstein, 1983). C700-5 was derived from C700 by using the EcoRI–Sal fragment from pJGgst1 (Reneke et al., 1988) to replace the BAR1 locus with the bar1Δ::hisG-URA3-hisG allele. The bar1Δ::hisG allele was generated from the resulting strain by selection on 5-fluoroorotic acid (Life Technologies, Grand Island, NY) medium (Boeke et al., 1994). This medium provides a positive selection for isolates in which the URA3 marker is excised by recombination within the direct hisG repeats (Alani et al., 1987). Strain C699-200 was derived from C699-5 by replacing the Fus3 locus with the fus3ΔΔ::LEU2 HindIII fragment from pYEE98 (Elion et al., 1990).

Strain C699-188 is a segregant resulting from a cross between strains C699-106 (Maleri et al., 2004) and C700-5. To make this cross, C699-106 was transformed with pNC752 (STE7) (Maleri et al., 2004) to complement the ste7Δ::hisG mutation. This complementation allowed selection of yagettes from mating mixtures with strain C700-5. Diploid isolates that lost the plasmid after nonselective growth were used for tetrad dissection and analysis of segregants from the cross. The presence of the STE7 allele in C699-188 was confirmed using yeast colony PCR analysis with oligonucleotide primers 350 and 1031 (Akada et al., 2000).

| Plasmid | Allele | Vector | Source or reference |
|---------|--------|--------|---------------------|
| pCORE-UK | kURA3::KanMX4 | | Stori and Resnick (2006) |
| pCR Blunt II Topo | | | Invitrogen Life Technologies |
| pGA1706 | PRE3-lacZ | pLGΔ178 | Baur et al. (1997) |
| pGA1895 | fusi750A, YIRF | pNC160 | Gartner et al. (1992) |
| pJGgst1 | bar1::hisG-URA3-hisG | Reneke et al. (1988) |
| pLGΔ178 | (CYC1Δ5A)-lacZ | Guarente et al. (1984) |
| pNC160 | TRP1-ARS1 CEN3 | Rhodes et al. (1990) |
| pNC343 | TCS-PRE-lacZ | pLGΔ178 | Baur et al. (1997) |
| pNC752 | STE7M | pNC160 | Maleri et al. (2004) |
| pGS5-CTM | Pgal1-STE5CTM | pRS314 | Pryciak and Huntress (1998) |
| pRS313 | HIS3 CEN6 ARSH4 | Sikorski and Hieter (1989) |
| pRS314 | TRP1 CEN6 ARSH4 | Sikorski and Hieter (1989) |
| pYEE81 | FUS3 | YCp50 | Elion et al. (1990) |
| pYEE98 | fus3ΔΔ::LEU2 | Elion et al. (1990) |
| YCp50 | URA3 ARS1 CEN4 | Johnston and Davis (1984) |

Table 2: Plasmids.
The following fus3A^{180}F^{182}, P_{GAL1}STE5-CTM, and GPA1E^{21}E^{22} strains were constructed in two steps using the "delitto perfetto" approach for in vivo site-directed mutagenesis as described by Storici and Resnick (2006). The first step for construction of fus3A^{180}F^{182} strains C699-192 and C699-207 was to replace the Fus3 locus of strains C699-188 and C699-5, respectively, with a fus3A^{180}F^{182}::CORE-UK allele. pCORE-UK (Storici and Resnick, 2006) was the template for the first round of PCR synthesis with primers 1218 and 1219. Colony PCR analysis using M13R and M13F primers to confirm the codon replacements and confirmed isolates was cloned into the pCR Blunt II Topo vector (Invitrogen Life Technologies, Grand Island, NY) and sequenced using M13R and M13F primers to confirm the codon replacements and the fidelity of the locus encompassing the region of mutagenesis.

| Strain a | Genotype | Source or reference |
|----------|----------|---------------------|
| C699     | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 | K. Nasmyth, University of Oxford, UK |
| C699-5   | MATa ade-2 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG | Esch and Errede (2002) |
| C600-106 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1Δ::HIS3MX6 ura3-1 bar1Δ::hisG STE7Δ4::hisG | Maleri et al. (2004) |
| C699-188 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1Δ::HIS3MX6 ura3-1 bar1Δ::hisG | This work |
| C699-192 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1Δ::HIS3MX6 ura3-1 bar1Δ::hisG | This work |
| C699-200 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG fusi3A^{180}F^{182} ::LEU2 | This work |
| C699-207 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::HisG fus3A^{180}F^{182} | This work |
| C699-214 | MATa ade-2 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG fusi3A^{180}F^{182} ::LEU2 P_{GAL1}STE5-CTM | This work |
| C699-218 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG FUS3 P_{GAL1}STE5-CTM | This work |
| C699-219 | MATa ade-2 can1-100 his3-11,15 leu2-3112 trp1Δ::HIS3MX6 ura3-1 bar1Δ::hisG fus3A^{180}F^{182} | This work |
| C699-221 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG fus3A^{180}F^{182} GPA1E^{21},E^{22} | This work |
| C699-223 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG FUS3 GPA1E^{21},E^{22} | This work |
| C699-331 | MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG fusi3A^{180}F^{182} ::LEU2 GPA1E^{21},E^{22} | This work |
| C700     | MATa ade-2 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 | K. Nasmyth |
| C700-5   | MATa ade-2 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG | This work |
| C700-29  | MATa ade-2 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG GPA1E^{21},E^{22} | This work |

*Strains C699-x and C700-x strains are isogenic to W303-1A.

**TABLE 3:** _S. cerevisiae_ strains.

This allele contains a silent mutation at codon 14 that introduces a BamHI site and codons for glutamate at positions 21 (GAA) and 22 (GAG). The allele was generated by annealing oligonucleotides 1218 and 1219 and extending them with primers 1220 and 1221 and extending them with primers 1222 and 1219. Colony PCR using primers 1223 and 1224 to amplify 1036 base pairs of Oxford, UK strains C699-200 (MATa ade-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::hisG) and C699-207 (MATa ade-2 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 bar1Δ::HisG) was used to confirm the codon replacements and the fidelity of the locus encompassing the region of mutagenesis. To make this cross, C699-221 was transformed with YpEE81 [FUS3 URA3] to complement the nonmating phenotype of the fusi3A^{180}F^{182} mutant strain. C699-205 was transformed with pRS313 [HIS3] to allow nutritional selection of diploids from mating mixtures on synthetic medium (SD) supplemented with adenine, leucine, and tryptophan. Diploid isolates that lost the [FUS3 URA3] and [HIS3] plasmids after nonselective growth were used for tetrad dissection and analysis of segregants from the cross. The a-mating (MATa FUS3) and α-mating (MATa FUS3) segregants from the cross were screened for the GPA1E^{21}E^{22} allele using colony PCR with primers 1223 and 1224 to amplify the 1036-base-pair GPA1 locus, followed by BamHI digestion as described. C699-331 is a MATa fus3A^{180}::LEU2 GPA1E^{21}E^{22} segregant from a cross between strains C699-200 (MATa fus3A^{180}::LEU2 GPA1) and C700-29 (MATa FUS3GPA1E^{21}E^{22}). To make this cross, C699-200...
was transformed with YpEE81 \([\text{FUS3 URA3]}\) to complement the nonmating phenotype of the fus3Δ mutant strain, and C700-29 was transformed with pRS313 \([\text{HIS3]}\) to allow nutritional selection of diploids from mating mixtures on SD supplemented with adenine and tryptophan. Diploid isolates that lost the \([\text{FUS3 URA3]}\) and \([\text{HIS3]}\) plasmids after nonselective growth were used for tetrads dissection and analysis of segregants from the cross. Nonmating Leu+ segregants \((\text{fus3}\Delta \text{6::LEU2})\) were screened for the \(\text{GPA1E}_{21}\text{E}_{22}\) allele using colony PCR with primers 1223 and 1224 to amplify the 1036-base pair \(\text{GPA1}\) locus, followed by BamHI digestion as
described. The MAT allele of these segregants was distinguished by colony PCR using MATα-specific primers 1250 and 1252 and MATα-specific primers 1251 and 1253.

**Reporter gene assays**
The expression of **PRE5-lacZ** (pGA1706) and **TCS-PRE-lacZ** (pNC343) reporter genes in strains with different **FUS3** and **GPA1** alleles was assessed by measuring β-galactosidase activity. Cultures for these assays were grown to a density of ~0.8–1.0) x 10^6 cells/ml in –URA medium to maintain selection for the reporter plasmids. Pheromone (alpha-factor; CHI Scientific, Maynard, MA) was added to 90-μl aliquots of cell culture in microtiter plate wells to final concentrations ranging from 1.0 x 10^-4 to 1.0 x 10^-9 M (log scale –6.0 to –9.0). No pheromone reference samples were included. After 90 min of incubation at 30°C, β-galactosidase activity was measured using fluorescein di-β-galactopyranoside (FDG; Marker Gene Technologies, Jacksonville, FL) as substrate. FDG assays in the microtiter plates were carried out as described previously, with activities reported in arbitrary fluorescence units (AU)/min OD_660 (Hoffman et al., 2002).

**Immune blotting conditions for analysis of dual-phosphorylated Fus3 and Kss1** Strains **C699-5** (**FUS3**), **C699-192** (**fus3A**Δ**F182**), and **C699-200** (**fus3Δ**) were grown in synthetic complete medium. When cultures reached a cell density of ~1 x 10^7 cells/ml, a 10-μl aliquot was removed to provide an uninduced (t = 0) reference sample. α-Factor was added to a final concentration of 100 nM in the remaining portion of the cultures, and 10-μl aliquots of cells were removed after 30 and 90 min of incubation. Cell lysis, extract preparation, and trichloroacetic acid precipitation were done according to the procedures described by Sabbagh et al. (2001). The precipitated protein was suspended in buffer (0.1 M Tris-HCl, pH 11.0, 3% SDS), boiled for 5 min, and centrifuged for 10 min to remove insoluble aggregates. A 40-μl amount of each sample was mixed with 8 μl of 6x SDS–PAGE loading buffer and boiled for 2 min before being loaded onto 10% SDS–PAGE gels for protein fractionation. Proteins were transferred from the gels to polyvinylidene fluoride membranes for detection of dual-phosphorylated Fus3 and Kss1 by using anti-phospho-p44/42 MAPK rabbit polyclonal antibodies (1:500; Cell Signaling Technologies, Danvers, MA) as primary antibody with horseradish peroxidase (HRP)–conjugated goat anti-rabbit immunglobulin G (IgG; 1:10,000; Jackson ImmoResearch, West Grove, PA) as secondary antibody. Antibodies were washed from the membranes by treatment with stripping buffer (Tris-HCl, pH 6.0, SDS, β-mercaptoethanol) and re probed for glucose-6-phosphate dehydrogenase (G6P) as the internal loading control by using rabbit monoclonal anti-G6P (1:20,000; Sigma-Aldrich, St. Louis, MO) primary antibody with HRP-conjugated goat anti-rabbit IgG (1:10,000; Jackson ImmoResearch) as the secondary antibody. Immunoreactive species were detected using ECLplus (GE Healthcare, Amersham, Pittsburgh, PA) and exposing the developed blots to x-ray film. Bands on the scanned x-ray films were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

**Time-lapse imaging to follow pheromone gradient tracking, morphology, and G1 arrest**
These analyses were done in 5–50 nM pheromone gradients using a microfluidic device and methods for cell culturing, cell loading, and gradient generation as described in the supplement to Hao et al. (2008). Differential interference contrast (DIC) images of cells from four to six different fields that span the entire gradient were captured at 5-min intervals for a 5-h time course.

Microscopy was performed with a Nikon Ti-E inverted microscope using a Plan Fluor ELWD 40×/0.6 Nikon objective and a Photometrics CoolSNAP HQ2 Monochrome camera. Acquisition was performed with MetaMorph software (Molecular Devices, Sunnyvale, CA). Image processing and cell scoring were aided by use of ImageJ software.

The morphological categories scored after 5 h of growth in the pheromone gradient are illustrated by micrographs in Figure 2D. Unbudded cells (whether vegetative, shmoo, or hyperelongated) in each of the fields were also scored to assess the percentage of G1 cells in the population. The distinction between mothers with large buds (G2/M) versus unbudded mother–daughter pairs (G1) was aided by time-lapse imaging. Cells were scored as G1 if a bud emerged from either within the next two 5-min intervals.

Gradient tracking was quantified in cells 4.5 h after growth in the pheromone gradient. The angle between the cell center and the furthest point of the leading edge was measured using the straight-line tool in FIJI (fiji.sc/Fiji; Schindelin et al., 2012). The measured angles were then normalized in relation to the direction of the gradient and the chamber orientation. A histogram of the angles was generated using Matlab (MathWorks, Natick, MA). The histogram was normalized to represent the percentage of cells in each bin and plotted as a figure using the Polar function in Matlab.

**P_GAL1-STE5-CTM morphology and G1 assays**

Cells were cultured for these assays in synthetic complete medium with 2% raffinose as the carbon source. Cells were loaded into the microfluidic chamber as described in the supplement to Hao et al. (2008) and exposed to medium with uniform 2% galactose to induce expression of Ste5-CTM from the galactose-inducible GAL1,10 promoter. DIC images of cells from four to six different fields were captured at 10-min intervals for a 12-h time course. Microscopy was performed as before except using a 60× Plan Apo 1.4 Nikon objective. Scoring of morphology before the switch to galactose and after 12 h of growth in galactose was done as described, and the categories are illustrated by micrographs in Figure 5A. Vegetative, shmoo, and hyperelongated cells that are unbudded were also scored to assess the percentage of G1 cells in the population as described earlier.

**Pheromone dose-response morphology and G1 assays**

Strains were exposed to uniform pheromone for dose-response comparisons. In these experiments, 50-ml cultures of each strain were grown in yeast extract/peptone/dextrose medium to ~2.5 x 10^6 cells/ml. The cultures were divided into 4-ml aliquots in culture tubes. Pheromone was added to each tube to final concentrations ranging from 3.16 x 10^-7 to 3.16 x 10^-9 M (log scale –6.5 to –8.5). After 5 h of incubation at 30°C, a 250-μl sample was removed to an Eppendorf tube, cells were sonicated mildly to disperse clumps, and formaldehyde solution was added a final concentration of 2% to fix the cells. Cell morphology was scored on the fixed cell suspensions with a hemocytometer and an aus Jena LaboScope microscope with a 40×/0.65 objective. Unbudded cells were also scored to assess the G1 population as described earlier.

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