# Phosphorylation of $\mathbf{G} \beta$ is crucial for efficient chemotropism in yeast 

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#### Abstract

Summary Mating yeast cells interpret complex pheromone gradients and polarize their growth in the direction of the closest partner. Chemotropic growth depends on both the pheromone receptor and its associated G-protein. Upon activation by the receptor, G $\alpha$ dissociates from $\mathrm{G} \beta \gamma$ and $G \beta$ is subsequently phosphorylated. Free $G \beta \gamma$ signals to the nucleus via a MAPK cascade and recruits Far1-Cdc24 to the incipient growth site. It is not clear how the cell establishes and stabilizes the axis of polarity, but this process is thought to require local signal amplification via the G $\beta \gamma-$ Far1-Cdc24 chemotropic complex, as well as communication between this complex and the activated receptor. Here we show that a mutant form of $\mathrm{G} \beta$ that cannot be phosphorylated confers defects in directional sensing and chemotropic growth. Our data suggest that phosphorylation of G $\beta$ plays a role in localized signal amplification and in the dynamic communication between the receptor and the chemotropic complex, which underlie growth site selection and maintenance.


Key words: $G \beta$ phosphorylation, Saccharomyces cerevisiae, Chemotropism, Polarized growth, Reorientation, Yeast mating response

## Introduction

In metazoans, directed cell movement in response to a chemical gradient (chemotaxis), plays a vital role in embryogenesis, postnatal development and immunity. Moreover, the survival of many single-celled organisms depends on their ability to detect and move in response to chemical stimuli. The related phenomenon, directed cell growth in response to a chemical gradient (chemotropism), is also essential in many species. In mammals, chemotropism is integral to axon guidance (Hong and Nishiyama, 2010; Tojima et al., 2011) and angiogenesis (English et al., 2001; Basile et al., 2004). Pollen tube guidance is also a chemotropic process (Palanivelu and Preuss, 2000; Kim et al., 2004), and there is an increasing appreciation for the role of chemotropism in the life cycles of fungal species, including plant and human pathogens (Snetselaar et al., 1996; Daniels et al., 2006).

Many chemosensing cells express G-protein-coupled receptors (GPCRs) to detect the presence of the extracellular signaling molecules and determine the direction of their source (Weiner, 2002). Using the spatial information encoded in the distribution of activated receptors, the cell establishes a landmark for polarization. Actin and/or microtubule cytoskeletal elements are then assembled at, or recruited to, this site to promote directional movement or growth. Additionally, the cell must be able to stabilize the axis of polarity, while retaining the plasticity to change its orientation in response to changes in the direction of the gradient. Because physiological gradients of chemoattractant are typically very shallow (Mato et al., 1975; Tranquillo et al.,

1988; Segall, 1993), establishment of the polarity site requires the conversion of small differences in receptor occupancy into a substantially steeper intracellular signaling gradient. This is thought to depend on feedback loops, such as those first discovered in Dictyostelium and neutrophils, that couple local excitation with global inhibition (LEGI) (Iijima et al., 2002; Weiner, 2002; Devreotes and Janetopoulos, 2003).

In comparison to chemotactic models, the amplification mechanisms that underlie directional sensing in chemotropic systems are less well understood. The best-characterized example of eukaryotic chemotropism to date occurs during the mating response of the budding yeast, Saccharomyces cerevisiae. In the haploid phase of its life cycle, budding yeast exist as two mating types, MATa and MATa. Each mating type constitutively secretes a peptide mating pheromone that binds to GPCRs on cells of the opposite type. Liganded receptors activate the mating-specific $\mathrm{G} \alpha$ protein, Gpa1 (henceforth $\mathrm{G} \alpha$ ), via guanine nucleotide exchange and the subsequent dissociation of G $\alpha-\mathrm{GTP}$ from the G $\beta \gamma$ dimer, Ste $4 /$ Ste 18. The signal is then transmitted by G $\beta \gamma$ via the Fus3 MAP kinase (MAPK) cascade that ultimately induces cell-cycle arrest, changes in gene expression and morphogenesis (Dohlman and Thorner, 2001; Bardwell, 2005). Cells polarize their growth and form mating projections, becoming pear-shaped 'shmoos', in response to both isotropic pheromone and pheromone gradients. In mating mixtures, cells find and contact the closest potential mating partner by determining the direction of the most potent pheromone source and growing toward it (Jackson and Hartwell, 1990).

Polarized growth in $S$. cerevisiae, like that in higher eukaryotes, requires the marking of a growth site at the cell cortex and alignment of the actin cytoskeleton towards it. Cargo bound for the polarized structures is transported along the actin cables (Pruyne and Bretscher, 2000a; Pruyne and Bretscher, 2000b). Actin polarization depends on Cdc42, which is thought to activate a formin protein, Bni1, that nucleates and tethers actin cables to the polarization site (Evangelista et al., 2002; Sagot et al., 2002). Bnil is part of the 'polarisome' complex, together with Spa2, Bud6 and Pea2 (Pruyne and Bretscher, 2000a). Cdc42 is activated by the exchange factor Cdc24, which itself undergoes localized activation during both vegetative budding and mating. In vegetative cells, cortical tags promote localized activation of the monomeric GTPase, Bud1/Rsr1 (hereafter Bud1), which binds directly to Cdc24 (Park et al., 1997) and Cdc42 (Kang et al., 2010). In cells exposed to pheromone, $\mathrm{G} \beta \gamma$ interacts with the Far1-Cdc24 complex (Butty et al., 1998; Nern and Arkowitz, 1998; Nern and Arkowitz, 1999). Interaction of G $\beta \gamma$ with Far1 is thought to activate Cdc24 (Wiget et al., 2004), leading to localized GTP-loading of Cdc42, recruitment of Bni1, nucleation of actin cables, and polarized growth, to form the mating projection. The $\mathrm{G} \beta \gamma-\mathrm{Far} 1-\mathrm{Cdc} 24$ complex is presumed to assemble in the region of the cell surface that experiences the highest concentration of pheromone and to mark this area for growth in preference to the predetermined bud site (Nern and Arkowitz, 2000). In this way, the cell is thought to orient its growth toward the source of pheromone, although the feedback loops that amplify the directional signal have not been well characterized. When cells are unable to sense a gradient of pheromone, on the other hand, they form a mating projection at the Bud1-marked site that would have been used for the next bud (Dorer et al., 1995; Nern and Arkowitz, 1999). This is called the default mating projection site.

Yeast exhibit a remarkable ability to interpret chemical gradients. It has been estimated that a $1 \%$ difference in receptor occupancy across the $5 \mu \mathrm{~m}$ length of a yeast cell in a pheromone gradient is sufficient to elicit robust orientation toward the pheromone source (Segall, 1993), and microfluidic studies suggest an even greater acuity (Moore et al., 2008). How is this very slight asymmetry in activated receptor and G-protein amplified internally to establish a unique shmoo site, and how is this positional information continually communicated to the polarisome to ensure properly oriented growth over time?

Since it was first reported that pheromone induces the phosphorylation of $\mathrm{G} \beta$ on multiple sites (Cole and Reed, 1991), the function of this modification has been elusive. A double point mutant form of $G \beta$ that cannot be phosphorylated was found to have no measurable effect on signal transmission, adaptation, or diploid formation (Li et al., 1998). More recently, we discovered that the activated forms of the mating-specific $G \alpha$ protein and the Fus3 MAPK interact directly. A mutant form of $\mathrm{G} \alpha$ that is severely defective in binding Fus3, $\mathrm{G} \alpha^{\mathrm{DSD}}$ ( $\mathrm{G} \alpha$-Docking Site Disrupted; previously $G \alpha^{\text {K21E R22E }}$ ), confers a defect in partner discrimination, indicating a problem in directional sensing and/or directed growth (Metodiev et al., 2002; Strickfaden and Pryciak, 2008; Yu et al., 2008). G $\alpha^{\text {DSD }}$ also results in hypophosphorylation and reduced levels of $G \beta$, as does fus $3 \Delta$. These observations raised the possibility that the phosphorylation of $G \beta$ plays a role in chemotropism. Here we show that $G \beta$ phosphorylation is critical for this process.

## Results

G $\alpha$ plays an essential role in the chemotropic shmoo pathway
The findings that $G \alpha^{\mathrm{DSD}}$ confers partial defects in partner discrimination, mating efficiency, shmooing and actin polarization (Metodiev et al., 2002; Matheos et al., 2004), suggest that the $G \alpha$-Fus 3 interaction plays a role in chemotropism. Moreover, $\mathrm{G} \alpha$ itself has been implicated in chemotropism (Strickfaden and Pryciak, 2008; Yu et al., 2008). We therefore asked whether $\mathrm{G}^{\mathrm{DSD}}$ bud $1 \Delta$ cells can shmoo. Cells can form mating projections if either the default or chemotropic shmoo pathways are inactivated, but are unable to polarize their growth in response to pheromone if neither pathway is functional (Nern and Arkowitz, 2000). Thus, components required for chemotropic growth can be uncovered by examining mutations in a budl $\Delta$ background. For example, $\operatorname{cdc} 24-m 1$ bud1 $\Delta$ and farl-H7 budl $\Delta$ cells cannot shmoo (Nern and Arkowitz, 2000) because Cdc24 and Far1 are essential chemotropic elements (Valtz et al., 1995; Nern and Arkowitz, 1998). Similarly, G $\alpha^{\text {DSD }}$ bud1 $\Delta$ cells were completely unable to form mating projections (Fig. 1A). Whereas the $\mathrm{G} \alpha$ bud1 $\Delta$ cells and $\mathrm{G} \alpha^{\mathrm{DSD}}$ BUD1 cells (not shown) formed normally shaped shmoos, the $\mathrm{G} \alpha^{\mathrm{DSD}}$ bud14 cells either enlarged uniformly, or exhibited highly aberrant morphologies, consistent with the idea that the G $\alpha$-Fus 3 interaction is required for chemotropic growth.

## G $\beta^{\mathrm{P}-}$ bud1d cells are defective in shmooing

As $G \beta$ phosphorylation and level are greatly reduced in pheromone-treated $\mathrm{G} \alpha^{\mathrm{DSD}}$ and fus $3 \Delta$ cells, $\mathrm{G} \beta$ may be a target of $\mathrm{G} \alpha$-Fus3. Because $\mathrm{G} \alpha^{\mathrm{DSD}}$ also confers defects in partner discrimination and in the genetic assay for 'chemotropic' shmooing described above, we wondered whether the phosphorylation of $\mathrm{G} \beta$ plays a role in the chemotropic response. As a first test of this idea, we created strains in which the native STE4 was replaced with ste $4^{\text {T320A/S335A }}$ (Li et al., 1998), which encodes a mutant form of $\mathrm{G} \beta$ that is not phosphorylated in vivo (henceforth $\mathrm{G} \beta^{\mathrm{P}-}$ ), in $B U D 1$ and bud $1 \Delta$ backgrounds. The $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta, \mathrm{G} \beta$ budl $\Delta, \mathrm{G} \beta^{\mathrm{P}-}$ BUD1 and G $\beta$ BUD1 (henceforth WT, for wild type) cells were treated with a range of pheromone concentrations and examined over 5 hours. In isotropic pheromone, $\mathrm{G} \beta^{\mathrm{P}-}$ BUD1 cells were indistinguishable from WT cells. In contrast, $\mathrm{G} \beta^{\mathrm{P}-}$ bud $1 \Delta$ cells exhibited a variety of shmoo abnormalities at all doses and time points. Representative images are shown in Fig. 1B.

A high proportion of the $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ shmoos were shorter and broader than those formed by the G $\beta$ bud $1 \Delta$ control cells, as if their growth was less well focused. Their mean lengths were 0.89 and 0.84 that of the G $\beta$ budld cells 4 and 5 hours after treatment, respectively ( $P<0.0001$; $n=300$ for each time point), and most were abnormally shaped. Instead of narrowing smoothly to a pointed tip, $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ mating projections often extended almost straight out from the cell body and terminated in a bulbous curve. Although aberrant shmoo morphologies were also seen in the G $\beta$ bud $1 \Delta$ culture, likely due to the absence of Bud1, their occurrence was greatly increased by $\mathrm{G} \beta^{\mathrm{P}-}(80.7 \%$ as compared to $16.7 \%$; $n \geq 260 ; P<0.0001$ ).

Wild-type cells treated with high pheromone concentrations form successive mating projections with regular periodicity such that the growth of the first projection ends as the second begins (Bidlingmaier and Snyder, 2004). In isotropic pheromone, the second growth site is usually established far from the first one, so


Fig. 1. Effect of G $\boldsymbol{a}^{\text {DSD }}$ and $\boldsymbol{G}^{\mathrm{P-}}$ on the chemotropic shmoo pathway. (A) $\mathrm{G} \alpha^{\mathrm{DSD}}$ bud1 $\Delta$ cells are unable to form mating projections. MATa gpal $\Delta$ budl $\Delta$ cells expressing either $\mathrm{G} \alpha$ or $\mathrm{G} \alpha^{\mathrm{DSD}}$ were exposed to 150 nM pheromone for 3 hours. (B) G $\beta^{\mathrm{P}-}$ $b u d 1 \Delta$ cells exhibit a variety of mating projection abnormalities. MATa G $\beta$ bud $1 \Delta$, $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta, \mathrm{G} \beta$ BUD1 and $\mathrm{G} \beta^{\mathrm{P}-}$ BUD1 cells were exposed to 30 nM pheromone for 3 hours. Representative images are shown. Lower right image is a composite. White arrowheads indicate cells scored as bulbous shmoos; white arrows indicate proximal double shmoos; and white asterisks mark SMP cells. (C) G $\beta^{\mathrm{P}-}$ bud $1 \Delta$ cells form projections proximal to the initial growth site. $M A T a \mathrm{G} \beta$ budl $\Delta$ and G $\beta^{\mathrm{P}-}$ budl $1 \Delta$ cells were exposed to 30 nM pheromone and the percentages of cells with a single projection, multiple distal projections, and multiple proximal projections were determined at each time point. The means $\pm$ s.e.m. of three independent experiments are shown ( $n \geq 200$ for each strain and time). $* P<0.0001$ comparing the higher and lower percentages of a given projection type. (D) Simultaneous localization of Spa2-GFP to multiple projection tips. MATa G $\beta$ bud $1 \Delta$ and G $\beta^{\mathrm{P}-}$ bud1 $\Delta$ cells expressing SPA2-GFP were exposed to 150 nM pheromone for 3 hours. Representative images are shown.
(E) Percentage of cells showing simultaneous localization of Spa2-GFP to multiple projection tips. The means $\pm$ s.e.m. of three independent experiments are shown $(n>120$ for each strain and time); $* P<0.0001$, $* * P<0.005$. Scale bars: $5 \mu \mathrm{~m}$.
that the two projections ultimately form a wide angle (distal projections). Another striking phenotype of the G $\beta^{\mathrm{P}-}$ bud1 $\Delta$ cells was their tendency to form second and third mating projections earlier than the control cells and in abnormal positions (Fig. 1C). In the control strain, we observed a small fraction of cells with two or more projections adjacent to one another (proximal projections). However, the occurrence of such cells was dramatically increased in the $\mathrm{G} \beta^{\mathrm{P}-}$ bud $1 \Delta$ strain, with the ratio of proximal to distal shmoos more than 10 -fold greater in $\mathrm{G} \beta^{\mathrm{P}-}$ as compared to control cells. Similar results were observed at all pheromone concentrations and incubation times. Interestingly, the Spa2-GFP marker of polarized growth simultaneously localized to more than one shmoo tip in a high proportion of $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ cells (Fig. 1D), suggesting that G $\beta$ phosphorylation is involved in switching growth from one site to another.

Although G $\beta^{\mathrm{P}-}$ budld cells were not completely incapable of polarized growth in isotropic pheromone, as are $\mathrm{G} \alpha^{\mathrm{DSD}}$ bud1 $\Delta$ and $c d c 24-m 1$ budl $\Delta$ cells, a significant fraction were unable to sustain unidirectional growth long enough to form a projection. Such cells were usually quite small, and formed multiple protrusions unlike true mating projections. In Fig. 1C, these cells are counted in the 'multiple proximal' category; we refer to them as small multiple protrusion (SMP) cells. [The SMP percentages (mean $\pm$ s.e.m.) were $16.2 \pm 1.4$ and $4.0 \pm 0.5$ for the $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ and $\mathrm{G} \beta$ budl $\Delta$ cultures, respectively; $n \geq 200$; $P<0.0001$.] Together, the results presented in Fig. 1B-D suggest that, in cells unable to shmoo via the Bud1-dependent default pathway, $G \beta$ phosphorylation is critical for stabilization of the axis of polarity (hence for focused growth of the mating projection) and for proper growth-site switching.

## The growth site of pheromone-treated $G \beta^{P-}$ bud14 cells wanders where the receptor is concentrated

Nern and Arkowitz (Nern and Arkowitz, 2000) found that double mutants lacking both the default and chemotropic pathways are unable to shmoo because they cannot stabilize their axis of polarity long enough to polarize their growth, not because they cannot establish polarity. The recent demonstration that the Bem1-GFP polarity marker wanders rapidly around the
membrane of pheromone-treated cdc24-ml bud1D cells (Dyer et al., 2013) supports this conclusion. Could the blunt shmoo and SMP phenotypes be the result of a less severe wandering-axis phenotype? To test this, we mixed MATa G $\beta^{\mathrm{P}-}$ bud $1 \Delta$ and MATa G $\beta$ bud $1 \Delta$ cells expressing Spa2-GFP with WT MAT $\alpha$ cells, and assayed reporter localization over time (Fig. 2A). In the control cells, Spa2-GFP localized tightly to the tip of the growing mating projection, moving steadily outward along the axis of polarity. In


G $\beta^{\text {p. }}$ bud1 $\Delta+$ BEM1-GFP-SNC2



D




Fig. 2. The growth site of pheromonetreated G ${ }^{\mathrm{P}-}$ bud1D cells wanders where the receptor is concentrated.
(A) Time-lapse images of Spa2-GFP in mating cells. MATa G $\beta$ budl $\Delta$ or $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ cells expressing SPA2-GFP were mixed with MATa cells and incubated for the indicated times. Final images show an overlay of Spa2-GFP movement from $60-180$ minutes; $\alpha$ indicates the position of the closest $M A T \alpha$ cell at 60 minutes. (B) Bem1-GFP-Snc2 rescues the $\mathrm{G} \beta^{\mathrm{P}-}$ bud $1 \Delta$ shmoo abnormalities. MATa G $\beta$ budl $1 \Delta$ or G $\beta^{\mathrm{P}-}$ budl $1 \Delta$ cells expressing BEM1-GFP-SNC2 were exposed to 30 nM pheromone for 3 hours. Representative DIC and fluorescent images are shown. (C) G $\beta^{\mathrm{P}-}$ bud $1 \Delta$ cells do not form proximal projections when receptor internalization is blocked. MATa $\mathrm{G} \beta$ budl $1 \Delta$, $\mathrm{G} \beta$ budl $1 \Delta$ ste $2^{7 X R / G P A A D}$, $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ and G $\beta^{\mathrm{P}-}$
bud1 $\Delta$ ste $2^{7 X R / G P A A D}$ cells were exposed to 25 nM pheromone for 4 hours. The percentages of cells with a single projection, multiple distal projections and multiple proximal projections were determined. The means $\pm$ s.e.m. of three independent experiments are shown ( $n=300$ for each strain and trial). $P<0.0001$ for the $\%$ single projections and \% proximal projections formed by the $\mathrm{G} \beta^{\mathrm{P}-}$ budl $1 \Delta$ strain as compared with the other three strains. (D) Representative images from the experiment quantified in C. White arrowheads indicate single shmoos; black arrows indicate distal double shmoos; white arrows indicate proximal double shmoos; and white asterisks mark SMP cells (which are counted in the proximal shmoo category). Scale bars: $5 \mu \mathrm{~m}$.
$\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ cells, Spa2-GFP moved more than twice as far, even though the mutant shmoos elongated at the same rate as the control cells. The increased mobility of Spa2-GFP correlated with an increased tendency to change direction (Table 1). Multiple Spa2-GFP spots were also commonly observed in the mutant, but rarely in control cells. Thus, the axis of polarity exhibited confined wandering within the broad mating projections formed by $\mathrm{G} \beta^{\mathrm{P}-}$ bud $1 \Delta$ cells during mating.

Essential regulators of actin cable polymerization (e.g. Cdc24Cdc42, Bem1, Spa2) cluster tightly in a patch at the tips of shmooing cells (Pruyne and Bretscher, 2000a; Arkowitz, 2009). In pheromone-treated WT cells, this 'polarity patch' wanders along the cell cortex, becoming restricted in its movement around the incipient growth site (Dyer et al., 2013). Polarity patch movement is thought to be retarded by free $G \beta \gamma$, whose concentration is ultimately determined by the local density of activated receptor. The role of the receptor and $G \beta \gamma$ in stabilizing the polarity patch can be bypassed by a chimeric protein consisting of Bem1 and the transmembrane domain of Snc2. Expression of Bem1-GFP-Snc2 dramatically slowed movement of the polarity patch, focusing the growth of WT cells and restoring the ability of $c d c 24-m 1$ budl $\Delta$ cells to shmoo (Dyer et al., 2013). If the shmoo morphology and maintenance defects displayed by $\mathrm{G} \beta^{\mathrm{P}-}$ bud $1 \Delta$ cells are also due to a wandering axis of polarity stemming from a weakened link between the receptor and polarity patch, we would expect these phenotypes to be suppressed by Bem1-GFP-Snc2 as well. This proved to be the case: The shmoos formed by $\mathrm{G} \beta$ budld and $\mathrm{G} \beta^{\mathrm{P}-}$ budld cells transformed with the Bem1-GFP-Snc2 construct were indistinguishable (Fig. 2B).

Pheromone triggers polarization of the receptor and G-protein to the mating projection (Suchkov et al., 2010) - the same area where the growth site wanders in $\mathrm{G} \beta^{\mathrm{P}-}$ budld cells. This confined wandering might underlie the formation of proximal projections. To determine whether receptor polarity influences the tendency of $\mathrm{G} \beta^{\mathrm{P}-}$ budld cells to form proximal mating projections, we constructed $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ and $\mathrm{G} \beta$ budl $\Delta$ strains expressing the Ste $2^{7 \mathrm{XR} / \mathrm{GPAAD}}$ mutant form of the receptor. Ste $2^{\text {7XR/GPAAD }}$ cannot be internalized (Ballon et al., 2006) and therefore does not polarize in response to pheromone (Suchkov et al., 2010). Although cells expressing $\operatorname{Ste} 2^{7 \mathrm{XR} / \mathrm{GPAAD}}$ also formed blunt shmoos, preventing receptor polarization suppressed the formation of proximal projections by $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ cells (Fig. 2C), consistent with our hypothesis.

Together, the data shown in Fig. 2 indicate that, although the axis of polarity wanders in $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ cells, the movement of the growth site is limited to the region of highest receptor density. This implies that $G \beta$ phosphorylation helps link the position of the polarity proteins to that of the receptor.

## Effect of G $\beta$ phosphorylation on $\mathbf{G} \beta$ localization

The phenotypes we observe in $G \beta^{P-}$ budl $\Delta$ cells could result from improper localization of the mutant $\mathrm{G} \beta$. To test this, we tagged the N -termini of WT G $\beta$ and G $\beta^{\mathrm{P}-}$ with GFP in situ. In isotropic pheromone, $\mathrm{GFP}-\mathrm{G} \beta^{\mathrm{P}-}$ polarized to the incipient shmoo site later and less stably than did GFP-G $\beta$. Similar but more pronounced defects were observed in mating mixtures (Table 2; Fig. 3). Notably, a substantial fraction of mating GFP-G $\beta$ cells switched from one polarization site to another, presumably to align with the strongest ambient gradient. In contrast, mating GFP-G $\beta^{P-}$ cells were defective in site switching, as well as in consolidating G $\beta$ in a single region of the membrane. These data suggest that $\mathrm{G} \beta$ phosphorylation plays an important role in the pheromone-induced redistribution of G $\beta$, particularly in gradient-stimulated cells.

## Effects of G $\beta^{P-}$ on $\mathrm{G} \beta$-Far1 interaction and Spa2 and Cdc42 dynamics

The inability to phosphorylate $G \beta$ could also impact the cell by affecting the strength of $G \beta$ interactions. For example, Ste 5 preferentially binds the phosphorylated form of G $\beta$ in yeast (Feng et al., 1998), and phosphorylation affects the activity of a mammalian $\mathrm{G} \beta$ (Chakrabarti and Gintzler, 2003). Given the phenotypes conferred by $G \beta^{P-}$, the interaction of $G \beta$ with the Far1-Cdc24 chemotropic complex is a good candidate to be regulated by $\mathrm{G} \beta$ phosphorylation. To test this, we used a wellestablished genetic assay (Whiteway et al., 1990; Cole et al., 1990; Bar et al., 2003; Draper et al., 2009) to ask whether Farl can discriminate between $\mathrm{G} \beta$ and $\mathrm{G} \beta^{\mathrm{P}-}$. The assay is based on the observation that by triggering the pheromone-responsive MAPK pathway, $G \beta$ overexpression blocks cell-cycle progression. Cooverexpression of a protein that binds $G \beta$ can prevent pathway induction and thereby rescue the vegetative growth of cells expressing excess $G \beta$. At the level of transcriptional induction, cell-cycle arrest and shmooing, $\mathrm{G} \beta$ and $\mathrm{G} \beta^{\mathrm{P}-}$ appear to be equally potent (Li et al., 1998). Nevertheless, Far1 overexpression specifically rescued the cell growth defect due to the overexpression of $\mathrm{G} \beta^{\mathrm{P}-}$, and not that of $\mathrm{G} \beta$ (supplementary material Fig. S1), suggesting that Far1 has a greater affinity for the unphosphorylated form of G $\beta$.

As a complementary way of assaying the effect of G $\beta$ phosphorylation on G $\beta \gamma$-Farl interaction, we performed pulldown assays. Cells overexpressing His-tagged $\mathrm{G} \gamma$ and either $\mathrm{G} \beta$ or $\mathrm{G} \beta^{\mathrm{P}-}$ were treated with pheromone to induce phosphorylation of WT $\mathrm{G} \beta$ before purifying $\mathrm{G} \beta \gamma$ and $\mathrm{G} \beta^{\mathrm{P}-} \gamma$ on nickel beads. Because Farl is a low-level protein (Ghaemmaghami et al., 2003), we used mass spectrometric analysis rather than western blotting to measure the amount of co-purifying Far1. Consistent with the results of the genetic assay, Far1 was detectable on the $\mathrm{G} \beta^{\mathrm{P}-} \gamma$ but not on the $G \beta \gamma$ beads (supplementary material Fig. S2).

Table 1. Spa2-GFP mobility

| Strain | Movement/15 minutes ( $\mu \mathrm{m})^{\text {a }}$ | Velocity ( $\mu \mathrm{m} /$ hour) ${ }^{\text {a }}$ | $\Delta$ Direction (\%) ${ }^{\text {a,b }}$ | Final length $(\mu \mathrm{m})^{\text {c }}$ | $\geq 2$ spots $^{\text {a,d }}$ | $n$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| $\mathrm{G} \beta^{\text {WT }}$ | $0.54 \pm 0.15$ | $2.15 \pm 0.61$ | $19 \pm 13$ | $8.07 \pm 0.97$ | 1 | 89 |
| bud1s |  |  |  |  |  |  |
| $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ | $1.13 \pm 0.24$ | $4.51 \pm 0.96$ | $52 \pm 15$ | $7.39 \pm 1.10$ | 18 | 38 |
| ${ }^{\mathrm{a}} P<0.0001$. |  |  |  |  |  |  |
| ${ }^{\mathrm{b}} \Delta$ Direction (\%) is the number of direction changes divided by the number of steps observed for each cell.${ }^{\mathrm{c}} P<0.0007$. |  |  |  |  |  |  |
| ${ }^{\text {d }}$ Number of G $\beta$-polarized cells in which the region of concentrated G $\beta$ was composed of two or more discreet spots. |  |  |  |  |  |  |

Table 2. Pheromone-induced polarization of $\mathbf{G} \boldsymbol{\beta}$

| Parameter | Isotropic |  |  | Mating |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | GFP-G $\beta$ | GFP-G $\beta^{\text {P- }}$ | $P$-value | GFP-G $\beta$ | GFP-G $\beta^{\text {P- }}$ | $P$-value |
| Polarized $n /$ total $n$ | 47/49 | 39/52 | - | 33/36 | 25/43 | - |
| \% Polarized G $\beta$ | 95.9 | 75 | $<0.0001$ | 91.7 | 58.1 | $<0.0001$ |
| Mean time of G $\beta$ polarization (minutes) ${ }^{\text {a }}$ | 1. $8 \pm 0.4$ | $17.3 \pm 1.73$ | $<0.0001$ | $-8.8 \pm 2.4$ | $16.8 \pm 4.2$ | $<0.0001$ |
| \% Polarized G $\beta$ pre-polarized growth ${ }^{\text {b }}$ | 55.3 | 38.4 | 0.034 | 84.8 | 28.0 | 0.0001 |
| \% Switch ${ }^{\text {c }}$ | NA | NA | - | 39.4 | 12.0 | 0.005 |
| \% Wandering ${ }^{\text {d }}$ | ND | ND | - | 9.1 | 36.0 | 0.0001 |
| \% Not consolidated ${ }^{\text {e }}$ | ND | ND | - | 34.4 | 92.0 | <0.0001 |
| \% Spots ${ }^{\text {f }}$ | ND | ND | - | 21.2 | 72.0 | $<0.0001$ |

${ }^{\mathrm{a}}$ For the cells that polarized $G \beta$, the mean $\pm$ s.e.m. of times of polarization relative to the onset of polarized growth are indicated.
${ }^{\mathrm{b}}$ Percentage of G $\beta$-polarized cells in which polarization occurred at or before morphogenesis.
${ }^{\text {c }}$ Percentage of G $\beta$-polarized cells that clearly switched polarization sites during the time-course.
${ }^{\text {d }}$ Percentage of G $\beta$-polarized cells in which the region of concentrated G $\beta$ appeared to wander.
${ }^{e}$ Percentage of $G \beta$-polarized cells in which the region of concentrated $G \beta$ was composed of two or more discreet spots.
${ }^{\text {f }}$ Percentage of G $\beta$-polarized cells in which high-intensity spots were observed well apart from the region of concentrated G $\beta$.
NA, not applicable; ND, not detectable.

To further investigate the possibility that $G \beta$ phosphorylation affects the dynamic interactions of the chemotropic complex, we used fluorescence photobleaching approaches. First, we compared recovery times of the Spa2-GFP signal after photobleaching the shmoo tips of G $\beta^{\mathrm{P}-}$ budl $1 \Delta$ and $\mathrm{G} \beta$ budl $1 \Delta$ cells. As a component of the polarisome, the positional stability of Spa 2 is likely linked to that of the chemotropic complex. In FRAP analysis, the recovery time is a function of the protein's dynamics: The slower the exchange into and out of a complex, the longer the signal will take to recover. As shown in Fig. 4A, the signal recovered in bleached G $\beta$ budld shmoo tips faster and to a greater degree than in bleached G $\beta^{\mathrm{P}-}$ budl $\Delta$ shmoo tips. This suggests that the duration of Spa2 interactions at the shmoo tip is increased when G $\beta$ cannot be phosphorylated.

To assess the effect of $\mathrm{G} \beta^{\mathrm{P}-}$ on the stability of the chemotropic complex itself, we performed iFRAP analysis of GFP-Cdc42 at the shmoo tips of G $\beta^{\mathrm{P}-}$ budld and G $\beta$ budl $\Delta$ cells. In this technique, the area that serves as a source of molecules bound for the complex of interest is photobleached, and the rate at which the signal intensity decreases at the site of complex formation is measured. The rate of signal loss is inversely related to the stability of the complex. In our experiment, the rate of GFP-Cdc 42 signal loss at the shmoo tip was determined after bleaching the cell body. As shown in Fig. 4C, the Cdc42-GFP signal decreased faster in G $\beta$ bud $1 \Delta$ than in the G $\beta^{\mathrm{P}-}$ bud1 $\Delta$ shmoo tips. Consistent with the Spa2-GFP FRAP results, this suggests that the duration of Cdc42 interactions at the shmoo tip are increased when $G \beta$ cannot be phosphorylated. Together, these data suggest that unphosphorylated $G \beta$ binds more tightly than phosphorylated $G \beta$ to components of the chemotropic complex and/ or polarisome. Thus, $G \beta$ phosphorylation may negatively affect the stability of the chemotropic complex and/or polarisome.

## G $\beta^{\mathrm{P}-}$ confers a defect in chemotropism

In budls cells, mating projection formation depends on chemotropic components (Nern and Arkowitz, 2000). Therefore, the observation that $\mathrm{G} \beta^{\mathrm{P}-}$ budld cells shmoo aberrantly in isotropic pheromone implicates $\mathrm{G} \beta$ phosphorylation in chemotropism. To test this, we set up dilute bilateral mating mixtures in which the cells best able to form stable, chemotropic mating projections were most likely to find and fuse with a partner, and analyzed images of newly formed zygotes (Fig. 5A). How
precisely a cell orients towards a potential partner can be inferred by measuring the angle created when two cells fuse. When orientation is optimal, the two cells of a mating pair grow directly toward one another, and consequently, their angle of fusion is $\sim 0^{\circ}$. In contrast, large fusion angles are indicative of poor orientation. In the zygote formation assay, $\mathrm{G} \beta^{\mathrm{P}-}$ cells exhibited a clear orientation defect (Fig. 5C).

After initial orientation toward a pheromone source, yeast can alter their growth in response to a directional change in the gradient (Segall, 1993; Moore et al., 2008) - either by initiating a second projection, or by bending the existing projection toward the new source (Moore et al., 2008). These phenomena are collectively referred to as reorientation. The mechanisms underlying reorientation are likely to overlap and/or interact with those involved in orientation and maintenance. As our data implicate G $\beta$ phosphorylation in initial orientation and stable chemotropic growth, we asked whether it is involved in reorientation as well. WT and $\mathrm{G} \beta^{\mathrm{P}-}$ bilateral mating mixtures were shaken vigorously to induce non-directional (default) shmooing while preventing cell fusion, then allowed to produce and respond to pheromone gradients on solid medium. $\mathrm{G} \beta^{\mathrm{P}-}$ conferred a reorientation defect (Fig. 5B,C). Whereas pre-stimulated WT cells often bent dramatically to contact a mating partner, $\mathrm{G} \beta^{\mathrm{P}-}$ cells formed zygotes that were predominantly the result of 'collision matings', i.e. two shmoos appeared to have simply grown into one another without having significantly changed their direction of growth.

One explanation for the results of the orientation and reorientation assays is that $\mathrm{G} \beta^{\mathrm{P}-}$ confers a defect in directional sensing and/or chemotropic shmooing. However, it is also possible that $\mathrm{G} \beta^{\mathrm{P}-}$ cells signal their positions less effectively than WT cells because their pheromone secretion is less focused. We distinguished these possibilities by comparing the ability of $\mathrm{G} \beta$ and $\mathrm{G} \beta^{\mathrm{P}-}$ cells to grow toward a pheromone source in vitro, using a microfluidic device (Brett et al., 2012). As previously reported, the $\mathrm{G} \beta$ control cells formed normal mating projections in the artificial gradient (Fig. 6A) and oriented toward its source with an accuracy similar to that observed in other published microfluidic experiments (Brett et al., 2012). Surprisingly, the $\mathrm{G} \beta^{\mathrm{P}-}$ cells were unable to sustain growth in a single direction. Rather, they formed multiple small protrusions, often without growing much in overall size - very much like the SMP cells in


Fig. 3. GFP-G $\boldsymbol{\beta}^{\mathrm{P}-}$ polarizes later and less stably than GFP-G $\boldsymbol{\beta}$ in both isotropic and gradient conditions. (A) Isotropic treatment: MATa GFP-G $\beta$ budl $\Delta$ and GFP-G $\beta^{\mathrm{P}-}$ budl $\Delta$ cells were exposed to 150 nM pheromone for the indicated times. (B) Mating mixtures: MATa GFP-G $\beta$ budl $\Delta$ and GFP-G $\beta^{\mathrm{P}-}$ budl $\Delta$ cells were mixed with MAT $\alpha$ cells, and incubated for the indicated times. For A and B, GFP-G $\beta$ concentration in arbitrary units was quantified along the major axis of each cell (line graphs) as described in the Materials and Methods; black arrowheads mark polarized growth and white arrowheads mark polarity. For B, white arrows mark spots and the white asterisk marks non-consolidated polarization. The G $\beta$ polarization data are summarized in Table 2 . Scale bars: $5 \mu \mathrm{~m}$.
$\mathrm{G} \beta^{\mathrm{P}}$ budl $1 \Delta$ cultures treated with isotropic pheromone (Fig. 1B). The first protrusions formed by the gradient-stimulated $G \beta^{P-}$ cells were positioned randomly. These data strongly support the idea that G $\beta$ phosphorylation plays a role in the positioning and maintenance of the chemotropic growth site.

The inability of $\mathrm{G} \beta^{\mathrm{P}-}$ cells to maintain a single axis of polarity in an artificial gradient, combined with their ability to form normal mating projections in isotropic pheromone, provides the first example, to our knowledge, of a gradient-specific shmoo phenotype. We therefore wished to confirm this observation using natural gradients. To look for gradient-dependent destabilization of polarized growth under physiological conditions, we compared WT and $\mathrm{G} \beta^{\mathrm{P}-}$ shmoos formed in liquid culture (isotropic treatment) with those formed in bilateral crosses. Although the $\mathrm{G} \beta^{\mathrm{P}-}$ cells in mating mixtures were not as severely compromised in chemotropic shmooing as the $\mathrm{G} \beta^{\mathrm{P}-}$ cells in the microfluidic device, they
exhibited a number of obvious shmoo defects (Fig. 6B,C). In fact, the response of $\mathrm{G} \beta^{\mathrm{P}-} B U D 1$ cells exposed to natural pheromone gradients was the same as that of $\mathrm{G} \beta^{\mathrm{P}-}$ budld cells exposed to isotropic pheromone: $\mathrm{G} \beta^{\mathrm{P}-}$ mating mixtures accumulated fewer shmoos, shorter shmoos (their mean length was 0.852 of the control cells after 5 hours of mating; $n \geq 105 ; P<0.0001$ ), and a significantly higher proportion of aberrant shmoos. In contrast, the $\mathrm{G} \beta^{\mathrm{P}-}$ and WT shmoos induced by isotropic treatment were indistinguishable at all concentrations and time points (Fig. 1B). These data suggest that the phosphorylation of G $\beta$ plays a critical role in chemotropic, but not default, shmooing.

## Discussion

The rapid phosphorylation of $\mathrm{G} \beta$ in yeast cells responding to mating pheromone was observed over 20 years ago (Cole and Reed, 1991), and yet its purpose has remained enigmatic. Two early studies failed


Fig. 4. Effect of G $\boldsymbol{\beta}^{\mathrm{P}-}$ on positional stability of Spa2 and Cde42. (A) FRAP analysis of Spa2-GFP. MATa G $\beta$ budl $\Delta$ and $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ cells expressing $S P A 2-$ $G F P$ were exposed to 30 nM pheromone for 1 hour prior to photobleaching. The data represent the means $\pm$ s.e.m. of two independent experiments. The FRAP $t_{1 / 2}$ and $\%$ recovery were $9.7 \pm 1.87$ seconds and $40.3 \pm 3.43 \%$, respectively, in $G \beta$ budl $\Delta$ cells ( $n=18$ ) and $19.3 \pm 2.32$ seconds $(P=0.003)$ and $24.6 \pm 1.73 \%$ $(P=0.0002)$ in $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ cells $(n=20)$. Images are shown from a representative FRAP experiment before, subsequent to photobleaching and after 26 seconds recovery. (B) iFRAP analysis of GFP-Cdc42. MATa G $\beta$ budl $\Delta$ and $G \beta^{\mathrm{P}-}$ budl $\Delta$ cells expressing $G F P-C D C 42$ were exposed to pheromone as above. Representative scatter plots of fluorescence loss and corresponding trend lines are shown. The iFRAP $t_{1 / 2}$ and $\%$ loss were $41.9 \pm 3.25$ seconds and $22.9 \pm 1.48 \%$, respectively, in $\mathrm{G} \beta$ bud $1 \Delta$ cells $(n=27)$, and $54.3 \pm 3.55$ seconds $(P=0.01)$ and $21.1 \pm 3.05 \%$ in $G \beta^{\mathrm{P}-}$ budl $\Delta$ cells ( $n=28$ ). Images are shown from a representative iFRAP experiment before and subsequent to photobleaching.
to elucidate its role (Cole and Reed, 1991; Li et al., 1998). We were impelled to revisit this issue by the discovery that the activated forms of $\mathrm{G} \alpha$ and Fus 3 interact, and that a double mutation that uncouples them, $\mathrm{G} \alpha^{\mathrm{DSD}}$, conferred defects in mating and partner discrimination (Metodiev et al., 2002). Indeed, we show here that $\mathrm{G} \alpha^{\mathrm{DSD}}$ budl $\Delta$ cells cannot form mating projections, which identifies $\mathrm{G} \alpha^{\mathrm{DSD}}$ as a chemotropic-defective allele (Fig. 1A). This suggests that the G $\alpha$-Fus3 interaction, like the Cdc 24 -Far1 interaction, is critical for chemotropism. Because $G \alpha^{D S D}$ also results in reduced $G \beta$ phosphorylation and protein levels in stimulated cells, we hypothesized that $\mathrm{G} \beta$ phosphorylation plays a role in chemotropism. Our results demonstrate that this is the case. Specifically, the modification of $G \beta$ appears to promote communication between activated receptors and downstream components essential for polarized growth. Cells unable to phosphorylate $G \beta$ exhibit defects in pheromone-induced morphogenesis, and both orientation and reorientation towards mating partners. We propose that $\mathrm{G} \beta$ phosphorylation provides a means to amplify the spatial signal and that the G $\beta$ phosphorylation/ dephosphorylation cycle is an updating mechanism that conveys the status of the receptor to the chemotropic complex.

## Aberrant shmoo morphology and proximal projections: wandering limited by high receptor and G $\beta \gamma$ density

Although not completely unable to form mating projections, $\mathrm{G} \beta^{\mathrm{P}-}$ budld cells exhibited a variety of shmoo abnormalities (Fig. 1B,C), including short and blunt shmoos, cells that formed multiple projections proximal to the first, and very small cells with multiple protrusions (SMP cells). Time-lapse imaging of

Spa2-GFP in these cells indicated that the axis of polarity wandered, albeit in a confined area (Fig. 2A). This broader movement of Spa 2 correlated with the generation of proximal projections in the region expected to be highest in receptor density. It is therefore of significance that preventing receptor polarization in $\mathrm{G} \beta^{\mathrm{P}-}$ budls cells dramatically suppressed the proximal-projection phenotype, and that expression of Bem1-GFP-Snc2, which slows polarity site wandering by a mechanism independent of the receptor and G-protein, restored their ability to shmoo normally (Fig. 2B,C). These observations suggest that although the axis of polarity is destabilized in pheromone-treated $\mathrm{G} \beta^{\mathrm{P}-}$ bud1 $\Delta$ cells, its wandering is limited by the high concentration of receptor and G $\beta \gamma$ in mating projections. This confined wandering likely underlies the formation of short, blunt shmoos, and in combination with the apparent defect in regulating growth-site switching (Fig. 1D; Table 2), may result in the emergence of multiple projections proximal to the first. The occurrence of $\geq 2$ Spa2-GFP spots in G $\beta^{\mathrm{P}-}$ budl $\Delta$ shmoos also correlates with the eventual formation of proximal projections (Table 1).

## Role of $\mathrm{G} \beta$ phosphorylation in receptor communication to actin cables

What causes confined wandering? Yeast chemotropism requires the translation of an extracellular pheromone gradient into a gradient of activated receptors across the cell surface, and the communication of this spatial information to the actin cytoskeleton. The receptor does not communicate with actin directly, but rather via its $\mathrm{G} \beta \gamma$, which recruits Far1-Cdc24.

A


C


Fig. 5. $G \boldsymbol{\beta}^{\mathrm{P}-}$ confers a defect in directional sensing. (A) Representative straight and angled zygotes from the orientation assays. Zygotes were analyzed from WT and G $\beta^{\mathrm{P}-}$ bilateral matings. The angle of orientation was measured by drawing a line from the base of each shmooing cell (white) to the zone of fusion (black). (B) Representative zygotes from the reorientation assays. Pre-stimulated MATa and MAT $\alpha$ cells were allowed to mate on agar media and scored for their ability to reorient growth of an existing mating projection. Zygotes were analyzed from WT and MATa G $\beta^{\mathrm{P}-}$ bilateral matings. Reorientation was defined as a change in direction in a line drawn from the base of a shmoo to the zone of fusion. The angle of reorientation for a given shmoo was measured by drawing a line from the original axis of polarity (white) to the zone of fusion (black). (C) Mean $\pm$ s.e.m. of the angles of orientation and reorientation. For orientation, $n>200$ for each strain in three trials; $P<0.0001$. For reorientation, $n=58$ for both strains; $P<0.0001$. Scale bars: $5 \mu \mathrm{~m}$.

Along with the polarisome, Cdc42 and Bem1, the G $\beta \gamma-$ Far1Cdc24 chemotropic complex ultimately nucleates actin cables at the incipient mating projection site. In $c d c 24-m 1$ and farl-H7 cells, the link between the receptor and chemotropic complex is completely severed. Polarization sites are established in such cells (Nern and Arkowitz, 2000), but they lack the constraint provided by interaction with receptor-activated G $\beta \gamma$ (Dyer et al., 2013). Hence, their position is not sufficiently stable to sustain polarized growth in one direction. $\mathrm{G} \beta^{\mathrm{P}-}$ budls cells exhibit a similar, although less severe, phenotype. Instead of global wandering and uniform growth, wandering is confined to the area of most concentrated receptor. This implies that the link between the receptor and chemotropic complex is weakened, but not broken.

We explored the effect of $G \beta$ phosphorylation on the stability of the chemotropic complex and polarisome. A genetic assay suggested that phosphorylation of $\mathrm{G} \beta$ reduces its affinity for Far1 (supplementary material Fig. S1), which was supported by the results of a G $\beta \gamma$ pull-down experiment (supplementary material Fig. S2). Photobleaching approaches indicated that both Spa 2 and Cdc42 are more mobile in WT cells than in $G \beta^{\mathrm{P}-}$ cells,
consistent with shorter-lived associations at shmoo tips (Fig. 4B,C). Thus, the phosphorylation of $G \beta$ appears to decrease its affinity for the chemotropic complex and destabilize the association of Spa2 with the polarisome. This suggests an explanation for the confined wandering observed in $\mathrm{G} \beta^{\mathrm{P}-}$ bud $1 \Delta$ cells.

Immediately after exposure to pheromone, the activated receptor and G-protein are distributed almost uniformly on the plasma membrane, as are the first chemotropic and polarisome complexes to be assembled. As the receptor polarizes, however, so do $\mathrm{G} \alpha$ and $\mathrm{G} \beta \gamma$, leading to the eventual concentration of the signaling proteins at the incipient shmoo site. The high density of free $G \beta \gamma$ in this area biases the recruitment of Far1-Cdc24, and thus the localized formation of the chemotropic complex. Moreover, as chemotropic complexes dissociate, they are much more likely to reassemble where free $G \beta \gamma$ is concentrated. The half-life of the chemotropic complex is critical. If it is too short, the cell will not be able to initiate polarized growth. If it is too long, the complex will wander out of the region of high-density receptor and G-protein before the axis of polarity is established. In WT cells, phosphorylation of $G \beta$ decreases the half-life of the complex, thereby limiting the distance it can move before dissociating. This increases the chance that the complex components will reassemble within the active growth area, where receptor and G-protein concentration are highest. In $\mathrm{G} \beta^{\mathrm{P}-}$ cells, on the other hand, the half-life of the complex is long enough to allow movement across the entire region of highdensity receptor and G-protein. Additionally, the zone of G $\beta$ appears to be less well consolidated in $G \beta^{\mathrm{P}-}$ cells (Table 2; Fig. 3). A key aspect in this scenario is the recycling of the Gprotein - the re-association of the heterotrimer with the receptor. G $\beta \gamma$ cannot report the position of active receptor until it releases from the chemotropic complex and binds inactive $\mathrm{G} \alpha$. Conversely, the chemotropic complexes can move away from the growth site, blind to local $\mathrm{G} \beta \gamma$ density, as long as they remain intact. Interestingly, mutations that disrupt the interaction of G $\beta$ with the N-terminal interface of $\mathrm{G} \alpha$, or prevent inactivation of $\mathrm{G} \alpha$, also implicate G-protein cycling in chemotropic shmooing (Strickfaden and Pryciak, 2008). We infer that by increasing the rate at which the position of the activated receptor is reported, the G $\alpha$-GDP/GTP and the G $\beta$-phosphorylation/dephosphorylation cycles strengthen the indirect link between the receptor and the chemotropic complex, and consequently influence the position of the polarity proteins. Posttranslational modification of G $\beta$ may therefore extend the well-established paradigm that cycling is central to the function of G-proteins.

## Polarization of G $\beta$

Our results suggest that $G \beta$ phosphorylation is critical for pheromone-induced $G \beta$ polarization (Fig. 3; Table 2). Although the GFP-G $\beta^{\mathrm{P}-}$ polarization defect could result simply from the drifting axis of polarity, a number of observations raise a more interesting possibility: $G \beta$ phosphorylation could play a key role in the genesis of pheromone-induced intracellular signaling gradients. Given that full pheromone-induced phosphorylation of G $\beta$ depends on Fus 3 and on $\mathrm{G} \alpha$-Fus 3 interaction (Metodiev et al., 2002), and that gradients of active Fus3 emanate from the tips of shmooing cells (Maeder et al., 2007), we propose that $\mathrm{G} \alpha$ recruits Fus3 to phosphorylate $G \beta$ at the incipient shmoo site. As it is also known that pheromone-induced polarization of $\mathrm{G} \alpha$ and G $\beta$ requires their co-internalization with the receptor (Suchkov


Fig. 6. $G \boldsymbol{\beta}^{\mathrm{P}-}$ confers chemotropic growth defects in both artificial and natural pheromone gradients. (A) $\mathrm{G} \beta^{\mathrm{P}-} B U D 1$ cells cannot properly orient and stabilize polarized growth in an artificial pheromone gradient. Representative time-lapse images of gradient-stimulated WT and G $\beta^{\mathrm{P}-}$ cells in a microfluidic device are shown. Black arrows indicate the direction of the gradient. The mean $\pm$ s.e.m.of the orientation angle for the first protrusions formed by G $\beta^{P-}$ cells was $89.9 \pm 4.2^{\circ}(n=314)$, as compared to $56.9 \pm 4.6^{\circ}(n=83)$ for WT shmoos (Brett et al., 2012), where $90^{\circ}$ indicates random orientation. (B) $\mathrm{G} \beta^{\mathrm{P}-} B U D 1$ cells in mating mixtures exhibit shmoo defects similar to $\mathrm{G} \beta^{\mathrm{P}-}$ budl $\Delta$ cells exposed to isotropic pheromone. Representative images of cells from bilateral $\mathrm{G} \beta B U D 1$ and $\mathrm{G} \beta^{\mathrm{P}-} B U D 1$ mating mixtures after 5 hours. Black arrowheads indicate cells scored as single shmoos; white arrowheads indicate distal double projections; and black arrows indicate proximal double projections. Small squares show examples of SMP cells. (C) Quantification of the shmoo types found in the WT and $\mathrm{G} \beta^{\mathrm{P}-}$ bilateral mating mixtures. The percentages of shmooed cells in each class are shown in the bar graph. * $P<0.0001$ for the comparison $\mathrm{G} \beta$ versus $\mathrm{G} \beta^{\mathrm{P}-}$ in each class; $n=300$ for both strains. Similar results were obtained in each of three trials. Scale bars: $5 \mu \mathrm{~m}$.
et al., 2010), it will be interesting to determine whether unphosphorylated $\mathrm{G} \beta$ is preferentially internalized along with $\mathrm{G} \alpha$ and the receptor while phosphorylated $\mathrm{G} \beta$ is left on the membrane. A synergistic combination of these two mechanisms localized $\mathrm{G} \beta$ phosphorylation at the shmoo site and preferential internalization of unphosphorylated $G \beta$ at the back of the cell would be expected to rapidly generate a steep gradient of phosphorylated $G \beta$ via what is essentially a LEGI-type mechanism. An intracellular gradient of phosphorylated $G \beta$ underlying yeast chemotropism would provide an interesting analogy to the intracellular gradient of the phosphorylated lipid, $\mathrm{PIP}_{3}$, found in numerous chemotactic systems (Jin et al., 2000; Servant et al., 2000; Comer and Parent, 2002).

## Role of $\mathbf{G} \beta$ phosphorylation in chemotropism

To determine whether $G \beta$ phosphorylation contributes to directional sensing in addition to its role in mating projection formation, we examined how $\mathrm{G} \beta^{\mathrm{P}-}$ affects chemotropism in both natural and artificial pheromone gradients. These experiments were performed with $B U D 1$ strains, as we wished to determine how well the chemotropic shmoo pathway would override the default shmoo pathway to orient and sustain growth up a gradient. In mating mixtures, $\mathrm{G} \beta^{\mathrm{P}-}$ conferred defects in initial orientation (Fig. 5A) and polarized growth (Fig. 6B). Similar but more pronounced defects were observed in artificial pheromone gradients (Fig. 6A). Remarkably, the polarized growth defects were gradient-specific. $G \beta^{P-}$ cells formed mating projections
when treated with isotropic pheromone, but could not maintain polarized growth -even at the default site - in either artificial or natural gradients. It is noteworthy that the types and proportions of aberrant shmoos formed by $\mathrm{G} \beta^{\mathrm{P}-}$ BUD1 cells in mating mixtures were very similar to those formed by $\mathrm{G} \beta^{\mathrm{P}-}$ bud $1 \Delta$ cells in isotropic pheromone (compare Fig. 6B,C and Fig. 1B,C). In both cases, the cells were forced to use the chemotropic pathway without being able to phosphorylate $\mathrm{G} \beta$. The resulting defects reveal that the cells attempt to shmoo chemotropically, but cannot maintain a stable axis of polarity. These data strongly suggest that $G \beta$ phosphorylation is critical for the initial positioning and maintenance of the chemotropic growth site.

In addition to mechanisms that establish and stabilize directional growth, chemotroping cells must have a means to alter their direction and track dynamic gradients in mating mixtures. To successfully fuse with the shmoo tip of a partner, a yeast cell must not only determine the direction of the strongest source of pheromone and orient its growth accordingly, it must continually reassess the position of the target cell while ignoring weaker signals. The shape of the gradient and concentration of pheromone most likely change as the two cells grow towards each other, and as zygotes form in the mating mixture. Although the mechanisms underlying reorientation are unknown, it is easy to appreciate that the establishment of a chemotropic growth site and ongoing adjustments to its position pose distinct challenges. To date, only one mutation that specifically affects reorientation has been reported: ste2 ${ }^{T 236}$ (Vallier et al., 2002). It is therefore of
considerable interest that $\mathrm{G} \beta^{\mathrm{P}-}$ confers a dramatic defect in reorientation. This suggests that $G \beta$ phosphorylation plays a critical role in gradient tracking.

In summary, we propose that the phosphorylation of $G \beta$ contributes to chemotropism in two ways: (1) G $\beta$ modification is used to rapidly generate a signaling gradient in which phosphorylated $\mathrm{G} \beta$ is concentrated at the incipient shmoo site. This may facilitate the amplification of other intracellular signaling gradients; (2) Phosphorylation of $G \beta$ decreases its affinity for Farl and perhaps other chemotropic components, thereby shortening the cycle time between the receptor and polarisome. More frequent updating of receptor status increases sensitivity to changes in the pheromone gradient.

## Materials and Methods

## Molecular and microbiological techniques

Standard methods were used for microbial and molecular manipulation (Sherman et al., 1986; Ausubel et al., 1994; Guthrie and Fink, 2002). Strains and plasmids used are listed in supplementary material Tables S1 and S2, respectively. Yeast cells were cultured in rich medium (YEPD) at $30^{\circ} \mathrm{C}$ unless noted otherwise.

## Spa2-GFP localization in isotropic pheromone

MATa budl $\Delta \mathrm{G} \beta$ or $\mathrm{G} \beta^{\mathrm{P}-}$ cells expressing SPA2-GFP were incubated together on s.d. complete agar pads containing $150 \mathrm{nM} \alpha$-factor; one strain was labeled with ConA-Alexa Fluor 594 (Molecular Probes). Six fields were imaged at 15 -minute intervals with $0.5 \mu \mathrm{~m} z$-sections acquired using a DeltaVision deconvolution system (Applied Precision) on an Olympus IX-70 microscope with an NA $1.4 \times 60$ objective. The images were deconvolved using Huygens Deconvolution Software V. 3.7 (Scientific Volume Imaging), sum projected (GFP), or average projected (DIC), and converted into 8 -bit TIF files using ImageJ. The scale bar equals $5 \mu \mathrm{~m}$ in all images.

## Spa2-GFP time-lapse microscopy in mating mixtures

WT MAT $\alpha$ cells were labeled with ConA-Alexa Fluor 594 prior to mixing with MATa bud $1 \Delta \mathrm{G} \beta$ or $\mathrm{G} \beta^{\mathrm{P}-}$ cells expressing SPA2-GFP. Mating mixtures were incubated on s.d. complete agar pads. Images were acquired 25 minutes after mixing and at 15 minute intervals thereafter. Image acquisition and analysis was as described above. Tracking analysis was performed using the ImageJ Manual Tracking plugin.

GFP-G $\beta$ time-lapse microscopy in isotropic pheromone conditions
$M A T \mathbf{a}$ budl $\Delta$ GFP-G $\beta$ and MATa budl $\Delta$ GFP- $\mathrm{G}^{\mathrm{P}-}$ cells were incubated together on s.d. complete agar pads containing $150 \mathrm{nM} \alpha$-factor. The strains were distinguished by labeling one or the other with ConA-Alexa Fluor 594. Cells were first imaged 15 minutes after exposure to pheromone and at 10 -minute intervals thereafter. Six fields were imaged at each time point with $150.3 \mu \mathrm{~m} z$-section acquired using an ANDOR Revolution XD spinning disk laser confocal system with a motorized Olympus IX-81 microscope, a Yokogawa CSU-X1 spinning disk unit, motorized XYZ control (piezo) and an iXon897 EMCCD camera, all controlled by Andor iQ2 software. A UplanSApo NA $1.4 \times 100$ objective was used with $488-\mathrm{nm}$ laser excitation. Voxel size was $133 \times 133 \times 280 \mathrm{~nm}$. An Okolab chamber was used to maintain cells at $30^{\circ} \mathrm{C}$. The images were then sum projected and converted into 8 -bit TIF files using ImageJ and false colored. The intensity profile along the major axis of the cells was quantified using BudPolarity (Vernay et al., 2012). The line graphs in Fig. 3 represent the output from this analysis. In Table 2, cells were scored as exhibiting polarized $G \beta$ if they met all of the following criteria: peak signal intensity $\geq 2 \times$ the baseline value over less than one half the cell surface for at least three consecutive time points. Cells were scored as exhibiting polarized growth when they were judged to be elongating predominantly along one axis at the center of the DIC z-stack. The ImageJ ellipse tool was used for this determination.

## GFP-G $\beta$ time-lapse microscopy in mating mixtures

WT MATa cells were mixed $1: 1$ with MATa budl $\Delta$ GFP-G $\beta$ or MATa budl $\Delta$ GFP-G $\beta^{\mathrm{P}-}$ cells. Mating mixtures were incubated on s.d. complete agar pads and images were acquired every 15 minutes. Image acquisition and analysis were identical to that described for isotropic pheromone conditions. In Table 2, cells were scored as exhibiting polarized $G \beta$ if they met all of the following criteria: peak signal intensity $\geq 1.5 \times$ the baseline value over less than one half the cell surface for at least three consecutive time points. Cells were scored as exhibiting polarized growth as described above.

## FRAP analysis of Spa2-GFP

MATa budl $\Delta \mathrm{G} \beta$ or $\mathrm{G} \beta^{\mathrm{P}-}$ cells expressing SPA2-GFP were grown to mid-log phase and then exposed to $30 \mathrm{nM} \alpha$-factor at $30^{\circ} \mathrm{C}$ for 1 hour. FRAP analysis was
carried out on a Zeiss LSM 510 META confocal Axiovert 200M microscope (Carl Zeiss) using an NA $1.4 \times 63$ Plan-Apo objective and 488 -nm LASER excitation. Images were captured every 1 second at 2-5\% maximum laser intensity and $10 \times 0.5$ milliseconds photobleaching scans at $100 \%$ laser intensity were performed on a circular area of $1 \mu \mathrm{~m}^{2}$ at the shmoo tip. Data analysis was carried out as described (Bassilana and Arkowitz, 2006).

## iFRAP analysis of GFP-Cdc42

$M A T \mathbf{a}$ budl $\triangle \mathrm{G} \beta$ or $\mathrm{G} \beta^{\mathrm{P}-}$ cells expressing GFP-CDC42 were grown to mid-log phase and exposed to $30 \mathrm{nM} \alpha$-factor at $30^{\circ} \mathrm{C}$ for 1 hour. iFRAP was carried out as described for FRAP analyses except that the cell body was photobleached rather than the shmoo tip. The average intensity of the bleached or unbleached area was normalized for photobleaching during image acquisition, using the average intensity of the cell with MatLab. Loss of fluorescence intensity was then fitted to an exponential curve.

## Mating projection assays

To study mating projection formation and maintenance under isotropic pheromone conditions, mid-log phase cells were exposed to $\alpha$-factor at the concentrations and times indicated. DIC images for experiments carried out in liquid cultures were acquired using a Zeiss Axioskop 2 microscope fitted with an NA $1.4 \times 63$ Plan-Apo oil immersion objective and a Zeiss AxioCam digital camera, and processed with Zeiss AxioVision software. To study mating projection formation and maintenance in physiological pheromone gradients, cultures were grown to mid-log phase at $30^{\circ} \mathrm{C}$ in YEPD. Dilute bilateral $\mathrm{G} \beta$ and $\mathrm{G} \beta^{\mathrm{P}-}$ mating mixtures containing $\sim 1 \times 10^{7}$ MATa cells and $\sim 1 \times 10^{7}$ MATa cells were then spread on YEPD agar. Time points were taken at 1 hour intervals for 5 hours, fixed with $3.7 \%$ formaldehyde, and $630 \times$ DIC images of shmoos were acquired using the Zeiss equipment described above. Mating projection lengths were measured using ImageJ. Percentages in each shmoo class were scored on a hemocytometer using a Leitz Laborlutz S phase-contrast microscope fitted with an NA $0.65 \times 40$ Plan objective.

## Orientation assays

To measure the ability of cells to orient their growth in physiological pheromone gradients, cultures were grown to mid-log phase. Dilute bilateral $\mathrm{G} \beta$ and $\mathrm{G} \beta^{\mathrm{P}-}$ mating mixtures containing $\sim 5 \times 10^{6}$ MAT $\alpha$ cells and $\sim 5 \times 10^{6}$ MATa cells were then incubated for 3 hours on filters ( $0.45 \mu \mathrm{~m}$ Millipore) placed on YEPD agar. Filters were washed in 1 ml sterile $\mathrm{H}_{2} \mathrm{O}$ to harvest cells. $630 \times$ DIC images of zygotes were acquired using the Zeiss Axioskop 2 microscope and camera described above. Zygote angles were measured using ImageJ. The response of cells to artificial pheromone gradients was assayed using a microfluidic device, as described (Brett et al., 2012).

## Reorientation assay

Mid-log phase liquid cultures containing $\sim 10^{7}$ MAT $\alpha$ cells and $\sim 10^{7}$ MATa cells were shaken at $250 \mathrm{r} . \mathrm{p} . \mathrm{m}$. at $30^{\circ} \mathrm{C}$ for 3 hours to induce shmooing prior to mating. Cultures of either WT MAT $\alpha$ and MATa cells or G $\beta^{\mathrm{P}-}$ MAT $\alpha$ and MATa cells were then spread onto YEPD agar and incubated for 3 hours. Images of zygotes were collected using an Olympus BH-2 inverted microscope fitted with a CDPlan 40 objective and an Olympus digital camera (magnification $400 \times$ ). Zygote angles were measured using ImageJ.

## Statistics

The $P$-values for the comparisons of length, angle and time measurements were determined using unpaired, two-tailed $t$-tests (Graphpad). The $P$-values for the comparisons of percentages were determined using the chi-square test (Graphpad).

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## Author contributions

R.D.F. participated in the experimental design, and performed experiments and data analysis, as well as constructing all of the figures; M.-E.B. performed the experiment shown in Fig. 6A along with D.E.; N.W. did the pull-down/mass spectrometry sample preparation for the experiments represented in supplementary material Fig. S2; M.V.M. ran and analyzed the mass spectrometry data and also first made the observation depicted in Fig. 1A; E.A. performed experiments and quantified the data; O.D. designed and performed the experiment shown in supplementary material Fig. S1; R.A. helped design and perform the experiments, as well
contributing to the formulation and editing of the manuscript; and D.S. directed the project and wrote the manuscript.

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