Pheromone Gradient Tracking Mechanisms During Yeast Mating

by

Allison Wolff McClure

Department of Pharmacology and Cancer Biology
Duke University

Date:_______________________

Approved:

___________________________
Daniel Lew, Supervisor

___________________________
Marc Caron

___________________________
Timothy Elston

___________________________
Steve Haase

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Thomas Petes

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Department of Pharmacology and Cancer Biology
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ABSTRACT

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Abstract

Many cell types are remarkably adept at tracking chemical gradients, but they use different mechanisms in order to properly migrate or grow up-gradient. Bacteria use a temporal sensing mechanism to determine if they are swimming up-gradient. In contrast, eukaryotes are thought to use spatial sensing mechanisms where they compare the chemical concentration on one side of the cell to the other. In the present study, we utilized budding yeast (Saccharomyces cerevisiae) mating as a model for gradient tracking. Yeast cells are thought to use a spatial gradient tracking mechanism to grow up the pheromone gradient created by their mating partners. However, yeast cells polarize their receptors towards the direction of growth thereby reducing the distance that they can use to compare pheromone concentrations.

Yeast cells grow towards their mating partners by establishing a polarity patch that concentrates the master regulatory GTPase Cdc42 and its associated polarity factors on the membrane. The Cdc42 polarity patch orients actin cables so vesicles trafficking along these cables fuse at the polarity patch. Therefore, the location of the polarity patch determines the direction of growth. During mating, the pheromone gradient is thought to bias the polarity patch to the up-gradient side of the cell, but especially in shallow gradients, sometimes yeast cells initially establish the polarity patch on the wrong side of the cell. Work from our lab has found that the polarity patch wanders along the cell cortex during pheromone gradient tracking, and suggests that wandering behavior could serve as a mechanism of reorientation for cells whose polarity patch is misaligned with the gradient.

In order for yeast cells to properly track the pheromone gradient, their polarity patch must spend more time on the up-gradient side of the cell. How does the pheromone gradient
bias wandering of the polarity patch to achieve this? We suggest that by polarizing their receptors and G proteins, yeast cells create a sensitized zone of the plasma membrane that can locally influence wandering of the polarity patch. As the polarity patch wanders along the cell cortex, so too does this zone of polarized receptors. If the patch wanders to a side of the cell with higher pheromone concentration, then more active receptors near the polarity patch could slow its wandering and allow more growth to occur in that direction.
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Acknowledgements

I would like to thank Tim Elston and Maria Minakova: our collaboration has been incredibly valuable. And, the computational modeling and simulations presented here were performed by Maria.

I would like to thank members of the Lew lab, past and present, for providing a wonderful environment to learn and do science. I thank Denise Ribar for always making the lab run smoothly, and Trevin Zyla for constructing yeast strains.

I am so grateful to Jayme Dyer for not only supporting me through my transition to the lab and teaching me everything about yeast, but also for being an amazing mentor and friend.

I would like to especially thank Danny for taking a risk on me in my fourth year. I am so thankful that he helped me step out of my comfort zone and pushed me to develop my critical thinking, presenting, writing, and mentoring skills. He has taught me how to do good science, how to be a scientist, and how to love science again.

I would like to thank Debbie Winter, Meghan Kapur, Julie Neubauer, Dave Rogers, Marcela Kokes, and many other friends and colleagues at Duke for countless lunches, cups of coffee, and conversations throughout my graduate school career.

I am so thankful for my parents, parents-in-law, grandparents, and family for their love and support especially when making tough decisions. I also deeply thank David McClure. I would probably still be coding without his help with image analysis. But more importantly, his endless patience, wisdom, encouragement, and love have been essential for me to be successful in my career.
1. Introduction

Many cell types track chemical gradients for a wide range of biological processes. For example, bacteria use gradient tracking to find food, sperm to find the egg, and neutrophils to chase bacteria (Alvarez et al., 2014; Rappel and Loomis, 2009; Sourjik and Wingreen, 2012; Swaney et al., 2010). These cells are all exhibiting chemotaxis, or the ability to swim or migrate towards a chemical cue. Other gradient tracking cells, such as the budding yeast, _Saccharomyces cerevisiae_, do not migrate and instead grow towards a chemical cue in a process called chemotropism (Arkowitz, 2009).

In order to properly align their movement or growth up-gradient, cells must sense differences in the chemical concentration around them. Two broad categories of gradient sensing have been described: temporal and spatial (Hoeller et al., 2014). In temporal sensing, a moving cell compares the concentration it detects now to the concentration it detected a time point ago to determine if it is moving up-gradient. In spatial sensing, the cell compares the concentration it detects on one side of the cell to the other to determine which direction is up-gradient.

1.1 Temporal gradient tracking: bacterial chemotaxis

_Escherichia coli_ are exquisitely adept at tracking nutrient gradients of aspartate and serine, and they have served as a primary model organism for understanding temporal gradient tracking mechanisms (Sourjik, 2004). Theoretical studies have suggested that _E. coli_ and other small and fast swimming bacteria could not use spatial gradient tracking mechanisms for two reasons. First, their movement would generate an apparent spatial gradient even when there is no gradient (Berg and Purcell, 1977). In addition, bacteria are very small (only about 2 μm in
length), so it would be extremely difficult, especially in shallow gradients that bacteria can readily track, for these cells to sense the difference in concentration across such a short distance (Macnab and Koshland, 1972). Instead, *E. coli* determine if they are moving up-gradient by comparing the chemoattractant concentration they sense now to the concentration they sensed approximately 1 second ago (Berg and Purcell, 1977). This temporal mechanism allows bacteria to track gradients over a wide range of mean concentration since they are sensing the difference in concentration and not the absolute concentration at any one moment (Vladimirov and Sourjik, 2009).

Bacteria alternate between two movement states: a “run” where the cell swims in one direction and a “tumble” where the cell stops swimming forward and changes direction (reviewed in Vladimirov and Sourjik, 2009)). Without a gradient, when cells are exposed to uniform serine or aspartate concentrations, the cells alternate between about 1 second-long runs and tumbles that reorient the cells to a random direction. This random walk process is then biased when cells are tracking a gradient. If the cells are moving up-gradient and sensing increases in concentration between time points, then the cells will run longer in that direction. However, if the cells are moving down-gradient, they will tumble much sooner and reorient. Thus, even though the cells move in random directions, their net movement over time will be up-gradient (Figure 1.1). A key to this process is the temporal gradient sensing by the cells. How do the cells sense they are moving up-gradient?
When bacteria are moving up-gradient, they swim in that direction for awhile before tumbling and reorienting to a random direction. If bacteria are moving down-gradient, they will not swim for long before tumbling. Together, this results in net movement up-gradient.

*E. coli* cells sense aspartate through the Tar receptor and serine through the Tsr receptor (reviewed in (Wadhams and Armitage, 2004)). In their active state, these receptors induce the CheA kinase to trans-autophosphorylate for activation. CheA then phosphorylates CheY that can bind a flagellar motor switch protein to induce tumbling (Figure 1.2). In the inactive state, the receptors do not activate CheA kinase, so the pathway no longer induces tumbling behavior. Instead, the flagella continue to rotate counter-clockwise to propel the bacteria forward.

In response to uniform chemoattractant conditions, ligand-binding of the receptor inhibits receptor activity resulting in longer runs (reviewed in (Vladimirov and Sourjik, 2009)). However, the cells will eventually adapt to their environment and produce consistent runs and tumbles, no matter the chemoattractant concentration. This adaptation is achieved through receptor methylation, which activates the receptor to produce a tumble (Borkovich et al., 1992). Receptors are methylated by the methyltransferase, CheR, and demethylated by the methylesterase, CheB. While CheR is constitutively active, CheB is activated through
phosphorylation by CheA (Figure 1.2). Therefore CheB forms a negative feedback loop: when the receptor is active, CheB demethylates the receptor favoring the inactive state. But when the receptor binds ligand and is inactive, CheR methylation of the receptor is unopposed, returning the receptor to the active state.

**Figure 1.2 Bacterial chemotaxis signaling pathway**

In their inactive state, the Tar or Tsr receptors activate CheA phosphorylation of CheY to bind to flagellar motor proteins and induce tumbling. When bound to ligand, the receptor is inactive allowing the flagella to continue propelling the bacteria forward. Constitutive CheR methylation of the receptor promotes its activity, but the CheA pathway also activates CheB demethylation of the receptor leading to inactivation of the receptor. Together, methylation provides a negative feedback and adaptation mechanism: after ligand-binding induced inactivation of the CheA pathway, receptor methylation accumulates and promotes its activation, so the pathway returns to the prestimulus state.

Adaptation is crucial for temporal gradient tracking. After a uniform stimulus, ligand binding of the receptor inhibits CheA activity until receptor methylation returns the receptor to the active state. But, if a cell is moving up-gradient, then the constant increase in
chemoattractant concentration will lead to receptor inhibition that outpaces receptor methylation. Together, this explains the longer runs exhibited by cells moving up-gradient.

While the main mechanisms of temporal gradient tracking by *E. coli* have been elucidated, many questions still remain. An active area of current research is the regulation and function of receptor clustering (reviewed in (Sourjik, 2004)). Receptor dimers have been considered the functional unit of signaling to promote CheA trans-autophosphorylation. But receptors exist in higher order clusters and structures mixed with other receptors. While receptor clustering is important for amplification of the CheA pathway (Sourjik and Berg, 2004), the mechanisms of clustering and of amplification remain unknown. Another remaining and controversial question is how receptor methylation promotes receptor activity. Many mechanisms have been suggested including receptor methylation reducing ligand affinity (Li and Weis, 2000), stabilizing receptor clusters (Levit et al., 1998; Shrout et al., 2003), and promoting amplification of the CheA pathway (Levit and Stock, 2002). More studies are needed to distinguish or reconcile these mechanisms.

1.2 **Spatial gradient tracking**

1.2.1 **Spatial sensing model systems**

In contrast to temporal sensing by bacteria, much less is understood about gradient tracking using spatial sensing. Many hypotheses have been suggested primarily utilizing neutrophils and *Dictyostelium discoideum* as model systems, but in order to truly decipher the mechanisms of spatial sensing and gradient tracking, protein and network connections will need to be uncovered.
Neutrophils have long been studied for their ability to track gradients of the chemicals released by bacteria or chemicals produced by other immune cells in order to recruit neutrophils to the site of infection (reviewed in (Rabiet et al., 2007)). Most studies have used either N-Formylmethionine leucyl-phenylalanine (fMLP) or complement component 5a (C5a) as a chemoattractant for neutrophils. Unstimulated neutrophils are round and do not extend membrane protrusions (reviewed in (Zigmond, 1978)). But once stimulated, neutrophils rapidly polarize to create a migrating cell with a defined “front” and “back”. The front is characterized by broad membrane extensions called lamellipodia or pseudopodia that are actin-rich. The back of the cell becomes a myosin-rich structure called a uropod that retracts as the cell migrates.

*Dictyostelium discoideum* (now on referred to as Dicty) is a social amoeba and transitions between unicellular vegetative growth and multicellular development (reviewed in (Loomis, 2014)). When starved, Dicty cells secrete the chemical cue cAMP, and cells migrate up the cAMP gradient towards one another forming an aggregate. This aggregate then specializes into a slug-like structure that settles in a dark place to further develop into a fruiting body, which secretes spores, and the cycle can begin again. Some gradient tracking studies use vegetative Dicty cells that migrate up folic acid gradients made by their bacterial food source. However, most studies utilize starving Dicty cells that migrate up cAMP gradients. Unlike neutrophils, Dicty cells polarize and migrate even in the absence of chemical cues. They migrate in random directions, and once exposed to a cAMP gradient, they bias their migratory direction up-gradient. Dicty cells extend actin-rich pseudopodia at the front that can vary in length and diameter. The back of the cell is myosin-rich like in neutrophils, but it does not specialize into a uropod.
1.2.2 Direct gradient sensing and amplification in neutrophil and Dicty cell chemotaxis

Both neutrophils and Dicty cells sense chemical cues through G-protein coupled receptors (GPCRs) (Figure 1.3). Prior to ligand binding, these seven transmembrane domain receptors are associated with a heterotrimeric G protein (reviewed in (McCudden et al., 2005)). Ligand binding triggers the receptor’s activity as a guanine nucleotide exchange factor (GEF): it catalyzes GDP/GTP exchange on the Gα subunit of the G protein. Gα-GTP then releases the other subunits as a free heterodimer, Gβγ. In GPCR pathways, both Gα-GTP and free Gβγ can bind effectors and stimulate downstream signaling. However, in neutrophil and Dicty cell chemotaxis, signaling primarily operates through free Gβγ, which activates a variety of pathways including Rho family GTPases to ultimately stimulate actin polymerization for membrane extensions at the front of the cell (Bourne, 1997; Peracino et al., 1998). The molecular details of how free Gβγ leads to actin polymerization in both neutrophils and Dicty cells remains elusive likely due to redundancies and complex protein networks (Yang et al., 2012).

![Figure 1.3 G-protein coupled receptor signaling mediating chemotaxis.](image_url)

After ligand binding, the GPCR catalyzes the exchange for GTP on its associated Gα protein. This frees Gβγ to then signal through many potential pathways that induce actin polymerization to generate membrane protrusions.
Both neutrophils and Dicty cells localize their receptors uniformly on their cell surface (Servant et al., 1999; Wang et al., 1988; Xiao et al., 1997). Dicty cells also localize their G proteins uniformly (Jin et al., 2000). Upon ligand binding, the $G_{\alpha}$ subunit dissociates from the $G_{\beta\gamma}$ dimer, and the dissociation occurs more on the up-gradient side of the cell as a reflection of receptor occupancy (Janetopoulos et al., 2001). If cells are exposed to uniform chemoattractant, then receptors are activated uniformly and produce free $G_{\beta\gamma}$ all over.

In attempts to understand how neutrophils and Dicty cells translate the external chemical gradient to an internal one, many studies have used GFP tagged plexstrin-homology (PH) domains that bind to phosphatidylinositol (3,4,5)-triphosphate ($\text{PIP}_3$) (Janetopoulos et al., 2004; Meili et al., 1999; Parent et al., 1998; Servant et al., 2000). $\text{PIP}_3$ is generated by the phosphatidylinositol 3-kinase (PI3K) and hydrolyzed by phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN), and is considered an early signaling protein downstream of free $G_{\beta\gamma}$. In cells exposed to chemoattractant gradients, the PH-GFP probes showed a steeper internal gradient of $\text{PIP}_3$, suggesting the presence of an amplification mechanism downstream of free $G_{\beta\gamma}$ but upstream of PI3K. The mechanisms of amplification leading to the internal $\text{PIP}_3$ gradient remain unclear; though, they likely include positive feedback loops. Several positive feedback loops amongst small GTPases and actin regulators have been proposed (Benard et al., 1999; Inoue and Meyer, 2008; Niggli, 2000; Weiner et al., 2002), and theoretical studies have shown that positive feedback loops could produce an amplification effect (Meinhardt, 1999; Postma and Van Haastert, 2001). Further complicating matters, the amplification mechanisms likely differ between Dicty cells and neutrophils. In Dicty cells, the $\text{PIP}_3$ gradient is independent of actin as seen in cells treated with latrunculin to depolymerize actin (Meili et al., 1999; Parent et
al., 1998), but the PIP\textsubscript{3} gradient is dependent on actin in neutrophils (Houk et al., 2012; Inoue and Meyer, 2008; Wang et al., 2002).

### 1.2.3 Adaptation and the local-excitation global-inhibition model for spatial sensing

When Dicty cells are exposed to a gradient of cAMP, they create an internal gradient of PIP\textsubscript{3} (discussed in previous section). However, if Dicty cells are exposed to a uniform concentration of cAMP, they localize Ras-GTP and PIP\textsubscript{3} uniformly on the membrane due to the activation of G protein signaling all over the membrane (Parent et al., 1998; Takeda et al., 2012). This response is only transient, and the Dicty cells return to their prestimulus state with no PIP\textsubscript{3} on the membrane. These results suggest that the Dicty spatial sensing mechanism contains adaptation: a stimulus is matched by a delayed negative regulation that returns the system to basal levels. It is less clear whether neutrophils display adaptation. In some studies where the uniform stimulus is a big change of concentration, neutrophils will initially uniformly recruit PH-GFP similar to Dicty cells (Servant et al., 2000). But following this initial burst when the neutrophils are migrating in random directions, about 50\% of cells showed PH-GFP at the leading edge of the pseudopod.

How does a Dicty cell both create a stable internal gradient in response to an external gradient and transiently respond to a uniform stimulus? One hypothesis that could reconcile these findings is the local-excitation global-inhibition (LEGI) model (figure 1.4)(reviewed in Iglesias and Devreotes, 2008)). In this model, local G protein activation leads to local signal activation (here, PIP\textsubscript{3}), but G protein activation also leads to a delayed global inhibitor that integrates the entire cell’s G protein activation and inhibits the PIP\textsubscript{3} signal. In a cell exposed to uniform concentration, the local activation is matched by the global inhibitor. A cell in a
gradient, however, will have more activation on the up-gradient side of the cell than the global inhibitor that matches the mean G protein activation. In contrast, the inhibitor will exceed the local activation on the down-gradient side of the cell.

![Diagram showing local receptor activation, inhibition based on total receptor activity, and net change over time for uniform and gradient conditions.]

**Figure 1.4 The local-excitation global-inhibition (LEGI) model**

The local excitation, global inhibition model accounts for both adaptation in uniform stimulation as well as sustained polarity during gradient stimulation.

A distinct advantage of the LEGI model is that it can account for the ability of Dicty cells to track gradients over a wide range of mean concentrations (Mato et al., 1975; Song et al., 2006). Because the amount of activation at the front will be reduced by the global inhibition, the cell effectively senses the difference in concentration between the up-gradient side and the mean concentration rather than the absolute concentrations.

While LEGI provides a potential explanation for how Dicty cells translate the external gradient to an internal gradient, it does not account for signal amplification. Therefore, the LEGI model has been combined with positive and negative feedback networks to produce both behaviors seen in cells (Huang et al., 2013; Xiong et al., 2010). However, more mechanistic...
protein interactions (including the global inhibitor of the LEGI model) must be uncovered in these chemotaxis systems in order to validate these models.

1.2.4 Cell polarization biases gradient sensing and signaling

When neutrophils or Dicty cells are migrating up chemical gradients, they display a dramatic polarized morphology. If the cells change direction, either in response to initially choosing the wrong direction or in response to a changing gradient, they will gradually turn the existing front instead of depolarizing and creating a new front in the new direction (Figure 1.5) (Zigmond, 1978).

Artificially depolarized Dicty cells can still sense and amplify the external chemical gradient to an internal PIP$_3$ gradient (Janetopoulos et al., 2004; Parent et al., 1998), demonstrating that polarity is not required for spatial sensing. However, if polarized Dicty cells are exposed to a change in gradient direction, they no longer show PIP$_3$ localization in line with the external gradient. Instead, PIP$_3$ localizes to the up-gradient side of the cell front (Figure 1.5) (Parent et al., 1998). This shows that cell polarity can bias the spatial sensing pathway.
Figure 1.5 Polarized cells turn their existing front rather than creating a new front

In response to a gradient change, a non-polarized cell (i.e. latrunculin-treated cells) will create a new “front” towards the new up-gradient direction. A polarized cell will instead turn its existing front to eventually orient up-gradient.

In addition, polarized cells display different sensitivities to the chemoattractant: the back of a migrating cell is less sensitive than the front (Ueda et al., 2001; Zigmond et al., 1981). This is surprising given that the receptor and G protein are uniformly localized on the plasma membrane. The back of the cell is characterized by PTEN, myosin, and Rho GTPase signaling, and some have suggested that these proteins inhibit the signaling pathways found at the front (Xu et al., 2003). It is possible that the back is therefore modified in a manner that is unfriendly to signaling: any signal would be rapidly quelled.

Alternatively, the morphology of the membrane at the front may account for some of the sensitivity discrepancy between the front and the back of a migrating cell. When cells are responding to chemoattractant, they display membrane “ruffling” due to flexible membrane extensions that will eventually lead to pseudopods (Zigmond, 1978). This ruffling behavior occurs at the front of a migrating cell, which means there will be more membrane at the front.
Therefore, even with a completely uniform concentration of receptor in the plasma membrane, simply having more membrane at the front will mean there are more receptors at the front (Onsum et al., 2006; Servant et al., 1999; Xiao et al., 1997). This is consistent with observations of a slight increase of receptor or Gβ fluorescence (using GFP fusions) at the cell front (Jin et al., 2000; Servant et al., 1999).

### 1.2.5 Properly migrating up-gradient with spatial sensing

Because of their polarized morphology, migrating cells only make turns rather than depolarizing and creating a new front in the new direction. But, how does a cell make sure it turns in the right direction? Two main ideas have emerged about how cells using spatial sensing to properly move up-gradient.

One proposed model suggests that local receptor activation and signaling at the leading edge of the cell results in small local membrane extensions that produce small turns in the overall direction or "compass" of the cell (Figure 1.6A) (Arrieumerlou and Meyer, 2005). If a cell is in a uniform concentration of the chemical cue, then the local membrane extensions will randomly occur on either side of the current cell front, leading to random direction changes. However, if a cell is in a gradient but not properly oriented up-gradient, then most of the local membrane extensions will occur on the up-gradient side of the leading edge resulting in a correction of the compass direction. This model is consistent with the finding of small asymmetries in downstream signaling within the front of a migrating cell (Parent et al., 1998).

An alternative mechanism has been proposed that focuses on pseudopod splitting (Figure 1.6B) (Andrew and Insall, 2007). Rather than small changes of the existing cell front, this model suggests that pseudopods split into two pseudopods in a chemoattractant-independent
manner. Whichever resulting pseudopod is more aligned with the gradient, will receive more signal and survive whereas the other pseudopod will retract. One attractive advantage of this model is that the two pseudopods could provide a wider comparison of receptor occupancies compared to the two sides of a leading edge. Therefore, this mechanism could potentially yield more accurate direction changes.

Figure 1.6 Models for changing direction using spatial sensing

(A) The first model involves small protrusions at the cell front on the side closer to the up-gradient direction. Over time, this will change the overall direction or “compass” of the cell. (B) The second model involves dividing or splitting the cell front into two. The new pseudopod that is better aligned with the gradient survives while the other retracts.

1.3 Size and speed considerations for gradient tracking mechanisms

Why do bacteria use a temporal mechanism whereas neutrophils and Dicty cells use a spatial mechanism for gradient tracking? Cell size and speed contribute to the use of either gradient tracking mechanism. Bacteria are very small cells, only a few microns in length, so the difference in chemical concentration at one end of the cell compared to the other is very small.
too (Berg and Purcell, 1977). In addition, bacteria are fast swimmers, reaching up to 30 \( \mu \text{m/sec} \). Theoretical studies have demonstrated that bacteria swim so quickly that they also distort the chemical gradient directly surrounding the cell (Berg and Purcell, 1977). In contrast, neutrophils and Dicty cells are much larger, about 8-15 microns in diameter, so they can detect a much larger difference in chemical concentration by direct sensing (Endres and Wingreen, 2008; Hu et al., 2010). Also, these cells migrate much slower, only 20 \( \mu \text{m/min} \), and they would need to move much faster in order for a temporal mechanism of gradient sensing to work (Berg and Purcell, 1977; Iijima et al., 2002).

These ideas have led to the generalization that fast and small cells use temporal mechanisms whereas large and slow cells use spatial mechanisms for gradient tracking (Figure 1.7). However, not all gradient tracking cells fit into one of these categories. For example, sperm are large cells that swim very fast up-gradient towards an egg. While sperm use a form of temporal sensing, they actually use a much more complex gradient tracking mechanism than bacteria (Alvarez et al., 2014).

![Figure 1.7 Cell size and speed are usually used to explain gradient sensing mechanisms.](image)

As small fast moving cells, bacteria use temporal sensing. As large slow cells, neutrophils use spatial sensing. But what about the fast and large sperm cell or the slow and tiny yeast cell?
Budding yeast, *Saccharomyces cerevisiae*, also do not fit into one of the generalized categories. During mating, a yeast cell will track the pheromone gradient to find its partner (reviewed in [Arkowitz, 2009](#)). Yeast gradient tracking is incredibly slow because they grow towards their partner instead of migrating. But, yeast cells are also small, approximately 5 μm. What type of mechanism does a small and slow yeast cell use?

Yeast mating is mediated through a GPCR pathway similar to neutrophil and Dicty cells. While many signaling pathways have been implicated in neutrophil and Dicty cell chemotaxis, there functional role in connecting free Gβγ to promote actin polymerization remains unclear. These systems are very complicated and redundant: a recent report suggests at least twenty-one small GTPases activate PI3K in neutrophils ([Yang et al., 2012](#)). In addition, the tools used to dissect the protein interactions have been blunt, resulting in some misleading conclusions ([Inoue and Meyer, 2008](#)). In contrast, yeast are genetically tractable and are extremely proficient at gradient tracking, so they are an ideal system to dissect the detailed mechanisms of GPCR mediated signaling and gradient tracking.

### 1.4 Chemotropism in yeast

#### 1.4.1 Yeast mating: GPCR-mediated pheromone sensing

When yeast are in their haploid state, they can take on one of two mating types: MATα or MATα. These cells secrete the pheromones, α-factor or α-factor, respectively. Both pheromones are small peptides, but α-factor is lipidated ([Adereteg et al., 1988; Caldwell et al., 1994](#)). For this reason, most studies of yeast mating have focused on MATα cells that respond to α-factor, which can be synthesized in vitro. MATα cells express the G-protein coupled receptor, Ste2, on their cell surface (reviewed in [Dohlman and Thorner, 2001](#)). Once bound to α-factor,
Ste2 triggers GDP/GTP exchange on the Ga subunit, which releases Gβγ. Free Gβγ then functions in two pathways: signaling and growth orientation (Figure 1.8). Pheromone G protein signaling is negatively regulated by Sst2, a regulator of G protein signaling (RGS) protein. Sst2 acts as a GTPase activating protein (GAP) for Ga, returning Ga to the inactive GDP-bound state, which binds Gβγ.

**Figure 1.8 G protein signaling during yeast mating**

Prior to ligand binding, the Ga subunit is bound to GDP and exists as a trimer with Gβγ. Following ligand binding, Ste2 GEF activity towards Ga is stimulated catalyzing GDP/GTP exchange and release of Gβγ.

Free Gβγ recruits the protein scaffold Ste5 to the plasma membrane to initiate the mitogen-activated protein kinase (MAPK) signaling cascade (Figure 1.9) (Pryciak and Huntress, 1998). Membrane recruitment of Ste5 brings the p21-activated kinase (PAK) protein Ste20 in proximity to its substrate Ste11 (Pryciak and Huntress, 1998). While Ste11 phosphorylation of Ste7 does not require Ste5, a domain of Ste5 is required for Ste7 phosphorylation of Fus3 (Choi et al., 1994; Good et al., 2009; Lamson et al., 2006; Zalatan et al., 2012).

Once active, the MAP kinase Fus3 phosphorylates many substrates including the transcriptional inhibitors Dig1 and Dig2. Phosphorylation of Dig1 and Dig2 releases their
inhibition on the transcription factor Ste12, therefore leading to induction of genes containing pheromone-responsive elements (Cook et al., 1996; Olson et al., 2000; Tedford et al., 1997).

Active Fus3 also phosphorylates the cyclin-dependent kinase (CDK) inhibitor, Far1 (Peter et al., 1993). During vegetative growth, Far1 is degraded just prior to START (Henchoz et al., 1997; McKinney et al., 1993). But when cells are exposed to pheromone, active Fus3 phosphorylates and stabilizes Far1 promoting cell-cycle arrest in G1. Pheromone signaling to G1 arrest promotes efficient mating between mating partners that are both unbudded and mononucleate (Hartwell, 1973).

In addition, the MAPK cascade initiates polarized growth (Pryciak and Huntress, 1998). Cells can be induced to express a membrane targeted version of Ste5 to activate the MAPK cascade without activation of G protein signaling. These cells arrest and induce transcription, and they form mating projections (Pryciak and Huntress, 1998; Strickfaden and Pryciak, 2008).

![Figure 1.9 Pheromone MAPK signaling](image)

Free Gβγ recruits the scaffold protein Ste5 that initiates the MAPK cascade. Once the MAP kinase Fus3 is phosphorylated and active, it phosphorylates targets important for pheromone-dependent transcription and induces cell cycle arrest.
1.4.2 Cdc42-mediated polarized growth

Polarized growth in yeast is regulated by Cdc42, the master regulatory GTPase (reviewed in (Bi and Park, 2012)). A patch of active Cdc42 at the cell cortex determines the direction of growth whether that be a bud during normal proliferative growth or a projection during mating. Both processes have spatial cues to bias the localization of Cdc42: during budding, Cdc42 localizes near the previous bud scar and during mating, Cdc42 localizes on the up-gradient side of the cell (Figure 1.10A). However, neither process requires a spatial cue to establish a Cdc42 patch (Arkowitz, 2009; Bi and Park, 2012). Instead, stochastic fluctuations in polarity factors are amplified by a positive feedback loop in a process called symmetry-breaking (Johnson et al., 2011).

The process of symmetry breaking centers around a scaffold protein, Bem1 (Irazoqui et al., 2003). During symmetry breaking, Bem1 brings together the Cdc42 effector p21-activated kinase (PAK) and the Cdc42 guanine nucleotide activator (GEF) Cdc24 (Kozubowski et al., 2008). In this way, Cdc24 is recruited to GTP-Cdc42 at the plasma membrane to activate neighboring Cdc42 molecules and form a patch of GTP-Cdc42 (Figure 1.10B).

In order to direct growth towards the Cdc42 patch, Cdc42 recruits and activates the formin actin nucleators, Bni1 and Bnr1 (Chen et al., 2012; Dong et al., 2003). By localizing Bni1 and Bnr1, actin cables will be oriented in one direction. Therefore, myosins traveling along the actin cables will deliver vesicles with membrane and cell wall constituents to the Cdc42 site (Figure 1.10C) (Harold, 1990; Pruyne and Bretscher, 2000).
During yeast mating, the pheromone gradient produces a spatial cue that biases the Cdc42 polarity patch, and therefore growth, on the up-gradient side of the cell (Arkowitz, 2009; Segall, 1993). This bias is mediated by the scaffold protein Far1 that binds to both Cdc24 and free Gβγ hence providing a connection between G protein signaling and the polarity patch (Figure 1.10D) (Butty et al., 1998; Nern and Arkowitz, 1999). The role of Far1 in growth orientation is independent from its role in cell cycle arrest since mutants that only prevent Far1 from binding Cdc24 do not affect cell cycle arrest (Valtz et al., 1995). Instead, these mutants direct polarized growth in a random orientation with respect to the gradient, illustrating the essential role of Far1 in gradient tracking (Nern and Arkowitz, 1998; Valtz et al., 1995).

Figure 1.10 Cdc42 polarity

(A) Spatial cues bias localization of the Cdc42 patch. During haploid budding, Cdc42 localizes next to the last bud scar. During mating, the pheromone gradient biases Cdc42 localization to the up-gradient side of the cell. (B) Cdc42 polarizes through a positive feedback loop that recruits Cdc24 to activate neighboring Cdc42 molecules. (C) The Cdc42 patch orients actin cables, so vesicles will be delivered and fuse at the Cdc42 patch. (D) Pheromone biases the Cdc42 polarity patch through Far1 that binds both free Gβγ and Cdc24.
1.4.3 Error correction: moving the Cdc42 polarity patch

Yeast cells are extremely proficient at gradient tracking, and they are thought to track gradients that produce only a 1% difference in receptor occupancy from front to back (Segall, 1993). Such a shallow gradient is likely blurred by the stochastic fluctuations or “noise” in receptor-ligand binding (Andrews et al., 2006; van Haastert and Postma, 2007; Miyanaga et al., 2007). Therefore, G protein signaling will not necessarily bias the polarity patch to the up-gradient side of the cell initially. Indeed, yeast cells sometimes initiate growth in the wrong direction, but they can correct their orientation over time and eventually grow properly up-gradient (Dyer et al., 2013; Moore et al., 2008). This error correction requires moving the Cdc42 polarity patch to a different side of the cell, but the positive feedback loop within the polarity regulators reinforces the current location of the polarity patch. How then do yeast cells overcome positive feedback to move the Cdc42 polarity patch?

Because Cdc42 determines the direction of growth as discussed above, the Cdc42 patch is constantly bombarded with vesicles. Mathematical modeling work from our lab has demonstrated that because vesicles do not carry most polarity factors, vesicle fusion events will cause a local depletion of the polarity factors where the vesicle-derived membrane has inserted into the plasma membrane (Figure 1.11) (Layton et al., 2011; Savage et al., 2012). The positive feedback loop of protein-protein interactions between the polarity regulators is very dynamic since these proteins rapidly dissociate, diffuse in the cytoplasm, and rebind the patch (Dyer et al., 2013; Howell et al., 2009). So, when a vesicle fusion event produces a local depletion of polarity regulators, they will rapidly adjust and reinforce the side of the dip that has more polarity regulators. Effectively, this moves the polarity patch away from the site of vesicle fusion (Figure 1.11) (Dyer et al., 2013; Meinhardt, 1999).
At the moment of vesicle fusion, the new membrane does not carry and polarity factors, thus creating a dip in the Cdc42 profile (right). The positive feedback loop then reestablishes on the higher concentrated side effectively moving the patch away from the site of fusion.

Using timelapse microscopy, our lab found that the polarity patch does, in fact, wander along the cell cortex during pheromone gradient tracking (Dyer et al., 2013). Though, concentrating the polarity scaffold protein Bem1 on vesicles should block the dilution effect of vesicle fusion and thus block wandering (Howell et al., 2009). Indeed, these cells showed very little patch wandering, and they could not correct their orientation if the direction of growth was initially incorrect (Dyer et al., 2013).

Consider a cell that originally established the Cdc42 polarity patch on the down-gradient side of the cell. Vesicles will effectively push the polarity patch along the cell cortex, so the cell can reorient in the proper direction. In order for the wandering of the polarity patch to be
effective in reorientation, somehow the pheromone gradient must bias the polarity patch to spend more time on the up-gradient side of the cell. How does the pheromone gradient influence polarity patch wandering?

1.4.4 Yeast spatial sensing: the problem of receptor polarity

Unlike neutrophils and Dicty cells, yeast do not distribute their pheromone receptors uniformly. Instead, they dramatically polarize their pheromone receptors in both uniform pheromone conditions as well as in gradients (Arkowitz, 2009; Ayscough and Drubin, 1998; Moore et al., 2008). Shortly after ligand binding, the receptor is phosphorylated then ubiquitinated, which triggers its endocytosis for delivery to the vacuole for degradation (figure 1.12A) (Hicke and Riezman, 1996; Hicke et al., 1998; Jenness and Spatrick, 1986; Schandel and Jenness, 1994). At the same time, recently synthesized receptors are delivered to the polarity site by vesicle trafficking. Before they diffuse far from the delivery site, the receptors bind ligand and are in turn internalized. Therefore the balance of delivery and internalization creates a polarized distribution (Figure 1.12B).

If yeast cells use a spatial sensing mechanism for gradient tracking, then they need to compare the pheromone concentration on one side of the cell to the other. How can this be accomplished if the receptors are only localized to one site on the plasma membrane?
Figure 1.12 Pheromone receptor polarity

(A) Once bound to pheromone, receptors are phosphorylated then ubiquitinated. This triggers their endocytosis where they are sent to the vacuole for degradation. (B) The balance of receptor endocytosis and delivery of newly synthesized receptors leads to a polarized distribution.
1.5 Thesis objectives

Many cells are incredibly adept at tracking gradients of chemical cues. Bacteria use a temporal gradient sensing mechanism where they compare the chemical concentration between time points, whereas most eukaryotes are thought to use a spatial sensing mechanism where they compare the chemical concentration between different sides of the cell. During spatial gradient tracking, cells polarize and establish a “front” that they turn in order to change direction. If the cell properly tracks a gradient, then somehow the gradient must influence the “front” turning so as to bias the cell up-gradient. How this is accomplished remains unknown.

While many pathways involved in neutrophil and Dicty cells chemotaxis have been identified, their functional significance remains unclear because of redundancies and complexities in both protein interactions and feedback loops. In contrast, yeast are both genetically tractable and have far less redundancies, so the pheromone signaling pathway is well characterized. This makes yeast a pivotal model for studying mechanisms of gradient tracking. In the present study, we used genetics, microscopy, and computational modeling based approaches to understand how yeast cells properly track the pheromone gradient. Specifically, we wanted to understand how pheromone biases polarity patch wandering to the up-gradient side of the cell to ensure growth occurs towards the mating partner. Also, we wanted to understand why yeast cells polarize their pheromone receptors and thereby reduce the cell’s ability to directly sense the gradient.
2. Role of polarized G protein signaling in yeast gradient tracking

2.1 Introduction

The ability to track chemical gradients underpins a multitude of cell and organismal behaviors. In humans, gradient tracking underlies axon guidance, homing of immune cells towards invaders, chemotaxis of fibroblasts towards wound sites, guidance of sperm towards the egg, and metastasis in cancer (Condeelis et al., 2005; von Philipsborn and Bastmeyer, 2007; Rappel and Loomis, 2009; Raz and Reichman-Fried, 2006; Schnorrer and Dickson, 2004; Swaney et al., 2010). For microorganisms, gradient tracking enhances the search for food or mating partners. Eukaryotic cells are thought to possess “spatial” gradient-sensing systems that discern small concentration differences across the cell diameter (reviewed in (Swaney et al., 2010)).

Ligands are sensed by G-protein-coupled receptors (GPCRs), which signal through heterotrimeric G proteins. Activated G proteins (Gα-GTP and free Gβγ) then signal through a variety of pathways to influence the actin cytoskeleton, which controls directional cell growth and migration. Despite much progress in identifying the molecular links between G proteins and the cytoskeleton, we still lack a clear view of how cells can effectively track shallow chemical gradients.

The accuracy of spatial gradient sensing is limited by the size of the cell: a larger cell can compare ligand concentrations across a longer distance. Budding yeast (Saccharomyces cerevisiae) are among the smallest eukaryotic cells, yet they are capable of tracking quite

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1 Chapters 2 and 3 are adapted from a manuscript that will be submitted as: Allison W. McClure*, Maria Minakova*, Jayme M. Dyer*, Trevin R. Zyla, Timothy C. Elston, and Daniel J. Lew. Role of polarized G protein signaling in tracking pheromone gradients.
shallow gradients of small peptide pheromones to locate mating partners (Moore et al., 2008; Segall, 1993). Remarkably, during gradient tracking, yeast pheromone receptors become polarized towards the growing “front” of the cell (Ayscough and Drubin, 1998; Jackson et al., 1991), thereby reducing the length scale that these tiny cells can use to compare pheromone concentrations. The benefit that cells get from polarizing their receptors must presumably outweigh the cost of reducing the sensitivity of gradient detection, but the nature of that benefit has been unclear. We now suggest that polarized receptor and G protein activity may enable gradient tracking through an unexpected mechanism.

Yeast cells are surrounded by a rigid cell wall and are not motile. Mating cells come into physical contact by growing a mating projection up the pheromone gradient towards the mating partner. Directional growth involves targeted delivery and fusion of secretory vesicles that contain cell wall-modifying enzymes and new cell wall constituents (Harold, 1990). Vesicles are delivered by myosin motors along actin “cables”, which are bundles of parallel actin filaments nucleated by formins (Pruyne and Bretscher, 2000). Formins are locally recruited to a patch of the cell cortex (the “front” of the cell) by the conserved Rho-family GTPase, Cdc42 (Figure 1.10) (Chen et al., 2012; Dong et al., 2003). Thus, actin cables are oriented towards Cdc42, and successful gradient tracking requires that the Cdc42 patch be located on the up-gradient side of the cell.

A pheromone gradient is not a prerequisite for cells to form a localized patch of Cdc42 at the cortex: even cells exposed to a uniform pheromone concentration polarize Cdc42 and exhibit polarized growth (Arkowitz, 2009). Such polarization can occur at a random location and involves an autocatalytic positive feedback loop among the polarity regulators (Dyer et al., 2013). When cells are exposed to a pheromone gradient, the gradient is thought to bias where
the Cdc42 patch is established. But in shallow gradients, cells sometimes polarize in the wrong direction (Dyer et al., 2013; Moore et al., 2008; Segall, 1993). Nevertheless, over time, the cells adjust their Cdc42 patch location to improve alignment with the external gradient (Dyer et al., 2013; Segall, 1993). Such ongoing gradient tracking must overcome the formidable obstacle imposed by the positive feedback loop, which reinforces the current location of the Cdc42 patch.

Theoretical studies have demonstrated that the addition of local negative feedback can allow cells to track gradients despite a positive feedback polarity system (Meinhardt, 1999). By perturbing the front (in this case, the Cdc42 patch), negative feedback could weaken the tendency of the positive-feedback system to continue in the same location and allow small signal asymmetries to influence the direction of growth. Recent studies have suggested a mechanism for such polarity perturbing negative feedback during yeast mating (Dyer et al., 2013). Secretory vesicles add new membrane at the polarity site, diluting key polarity factors and providing a local perturbation (Figure 1.11) (Layton et al., 2011). When combined with positive feedback among polarity regulators, that perturbation can “nudge” the polarity patch away from the site of vesicle fusion. Stochastic vesicle fusion in the vicinity of the Cdc42 patch can therefore cause the polarity site to gradually “wander” along the cell cortex (Dyer et al., 2013). If pheromone gradients were able to bias such wandering, then that would provide a basis for gradient tracking.

Polarity patch wandering has been documented in cells tracking pheromone gradients, as well as in cells exposed to uniform and non-saturating concentrations of pheromone (Dyer et al., 2013). However, at saturating pheromone concentrations, wandering was severely reduced (Dyer et al., 2013). The constraint of wandering in cells exposed to higher levels of pheromone
suggests a mechanism of gradient tracking: when the polarity patch is located on the down-gradient side of the cell, it wanders more, whereas when the polarity patch is located on the up-gradient side of the cell, it wanders very less.

In this chapter, we address the mechanism(s) whereby higher pheromone levels lead to reduced wandering of the polarity patch. We show via computational analyses that polarized G protein signaling can constrain wandering by locally generating GTP-Cdc42. Thus, cells polarize both the GPCR reducing the cell surface area in which pheromone concentrations can be sensed. In return, polarized G protein signaling enables a pheromone dose-dependent constraint of polarity patch wandering that can contribute to gradient tracking.

2.2 Results

2.2.1 Pheromone dose-dependent constraint of polarity patch wandering

Previous work has indicated that the bud-site-selection regulator Rsr1 can bias the polarity axis towards a “default” predetermined landmark and that cells tracking pheromone gradients override this bias (Dorer et al., 1995; Madden and Snyder, 1992; Nern and Arkowitz, 2000; Valtz et al., 1995). In all of our experiments, we used rsr1Δ mutants lacking this default pathway.

We investigated whether constraint of polarity patch wandering by high pheromone concentrations was simply a consequence of high MAPK activity. Cells responding to pheromone produce free Gβγ that recruits the MAPK scaffold protein Ste5 to the membrane, leading to pheromone dose-dependent activation of the MAP kinases Fus3 (Figure 2.1A) (Yi et al., 2003). MAPK activation can be induced downstream of the GPCR and G protein by
expressing a fusion protein, Ste5-CTM, that artificially targets Ste5 to the plasma membrane (Figure 2.1B) (Pryciak and Huntress, 1998).

Induction of Ste5-CTM led to high levels of MAPK activation that were not further elevated by addition of pheromone (Figure 2.1C), and triggered several mating pathway outputs including cell-cycle arrest in G1 (unbudded cells) and polarization of the polarity marker Spa2-mCherry (Figure 2.1D,E).
Figure 2.1 Ste5-CTM induces maximal MAPK activation, cell cycle arrest, and polarization without pheromone

(A) Upon binding pheromone, the pheromone receptor activates Gα, which releasing Gβγ. Free Gβγ recruits the scaffold protein Ste5 which activates MAPK signaling. (B) The inducible Ste5-CTM construct (Pryciak and Huntress, 1998) is targeted to the plasma membrane and initiates the MAPK cascade even without the addition of pheromone. (C) Pheromone does not further increase MAPK signaling following Ste5-CTM induction. Cells (DLY18172) were treated with β-estradiol to induce Ste5-CTM for 4 h, and then with the indicated α-factor dose for 30 min. Control STE5 (DLY15596) and ste5Δ (DLY18172) cells were treated with α-factor for 30 minutes without β-estradiol pretreatment. Samples were harvested and immunoblotted for phospho-Fus3 (phospho-MAPK) and actin. Graph shows quantification of the P-Fus3 signal normalized to the actin loading control. (D) Ste5-CTM expression leads to cell cycle arrest and polarization.
Cells (DLY18172) were treated with β-estradiol for 4 h, then imaged using DIC and fluorescence microscopy to detect the polarity marker Spa2-mCherry (maximum projection). (E) Graph shows the percentage of cells that were unbudded (left) or unbudded and polarized (right) after 4 h of Ste5-CTM induction (N=256 cells).

Wandering of the polarity site was quantified in cells expressing Ste5-CTM by tracking the position of the Spa2-mCherry patch centroid and calculating its mean-squared displacement (MSD). In the absence of pheromone, cells displayed highly mobile polarity patches (Figure 2.2A). Thus, a high level of MAPK activation is not sufficient to constrain wandering of the polarity patch.

When cells expressing Ste5-CTM were exposed to uniform pheromone concentrations, there was a dose-dependent reduction in polarity patch wandering (Figure 2.2B), which we also quantified by calculating an effective diffusion coefficient for the polarity patch (D_{patch}) (Figure 2.2C). Corresponding to the dose-dependent reduction in wandering, the higher pheromone concentrations produced more “pointy” mating projections (Figure 2.2D). Even low concentrations of pheromone, which would not have been sufficient to cause G1 arrest without Ste5-CTM expression, resulted in a significant decrease in wandering. Thus, pheromone treatment provides dose-dependent constraint of wandering under conditions of high MAPK activity.
Figure 2.2 Pheromone constrains polarity patch wandering independently of MAPK signaling

(A) Cells (DLY18172) were treated with β-estradiol for 4 h to induce Ste5-CTM, loaded on a slab with the indicated dose of α-factor, and imaged 20 min later. Images are shown as maximum projections. Right: example Spa2-mCherry centroid tracks. (B) Mean squared displacement (MSD) of the Spa2-mCherry centroid (n>200 cells) in cells imaged as in (A). (C) Effective diffusion coefficient of the polarity patch was calculated from the slope of the graphs in (B). Mean +/- SEM for 4 independent biological replicates. (D) DIC images of representative cells from each α-factor concentration, illustrating that constraining wandering produces polarized morphology.
2.2.2 Constraint of polarity patch wandering operates through a Gβγ-Far1-Cdc24 pathway

Previous studies have identified interactions between free Gβγ and the scaffold protein Far1, as well as between Far1 and the Cdc42-directed GEF, Cdc24 (Figure 2.3A) (Butty et al., 1998; Nern and Arkowitz, 1999). These interactions provide a way for the activated G protein to influence the localization of GTP-Cdc42. Mutations that disrupt the Far1-Cdc24 interaction blocked the ability of yeast cells to track pheromone gradients (Nern and Arkowitz, 1998). Such mutations also allowed polarity patch wandering to continue even at high pheromone doses (Dyer et al., 2013; Nern and Arkowitz, 2000). We found that in cells expressing Ste5-CTM, the ability of pheromone to constrain wandering was abolished by deletion of STE4 (encoding Gβ) (Figure 2.3B) and was severely reduced by a point mutation, cdc24-m1 (Nern and Arkowitz, 1998), that impairs the interaction of Cdc24 with Far1 (Figure 2.3C). Thus, constraint of polarity patch wandering in uniform pheromone conditions operates through a Gβγ-Far1-Cdc24 pathway.
Figure 2.3 The Gβγ-Far1-Cdc24 connection is required for pheromone to constrain polarity patch wandering

(A) Free Gβγ recruits a Far1-Cdc24 complex from the cytoplasm, and Cdc24 is the GEF for Cdc42. The cdc24-m1 mutation reduces Far1-Cdc24 binding, which yields cells that are unable to track gradients (Nern and Arkowitz, 1998). (B) Cells lacking Gβ (ste4Δ, DLY18425) cells and (C) cdc24-m1 cells (DLY18478) were treated with β-estradiol for 4 h to induce Ste5-CTM expression, imaged and their polarity patch wandering was quantified as an effective diffusion constant for different pheromone concentrations (n>59 cells, n>41 cells, respectively). Constraint of polarity patch wandering by pheromone was much weaker in these mutants than wild-type cells.
2.2.3 Computational modeling suggests that uniform recruitment of GEF would not constrain polarity patch wandering\(^2\)

To investigate how the Gβγ-Far1-Cdc24 pathway might constrain polarity patch wandering, we explored several potential hypotheses using computational modeling. Our model incorporated the positive feedback loop among polarity regulators, Cdc42, Bem1, and Cdc24 (GEF) (Figure 2.4A), yielding a polarized patch of Cdc42. We also included the stochastic fusion (exocytosis) and fission (endocytosis) of vesicles, which perturbs the patch to yield wandering (Dyer et al., 2013). Our model builds on previous models by refining how endocytosis and exocytosis locations are specified. For exocytosis, we assumed that vesicles fuse at locations specified by actin cables and that actin cable locations are in turn specified by formin nucleation, which depends on the local concentration of GTP-Cdc42. Based on recent findings indicating that yeast formin recruitment by GTP-Cdc42 can occur through several distinct interactions (Chen et al., 2012; Evangelista et al., 1997; Fujiwara et al., 1998; Sheu et al., 1998), the probability of actin cable nucleation by formins at any given location was modeled as a Hill function of the GTP-Cdc42 concentration (Figure 2.4B).

For endocytosis, we assumed that endocytic sites form at locations that are influenced by the local concentration of endocytic cargo proteins. We modeled a representative endocytic cargo with characteristics of a transmembrane v-SNARE, which recycled between the plasma membrane and a well-mixed internal compartment, and actively concentrated into both exocytic and endocytic vesicles (Valdez-Taubas and Pelham, 2003). The probability of endocytic site nucleation at any given location was modeled as a Hill function of the v-SNARE

\(^2\) Computational modeling, simulations, and analysis were performed by Maria Minakova.
concentration (Figure 2.4C). With this model, we simulated the time evolution of polarity protein concentrations on the plasma membrane, allowing us to track the centroid of the polarity patch (GTP-Cdc42) and calculate its MSD and effective diffusion constant for comparison with movies from live cells (Figure 2.4D).
Figure 2.4 Computational model of polarity patch wandering

(A) Schematic of model incorporating the protein interactions that mediate positive feedback and Cdc42 clustering. Bem1-complexes containing Cdc24 (GEF for Cdc42) can associate with pre-existing GTP-Cdc42 at the membrane, promoting activation of neighboring GDP-Cdc42. The resulting local depletion of GDP-Cdc42 leads to net delivery of Cdc42 from the cytoplasm by the GDI, leading to further GTP-Cdc42 accumulation. (B) GTP-Cdc42 promotes nucleation of actin cables through formins, so the local probability of actin cable nucleation (green) is modeled as a Hill function of GTP-Cdc42 concentration (blue). (C) Exocytosis near the GTP-Cdc42 cluster and subsequent retrieval by endocytosis leads to an enrichment of vesicular cargo (represented by v-SNARE) surrounding the polarity patch. The local probability of endocytic patch nucleation (green) is modeled as a Hill function of cargo (v-SNARE) concentration (blue). (D) The combination of reactions, diffusion, and vesicle traffic yields a model of the polarity patch that wanders around the cortex. Left: example centroid trace. Right: MSD of the polarity patch centroid.
To better illustrate how vesicle traffic causes patch wandering in the model, we generated simulations in which all actin cables (and hence exocytic events) were artificially constrained to occur on a quadrant northeast of the polarity patch. Exocytic vesicle membranes contained only low levels of Cdc42 relative to the polarity site and no Bem1-Cdc24 complexes (Layton et al., 2011; Savage et al., 2012). Thus, fusion of vesicles near the polarity peak locally diluted polarity factors: the resulting GTP-Cdc42 distribution was asymmetric, and actin cables (indicated by the exocytosis probability distribution) trailed behind the moving patch. (Figure 2.5). Constant fusion of vesicles northeast of the patch caused the patch to move consistently southwest. In agreement with the previous model (Dyer et al., 2013), our findings show that vesicle delivery acts as a negative feedback loop on polarity with a time delay due to the time required to recruit new actin cables to the shifting peak of GTP-Cdc42.

Figure 2.5 Computational model illustrates mechanism of polarity patch wandering

Simulations where actin cable nucleation was constrained to the northeast quadrant leads to polarity patch movement southwest. Distributions of GTP-Cdc42 (left) and the locations of exocytosis (middle) were rotated to display patch migration to the left (arrows). Right: Superposition of distributions in the direction of motion illustrates how exocytosis occurs behind the moving patch, driving polarity patch wandering.
To simulate addition of uniform pheromone, we initially assumed that uniform receptor activation would lead to free Gβγ all over the cortex. In turn, Gβγ would recruit Far1-Cdc24 complexes to the cortex, generating a uniform cortical GEF activity in addition to the Bem1-associated GEF already incorporated in the model. When we added increasing amounts of uniform GEF activity on the cortex, basal levels of GTP-Cdc42 rose, eventually disrupting the ability of the positive feedback loop to maintain polarity (Figure 2.6A). However, as long as the model was able to maintain polarity, the uniform GEF had little effect on polarity patch wandering (Figure 2.6B). Similarly, simulations restricting cable nucleation to occur on one quadrant showed no change in patch speed upon addition of uniformly distributed GEF (Figure 2.6C). These simulations suggest that a uniformly distributed cortical GEF activity would not be sufficient to constrain polarity patch wandering in response to pheromone.
Figure 2.6 Modeling predicts that uniform GEF recruitment would be unable to constrain polarity patch wandering

(A) A uniformly membrane-localized GEF was added to the model to simulate recruitment of Far1-Cdc24 by free Gβγ in response to exposure to uniform pheromone. Graph shows averaged peak and basal GTP-Cdc42 concentrations. Increasing GEF activity raised basal GTP-Cdc42 levels, eventually causing loss of polarity. (B) Simulated patch wandering was quantified by estimating the effective patch diffusion coefficient. Increasing uniform GEF activity did not constrain wandering. (C) Simulations where cabin nucleation was restricted to the northeast quadrant results in polarity patch movement at constant speed. Increasing uniform GEF activity did not slow the speed of patch movement.
2.2.4 Unpolarized free Gβγ does not constrain polarity patch wandering

Like high levels of pheromone, deletion of GPA1 (encoding Gα) releases free Gβγ at the membrane. In \( gpa1Δ \) cells expressing Ste5-CTM, GFP-Ste4 localized mostly uniformly on the membrane (Figure 2.7A). This provided an opportunity to examine polarity patch wandering in cells with free Gβγ all over the membrane similar to our computational simulations. We found that \( gpa1Δ \) cells expressing Ste5-CTM displayed rampant patch wandering, comparable to cells expressing Ste5-CTM but not treated with pheromone (Figure 2.7B). Thus, as predicted by our computational model, the ability of cells to polarize Gβγ is correlated with their ability to constrain polarity patch wandering.

**Figure 2.7 Unpolarized free Gβγ cannot constrain polarity patch wandering**

(A) Wild-type (DLY18172) or \( gpa1Δ \) (DLY18559) were treated with β-estradiol for 4 h to induce Ste5-CTM, placed on β-estradiol slabs for 20 min and imaged for GFP-Ste4 (medial confocal plane). (B) Polarity patch wandering for cells treated as in (A) was calculated as MSD (n>150 cells).
2.2.5 Polarization of a pheromone-recruited GEF would constrain polarity patch wandering

Although exposure of yeast cells to uniform pheromone would initially produce uniformly distributed free Gβγ, over time the distribution would change because the pheromone receptors polarize (Arkowitz, 2009; Ayscough and Drubin, 1998; Kim et al., 2000; Moore et al., 2008). Pheromone binding triggers endocytosis and degradation of the pheromone receptor, Ste2 (Hicke et al., 1998), accompanied by delivery of newly synthesized Ste2 to the polarity patch on secretory vesicles. As diffusion of proteins in the yeast plasma membrane is slow (Valdez-Taubas and Pelham, 2003), continued pheromone exposure leads to a polarized steady state in which Ste2 concentration is highest near the polarity patch. The precise distribution of Ste2 is expected to depend on the pheromone concentration: in high pheromone, rapid ligand binding and internalization would produce a steep Ste2 distribution, while in low pheromone, Ste2 would diffuse further before being endocytosed, producing a shallower distribution. The G protein subunits are thought to traffic together with the receptor (Suchkov et al., 2010), and using a functional GFP-Ste4 (Gβ) probe (Arkowitz, 2009; Suchkov et al., 2010), we confirmed that the Gβγ distribution followed the predicted Ste2 distribution and became more polarized at higher pheromone concentrations (Figure 2.8A,B).
Figure 2.8 Gβγ polarizes in a pheromone dose-dependent manner

(A) Cells (DLY18172) were treated with β-estradiol for 4 h to induce Ste5-CTM, placed on agarose slab for 20 minutes, then imaged for GFP-Ste4 (medial confocal plane). (B) GFP-Ste4 distribution (n>50 cells) along the cell perimeter for the indicated pheromone dose.
By varying pheromone concentration, we not only changed the rate of ligand binding, but we also changed polarity patch wandering. To ask whether Gβγ polarization was simply a consequence of constraining wandering, we artificially altered polarity patch wandering behavior. Using the cdc24-m1 mutant, which exhibits high polarity patch wandering even in high pheromone concentrations (Figure 2.3)(Dyer et al., 2013; Nern and Arkowitz, 2000), we found that GFP-Ste4 still exhibited a dose-dependent distribution (Figure 2.9). We conclude that Gβγ polarity is regulated by pheromone concentration and not indirectly through effects on polarity patch wandering.

**Figure 2.9 Pheromone dose-dependent polarity of Gβγ is independent of polarity patch wandering**

Cells with either CDC24 (DLY18172) or cdc24-m1 (DLY18478) were treated with β-estradiol for 4 h to induce Ste5-CTM, then loaded on an α-factor slab, and imaged (medial confocal plane). Right: GFP-Ste4 distributions (n>58 cells) along the cell perimeter.
Given receptor and G protein polarization, exposure of cells to uniform pheromone would lead to a polarized distribution of free Gβγ and a polarized recruitment of Far1-Cdc24 GEF complexes. To ask whether such a polarized GEF would constrain wandering, we added a new coarse-grained molecular species, RecGEF, to represent the aggregate behavior of the pheromone receptor (Rec), the G protein, and the Far1-Cdc24 complex (GEF) (Figure 2.1A). RecGEF was delivered on secretory vesicles to the plasma membrane, where it diffused slowly and could bind to extracellular pheromone (Kᵦ 6 nM) to turn on its GEF activity, now denoted as RecGEF*. RecGEF* activity represents Far1-Cdc24 recruitment by Gβγ freed upon pheromone binding, and we assumed that this GEF activity was proportional to the local amount of ligand bound receptor. RecGEF* was actively concentrated into endocytic vesicles, as modeled for v-SNARE proteins, and RecGEF* activity was terminated upon endocytosis. We assumed that exocytosis and endocytosis rates were balanced so that total membrane area and total receptor concentration were constant. This simplified strategy provided a receptor-associated GEF activity with a distribution that varied with the pheromone concentration (Figure 2.10B) consistent in a manner similar to that observed for Gβγ in cells (Figure 2.8).

Unlike uniform GEF activity (Figure 2.4), the polarized RecGEF* provided a robust constraint on polarity patch wandering in the presence of pheromone (Figure 2.10C). This striking result suggests that trafficking and consequent polarization of the receptor and G-protein signaling is sufficient to constrain polarity patch wandering.
Figure 2.10 Pheromone constrained polarity patch wandering by producing a polarized distribution of $G\beta\gamma$

(A) The pheromone-sensing machinery in cells (top) was represented by a single receptor-GEF species in the model (bottom). (B) RecGEF* (ligand-bound model receptor with GEF activity) distributions from simulations conducted at the indicated doses of $\alpha$-factor. Arrow indicates the direction of polarity patch movement. (E) RecGEF* constrained polarity patch wandering in a pheromone dose-dependent manner.
Why does the polarized RecGEF* constrain patch wandering when uniform GEF does not? As discussed above, patch movement occurs when off-center vesicle fusion dilutes polarity factors on one side of the patch, “pushing” the patch centroid away from the vesicle fusion site. But vesicles also deliver fresh RecGEF to the cortex. Once pheromone binds to the newly arrived RecGEF, the resulting active RecGEF* raises the GTP-Cdc42 concentration “behind” the moving patch, thereby reducing patch movement. In effect, RecGEF provides a delayed positive feedback that counteracts the dilution-mediated negative feedback from vesicle fusion.

To better illustrate how RecGEF* reduces wandering, we added the RecGEF species to the simulations where actin cable attachment was constrained to the northeast quadrant driving the patch southwest. As expected, RecGEF* was progressively more polarized at higher pheromone levels, and, notably, the peak of the RecGEF* distribution lagged behind the polarity patch (Figure 2.1A,B).

To test the prediction that receptor and Gβγ would lag behind the polarity patch during wandering, we imaged cells harboring cdc24-m1 in a high pheromone concentration to allow the polarity patch to wander yet show a polarized Gβ distribution. In favorable instances when the polarity patch migrated for several minutes in a consistent direction, the polarized Gβ crescent could be seen to lag behind the Spa2-marked polarity patch (Figure 2.1D). Thus, consistent with the wandering simulations, there is a spatial offset between the G protein and polarity peaks.
Figure 2.11 Basis for constraint of polarity patch movement by RecGEF*

(A) Simulations where actin cable attachment was constrained to the northeast quadrant. Left: averaged RecGEF* distribution at 10 nM α-factor concentration. White dot indicates centroid of Cdc42 patch and arrow indicates direction and speed of patch movement. Right: one-dimensional GTP-Cdc42 and RecGEF* distributions along the direction of movement (arrow). (B) Simulations as in (A) but at 100 nM α-factor. (C) Cells (DLY18478) with cdc24-m1 mutation to allow high polarity patch wandering were treated with β-estradiol for 4 h to induce STE5-CTM, placed on a slab and imaged for GFP-Ste4 and Spa2-mCherry. In the rare cells that exhibited prolonged patch movement in a consistent direction, GFP-Ste4 (Gβγ) clearly lagged behind Spa2-mCherry (polarity patch). Montage of medial slice images of GFP-Ste4 and maximum projection images of Spa2-mCherry.
To understand why the RecGEF* peak lags behind the Cdc42 patch, we considered the time lag between the arrival of a RecGEF at the membrane and its binding to pheromone to generate RecGEF*. During this time lag, the Cdc42 patch moved away from the exocytosis site where the RecGEF was deposited, so the RecGEF* peak is located where the Cdc42 patch was before the time lag. In that way, the time lag in pheromone binding is converted into a spatial offset in the direction of patch movement. Furthermore, the spatial offset between the Cdc42 and RecGEF* peaks decreased with higher pheromone concentrations (Figure 2.1A,B).

We considered two explanations for why the spatial offset was pheromone dose-dependent. First, high pheromone concentrations resulted in a shorter time lag between the appearance of RecGEF on the membrane and ligand binding (time lag is inversely proportional to pheromone concentration). Second, because the polarity patch moves more slowly at higher pheromone concentrations (Figure 2.1C), a given time lag produces a smaller spatial offset. To isolate these two effects, we simulated a “neutral” Rec/Rec* that trafficked on vesicles and bound pheromone in the same manner as RecGEF/RecGEF* but lacked GEF activity. There was still a spatial offset between the Cdc42 and Rec* peaks, and the offset decreased at higher pheromone concentrations indicating that faster pheromone binding to the receptor can reduce the spatial offset (Figure 2.12). However, the spatial offset between the Cdc42 and Rec* peaks was larger than that between the Cdc42 and RecGEF* peaks for a given pheromone concentration indicating that slower patch movement (from RecGEF* activity generating GTP-Cdc42) also contributes to reducing the offset at high pheromone concentrations.
The spatial lag between the GTP-Cdc42 and RecGEF* peaks is plotted from simulations at each dose. Green: data from simulations with RecGEF*. Blue: data from simulations with a “neutral” Rec* that does not constrain patch movement.

In summary, our simulations suggest that when the Gβγ-recruited GEF is polarized due to vesicle trafficking of the receptor and G protein, then it acquires the ability to constrain polarity patch wandering in a pheromone dose-dependent manner. With enough pheromone, the patch simply wiggles in place: any movement induced by vesicle fusion is rapidly reversed by the lagging Gβγ-recruited GEF. In contrast, a uniformly-localized receptor and G protein system would be unable to influence patch wandering to a significant degree. These simulations provide an attractive explanation for why it would be advantageous for yeast cells to polarize their pheromone-sensing machinery during mating.
2.3 Discussion: Why do yeast cells polarize their G protein signaling?

Cells that utilize spatial sensing mechanisms to accurately detect a gradient compare the chemical concentration on different sides of the cell. The best way to do this, in theory, is to localize receptors all over the cell surface that bind ligand and signal to the cell (Berg and Purcell, 1977; Endres and Wingreen, 2008). Dictyostelium discoideum cells are thought to use such a mechanism, and they localize their receptors all over the membrane, which activate the G protein as a proper reflection of the gradient (Janetopoulos et al., 2001). However, yeast polarize their receptors and G proteins, so they do not directly detect the pheromone gradient. Though seemingly counterproductive, we suggest that polarized pheromone signaling could actually be useful for yeast cells tracking the pheromone gradient.

We recently characterized a wandering behavior exhibited by the polarity patch that results from both the positive feedback of Cdc42 polarity with the localized negative feedback of vesicle fusion (Dyer et al., 2013). Because pheromone receptors are delivered via vesicular trafficking near the wandering Cdc42 patch, the receptors too will wander. In essence, this creates a sensitized zone that continually checks the concentration of pheromone as it moves along the cell cortex. As the zone of receptors detects higher pheromone concentrations, the wandering decreases, so the polarity site spends more time – and thus the cell grows more – in that direction (Figure 2.13). We suggest this mechanism as a potential benefit that would outweigh the costs of reduced gradient sensing by polarized receptors.
Figure 2.13 Model for gradient tracking with polarized G protein signaling

(A) The polarity patch forms through a positive feedback mechanism. (B) Vesicle fusion drives polarity patch wandering along the cell cortex. (C) When the patch is on the down-gradient side of the cell, the G protein signaling has a wide distribution and is more offset from the polarity patch allowing the patch to wander more. (D) The GTP-Cdc42 generated by a closer and more polarized distribution of G protein signaling slows polarity patch wandering so more growth occurs in the proper direction.
3. Mechanisms of polarized G protein signaling

3.1 Introduction

Many eukaryotic cells track chemical gradients using spatial sensing whereby they compare the chemical concentration on one side of the cell compared to the other. The chemical cue is sensed through G-protein-coupled receptors (GPCRs) that signal to lipid kinases, protein kinases, and small GTPases to eventually induce actin polymerization for membrane extension during migration or growth.

Polarized growth in yeast is regulated by the GTPase Cdc42 that accumulates as a patch on the cell cortex by positive feedback mechanisms (Figure 1.10) (Johnson et al., 2011). This Cdc42 patch recruits formin actin nucleators (Chen et al., 2012; Dong et al., 2003), so actin cables are oriented towards the patch. Vesicles being carried along these actin cables are then delivered to the patch where they fuse and add new membrane and cell wall components for cell growth. However, because vesicles themselves do not carry many polarity regulators, the event of vesicle fusion is actually disruptive to the Cdc42 patch and effectively pushes the patch away from the fusion site (Figure 1.11) (Dyer et al., 2013; Layton et al., 2011; Savage et al., 2012). This vesicle fusion-mediated wandering of the polarity patch helps cells with error correction: if the cell initially polarizes Cdc42 on the wrong side of the cell, then vesicles can push the patch to reorient Cdc42 and the direction of growth.

If a yeast cell is properly tracking the pheromone gradient, then the Cdc42 patch must spend more time on the up-gradient side of the cell so that growth occurs primarily in the up-
gradient direction. So, how does the pheromone gradient bias the Cdc42 patch location?

Previous studies have identified the scaffolding protein Far1 as an essential regulator of gradient tracking (Chenevert et al., 1994; Valtz et al., 1995). Once bound to pheromone, the receptor activates the Gα subunit of the G protein, which frees the heterodimer Gβγ. This free Gβγ can then bind Far1, which also binds to Cdc24, the activator of Cdc42. Mutants that prevent Far1-Cdc24 binding cannot track the pheromone gradient (Butty et al., 1998; Nern and Arkowitz, 1998, 1999).

While most cells using spatial gradient sensing localize their receptors all over the membrane to maximize their ability to sense differences in chemical concentration (Servant et al., 1999; Wang et al., 1988; Xiao et al., 1997), instead, yeast localize their receptors to a patch on the membrane when exposed to their chemoattractant (Ayscough and Drubin, 1998; Jackson et al., 1991). Although seemingly counterintuitive for yeast to reduce their spatial resolution of the gradient, we propose a model (see Chapter 2) where polarized receptors could locally influence the direction of growth. When a yeast cell is properly oriented up-gradient, the polarized and activated receptors generate local active Cdc42 that, in essence, counteracts vesicle-mediated pushing of the polarity patch. But when the polarity patch is on the down-gradient side of the cell, then the receptors are less active and do not counteract the vesicle-mediated pushing, resulting in wandering of the polarity patch.

Consistent with our model, we found that, in cells exposed to uniform pheromone conditions, the polarity patch wandered more in low pheromone concentrations, but wandering was constrained in high pheromone concentrations (Figure 2.2). The ability of high pheromone concentrations to constrain polarity patch wandering correlated with polarized Gβγ in agreement with our mathematical model (Figure 2.8, 2.10). But in order to test whether
polarized G protein signaling is necessary for pheromone to constrain polarity patch wandering, we sought to understand how the receptor and Gβγ are polarized. In this chapter, we show that receptor polarity is not required for pheromone to constrain polarity patch wandering or for yeast gradient tracking. But, we show that Gβγ can polarize independently of the receptor, and we suggest that free Gβγ polarizes through vesicle trafficking to constrain polarity patch wandering.

3.2 Results

3.2.1 Effect of blocking pheromone receptor endocytosis on polarity patch wandering

Our model demonstrated that polarized G protein signaling can constrain polarity patch wandering in high uniform pheromone conditions (Figure 2.10). We therefore predicted that reducing receptor polarization by blocking its endocytosis should allow polarity patch wandering to continue even at high pheromone concentrations. The pheromone receptor, Ste2, is rapidly internalized following binding to pheromone, which triggers phosphorylation and ubiquitination of the C-terminal tail (Figure 1.12) (Chen and Konopka, 1996; Hicke et al., 1998). To block receptor endocytosis, we utilized a mutant receptor (designated Ste2<sup>7KR</sup>) in which 7 lysines (the ubiquitination sites) are mutated to arginines and the NPF endocytosis motif is mutated (Terrell et al., 1998).

Previous studies have reported Ste2<sup>7KR</sup>-mCherry localization at the plasma membrane but exclusion from the mating projection tip (Ballon et al., 2006). When we tagged Ste2<sup>7KR</sup>, we noticed different localizations depending on the fluorophore tag used. Ste2<sup>7KR</sup>-mCherry was slightly excluded from the tip of the mating projection, but Ste2<sup>7KR</sup>-GFP showed a slight enrichment at the tip of the mating projection (Figure 3.1A). We wondered whether this could
be due to the difference in maturation time between the fluorophores: mCherry has a half-time of around 40 minutes whereas GFP has a half-time around 8 minutes (Jackson et al., 2006; Merzlyak et al., 2007). To test this, we fused both GFP and mCherry to Ste2^{7KR} and found that mCherry was still excluded from the mating projection tip (Figure 3.1B). Therefore, in this context, mCherry is indeed maturing slower than GFP, and Ste2^{7KR} is mostly uniform on the membrane. We speculate that the slight enrichment of Ste2^{7KR}-GFP in the mating projection tip is due to delivery of new receptors outpacing the slow diffusion of the receptor in the membrane (Valdez-Taubas and Pelham, 2003).

**Figure 3.1** Ste2^{7KR} visualization with different fluorophore tags

(A) Cells expressing Ste2^{7KR}-mCherry (DLY16074) or Ste2^{7KR}-GFP (DLY15656) were treated with 400 nM α-factor for 2 h, loaded on an α-factor slab, and imaged (medial confocal plane). (B) Cells expressing Ste2^{7KR}-mCherry-GFP (DLY16185) were treated as in (A).
We found that despite a much less polarized distribution of the receptor (Figure 3.2A), polarity patch wandering was still constrained in Ste2\textsuperscript{7KR} cells upon exposure to a high pheromone concentration (Figure 3.2B). Moreover, Ste2\textsuperscript{7KR} cells exhibited a mating efficiency comparable to wild-type cells (Figure 3.2C), suggesting that they were competent to detect pheromone gradients and bias growth of the projection towards mating partners. Thus, contrary to our predictions, polarization of pheromone receptors is not necessary to constrain polarity patch wandering in response to pheromone.

![Figure 3.2 Pheromone constrains polarity patch wandering in cells with non-endocytosable receptor](image)

(A) Cells expressing Ste2-GFP (DLY15655) or Ste2\textsuperscript{7KR}-GFP (DLY15655) were treated with 300 nM α-facto for 2.5 h then imaged (medial confocal plane). (B) Cells harboring STE2\textsuperscript{7KR} (DLY15685) or STE2 (DLY11065) were pretreated with 300 nM α-factor for 1 h, loaded on an α-factor slab, then imaged. Polarity patch wandering was calculated as MSD (n>37 cells). (C) Cells harboring STE2\textsuperscript{7KR} (DLY15685) or STE2 (DLY11740) were assayed for mating efficiency in the presence or absence of excess α-factor to obscure the gradient.
3.2.2 Gβγ can polarize independently of receptor endocytosis

Although receptor polarization is not necessary to constrain polarity patch wandering, we wondered if Gβγ polarization is nevertheless required. Because previous studies found that both the receptor and the G protein subunits were internalized and polarized following exposure to pheromone (Arkowitz, 2009; Suchkov et al., 2010), we predicted that Gβγ would be less polarized in cells harboring the non-endocytosable Ste27KR mutant. However, we found that following prolonged exposure to pheromone, GFP-Ste4 (Gβ) became polarized in Ste27KR cells (Figure 3.3A,B). Thus, even though receptor is present all around the cell, G protein signaling is still polarized. This unexpected finding suggests that Gβγ can be internalized and recycled to the membrane independently of the receptor.

Figure 3.3 Gβγ polarization independent of receptor endocytosis

(A) Cells harboring STE2 (DLY15596) or STE27KR (DLY15717) were pretreated with 300 nM α-factor for 2.5 h, loaded on α-factor slabs, then imaged for GFP-Ste4. (B) Cells were treated as in (A) except the pretreatment was shortened to 1 h to reduce morphology variations. Graph shows GFP-Ste4 distributions (n>48 cells).
While Ste2\(^{7KR}\) cannot be internalized, cells with this mutant still induce receptor expression, and newly synthesized receptors are trafficked to the plasma membrane. We wondered whether G\(\beta\gamma\) could internalize independently of the receptor but still require receptor delivery in order to be recycled back to the membrane. To test this idea, we replaced the STE2 promoter with the inducible GAL promoter. We grew \(P_{\text{GAL}}\text{-}\text{STE2}^{7KR}\) cells in galactose media and pretreated them with \(\alpha\)-factor. Then, we switched the cells to dextrose media to stop transcription of \(\text{STE2}^{7KR}\) and examined GFP-Ste4. Even though receptor delivery and internalization was blocked in these conditions, GFP-Ste4 was still polarized further demonstrating that G\(\beta\gamma\) can polarize independently of the receptor (Figure 3.4).

**Figure 3.4 G\(\beta\gamma\) does not require receptor delivery to maintain polarity**

Cells expressing Ste2\(^{7KR}\)-GFP (DLY16078) or harboring \(P_{\text{GAL}}\text{-}\text{STE2}^{7KR}\) and expressing GFP-Ste4 (DLY16120) were grown in galactose media overnight and pretreated with 400 nM \(\alpha\)-factor for 2 h. Cells were then switched to dextrose media to halt transcription of Ste2\(^{7KR}\) for 2 h. Cells were then loaded on a slab and imaged (medial widefield plane).
Gβγ associates with membranes through the Gγ subunit, which undergoes both prenylation and palmitoylation (Hirschman and Jenness, 1999). In principle, Gβγ could become polarized in the same way as a transmembrane protein like v-SNARE: through endocytic internalization and exocytic delivery to the polarity site (Grote et al., 2000; Lewis et al., 2000; Valdez-Taubas and Pelham, 2003). Proteins that polarize by vesicle trafficking exhibit a slow fluorescence recovery after photobleaching (FRAP) rate (Valdez-Taubas and Pelham, 2003), and we found that GFP-Ste4 had a similar FRAP curve as the v-SNARE GFP-Snc2 (Figure 3.5) consistent with a vesicle trafficking polarization mechanism for Gβγ.

**Figure 3.5 FRAP recovery of polarized GFP-Ste4 and GFP-Snc2**

Cells expressing GFP-Ste4 (DLY13968) and GFP-Snc2 (DLY16164) were pretreated with 300 nM α-factor for 1 h then loaded on an α-factor slab and imaged with the FRAP protocol (see Chapter 5: Materials and Methods).
Alternatively, depalmitoylation of Gγ could release Gβγ from the membrane to the cytoplasm, and subsequent palmitoylation could return Gβγ to the plasma membrane, as has been suggested for mammalian Gβγ and other similarly modified proteins (Chisari et al., 2007; Goodwin et al., 2005; O’Neill et al., 2012). To assess whether Gβγ polarization required its release to the cytoplasm, we replaced the Gβγ prenylation motif with a transmembrane domain, forcing Gβγ to remain associated with membranes. The transmembrane domain we used is derived from the v-SNARE Snc2. Trafficking of Snc2 has been extensively characterized, and the sequence we used (SNC2^{V39M,M42A}) lacks the endogenous Snc2 endocytosis motif (Grote et al., 2000): it is delivered to the plasma membrane but is not internalized and does not become polarized (Figure 3.6A). When we fused Ste18 (Gγ) to the Snc2^{V39M,M42A} anchor and visualized Gβγ localization with GFP-Ste4 (Gβ), Gβγ still polarized (Figure 3.6C) suggesting that Gβγ contains an endocytosis signal that can induce vesicular recycling leading to polarization. This was the case even in Ste2^{7XR} cells (Figure 3.6C), in which Gβγ cannot be internalized due to association with endocytosing receptors. These results demonstrate that Gβγ polarization does not require its release to the cytoplasm, although it is possible that such a release may occur in wild-type cells.
Figure 3.6 Gβγ can polarize without releasing from the plasma membrane

(A) The v-snare Snc2 has a well-characterized endocytosis motif that is mutated in GFP-Snc2^V39A,M42A, yielding an unpolarized membrane protein. Cells (DLY17966) were grown with β-estradiol overnight to induce GFP-Snc2^V39A,M42A, then treated with or without 300 nM α-factor for 2.5 h prior to loading on an α-factor slab and imaged (medial confocal plane). (B) Cells harboring STE18-SNC2^V39A,M42A (Gγ fused to the mutant Snc2) and GFP-Ste4 (Gβ) were treated with or without 300 nM α-factor for 2.5 h and imaged for GFP-Ste4 (medial confocal plane). (C) Cells harboring STE18-SNC2^V39A,M42A with STE2 (DLY17437) or STE2^7KR (DLY17520) were treated as in (B).
We also found that Gβγ co-localized with markers of endocytic membranes and not other internal markers including Golgi and ER markers (Figure 3.7A), supporting an endocytosis and vesicle recycling mechanism of Gβγ polarity. But, what signal is responsible for Gβγ endocytosis? Gβγ polarization was not observed in the absence of pheromone (Figure 3.3) suggesting that the endocytosis signal is triggered by the pheromone response. Previous studies have indicated that, like the pheromone receptor, Gβ becomes phosphorylated and ubiquitinated following exposure to pheromone (Li et al., 1998; Zhu et al., 2011). However, we found that GFP-Ste4 (Gβ) mutants that could not be phosphorylated or ubiquitinated still became polarized in response to pheromone, even in Ste2_{7KR} cells (Figure 3.7B). We conclude that Gβγ displays a previously unsuspected endocytosis signal following exposure to pheromone, which allows Gβγ polarization independent of receptor endocytosis. The nature of the endocytosis signal remains to be determined.
Figure 3.7 Internal Gβγ colocalization and role of known post-translational modifications

(A) Cells expressing different internal compartment markers (DLY17257: Snf7-mCherry, DLY17883: mCherry-Tlg1, DLY17206: Cop1-mCherry, DLY17209: Sec13-mCherry, DLY17254: Anp1-mCherry, DLY17754: DsRED-HDEL) were treated with 400 nM α-factor for one hour, loaded on an α-factor slab, and imaged for GFP-Ste4 (maximum projections and images are not equally scaled). (B) GFP-Ste4 polarization does not require phosphorylation of T320/S335 or ubiquitination of K340. Cells harboring STE4 (left, DLY15596), STE4T320A,S335A (middle, DLY16191), or STE4K340R (right, DLY15927), were treated with 300 nM α-factor for 2.5 h and imaged (medial confocal plane). Representative cells are also shown for strains harboring STE27KR (DLY15717, DLY16189, DLY16016, respectively).
3.2.3 Parallel pathways for Gβγ polarization

Although Gβγ can polarize independently of receptor endocytosis, we fortuitously discovered that in cells expressing Ste5-CTM, Gβγ polarization required receptor endocytosis. In particular, exposure to pheromone induced Gβγ polarization in Ste2<sup>7KR</sup> cells and in Ste5-CTM cells but much less effectively in cells with both Ste2<sup>7KR</sup> and Ste5-CTM (Figure 3.8). Thus, the presence of Ste5-CTM must somehow block the receptor-independent Gβγ internalization pathway. The basis for this effect is unclear, but we note that Ste5 itself binds directly to free Gβγ, and that the Ste5-CTM fusion is expressed at high levels relative to endogenous Ste5. Thus, one possibility is that Ste5-CTM blocks Gβγ endocytosis by binding Gβγ and masking the endocytic signal. Consistently, we found that expressing Ste5ΔN-CTM, a truncated version of Ste5-CTM that cannot bind Gβγ (Pryciak and Huntress, 1998; Whiteway et al., 1995), no longer prevented Gβγ polarity in the presence of Ste2<sup>7KR</sup> (Figure 3.8).

![Figure 3.8 Parallel pathways for Gβγ polarization](image)

Left: cells harboring STE2 (DLY15596) or STE2<sup>7KR</sup> (DLY15717) were pretreated with 400 nM α-factor for approximately 2 h, loaded on an α-factor slab, then imaged. Middle: cells harboring STE2 (DLY17029) or STE2<sup>7KR</sup> (DLY17432) were treated with β-estradiol to induce Ste5-CTM for 4 hours, then loaded on an α-factor slab for 2 h, and imaged for GFP-Ste4. Right: cells harboring STE2 (DLY17837) or STE2<sup>7KR</sup> (DLY17868) were treated with β-estradiol to induce Ste5ΔN-CTM for 4 h, then loaded on an α-factor slab for 2 h, and imaged. Medial confocal plane images of GFP-Ste4 are presented.
The fact that Gβγ polarization was not blocked by Ste5-CTM in cells with wild-type receptor (Figure 3.8) suggests that there are two parallel pathways to polarize Gβγ: one that is independent of receptor internalization and can be blocked by Ste5-CTM, and a second one that is insensitive to Ste5-CTM but requires receptor internalization (Figure 3.9). This second pathway may correspond to the “piggyback” mechanism previously envisaged for G protein polarization, in which interactions between the receptor and the G protein subunits promotes Gβγ internalization via receptor endocytosis.

![Figure 3.9 Schematic of parallel pathways for Gβγ polarization](image)

Gβγ can be internalized by two mechanisms: one that requires receptor endocytosis (left) and one that we suggest relies on endocytosis and is blocked by Ste5-CTM (right).
3.2.4 Role of Gα in Gβγ polarity

Considering the receptor endocytosis-mediated pathway for Gβγ internalization, we wondered whether Gα might be required, perhaps as a connection between the receptor and Gβγ (Strickfaden and Pryciak, 2008). Indeed, we found that Gα was required for the receptor-mediated Gβγ polarization pathway: cells expressing Ste5-CTM (to block the receptor-independent pathway) required Gα in order to polarize Gβγ (Figure 3.10). We confirmed this result using a mutant of STE4 that prevents Gα-Gβγ binding (Strickfaden and Pryciak, 2008). Similarly, the GFP-Ste4L117R mutant did not polarize in Ste5-CTM cells (Figure 3.10).

![Figure 3.10 Gβγ-Gα interaction regulates receptor endocytosis-dependent Gβγ polarization](Figure)

Cells harboring GPA1 (DLY18172) or gpa1Δ (DLY18559) or STE4L117R (DLY18195) were treated with β-estradiol to induce Ste5-Ctm for 4 h, then loaded on a slab with or without 300 nM α-factor, and imaged for GFP-Ste4 or GFP-Ste4L117R (medial confocal plane).
We also tested whether Gα is required for the receptor endocytosis-independent Gβγ polarity pathway. In cells expressing the Ste5ΔN-CTM to allow Gβγ to polarize independently of the receptor, we asked whether Gα was required for polarizing Gβγ. To our surprise, GFP-Ste4 was strongly localized to internal puncta and showed very little signal at the plasma membrane in gpa1Δ cells whether or not pheromone was added (Figure 3.11A). The Gα binding-deficient mutant, GFP-Ste4L117R (Strickfaden and Pryciak, 2008) also showed the same localization (Figure 3.11B). All together, we conclude that Gα either slows Gβγ internalization or promotes Gβγ recycling to the plasma membrane, or both.

**Figure 3.11** Gβγ-Gα interaction regulates Gβγ localization at the plasma membrane

(A) Cells with GPA1 (DLY17837) or gpa1Δ (DLY18365) were treated with β-estradiol to induce Ste5ΔN-CTM for 4 h, then loaded on a slab with or without 300 nM α-factor for 20 min, and imaged for GFP-Ste4 (medial confocal plane). (B) Cells expressing GFP-Ste4 (DLY17837) or GFP-Ste4L117R (DLY17825) were treated as in (A), but loaded on the slab for 2 hours.
3.2.5 Free G\(\beta\gamma\) on vesicles can slow polarity patch wandering

In \(gpa1\Delta\) cells, all G\(\beta\gamma\) is free and activates the MAPK cascade leading to permanent cell arrest (Dietzel and Kurjan, 1987). (Our cells are \(ste5\Delta\), so they do not arrest until \(STE5\)-CTM or \(STE5\DeltaN\)-CTM expression is induced with the GAL promoter). This implies that G\(\beta\gamma\) must be able to reach the plasma membrane to initiate the MAPK cascade independently of G\(\alpha\). Together with our result of G\(\beta\gamma\) localization to internal structures in \(STE5\DeltaN\)-CTM \(gpa1\Delta\) cells (Figure 3.11), we hypothesized that G\(\beta\gamma\) was still being delivered to the plasma membrane but quickly internalized.

If free G\(\beta\gamma\) is indeed trafficking on vesicles to the polarity site, then it is possible that free G\(\beta\gamma\) is interacting with Far1-Cdc24 complexes while on these vesicles. Previous work from our lab has shown that if polarity proteins traffic on vesicles, then the perturbation of vesicle fusion on the polarity patch is reduced (Dyer et al., 2013; Howell et al., 2009; Layton et al., 2011; Savage et al., 2012). Therefore, free G\(\beta\gamma\) trafficking on vesicles in \(gpa1\Delta\) cells may lead to reduced polarity patch wandering. Indeed both \(STE5\DeltaN\)-CTM \(gpa1\Delta\) and the G\(\alpha\) binding-deficient mutant \(GFP\)-\(STE4^{L117R}\) cells showed reduced polarity patch wandering compared to cells only expressing \(STE5\DeltaN\)-CTM (Figure 3.12).
Figure 3.12 Free Gβγ on vesicles can slow polarity patch wandering

(A) Cells harboring GPA1 (DLY17837) or gpa1Δ (DLY18365) were treated with β-estradiol to induce Ste5ΔN-CTM for 4 h, then loaded on a slab, and imaged for Spa2-mCherry. Patch wandering of the Spa2-mCherry centroid was calculated as MSD (n>59 cells). (B) GFP-Ste4 (DLY17837) or GFP-Ste4L117R (DLY17825) cells were treated as in (A) (n>53 cells).
3.3 Discussion

In Chapter 2, we suggested a mechanism for yeast gradient tracking where cells produce a polarized distribution of receptors to create a sensitized zone that determines the local pheromone concentration as it wanders. If the zone detects high pheromone concentrations, the receptors will generate local active Cdc42 that can counteract vesicle-mediated polarity patch wandering. In this way, the patch will wander less on the up-gradient side of the cell but wander more on the down-gradient side. To test this hypothetical mechanism, we wanted to prevent G protein polarity. We predicted that depolarized G protein signaling would lead to rampant patch wandering even in high pheromone concentrations.

3.3.1 Polarized pheromone signaling is enforced by Gβγ recycling

Both the pheromone receptor and the G proteins are polarized in cells responding to pheromone (Arkowitz, 2009). While the mechanism of receptor polarity is established as a balance between delivery of newly made receptors to the polarity site and ligand-induced internalization and degradation (Hicke and Riezman, 1996; Hicke et al., 1998; Jenness and Spatrick, 1986; Schandel and Jenness, 1994), the exact mechanism of Gβγ polarity was not well-characterized. Previous work suggested Gβγ is internalized with the receptor after short stimulation with pheromone (Suchkov et al., 2010), and we now show that Gβγ can also polarize independently of receptor endocytosis. In mammalian cells, Gβγ has been shown to localize to internal structures (Ajith Karunaratne et al., 2012; Saini et al., 2007), and the kinetics of Gβγ translocation vary depending on the C-terminal residues and modifications of the Gγ subunit suggesting membrane affinity plays a role in Gβγ localization (O’Neill et al., 2012). We show that free Gβγ in yeast likely polarizes through an endocytosis and vesicle recycling mechanism and
not a depalmitoylation cycle like the one proposed for mammalian Gβγ and other palmitoylated proteins such as Ras (Goodwin et al., 2005).

Using the Ste5-CTM system, we were able to block the receptor-independent mechanism for Gβγ polarity and therefore isolate the receptor endocytosis-dependent mechanism. We found that Gα is required for this pathway consistent with previous work suggesting that Gα might serve as a connection between Gβγ and the receptor for proper gradient tracking (Strickfaden and Pryciak, 2008).

We were initially surprised to find that cells with receptor all over the membrane (STE27KR) still constrained wandering in response to high pheromone concentrations given the results of our computational simulations. But considering that Gβγ and thus downstream signaling still polarized in these cells, there is still a sensitized zone that can respond to pheromone changes and bias polarity patch wandering. As the polarity patch wanders along the cell cortex, the ligand-bound receptors can activate the polarized pool of Gβγ to slow down patch wandering.

### 3.3.2 Free Gβγ on vesicles can slow polarity patch wandering

Given our finding that free Gβγ likely polarizes by vesicle trafficking, we wondered if free Gβγ could bind to Far1-Cdc24 complexes while on vesicles. If polarity factors are indeed recruited to vesicles, then we predicted that fusion at the polarity patch by these free Gβγ-containing vesicles would not dilute the polarity factors and thus not “push” the polarity patch as much as vesicles without free Gβγ. Indeed, the polarity patch showed reduced wandering in cells with free Gβγ trafficking on vesicles. However, while polarity patch wandering was reduced in these cells, the patch still wandered more than in wild-type cells treated with high
pheromone concentrations, and these cells were not able to form pointy mating projections. Therefore, simply producing free Gβγ that traffics on vesicles is not sufficient to explain how pheromone constrains polarity patch wandering, and we suggest that polarized free Gβγ must also contribute to pheromone constraint of wandering (as discussed above and in Chapter 2).
4. Conclusions and future directions

4.1 Conclusions

Many mechanisms have been proposed to explain how cells can track gradients so efficiently. Work in the bacteria model, *E. coli*, has uncovered temporal gradient tracking mechanisms whereby a cell compares the chemoattractant concentration it currently senses to the concentration it sensed a second ago (Berg and Purcell, 1977). *E. coli* cells swim longer if they are moving up-gradient, but if they are not moving up-gradient, then they tumble and reorient in a random direction (Berg and Brown, 1972). In contrast, work in neutrophil and Dicty cells suggests that eukaryotic chemotaxis operates with a direct spatial sensing mechanism (Jin, 2013; Swaney et al., 2010). These cells localize their receptors uniformly on the plasma membrane and reflect the gradient in G protein activation (Janetopoulos et al., 2001; Jin et al., 2000; Servant et al., 1999; Wang et al., 1988; Xiao et al., 1997). During chemotaxis, these cells polarize their morphology and create a “front” through actin polymerization (Swaney et al., 2010; Zigmond, 1978). If these cells are not properly migrating up-gradient, these cells turn their front and reorient their polarity in accordance with the gradient. Despite many hypotheses that have been suggested and explored computationally, the mechanistic basis for how eukaryotes use spatial gradient sensing to properly migrate up-gradient remains unknown.

The budding yeast, *Saccharomyces cerevisiae*, can track gradients during mating remarkably well (Moore et al., 2008; Segall, 1993). And, unlike other eukaryotic gradient tracking systems, the molecular details connecting G protein coupled receptor activation and actin polymerization are well characterized (Dohlman and Thorner, 2001). In response to mating pheromones, yeast cells polarize Cdc42 and its associated polarity proteins to a patch on
the cortex that dictates the site of growth (Arkowitz, 2009). Previous work from our lab and others showed that vesicle fusion mediates polarity patch wandering along the cell cortex in order to reorient the direction of growth (Dyer et al., 2013), but how the pheromone gradient influenced this wandering behavior to ensure that growth occurs primarily on the up-gradient side of the cell was unknown.

In addition, yeast cells polarize their G protein-coupled receptors and G proteins when exposed to pheromone (Ayscough and Drubin, 1998; Jackson et al., 1991; Moore et al., 2008). Yeast cells are thought to use a spatial sensing mechanism rather than a temporal mechanism, so how can they directly sense the gradient if their receptors are not uniformly distributed on the plasma membrane?

In the present study, we suggest that by polarizing their pheromone receptors and G proteins, yeast cells create a sensitized zone of the membrane that senses the local concentration of pheromone. As the polarity patch wanders up-gradient, the receptors sense a higher concentration of pheromone and locally influence the polarity patch to reduce its wandering.

We show that cells in high uniform pheromone concentrations produce a highly polarized Gβγ distribution (Figure 2.8) and exhibit little polarity patch wandering (Figure 2.2). Using computational approaches, we explain how delivery of receptors on vesicles to the polarity patch combined with delayed ligand binding results in a spatial offset between the polarity patch and the receptors (Figure 2.11). In effect, active receptors follow behind the wandering polarity patch. These active receptors can generate local GTP-Cdc42 that counteracts the vesicle fusion mediated pushing of the polarity patch and therefore slows patch wandering.

But in low doses of pheromone, the receptors are less active and further offset from the polarity
patch. The local GTP-Cdc42 produced is therefore much less and further away, meaning it has little effect on polarity patch wandering.

Our proposed mechanism for gradient tracking in yeast centers on the sensitized zone of the membrane composed of the polarized receptors and G proteins. But do yeast cells require such a polarization for effective gradient tracking? Surprisingly, we found that cells harboring endocytosis-deficient receptors were able to mate just as well as wild-type cells despite a more uniform distribution of receptors (Figure 3.2). However, Gβγ still polarized in these cells, so we speculate that the sensitized zone still holds in these cells because only active receptors near the polarized pool of Gβγ will be able to activate G protein signaling.

In order to ask whether polarized Gβγ was required for gradient tracking, we needed to better understand how Gβγ polarizes during yeast mating. We found that, unlike mammalian Gβγ, yeast free Gβγ likely polarizes by endocytosis and vesicle recycling to the plasma membrane (Figure 3.5,3.6). Further, Gβγ can polarize through two parallel pathways: one that is dependent on receptor endocytosis and one that is independent (Figure 3.9). Both pathways were dependent on Gα, and we speculate that Gα regulates Gβγ localization at the plasma membrane by preventing Gβγ internalization, encouraging Gβγ recycling from an internal compartment, or both. Further studies are required to clarify the role of Gα in regulating Gβγ localization.

In addition to the role of polarized Gβγ, we also found that free Gβγ likely slows polarity patch wandering simply due to trafficking on vesicles. Vesicle fusion locally dilutes the polarity patch to drive polarity patch wandering. But if free Gβγ could bind Far1-Cdc24 complexes while trafficking on vesicles, then vesicle fusion would not be as disruptive resulting in less wandering of the polarity patch (Figure 3.12). Together, we propose that polarized Gβγ behind the polarity
patch generating local GTP-Cdc42 and free Gβγ on vesicles preventing vesicle mediated pushing of the polarity patch both contribute to constraint of polarity patch wandering. These mechanisms would in principle reduce polarity patch wandering if the patch is on the up-gradient side of the cell and allow wandering if the patch is on the down-gradient side of the cell. We propose this as a gradient tracking mechanism that clarifies why yeast polarize their receptors and how pheromone could bias polarity patch wandering.

4.2 Future directions

4.2.1 Tracking gradients over a wide range of mean concentration

In *E. coli* chemotaxis, adaptation is a key component to the temporal gradient tracking mechanism (reviewed in (Vladimirov and Sourjik, 2009)). As a cell moves up-gradient, receptor inhibition by ligand-binding outpaces receptor activation through methylation allowing the cells to swim longer before tumbling and reorienting to a random direction. Receptor methylation serves as an adaptation mechanism that counteracts the ligand-induced signal. In uniform nutrient conditions, cells match the external stimulation with receptor methylation returning the cells to the normal cadence of runs and tumbles.

Similarly, Dicty cells adapt following uniform stimulation with cAMP. PIP$_3$ and Ras-GTP only transiently localize uniformly on the plasma membrane (Parent et al., 1998; Takeda et al., 2012). The local excitation-global inhibition model has been proposed and reconciles adaptation to uniform conditions with sustained polarized signaling in gradients (Iglesias and Devreotes, 2008).

In both *E. coli* and Dicty, adaptation can explain why these cells are so adept at tracking gradients over a wide range of mean concentration. The cells are comparing concentrations of
chemoattractant rather than sensing the absolute concentrations in time or space. Yeast are also proficient at tracking gradients over a wide range of mean concentration (Moore et al., 2008; Segall, 1993), but they do not seem to show adaptation in uniform pheromone conditions. Yeast signal through the MAP kinase cascade and polarize Cdc42 just as in gradient conditions (Arkowitz, 2009; Behar et al., 2008). However, because our experiments on polarity patch wandering were performed in uniform pheromone conditions, it remains possible that polarity patch wandering shows adaptation when cells are in a gradient. In order to detect this sort of adaptation, experiments could be performed where uniform pheromone concentrations are slowly raised or lowered to simulate polarity patch movement to the up- or down-gradient side of the cell. If yeast cells do adapt, then the polarity patch may exhibit different wandering behavior depending on the previous pheromone concentration.

### 4.2.2 MAP kinase and Rsr1 constraint of polarity patch wandering

Almost all of the experiments performed in this study used yeast strains with a bud site selection mutation: rsr1Δ. During vegetative growth, haploid yeast cells bud towards a previous bud scar through Rsr1 binding to Cdc24, the GEF for Cdc42 (Park and Bi, 2007). In the absence of Rsr1, cells establish a Cdc42 polarity patch and bud in a random direction. When in a pheromone gradient, yeast cells polarize and direct polarized growth up-gradient (Madden and Snyder, 1992). But when exposed to uniform pheromone conditions, cells instead polarize towards the bud scar (Arkowitz, 2009; Madden and Snyder, 1992). How does Rsr1 bias Cdc42 localization when cells are in uniform conditions but not when cells are in a pheromone gradient? In other words, how does a pheromone gradient bias the polarity patch to overcome the Rsr1 signal from the bud scar?
One potential mechanism is through MAP kinase regulation of Rsr1 activity. Wild-type cells with intact bud site selection still display pheromone dose-dependent constraint on polarity patch wandering in uniform conditions, though the wandering is overall lower compared to rsr1Δ cells (Dyer et al., 2013). Also, when cells express Ste5-CTM with high MAP kinase activity, they produce narrow mating projections suggesting the polarity patch wanders very little (Pryciak and Huntress, 1998; Strickfaden and Pryciak, 2008). Together, we speculate that Rsr1-dependent constraint of the polarity patch is regulated by MAP kinase activity.

Further, Rsr1-dependent constraint of polarity patch wandering is independent of pheromone activation of the free Gβγ-Far1-Cdc24 constraint pathway described in this study. Mutant cdc24-m1 cells form mating projections towards the bud scar and Ste5-CTM cells not treated with pheromone do not have free Gβγ and thus do not activate the pathway (Nern and Arkowitz, 1998; Pryciak and Huntress, 1998).

Now, consider a cell in a gradient whose bud scar is on the down-gradient side of the cell. Rsr1’s influence on the polarity patch could be reduced because the down-gradient side of cell has less MAP kinase activation, thus allowing the polarity patch to wander to the up-gradient side of the cell. This idea requires a local influence of the MAP kinase pathway to Rsr1, and future studies would need to identify the molecular connection to substantiate this potential mechanism.

Another way in which wild-type cells in a pheromone gradient could overcome the Rsr1 signal would be to “move” the Rsr1 signal to the current patch location. Cells in uniform pheromone produce a mating projection adjacent to the bud scar, and as the mating projection grows, the landmark protein Axl1, which activates Rsr1 during vegetative growth, localizes to the mating projection tip (Lord et al., 2002; Park and Bi, 2007). The Rsr1 GEF, Bud5, is recruited
to the bud scar by landmark proteins and also localizes to the tip of the mating projection (Figure 4.1). However, we found that Bud5 localization to the mating projection does not require the Axl2, Bud8, or Bud9 landmarks (Figure 4.1), so how Bud5 localization is regulated differently during mating remains a mystery. Nonetheless, because Bud5 localized to the tip of the mating projection, GTP-Rsr1 is likely localized to the mating projection and no longer at the bud scar. Therefore, Rsr1 could reduce wandering of the polarity patch by locally binding Cdc24.

![Figure 4.1 Bud5-GFP localization to the mating projection is independent of landmarks](image)

Cells harboring \textit{AXL2, BUD8, BUD9} (DLY17178) or landmark-deficient cells harboring \textit{axl2Δ, bud8Δ, bud9Δ}, (DLY17497) were pretreated with 300 nM α-factor for 2 h, then loaded on an α-factor slab, and imaged for Bud5-GFP (maximum projection). Bud5-GFP localized to the bud and neck (upper left) in a landmark-dependent manner (lower left), but Bud5-GFP localized to the mating projection independent of landmarks (right).
5. Materials and methods

5.1 Strains and plasmid construction

Standard molecular genetic procedures were employed to generate yeast strains, listed in Table 5.1, and plasmids, listed in Table 5.2 (Guthrie and Fink, 1991). All strains are in the BF264-15Du background (ade1 his2 leu2-3,112 trp1-1 ura3Δns) (Richardson et al., 1989).

rsr1::kan\(^\text{R}\) was made by the one-step PCR based method (Baudin et al., 1993) using genomic DNA from the ResGen deletion collection as a template. rsr1::URA3, gpa1::URA3, bud8::URA3, and ste4::URA3 deletions were made by the same method using pRS306 as a template, and bud9::TRP1 was made with pRS304 as a template (Sikorski and Hieter, 1989). ste5::nat\(^\text{R}\) and axl2:: nat\(^\text{R}\) deletions were made using pRS40N as a template (Chee and Haase, 2012). BUD5-GFP was made by the PCR-based C-terminal tagging method (Bahler et al., 1998).

BEM1-GFP and SPA2-mCherry were introduced into strains as in (Howell et al., 2009). cdc24-m1 was introduced as in (Dyer et al., 2013). GFP-STE4 and GFP-STE4\(\text{T320A,S335A}\) were crossed in from the strains RDY126 (DLY13968) (Suchkov et al., 2010) and RDY139 (Deflorio et al., 2013).

pRS304-\(P_{\text{ADH1}}\)-GAL4BD-\(hER\)-VP16 (pPP1557 from (Takahashi and Pryciak, 2008)) was cut with SnaBI to target integration at TRP1. pRS305-\(P_{\text{GAL1}}\)-STE5-CTM (Pryciak and Huntress, 1998) was cut with PpuMI to integrate at LEU2. pRS305-\(P_{\text{GAL1}}\)-STE5ΔN-CTM was made by cutting pRS303-\(P_{\text{GAL1}}\)-STE5ΔN-CTM (Pryciak and Huntress, 1998) with PvuI and ligating into pRS305, and then cut with Hpal to target integration at LEU2.

STE2\(7\text{KR}\) was derived from LHP507 containing pRS426-STE2\(7\text{KR,GPAAD}\) (a gift from Linda Hicke). The STE2\(7\text{KR,GPAAD}\) gene was amplified using primers that added BamHI sites to 208 bp
downstream of the *STE2* start codon and 200 bp downstream of the stop codon, then ligated into pRS304 to make DLB3217. DLB3217 was then cut with BsmI to target integration at *STE2*.

*STE2-GFP* and *STE27KR-GFP* were made by first constructing a plasmid with bp 600–974 of *STE2* and 198 bp downstream of the *STE2* stop codon that added HindIII and NotI sites between the two regions and added flanking XhoI and SacI sites, used to ligate into pRS306. Next, the C-terminal fragment of *STE2* (from genomic DNA) or *STE27KR* (from DLB3217) were amplified using primers that added flanking HindIII and NotI sites, then used to ligate into the previous plasmid. Finally, *GFP* was amplified using primers that added NotI sites and was added to both plasmids yielding DLB3849 and DLB3850, which were cut with ClaI to target integration at *STE2*. *STE27KR-mCherry-GFP* and *STE27KR-mCherry* were made by amplifying mCherry using primers that added flanking NotI sites and ligating into DLB3859.

*PGAL1-STE27KR-GFP* was made by the PCR-based C-terminal tagging method (Bahler et al., 1998) and targeted for integration in a *STE27KR-GFP* strain. *PGAL1-STE27KR* was constructed similarly but targeted for integration in a *STE27KR* strain.

*STE41117R* was derived from pPP2977 (Strickfaden and Pryciak, 2008). The *STE41117R* gene was amplified using a primer upstream of the *STE4* start codon and a primer downstream of the stop codon that added a NotI site. The PCR product was cut with PstI (90 bp downstream of the start codon) and NotI (306 bp downstream of the stop codon) and ligated into pRSIII06 (Chee and Haase, 2012) to make DLB4066. DLB4066 was cut with MscI to target integration to *STE4* in *GFP-STE4* strain to make *GFP-STE41117R:URA3*.

*STE18-SNC2V39A,M42A* was made by the PCR-based C-terminal tagging method (Bahler et al., 1998) using DLB4069: pFA6a-SNC2V39A,M42A-nat* R* as a template to target *SNC2V39A,M42A-nat* R* to the *STE18* locus. DLB4069 was constructed first by creating DLB4064: pFA6a-GFP-nat* R*. The nat* R*
gene was amplified from pRS40N (Chee and Haase, 2012) using primers that added flanking NcoI and EcoRI sites, which were then used to replace the \textit{kan}^R gene in the pFA6a-GFP-\textit{kan}^R plasmid with \textit{nat}^R. Then the \textit{SNC2}^{V39A,M42A} gene was amplified from DLB2920 (Howell et al., 2009) using primers that added flanking PacI and Ascl sites and was used to replace the \textit{GFP} from DLB4064.

\textit{STE4}^{K340R} was amplified from genomic DNA using a primer that added a PstI site 588 bp downstream of the \textit{STE4} start codon and a primer that introduced the K340R mutation and extended past the XbaI site 1038 bp downstream of the start codon. This PCR product was then cut with PstI and XbaI and ligated into pRS305-\textit{STE4}, which was made by amplifying \textit{STE4} from genomic DNA using primers that added a PstI site 588 bp downstream of the \textit{STE4} start codon and a NotI site 306 bp downstream of the stop codon prior to ligation into pRS305. DLB3906: pRS305-\textit{STE4}^{K340R} was cut with Nhel to target integration at \textit{STE4} in a \textit{GFP-STE4} strain.

\textit{COP1}-mCherry:hyg^R, \textit{SEC13}-mCherry:hyg^R, \textit{ANP1}-mCherry:hyg^R, and \textit{SNF7}-mCherry:hyg^R, were generated using the PCR-based C-terminal tagging method (Bahler et al., 1998). pRS305-\textit{DsRED-HDEL} (Manford et al., 2012) was cut with EcoRV to target integration at \textit{LEU2}. pRS315-\textit{P}_{\text{TPI1}}-mCherry-TLG1 was from (Xu et al., 2013).

\textit{P}_{\text{ADH1}}-\textit{GFP-SNC2} was generated using the PCR-based C-terminal tagging method (Bahler et al., 1998) with DLB3934: pFA6a-\textit{kan}^R-\textit{P}_{\text{ADH1}}-\textit{GFP} as a template. DLB3934 was constructed first by amplifying 500 bp upstream of the \textit{ADH1} stop codon using primers that added flanking BglII and PacI sites and replacing \textit{P}_{\text{GAL1}} from pFA6a-\textit{TRP1-P}_{\text{GAL1}}-\textit{GFP} (Bahler et al., 1998). Then, the \textit{TRP1} gene was replaced with \textit{kan}^R from pFA6a-\textit{kan}^R-\textit{P}_{\text{GAL1}}-3HA (Bahler et al., 1998) using EcoRI and BglII.

\textit{P}_{\text{GAL1}}-\textit{GFP-SNC2}^{V39A,M42A} was made by cutting DLB4144 with ClaI to target integration at \textit{LEU2}. DLB4144 was made first by amplifying the 541 bp upstream of the \textit{GAL1} start codon using
primers that added flanking XhoI and HindIII sites, amplifying GFP with primers that added flanking BamHI sites, and amplifying 350 bp downstream of the ADH1 stop codon with primers that added flanking SacII and SacI sites. Then the \( \text{SNC2}^{V39A,M42A} \) gene was amplified from DLB2920 (Howell et al., 2009) with primers that added flanking SacII sites. These genes were then all ligated sequentially into pRS305.

**Table 5.1 Yeast strains**

<table>
<thead>
<tr>
<th>strain*</th>
<th>genotype**</th>
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<tbody>
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<td>DLY11065</td>
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<td>DLY13968</td>
<td>( \text{MATa, bar1, GFP-STE4} )</td>
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*all strains in the BF264-15Du background (<i>ade1</i>,<i>his2</i>,<i>leu2</i>-3,112 <i>trp1</i>-1, <i>ura3</i>Δns)

**STE2<sup>7KR</sup>** strains contain K337R, K352R, K358R, K374R, K387R, K400R, K422R lysine mutations as well as the NPF motif mutation F394A

Table 5.2 Plasmids
5.2 Microscopy

5.2.1 Live cell microscopy

Cells were grown overnight at 30°C to mid-log phase in Complete Synthetic Media (CSM, MP Biomedicals, Santa Ana, CA) supplemented with 0.67% Yeast Nitrogen Base, 2% dextrose, 0.01% adenine. Cultures were then diluted to OD$_{600}$=0.1 just prior to treatment. For Ste5-CTM experiments, cells were treated with 20 nM β-estradiol (Sigma, St. Louis, MO) for 4 h, then mounted on a 2% agarose (Denville Scientific, South Plainfield, NJ) slab with β-estradiol and α-factor (Genway Biotech), and incubated for 20 min at room temperature prior to imaging. Slab edges were sealed with petroleum jelly. For STE5 experiments, cells were pre-treated with α-factor in culture for 1 h for MSD experiments or 1-2.5 h for still images of cells with fully formed mating projections.

All imaging (except for Figure 3.4 and Figure 3.5) was performed using an Andor Revolution XD spinning-disk confocal microscope with an Andor Ixon3 897 512 EMCCD camera (Andor, Belfast, UK). Timelapse images were acquired every 90 sec for 30 min total. 30 z-sections 0.24 μm apart were acquired with 50 ms exposure for DIC and 200 ms exposure in both 488 nm and 561 nm channels. All channels used 200 EM gain, and laser power was reduced to 15% for both wavelengths during live cell imaging. Still images were acquired as 47 z-sections 0.13 μm apart with laser power of 25% for the 488 nm laser and 35% for the 561 nm laser.

Image processing was performed using FIJI software. Image stacks were processed with the 3D hybrid median filter plugin (http://rsb.info.nih.gov/ij/plugins/hybrid3dmedian.html), scaled, and inverted. Images within each figure are scaled the same unless otherwise noted.
Within each figure, the scale bar = 5 μm. Images used for MSD or linescan analysis (see below) were not processed.

Imaging for Figure 3.4 was performed using an Axio Imager widefield fluorescence microscope (Zeiss, Thornwood, NY) with a 100x/1.4 objective and a Hamamatsu Orca CCD camera (Hamamatsu, Bridgewater, NJ). Images were acquired with 1 s exposure using a Filter Set 38 HE (Zeiss).

5.2.2 Fluorescence recovery after photobleaching (FRAP)

Cells were pretreated with 300 nM α-factor for 1.5 hours, then loaded on an α-factor slab prior to the experiment. FRAP was performed using an Olympus IX-71 wide-field fluorescent microscope using a 100x/1.4 oil objective. Images were acquired with an Evolve back-thinned EM-CCD camera (Photometrics®) connected to a Deltavision Imaging System with SoftWoRx software (Applied Precision). Photobleaching was achieved by a single 0.1 s laser pulse at 488 nm (25% laser power), and 30 subsequent images were acquired at adaptive time intervals. Mean fluorescence intensity was normalized to prebleach peak intensity.

5.2.3 Mean squared displacement (MSD) analysis

Polarity patch tracking was performed using Volocity software (Improvision, Waltham, MA). The three-dimensional centroid of each patch was calculated after thresholding the SPA2-mCherry signal (or BEM1-GFP for Figure 3.2). To account for stage drift, TetraSpeck beads (Invitrogen, now Thermo Fisher Scientific, Waltham, MA) were added to the slab, and the centroid of the bead was subtracted from each patch centroid. MSD was calculated for each cell as in (Dyer et al., 2013).
5.2.4 GFP-Ste4 distribution analysis

GFP-Ste4 distribution analysis was performed using FIJI software. Using the freehand line tool, the average intensity of a 3 pixel wide line drawn on the perimeter of the cell was measured. Location along the perimeter was normalized to values between 0 and 1, then the distribution for each cell was fit with a spline using the smooth.spline function in the statistical software R with a smoothing parameter of 0.7. The fluorescence intensity values of the spline function were normalized to an integral of 1, then a subsequent spline was fit to all the cells in the treatment group.

5.3 Western blotting

Cells were grown overnight at 30°C to mid-log phase in CSM, then diluted to OD$_{600}$=0.3 prior to treatment. Ste5-CTM cells were treated with 20 nM β-estradiol for 4 h then treated with α-factor for an additional 30 min. Samples were then prepared using TCA, separated using SDS-PAGE, and transferred to nitrocellulose as in (Keaton et al., 2008). Blots were probed using the anti-phospho-MAPK rabbit polyclonal antibody used at 1:500 dilution (cat# V803A, Promega, Madison, WI) and anti-actin mouse monoclonal antibody used at 1:2000 dilution (clone C4, MP Biomedicals).

5.4 Mating assay

Cells were grown overnight at 30°C in YEPD to mid-log phase. 20,000 $MAT\alpha$ cells were plated on either a YEPD plate or a YEPD plate supplemented with 200nM alpha-factor. Then, 3 μl drops containing either 5,000 500 cells of $MAT\alpha$ cells were spotted on each plate. Calibration drops containing 10 cells each were spotted on a separate YEPD plate to calculate the actual number of $MAT\alpha$ cells spotted on the mating plates. After approximately 16 h at 30°C, the
mating plates were replica plated to selective media plates allowing only diploids to grow.

Mating efficiency was calculated as the number of diploids per spot divided by the number of \textit{MAT}^a cells spotted.

5.5 Computational modeling

We used a mathematical model that incorporates the diffusion and protein interactions of Cdc42 as described in Figure 2.4 and modeled in (Goryachev and Pokhilko, 2008). Vesicle trafficking described in (Layton et al., 2011) was integrated into the model as in (Savage et al., 2012). The model was then adapted to simulate polarity patch wandering during yeast mating in (Dyer et al., 2013).
References


Biography

Allison Wolff McClure (née Wagner) was born on May 27, 1986 in St. Louis Park, MN. She attended St. Olaf College in Northfield, MN from 2004-2008 where she received a Bachelor of Arts, magna cum laude, in Biology and in Mathematics. She matriculated at Duke University in the Cell and Molecular Biology Program in 2008 and earned a Doctor of Philosophy in the Program in Molecular Cancer Biology in 2014.

Allison is a member of the American Society for Cell Biology, the Genetics Society of America, Graduate Women in Science (Rho Tau Chapter), and the Women in Science and Engineering group at Duke University. She has presented her work at several national meetings including the American Society for Cell Biology annual meeting (2012, 2013), the Cell Biology of Yeasts at Cold Spring Harbor (2013), and the Yeast Genetics Meeting (2014). She earned the Duke University Graduate School Travel Fellowship in 2012, 2013, and 2014.

Allison has published the following article: