Phosphorylated G $\beta\gamma$ is a leading marker and positional cue during yeast gradient tracking

Running title: Phosphorylated G $\beta\gamma$ directs yeast gradient tracking

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Abstract

Budding yeast cells interpret shallow pheromone gradients from cells of opposite mating type, polarize their growth toward the pheromone source, and fuse at the chemotropic growth site. We have proposed a deterministic gradient sensing model that explains how yeast cells switch from the intrinsically positioned default polarity site (DS) to the gradient-aligned chemotropic site (CS). Phosphorylation of the mating-specific G β is thought to be important for this process, yet no tools are available to monitor the spatiotemporal dynamics of phosphorylated G β in living cells. Here, we describe the development of a biosensor that binds to phosphorylated but not unphosphorylated G β and test key predictions of our gradient sensing model. In mating cells, the biosensor colocalized with G β and receptor reporters at the DS, then tracked with them to the CS. Notably, the biosensor concentrated on the leading side of the tracking G β and receptor peaks and was the first to arrive and stop tracking at the CS. Our data show that the concentrated localization of phosphorylated G β correlates with the tracking-direction and final position of the G-protein and receptor, consistent with the idea that gradient-regulated phosphorylation and/or dephosphorylation of G β contributes to gradient sensing. Cells expressing a non-phosphorylatable mutant form of G β exhibited defects in gradient tracking, orientation toward mating partners, and mating efficiency.

Introduction

Chemical gradient sensing is essential for cell function across eukaryotic phyla. A wide range of developmental and homeostatic processes depend on chemotaxis (directed movement) and chemotropism (directed growth). Examples include immune and inflammatory responses, tumor metastasis (Iijima et al., 2002), axonal guidance (Hong and Nishiyama, 2010; Tojima et al., 2011), angiogenesis (Basile et al., 2004), and fungal life cycles (Daniels et al., 2006; Snetselaar et al., 1996).

In the haploid phase of its lifecycle, the unicellular eukaryote *Saccharomyces cerevisiae* (budding yeast) exists as two mating types, *MATa* and *MAT α* , which can proliferate by mitosis or mate to produce *MATa/MAT α* diploid zygotes (Jackson and Hartwell, 1990). Because yeast cells are not motile, they depend on chemotropism to locate and grow toward potential mating partners, and thus to mate efficiently. Haploid cells of each mating type secrete a peptide pheromone that binds to G-protein coupled receptors (GPCRs) expressed by cells of the opposite type. Pheromone-bound receptor activates its cognate heterotrimeric G protein, encoded by *GPA1* ($G\alpha$), *STE4* ($G\beta$), and *STE18* ($G\gamma$). Upon dissociation from $G\alpha$ -GTP, $G\beta\gamma$ signals via a mitogen-activated protein kinase (MAPK) cascade to the nucleus, where the activated Fus3 MAPK induces cell-cycle arrest in late G1 and mating-specific changes in gene expression. Following cell-cycle arrest, pheromone-stimulated cells polarize their growth to form mating projections (also called shmoo) by focusing actin-cable-directed secretion to a single site (Jackson and Hartwell, 1990). In mating mixtures, partners shmoo toward each other at a position known as the chemotropic growth site (CS), growing up the pheromone gradient until they meet and fuse. Pheromone-stimulated cells that cannot sense a gradient (e.g., under

isotropic or saturating-dose conditions) also form mating projections: they shmoo where they would have budded in the next cell cycle at a position known as the default polarity site (DS) (Dorer et al., 1995).

Although the yeast mating response is one of the best characterized models of chemotropism to date (Arkowitz, 2009), many fundamental questions remain. It has been estimated that yeast cells can accurately respond to pheromone gradients as shallow as 1% (Segall, 1993). Historically, it has been difficult to explain how yeast cells decode such subtle spatial signals, especially when there are multiple gradient sources, as in a mating mixture. An additional challenge has been to understand how the chemotropic growth site is aligned with the external chemical gradient despite the strong intrinsic polarity of the default site. We have recently proposed a model that addresses these problems (Wang et al., 2019). Our data suggest that, in mating cells, signaling, polarity, and trafficking proteins co-localize at the DS, where they assemble into what we call the gradient tracking machine (GTM). Differential activation of the receptor triggers feedback mechanisms that bias exocytosis upgradient and endocytosis downgradient, thus effecting redistribution, or tracking, of the GTM toward the pheromone source. The position of the GTM stabilizes when it reaches the eventual site of chemotropic growth.

According to our model, the feedback mechanisms that enable yeast cells to amplify shallow pheromone gradients into robust directional responses involve the receptor, its G protein, the Yck1 and Yck2 receptor kinases (hereafter, Yck1/2), and the Fus3 MAPK (Ismael and Stone, 2017; Ismael et al., 2016). Like GPCRs in higher eukaryotes, the yeast pheromone receptors are phosphorylated, ubiquitinated, and internalized when activated (Ayscough and Drubin, 1998; Moore et al., 2008; Suchkov et al., 2010). Pheromone also induces rapid Fus3- and Yck1/2-

dependent phosphorylation of G β on multiple residues (Ismael et al., 2016; Li et al., 1998; Metodiev et al., 2002) Active G α is thought to recruit Fus3 to the cell cortex where it phosphorylates G β (hereafter, G β^P), and thereby enhances the affinity of G $\beta\gamma$ for Yck1/2 (Ismael et al., 2016; Metodiev et al., 2002). Our data suggest that the interaction of G $\beta^P\gamma$ with Yck1/2 inhibits receptor phosphorylation, which slows receptor and G-protein internalization (Ismael et al., 2016). Because G-protein activation increases as pheromone concentration increases, generation of G $\beta^P\gamma$, and consequently, receptor protection, are maximized upgradient. Conversely, less G $\beta^P\gamma$ is generated as the pheromone concentration decreases, leading to a higher rate of receptor and G-protein internalization downgradient. We have shown that receptor phosphorylation is essential for yeast gradient tracking (Wang et al., 2019). Thus, a central postulate of our model is that yeast gradient tracking depends on differential phosphorylation of the receptor, which in turn, depends on localized phosphorylation and/or dephosphorylation of G $\beta\gamma$. If this postulate is correct, we would expect G $\beta^P\gamma$ to concentrate on the leading side of the total G $\beta\gamma$ and receptor distributions during tracking, and to anticipate the GTM transitions from assembly to tracking and from tracking to stabilization. To test these predictions, we developed a biosensor that distinguishes the phosphorylated and unphosphorylated forms of G β .

Here, we describe the isolation and characterization of a Forkhead Associated (FHA1) domain variant, affinity-selected from a phage-display library against a phosphorylated G β peptide. This FHA1 variant distinguishes between mono, dual, and triple phosphorylated G β peptides in vitro, and provides the binding specificity for a fluorescent biosensor that distinguishes between G $\beta\gamma$ and G $\beta^P\gamma$ in vivo. We show that in yeast cells treated with isotropic pheromone, the G β^P biosensor translocates from the cytoplasm to the plasma membrane (PM) at the DS and remains at the site of polarized growth as cells shmoo. In mating cells, the G β^P

biosensor mimicked the behavior of GFP-G β , redistributing upgradient from the default site to the chemotropic site; in mating cells expressing both the G β^P biosensor and either a G β reporter or a receptor reporter, G β^P concentrated upgradient relative to total G $\beta\gamma$ and total receptor, consistent with the proposed role for G β phosphorylation as a determinant of GTM positioning. Mating phenotypes conferred by a non-phosphorylatable form of G β further support this conclusion.

Results

Isolation of FHA1 variants against a dually phosphorylated G β peptide

The highly conserved primary structure of G β proteins consists of eight repeats of approximately forty residues that fold to form a seven-bladed β -propeller. Ste4 has two unique insert domains, one at its N-terminus (residues 1-35) and one between the sixth and seventh repeats (residues 310-346) (Cole and Reed, 1991). To date, the crystal structure of Ste4 has not been solved. By taking advantage of the high sequence similarity between mammalian and yeast G β proteins, we obtained a predicted Ste4 structure in which the internal insert domain is a flexible loop, comprising residues 310-348, between the sixth and seventh blades of the β -propeller (Fig. 1A) (Kelley et al., 2015). This region (hereafter, the G β phosphorylation loop) contains all of the known Ste4 phosphorylation sites (Cole and Reed, 1991; Ismael et al., 2016; Li et al., 1998).

Given that phosphorylation of G β occurs on T320, T322, and S335 (Ismael et al., 2016; Li et al., 1998), and that the engineered FHA1 reagents are both pThr specific and dependent (Venegas et al., 2016), we sought a recombinant affinity reagent that recognizes G $\beta^{pT320 pT322}$ but not unphosphorylated G β . Synthetic peptides corresponding to G β residues 314-328, with and without phosphorylation of residues T320 and T322, were used for affinity selection and as a

negative control, respectively (Fig. 1B). After three rounds of selection using the previously generated FHA1G2 library (Pershad et al., 2012), 96 clones were examined by phage ELISA. Of these, fifteen clones exhibited at least 1,000-fold greater binding to the phosphorylated peptide than to the non-phosphorylated peptide. DNA sequencing of the fifteen G β phosphothreonine-binding domains (pTBDs) revealed that there were four unique clones (Fig. 1C). The G2 clone showed the highest relative binding to the G β phosphopeptide (Fig. 1D).

Pheromone induces the binding of a G β pTBD to cortical G β at sites of polarized secretion

To assess the potential for using pTBD variants as in-cell sensors of phosphorylated G β , we constructed a centromeric plasmid containing G2 (the variant that showed the highest relative binding to the G β -pT320/pT322 phosphopeptide in vitro – hereafter, G2-pTBD) fused to GFP and driven by the *ADHI* promoter (hereafter, the biosensor). As compared to control cells, wild type (WT) cells transformed with the biosensor exhibited similar sensitivity to pheromone-induced cell-cycle arrest (Fig. S1) and identical shmoo morphology, and mated with the same efficiency (mean percentage zygotes formed $\pm$ SEM: WT *MATa BARI* = 63 ± 5.8 , *MATa BARI* + biosensor = 59 ± 8.4 ; $n = 6$; $p = 0.4090$). Using time-lapse imaging, we compared the localization of the putative G β^P biosensor to that of an in-situ tagged GFP-G β reporter in vegetative and pheromone-treated cells (Fig. 2A). As previously observed (Suchkov et al., 2010), GFP-G β localized uniformly at the plasma membrane (PM) of vegetative cells through most of the cell cycle, consistent with the known cortical localization of G $\beta\gamma$, but dramatically polarized to the tip of emerging buds (DeFlorio et al., 2013) and the mother-daughter neck shortly before abscission (Wang et al., 2019). In response to isotropic pheromone-treatment, the GFP-G β signal moved from the cytokinesis site to the default polarity site prior to morphogenesis (DeFlorio et al., 2013), and subsequently formed a polarized crescent at the shmoo tip. In contrast, the

biosensor did not localize to the PM of vegetative cells, although it did transiently concentrate at the bud tip and mother-daughter neck, sites of stable and robust polarized secretion. In pheromone-treated cells, the biosensor mimicked the dynamic PM-localization pattern of GFP-G β , moving from the cytokinesis site to the DS and subsequently polarizing at the shmoo tip, consistent with the pheromone-induced phosphorylation of G β . The biosensor also concentrated in the nuclei of both vegetative and pheromone-treated cells (Fig. S2), which was expected, given that FHA domains are known to localize to the nucleus via a bipartite NLS (Durocher et al., 1999; Hofmann and Bucher, 1995).

To confirm the specificity of the biosensor for pThr in vivo, we made a substitution mutation in the pTBD moiety (R70A) that prevents its interaction with the γ -phosphoryl group on pThr (Durocher et al., 1999). As expected, the mutant biosensor (biosensor^{R70A}) showed no discrete localization in either vegetative or pheromone-treated cells (Fig. 2A). To test the specificity of the biosensor for G β ^P, we assayed its localization in cells expressing Ste4^{T320A/S335A}, a mutant form of G β previously reported to remain unphosphorylated in pheromone-treated cells (Li et al., 1998). Contrary to our expectations, the localization patterns of the biosensor in WT and G β ^{T320A/S335A} cells were indistinguishable (Fig. 2A).

Failure of the biosensor to distinguish between G β and G β ^{T320A/S335A} could be due to a lack of specificity for phosphorylated G β in vivo, or to previously undetected phospho-threonine sites in G β . Alternatively, the biosensor might cross-react with an unknown yeast protein that concentrates at sites of polarized secretion. To distinguish these possibilities, we examined the localization of the biosensor in cells lacking *STE4*, the gene that encodes G β . In vegetative *ste4* Δ cells, the biosensor polarized to the mother-daughter neck and apical bud tip, indicating that one or more unidentified secretory proteins bind the sensor at specific times during the cell cycle

(Fig. 2B). Importantly, no cortical localization of the sensor was detected in G1 cells, which do not polarize their secretion. To determine whether the sensor binds to G β^P in addition to the unknown secretory protein(s), we asked whether PM-localization of the sensor would be higher in cells producing increased levels of the target. Indeed, the biosensor hyper-localized to both the shmoo tip and the surrounding PM in pheromone-treated cells overexpressing *STE4* (Fig. 2, C and D). That this effect was dependent on pheromone is consistent with the idea that the biosensor binds to phosphorylated but not unphosphorylated G β . Together, these results indicate that the biosensor binds to PM-localized G β in response to pheromone stimulation and to at least one other pThr-containing protein that concentrates at sites of polarized secretion.

The biosensor physically interacts with G β but not with G $\beta^{T318A/T320A/T322A}$

G $\beta^{T320A/S335A}$ was originally thought to remain unphosphorylated in pheromone-treated cells on the basis of gel-shift and in-vivo labeling assays (Li et al., 1998). Subsequently, mass spec analysis of phospho-peptides purified from vegetative and pheromone-treated cells indicated that pheromone induces phosphorylation of residues T318, T320 and/or T322, and S335 (Ismael et al., 2016). To further characterize the phospho-specific binding preferences of the G2-pTBD clone within the G β phosphorylation loop, we compared its binding to the peptide used in the library screen (G β -pT320/pT322) to peptides of identical sequence but differing in phosphorylation pattern (Fig. 3, A and B). In ELISA assays, the relative binding of G2-pTBD to the triply phosphorylated peptide (G β -pT318/pT320/pT322) was about 1.5-fold greater as compared to the screen peptide. To our knowledge, this is the first example of an FHA domain that recognizes more than two pThr residues on a single peptide target. The ELISA data also suggest that pT322 is the “anchor” residue (Pershad et al., 2012) that fits into the pThr-recognition pocket of the pTBD, as the pT322 peptide is the only mono-phosphorylated variant

that showed significant interaction with G2-pTBD. These findings raised the possibility that $G\beta^{T320A/S335A}$, which we presumed would serve as a negative control, could be phosphorylated on either T318 or T322 and thereby bind the biosensor in pheromone-treated cells. Therefore, we created a strain in which the native *STE4* locus was replaced with a *ste4*^{T318A/T320A/T322A} triple mutant allele (hereafter, *ste4*^{3TA} for the allele and $G\beta^{3TA}$ for the protein).

To test the ability of the biosensor to distinguish phosphorylated from unphosphorylated $G\beta$ in vivo, we used the Bimolecular Fluorescence Complementation (BiFC) assay. We imaged cells transformed with BiFC constructs encoding the N-terminal half of Venus fluorescent protein fused to the biosensor and the C-terminal half of Venus fused to either $G\beta$, $G\beta^{T320A/S335A}$ or $G\beta^{3TA}$, before and after isotropic pheromone treatment. Although pheromone induced polarized BiFC signals at the bud neck and at the shmoo tip of cells co-expressing the biosensor construct and either the $G\beta$ or the $G\beta^{T320A/S335A}$ constructs (Fig 4, A-C), no BiFC signal was detected in cells co-expressing the biosensor and $G\beta^{3TA}$ constructs (Fig 4, A and D) or in the negative control strains (Fig. 4A). Moreover, none of the BiFC strains produced a detectable signal when grown in the absence of pheromone (Fig. 4A). These results show that the biosensor specifically binds to phosphorylated forms of $G\beta$ in vivo, and that $G\beta^{T320A/S335A}$ but not $G\beta^{3TA}$ can bind the sensor in pheromone-treated cells.

The biosensor mimics GFP- $G\beta$ tracking in mating cells

In a recent study of yeast gradient sensing in vivo, we characterized the spatiotemporal dynamics of the pheromone receptor (Ste2-GFP) and G protein (GFP- $G\beta$) in *MATa* cells orienting towards their mating partners (Wang et al., 2019). The receptor and $G\beta$ reporters behaved similarly. Both concentrated at the mother-daughter neck of cells in late M before relocating as polarized crescents to the DS following cytokinesis. After a pause of about ten minutes, the polarized

receptor and G β crescents redistributed upgradient and ultimately stabilized at the CS in advance of morphogenesis and cell fusion. For convenience, we call this redistribution “tracking.”

Tracking begins after cytokinesis and ends with the pre-morphogenic establishment of the CS.

To test our ability to follow G β^P during the gradient tracking phase in mating cells, we took time-lapse images of mating mixtures in which wild type *MAT α* cells were crossed to *MAT α* cells expressing the GFP-G β reporter, or to *MAT α* *STE4* (wild type G β) and *MAT α* *ste4^{3TA}* (G β^{3TA}) cells expressing the biosensor. Like GFP-G β , the biosensor moved from the bud neck to the DS after cytokinesis in wild type cells, then tracked to the CS (Fig. 5, A and B). In contrast, the biosensor gradually disappeared from the bud neck of G β^{3TA} cells; neither localization to the DS after cytokinesis nor tracking along the PM could be detected, although a highly focused biosensor signal could be seen in deconvolved images concomitant with morphogenesis (Fig. 5C). To assay the expression and localization of G β^{3TA} in mating cells, we crossed *MAT α* cells to *MAT α* cells expressing both *ste4^{3TA}* tagged with RFP in situ and the biosensor. As shown in Fig. 5D, the failure of the biosensor to localize to the PM of gradient-tracking *MAT α* RFP-*ste4^{3TA}* cells cannot be attributed to poor expression or mislocalization of RFP-G β^{3TA} . Moreover, population measurements showed no significant difference between the mean PM levels of RFP-G β and RFP-G β^{3TA} in vegetative cells, and a decrease in the mean PM level of RFP-G β^{3TA} in mating cells too small to account for the absence of biosensor PM localization (Fig. S3).

The biosensor colocalizes with GTM markers during assembly, centers upgradient during tracking, and leads stabilization

We have proposed that the assembly, tracking, and stabilization of the GTM underlie the ability of yeast cells to locate and orient toward mating partners (Wang et al., 2019). During the

gradient tracking phase, signaling-competent (active-unphosphorylated) receptors and active G proteins predominate on the upgradient side of the GTM, while signaling-incompetent (inactive and active-phosphorylated) receptors and inactive G proteins predominate downgradient. During the stabilization phase, signaling-competent receptors and active G proteins concentrate at the center of the CS, which is corralled by signaling-incompetent receptors and inactive G proteins. We have also proposed that active $G\alpha$ recruits the Fus3 MAPK to phosphorylate $G\beta$, which promotes subsequent phosphorylation of $G\beta$ by Yck1/2 (Ismael et al., 2016; Li et al., 1998; Metodiev et al., 2002). Based on these postulates, we expect maximal generation of $G\beta^P\gamma$ to correspond with the peaks of active receptor and $G\alpha$ -GTP – i.e., at least slightly upgradient relative to the peaks of total receptor and total $G\beta$ during tracking, and at the center of the stabilized GTM. Indeed, output from our computational model shows the peak of activated $G\alpha$ tracking about 0.1 μm ahead of the total receptor peak (Wang et al., 2019; Video S2). To test these predictions, we assayed the localization of $G\beta^P$ relative to that of total $G\beta$ and total receptor in mating cells expressing both the biosensor and either in-situ tagged $G\beta$ (RFP- $G\beta$) or receptor (Ste2-GFP). Because slightly fewer cells co-expressing the biosensor and $G\beta$ reporter exhibited gradient tracking (55%; $n = 31$) as compared to control cells (76%; $n = 36$; $p = 0.01$), we limited our analysis of doubly-labeled biosensor/ $G\beta$ cells to those that oriented toward a partner and mated. Gradient tracking was unaffected in cells co-expressing the biosensor and receptor reporter. During assembly, the biosensor colocalized with the $G\beta$ and receptor reporters at the DS, although it was less broadly distributed than the GTM markers (Fig. 6, A-C; Fig. 7, A-C). Notably, the biosensor concentrated upgradient relative to the distribution midpoints of the GTM markers during tracking (Fig. 6, B and C; Fig. 7, B and C), became more focused as it redistributed upgradient (Fig. 6D; Fig. 7D), and stabilized at the

eventual CS 4.5 ± 0.5 minutes and 5.3 ± 0.5 minutes (mean $\pm$ SEM) before the polarized G β and receptor reporters reached their final positions, respectively ($n \geq 50$; $p < 0.001$). When ultimately stabilized at the CS, the centers of the G β and receptor crescents aligned with the center of the biosensor crescent (see “End” time point in Figures 6C and 7C). That the biosensor crescent was significantly smaller than both the GFP-G β and Ste2-GFP crescents during tracking suggests a concentrated region of G β phosphorylation within the broader distributions of total G β and receptor. The earlier arrival of the biosensor at the CS was also seen in cells that appeared to be choosing between two mating partners. In the *MATa* cell shown in Figure S4, for example, the biosensor vacillated between the first and second *MATa* cells before aligning with the second partner at 75 minutes, whereas the G β crescent continued to shift between the two potential partners until fusion. Similarly, the biosensor first polarized toward two proximal *MATa* cells in the *MATa* cell shown in Figure 6E before concentrating at the eventual CS. In contrast, the G β reporter polarized to the entire PM region between the two potential partners, and only concentrated at the CS after the biosensor had stabilized there. These data show that the concentrated localization of G β^P correlates with GTM tracking direction and final position, consistent with the idea that gradient-regulated phosphorylation and/or dephosphorylation of G β contributes to gradient sensing.

***ste4^{3TA/SA}* confers defects in gradient tracking and mating efficiency**

As revealed by the biosensor, the spatiotemporal dynamics of G β^P are consistent with our hypothesis that localized phosphorylation of G β contributes to gradient sensing. If this is correct, cells unable to phosphorylate G β should show defects in gradient sensing and mating. Because pheromone is thought to stimulate the phosphorylation of G β on residue S335 in addition to

residues T318, T320, and T322 (Li et al. 1998; Ismael et al., 2016), we created a strain in which the native *STE4* locus was replaced with a *ste4*^{T318A/T320A/T322A/S335A} quadruple mutant allele (hereafter, *ste4*^{3TA/SA} for the allele and Gβ^{3TA/SA} for the protein). By two measures of the pheromone response, *ste4*^{3TA/SA} cells were indistinguishable from WT cells: the mutant cultures were as sensitive to pheromone-induced cell-cycle arrest as the control cultures (Fig. S1) and formed a similar percentage of shmooos when challenged with a sub-saturating pheromone dose (Fig. 8A), indicating no deficiency in Gβ^{3TA/SA} signaling to the nucleus. Interestingly, a significant fraction of the *ste4*^{3TA/SA} cells lysed after shmooing, consistent with a defect in cell wall remodeling at the polarized growth site (Barral et al., 2000; Huberman et al., 2014; Tartakoff, 2015). To study the spatiotemporal dynamics of Gβ^{3TA/SA} while assessing its effects on mating, we tagged *ste4*^{3TA/SA} with RFP in situ. In mating assays, *MATa* RFP-*ste4*^{3TA/SA} cells formed significantly fewer zygotes than the *MATa* RFP-*STE4* control cells (mean percentage ± SEM: 46 ± 12.5 in four independent experiments vs. 63 ± 5.8 in six independent experiments; $p < 0.001$). Although this decrease in mating efficiency could be at least partially due to shmoo lysis, it also correlated with a defect in the ability of the mutant cells to orient toward their partners (Fig. 8, B and C) and with a smaller proportion of cells that exhibited RFP-Gβ^{3TA/SA} gradient-tracking behavior (Fig. 8D). In the control cross, RFP-Gβ localized to the DS and tracked to the CS in 74% of the mating cells examined, while the remaining 26% mated at the DS (i.e., no tracking). In contrast, tracking was observed in only 46% of the mating cells expressing RFP-Gβ^{3TA/SA} ($n = 50$; $p < 0.0001$) while 26% mated at the DS, the same fraction as in the control culture; the remaining 26% showed a novel phenotype characterized by initial RFP-Gβ^{3TA/SA} localization to a CS positioned distal to the cytokinesis site rather than initial localization to the DS followed by tracking to variably positioned chemotropic sites. This

“jumping” phenotype could be produced by an as yet uncharacterized global sensing mechanism. Alternatively, it could reflect polarization at the distal bud site, which can substitute for the axial bud site (i.e., the DS) in haploid cells under certain conditions (Cullen and Sprague, 2002; Vasen et al., 2020). Together, these data strongly suggest that the phosphorylation of G β contributes to the decoding of pheromone gradients.

Discussion

Budding yeast exhibit remarkable chemosensing abilities. In mating mixtures, partners accurately locate and orient their growth towards one another despite having to do so by decoding shallow, competing, and dynamic pheromone gradients. How they accomplish this has been explained most recently by two distinct types of model. The stochastic local sampling models suppose that the Cdc42-Cdc24-Bem1 polarity complex moves from random cortical positions toward the eventual CS by a biased random walk; its mobility decreases as it moves in the direction of the pheromone source, biasing its displacement upgradient (Dyer et al., 2013; Hegemann and Peter, 2017; Hegemann et al., 2015; McClure et al., 2015). In contrast, we have proposed a deterministic model (Wang et al., 2019). Following global internalization of the receptor and G protein, the GTM is assembled at the DS. The slope of activated receptor and G protein across the GTM initially reflects the external pheromone gradient but is rapidly increased by interconnected feedback mechanisms involving the receptor, G protein, receptor kinases, MAPK, and protein trafficking systems. Consequently, the generation and maintenance of signaling species (active-unphosphorylated receptor, G α -GTP/MAPK, and G $\beta^P\gamma$) and vesicle delivery are favored upgradient, whereas the generation of non-signaling species (inactive receptor, active-phosphorylated receptor, and heterotrimeric G protein) and their removal are favored downgradient. In short, the pheromone gradient elicits asymmetric signaling and

trafficking activities across the GTM, which underlie its directed redistribution toward the gradient source. Here we sought to visualize the asymmetry of $G\beta^P\gamma$, which we propose to be important for GTM function (Fig. 9).

Development and characterization of a phospho-specific $G\beta$ biosensor

At the outset of this work, it was known that pheromone induces the release of $G\beta\gamma$ from $G\alpha$ and concomitant phosphorylation of at least two threonine residues in the $G\beta$ phosphorylation loop. To develop an in-cell reporter of $G\beta^P$, we used a phage-displayed FHA1 library designed to detect phosphothreonine-containing peptides. Affinity-selection/ELISA screening of the FHA1 library yielded four clones that specifically recognized a dually phosphorylated $G\beta$ peptide with relative binding that varied over a 5-fold range.

To explore the potential utility of the selected FHA1 variants as $G\beta^P$ biosensors, we constructed a centromeric plasmid containing the clone exhibiting the highest relative binding, fused to GFP. We chose to express the biosensor from an autonomously replicating centromeric plasmid because such plasmids are maintained at variable copy number in populations of transformed yeast and therefore allow for a range of expression levels. Although the biosensor cross-reacted with an unknown protein or proteins that localize to sites of highly polarized secretion, our data clearly indicate that it specifically binds to phosphorylated forms of $G\beta$. First, localization of the biosensor to the PM in the absence of stable and robust polarized secretion was only observed in cells expressing a phosphorylatable form of $G\beta$ when stimulated by pheromone. Second, BiFC interaction between the biosensor and $G\beta$ required phosphorylation of Thr 318 and/or Thr 322. Third, the biosensor behaved similarly to GFP- $G\beta$ in mating cells – localizing to the PM at the DS then tracking to and stabilizing at the CS – but no such localization was seen in mating cells expressing $G\beta^{3TA}$.

Surprisingly, the biosensor bound as well to $G\beta^{T320A/S335A}$ as to $G\beta$ in pheromone-treated cells. Previous genetic and labeling results led us to conclude that $G\beta^{T320A/S335A}$ could not be phosphorylated in vivo (Li et al., 1998). However, subsequent mass spec analysis indicated that pheromone induces phosphorylation of residues T318, T320 and/or T322, and S335 (Ismael et al., 2016). Indeed, additional in vitro analysis showed that the FHA1 moiety of our biosensor binds to and distinguishes single-, double-, and triple-phosphothreonine forms of the cognate peptide. This is the first example of an FHA variant that binds a triply phosphorylated peptide. Consistent with the idea that $G\beta^{T320A/S335A}$ is phosphorylated in vivo, $G\beta^{T318A/T320A/T322A}$ ($G\beta^{3TA}$) showed no pheromone-induced binding of the biosensor.

In addition to binding $G\beta^P$, the biosensor localized to the mother-daughter neck and bud tip of vegetative cells, and to the shmoo tip of pheromone-treated cells, all target sites of stable and robust polarized secretion. This is not unexpected, as FHA variants are known to exhibit cross-reactivity with peptide targets in vitro (Venegas et al., 2018). Because our goal here was to study the spatiotemporal dynamics of $G\beta^P$ on the PM of mating cells prior to morphogenesis, this cross-reactivity was not problematic, as the cross-reacting protein(s) and $G\beta^P$ do not colocalize in pre-morphogenic mating cells.

GTM positioning and mobility correlates with that of $G\beta^P\gamma$

In mating cells, the biosensor behaved like a component of the GTM: it polarized to the DS following cytokinesis, then tracked to the CS, where it stabilized prior to morphogenesis. In contrast to the total $G\beta$ and receptor reporters, however, the biosensor exhibited a much more focused distribution. Time-lapse images of doubly labeled mating cells showed that the biosensor tracks upgradient of the GTM center, narrows as it progresses, and stabilizes at the CS significantly earlier than $G\beta$ and the receptor. In fact, the concentrated patch of $G\beta^P\gamma$ appeared to

act as a highly sensitive gradient detector. This was most apparent when doubly labeled *MATa* cells were choosing between two proximal *MATα* partners. The case that $G\beta^P\gamma$ is an important element of the GTM is further supported by our finding that *ste4^{3TA/SA}*, which encodes a non-phosphorylatable form of $G\beta$, confers decreased gradient tracking, cell orientation, and mating efficiency, but does not affect pheromone-induced cell cycle arrest or shmoo emergence. These observations raise two questions: how does the phosphorylation of $G\beta$ contribute to gradient sensing, and how is the concentrated localization of $G\beta^P\gamma$ generated and maintained?

In previous studies, we have shown the following: (1) Pheromone-induced receptor phosphorylation is essential for gradient sensing, whereas receptor internalization contributes to the accuracy of alignment with the gradient but is not required for tracking (Ismael et al., 2016; Wang et al., 2019); (2) $G\beta\gamma$ inhibits receptor phosphorylation by binding to the receptor kinases, Yck1/2, and the phosphorylation of $G\beta$ increases $G\beta\gamma$ -Yck1/2 interaction (Ismael et al., 2016); (3) The full phosphorylation of $G\beta$ depends on the Fus3 MAPK, on the interaction of active $G\alpha$ with Fus3, and on Yck1/2 (Ismael et al., 2016; Li et al., 1998; Matheos et al., 2004; Metodiev et al., 2002). These findings suggest that yeast gradient sensing depends on differential phosphorylation of the receptor and that feedback mechanisms between the receptor and its G protein amplify this differential, thereby helping the cell decode shallow pheromone gradients. According to our model, the initially slight asymmetry in activated receptor across the GTM is increased by localized $G\alpha$ -Fus3 phosphorylation of $G\beta$ upgradient, which increases the lifetime of nearby signaling-competent receptors by inhibiting their phosphorylation. This scenario places $G\beta^P\gamma$ at the center of the feedback interactions that amplify the signaling gradient: it is both downstream (an output) and upstream (an enhancer) of the active-unphosphorylated receptor. We have also proposed that $G\beta\gamma$ directs secretion toward the upgradient side of the GTM via its

known interaction with Far1-Cdc24 (and hence Cdc42-Sec3), thus preferentially increasing receptor and G-protein delivery in the direction of the pheromone source (Wang et al., 2019). Like the biosensor, the peak of Sec3 also tracks ahead of the total receptor peak and narrows as it approaches the CS in mating cells (Wang et al., 2019; Fig. 6, A and C). It will be interesting to determine whether the phosphorylation of G β increases the affinity of G $\beta\gamma$ for Far1, as this would implicate G $\beta^P\gamma$ in an additional positive feedback loop that steepens the signaling gradient.

The focused localization of G $\beta^P\gamma$ is consistent with and integral to its postulated role in positioning the GTM. How is this tight distribution generated and maintained? Although our model can account for the local production of G $\beta^P\gamma$ – the G α -Fus3 and Yck1/2 activities track with the peak of active-unphosphorylated receptor – we do not know how its localization is constrained. What prevents G $\beta^P\gamma$ from broadly diffusing from its point of origin? A priori, it is likely that a G β phosphatase is involved. Localized production of G $\beta^P\gamma$ coupled with a G β phosphatase that surrounds the tracking GTM peak would be expected to result in the distribution pattern we see. Alternatively, a uniformly localized G β phosphatase with a high catalytic rate could rapidly dephosphorylate G $\beta^P\gamma$ and thereby limit its diffusion. A distinct but not mutually exclusive possibility is that the localization of G $\beta^P\gamma$ is affected by its binding partners. For example, interaction with effectors such as the Ste5 kinase-scaffold could restrict its mobility. Additional studies will be required to answer this question.

General utility of the FHA1G2 library

In this study, we have demonstrated that an FHA1 variant can serve as a phosphothreonine-specific binding moiety in vivo, an activity that can be exploited for a variety of purposes. These include biosensors, bio-inhibitors, and optogenetic tools. Biosensors must meet three

requirements to be useful in studying living cells. First, the subcellular localization of the biosensor must allow it access to the target molecule. Second, the affinity of the biosensor for its target must be high enough to report its location or activity, but not so high as to inhibit its function. Third, “false positive” signals due to cross-reactivity of the biosensor with molecules other than the target – i.e., imperfect specificity – must be distinguishable from the signal of interest.

To date, there is only one report of biosensors that have been successfully used to monitor phosphorylation of a native protein in vivo: Tiruthani et al. (2019) developed probes that specifically bound to pTyr⁹⁹² and pTyr¹¹⁴⁸ of the epidermal growth factor receptor, EGFR, without inhibiting agonist-induced receptor internalization. Although pThr is at least ten times more abundant than pTyr in the eukaryotic phosphoproteome (Venegas et al., 2016), there are thus far no examples of live-cell sensors that directly detect the phosphorylation of specific threonines in a protein of interest. Rather, FRET-based sensors have been used to monitor the activity of pThr kinases. For example, a FRET-based sensor of aurora B kinase activity relied on an FHA2 domain to bind a phosphorylated threonine in a substrate peptide; however, the pT+3 site of the target sequence had to be mutated to promote FHA2 binding (Fuller et al., 2008). Instead of modifying the sequence of interest, a more powerful and adaptable approach is to affinity-select specific binders to the target peptide from a diverse library of FHA variants. The FHA1G2 library is ideal for this purpose. It contains 2×10^9 variants and shows no cross-reactivity with phosphoserine- or phosphotyrosine-containing peptides (Pershad et al., 2012; Venegas et al., 2016). Because the FHA motif is small and soluble, it can be directed to any cellular compartment or domain for which a localization tag is available. If the utility of a selected FHA1 variant as a biosensor is limited because it binds too tightly or not tightly enough

to the protein of interest, or because it exhibits problematic cross-reactivity, its β 4-5 and β 10-11 loops can be randomly mutagenized and the resulting derivative library screened for clones with optimal properties. Manipulation of biosensor levels using a tunable promoter is another way to optimize visualization of the target protein.

Materials and Methods

Molecular and microbiological techniques. Standard methods were used for microbial culture and molecular manipulation (Ausubel et al., 1994; Guthrie and Fink, 2002; Sherman, 1986).

Yeast strain construction. All yeast strains used in this study (Table S1) were derived by transformation of the parent strain DSY129 (*MATa adel his2 leu2-3,112 trp1-1a ura3 Δ*), which is congenic with strain BF264-15D (Reed et al., 1985). Strain RGY062 was generated by transforming the centromeric plasmid RGB024 into strain DSY245. Strain RGY065 was generated by transforming the centromeric plasmid MCB26 into strain RGY062. Strains RGY068 and RGY069 were generated by transforming the centromeric plasmid RGB024 into strains DSY129 and EBY217, respectively. Strain RGY071 was generated by transforming the centromeric plasmids AIB201 and RGB027 into strain AIY279. Strain RGY072 was generated by transforming the centromeric plasmids p415-VF2 and RGB027 into strain AIY279. Strain RGY073 was generated by transforming the centromeric plasmids RGB029 and AIB201 into strain AIY279. Strain RGY074 was generated by transforming the centromeric plasmids RGB027 and RGB028 into strain AIY279. The DNA fragments for *URA3* replacement were PCR-amplified from RGB025 and RGB026 with oligomers

5'-TACTCAAAAACTGTACAGCTCAATCAGGTACACATTACGATGGCAGCACATCA
GATGGA-3' and 5'-TCCATTGATAATTACTGCTCACAGTATTTCCAATTCTGAAGCTATT

GATAACCTGGAGACC-3', in which the underlined sequences direct recombination at the 5' and 3'-ends of the *STE4* gene, respectively. Strains RGY075 and RGY076 were generated by replacing *URA3* at the *STE4* locus in RDY102 with the *ste4*^{T318A/T320A/T322A}(*ste4*^{3TA}) and *ste4*^{T318A/T320A/T322A/S335A}(*ste4*^{3TA/SA}) PCR fragments described above, respectively. The DNA fragment for *STE4* replacement was PCR-amplified from Ycplac33 with oligomers

5'-ATGGCAGCACATCAGATGGACTCGATAACGTATTCTAATAATGACAAATAGGGG

TTCCGC-3' and 5'-CTATTGATAA CCTGGAGACCATATTTTGGTTGAGTCCCATGAACA

AACATGAGAATTGGG-3', in which the underlined sequences direct recombination at the 5' and 3'-ends of the *STE4* gene, respectively. Strain RGY079 was generated by replacing *STE4* in DSY129 with the *URA3* PCR fragment described above. Strain RGY080 was generated by transforming the centromeric plasmid RGB032 into strain DSY245. Strain RGY082 was generated by replacing *URA3* at the *STE4* locus in RGY079 with the *ste4*^{3TA} PCR fragment described above. Strain RGY083 was generated by transforming the centromeric plasmid RGB024 into strain RGY082. Strain RGY084 was generated by transforming the centromeric plasmids RGB027 and AIB153 into strain AIY279. Strain RGY086 was generated by transforming the centromeric plasmid RGB024 into strain RDY114. Strain RGY087 was generated by transforming the centromeric plasmid RGB032 into strain DSY245. Strains RGY088 and RGY090 were generated by integrating PstI-cut XWB111 into strains DSY129 and RGY082, respectively. Strains RGY093 and RGY095 was generated by transforming the centromeric plasmid RGB024 into strains RGY088 and RGY090, respectively. Strain RGY098 was generated by replacing *URA3* at the *STE4* locus in RGY079 with the *ste4*^{3TA/SA} PCR fragment described above. Strain RGY099 was generated by integrating PstI-cut XWB111 into strain RGY098. Strain XWY173 was generated by transforming the centromeric plasmid

XWB149 into strain XWY065. All genomic modifications were confirmed by sequencing (UIC Research Resource Center Sequencing Core).

Plasmid construction. The plasmids used in this study are listed in Table S2. Phagemid pKP700 was obtained from the FHA1G2 library (Pershad et al., 2012). FHA3^{R70A} gene flanked by NcoI and NotI restriction sites at the 3'- and 5'-ends, respectively, was synthesized as a gBlock® gene fragment by Integrated DNA Technologies. Phagemid pKP700-FHA3^{R70A} was constructed by inserting the NcoI- and NotI-digested FHA3^{R70A} gene fragment into the phagemid pKP700. To construct RGB024, GFP was PCR-amplified from RDB143 with the oligomers 5'-ACGTACCGCGGATGGTTTCAAAAGGTGAAGAAGA TAATATG-3' and 5'-ATCAAGAGCTCTTATTTATATAAT TCATCCATACCACCAGTTG-3'. NcoI- and NotI-digested pKP700-FHA3 and the SacII- and SacI-digested GFP product were inserted into pRS316-GC/CUP1. Plasmid RGB027 was constructed by PCR amplifying the FHA3 gene from plasmid RGB024 with the oligomers 5'-TGCGGATCCATG GATTACAAGGACGACGATGACAAGC-3' and 5'-TGCTATGAATTCGGTATTTTT AAGATTTGAACAGATACGATCGAC-3'. The BamHI- and EcoRI-digested PCR fragment was inserted into p416-VF1. Plasmid RGB029 was constructed by PCR amplifying the FHA3^{R70A} gene from plasmid pKP700-FHA3^{R70A} with the oligomers 5'-TGCGGATCCATGG ATTACAAGGACGACGATGACAAGC-3' and 5'-TGCTATGAATTCGGTATTTTTTAAG ATTTGAACAGATACGATCGAC-3'. The BamHI- and EcoRI-digested FHA3^{R70A} PCR product was inserted into p416-VF1. Plasmid RGB032 was constructed by PCR amplifying the FHA3^{R70A} gene from pKP700-FHA3^{R70A} with the oligomers 5'-TGCTATGGATCCATGGATTACAAGGACGACGATGACAAGC-3' and

5'-TATGCGGCCGCGGTATTTTAAAGATTGTAACAGATACGATCGAC-3' and inserting the BamHI- and NotI-digested product into EDB53. To construct plasmid XWB111, the *STE4* promoter, the RFP gene, and the N-terminal half of *STE4* were PCR-amplified with the following pairs of oligomers: 5'-CAGTGAATTCTCCTCAAAGAGTACACTAGATCCATTC-3' and 5'-CAGTGGATCCCGTAATGTGTACCTGATTGAGCTG-3' (*STE4* promoter); 5'-ATCGGGATCCATGGTTTCAAAAGGTGAAGAAGATAATATG-3' and 5'-ATCAGTCGACTTTATATAATTCATCCATACCACCAGTTG-3' (RFP); 5'-CAGTGTCGACATGGCAGCACATCAGATGGACTC-3' and 5'-CAGTAAGCTTCTGGAATAAGCTTGCATCTTGTATC-3' (N-terminal *STE4*). The EcoRI- and BamHI-digested *STE4* promoter, BamHI- and SalI-digested RFP, and SalI- and HindIII-digested *STE4* PCR products were then inserted into plasmid YIplac128. Plasmid XWB149 was constructed by PCR-amplifying the RFP gene with the oligomers 5'-ACGTACCGCGGATGGTTTCAAAAGGTGAAGAAGATAATATG-3' and 5'-ATCAAGAGCTCTTATTTATATAATTCATCCATACCACCAGTTG-3' and inserting the SacII- and SacI-digested product into the plasmid pRS316-GC/CUP-FHA3-GFP, thereby replacing GFP with RFP. All constructions were confirmed by sequencing.

Peptides. Peptides were synthesized with > 90% purity by the UIC Research Resource Center.

All peptides were biotinylated at their N-terminus and amidated at their C-terminus.

N-YEERTP**p**TP**p**TYMAANM-C (Gβ-pT320/pT322) was used as the peptide target in the library screen; N-YEERTPTPTTYMAANM-C was used as the negative control. The following peptides were probed to test the specificity of G2-pTBD: YEER**p**TPTPTTYMAANM (Gβ-pT318), YEERTP**p**TPTTYMAANM (Gβ-pT320), YEER- TPTP**p**TYMAANM (Gβ-pT322), YEERTP**p**TP**p**TYMAANM (Gβ-pT318/pT320/pT322/).

Phage display affinity selection. The phage display affinity selection was performed using a standard protocol (Pershad et al., 2012). Three rounds of affinity selection were performed with the FHA1G2 library and the G β -pT320/pT322 peptide. All the selection steps were performed at room temperature using a KingFisher™ mL Purification System (ThermoFisher Scientific). The biotinylated peptide (3 ng/ μ l in 400 μ l) was immobilized using Dynabeads™ M-270 Streptavidin (ThermoFisher Scientific) and blocked with 2% skim milk in phosphate buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄). The phage library (2.8×10^9 phage particles) was incubated with the blocked target for 1 hour, followed by three washes with PBS containing 0.1% Tween-20 (PBST) and three washes with PBS. Phage particles bound to the target were eluted using trypsin (100 μ g/ml), and used to infect 0.8 ml of TG1 cells at mid-logarithmic phase for 40 minutes (min) at 37°C. The cells were plated after infection, scraped the next day, and phage amplified. Phage particles were precipitated using 24% PEG₈₀₀₀ and 3M NaCl, and the pellet was resuspended in 0.6 ml PBS. The second and third rounds of affinity selection were conducted in the same manner with minor exceptions. The target concentration for round two was 1.5 ng/ μ l in 400 μ l and the beads were washed six times with PBST followed by six times with PBS. After the third round of affinity selection, 96 individual clones were propagated as phage, followed by phage-ELISA (Kierny et al., 2015; Pershad et al., 2012) to identify functional clones that recognized the peptide target. Binding clones were sequenced.

Phage ELISA. The phage ELISAs were performed using a modified version of an established protocol (Pershad et al., 2012). Phage particles displaying G2-pTBD were amplified using TG1 bacterial cells harboring phagemid DNA. TG1 cells were grown to mid-log and infected with the M13K07 helper phage (New England Biolabs) at a multiplicity of infection of 20 for 1 hour at 37°C with orbital shaking at 150 rpm. Infected cells were centrifuged, pelleted, and resuspended

in 2xYT medium supplemented with 50 µg/ml Carbenicillin and 50 µg/mL Kanamycin. Phage were amplified overnight at 25°C with orbital shaking at 200 rpm. Biotinylated peptides (5 ng/µl final concentration) were immobilized on Nunc MaxiSorp flat-bottom 96 well-plates (Thermo Fisher Scientific) using 1 µg/ml Neutravidin (Thermo Fisher Scientific) and blocked for 2 hours at 25°C using 2% Skim milk in PBS. Wells were then washed with PBST. G2-pTBD-displaying phage particles were diluted in wash buffer, added to wells, and allowed to incubate for 1 hour at 25°C. Wells were washed three times with PBST. Anti-M13 antibody conjugated to Horseradish Peroxidase (HRP) (GE Healthcare) was added to detect binding phage particles. After washing the wells three times with PBST, the 2,2'-Azinobis (3-ethylbenzothiazoline-6-Sulfonic Acid) diammonium salt (Thermo Fisher Scientific) substrate supplemented with hydrogen peroxide was added to each well and the absorbance was read at 405 nm wavelength in 10 min intervals, for a total of 40 min on a POLARstar OPTIMA microtiter plate reader (BMG Labtech). Binding signals were normalized for phage titer, which was determined as follows. Plates were coated with 5ng/ul Neutravidin and biotinylated anti-Flag antibody diluted in PBS and blocked with 2% skim milk. Phage was added at dilutions of 1:10 and 1:100. Secondary antibody (anti-M13-HRP, diluted 1:5000 in PBST) and substrate were then added and the A₄₀₅ taken. All experiments were performed in triplicate and repeated at least three times.

Ste4 structure prediction. The 3D structure of Ste4 was predicted using the Phyre2 software (Kelley et al., 2015) and represented using PyMOL v.1.8.6.0 (Shrödinger LLC).

Cell imaging. In preparation for imaging, most strains were grown to mid-log phase in synthetic 2% dextrose medium and spread at a density of 14,000 cells/mm² on agarose pads made from synthetic dextrose medium. For experiments requiring the induction of *GALI-STE4* (Fig. 2, C and D), the control and experimental strains were grown to mid-log phase in synthetic

2% sucrose medium; 2% galactose was added 5 hours before spreading the cells as above on agarose pads made from synthetic 2% galactose medium. In pheromone response experiments, the pads contained 90 μ M α -factor. The shmooes shown in Fig. 2C and quantified in Fig. 2D were exposed to pheromone for 2 hours. With the exception of Fig. 6A, all DIC and fluorescent images were acquired at 5-minute intervals from 10 fields using a DeltaVision Elite Deconvolution Microscope (GE Healthcare Bio-Sciences) with a 60x oil immersion objective and a Front Illuminated sCMOS camera. To minimize phototoxicity, 5 z-sections 0.5 μ m apart were acquired around the center slice of each cell at each time point, which were taken at 5-minute intervals. Cultures were maintained at 30°C using a DeltaVision environment control chamber. The light-emitting diode intensities and exposure times used to image cells expressing the GFP-tagged reporters Figs. 2 and 3 were as follows: GFP-G β (100% maximum at 461-489 nm for 50 msec); the GFP biosensor (10% maximum at 461-489 nm for 200 msec). Time-lapse imaging of mating mixtures was performed as previously described (Wang et al., 2019) using the following light intensities and exposure times. For Fig. 5: GFP-G β (10% maximum at 461-489 nm for 200 msec); the GFP biosensor (100% maximum at 529-556 nm for 50msec). For Figs 6B and 7: RFP-G β (10% maximum at 563-588 nm for 400 msec); GFP biosensor (10% maximum at 461-489 nm for 150 msec); Ste2-GFP (10% maximum at 461-489 nm for 200 msec); RFP biosensor (32% maximum at 563-588 nm for 250 msec). The cells shown in Fig. 6A were imaged at 5-minute intervals using a Spinning disk confocal on an inverted IX81 Olympus microscope (Olympus Corporation of the Americas, Center Valley, Pa), equipped with a motorized XY stage (Prior scientific, Cambridge, UK), a Yokogawa CSU-X1 confocal head (Yokogawa Electric Corporation, Tokyo, Japan), and a sensitive iXON DU-897-BV EMCCD camera (Andor technology, Belfast, UK). Both RFP-G β and the GFP biosensor were imaged

using 20% laser at 561nm and 488nm respectively for 300 ms and camera gain of 600. With the exceptions of Figs. 4 and 6A, all representative fluorescent images were deconvolved using Huygens Essential software (Scientific Volume Imaging) in standard mode. Images in Fig. 4 were obtained by adjusting the brightness and contrast of the center slices using ImageJ. Images in Fig. 6A were deconvolved using Huygens Professional software with a maximum of 100 iterations and a signal to noise ratio of 10.

Image analysis. *MATa* cells were randomly chosen for analysis by scanning the images for cells that completed a division cycle and either formed a shmoo in response to isotropic pheromone treatment (Fig. 2D) or formed a zygote in a mating mixture (Figs. 5-7). The endpoints of the GFP-biosensor shmoo-tip crescents (Fig. 2D), and the sizes and relative positions of the reporter crescents in Figures 6C and 7C at the time points indicated in the figure legends were determined visually using the center-slice fluorescent images. The segmented line tool of ImageJ (National Institutes of Health) was used to measure the crescent sizes and the cell circumferences. PM fluorescent signal intensities were quantified by tracing the circumference of the center-slice DIC images using the segmented line tool of ImageJ; pixel values were determined from the corresponding raw fluorescent images after subtracting the background. In Fig. 2D, the biosensor fluorescent intensity for a given cell was measured as the mean PM signal intensity within the crescent or surrounding PM minus the background signal. The averaged plots of BiFC signals on the PM of shmooing cells (Fig. 4, B-D) were normalized for cell size by consolidating the circumference of each cell to 100 evenly-spaced points with the signal value for each point determined by the original curve, as described (Wang et al., 2019). Orientation angles and zygote fusion angles were determined essentially as described (Ismael et al., 2016). The direction of

orientation was determined by examining the change in position of the RFP-G β reporters over time. P values were calculated using unpaired t-tests and binomial tests (Prism GraphPad).

G β -Biosensor split Venus BiFC assay. Because constitutive overexpression of G β causes permanent cell cycle arrest, the G β -Biosensor BiFC assay was performed in a strain that enabled conditional expression of *STE7*. The assay was performed as previously described (Ismael et al., 2016) with the following changes. Cells grown in synthetic 2% galactose medium were treated with 120 μ M α -factor for 3 hours. Images were acquired with the DeltaVision Elite Deconvolution Microscope as described above using 10% maximum light intensity at 505-515 nm for 200 msec. PM fluorescent signal intensities were quantified as described above.

Quantitative mating assays. Quantitative mating assays were performed as previously described (Guthrie and Fink, 1991) with the following modifications. Mid-logarithmic phase cultures of *MATa* and *MAT α* cells were mixed in a 1:1 ratio (10^6 cells each) and filtered onto nitrocellulose filters (25mm diameter, 0.45 μ m pore size). The filters were placed on YEPD plates and incubated at 30°C for 4 hours to allow mating. Cells were then washed off filters into 1ml of deionized water and the total number of zygotes was determined using a hemocytometer. Counting was performed in duplicate and averaged for each biological replicate. Mating efficiency was determined by dividing the average number of zygotes formed by the number of *MATa* cells in the mating mixtures.

Halo assays. Pheromone-induced growth arrest was measured using the standard halo assay as previously described (Cole et al., 1990). Briefly, *MATa bar1 Δ* strains were grown to saturation in YEPD or selective SD medium. Approximately 10^5 cells were then resuspended in 7ml of 1% top agar and spread onto YEPD or selective SD plates. Upon hardening of the top agar, 4 μ l of

12ng/ul or 60ng/ul synthetic α -factor was spotted onto the center of each plate and allowed to dry. Plates were incubated at 30°C for 2 nights and halos measured with a ruler. All halo assays were performed in triplicate.

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The authors declare no competing financial interests.

A

B

N-YEERTP_{pT}P_{pT}YMAANM-C

N-YEERTP_TP_TYMAANM-C

C

Unique pTBD Clones	Number of sub-clones	β 4- β 5 (82-84)	β 10- β 11 (133-139)
pTBD (WT)		SRL	GVGVESD
A8	2	RSI	ARTHKHA
G2	1	KWV	AACPLEQ
A11	1	RWV	FRPRDTS
B6	11	RWV	FTPRDTS

D

Figure 1. Affinity selection of FHA variants against the dually phosphorylated G β -pT320/pT322 peptide. (A) Predicted three-dimensional structure of Ste4 depicted in a ribbon diagram. The β -propeller, the N-terminal alpha-helical domain, and the phosphorylation loop are colored pink, cyan, and grey, respectively. The inset shows an enlarged representation of the phosphorylation loop with the positions of Thr 318, Thr 320, and Thr 322 highlighted in red.

(B) Synthetic peptides used in affinity selection. Phosphorylated G β peptide with pThr at positions 320 and 322 (top) and unphosphorylated G β peptide (bottom). N- and C- correspond to residues 314 and 328, respectively.

(C) Amino acid sequences of the two variable loops in the clones isolated by affinity selection. The sequence of the β 4-5 and β 10-11 loops in the selected FHA1 variants (red) is compared to the corresponding sequence of the WT FHA1 (black).

(D) Relative binding and specificity for the phosphorylated G β peptide of phage clones representing the four FHA-variant classes identified by affinity selection. Binding to the biotinylated G β -pT320/pT322 and G β -T320/T322 peptides was measured by ELISA. The A₄₀₅ of the bound phage was normalized by the phage titer as described in Materials and Methods. Negative control wells contained only the blocking reagent.

Figure 2. Relationship between the localization of the biosensor and the expression, localization, and phosphorylation of Gβ in vegetative and pheromone-treated cells.

(A) Representative images of vegetative *MATa* cells at the indicated points in the cell cycle and of time-lapse images at the indicated times after pheromone treatment. The following strains are shown: i) *MATa* cells expressing in-situ tagged GFP-Gβ; ii) *MATa STE4* cells expressing the biosensor; iii) *MATa STE4* cells expressing the R70A mutant biosensor; iv) *MATa ste4*^{T320A/S335A}

cells expressing the biosensor. The localization patterns shown are typical of at least 95% of the cells examined ($n \geq 50$). (B) Representative images of vegetative *MATa ste4Δ* cells. The biosensor did not localize to the PM of the vegetative cells except at the apical bud tip and the mother-daughter neck, both target sites of polarized secretion ($n = 240$). (C) Representative images of the biosensor in *MATa* cells overexpressing *STE4* (OE-*STE4*) and in pheromone-treated cells overexpressing *STE4* (OE-*STE4* + pheromone). The dark and light green lines in the DIC image indicate the region quantified as shmoo-tip signal and surrounding PM signal, respectively. Pink arrowheads indicate the location of the shmoo-tip GFP-signals. Scale bars, 5 μ m (D) Effect of G β overexpression and pheromone treatment on the biosensor PM signal intensity. The data points show the mean fluorescent intensity (F.I.) in arbitrary units (au) of the biosensor in the polarized PM crescent (shmoo-tip signal) and around the rest of the PM (surrounding PM) in untreated and pheromone-treated cells expressing the native level or excess G β (OE-G β), as indicated. Each data point represents a cell from one of three independent experiments, indicated by shading. The horizontal lines indicate the means $\pm$ SEM. $n = 45$ for all conditions and measurements; * $p \leq 0.05$; ** $p \leq 0.0001$; ns, not significant. Mean biosensor F.I. at shmoo-tip $\pm$ SEM: Endogenous *STE4* + pheromone = 850.4 ± 115.9 ; Overexpressed (OE) *STE4* = 1215 ± 151.1 ; Overexpressed *STE4* + pheromone = 1429 ± 187.4 . Mean biosensor F.I. outside of shmoo-tip $\pm$ SEM: Endogenous *STE4* + pheromone = 441.2 ± 74.7 ; Overexpressed *STE4* = 559.7 ± 61.1 ; Overexpressed *STE4* + pheromone = 651.2 ± 71.8 .

Figure 3. Effect of phosphorylated threonine residues within the Gβ phosphorylation loop on the binding of G2-pTBD in vitro and in vivo. (A) Mono and tri-phosphothreonine Gβ peptides (residues 314-328) used in ELISAs. The phosphorylated Thr residues are shown in red. The cognate peptide was used in the affinity selection. (B) Bar graph showing the binding of Gβ-pTBD to mono- and tri-phosphothreonine variants of Gβ-pT320/pT322 normalized to its binding of the cognate peptide, measured in ELISA assays.

Figure 4. BiFC assay of the interaction between G β and the biosensor. (A) Representative images of vegetative and phormone-treated cells expressing the indicated BiFC constructs. Pink arrowheads indicate PM BiFC signals. (B-D) Average PM distribution of BiFC signals in shmooing cells. The PM signals of shmooing cells from phormone-treated cultures of the

indicated strains were quantified with ImageJ, normalized for cell size, and averaged as described in Material and Methods. The plots show the mean BiFC signal distribution $\pm$ SEM (green shadow). The x-axes represent distance along the PM; the y-axes indicate F.I. in arbitrary units (au). The tip of the shmoo was set at position 0.5 on the x-axis (dashed lines). $n \geq 20$ cells in two independent experiments for each strain. Average BiFC signal distribution in cells expressing VF1-G2-pTBD and (B) STE4-VF2; (C) $G\beta^{T320A/S335A}$ -VF2Ste2-GFP; (D) $G\beta^{3TA}$ -VF2. Scale bars, 5 μ m

Figure 5. Localization of GFP-G β and the biosensor in mating cells. (A-D) Representative time-lapse images. *MATa* cells expressing the indicated constructs were mixed with *MAT α* cells in equal proportions and imaged at 5 min intervals. The mating partners are labeled **a** and α in the DIC images. The fluorescent images show the localization of GFP-G β (A), the biosensor (B-C), or the biosensor and RFP-G β^{3TA} (D) as *MATa* cells orient toward their mating partners. Asterisks indicate polarized signals at the mother-daughter necks. Dashed, closed, and filled arrowheads indicate the peaks of the default, tracking, and stabilized signals, respectively. The plots show the distribution of the GFP-G β reporter and the biosensor on the PM at the indicated time points (10-point rolling average). The x-axes represent distance along the PM; the y-axes indicate fluorescent intensity (F.I.) in arbitrary units (au). The dashed blue, pink and red lines on the plots in A and B indicate the DS peak, the leading peak and the CS peak, respectively.

Localization of (A) in-situ tagged GFP-G β ; (B) biosensor in WT (*STE4*) cells; (C) biosensor in *ste4^{3TA}* cells. Percentage of cells showing tracking fluorescent signals: in-situ tagged GFP-G β = 83.7%; biosensor (*STE4*) = 71.4%, $p=0.06$; biosensor (*ste4^{3TA}*) = 2.3%. $p < 0.0001$; $n = 49$ for all strains from at least two independent experiments. (D) Expression and localization of G β^{3TA} and the biosensor in double-labeled cells. The RFP-G β^{3TA} and biosensor signal patterns in the two highlighted cells are typical of 30 mating *MATa* cells examined in two independent experiments. Scale bars, 5 μ m

Figure 6. Relative distribution of gradient-tracking $G\beta\gamma$ and the biosensor in WT cells.

MATa cells co-expressing RFP- $G\beta$ and the biosensor were mixed with *MAT α* cells in equal proportions and imaged at 5-minute intervals. The mating partners are labelled **a** and α in the DIC images. Fluorescent images show the localization of $G\beta$ (magenta) and the biosensor (green) during assembly, tracking, stabilization, and fusion. Asterisks indicate polarized signals at the mother-daughter necks. Dashed, closed, and filled arrowheads indicate the peaks of the default, tracking, and stabilized signals, respectively; they point to the leading peak when more than one peak is apparent. The dotted blue outlines represent *MAT α* cells that appear to be competing to mate with the indicated *MATa* cells. (A) Representative time-lapse images of a *MATa* cell orienting toward a single partner. The plots show the relative distribution of $G\beta$ (magenta) and the biosensor (green) on the PM at the indicated time points (10-point rolling average). The dashed blue, pink, and red lines mark the DS, tracking, and CS peak, respectively. The images are representative of 30 matings examined. Scale bars, 5 μ m (B) Schematic diagram of how the size and position of the biosensor crescent relative to the total $G\beta$ crescent was measured during gradient sensing. With the size of each $G\beta$ crescent (magenta arc) normalized to 100, the size and midpoint (y) of its corresponding biosensor crescent (green arc) are given by $z-x$ and $(z-x)/2 + x$, respectively. (C) Box plots showing the mean size and position of the biosensor crescents relative to the total $G\beta$ crescents during gradient sensing. The boxes show the mean downgradient ends, midpoints (horizontal line), and upgradient ends of biosensor crescents $\pm$ SEM superimposed on the $G\beta$ crescents, which have been normalized to 100 at each time point. Start, the last time point before tracking begins; 1/3 and 2/3, fraction of complete distance tracked; SS, biosensor stabilization at the CS; End, $G\beta$ stabilization at the CS. $n = 20$ from three independent experiments. Mean crescent midpoints $\pm$ SEM; Start = 42.3 ± 3.3 ; One-

third = 53.9 ± 4.0 ; Two-thirds = 62.6 ± 3.3 ; SS = 63.4 ± 3.4 ; End = 54.0 ± 2.4 ; * $p < 0.05$;

** $p < 0.005$; *** $p < 0.0005$; ns, not significant. (D) Biosensor crescent size during tracking in cells co-expressing RFP-G β and the GFP-biosensor. Mean crescent size $\pm$ SEM; n = 20.

(E) Representative time-lapse images of *MATa* cells orienting toward two potential partners of equal size. Scale bars, 5 μ m

Figure 7. Relative distribution of gradient-tracking receptor and the biosensor in WT cells.

MATa cells co-expressing Ste2-GFP and the biosensor fused to RFP were mixed with *MATα* cells in equal proportions and imaged at 5-minute intervals. The mating partners are labeled **a** and **α** in the DIC images. Fluorescent images show the localization of the Ste2 receptor (green) and the biosensor (magenta) during assembly, tracking, stabilization, and fusion. Asterisks indicate polarized signals at the mother-daughter necks. Dashed, closed, and filled arrowheads indicate the peaks of the default, tracking, and stabilized signals, respectively; they point to the leading peak when more than one peak is apparent. (A) Two representative *MATa* cells orienting toward a partner. The plots show the relative distribution of the receptor (green) and the biosensor (magenta) on the PM at the indicated time points (10-point rolling average). The dashed blue, pink, and red lines mark the DS, tracking, and CS peak, respectively. Multiple *MATα* cells are labeled around Cell 2, as competition between them may have caused the biosensor signal to split into two peaks at the 30-minute time point. The images are representative of 29 matings examined. Scale bars, 5μm. (B) Schematic diagram of how the size and position of the biosensor crescent relative to the receptor crescent was measured during gradient sensing. With the size of each receptor crescent (green arc) normalized to 100, the size and midpoint (y) of its corresponding biosensor crescent (magenta arc) are given by $z-x$ and $(z-x)/2 + x$, respectively. (C) Box plots showing the mean size and position of the biosensor crescents relative to the receptor crescents during gradient sensing. The boxes show the mean downgradient ends, midpoints (horizontal line), and upgradient ends of biosensor crescents $\pm$ SEM superimposed on the receptor crescents, which have been normalized to 100 at each time point. Start, the last time point before tracking begins; 1/3 and 2/3, fraction of complete distance tracked; SS, biosensor stabilization at the CS; End, receptor stabilization at the CS. n = 20 from

three independent experiments. Mean crescent midpoints $\pm$ SEM; Start = 51.1 ± 2.4 ; One-third = 71.0 ± 3.4 ; Two-thirds = 73.2 ± 2.7 ; SS = 72.2 ± 2.5 ; End = 51.87 ± 1.3 ; *** $p < 0.0001$; ns, not significant. (D) Biosensor crescent size during tracking in cells co-expressing Ste2-GFP and RFP-Biosensor. Mean crescent size $\pm$ SEM; $n = 20$.

Figure 8. Effect of *ste4*^{3TA/SA} on shmooing, orientation toward mating partners, and gradient tracking

(A) *MATa ste4*^{3TA/SA} cells shmoo with normal efficiency but subsequently show a defect in viability. *MATa* WT and *ste4*^{3TA/SA} cells were exposed to a sub-saturating dose of pheromone on agarose pads and scored for shmooing after two hours. For the proportion of cells that shmooed

(80% vs. 67%), $p = 0.31$; for the proportion of shmooed cells that lysed (1% vs. 10%), $p < 0.0001$; $n = 100$ in two independent experiments. (B-D) Effect of *ste4^{3TA/SA}* on cell orientation and gradient tracking. *MATa RFP-STE4* and *MATa RFP-ste4^{3TA/SA}* cells were mixed with *MATa* cells in equal proportions and imaged at 5-minute intervals. Each data point represents a *MATa* cell that completed cytokinesis and formed a zygote. For each measurement, $n = 50$ in two independent experiments, indicated by shading in panels B and C. The horizontal lines indicate the means $\pm$ SEM. (B) *MATa RFP-ste4^{3TA/SA}* cells are more likely than *MATa RFP-STE4* cells to mate with partners close to their default site. Orientation angles (δ) were measured as illustrated in the cartoon at the prezygote stage using DIC images. The direction of orientation was determined by following the RFP-G β reporters. Fusion within 45° of the cytokinesis site (red bar) was presumed to be at the DS, demarcated by the blue lines. Mean orientation angle $\pm$ SEM in degrees = 105.3 ± 10.4 for RFP-G β cells and 88.33 ± 6.9 for RFP-G $\beta^{3TA/SA}$ cells. * $p < 0.05$. (C) *MATa RFP-ste4^{3TA/SA}* cells are more likely than *MATa RFP-STE4* cells to form angled zygotes. Fusion angles (γ) were measured as illustrated in the cartoon upon zygote formation using DIC images. Mean fusion angle $\pm$ SEM in degrees = 7.0 ± 1.3 for RFP-G β cells and 12.5 ± 1.7 for RFP-G $\beta^{3TA/SA}$ cells. Higher fusion angles correlate with compromised gradient sensing (Ismael et al., 2016). ** $p < 0.001$. (D) *MATa RFP-ste4^{3TA/SA}* cells are less likely than *MATa RFP-STE4* cells to exhibit gradient tracking behavior. *MATa* cells that completed cytokinesis and formed zygotes were scored as having mated at the default polarity site (DS mated), tracked to a chemotropic site (Trackers), or localized directly to the fusion site (Jumpers) based on the spatiotemporal dynamics of the RFP-G β reporters in time-lapse fluorescent images. *** $p < 0.0001$ for the percentage of trackers.

Figure 9. Role of $G\beta^P\gamma$ in local gradient sensing. In preparation for mating, the gradient tracking machine (GTM) is assembled (i) at the default polarity site (DS, blue) following cytokinesis, according to the model we proposed in Wang et al. (2019). During tracking (ii), free $G\beta^P\gamma$ concentrates on the upgradient side of the GTM (green), where we postulate that it targets vesicle delivery via its interaction with Far1-Cdc24 (Nern and Arkowitz, 1999; Pruyne and Bretscher, 2000) and/or Rho1 (Bar et al., 2003), while inhibiting phosphorylation and internalization of activated receptors via its interaction with Yck1/2 (Ismael et al., 2016). During stabilization (iii), $G\beta^P\gamma$ concentrates vesicle delivery and receptor protection at the center of the incipient chemotropic site (CS, red), thereby directing chemotropic growth toward the mating partner.

Figure S1. Effect of the biosensor, *ste4*^{3TA}, and *ste4*^{3TA/SA} on pheromone-induced cell cycle arrest. Standard halo assays were performed as described in Materials and Methods. Mean halo sizes $\pm$ SEM for the indicated strains at the indicated pheromone concentrations are shown.

*p < 0.05; **p < 0.0001; ns, not significant. n = 6 from two independent experiments for each strain.

Figure S2. Nuclear localization of the biosensor in vegetative and pheromone-treated cells.

Representative images of vegetative and pheromone-treated *MATa* cells expressing the biosensor. Vegetative or pheromone-treated cells were stained with DAPI at 10ug/ml and imaged. Arrowheads indicate the nuclear biosensor signal as seen in the DAPI-stained and merged images.

Figure S3. Effect of the *ste4*^{3TA} mutations on RFP-Gβ expression levels in vegetative and mating cells. The data points show the mean PM fluorescent intensity (F.I.) in arbitrary units (au) of RFP-Gβ and RFP-Gβ^{3TA} in vegetative and mating cells, as indicated. Each data point represents a cell from one of two independent experiments, indicated by shading. The horizontal lines indicate the means ± SEM. n = 40 and n = 20 for vegetative and mating cells, respectively; ****p < 0.0001; ns, not significant. Mean PM F.I. ± SEM for vegetative cells: 70 ± 2.2 (RFP-Gβ) and 60 ± 2.0 (RFP-Gβ^{3TA}). Mean PM F.I. ± SEM for mating cells: 98.2 ± 6.3 (RFP-Gβ, DS) and 53.7 ± 1.8 (RFP-Gβ^{3TA}, DS); 74.6 ± 4.7 (RFP-Gβ, tracking) and 45.2 ± 1.9 (RFP-Gβ^{3TA}, tracking).

Figure S4. Representative images of *MATa* cells choosing between two potential partners of different size. The plots show the relative distribution of Gβ (magenta) and the biosensor (green) on the PM at the indicated time points (10-point rolling average). The dashed blue, pink, and red lines mark the DS, tracking, and CS peak, respectively. Scale bars, 5μm

Supplementary Materials

Table S1: Yeast strains

Strain	Background	Genotype	Source
DSY129	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ BAR1</i>	Stone lab
DSY245	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ</i>	Stone lab
DSY246	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ</i>	Stone lab
EBY217	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste4Δ::KanMX4</i>	Stone lab
RDY102	BF264-15D	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste4Δ::URA3</i>	Stone lab
RDY114	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ STE4::ste4^{T320A/S335A}</i>	(DeFlorio et al., 2013)
RDY126	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ GFP-STE4</i>	(DeFlorio et al., 2013)
AIY272	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste7Δ::KanMX4 YCPlac22/GAL-STE7 pRS416/ADHI-VF1 pRS415/ADHI-VF2</i>	Stone lab
AIY275	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste7Δ::KanMX4 YCPlac22/GAL-STE7 pRS416/ADHI-VF1 pRS415/ADHI-STE4-VF2</i>	(Ismael et al., 2016)
AIY279	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste7Δ::KanMX4 YCPlac22/GAL-STE7</i>	Stone lab
XWY096	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ BAR1 GFP-STE4</i>	(Wang et al., 2019)
RGY062	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ pRS316-CG/CUP1-FHA3-GFP</i>	This study
RGY065	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ Ycplac111-Gal-STE4 pRS316-CG/CUP1-FHA3-GFP</i>	This study
RGY068	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ BAR1 pRS316-CG/CUP1-FHA3-GFP</i>	This study
RGY069	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste4Δ::KanMX4 pRS316-CG/CUP1-FHA3-GFP</i>	This study
RGY071	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste7Δ::KanMX4 YCPlac22/GAL-STE7 pRS416/ADHI-VF1-FHA3 pRS415/ADHI-STE4-VF2</i>	This study
RGY072	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste7Δ::KanMX4 YCPlac22/GAL-STE7 pRS416/ADHI-VF1-FHA3 pRS415/ADHI-VF2</i>	This study
RGY073	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste7Δ::KanMX4 YCPlac22/GAL-STE7 pRS416/ADHI-VF1-FHA3^{R70A} pRS415/ADHI-STE4-VF2</i>	This study
RGY074	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste7Δ::KanMX4 YCPlac22/GAL-STE7 pRS416/ADHI-VF1-FHA3 pRS415/ADHI-ste4^{T318A/T320A/T322A}-VF2</i>	This study
RGY075	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste4::URA3::ste4^{T318A/T320A/T322A}</i>	This study
RGY076	BF264-15D	<i>MATa ade1 his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste4::URA3::ste4^{T318A/T320A/T322A/S335A}</i>	This study

RGY079	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 ste4Δ::URA3</i>	This study
RGY080	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ bar1Δ pRS316-CG/CUP1-FHA3^{R70A}-GFP</i>	This study
RGY082	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 ste4Δ::URA3::ste4^{T318A/T320A/T322A}</i>	This study
RGY083	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 ste4::URA3::ste4^{T318A/T320A/T322A} pRS316-CG/CUP1-FHA3-GFP</i>	This study
RGY084	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ bar1Δ ste7Δ::KanMX4 YCPlac22/GAL-STE7 pRS416/ADH1-VF1-FHA3 pRS415/ADH1-ste4^{T320A/S335A}-VF2</i>	This study
RGY086	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ bar1Δ STE4::ste4^{T320A/S335A} pRS316-CG/CUP1-FHA3-GFP</i>	This study
RGY087	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ bar1Δ pRS316CG-CUP1-FHA3^{R70A}-GFP</i>	This study
RGY088	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 RFP-STE4</i>	This study
RGY090	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 RFP-ste4^{T318A/T320A/T322A}</i>	This study
RGY093	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 RFP-STE4, pRS316-CG/CUP1-FHA3-GFP</i>	This study
RGY095	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 RFP-ste4^{T318A/T320A/T322A}, pRS316-CG/CUP1-FHA3-GFP</i>	This study
RGY098	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 ste4Δ::URA3::ste4^{T318A/T320A/T322A/S335A}</i>	This study
RGY099	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 RFP-ste4^{T318A/T320A/T322A/S335A}</i>	This study
XWY173	BF264-15D	<i>MATa adel his2 leu2-3,112 trp1-1a ura3Δ BAR1 STE2-GFP, pRS316-CG/CUP1-FHA3-RFP</i>	This study

Table S2: Plasmids

Plasmid #	Plasmid construction	Marker/type	Source
p415-VF2	pRS415/ADH1-Venus Fragment 2	LEU2/2μm	(Paquin et al., 2007)
p416-VF1	pRS416/ADH1-Venus Fragment 1	URA3/2μm	(Paquin et al., 2007)
AIB201	pRS415/ADH1-Ste4-VF2	LEU2/2μm	(Ismael et al., 2016)
AIB153	pRS415/ADH1-Ste4 ^{T320A/S335A} -VF2	LEU2/2μm	Stone lab
MCB26	Ycplac111/ GAL1-STE4	LEU2/2μm	(Cismowski et al., 2001)
EDB52	pYES2/pGAL1-STE11ΔN	URA3/2μm	Stone lab
pKP700-FHA3	pKP700/ FHA3		This study
RGB024	pRS316-CG/CUP1-FHA3-GFP	URA3/CEN	This study
pKP700-FHA3^{R70A}	pKP700/ FHA3 ^{R70A}		This study
RGB025	Ycplac111/PSste4-5XHA-STE4 ^{T318A/T320A/T322A}	LEU2/ CEN	This study
RGB026	Ycplac128/PSste4-5XHA-STE4 ^{T318AT320A/T322A/S335A}	LEU2/INT	This study
RGB027	pRS416/ADH1-VF1-FHA3	URA3/2μm	This study
RGB028	pRS415/ADH1-STE4 ^{T318A/T320A/T322A} -VF2	LEU2/2μm	This study
RGB029	pRS416/ADH1-VF1-FHA3 ^{R70A}	URA3/2μm	This study

RGB032	pRS316CG-CUP1-FHA3 ^{R70A} -GFP	URA3/CEN	This study
XWB111	YIplac128/ PSte4-RFP-STE4 ¹⁻²⁰⁰	LEU2/INT	This study
XWB149	pRS316-CG/CUP1-FHA3-RFP	URA3/CEN	This study

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