Published in final edited form as:

Sci Signal. ; 9(423): ra38. doi:10.1126/scisignal.aad4376.

G β promotes pheromone receptor polarization and yeast chemotropism by inhibiting receptor phosphorylation

Amber Ismael¹, Wei Tian^{2,*}, Nicholas Waszczak^{1,*}, Xin Wang^{1,*}, Youfang Cao^{2,†}, Dmitry Suchkov^{1,‡}, Eli Bar^{1,‡}, Metodi V. Metodiev³, Jie Liang², Robert Arkowitz⁴, and David E. Stone^{1,§}

¹Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL 60607, USA

²Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607

³School of Biological Sciences, University of Essex, Essex CO4 3SQ, United Kingdom

⁴CNRS UMR7277 / INSERM UMR1091 / Université de Nice - Sophia Antipolis, Institute of Biology Valrose, 06108 Nice Cedex 2, France

Abstract

Gradient-directed cell migration (chemotaxis) and growth (chemotropism) are universal processes, which are essential to the development and life cycles of all species. Cells use surface receptors to sense the shallow chemical gradients that elicit chemotaxis and chemotropism. Slight asymmetries in receptor activation are amplified by downstream signaling systems, which ultimately induce dynamic reorganization of the cytoskeleton. During the mating response of budding yeast, a model

[§]Corresponding author. dstone@uic.edu.

Current address: Theoretical Biology and Biophysics Group (T-6), Center for Nonlinear Studies (CNLS), Los Alamos National Laboratory, Los Alamos, NM 87545

[†]Current address: Collecta Incorporated, Mountain View, CA 94043, USA.

[‡]Current address: Department of Neurological Surgery, Case Western Reserve University, Cleveland, OH 44106, USA.

*These authors contributed equally to this work.

SUPPLEMENTARY MATERIALS

Fig. S1. Examples of mass spectra used to map the sites of pheromone-induced G β phosphorylation.

Fig. S2. Dynamic localization of the pheromone receptor as cells orient toward mating partners.

Fig. S3. Exogenous pheromone induces the formation of angled zygotes.

Fig. S4. Time-lapse images of elongating and turning *MATa STE2^{7XR} 6SA* and *MATa STE2^{7XR} 6SD* cells in mating mixtures.

Fig. S5. Surface distribution of the pheromone receptor as assayed by Alexa Fluor 594– conjugated α -factor binding.

Fig. S6. Receptor internalization assay.

Fig. S7. Spatial model of the computational yeast cell.

Fig. S8. Computational model of receptor dynamics.

Fig. S9. Computational model of G protein dynamics.

Fig. S10. Computational model of Yck dynamics.

Table S1. Yeast strains used in this study.

Table S2. Plasmids used in this study.

Table S3. Equations used for the spatial model of the yeast cell.

Table S4. Definitions and parameters.

Table S5. Reaction formulae

Table S6. Partial differential equations.

References (63–68)

Author contributions: D.S. and E.B. performed experiments; A.I., N.W., X.W., and M.M. performed experiments and analyzed data; Y.C., W.T., and J.L. did the mathematical modeling and analyzed data; D.E.S. and R.A. analyzed data; and D.E.S. wrote the manuscript.

Competing interests: The authors declare that they have no competing interests.

chemotropic system, the pheromone receptor on the plasma membrane polarizes to the side of the cell closest to the stimulus. Although receptor polarization occurs before and independently of actin-cable dependent vesicle delivery (directed secretion), it requires receptor internalization. Phosphorylation of pheromone receptors by yeast casein kinase 1 or 2 (Yck1/2) stimulates their internalization. We showed that the pheromone-responsive G $\beta\gamma$ dimer promotes the polarization of the pheromone receptor by interacting with Yck1/2 and locally inhibiting receptor phosphorylation. We also found that receptor phosphorylation is essential for chemotropism, independent of its role in inducing receptor internalization. A mathematical model supports the idea that the interaction between G $\beta\gamma$ and Yck1/2 results in differential phosphorylation and internalization of the pheromone receptor and accounts for its polarization before the initiation of directed secretion.

INTRODUCTION

Polarized cellular growth in response to a chemical gradient (chemotropism) is a fundamental process that is required for a broad range of phenomena, including angiogenesis, axon growth cone guidance, pollen tube guidance, fungal life cycles, and fungal pathogenicity. Similar to chemotaxing cells, chemotroping cells must be able to determine the direction of dynamic chemical gradients, establish a stable axis of polarity, and realign that axis as they track changes in the gradient.

The ability of the budding yeast, *Saccharomyces cerevisiae*, to mate efficiently depends on what is to date the best-understood chemotropic response. During the sexual reproduction phase of their life cycle, haploid yeast cells of the opposite mating types *MATa* and *MAT α* signal their position to one another by secreting peptide pheromones. The binding of pheromone to heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) on the surface of each cell type — Ste2 in *MATa* cells and Ste3 in *MAT α* cells — and the consequent activation of their cognate G proteins, induces cell cycle arrest, changes in gene expression, and the formation of mating projections, commonly called shmoos. Cells grow up the pheromone concentration gradient toward a potential mating partner, leading to the eventual fusion of the partners at their growth tips (1). The polarization of cell growth (morphogenesis) is accomplished by the directed movement of secretory vesicles along actin cables oriented toward the growth site (2, 3). The G $\beta\gamma$ subunit of the heterotrimeric G protein serves as a positional determinant for the chemotropic growth site by linking the pheromone receptor to the machinery that nucleates actin cables (4, 5).

As the most upstream elements in the mating response pathway, the pheromone receptor and its G protein are the primary gradient sensors. Unlike most chemotaxing cells, whose surface receptors are uniformly distributed within the plasma membrane, pheromone-stimulated yeast cells polarize their GPCR. In response to isotropic pheromone treatment, the receptor concentrates around the predetermined bud site, which is also known as the default polarity site. In mating mixtures, the receptor polarizes towards proximal mating partners, presumably where the pheromone concentration is highest, and this phenomenon is detectable before morphogenesis occurs (6). The phenomenology and regulation of

pheromone-induced receptor polarization in *MATa* cells is well-documented. During vegetative growth, the *MATa*-specific receptor Ste2 appears uniformly distributed on the plasma membrane. When activated by ligand, however, the receptor is sequentially phosphorylated and ubiquitylated on its C-terminal cytoplasmic domain (7, 8). This stimulates its global internalization, after which it reappears as a polarized crescent on the cell surface (9, 10). Although receptor polarity could arise from the directed delivery of nascent receptors to the incipient shmoo site, several observations argue for a distinct mechanism. First, Ste2 becomes polarized before morphogenesis occurs. Second, establishment of receptor polarity does not depend on the directed delivery of vesicles along actin cables (6). Third, the genesis of receptor polarity absolutely depends on receptor internalization (6).

How does the receptor polarize towards the gradient source before and independently of actin-cable directed delivery of secretory vesicles? One possibility is that the pheromone-induced global internalization of the receptor is slower on the up-gradient side of the cell such that relative receptor density increases at the future shmoo site. This is consistent with the observation that the redistribution of the receptor depends on its internalization, but at the same time, it introduces a paradox: Because only activated receptors are phosphorylated, ubiquitylated, and internalized, the rate at which the receptor is removed from the cell surface should be greatest where the pheromone concentration is highest. In principle, therefore, the establishment of the chemotropic growth site through differential internalization of the receptor requires a mechanism to locally inhibit receptor internalization.

In a genetic screen for proteins that interact directly with G β (11), we identified Yck1, one of a pair of sister casein kinases (CKs) (12) that are essential for the phosphorylation of the pheromone receptor on its C-terminal cytoplasmic domain (8), and hence for its internalization (7, 8). Here, we showed that G β interacted with Yck1 at the plasma membrane and that G β locally inhibited phosphorylation of the receptor, consistent with the idea that differential phosphorylation and internalization of the receptor play a role in the establishment of polarity. In addition, imaging and genetic data suggest that differential receptor phosphorylation contributes to chemotropic function. A mathematical model that incorporates the G β γ -dependent inhibition of receptor phosphorylation mimics key aspects of gradient-induced receptor polarization.

RESULTS

G β interacts with Yck1 at the plasma membrane and is a candidate Yck substrate

To identify candidate interactors of G β , we previously conducted an allele-specific dosage suppressor screen in yeast in which we took advantage of the observation that the overexpression of G β induces the mating signal, and thereby confers cell cycle arrest (13, 14). A high-copy yeast complementary DNA (cDNA) library was screened for plasmids that could rescue the overexpression of wild-type G β , but not that of an adaptive-defective mutant form of G β , encoded by *STE4^{A405V}*. Ten genes were identified, including those encoding Rho1 (11), Dse1 (15), and Yck1. In addition to the G β -Yck1 genetic interaction, here we found that a myc-tagged form of Yck1 expressed in yeast specifically bound to a

Gβγ-affinity column, as evidenced by both Western blotting and mass spectrometric analyses (Fig. 1, A and B, and Table 1). Moreover, a direct physical interaction between Gβ and Yck1 in yeast was shown by bimolecular fluorescence complementation (BiFC) assay (Fig. 1C and Table 2). The Gβ-Yck1 BiFC signal was detectable on the plasma membrane of pheromone-treated cells before morphogenesis occurred and was concentrated on the plasma membrane of mating projections in shmooing cells.

Because Gβ is phosphorylated on multiple sites in pheromone-treated cells (16), and because its sequence contains potential CK1 sites (17–19), we asked whether Gβ phosphorylation depended on Yck activity. Pheromone did not induce full phosphorylation of Gβ in cells lacking functional Yck1 and Yck2 (hereafter, Yck1/2) (Fig. 1D). Previous genetic analyses suggested that Gβ residues Thr³²⁰ (T320), Thr³²² (T322), and Ser³³⁵ (S335) are phosphorylated, and that full Gβ phosphorylation is dependent on the pheromone-responsive mitogen-activated protein kinase (MAPK) Fus3 (20). Mass spectrometric analysis of Gβ phosphorylated peptides purified from pheromone-treated cells confirmed these phosphorylation sites and revealed an additional site at Thr³¹⁸ (Fig. 1E and fig. S1). Study of mammalian CK1s suggests that they require a phosphorylated substrate and that S/T^PX_n/T (where n = 1 to 3 and X is any amino acid residue) is the consensus motif (17–19). If this holds true for yeast, the phosphorylation of Thr³¹⁸ or Thr³²⁰ would convert Gβ to a Yck1 substrate (Fig. 1E). Conversely, unphosphorylated Gβ would be expected to interact with Yck1 to a lesser degree than would the partially phosphorylated species. We tested this with a mutant form of Gβ, *ste4^{T320A/S335A}* (henceforth Gβ^{P-}), which cannot be phosphorylated at Thr³²⁰ or Ser³³⁵ (20). In pull-down experiments in which the relative amount of Yck1 that bound to Gβγ or Gβ^{P-}γ beads was quantified both by Western blotting and mass spectrometric analyses (Fig. 1, A and B, and Table 1), the apparent affinity of Yck1 for Gβ^{P-} was less than that for Gβ. Note that endogenous Yck2 (that is, untagged native protein) was also identified by mass spectrometry as specifically binding to the Gβγ beads, and, like Yck1, it showed a weaker apparent affinity for Gβ^{P-} than for Gβ (Table 1).

Gβ overexpression inhibits phosphorylation of the pheromone receptor and promotes its polarized localization

Because Gβ adopts a polarized localization before pheromone-induced morphogenesis occurs (21), its direct interaction with Yck1 raises the possibility that Gβγ promotes receptor polarization by locally inhibiting receptor phosphorylation. Consistent with this, a region of the plasma membrane enriched in hypo-phosphorylated receptor arises in response to isotropic pheromone treatment (6, 22). This was demonstrated with strain YDB111 (22), which expresses a GFP-tagged form of the RGS protein Sst2, and Ste2^{7XR}, a mutant form of the *MATa* receptor that cannot be internalized. The Sst2-GFP reporter binds specifically to the unphosphorylated form of the Ste2 receptor (22). YDB111 cells treated with latrunculin A (LatA, which inhibits polymerization of actin) and pheromone cannot redistribute the receptor, nevertheless they exhibit asymmetric receptor phosphorylation (6).

To determine whether Gβ could inhibit receptor phosphorylation, we examined pheromone- and LatA-treated YDB111 cells overexpressing either Gβ or Gβ^{P-}, the weaker Yck1/2-binding form of Gβ (Fig. 2, A to F). Overexpression of Gβ correlated with an increase in

both the amount of Sst2-GFP that was localized to the plasma membrane and the extent of Sst2-GFP polarity. In contrast, overexpression of $G\beta^{P-}$ did not affect the plasma membrane localization of Sst2-GFP. To further test the effect of weakening the $G\beta$ -Yck1/2 interaction on receptor phosphorylation, we replaced *STE4* ($G\beta$) with *ste4^{T320A/S335A}* ($G\beta^{P-}$) in the YDB111 strain. Treatment with pheromone and LatA stimulated significantly less plasma membrane localization of Sst2-GFP in cells expressing the native amount of $G\beta^{P-}$, compared to that in control cells (Fig. 2, F and G). Together, these data support the idea that $G\beta\gamma$ protects the pheromone receptor from Yck1/2-dependent phosphorylation, and that it does so most effectively in a discrete region of the plasma membrane.

Local inhibition of receptor phosphorylation by $G\beta\gamma$ helps to establish and maintain the chemotropic growth site

In time-lapse images of mating mixtures, we found that the polarized receptor crescent frequently relocated from the default polarity site to the chemotropic site in cells that had not yet shmooed (fig. S2). Could anisotropic receptor phosphorylation play a role in this process? In mating mixtures of YDB111 cells, the Sst2-GFP crescent was almost always visible before morphogenesis occurred and either formed initially at the eventual shmoo site or relocated from the presumptive default site to an apparent chemotropic site before morphogenesis (Fig. 3). Thus, the position of minimal receptor phosphorylation anticipated the eventual site of polarized growth. Although the Sst2-GFP crescent in YDB111 cells expressing $G\beta^{P-}$ also anticipated the shmoo site, the $G\beta^{P-}$ cells shmooed about 1 time point earlier (15 s) than did the $G\beta$ cells ($n = 30$ cells; $P = 0.05$), and did so markedly closer to the presumptive default site (Fig. 3). These data suggest two conclusions. First, cells that are unable to internalize receptors, and thereby polarize receptor distribution, are nevertheless able to establish a chemotropic growth site by local inhibition of receptor phosphorylation. Second, the role $G\beta$ plays in positioning and stabilizing the chemotropic growth site correlates with its effect on receptor phosphorylation.

To further examine the possibility that receptor phosphorylation plays a role in chemotropism, apart from triggering receptor internalization, we replaced native *STE2* with *STE2^{7XR}*, *STE2^{7XR 6SA}*, or *STE2^{7XR 6SD}* in an otherwise wild-type *MATa* strain. These alleles all encode forms of the receptor that cannot be internalized (7XR) (23), and which are either barely phosphorylated (6SA), or which have phosphomimetic substitutions (6SD) (24). We then assessed the effects of the mutant receptors on chemotropism in time-lapse images of mating mixtures (Fig. 4). Cells that were fully competent to mate typically shmooed directly towards their mating partner, grew chemotropically about the same distance to their point of contact, and ultimately fused at an angle near 0° (Fig. 4, B and D). The resulting zygotes were straight and symmetrical. In contrast, cells defective in gradient sensing or chemotropism, or wild-type cells confused by exogenous pheromone (fig. S3), did not orient directly toward their partners, and ultimately formed angled zygotes (21).

When chemotropism was assayed by observing mating pairs directly rather than by quantifying the efficiency of diploid formation by genetic markers, as performed by McClure *et al.* (25), it was apparent that *STE2^{7XR}* conferred a defect in initial orientation (Fig. 4B). The mean orientation angle of the mutant cells was considerably smaller than that

of the control cells, and the mean fusion angle and the mean time to fusion were markedly greater in the WT *MAT α* X *MAT α STE2^{7XR}* mating mixtures than in the WT $\times$ WT crosses (Fig. 4, C to E). Note that *STE2^{7XR}* conferred an increase in the mean fusion angle that was equivalent to the addition of exogenous pheromone to wild-type mating mixtures (fig. S3). We infer that the inability to internalize and thereby redistribute the mutant receptor compromised directional sensing. When cells could not either polarize the distribution of the pheromone receptor, or locally affect its phosphorylation state, they were unable to grow chemotropically. In mating mixtures, the *MAT α STE2^{7XR} δ SD* cells that ultimately formed zygotes with wild-type *MAT α* cells always polarized their growth adjacent to their last bud site (Fig. 4B). Similarly, all *MAT α STE2^{7XR} δ SA* cells that ultimately formed zygotes polarized their growth—or mated without undergoing morphogenesis—proximal to their last bud site. Thus, *MAT α STE2^{7XR} δ SA* and *MAT α STE2^{7XR} δ SD* cells could not switch from the presumptive default polarity site to a chemotropic growth site regardless of the position or proximity of potential mating partners (Fig. 4C).

In addition to eliminating gradient-sensing in situ, the *STE2^{7XR} δ SA* and *STE2^{7XR} δ SD* alleles conferred pronounced defects in mating projection formation (Fig. 4F). Although most of the mating *MAT α STE2^{7XR} δ SA* cells and all of the mating *MAT α STE2^{7XR} δ SD* cells exhibited robust polarized growth, a substantial fraction of the mutants apparently could not stabilize their axis of polarity well enough to generate a tapered shmoo tip. Such cells elongated along very broad growth zones, the shape of which was easily distinguished from that of a normal mating projection. Of particular note, the broadly polarized *MAT α STE2^{7XR} δ SA* and *MAT α STE2^{7XR} δ SD* cells persistently turned in one direction as they elongated (Fig. 4B and fig. S4), a behavior also seen in *sst2* cells (26). Because some of these cells eventually mated, albeit over abnormally long times (Fig. 4E), their curving growth might be mistaken for chemotropism. Is the persistent turning toward the eventual fusion site a dramatic example of reorientation? Our data indicate that it is not. The broadly polarized and curving *MAT α STE2^{7XR} δ SA* and *MAT α STE2^{7XR} δ SD* cells showed no sign of gradient sensing. Their initial direction of growth was determined by default polarity (Fig. 4C), and they continued to elongate and turn until they encountered a mating-competent *MAT α* cell directly in their path, often after having failed to mate with nearer potential partners (Fig. 4B). Only 26% of the *MAT α STE2^{7XR} δ SA* and *MAT α STE2^{7XR} δ SD* cells that elongated and turned, and 42% of those that were initially positioned within 1 μ m of one or more potential partners, formed zygotes within the six-hour time course (fig. S4). Based on our analysis of cells expressing mutant receptors in physiological gradients, we conclude that receptor internalization is required for full chemotropic function, and that receptor phosphorylation plays a critical role in chemotropism that is distinct from its established function as a stimulus for internalization.

The pheromone receptor becomes polarized before Cdc42 is activated

In pheromone-treated cells that were first allowed to internalize the receptor for 15 min, and then treated with LatA, a polarized receptor crescent subsequently formed on the plasma membrane within about 30 min, even though ongoing actin-dependent secretion and endocytosis were blocked (Fig. 6A and B; 6, 27). A likely explanation for this is that, at the time of LatA treatment, a preexisting polarity site—in this case, one that was generated as

the receptor was internalized—was amplified by uniform delivery of secretory vesicles together with biased docking and fusion at the plasma membrane, as proposed by Sahin *et al.* (28). The receptor itself could be the primary determinant of this site. Although the membrane localization of the Ste2-GFP reporter was undetectable after 15 min of induced internalization (6), a concentrated receptor patch was seen when such cells are labeled with Alexa Fluor 594–tagged pheromone (fig. S5). Alternatively, cortical polarity could be generated by actin-independent, Cdc42-dependent exocytosis, as described by Bendezu *et al.* (29), or by endocytosis and recycling of Cdc42, as proposed by Yamamoto *et al.* (30). To distinguish between these possibilities, we investigated whether pheromone-induced receptor (Ste2-GFP) polarity was detectable before Cdc42-GTP (Gic2-PBD-RFP) polarity occurred, or vice versa. Before they were treated with pheromone, about half of the cells in G1 exhibited Gic2-PBD-RFP polarity, but not Ste2-GFP polarity, whereas none showed receptor polarization alone, consistent with the essential role of Cdc42-GTP in bud emergence (Fig. 5, A and B). Conversely, about half of the cells in G1 that were not yet shmooing after 60 min of pheromone treatment exhibited receptor crescents before Cdc42-GTP polarity was detectable, even though the reporter signals were of similar intensity. No pheromone-treated cells were observed that exhibited polarized Gic2-PBD-RFP before exhibiting polarized Ste2-GFP (Fig. 5, A and B).

Changes in the localization of the receptor also preceded the redistribution of active Cdc42, and morphogenesis, in gradient-stimulated cells (Fig. 5, C and D). In time-lapse images of *MATa* cells that switched from their presumptive default polarity site to a presumptive chemotropic growth site in mating mixtures, both Ste2-GFP and Gic2-PBD-RFP moved slowly from their initial sites to final sites of polarization, consistent with results reported by Hegemann *et al.* (31). The pheromone receptor was clearly detectable and centered around the eventual polarized growth site before Gic2-PBD-RFP was similarly redistributed in 13 of 19 scored cells (68%), and was the first to fully re-localize in 13 of 16 scored cells (81%). Redistribution of Gic2-PBD-RFP was never seen to precede that of Ste2-GFP. These data suggest that pheromone-induced polarity of the receptor determines the position of the chemotropic shmoo site upstream of active Cdc42.

Gβ^P– cells are defective in receptor polarization

Our biochemical data suggest that Gβ^P– has a reduced affinity for Yck1/2 compared to that of Gβ. We also previously showed that the pheromone-induced polarization of GFP-Gβ^P– before morphogenesis occurs is moderately defective (21). If either early Gβ polarization or the Gβ-Yck1 interaction plays an important role in establishing receptor polarity, then Gβ^P– would be expected to adversely affect the formation of receptor crescents. To test this, we compared the localization of GFP-tagged receptor in Gβ and Gβ^P– cells treated with LatA 15 min after pheromone stimulation. We found that Gβ^P– cells exhibited reduced receptor polarization as compared to that of Gβ cells (Fig. 6, A and B), suggesting a role for Gβ phosphorylation in the establishment of the polarity site as the receptor undergoes global internalization. A similar result was observed under physiological conditions: Gβ^P– cells exposed to pheromone gradients in mating mixtures also exhibited a defect in receptor polarization (Fig. 6, C and D).

Pheromone-induced internalization can result in the polarization of activated receptors

To follow the internalization of ligand-bound receptors as distinct from the trafficking of inactive receptors, we treated cells with fluorescent pheromone. Cells were labeled on ice in medium lacking essential nutrients and were imaged at room temperature 1 to 2 min after being re-fed. Although the cells were not exposed to a pheromone gradient, approximately 25% exhibited an asymmetry in the rate of receptor internalization, leading to enhanced receptor polarity (fig. S6). Thus, the surface distribution of the activated receptor changed as the receptors were internalized. We speculate that the cell cycle-dependent polarization of the G protein (6) mimics the early response of cells to stimulation by a gradient, revealing the possibility of polarization through differential internalization.

The $G\beta^P\gamma$ -Yck interaction is critical in a reaction-diffusion model that mimics gradient-induced receptor polarization upstream of directed secretion

The observations presented earlier are suggestive of a previously uncharacterized mechanism for the establishment of pheromone-induced receptor polarity. By interacting with Yck1/2, $G\beta\gamma$ inhibits phosphorylation of the pheromone receptor, which in turn slows receptor internalization. The interaction between $G\beta\gamma$ and Yck1/2 interaction would also be expected to inhibit endocytosis of the G protein, because we previously found that both $G\alpha$ and $G\beta\gamma$ internalize with the receptor (6). Initially, a shallow gradient of pheromone is mirrored by a similarly shallow gradient of occupied receptor across the cell. This slight differential in activated receptor should lead to a corresponding differential of activated $G\alpha$ and free $G\beta\gamma$. Because the receptor and G protein in this area are less likely to be internalized, a positive feedback loop is generated which, in combination with other mechanisms, rapidly amplifies the intracellular signaling gradient before actin cables become nucleated (Fig. 7A). Previously published data suggest that $G\alpha$ recruits the activated MAPK Fus3 to phosphorylate cortical $G\beta$ (32, 33) and demonstrate roles for the $G\alpha$ -Fus3 interaction in directional sensing and chemotropic shmooing (21, 33, 34). Given the data reported here, phosphorylation of $G\beta$ by a MAPK would be expected to enhance its interaction with Yck1/2. This raises the possibility that two positive feedback loops act synergistically to promote receptor polarization: (i) $G\alpha$ /Fus3-mediated phosphorylation of $G\beta$, and (ii) inhibition of the Yck-dependent internalization of the receptor and G protein by phosphorylated $G\beta$.

To determine whether our postulated feedback mechanisms could explain how receptor polarity is generated in response to a pheromone gradient, we developed a reaction-diffusion model which, unlike other mathematical models of yeast chemotropism (35–37), is based on mechanisms that are independent of Cdc42 (Fig. 7, B to D, fig. S7, and tables S3 to S6). We first generated a core network that included only the best-characterized components. Network 1 consists of the receptor-pheromone interaction, the G protein cycle, and the Yck-dependent internalization of the receptor and G protein. Network 2 adds the $G\beta^P\gamma$ -Yck interaction, whereas Network 3 adds the $G\alpha$ -Fus3 interaction. A 10 to 5 nM pheromone gradient induced a substantial reduction in the number of receptors on the plasma membrane of a computational yeast cell lacking downstream regulation of receptor internalization (Fig. 7E), with no resulting polarity (Network 1). In contrast, a computational cell that differed only in the addition of the $G\beta^P\gamma$ -Yck interaction (Network 2; Fig. 7F) responded to the same

gradient by robustly polarizing its receptor. Addition of the G α -Fus3 feedback loop (Network 3; Fig. 7G) resulted in faster receptor polarization. Note that the rate of receptor internalization was greatest on the side of the cell facing the gradient immediately after it was stimulated with pheromone, which was expected because of the higher receptor occupancy in this area (fig. S8). Within 12 min (Network 3) or 15 min (Network 2), however, iteration of the feedback loops reversed the differential so that receptor internalization was slowest at the front of the cell, where the receptor eventually became polarized. Output from the mathematical model also yielded testable predictions about the spatiotemporal dynamics of other key signaling elements in the response networks. Whereas the heterotrimeric G protein remained largely uniform in distribution on the surface of the gradient-stimulated computational cell, activated G α and free G $\beta\gamma$ polarized together with the receptor (fig. S9). At the same time, inverse intracellular gradients of free Yck (back) and G $\beta\gamma$ -bound Yck (front) were generated (fig. S10).

DISCUSSION

Although both chemotactic and chemotropic cells use GPCRs to detect and respond to chemical gradients, they differ in how they deploy their surface receptors. With few exceptions, chemotactic cells exhibit a uniform surface distribution of receptors. This enables them to rapidly sense changes in ligand concentration in three dimensions. Although the surface distribution of receptors is uniform, the distribution of activated receptors reflects changes in the gradient within seconds. Local receptor activation is amplified by downstream signaling mechanisms, which leads to polarized cellular outputs (38, 39). By comparison, chemotropism is a slower and more two-dimensional process. As exemplified by budding yeast, chemotropic growth occurs on a timescale of tens of minutes rather than seconds, and on solid surfaces rather than in fluid or tissues. These spatiotemporal differences may underlie the redistribution of receptor to the region of polarized growth in pheromone-stimulated cells, as compared to the invariant receptor displayed on the surface of chemotactic cells.

The formation of polarized receptor crescents in shmooing yeast was first reported in 1991 (40), and its importance has been debated ever since. Is receptor polarization simply the result of global internalization followed by actin-cable directed delivery of secretory vesicles to the plasma membrane, or does the local concentration of receptor determine the position of the chemotropic growth site? A number of results indicate that the initial polarization of surface receptor is established independently of actin-cable directed secretion. Polarized receptor crescents are easily visualized before morphogenesis (6, 31). Pheromone-treated cells in which actin cable-directed secretion is disabled by mutation or drug nevertheless form robust receptor crescents (6, 31). Here, we report the formation of receptor crescents before the polarization of active Cdc42 in pheromone-treated G1 cells (Fig. 5), which implies that the receptor polarizes before the actin cables became nucleated.

In contrast to the robust receptor polarization that we observed in the absence of directed secretion, the receptor cannot polarize if its internalization is blocked by mutation or by treatment with LatA together with pheromone (6). However, when pheromone-induced receptor internalization was allowed to continue for 15 min before actin was depolymerized

with LatA, robust receptor crescents subsequently appeared (Fig. 6) (6). We infer that a polarity site is established while the receptor is being internalized, and that the position of this site is most likely determined by the receptor itself. A mutant form of the receptor that cannot be internalized, and is thus unable to polarize (Ste2^{7XR}), conferred a defect in gradient sensing, as was measured in low-density unilateral mating mixtures (Fig. 4). Moreover, the receptor crescent shifted from the default polarity site to the chemotropic growth site in wild-type cells that had not yet changed shape (fig. S2). Together, these data indicate that receptor polarization occurs earlier than directed secretion and contributes to gradient sensing.

In addition to suggesting that the distribution of the receptor and G protein can be polarized by differential internalization, our data indicate that differential phosphorylation of the receptor alone can support a chemotropic response. In mating cells that could internalize the pheromone receptor, the unphosphorylated receptor crescent was often seen to move from the presumptive default polarity site to an apparent chemotropic site in advance of morphogenesis; that is, the unphosphorylated receptor crescent responded to the gradient and anticipated the eventual shmoo site (Fig. 3). Because the distribution of unphosphorylated receptor could not be determined with the Sst2-GFP reporter in wild-type cells, we measured the effects of preventing receptor internalization and phosphorylation on chemotropism in otherwise wild-type cells stimulated by natural gradients. In contrast to the moderate orientation defect seen in Ste2^{7XR} cells, Ste2^{7XR 6SA} cells and Ste2^{7XR 6SD} cells that could neither internalize nor phosphorylate their receptor were unable to switch from default to chemotropic polarity, and were compromised in their ability to form mating projections (Fig. 4 and fig. S4). These data suggest that receptor phosphorylation plays an essential role in chemotropism that is distinct from its role as a stimulus for receptor internalization, and suggest that the localized concentration of unphosphorylated receptor is critical to establishing the chemotropic growth site.

The processes that enable yeast cells to align their mating projection sites with pheromone gradients are not well understood; however, two relevant mechanisms have been identified. The binding of free G $\beta\gamma$ to the Far1-Cdc24-Bem1 protein complex was established early in the study of yeast chemotropism (4, 5). It was subsequently shown that disrupting either the G $\beta\gamma$ -Far1 interaction (4) or the G $\beta\gamma$ -Cdc24 interaction (5) prevents chemotropic shmooing. Furthermore, Dyer *et al.* (35) and Hegemann *et al.* (31) used live-cell imaging to show that a dynamic polarity complex, which is likely composed of the core constituents Bem1, Cdc24, Cdc42, and Bni1, moves in a random walk around the cortex of vegetative cells. When cells are exposed to pheromone, the movement of the polarity complex is biased toward the incipient shmoo site through the interaction of Far1-Bem1-Cdc24 with free G $\beta\gamma$ (27, 34). In principle, therefore, yeast chemotropism can be explained as follows: A gradient of pheromone induces a gradient of activated receptor on the surface of the cell, which in turn, is mirrored by a gradient of activated G protein. Free G $\beta\gamma$ then restricts the movement of the polarity patch along the cell cortex on the side of the cell exposed to the highest concentration of pheromone, and ultimately stabilizes its position at the incipient growth site. Actin cables are subsequently nucleated at this site.

As the primary determinant of the chemotropic growth site downstream of the receptor, $G\beta\gamma$ must be locally concentrated where the pheromone concentration is greatest. Polarization of $G\beta\gamma$ is required to constrain the polarity patch and nucleate actin cables. There are two challenges to explaining how $G\beta\gamma$ polarizes before polarity patch stabilization and actin-cable nucleation occur. First, physiological gradients are shallow. It has been estimated that a 1% difference in receptor occupancy across the 5- μm length of a yeast cell in a pheromone gradient is sufficient to elicit robust orientation of cell growth toward the pheromone source (42), and microfluidic studies suggest an even greater acuity (36). Thus, although there is a slight excess of free $G\beta\gamma$ on the side of the cell exposed to the highest concentration of pheromone, there is almost as much on the opposite side. Second, because activated receptors are targeted for phosphorylation and internalization, the density of receptor and G protein at the plasma membrane is expected to be least where the pheromone concentration is greatest, the opposite of what would be expected to drive chemotropism. The $G\beta\gamma$ -Yck interaction that we demonstrated here (Fig. 1, A to C; and Tables 1 and 2) provides a means to invert the relationship between receptor activation and receptor internalization. From first principles, the proportion of activated receptors on the surface of a cell subjected to a pheromone gradient should increase as a function of pheromone concentration. Based on the established model of pheromone-induced receptor modification and endocytosis, the initial rate of receptor internalization is expected to be greatest where the density of activated and phosphorylated receptors is greatest, as is the initial rate of heterotrimeric G protein activation. We postulate that free $G\beta\gamma$ protects proximal receptors from Yck-dependent phosphorylation and internalization, which in turn preserves heterotrimeric G proteins at the plasma membrane (6). Thus, the slightly greater burst of $G\beta\gamma$ released from $G\alpha$ on the up-gradient side of the cell triggers a feedback loop that amplifies signaling at the incipient shmoo site. As yet, the most upstream event in this pathway is the induction of anisotropic receptor phosphorylation such that the most concentrated region of activated, unphosphorylated receptor marks the direction of the gradient source.

Based on published results and the observations presented here, we also postulate a feedback loop that promotes the $G\beta\gamma$ -Yck interaction. Activated $G\alpha$ is thought to recruit active Fus3 to the cell cortex (32, 33, 43), where it phosphorylates $G\beta$ (20) on one or more MAPK consensus sites (Fig. 1E). Given the CK1 consensus motifs in the $G\beta$ sequence (17–19), phosphorylation of either Thr³¹⁸ or Thr³²⁰ by a MAPK could convert $G\beta$ to a Yck substrate. Indeed, $G\beta$ was hypo-phosphorylated in cells lacking Yck1/2 function (Fig. 1D). These various points can be unified in a number of ways: Yck has a basal affinity for unphosphorylated $G\beta$, which is increased when $G\beta$ is phosphorylated by $G\alpha$ -recruited Fus3. Thereby marked as a Yck substrate, $G\beta$ acts as a competitive inhibitor of receptor phosphorylation. Alternatively, $G\beta$ might allosterically decrease the catalytic activity of Yck. Although it will be of interest to distinguish between these possibilities, the protection of proximal receptors by $G\beta$ could involve either or both mechanisms.

We conclude that, similar to chemotactic cells, yeast cells unable to polarize the distribution of receptor can nevertheless orient in a gradient, albeit not as well as do wild-type cells. How do they do this? A likely explanation is that, as the distribution of the phosphorylated receptor becomes polarized, G protein activation is favored where the active, unphosphorylated receptor is concentrated. This requires that the G protein is able to freely

diffuse along the plasma membrane and be subject to activation by coupling with ligand-bound receptors, as has been reported for chemotactic cells. In *Dictyostelium*, for example, activated $G\alpha$ and free $G\beta\gamma$ are thought to be concentrated on the leading edge of chemotaxing amoebae when freely diffusing heterotrimeric G proteins couple with ligand-bound receptors on the side of the cell facing the gradient (44, 45). The output from our mathematical model is consistent with this hypothesis: Although the heterotrimeric G protein is almost uniformly distributed on the surface of the gradient-stimulated computational cell, activated $G\alpha$ and free $G\beta\gamma$ markedly polarize together with the active receptor (figs. S7 and S8). It remains to be determined whether the ligand-bound, unphosphorylated receptor activates the G protein more effectively than does the ligand-bound phosphorylated receptor, and if so, how it does so. If confirmed, however, a confined G protein activation center would add another powerful feedback mechanism to our proposed networks. In this scenario, activated receptor would generate activated $G\alpha$ and free $G\beta\gamma$, which would increase the local density of activate, unphosphorylated receptor and G protein, which would further increase the local density of activated $G\alpha$ and free $G\beta\gamma$, and so on. As discussed earlier, the rapid localization of $G\beta\gamma$ toward the source of the gradient is essential for positioning the chemotropic growth site.

Together, our data suggest that polarity is first established in response to a pheromone gradient by differential phosphorylation and internalization of the receptor, which depends, in turn, on anisotropic protection of the receptor by its G protein. At least two interconnected positive feedback loops are at the heart of the localized amplification mechanism underlying the yeast chemotropic response. Similar processes may contribute to directional sensing in other systems, because a role for GPCR phosphorylation other than desensitization has been reported in *Dictyostelium* chemotaxis (46).

MATERIALS AND METHODS

Molecular and microbiological techniques

Standard methods were used for microbial culture and molecular manipulation, which were performed as described previously (47–49).

Yeast strain construction

The yeast strains used in this study are listed in table S1. Unless noted otherwise, they were derived by transformation from strain 15Dau (*MATa bar1 ade1 his2 leu2-3, -112 trp1 ura3*), which is congenic with strain BF264-15D (50). To generate strains that could be heavy-labeled for mass spectrometric analysis, *ARG5,6* was knocked out in strains 15Dau and RDY114 by transplacement with a fragment containing KanMX4 G418, PCR-amplified from pFA6-kanMX4 (51), flanked by ends homologous to *ARG5,6*, with the oligomers 5'-TCCAAATTCCAAAAATTTGTGTCTTCATTAAACAAATCCACCATAGCAGGCGTACG CTGCAGGTCGAC-3' and 5'-TCAGGGATACCAGCATACTCTCCATAACCCATAGCAAGATTAATATTTTGATCGATG AATTTCGAGCTCG-3'. Integrants were selected on medium containing geneticin (G418, 200 mg/l). *LYS1* was then transplaced with a TEF promoter-hph-TEF terminator fragment amplified from the pAG32 plasmid (EUROSCARF) and flanked by ends homologous to

LYSI with the oligomers 5'-GCTGCCGTACATTACATCTAAGAGCTGAACTAAACCCCTAGAGGCTCTGTTTAGCTTGCCCTTGTC-3' and 5'-GTACCAGAACGGTAGGTTTGTAAACACAGTAGCCACAGTGTATATGCTCGTTTTTCGACACTGGAT-3'. Integrants were selected on medium containing hygromycin B. To enable exclusive expression of 6xHis-Ste18 and 3xHA-Ste18, native *STE18* (G γ) was transplacated with a fragment containing *URA3* as described previously (52). Strain AIY109 was generated by integrating *ste4*^{T320A S335A} into strain YDB111 (22) in situ with Msc1-cut YIplac128/*ste4*^{T320A S335A} N¹¹² (pAIB130). Strain AIY197 was generated by integrating HpaI-cut LHP1921 (53) into RDY114 (21). Strain AIY301 was generated by integrating ApaI-digested YIplac211-GIC2-PBD-RFP (54) into DMY169 (6). Strains XWY005, XWY008, and XWY018 were generated by integrating ClaI-digested DLB3850, DLB3784, and DLB3851, respectively, into strain DSY257. The fluorescent marked *MAT α* mating tester strain, XWY027, was generated by integrating Bsu36I-digested pRS406/GFP-BUD1 at the *BUD1* locus of DSY129. All integrations intended to cause gene deletion, replacement, or in-situ tagging were checked by genomic sequencing.

Plasmid construction

The plasmids used in this study are listed in table S2. YCplac22/*GAL1*-3xHA-STE18 was constructed by PCR-amplifying *STE18* from pGEX-KG-STE18 (MCB35) with the oligomers 5'-TGCTATCCAGTCGACATGTTACCCATACGACGTCCCAGACTACGCTTACCCATACGACGTCCCAGACTACGCTTACCCATACGACGTCCCAGACTACGCTTACCCATACGACGTCCCAGACTACGCTATGACATCAGTTCAAAATC-3', in which the underlined sequence encodes the N-terminal 3X-HA tag, and 5'-TGGACCGCCAAGCTTTTACATAAGCGTACAACAAA-3'. The Sal I- and Hind III-digested PCR product was inserted into YCplac22/*GAL1* (55). YIplac128/*ste4*^{T320A S335A} N¹¹² was constructed by PCR-amplifying *ste4*^{T320A S335A}, minus the first 112 bases of the coding region, from RDY120 (21) with the oligomers 5'-CGCGAATTCTGCGCTTCCACAGAACTAATG-3' and 5'-CGCGGATCCAAATAGAGGCCGCCAGACAAG-3'. The PCR fragment digested with Eco RI and Bam HI was subcloned into YIplac128 (55) to generate AIB130. pRS415/ADH1-STE4-VF2 was constructed by PCR-amplifying genomic *STE4* lacking the stop codon with the oligomers 5'-GCAGGATCCGATGGCAGCACATCAGATGG-3' and 5'-GCTGGATCCTTGATAACCTGGAGACCAT-3'. The Bam HI-digested PCR fragment was subcloned into p415-VF2 (56) in frame with the C-terminal Split-Venus fragment under the control of the *ADH1* promoter to generate AIB201.

Gβ-Yck1 Split-Venus bimolecular fluorescence complementation (BiFC) assay

Because constitutive overexpression of G β causes permanent cell cycle arrest, the G β -Yck1 BiFC assay was performed in a strain that enabled conditional expression of *STE7*. *MATa STE7 ::KanMX*YCplac22/GAL1-STE7 cells transformed with pRS416/ADH1-VF1-YCK1 and pRS415/ADH1-STE4-VF2 (AIY273), pRS416/ADH1-VF1-YCK1 and pRS415/ADH1-VF2 (AIY276), or pRS415/ADH1-STE4-VF2 and pRS416/ADH1-VF1 (AIY275) were grown to mid-log phase in selective medium containing 2% sucrose, and were induced with 2% galactose for 1 hour before being treated with 150 nM α -factor. Images were acquired

2.5 hours after pheromone treatment with an ANDOR Revolution XD spinning disk laser confocal system with a motorized Olympus IX-81 microscope, a Yokogawa CSU-X1 spinning disk unit, a motorized XYZ control (piezo), and an iXon897 EMCCD camera controlled by Andor iQ2 software. A UplanSApo NA 1.4×100 objective was used with a laser excitation of 488 nm. The mean plasma membrane fluorescence and mean cytoplasmic fluorescence were quantified with the ImageJ segmented line tool and selection brush tool, respectively.

Gβ phosphorylation assay

DMY222 (*MATa his3 leu2 ura3-52 yck1-D1::ura3 yck2-2^{ts} leu2:STE2-GFP::LEU2*) and the congenic strain DMY224 (*MATa his3 leu2 ura3-52 leu2:STE2-GFP::LEU2*) were grown to mid-log phase at room temperature in rich medium (YEPD), shifted to 37°C (restrictive temperature), or maintained at room temperature, and treated with either 150 nM α-factor or 1.2 μM α-factor for 1 hour. Cell equivalents were collected from all cultures, and the phosphorylation state of Gβ was assessed by gel-shift Western blotting assay as described previously (20) using affinity-purified antisera raised against full-length Gβ (57).

Gβ and Gβ^P- overexpression assays

Strains XWY011 (Gal1-Gβ), XWY033 (Gal1-Gβ^P-), and RDY102 (Gβ⁻) were grown to mid-log phase at 30°C in selective medium containing 2% sucrose, and galactose was added to a final concentration of 2%. Cell equivalents were collected at time zero and 2 hours after induction, and protein concentrations in the cell lysates were determined by Bradford assay. Total protein (30 μg per sample) was resolved on a 4 to 20% gradient gel (Bio-Rad), and the amount of Gβ was assayed by Western blotting as described earlier.

Gβγ and Gβ^P-γ affinity beads and pull-down analysis

To prepare Gβγ-affinity beads and negative control beads, strains NWY069, NWY068, and NWY071 were grown to mid-log phase in selective medium containing 2% sucrose and 0.1% dextrose, induced with 2% galactose for 5 hours, and then treated with 150 nM α-factor for 1 hour. Cells were harvested at 2300g at room temperature, washed once with ice-cold deionized water, and frozen in dry-ice ethanol. Cell pellets were lysed at 4°C with 0.5-mm silica beads in 1× tris-buffered saline (TBS) buffer containing protease inhibitors [150 mM NaCl, 10 mM Tris-HCl (pH 8), 100 mM PMSF, 2 mM aprotinin, 2 mM pepstatin, and 2 mM leupeptin]. Crude lysates were centrifuged at 16,100g at 4°C for 20 min, and protein concentrations were determined with the Pierce 660nm assay kit (ThermoFisher). Each cell lysate (7.3 mg) was mixed with 30 μl of anti-HA agarose beads (ThermoFisher), adjusted to a total volume of 1.25 ml with 1× TBS buffer containing protease inhibitors, and incubated for 2 hours at 4°C with end-over-end rotation. The beads were washed 3 times with 1× TBS-T buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8), 0.1% Tween-20]. To obtain cell lysates containing myc-Yck1, strain NWY073 was cultured and processed as described earlier except that it was induced with galactose for 3 hours. Low (L, 225 μg) and high (H, 900 μg) amounts of total protein from the NWY073 lysate were added to the Gβγ or Gβ^P-γ affinity beads. Volumes were adjusted to 1.25 ml with 1× TBS buffer containing protease inhibitors, and the slurries were incubated at 4°C for 1 hour with end-over-end rotation. The beads were

washed three times with 1× TBS-T buffer, and then were reduced in 1× SDS sample buffer. The supernatant was split into two fractions for Western blotting and mass spectrometric analyses. For Western blotting analysis, myc-Yck1 was detected with a horseradish peroxidase (HRP)-conjugated mouse anti-c-myc antibody (1:900, ThermoFisher); 3x-HA-G γ was detected with a primary mouse anti-HA antibody (1:900, Covance) and a HRP-conjugated secondary goat anti-mouse IgG₁ antibody (1:400,000, Jackson ImmunoResearch Inc.). Densitometric analysis of scanned Western blotting films was performed with the ImageJ gel analysis tool, which provided absolute intensity (AI) values for each band. The relative amount of myc-Yck1 binding to G β γ as compared to G β^P - γ was determined by subtracting the corresponding negative control signal (L or H), and then taking the ratio of the resulting signal-above-noise (SAN) values. Normalization to HA-G γ was not necessary, because the signal detected with the anti-HA antibody did not vary from lane to lane. For the mass spectrometric analysis, 225 μ g of total protein from NWY073 lysates was incubated with the affinity beads. Eluted proteins were alkylated, and resolved by SDS-PAGE. The ~55 to 70 kD (for myc-Yck1 and Yck2) and 45 to 55 kD (myc-Yck1 cleavage products; G β) regions of the gel were then excised, the proteins were in-gel digested with sequencing-grade modified trypsin (Promega), and the resulting trypsin fragments were analyzed by nanoscale liquid chromatography combined with mass spectrometry (LC-MS/MS), as previously described (58, 59).

Analysis of G β phosphopeptides

NWY052 cells were grown to mid-log phase in selective synthetic 2% sucrose medium containing natural arginine and lysine (normal Arg/Lys), and induced with 2% galactose for 5.5 hours (light culture). A parallel culture of NWY052 cells was labeled in medium containing heavy Arg/Lys (20 mg/l, L-lysine:2HCL, U-13C6 and L-Arginine: HCL U13C6, U-15N2, Cambridge Isotope Labs), and treated with 150 nM α -factor for 1 hour after galactose induction. The light and heavy cultures were prepared for mass spectrometric analysis in parallel. Cells were harvested at 2300g at room temperature, washed once with ice-cold deionized water, and frozen in dry-ice ethanol. Cell pellets were lysed at 4°C with 0.5-mm silica beads in 1× TBS buffer containing protease and phosphatase inhibitors [150 mM NaCl, 10 mM Tris-HCl (pH 8), 100 mM PMSF, 2 mM aprotinin, 2 mM pepstatin, 2 mM leupeptin, 1 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM β -glycerol phosphate]. Crude lysates were centrifuged at 16,100g at 4°C for 20 min and protein concentrations were determined with the Pierce 660-nm assay kit. Total protein (25 mg) from each lysate was mixed with 100 μ l of Ni-NTA beads (Qiagen). Volumes were adjusted to 1.25 ml with 1× TBS buffer, containing protease and phosphatase inhibitors, and the slurries were incubated at 4°C with end-over-end rotation for 16 hours. The beads were washed 10 times with 1× TBS-T buffer [150 mM NaCl, 10 mM Tris (pH 8), 0.1% Tween-20], and then reduced in 1× SDS sample buffer. Equal volumes of supernatant from heavy and light cultures were mixed. The proteins were alkylated and separated by PAGE. The ~45- to 50-kD region of the gel was excised and subjected to in-gel digestion with sequencing grade–modified trypsin. Peptides were concentrated under vacuum and enriched for phospho-peptides with a TiO₂ phosphopeptide enrichment and clean-up kit (Pierce biotechnology). Phospho-peptides were concentrated under vacuum and analyzed by nanoscale LC-MS/MS as previously described (58–60). A peptide search engine using a

probabilistic scoring model (Andromeda) was used to analyze the resulting spectra and obtain assignments for sites of phosphorylation (61a).

Analysis of the localization of Sst2-GFP, Ste2^{7XR}-mCherry, and Ste2-GFP in G1-synchronized cells

G1-synchronized cells were purified by elutriation as previously described (6). Cells used to study the localization of Sst2-GFP and Ste2^{7XR}-mCherry (strains AIY100, AIY101, and AIY221) were spun down and resuspended in selective synthetic medium containing 2% galactose, cultured for 1 hour at 30°C, and then treated with 1.2 μ M α -factor and 200 μ M latrunculin A (LatA). Cells used to study the localization of Ste2-GFP (strains DMY169 and AIY197) were spun down and resuspended in synthetic medium containing 1.5% sucrose and 0.5% dextrose, treated with 9 nM α -factor at time zero and with 200 μ M LatA 15 min later. All cultures were maintained at 30°C. Images were acquired at 15-min intervals with an Axioskop 2 microscope (Carl Zeiss) with a 100 $\times$ oil immersion objective and a digital AxioCam camera. Laser excitation was at 488 nm. Images were processed with Zeiss AxioVision software (Carl Zeiss).

Time-lapse analysis of Sst2-GFP and Ste2-GFP localization in mating mixtures

Wild-type (WT) *MAT α* cells and experimental *MAT α* cells were grown to mid-log phase in synthetic 2% dextrose medium, mixed at a 1:1 ratio, and spread at a density of 14,000 cells/mm² on agarose pads made from synthetic dextrose medium. Mating mixtures were maintained at 30°C with an Okolab chamber. Differential interference contrast (DIC) and fluorescent (488-nm excitation) images were acquired from 6 fields at 15-min intervals with the spinning disk microscope described earlier. To follow the localization of Sst2-GFP, 21 z-sections were acquired over 6 μ m. Images were sum-projected with ImageJ software, and the orientation and reorientation angles were quantified as described in Fig. 2. To follow the localization of Ste2-GFP, 15 z-sections were acquired over 4.2 μ m. Images were sum-projected with ImageJ and the signal intensity along the long axis of the cell was quantified using the BudPolarity Matlab program (61b).

Time-lapse analysis of zygote formation in mating mixtures

GFP-Bud1 labeled *MAT α* cells (XWY028) and experimental *MAT α* cells were grown to mid-log phase in synthetic 2% dextrose medium, mixed at a 1:1 ratio, and spread at a density of 14,000 cells/mm² on agarose pads made from synthetic dextrose medium. Mating mixtures were maintained at 30°C with a DeltaVision environment control chamber (GE Healthcare Bio-Sciences). Images were acquired from 20 fields at 15-min intervals with a DeltaVision Elite microscope (GE Healthcare Bio-Sciences) with a 60 $\times$ oil immersion objective and a Front Illuminated sCMOS digital camera. To follow details of cell growth and zygote formation, DIC images were acquired in 31 z-sections over 6 μ m. To identify the *MAT α* cells, fluorescent images (461 to 489 nm excitation) were acquired at the center slice. Images were processed with ImageJ software. Initial orientation angles, fusion angles, time of fusion, and morphologies of *MAT α* cells were quantified and analyzed as described in Fig. 4.

Analysis of Ste2-GFP and Gic2-PBD-RFP localization in asynchronous cells

AIY301 cells were grown to mid-log phase at 30°C in selective medium containing 2% sucrose and were treated with 15 nM α -factor. Images were acquired 1 and 2 hours later with an Axioskop 2 microscope as described earlier. RFP and GFP were visualized with laser excitations of 561 and 488 nm, respectively. Exposures and processing were identical for all images.

Time-lapse analysis of Ste2-GFP and Gic2-PBD-RFP localization in mating mixtures

Wild-type *MATa* (DSY129) cells and experimental *MATa* cells (AIY301) were grown to mid-log phase in synthetic 2% dextrose medium, mixed at a ratio of 1:1, and spread at a density of 14,000 cells/mm² on agarose pads made from synthetic dextrose medium. Mating mixtures were maintained at 30°C with a DeltaVision environment control chamber. Images were acquired from 15 fields at 7.5-min intervals with a DeltaVision Elite microscope with a 60 $\times$ oil immersion objective and a Front Illuminated sCMOS camera. To follow the localization of the Ste2-GFP and Gic2-PBD-RFP reporters, fluorescent images were acquired in 3 z-sections over 1 μ m with LED excitations of 461 to 489 nm and 529 to 556 nm, respectively. The exposures were the same for all images. Images were processed with ImageJ software. Processing was the same for all Ste2-GFP images in a given series, and all Gic2-PBD-RFP in a given series..

Receptor labeling and internalization assays

The surface-expressed α -factor receptor Ste2 was labeled with Alexa Fluor 594–conjugated α -factor according to the method of Toshima *et al.* (62). To determine how the density and distribution of the receptor changed over time in response to pheromone, DMY169 cells were G1-synchronized by elutriation and treated with 6 nM α -factor at 30°C in YEPD. Aliquots were taken at 5-min intervals, treated with 10 mM sodium azide, and incubated with the labeled α -factor (a generous gift from Dr. David King, University of California, Berkeley). The *MATa ste2* strain (EDY208) was used as a negative control for receptor labeling. Images were acquired with an ANDOR Revolution WD spinning disk laser confocal system with a motorized Olympus IX-81 microscope, a Yokogawa CSU-W1 spinning disk unit, a Prior motorized stage, and a neo sCMOS camera controlled by Andor iQ2 software. A UPLSAPO 60 $\times$ Silicon immersion objective (NA 1.3) was used and nine z-sections were acquired over 4.8 μ m with a laser excitation of 561 nm; one center slice was imaged with DIC. Images were sum-projected with ImageJ. To assay the relative rate of receptor internalization as a function of position on the plasma membrane, *MATa* cells (RDY126) were grown to mid-log phase in YEPD medium, labeled with Alexa Fluor 594–conjugated α -factor, and imaged at 1-min intervals at room temperature as described above. Nine z-sections were acquired over 5.6 μ m with a laser excitation of 561 nm and one center slice was imaged with DIC. Images were sum-projected and membrane fluorescence was obtained with the segmented line tool of ImageJ.

Relative rates of signal decay for labeled receptors at the plasma membrane

The variation in fluorescence intensity measured by ImageJ from pixel to pixel along the plasma membrane was smoothed using a moving average function with the sliding window

equal to $1/10^{\text{th}}$ of the membrane perimeter. For position x at time t in cell c with a window size w , the fluorescence intensity was calculated by the following formula:

$$FI_n(x, t, c) = \text{avg} \left[FI_o(x - \frac{w}{2}, t, c), \dots, FI_o(x + \frac{w}{2}, t, c) \right]$$

in which FI_n denotes the new value after filtering, and FI_o represents the original raw value. The background was removed by subtracting the minimum value from each data set:

$$FI(x, t, c) = FI_n(x, t, c) - \min_{x,t} FI_n(x, t, c).$$

Assuming that the kinetics of internalization resembled a first-order chemical reaction, we calculated the relative fluorescence intensity of time point t compared to time point 0 to determine how membrane receptor density changed with time using the following formula:

$$FI_r(x, t, c) = FI(x, t, c) / FI(x, 0, c)$$

Finally, we used the data set for each cell to determine whether a discrete region of the plasma membrane exhibited a statistically significantly slower rate of signal loss than did the remainder of the plasma membrane. This was determined by fitting the 10 values for each pixel (time points 0 to 9) to the exponential decay formula:

$$FI(x, t, c) = A \exp(-\lambda t)$$

in which the decay rate λ and that initial quantity A are the two parameters to be fitted. A putative area of receptor protection was defined as the points that were in the 30th percentile for decay rate (that is, the slowest 30%). We calculated the average FI_r of the “protected region” and compared it to the average of the rest of the cell. In some cases, the protected region was fragmented. The regions were connected if the distance between them was less than $1/10$ of the perimeter of the cell and the average decay rate of region after connecting was still above the threshold.

Computational model of pheromone-induced receptor polarization

To model the plasma membrane of a yeast cell, we used a sphere, the surface of which was partitioned into patches by uniformly space n latitude and m longitude lines (fig. S7). The center of each patch was used to represent its position. The surface distances between neighboring patches are given by equations (Eq.) 1 and 2 (table S3). The j -th patch in the i -th band can be denoted by an index pair (i, j) . The position of the (i, j) patch, (x_{ij}, y_{ij}, z_{ij}) , is given by Eq. 3 (table S3). In each patch, we stimulated the reaction network model with the pheromone concentration appropriate to its position. Because the pheromone gradient is aligned along the x -axis, and the pheromone concentration is assumed to change linearly with x , only x_{ij} is needed to determine the local pheromone concentration of the (i, j) patch (Eq. 4). All proteins were assumed to diffuse laterally, because only the plasma membrane was modeled in this analysis. If the effect of surface curvature is ignored, the diffusion of

each molecular species can be obtained from Eq. 5 to Eq. 7 (table S3). To model pheromone-induced receptor polarization that occurs before directed secretion, and to evaluate how our postulated feedback mechanisms affect the establishment of receptor polarity, we first created a core network that includes only the most basic and best-characterized components. Network 1 comprises the receptor-pheromone interaction, G protein cycle, and Yck-dependent internalization of the receptor and G protein (Fig. 4B). Network 2 adds the $G\beta^P\gamma$ -Yck interaction (Fig. 4C) and Network 3 adds the $G\alpha$ -Fus3 interaction (Fig. 4D). The mathematical representation of these networks is detailed in tables S4 to S6. A common set of coupled partial differential equations (PDEs) was derived from their corresponding reaction formulae. Each PDE describes how the concentration of a given molecule changes over time and space. Differences in the topology of the three networks were accounted for by varying the initial values of the relevant parameters (color-coded in table S4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank D. King for the generous gift of Alexa Fluor 594-conjugated α -factor.

Funding: This work was supported by NSF grants 1024718 and 1415589 (to D.E.S.), CNRS grant PICS06228 and Fondation ARC grant SFI20121205755 (to R.A.), NIH grant GM079804 and NSF grant DBI 1062328 (to J.L.), and NIH grant IRO3CA150131 (to M.V.M.). This work was also funded by the Chicago Biomedical Consortium with support from the Searle Funds at The Chicago Community Trust.

REFERENCES AND NOTES

1. Bardwell L. A walk-through of the yeast mating pheromone response pathway. *Peptides*. 2005; 26:339–350. [PubMed: 15690603]
2. Pruyne D, Bretscher A. Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *J Cell Sci*. 2000; 113(Pt 3):365–375. [PubMed: 10639324]
3. Pruyne D, Bretscher A. Polarization of cell growth in yeast. *J Cell Sci*. 2000; 113(Pt 4):571–585. [PubMed: 10652251]
4. Butty AC, Pryciak PM, Huang LS, Herskowitz I, Peter M. The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. *Science*. 1998; 282:1511–1516. [PubMed: 9822386]
5. Nern A, Arkowitz RA. A Cdc24p-Far1p- $G\beta\gamma$ protein complex required for yeast orientation during mating. *J Cell Biol*. 1999; 144:1187–1202. [PubMed: 10087263]
6. Suchkov DV, DeFlorio R, Draper E, Ismael A, Sukumar M, Arkowitz R, Stone DE. Polarization of the yeast pheromone receptor requires its internalization but not actin-dependent secretion. *Mol Biol Cell*. 2010; 21:1737–1752. [PubMed: 20335504]
7. Hicke L, Riezman H. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell*. 1996; 84:277–287. [PubMed: 8565073]
8. Hicke L, Zanolari B, Riezman H. Cytoplasmic tail phosphorylation of the α -factor receptor is required for its ubiquitination and internalization. *J Cell Biol*. 1998; 141:349–358. [PubMed: 9548714]
9. Jackson CL, Konopka JB, Hartwell LH. *S. cerevisiae* a pheromone receptors activate a novel signal transduction pathway for mating partner discrimination. *Cell*. 1991; 67:389–402. [PubMed: 1655282]

10. Jenness DD, Spatrick P. Down regulation of the alpha-factor pheromone receptor in *S. cerevisiae*. *Cell*. 1986; 46:345–353. [PubMed: 3015412]
11. Bar EE, Ellicott AT, Stone DE. G β recruits Rho1 to the site of polarized growth during mating in budding yeast. *J Biol Chem*. 2003; 278:21798–21804. [PubMed: 12660244]
12. Robinson LC, Hubbard EJ, Graves PR, DePaoli-Roach AA, Roach PJ, Kung C, Haas DW, Hagedorn CH, Goebel M, Culbertson MR, et al. Yeast casein kinase I homologues: an essential gene pair. *Proc Natl Acad Sci USA*. 1992; 89:28–32. [PubMed: 1729698]
13. Cole GM, Stone DE, Reed SI. Stoichiometry of G protein subunits affects the *Saccharomyces cerevisiae* mating pheromone signal transduction pathway. *Mol Cell Biol*. 1990; 10:510–517. [PubMed: 2105453]
14. Whiteway M, Hougau I, Thomas DY. Overexpression of the STE4 gene leads to mating response in haploid *Saccharomyces cerevisiae*. *Mol Cell Biol*. 1990; 10:217–222. [PubMed: 2104659]
15. Draper E, Dubrovskiy O, Bar EE, Stone DE. Dse1 may control cross talk between the pheromone and filamentation pathways in yeast. *Curr Genet*. 2009; 55:611–621. [PubMed: 19820940]
16. Cole GM, Reed SI. Pheromone-induced phosphorylation of a G protein β subunit in *S. cerevisiae* is associated with an adaptive response to mating pheromone. *Cell*. 1991; 64:703–716. [PubMed: 1900039]
17. Flotow H, Graves PR, Wang AQ, Fiol CJ, Roeske RW, Roach PJ. Phosphate groups as substrate determinants for casein kinase I action. *J Biol Chem*. 1990; 265:14264–14269. [PubMed: 2117608]
18. Flotow H, Roach PJ. Synergistic phosphorylation of rabbit muscle glycogen synthase by cyclic AMP-dependent protein kinase and casein kinase I. Implications for hormonal regulation of glycogen synthase. *J Biol Chem*. 1989; 264:9126–9128. [PubMed: 2498326]
19. Meggio F, Donella-Deana A, Pinna LA. Studies on the structural requirements of a microsomal cAMP-independent protein kinase. *FEBS Lett*. 1979; 106:76–80. [PubMed: 227729]
20. Li E, Cismowski MJ, Stone DE. Phosphorylation of the pheromone-responsive G β protein of *Saccharomyces cerevisiae* does not affect its mating-specific signaling function. *Mol Gen Genet*. 1998; 258:608–618. [PubMed: 9671029]
21. DeFlorio R, Brett ME, Waszczak N, Apollinari E, Metodiev MV, Dubrovskiy O, Eddington D, Arkowitz RA, Stone DE. Phosphorylation of G β is crucial for efficient chemotropism in yeast. *J Cell Sci*. 2013; 126:2997–3009. [PubMed: 23613469]
22. Ballon DR, Flanary PL, Gladue DP, Konopka JB, Dohlman HG, Thorner J. DEP-domain-mediated regulation of GPCR signaling responses. *Cell*. 2006; 126:1079–1093. [PubMed: 16990133]
23. Howard JP, Hutton JL, Olson JM, Payne GS. Sla1p serves as the targeting signal recognition factor for NPFX(1,2)D-mediated endocytosis. *J Cell Biol*. 2002; 157:315–326. [PubMed: 11940605]
24. Toshima JY, Nakanishi J, Mizuno K, Toshima J, Drubin DG. Requirements for recruitment of a G protein-coupled receptor to clathrin-coated pits in budding yeast. *Mol Biol Cell*. 2009; 20:5039–5050. [PubMed: 19828733]
25. McClure AW, Minakova M, Dyer JM, Zyla TR, Elston TC, Lew DJ. Role of Polarized G Protein Signaling in Tracking Pheromone Gradients. *Dev Cell*. 2015; 35:471–482. [PubMed: 26609960]
26. Kelley JB, Dixit G, Sheetz JB, Venkatapurapu SP, Elston TC, Dohlman HG. RGS proteins and septins cooperate to promote chemotropism by regulating polar cap mobility. *Curr Biol*. 2015; 25:275–285. [PubMed: 25601550]
27. Ayscough KR, Drubin DG. A role for the yeast actin cytoskeleton in pheromone receptor clustering and signalling. *Curr Biol*. 1998; 8:927–930. [PubMed: 9707405]
28. Sahin A, Daignan-Fornier B, Sagot I. Polarized growth in the absence of F-actin in *Saccharomyces cerevisiae* exiting quiescence. *PLoS One*. 2008; 3:e2556. [PubMed: 18596916]
29. Bendezu FO, Martin SG. Actin cables and the exocyst form two independent morphogenesis pathways in the fission yeast. *Mol Biol Cell*. 2011; 22:44–53. [PubMed: 21148300]
30. Yamamoto T, Mochida J, Kadota J, Takeda M, Bi E, Tanaka K. Initial polarized bud growth by endocytic recycling in the absence of actin cable-dependent vesicle transport in yeast. *Mol Biol Cell*. 2010; 21:1237–1252. [PubMed: 20147449]

31. Hegemann B, Unger M, Lee SS, Stoffel-Studer I, van den Heuvel J, Pelet S, Koepl H, Peter M. A Cellular System for Spatial Signal Decoding in Chemical Gradients. *Dev Cell*. 2015; 35:458–470. [PubMed: 26585298]
32. Matheos D, Metodiev M, Muller E, Stone D, Rose MD. Pheromone-induced polarization is dependent on the Fus3p MAPK acting through the formin Bni1p. *J Cell Biol*. 2004; 165:99–109. [PubMed: 15067022]
33. Metodiev MV, Matheos D, Rose MD, Stone DE. Regulation of MAPK function by direct interaction with the mating-specific Gα in yeast. *Science*. 2002; 296:1483–1486. [PubMed: 12029138]
34. Errede B, Vered L, Ford E, Pena MI, Elston TC. Pheromone-induced morphogenesis and gradient tracking are dependent on the MAPK Fus3 binding to Gα. *Mol Biol Cell*. 2015 Epub ahead of print.
35. Dyer JM, Savage NS, Jin M, Zyla TR, Elston TC, Lew DJ. Tracking shallow chemical gradients by actin-driven wandering of the polarization site. *Curr Biol*. 2013; 23:32–41. [PubMed: 23200992]
36. Moore TI, Chou CS, Nie Q, Jeon NL, Yi TM. Robust spatial sensing of mating pheromone gradients by yeast cells. *PLoS One*. 2008; 3:e3865. [PubMed: 19052645]
37. Yi T-M, Chen S, Chou C-S, Nie Q. Modeling yeast cell polarization induced by pheromone gradients. *J Stat Phys*. 2007; 128:193–207.
38. Jin T. Gradient sensing during chemotaxis. *Curr Opin Cell Biol*. 2013; 25:532–537. [PubMed: 23880435]
39. Wang Y, Chen CL, Iijima M. Signaling mechanisms for chemotaxis. *Dev Growth Differ*. 2011; 53:495–502. [PubMed: 21585354]
40. Jackson CL, Konopka JB, Hartwell LH. *S. cerevisiae* [α] pheromone receptors activate a novel signal transduction pathway for mating partner discrimination. *Cell*. 1991; 67:389–402. [PubMed: 1655282]
41. Vallier L, Jeffrey E, Segall Michael Snyder, The alpha-factor receptor C-terminus is important for mating projection formation and orientation in *Saccharomyces cerevisiae*. *Cell Motil Cytoskeleton*. 2002; 53:251–266. [PubMed: 12378535]
42. Segall JE. Polarization of yeast cells in spatial gradients of alpha mating factor. *Proc Natl Acad Sci USA*. 1993; 90:8332–8336. [PubMed: 8397402]
43. Blackwell E, Halatek IM, Kim HJ, Ellicott AT, Obukhov AA, Stone DE. Effect of the pheromone-responsive Gα and phosphatase proteins of *Saccharomyces cerevisiae* on the subcellular localization of the Fus3 mitogen-activated protein kinase. *Mol Cell Biol*. 2003; 23:1135–1150. [PubMed: 12556475]
44. van Hemert F, Lazova MD, Snaar-Jagaska BE, Schmidt T. Mobility of G proteins is heterogeneous and polarized during chemotaxis. *J Cell Sci*. 2010; 123:2922–2930. [PubMed: 20682639]
45. Xu X, Meckel T, Brzostowski JA, Yan J, Meier-Schellersheim M, Jin T. Coupling mechanism of a GPCR and a heterotrimeric G protein during chemoattractant gradient sensing in *Dictyostelium*. *Sci Signal*. 2010; 3:1–11. [PubMed: 20940426]
46. Brzostowski JA, Sawai S, Rozov O, Liao XH, Imoto D, Parent CA, Kimmel AR. Phosphorylation of chemoattractant receptors regulates chemotaxis, actin reorganization and signal relay. *J Cell Sci*. 2013; 126:4614–4626. [PubMed: 23902692]
47. Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. *Current Protocols in Molecular Biology*. In: Janssen, K., editor. *Current Protocols in Molecular Biology*. John Wiley and Sons, Inc.; 1994.
48. Guthrie, C.; Fink, GR. *Guide to Yeast Genetics and Molecular Biology*., *Methods Enzymol*. Vol. 351. San Diego, CA: Academic Press; 2002. p. 325-456.
49. Sherman, F.; Fink, GR.; Hicks, JB., editors. *Laboratory Course Manual For Methods in Yeast Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1986.
50. Reed SI, Hadwiger JA, Lorincz AT. Protein kinase activity associated with the product of the yeast cell division cycle gene CDC28. *Proc. Natl. Acad. Sci. USA*. 1985; 82:4055–4059. [PubMed: 3889921]
51. Wach A. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast*. 1996; 12:259–265. [PubMed: 8904338]

52. Cismowski MJ, Metodiev M, Draper E, Stone DE. Biochemical analysis of yeast G α mutants that enhance adaptation to pheromone. *Biochem Biophys Res Commun*. 2001; 284:247–254. [PubMed: 11394869]
53. Dunn R, Klos DA, Adler AS, Hicke L. The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. *J Cell Biol*. 2004; 165:135–144. [PubMed: 15078904]
54. Tong Z, Gao XD, Howell AS, Bose I, Lew DJ, Bi E. Adjacent positioning of cellular structures enabled by a Cdc42 GTPase-activating protein-mediated zone of inhibition. *J Cell Biol*. 2007; 179:1375–1384. [PubMed: 18166650]
55. Gietz RD, Sugino A. New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene*. 1988; 74:527–534. [PubMed: 3073106]
56. Remy I, Montmarquette A, Michnick SW. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat Cell Biol*. 2004; 6:358–365. [PubMed: 15048128]
57. Cole GM, Reed SI. Pheromone-induced phosphorylation of a G protein [beta] subunit in *S. cerevisiae* is associated with an adaptive response to mating pheromone. *Cell*. 1991; 64:703–716. [PubMed: 1900039]
58. Alldridge L, Metodieva G, Greenwood C, Al-Janabi K, Thwaites L, Sauven P, Metodiev M. Proteome profiling of breast tumors by gel electrophoresis and nanoscale electrospray ionization mass spectrometry. *J Proteome Res*. 2008; 7:1458–1469. [PubMed: 18257521]
59. Metodiev MV. Applications of nanoscale liquid chromatography coupled to Tandem mass spectrometry in quantitative studies of protein expression, protein-protein interaction, and protein phosphorylation. *Methods Mol Biol*. 2011; 790:99–113. [PubMed: 21948409]
60. Metodieva G, Nogueira-de-Souza NC, Greenwood C, Al-Janabi K, Leng L, Bucala R, Metodiev MV. CD74-dependent deregulation of the tumor suppressor scribble in human epithelial and breast cancer cells. *Neoplasia*. 2013; 15:660–668. [PubMed: 23730214]
- 61a. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res*. 2011; 10:1794–1805. [PubMed: 21254760]
- 61b. Vernay A, Schaub S, Guillas I, Bassilana M, Arkowitz RA. A steep phosphoinositide bis-phosphate gradient forms during fungal filamentous growth. *J Cell Biol*. 2012; 198:711–730. [PubMed: 22891265]
62. Toshima JY, Toshima J, Kaksonen M, Martin AC, King DS, Drubin DG. Spatial dynamics of receptor-mediated endocytic trafficking in budding yeast revealed by using fluorescent alpha-factor derivatives. *Proc Natl Acad Sci USA*. 2006; 103:5793–5798. [PubMed: 16574772]
63. Gietz RD, Sugino A. New yeast—Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene*. 1988; 74:527–534. [PubMed: 3073106]
64. Paquin N, Ménade M, Poirier G, Donato D, Drouet E, Chartrand P. Local activation of yeast ASH1 mRNA translation through phosphorylation of Khd1p by the casein kinase Yck1p. *Mol Cell*. 2007; 26:795–809. [PubMed: 17588515]
65. Yi TM, Kitano H, Simon MI. A quantitative characterization of the yeast heterotrimeric G protein cycle. *Proc Natl Acad Sci USA*. 2003; 100:10764–10769. [PubMed: 12960402]
66. Chou CS, Nie Q, Yi TM. Modeling robustness tradeoffs in yeast cell polarization induced by spatial gradients. *PLoS One*. 2008; 3:e3103. [PubMed: 21267054]
67. Maeder CI, Hink MA, Kinkhabwala A, Mayr R, Bastiaens PI, Knop M. Spatial regulation of Fus3 MAP kinase activity through a reaction-diffusion mechanism in yeast pheromone signalling. *Nat Cell Biol*. 2007; 9:1319–1326. [PubMed: 17952059]
68. Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N, O'Shea EK, Weissman JS. Global analysis of protein expression in yeast. *Nature*. 2003; 425:737–741. [PubMed: 14562106]

Fig. 1. Physical and genetic interaction between Gβ and Yck1/2

(A) Binding of Yck1-myc to GβHA-γ and Gβ^P-HA-γ affinity beads. H and L indicate high and low input of lysate containing Yck1-myc, respectively (see Materials and Methods). In the control lanes, the beads were not loaded with G protein. (B) Quantification of the association of Yck1 with the Gβγ-affinity beads. The absolute intensity (AI) of the myc-Yck1 bands in the low input lanes of the Western blot shown in (A) was measured with ImageJ software. The signal above noise (SAN) for myc-Yck1 was calculated by subtracting the AI of the negative control (empty) from the AIs of Gβγ and Gβ^Pγ. The relative amount of myc-Yck1 that bound to Gβγ vs. Gβ^Pγ is expressed as the Gβ/Gβ^Pratio. Data are representative of three experiments. (C) Phormone-treated 15Dau cells expressing the indicated constructs were analyzed by BiFC before morphogenesis (top) and after mating projection formation (bottom). Some fluorescence was detectable at the tips of shmooing negative control cells (bottom middle), but this was of lower intensity and distinct localization compared to that in the experimental cells (see Table 2). Images are representative of two experiments. (D) *YCK1 YCK2* control cells and *yck1 yck2^{ts}* mutant cells were left untreated or were exposed to phormone (αF) at a permissive temperature (room temperature, RT) or a restrictive temperature (37°C). The indicated cell lysates were then analyzed by Western blotting to detect Gβ. Lysate from *ste4* cells (Gβ) provided a negative control for antibody specificity. The bracket indicates the positions of the Gβ bands. The lowest band is unphosphorylated Gβ; the higher bands are the phosphorylated species. The asterisks indicate two background bands that cross react with the Gβ antibody. Western blots are representative of three experiments. (E) Map of phosphorylation sites among amino acid residues 317 to 337 of Gβ. Residues in blue and green indicate sites identified by mass

spectrometry analysis (see fig. S1) or genetic analysis (20), respectively, whereas residues in red indicate sites identified by both methods. Arrows indicate putative MAPK and CK1 sites.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2. Effects of Gβ and Gβ^{P-} on receptor phosphorylation

(A to C) Overexpression of Gβ, but not Gβ^{P-}, inhibits phosphorylation of the pheromone receptor. (A) YDB111 cells expressing Gβ at an amount equivalent to endogenous protein, excess Gβ, or excess Gβ^{P-} (excess indicated by double arrows) were treated with pheromone and LatA at time zero. The cells were then imaged by epifluorescence microscopy to visualize the indicated proteins. The intensity of the Sst2-GFP signal on the plasma membrane corresponds to the location and quantity of the unphosphorylated pheromone receptor, whereas that of the Ste2^{7XR}-mCherry signal corresponds to the location and

quantity of total pheromone receptor. The arrowhead indicates a polarized Sst2-GFP crescent. Relative signal intensities are indicated by the color bar. Images are representative of three or two (for the control strain) experiments. (B) Western blotting analysis of the relative amounts of G β and G β^{P-} in lysates of 15Dau cells expressing the indicated constructs after 0 and 2 hours of exposure to galactosidase to induce *GAL1* expression. The topmost band corresponds to G β ; asterisks indicate G β degradation products. Western blots are representative of four experiments. (C) Normalized quantification of the amount of Sst2-GFP localized to the plasma membrane. The relative amount of Sst2-GFP localized to the plasma membrane at the indicated times after treatment with pheromone and LatA at time zero was normalized to total receptor abundance by dividing the mean plasma membrane (PM) Sst2-GFP signal by the mean plasma membrane Ste2^{7XR}-mCherry signal. Data are mean ratios $\pm$ SEM of three or two (for the G β^{P-} -overexpressing strain) experiments. **P* = 0.003 compared to control. *n* = 44 cells for control and G β -overexpressing strains; *n* = 15 cells for the G β^{P-} -overexpressing strain. (D and E) Overexpression of G β enhances polarity of unphosphorylated receptor in pheromone- and LatA-treated cells. (D) YDB111 cells expressing the native amount of G β excess G β , or excess G β^{P-} (excess indicated by double arrows) were treated with pheromone and LatA at time zero. The cells were then imaged by epifluorescence microscopy to visualize Sst2-GFP. Arrowheads indicate polarized Sst2-GFP crescents. (E) The degree of polarization of Sst2-GFP to the plasma membrane of each cell is indicated by the Polarity Index (PI): the mean fluorescence in the brightest third of the plasma membrane divided by the mean signal in the rest of the plasma membrane. Data are means $\pm$ SEM of at least two experiments per strain. **P* < 0.02, ***P* = 0.002, †*P* = 0.0003 compared to cells expressing excess G β . *n* = 20 cells for each strain and time point. (F and G) The pheromone receptor is hyper-phosphorylated in G β^{P-} cells. (F) YDB111 cells expressing the native amount of G β or G β^{P-} were treated with pheromone and LatA at time zero. The cells were then imaged by epifluorescent microscopy to visualize the indicated proteins. (G) Quantification of the extent of Sst2-GFP localization to the plasma membrane. Data are means $\pm$ SEM of one experiment. **P* = 0.005 for G β^{P-} compared to G β cells at 90 min. *n* = 15 cells for each strain and time point. PM Ste2^{7XR}-mCherry levels did not differ significantly in the control and experimental cells.

Fig. 3. Effects of $G\beta$ and $G\beta^{P-}$ on receptor phosphorylation and gradient sensing in mating mixtures

YDB111 cells expressing Sst2-GFP and either $G\beta$ or $G\beta^{P-}$ were exposed to natural pheromone gradients in mating mixtures, and mating cells were analyzed by time-lapse imaging to follow the relocation of Sst2-GFP from the presumptive default polarity site to the presumptive chemotrophic site. The degree of movement is described by the orientation angle, δ (yellow arc). Mean orientation angles $\pm$ SEM were 82.6 ± 8.0 for $G\beta$ -expressing cells compared to 52.0 ± 8.4 for $G\beta^{P-}$ -expressing cells; $P = 0.01$. $n = 33$ cells for each strain from two independent experiments.

Fig. 4. Effects of receptor internalization and phosphorylation on chemotropism

(A) Illustration of how the initial orientation angles (left, δ) and the fusion angles (right, γ) were measured. Polarized growth initiated within 45° of the cytokinesis site (CKS, red bar) was presumed to be at the default polarity site, demarcated by dotted blue lines. (B) Time-lapse DIC and fluorescent images of the indicated mating mixtures taken at 15-min intervals after spreading on agarose pads. The GFP-Bud1 signal at the plasma membrane was used to distinguish the *MAT α* cells and as a marker for cell fusion. The initial orientation, reorientation, and fusion angles are indicated by δ , δ' , and γ , respectively. (i) Representative

wild-type (WT) X WT mating. The *MAT α* cell shmooed opposite to its CKS. (ii) Example of a mating cell reorienting by initiating a second projection in a WT X WT cross. (iii) Representative WT *MAT α* X *MAT α* *STE2^{7XR}* mating showing defective initial orientation and the formation of an angled zygote after 90 min. (iv) Two representative WT *MAT α* X *MAT α* *STE2^{7XR} 6SA* matings showing a *MAT α* cell fusing adjacent to the CKS of a mutant cell that failed to polarize its growth (top), and a mutant cell that broadly polarized its growth in the default region, but failed to fuse with an adjacent partner until it had elongated over an abnormally long time and distance (bottom). (v) Two representative WT *MAT α* X *MAT α* *STE2^{7XR} 6SD* matings showing mutant cells that broadly polarized their growth at the default site (top), or presumptive default site (bottom), and elongated while persistently turning until their growth zones were apposed to a partner. (C) Quantification of initial orientation angles [purple lines in (A)]. * $P < 0.06$; ** $P = 0.003$; *** $P < 0.0001$. (D) Quantification of fusion angles [aqua lines in (A)]. * $P < 0.0001$. (E) Time to fusion. Diffusion of GFP-Bud1 from the *MAT α* partner to the *MAT α* partner was used as a marker for the completion of cell fusion. * $P = 0.01$; ** $P < 0.0001$. (F) Effect of receptor mutants on morphogenesis. *MAT α* cells that mated were placed in three classes based on their ability to form mating projections: tapered growth (normal shmoos), broadly polarized growth, and no polarized growth, as indicated. The proportion of normal shmoos was compared by chi-square test. * $P < 0.0001$. The indicated n values represent two experiments for the *STE2^{7XR} 6SA* and *STE2^{7XR} 6SD* strains and one experiment for the WT and *STE2^{7XR}* strains.

Fig. 5. Localization of receptor and Cdc42-GTP reporters in vegetative and pheromone-stimulated G1 cells

(A and B) Sequential polarization of the receptor and active Cdc42 in cultured cells. Strain AIY301 was grown to log-phase, and G1 (round, unbudded) cells were scored for polarization of the Ste2-GFP (receptor) and Gic2-PBD-RFP (Cdc42-GTP) reporters before and after pheromone treatment. (A) Representative images 60 min after treatment with pheromone. The exposures and processing were the same for all images. (B) Relative proportions of cells showing Ste2-GFP and Gic2-PBD-RFP polarity before and at the

indicated times after pheromone-induced morphogenesis. RNP: round cell, neither reporter polarized; RAC: round cell, activated Cdc42 polarized; RR: round cell, receptor reporter polarized; RB: round cell, both reporters polarized; SB: shmooing cell, both reporters polarized. $n = 20, 35,$ and 56 cells at the $0, 1,$ and 2 hour time points, respectively, from two experiments. (C and D) Sequential polarization of the receptor and active Cdc42 in mating cells. (C) Time-lapse images of representative *MATa* cells that switched from their presumptive default polarity site to a presumptive chemotropic growth site in mating mixtures. Polarity of the Ste2-GFP and Gic2-PBD-RFP reporters is categorized in one of three ways: polarity established at the initial site (PE, dotted arrowheads), polarity switching to the future growth site (PS, solid arrowheads), and polarity fully switched and fixed at the final site (PF, filled arrowheads). (D) Percentages of cells that showed sequential re-localization of the Ste2-GFP and Gic2-PBD-RFP reporters in mating mixtures. Time of Polarity Switch (PS) corresponds to the solid arrowheads in (D) ($n = 19$ cells). Time of Polarity Fixed (PF) corresponds to the filled arrowheads in (D) ($n = 16$ cells). Two experiments were performed.

Fig. 6. Effect of Gβ^{P-} on receptor polarization

(A and B) Isotropic conditions. Gβ cells and Gβ^{P-} cells expressing the receptor reporter Ste2-GFP were treated with pheromone at time zero and with LatA after global receptor internalization (15 min). (A) Representative images of the indicated cells to visualize Ste2-GFP. Arrowheads indicate Ste2-GFP crescents. (B) Polarity Indices (PIs) for the cells represented in (A) were measured as described for Fig. 2D. Data are means ± SEM of at least 30 cells of each strain from three experiments. **P* < 0.03. (C and D) Mating mixtures. (C) Time-lapse images of mating Gβ cells and Gβ^{P-} cells showing Ste2-GFP localization.

Insets show heat map images analyzed using the BudPolarity Matlab program (61), which quantifies signal intensity along the long axis of the cell. (D) Output BudPolarity trace of the pictured cell (inset). Receptor polarization in the cells represented in (C) was quantified by dividing the peak fluorescence signal at the plasma membrane by the minimum fluorescence at the plasma membrane before morphogenesis became apparent. Mean polarization values $\pm$ SEM were 1.76 ± 0.14 and 1.28 ± 0.07 for G β cells and G β^{P-} cells, respectively ($P < 0.007$). Data are means $\pm$ SEM of at least 17 cells of each strain from two independent experiments.

Fig. 7. Proposed feedback loops that drive receptor polarization upstream of directed secretion
(A) Cartoon depiction of the model. We propose that the establishment of pheromone-induced cell polarity before the initiation of actin-cable directed secretion depends on two interconnected positive feedback loops. Initially, a shallow gradient of pheromone is mirrored by a similarly shallow gradient of occupied receptor across the cell. This slight differential in activated receptor leads to a corresponding differential in activated G protein. $G\alpha$ recruits the MAPK Fus3 to phosphorylate $G\beta$ (loop 1), which augments its interaction with Yck1/2 while preventing its interaction with $G\alpha$. Because $G\beta^P$ inhibits receptor

internalization by interacting with Yck1/2, the signaling apparatus preferentially accumulates on the side of the cell closest to the gradient of pheromone (loop 2). The two loops act synergistically to promote local signaling while protecting $G\beta^P\gamma$ from internalization, thereby generating a concentration gradient of free $G\beta^P\gamma$. Ultimately, the localized increase in $G\beta\gamma$ is sufficient to stimulate the nucleation of actin cables. Directed secretion then reinforces the spatial signal and drives mating projection formation. **(B to D)** Network diagrams. **(B)** Network 1. Yck1/2 (Yck) stimulates the internalization of inactive receptors (R) and active receptors (RL), represented by the green and blue bars, respectively. One heterotrimeric G protein is removed along with each receptor. **(C)** Network 2. Phosphorylation of $G\beta$ and the $G\beta^P\gamma$ -Yck interaction are included. The binding of $G\beta^P\gamma$ (GbgP) to Yck inhibits receptor and G protein internalization. **(D)** Network 3. The $G\alpha$ -Fus3 feedback loop is included. Active $G\alpha$ recruits active Fus3 to phosphorylate $G\beta$, which augments the interaction of $G\beta$ with Yck and enhances the $G\beta\gamma$ -dependent activation of Fus3 through the MAPK cascade (not shown). **(E to G)** Comparison of network outputs. The graphs show the spatio-temporal dynamics of the total receptor (both active and inactive). **(E)** Network 1. Without downstream regulation, the pheromone gradient induces complete removal of the receptor from the plasma membrane; no polarity is generated. **(F)** Network 2. With the addition of the $G\beta^P\gamma$ -Yck interaction, which slows the internalization of receptor and G protein, the pheromone gradient induces receptor polarity. **(G)** Network 3. The recruitment of Fus3 by $G\alpha$ leads to locally enhanced phosphorylation of $G\beta$, and consequently, faster receptor polarization.

Table 1
Mass spectrometric quantification of Yck1 and Yck2 pull-downs by Gβγ-affinity beads

Total protein (225 µg, low input) from cells expressing endogenous Yck2 and overexpressing myc-Yck1 were incubated with the indicated Gβγ-affinity beads or negative control beads (empty). Bound proteins were resolved by PAGE, processed, and analyzed by mass spectrometry as described in Materials and Methods.

Detected protein	Affinity bead type	Intensity 70-55 kD	Intensity 55-45 kD	Total intensity	Gβ/Gβ ^P -ratio
Yck1	Gβγ	3.16E+07	2.25E+07	5.42E+07	2.25
	Gβ ^P -γ	6.88E+06	1.72E+07	2.41E+07	
	Empty	0	0	0	
Yck2	Gβγ	2.39E+07	0	2.39E+07	2.09
	Gβ ^P -γ	1.15E+07	0	1.15E+07	
	Empty	0	0	0	

Quantification of BiFC results

Table 2

No background signal was seen in the VF1-YCK1 + VF2 negative control cells. Detectable fluorescence was observed on the tips of a substantial percentage of the shmooing Gβ-VF2 + VF1 negative control cells. This was attributable to the direct, irreversible interaction between the VF1 and VF2 fragments, where they were concentrated together by directed secretion, and was clearly distinguishable from the signal from the interaction between Gβ-VF2 and VF1-Yck1 by the measures shown here. PM, plasma membrane; n.d., not determined.

	% cells with PM signal ^a		PM/Cyto ± SEM ^b		% PM ± SEM Cyto ^c	
	Pre-shmoo	Shmooed	Pre-shmoo	Shmooed	Pre-shmoo	Shmooed
Gβ-VF2 + VF1-Yck1	28.5	15.2	n.d.	1.46 ± 0.09 *	n.d.	50.5 ± 5.4 *
Gβ-VF2 + VF1	3.4	27.4	n.d.	0.95 ± 0.03	n.d.	17.0 ± 1.9

^a Cells with a clear plasma membrane signal were scored; *n* = 45 cells.

^b The plasma membrane signal was normalized to the cytoplasmic signal in each cell by dividing the mean plasma membrane fluorescence by the mean cytoplasmic fluorescence.

^c The percentage of the plasma membrane that showed a signal at least 25% greater than the mean cytoplasmic signal in each cell was determined.

* As compared to the corresponding measure for Gβ-VF2 + VF1 cells; *P* < 0.0001 (*n* = 19 cells).